Effect of Potassium Bicarbonate and Vitamin D Insufficiency on Aging Skeletal Muscle

A thesis submitted by
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Abstract

**Background:** Age-associated, low-grade metabolic acidosis and vitamin D insufficiency are potential contributors to skeletal muscle loss and impairment. Reducing endogenous acid production with alkali therapy lowers markers of muscle protein breakdown, but effects on muscle morphology are unknown. Also, whether alkali therapy modifies the effect of vitamin D insufficiency on muscle has not been studied.

**Objective:** We conducted a pilot study to investigate the impact of an alkaline salt, potassium bicarbonate (KHCO$_3$), and a vitamin D$_3$-deficient diet, alone and in combination, on intermediate biomarkers of muscle protein metabolism and muscle fiber size in rats on acid-producing diets for 12 weeks.

**Design:** Thirty-six 20-month-old rats on acid-producing diets were weight-matched and randomly assigned in a 2x2 factorial design to one of two KHCO$_3$-supplemented diets (with or without vitamin D$_3$) or one of two diets without KHCO$_3$ (with or without vitamin D$_3$) and pair fed for 12 weeks.

**Main Outcomes:** At 12 weeks, we measured 24h urinary nitrogen-to-creatinine ratio (UNi/Cr), a ratio of type I muscle fiber cross-sectional area (CSA) to soleus weight (CSA1/soleus), a ratio of type II fiber CSA to extensor digitorum longus (EDL) muscle (CSA2/EDL), serum IGF-1 and IGFBP-3 levels.

**Results:** KHCO$_3$ supplementation, adjusted for vitamin D$_3$ group, resulted in lower mean UNi/Cr (11.99 mg/mg [95%CI 8.16-15.83] with KHCO$_3$; 19.17 mg/mg [95%CI 15.46-22.89] without KHCO$_3$; P=0.01), higher mean CSA1/soleus (123.78 µm$^2$/mg [95%CI 116.80-130.76] with KHCO$_3$; 112.14 µm$^2$/mg [95%CI 105.39-118.89] without KHCO$_3$; P=0.02), similar mean CSA2/EDL, and similar IGF-1 and IGFBP-3 levels when
compared to groups without KHCO$_3$. Vitamin D$_3$-deficient diets, adjusted for KHCO$_3$ group, had no significant effects on muscle endpoints. We did not detect statistically significant interactions between the KHCO$_3$ and vitamin D$_3$ groups.

**Conclusion:** A 12-week alkalinization of the diet with KHCO$_3$ promoted nitrogen sparing and larger type I fibers in aged rats on acid-producing diets. Conversely, vitamin D$_3$-deficient diets had no significant effect on these muscle outcomes over a 12-week period.
Acknowledgements

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**Introduction**

With advancing age, there is a progressive loss of skeletal muscle mass, strength, and performance, also referred to as sarcopenia [1-3]. Histological findings from muscle specimens in older adults reveal muscle fiber atrophy with a preferential loss of type II (fast-twitch) skeletal muscle fibers [4-6]. These muscle changes associated with aging result in a higher risk of physical disability, falls, and fractures in older adults [3, 7]. Among the various mechanisms proposed for the development of sarcopenia are an age-related, low-grade, chronic metabolic acidosis [8] and a low vitamin D status [9]. Both factors are commonly found in community-dwelling older individuals.

Chronic metabolic acidosis in renal failure [10] and in obese subjects on very low energy protein diets [11], is associated with increased muscle protein degradation as measured by nitrogen excretion in the urine. A low-grade, chronic metabolic acidosis reported in healthy older adults results from age-associated declines in renal function and ingestion of diets rich in protein and cereal grains [12-13]. Placebo-controlled trials demonstrate that reducing dietary acid load with an alkaline salt decreases urinary nitrogen excretion [14-16] and improves muscle power [16] in older individuals. Yet, effects of alkaline salts on biochemical markers of muscle hypertrophy, muscle morphology, and muscle mass are lacking.

Studies indicate that low vitamin D status, as measured by serum levels of 25-hydroxyvitamin D (25OHD), also contributes to muscle atrophy and functional impairment in older adults [4-6, 9, 17-18]. The underlying mechanisms to explain vitamin D’s muscle effects have not been well-characterized, and data on the impact of lowering 25OHD levels on intermediate indices of muscle conservation and muscle fiber size are
limited. More than 3 decades ago, a series of studies in experimental animals described a potential link between vitamin D deficiency and acid-base status by means of changes in renal bicarbonate reabsorption [19-21]. Depletion of vitamin D in animals resulted in a metabolic acidosis whereas repletion resulted in a metabolic alkalosis [19, 21]. To our knowledge, there are no data examining whether a change in acid-base status could alter the effect of vitamin D on skeletal muscle.

Utilizing an aging rodent model on a mildly acid-producing diet to represent a healthy older adult, we conducted a pilot study to investigate whether reduction in dietary acid load through supplementation with KHCO$_3$ and a moderate decrease in vitamin D status affect urine nitrogen loss, muscle fiber size, and muscle mass. Furthermore, given a potential mechanistic link between acid-base status and vitamin D status, we investigated whether an alteration in acid-base balance could modify the effect of inadequate vitamin D on these skeletal muscle outcomes.

**Materials and Methods**

*Animals and Experimental Protocol*

Thirty-six male, Fischer 344/Brown-Norway rats (20-month old) were purchased from the National Institute on Aging. The rats were housed individually in plastic cages at 25 degrees C, in 12-h-light/12-h-dark cycles and had free access to water. We measured body weight weekly and food intake daily. Male rats were chosen to eliminate the potential confounding effect of hormonal fluctuations. Twenty-month old rats were chosen to represent an older human adult (age 65 years and older).
Upon arrival, all rats were placed on a 2-week period of acclimation on the AIN-93M diet [22] with standard mineral and vitamin mix added. Based on prior work in our laboratory (Appendix A), 18 to 20-month-old, male, Fischer 344/Brown-Norway rats on this same diet had mildly acidic 24-h urine pH between 6 and 7.

Following the 2-week run-in phase, the rats were weighed and ranked by weight from lightest to heaviest. From the weight-ranked list of animals, each block of 4 rats was randomly assigned to 1 of 4 groups using a random number list. Two groups [KHCO$_3$+/D$_3$+ (n=8); KHCO$_3$+/D$_3$- (n=10)] received AIN-93M plus KHCO$_3$ (0.4 mol/kg [23]) with a mineral mix containing either the standard vitamin D$_3$ supplementation (100,000 IU/kg, usual content in vitamin mix [22]) or no vitamin D$_3$, respectively (Table 1). The other two groups [KHCO$_3$-/D$_3$+ (n=8); KHCO$_3$-/D$_3$- (n=10)] received a vitamin D$_3$-replete or vitamin D$_3$-deficient AIN-93M diet with no added KHCO$_3$ (Table 1). All diets were purchased from Harlan Laboratories, Inc. All rats were fed the same measured amount of diet for a total study period of 12 weeks. A pair-feeding protocol allowed us to match the energy intakes of the 4 groups. The average daily intake in all groups over the 12 weeks was 18.8 ± 0.9 g/d. Sample size differences between the vitamin D$_3$-replete and -deficient groups were based on a decision to sacrifice two rats from each of the vitamin D$_3$-deficient groups mid-study (6 weeks) to confirm low serum 25OHD levels. At week 3 of the intervention, one rat in the KHCO$_3$+/D$_3$+ group was found to have a large abdominal mass and reduced feeding; therefore, this rat was sacrificed and not included in the study results. Given the early loss of one rat and the potential for additional unforeseen medical complications in these aged rats, we sacrificed one rat (rather than two) from the KHCO$_3$+/D$_3$- and KHCO$_3$-/D$_3$- groups to verify low serum 25OHD level (9.3 ng/ml and 5.9 ng/ml, respectively) at week 6.
At weeks 6 and 12, the rats were transferred to individual metabolic cages and a 24-h urine sample was collected. At 12 weeks, thirty-three remaining rats were fasted for 12 hours, anesthetized by isoflurane inhalation, and then sacrificed by terminal exsanguination. This technique provided a large amount of blood and minimized distress associated with hypovolemia prior to exsanguination while simultaneously sacrificing the animal. Blood was collected via tail artery puncture and frozen at -20 degrees C until analysis. Soleus (predominantly type I muscle fibers) and extensor digitorum longus (EDL; predominantly type II muscle fibers) muscles of the rat hind limb were excised and weighed. Muscles were cut at the mid-belly and then frozen in isopentane liquid nitrogen “slurry” and stored at -80 degrees C for later histological analysis. The study was approved by the Institutional Animal Care and Use Committee at Tufts University.

**Biochemical Measurements**

All samples were batched for analyses. Serum 25OHD was measured with RIA kits from Diasorin with coefficients of variation (CVs) of 5.6-7.7%. Serum IGF-1 and IGF-BP3 levels were measured by chemiluminescent immunoradiometric assays on an automated immunoassay system with CVs from 3-9%. Urinary creatinine was measured on an automated clinical chemistry analyzer with CVs from 3-6%. Urinary nitrogen was measured with a model FP-2000 nitrogen/protein determinator, which employs a Dumas combustion method and detection using a thermal conductivity cell with intra- and inter-assay CVs of 6.5% and 8.6%, respectively. Urine pH was measured using a pH meter with a resolution of 0.1/0.01 and relative accuracy ± 0.1/0.01.

**Histological Analysis**

As part of the immunohistochemical analysis, 7 µm cryostat cross-sections from frozen soleus and EDL muscles were incubated with a primary antibody against laminin to
facilitate identification and measurement of individual muscle fibers. The same cryosections were then incubated for myofibrillar ATPase after pre-incubation at pH 4.35 [24] to identify muscle fiber types I (slow-twitch) and II (fast-twitch). Slide preparations were analyzed under bright field and fluorescent microscopy and captured by a digital camera. With the aid of an image morphometry program (ImageJ 1.32j, NIH, Bethesda, MD), the outline of the individual fibers was traced. The fiber cross-sectional area (CSA) was calculated and expressed in µm². A total of 150 type I fibers from soleus muscle and 150 type II muscle fibers from EDL muscle were measured for CSA per rat. A sample size of 150 is considered on the upper range of sample sizes used in previous rat studies [25-26]. To reduce bias in the selection of fibers for measurement, fibers in the entire section were numbered and 150 were selected at random based on a web-based sequence generator (www.random.org). All area measurements were performed by two co-authors (BCL and LC) who were both blinded to group assignment. The coefficient of variation for the area measurement of individual fibers was under 3%.

Statistical Analysis

Sample size was based on a previous study investigating the effect of acidosis on urinary nitrogen excretion [27], and found that urinary nitrogen excretion was 16.5±0.8 mmol/day in 8 acidotic rats (urine pH 5.3) compared to 12.5±1.6 mmol/day in 8 control rats (urine pH 8) after 2 weeks. Assuming a pooled standard deviation of 1.2 mmol/day, 8 rats/group provided >90% power at the two-tailed 0.05 alpha level. Based on a prior study on the effect of growth hormone on muscle fiber diameter [28], 7 rats treated with growth hormone for 5 weeks had a type IIa muscle fiber diameter of 54±3 µm compared to 46±3 µm in 7 control rats. Assuming a pooled standard deviation of 3 µm, 8 rats/groups also provided >90% power at the two-tailed 0.05 alpha level.
One-way analysis of variance (ANOVA) with Tukey’s multiple comparison test was used to determine significant differences in baseline and dietary intervention characteristics of the rats in the four treatment groups. Homogeneity of variance was tested using the Levene test. Serum 25OHD level was the only variable that did not meet the homogeneity of variance assumption of the one-way ANOVA; therefore, a Kruskall-Wallis test was used. We did not adjust for multiple comparisons when evaluating differences in 25OHD level in the 4 groups, but performed pairwise comparisons using the Wilcoxon-Rank Sum test. P values of <0.05 were considered statistically significant.

Urinary nitrogen was normalized to creatinine to control for body size of the animal [29] and incomplete 24h urine collections [30]. Furthermore, type I and II muscle fiber CSAs were associated with soleus (r = 0.40, P = 0.02) and EDL (r = 0.44, P = 0.01) wet weights, respectively. Therefore, we analyzed type I CSA as a ratio to soleus muscle weight (CSA1/soleus) and type II CSA as a ratio to EDL muscle weight (CSA2/EDL).

The main effects of KHCO$_3$ and a vitamin D$_3$-deficient diet and their potential interaction on urinary nitrogen-to-creatinine ratio (UNi/Cr), CSA1/soleus, CSA2/EDL, soleus and EDL muscle wet weight, and serum IGF-1, and IGF-BP3 levels were analyzed by two-factor analysis of variance. P values of <0.05 were considered statistically significant. Statistical analyses were conducted with SAS 9.2.

Two-factor analyses of variance did not demonstrate a statistically significant interaction for any of the skeletal muscle endpoints; thus, the main effects of KHCO$_3$ and vitamin D$_3$-deficient diet were analyzed separately. Least-square means were used to describe the relationship between KHCO$_3$-supplemented and no KHCO$_3$ groups, adjusted for vitamin D$_3$-deficient and –replete groups. Likewise, least-square means were used to
describe the relationship between vitamin D₃-deficient and -replete groups, adjusted for KHCO₃ group. P values of <0.05 were considered statistically significant.

Results

Body Weight, Acid-Base Status, and Vitamin D Status

Mean baseline and final body weights did not differ significantly across the four groups (Table 2; P > 0.66). During the 12-week intervention, mean body weight fluctuated similarly across groups (data not shown). Supplementation with KHCO₃ resulted in significantly higher 24h urine pH after 6 and 12 weeks (all P < 0.01; Table 2). We did not detect any statistically significant differences in urine pH in rats on the vitamin D₃-deficient as compared to the vitamin D₃-replete diet (Table 2). However, at 12 weeks, 25OHD levels were significantly lower in the rats on vitamin D₃-deficient diet compared to those on the vitamin D₃-replete diet (all P < 0.01; Table 2).

KHCO₃ Supplementation

Analyses for the main effect of KHCO₃ supplementation were performed after adjustment for vitamin D₃ group. KHCO₃ supplementation resulted in significantly lower mean UNi/Cr ratio as compared to no KHCO₃ supplementation (P = 0.01, Table 3, Figure 1b). Rats on KHCO₃ supplementation also had a higher mean CSA1/soleus ratio than those who were not on KHCO₃ (P = 0.02; Table 3, Figure 1c). Mean soleus wet weight was lower in the KHCO₃-supplemented groups as compared to the non-KHCO₃-supplemented groups; however, this difference was reduced after correcting for final body weight (Table 3). Mean CSA2/EDL ratio did not differ significantly by KHCO₃ group (Table 3, Figure 1d). Similarly, KHCO₃ had no effect on mean EDL wet weight (Table...
Mean IGF-1 and IGF-BP3 levels were also not statistically significantly different in rats consuming KHCO₃ versus no KHCO₃ (Table 3).

### Vitamin D₃-Deficient Diet

The main effect of the vitamin D₃-deficient diet was examined following adjustment for KHCO₃ supplementation. The vitamin D₃-deficient diet did not significantly affect mean UNi/Cr ratio (P = 0.15, Table 4). Mean CSA1/soleus and CSA2/EDL ratios in rats on vitamin D₃-deficient diets did not differ significantly from those on vitamin D₃-replete diets (Table 4). There were also no significant differences in mean soleus and EDL wet weights (Table 4). Lastly, IGF-1 and IGF-BP3 levels were similar in the vitamin D-deficient and -supplemented groups (Table 4).

### Discussion

Our study demonstrated that a net alkali-producing dietary load due to KHCO₃ supplementation led to more than a 35% lower UNi/Cr ratio as compared to an acidogenic diet. This finding was independent of vitamin D status. Of note, our study rats were on identical protein and calorie intakes and had similar body weight and activity levels; thus, the lower UNi/Cr can be considered an indicator of reduced muscle breakdown. These results lend support to prior human studies in our laboratory indicating nitrogen sparing in older adults on KHCO₃ supplementation for 2-3 month periods [15-16]. In a 6 week study in 19 healthy older adults (average age 62 years), KHCO₃ supplementation attenuated a protein-induced rise in urinary nitrogen-to-creatinine excretion by over 50% compared to placebo [15]. A larger study in 162 older subjects (average age 62 years) given a bicarbonate supplement or no bicarbonate, also demonstrated a decline of 6% in nitrogen-to-creatinine excretion and a 13% increase in
lower extremity muscle power in the women over a 12-week period of supplementation [16].

Histological analyses of muscle specimens in sarcopenic individuals have typically shown atrophy predominantly in type II fibers versus the type I [7]. Yet, numerous interventions ranging from administration of growth hormone, testosterone, and protein supplementation to resistance exercise have resulted in hypertrophy of type I as well as type II muscle fibers [28, 31-33]. Following correction for soleus wet weight, we found that the KHCO$_3$-supplemented rats had 10% larger type I muscle fibers than those on an acid-producing diet after only 12 weeks. KHCO$_3$ supplementation, however, did not impact type II muscle fiber size corrected for EDL wet weight during this period. To our knowledge, there are no prior published data on KHCO$_3$’s effects on skeletal muscle fiber size. The reason for a selective effect of alkalinization on type I but not type II fibers is uncertain, but may be in part related to higher than expected variability in type II fiber CSA measurements or to the short 12-week intervention period. A larger and longer-term study is needed to fully characterize effects of this dietary intervention on muscle morphology.

The results of our study also suggest that a vitamin D$_3$-deficient diet, after adjustment for acid-base status, resulted in a near 25% increase in UNi/Cr as compared to a vitamin D$_3$-replete diet, but this difference was not large enough to reach statistical significance in this study. Vitamin D status was not predictive of either type I or II fiber size. These null findings may be partially attributed to the fact that the rats did not reach sufficiently low serum 25OHD levels (despite having declined by more than 50%) to affect muscle size. Alternatively, a 12-week vitamin D$_3$-deficient diet may not have been a long enough period to detect effects on muscle size. According to a prior report, rats with
undetectable 25OHD levels and concurrent hypocalcemia demonstrated clear signs of muscle degradation as measured by increased urinary methylhistidine excretion, a marker of myofibrillar breakdown [34]. In addition, case series of a proximal myopathy and atrophy of type II muscle fibers on a muscle biopsy specimen were reported in individuals with osteomalacia from profound vitamin D deficiency, not vitamin D insufficiency [4, 6, 17]. On the other hand, these published papers were, in fact, case reports, cross-sectional in terms of their observations between vitamin D status and muscle morphology, and uncontrolled in their design. Thus, these factors allow for the introduction of potential confounding factors and sampling biases, and our intervention study in the aged rat model does not support these findings. The distinction between profound deficiency, insufficiency, and sufficiency in vitamin D status is an area of active research and debate in older adults. The results of the present study raise the possibility that mild reductions in 25OHD status in the short-term may not have dramatic effects on muscle protein metabolism.

IGF-1, a protein growth factor, stimulates skeletal muscle protein synthesis and muscle fiber hypertrophy [35-37]. It was recently proposed that expression of its binding protein, IGF-BP3, is upregulated by 1,25-dihydroxyvitamin D [38]. Furthermore, metabolic acidosis in rats can decrease serum IGF-1 and IGF-BP3 levels [39]. In our study animals; however, KHCO$_3$ supplementation and vitamin D insufficiency did not significantly affect serum IGF-1 or IGF-BP3 levels.

Lastly, a 12-week vitamin D$_3$-deficient diet did not significantly lower urine pH in these aged rats, and we did not identify a significant interaction between the two dietary interventions on any of the muscle endpoints in this pilot study sample of rats. However, the potential relationship between vitamin D and acid-base balance may be worth
investigating further in a larger study with adequate statistical power to detect an interaction.

This study had some important strengths. We administered an adequate dose of KHCO$_3$ that effectively alkalinized the acid dietary load. The study achieved a moderate reduction in 25OHD level in the vitamin D$_3$-deficient dietary groups as planned. Our rats tolerated the pair-fed dietary interventions and maintained their weights fairly stable during the 12-week study period. Limitations of this study included that the design precluded baseline biochemical and muscle samples in all groups since it would involve a larger sample to sacrifice at baseline; the small sample size may have prevented us from detecting some meaningful interaction effects; the variability in type II muscle fiber CSA within and across animals in this study was larger than we had anticipated; and this being a pilot study with multiple endpoints being explored, additional research is needed to confirm these findings.

In conclusion, supplementation with KHCO$_3$ attenuated urinary nitrogen loss that accompanies a mildly acidic dietary load, suggesting that the net effect of KHCO$_3$ on skeletal muscle may be muscle protein-sparing in aging rats. KHCO$_3$ supplementation resulted in larger type I muscle fibers following adjustment for muscle wet weight, but no difference in type II muscle fiber size. Contrary to our expectations, a moderate reduction in vitamin D status did not result in higher urinary nitrogen loss nor did it affect muscle fiber size in this aging rat sample. Neither dietary intervention altered serum levels of IGF-1 or IGF-BP3. These findings provide further support for alkali supplementation as a promising therapeutic intervention to promote preservation of skeletal muscle mass in older adults. Larger and longer-term studies are needed to better define the mechanisms by which KHCO$_3$ supplementation reduces age-related
muscle wasting and to determine whether these muscle mass effects translate into improved physical function in an older adult population.
Table 1. Composition of the four dietary interventions

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>KHCO$<em>{3}$/D$</em>{3}^+$</th>
<th>KHCO$<em>{3}$/D$</em>{3}^-$</th>
<th>KHCO$<em>{3+}$/D$</em>{3}^+$</th>
<th>KHCO$<em>{3+}$/D$</em>{3}^-$</th>
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<tr>
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<tr>
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<tr>
<td>Vitamin mix (without D$_{3}$), g/kg</td>
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<td>Vitamin D$_{3}$, IU/kg</td>
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<td>KHCO$_{3}$, mol/d</td>
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</table>
Table 2. Body weight, biochemical and muscle parameters in the four dietary intervention groups

| Variable (units) | Study Week (weeks) | Acidic | | | Alkaline |
| | | KHCO₃/D₃+ Mean ± SD (n=8) | KHCO₃/D₃- Mean ± SD (n=9) | KHCO₃+/D₃+ Mean ± SD (n=7) | KHCO₃+/D₃- Mean ± SD (n=9) |
| Body weight (g) | 0 | 563 ± 51 | 560 ± 39<sup>a</sup> | 556 ± 48 | 562 ± 43<sup>a</sup> |
| | 12 | 572 ± 30 | 562 ± 33 | 555 ± 33 | 552 ± 44 |
| 24h urine pH | 6 | 6.39 ± 0.25<sup>a</sup> | 6.53 ± 0.31<sup>b</sup> | 8.39 ± 0.11 | 8.12 ± 0.71<sup>a</sup> |
| | 12 | 6.35 ± 0.65<sup>c</sup> | 6.22 ± 0.30<sup>c</sup> | 8.52 ± 0.27 | 8.29 ± 0.42 |
| 24h UNi/Cr (mg/mg) | 12 | 15.95 ± 10.40<sup>a</sup> | 22.25 ± 8.00<sup>a</sup> | 11.49 ± 5.09 | 12.82 ± 5.07 |
| IGF-1 (ng/ml) | 12 | 1216 ± 172 | 1107 ± 265 | 1124 ± 188 | 1088 ± 153 |
| IGF-BP3 (ng/ml) | 12 | 131 ± 21 | 117 ± 19 | 123 ± 21 | 129 ± 24 |
| CSA1/soleus (µm²/mg) | 12 | 113.43 ± 11.74 | 110.86 ± 15.51 | 125.16 ± 9.81 | 122.41 ± 16.18 |
| CSA2/EDL (µm²/mg) | 12 | 90.87 ± 12.26 | 93.52 ± 12.75 | 86.37 ± 9.95<sup>d</sup> | 93.09 ± 10.48 |
| Soleus wet weight (mg) | 12 | 198 ± 25 | 199 ± 26 | 171 ± 15 | 187 ± 22 |
| Soleus/body weight (mg/g) | 12 | 0.346 ± 0.037 | 0.353 ± 0.044 | 0.309 ± 0.019 | 0.340 ± 0.032 |
| EDL wet weight (mg) | 12 | 194 ± 13 | 189 ± 15 | 191 ± 12<sup>d</sup> | 187 ± 20 |
| EDL/body weight (mg/g) | 12 | 0.340 ± 0.027 | 0.337 ± 0.026 | 0.344 ± 0.011<sup>e</sup> | 0.341 ± 0.053 |
| Median [25<sup>th</sup>, 75<sup>th</sup> percentile] | | | | | |

<sup>a</sup> n= 10, this includes 1 rat which was later sacrificed at week 6 to confirm low 25OHD level
<sup>b</sup> Pairwise comparisons showing that groups without KHCO₃ differ from the KHCO₃-supplemented groups, with P<0.05
<sup>c</sup> Pairwise comparisons showing that groups without vitamin D₃ differ from the vitamin D₃-replete groups, with P<0.05
<sup>d</sup> n=6, one EDL muscle was not measured
Table 3. LS-mean [95% CI] of body weight, biochemical, and muscle parameters by KHCO₃ supplementation status adjusted for vitamin D₃ diet at 12 weeks

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Acidic KHCO₃- LS-Mean [95% CI] (n=17)</th>
<th>Alkaline KHCO₃+ LS-Mean [95% CI] (n=16)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>567 [549-585]</td>
<td>553 [535-572]</td>
<td>0.28</td>
</tr>
<tr>
<td>24h urine pH</td>
<td>6.28 [6.07-6.49]</td>
<td>8.39 [8.17-8.61]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UNi/Cr (mg/mg)</td>
<td>19.17 [15.46-22.89]</td>
<td>11.99 [8.16-15.83]</td>
<td>0.01</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>1160.9 [1062.6-1259.1]</td>
<td>1108.2 [1006.7-1209.8]</td>
<td>0.45</td>
</tr>
<tr>
<td>IGF-BP3 (ng/ml)</td>
<td>123.7 [112.9-134.4]</td>
<td>126.9 [115.7-138.0]</td>
<td>0.68</td>
</tr>
<tr>
<td>CSA1/soleus (µm²/mg)</td>
<td>112.14 [105.39-118.89]</td>
<td>123.78 [116.80-130.76]</td>
<td>0.02</td>
</tr>
<tr>
<td>CSA2/EDL (µm²/mg)</td>
<td>92.14 [86.49-97.79]</td>
<td>89.95 [83.88-96.02] *</td>
<td>0.59</td>
</tr>
<tr>
<td>Soleus wet weight (mg)</td>
<td>198 [187-210]</td>
<td>180 [168-191]</td>
<td>0.03</td>
</tr>
<tr>
<td>Soleus/body weight (mg/g)</td>
<td>0.349 [0.332-0.367]</td>
<td>0.325 [0.307-0.343]</td>
<td>0.05</td>
</tr>
<tr>
<td>EDL wet weight (mg)</td>
<td>192 [184-200]</td>
<td>189 [180-197]</td>
<td>0.59</td>
</tr>
<tr>
<td>EDL/body weight (mg/g)</td>
<td>0.339 [0.322-0.356]</td>
<td>0.343 [0.324-0.361] *</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*a n=15, one EDL muscle was not measured
Table 4. LS-mean [95% CI] of body weight, biochemical, and muscle parameters by vitamin D₃ diet adjusted for KHCO₃ supplementation at 12 weeks

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Vitamin D₃-Deficient Diet LS-Mean [95% CI] (n=17)</th>
<th>Vitamin D₃-Replete Diet LS-Mean [95% CI] (n=16)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>557 [540-574]</td>
<td>564 [545-583]</td>
<td>0.60</td>
</tr>
<tr>
<td>25OHD (ng/ml)</td>
<td>6.1 [4.9-7.2]</td>
<td>11.7 [10.5-13.0]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UNi/Cr (mg/mg)</td>
<td>17.54 [13.93-21.94]</td>
<td>13.63 [9.68-17.58]</td>
<td>0.15</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>1097.8 [1002.4-1193.2]</td>
<td>1171.3 [1066.7-1275.9]</td>
<td>0.30</td>
</tr>
<tr>
<td>IGF-BP3 (ng/ml)</td>
<td>122.9 [112.5-133.4]</td>
<td>127.6 [116.1-139.0]</td>
<td>0.55</td>
</tr>
<tr>
<td>CSA1/soleus (µm²/mg)</td>
<td>116.63 [110.08-123.19]</td>
<td>119.29 [112.10-126.48]</td>
<td>0.58</td>
</tr>
<tr>
<td>CSA2/EDL (µm²/mg)</td>
<td>93.31 [87.82-98.79]</td>
<td>88.78 [82.53-95.03]*a</td>
<td>0.28</td>
</tr>
<tr>
<td>Soleus wet weight (mg)</td>
<td>193 [182-204]</td>
<td>185 [173-197]</td>
<td>0.34</td>
</tr>
<tr>
<td>Soleus/body weight (mg/g)</td>
<td>0.347 [0.330-0.363]</td>
<td>0.328 [0.309-0.346]</td>
<td>0.14</td>
</tr>
<tr>
<td>EDL wet weight (mg)</td>
<td>188 [180-196]</td>
<td>193 [184-201]*a</td>
<td>0.41</td>
</tr>
<tr>
<td>EDL/body weight (mg/g)</td>
<td>0.339 [0.323-0.355]</td>
<td>0.342 [0.324-0.361]*a</td>
<td>0.79</td>
</tr>
</tbody>
</table>

*a n=15, one EDL muscle was not measured
Figure 1. a) Mean urine pH at 12 weeks by KHCO₃ group (P<0.01); b) Mean UNi/Cr ratio by KHCO₃ group (P=0.01); c) CSA1/soleus ratio by KHCO₃ group (P=0.02); d) CSA2/EDL ratio by KHCO₃ group (P=0.59)
Appendix A

Background

A) Sarcopenia

With aging, there is a loss of muscle mass and strength, also known as sarcopenia. Even in healthy older people, aging is accompanied by a decline in muscle mass at an average rate of 1.6 kg per decade for men and 0.6 kg for women [40]. The prevalence is about 25% in adults between the ages of 50 and 70 years, but increases to 40% in those 80 years or older [41]. Sarcopenia leads to an increased risk for falls, injuries, and loss of independence in performing daily activities [41]. These factors in turn predict physical disability and mortality [42]. The US population of persons aged 65 years and older is expected to nearly double in the next 25 years [43]. Sarcopenia is, therefore, a growing problem in this age group. Understanding the underlying mechanisms and determining broad-based interventions to slow or arrest this age-related muscle wasting are essential to reduce morbidity and mortality in the elderly and improve quality of life.

B) Skeletal Muscle Morphology in Sarcopenia

A skeletal muscle is composed of bundles (fascicles) of muscle fibers. Muscle fibers are each individual muscle cells. There are two principal ways to categorize muscle fibers: the type of myosin (motor protein of the muscle contractile element) present, and the degree of oxidative phosphorylation that the fiber undergoes. Skeletal muscle can thus be broken down into two broad categories: Type I and Type II. Type I fibers appear red due to the presence of the oxygen binding protein myoglobin. These fibers are suited for endurance and are slow to fatigue because they use oxidative metabolism to generate ATP. Type II fibers are white due to the absence of myoglobin and a reliance on glycolytic enzymes. These fibers are efficient for short bursts of speed and power and use both oxidative metabolism and anaerobic metabolism depending on the particular sub-type. These fibers are quicker to fatigue. Type II muscle fibers are also the first to be recruited to prevent a fall [44].
The decline in muscle mass with ageing is caused by muscle fiber atrophy and a loss of the number of fibers in a whole muscle. Needle biopsies of skeletal muscle have provided some insight into the histological findings associated with age-related muscle fiber atrophy. Studies show that average type II fiber size as measured by the cross-sectional area of a muscle fiber, diminishes with age, whereas the size of type I fibers is less affected [45-46]. These studies have also demonstrated lower numbers of muscle fibers in older versus younger individuals [47].

C) Animal Model of Sarcopenia

The Fischer 344/Brown Norway rat is a validated model of age-associated changes seen in human skeletal muscle [48]. It has been used to study muscle in aging rats in previous studies [49]. An older male Fischer 344/Brown Norway rat rather than a female, is also considered a model less affected by rapid hormonal fluctuations such as those seen in menopause. The sharp decline in estrogen level as occurs in menopause, is known to impact muscle mass and serum 25-hydroxyvitamin D level [25, 50]. Twenty-month old rats reflect an older-aged human.

D) Acid-base Balance – Potential Contributor to Sarcopenia

Aging results in a mild chronic metabolic acidosis [51] due to both a decline in renal function that limits the capacity to excrete hydrogen ions, and the composition of diets with respect to acid-base balance [12]. Protein and cereal grains are metabolized to acidic residues, mainly sulfuric acid, and fruit and vegetables are metabolized to alkaline residues, mainly potassium bicarbonate [52]. In general, American diets are acidogenic, generating 75–100 mEq acid/d [53]. In our laboratory, in a preparatory phase 1 pilot study (unpublished data), we found that 18-20-month-old, Fischer 344/Brown Norway, male rats on an AIN-93M rat diet, a maintenance casein-rich diet [22], resulted in a mildly acidic urine pH between 6 and 7.
Metabolic acidosis has been linked to muscle wasting in chronic renal failure [10] and in obese subjects who were acidic while following weight-loss diets [11]. In rats, inducing a metabolic acidosis promoted urinary nitrogen excretion, an indicator of muscle wasting [54]. In experimental animals, the proposed underlying mechanism is that metabolic acidosis stimulates muscle protein degradation pathways, which consist of enzymes that function to recognize and label proteins destined for degradation or function as proteases [55].

A few studies in human adults have shown that daily treatment with alkaline salts of potassium reduces urinary nitrogen excretion [15, 56-57]. A metabolic study in postmenopausal women on high protein, acidogenic diets demonstrated that a daily potassium bicarbonate supplement lowered nitrogen wasting, whereas discontinuation of the alkali therapy resulted in a rapid increase in urinary nitrogen excretion toward baseline [56]. In our laboratory, we found that neutralizing age-related acidosis with a daily potassium bicarbonate supplement decreased urinary nitrogen loss and improved muscle power in older women [57]. Effects of a net alkali-producing intake on muscle fiber composition and morphology are not known.

E) Vitamin D Status – Potential Contributor to Sarcopenia

Older adults are at risk of vitamin D insufficiency because of reduced production in the skin (decreased exposure and reduction in the skin’s ability to synthesize vitamin D [58]) and inadequate dietary intake [59]. Reports on the prevalence of vitamin D insufficiency have varied depending on serum 25-hydroxyvitamin D levels (25OHD) used to define it. The NHANES III survey estimated the prevalence of vitamin D insufficiency in US adults age 60 and older to be up to 30% in the winter/low latitude subpopulation and up to 26% in the summer/high latitude subpopulation [60]. The prevalence of vitamin D insufficiency has been reported to be present in 54% of homebound elderly Baltimore residents [61] and up to 86% in institutionalized women [62].
It has long been recognized that vitamin D affects muscle performance and risk of falls [5, 63-66]. Reports have described a link between osteomalacia and a vitamin D-reversible myopathy [4, 17, 67-68]. The presentation has been described as ranging from a proximal muscle weakness to a diffuse skeletal or muscle pain [5, 69]. Yet, more recent data indicate that milder reductions in vitamin D status, common in healthy older adults, are associated with muscle impairment and reduced physical function. The NHANES III survey reported a positive association between serum 25OHD levels and performance in the 8-foot walk and timed sit-to-stand tests (26). In the Longitudinal Study of Aging Amsterdam (LASA), lower 25OHD levels were associated with decreased grip strength and appendicular muscle mass in older adults (13). In the LASA study, low 25(OH)D levels (less than 10 ng/ml) were associated with an increased risk of repeated falling over the subsequent year, particularly in persons under 75 years of age [18]. A similar finding was shown in a large cohort study of older community-dwelling women where higher 25(OH)D levels were associated with a lower rate of falls over a 4 year period [70]. Other observational data in older adults have demonstrated similar results [71-74].

Histological analysis of muscle biopsy specimens in adults with profound vitamin D deficiency reveals a predominance of type II muscle fiber atrophy. These studies have been cross-sectional. To our knowledge, there are no longitudinal studies on the morphological changes in muscle following a reduction in vitamin D status.

There is incomplete understanding of how vitamin D acts on skeletal muscle. Thus far, several studies have reported on the potential role of the vitamin D receptor (VDR) which has been identified in myonuclei (9). Data in the VDR knockout mouse model (29, 30) suggest alterations in a transcription factors involved in muscle development and terminal differentiation of myofibers. Other studies in experimental animals suggest an additional mechanism whereby vitamin D may affect acid-base balance via regulation of renal bicarbonate reabsorption. These studies have shown that vitamin D deficiency results in a metabolic acidosis via renal bicarbonate wasting, whereas acute administration
of vitamin D results in a metabolic alkalosis by the reverse effect in the kidney [19-21]. Thus, given our knowledge of the effect of acidosis on skeletal muscle, this may potentially be an alternate pathway to muscle degradation from a vitamin D deficient state. Yet, no study to date has investigated whether altering the acid-base status with an alkaline salt may modulate the effect of vitamin D deficiency on skeletal muscle mass and metabolism.
Appendix B
Regression Diagnostics

The study aimed to determine the impact of an alkaline salt, potassium bicarbonate (KHCO$_3$), and a vitamin D$_3$-deficient diet, alone and in combination, on intermediate biomarkers of muscle protein metabolism and muscle fiber size in rats on acid-producing diets for 12 weeks. Thirty-six 20-month-old rats on acid-producing diets were randomly assigned in a 2 × 2 factorial design to receive either a KHCO$_3$-supplemented diet (with or without vitamin D$_3$), or no KHCO$_3$ (with or without vitamin D$_3$) and pair fed for 12 weeks. The main outcomes at 12 weeks were 24h urinary nitrogen-to-creatinine ratio (UNi/Cr), a ratio of type I muscle fiber cross-sectional area (CSA) to soleus weight (CSA1/soleus), and a ratio of type II fiber CSA to extensor digitorum longus muscle (CSA2/EDL).

We performed a two-factor analysis of variance. The study factors were the main effects of KHCO$_3$ versus no KHCO$_3$, vitamin D$_3$ versus no vitamin D$_3$, and the interaction between the two factors. There were no added covariates, because we utilized 1) 20-month-old, Fisher-344/Brown-Norway, male rats from the same colony, 2) a pair-feeding protocol, and 3) a randomized 2x2 factorial experimental design with identical conditions except the specific study dietary intervention. Interactions were not statistically significant based on p-value less than 0.05 and were not included in our final model.

Model Evaluation
A distribution analysis was performed on residuals to evaluate for normality assumptions. The data points were analyzed graphically using frequency histograms. As shown in Figure 1 below (based on model CSA1/soleus = KHCO$_3$ + vitamin D$_3$ deficient diet), the histogram suggests a normal distribution.
**Figure 1.** Histogram of residuals for CSA1/soleus ratio

The Normal quantile plot (Q-Q plot; **Figure 2**) was also analyzed to evaluate how the data compared against the expected normal quantile. As shown below, there was minimal deviation from the line.

**Figure 2.** Normal quantile plot for CSA1/soleus ratio
When analyzing diagnostics such as Cook’s D and DFFITS (Figures 3 and 4, respectively), we looked at plots for values that appeared much larger than the other values since cutoffs vary. When we found an extreme value which could be an outlier or influential point, we ran the model with and without that extreme observation. In all cases, results were similar so no values were removed from the final data set.

**Figure 3.** Cook’s D dot plot for CSA1/soleus ratio

![Cook's D Dot Plot for CSA1/soleus](image)

**Figure 4.** DFFITS dot plot for CSA1/soleus

![DFFITS Dot Plot for CSA1/soleus](image)
In addition, during analysis of rat body weights at 12 weeks, we noted that a different rat than the one described in the graphs above, had a low body weight and serum IGF-1 level compared to the other rats in the study. These findings suggested that this rat may have had an underlying sickness with poor food intake. We ran our analyses with and without this rat but we detected no differences in our data results. Therefore, no rats were excluded from the final analysis.

**Transformations**

We did not detect any skewness of residuals that required transformations of the outcome variables.

**Predictor variables**

The predictor variables, KHCO$_3$ and vitamin D$_3$ deficiency, were coded as indicator variables (0/1).
Appendix C – Poster on Human Pilot Study

This poster was presented at the CTSI Symposium in May 2010.
Effect of vitamin D₃ supplementation on muscle fiber size in older women

Lisa Ceglé1, Mauricio D. S. Morais2, Susan S. Harris1, Heike Bischoff-Ferrari1, Roger A. Fielding2, Bess Dawson-Hughes1

1Bone Metabolism Laboratory and 2Nutrition, Exercise Physiology and Sarcopenia Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA 2Center on Aging and Mobility, University of Zurich and Department of Rheumatology and Institute of Physical Medicine, University Hospital Zurich, Switzerland

Abstract

Histological analysis of muscle biopsies in adults with vitamin D deficiency reveals a predominance of type II (TII) muscle fiber atrophy. We sought to determine the impact of supplementation with vitamin D₃ in older vitamin D insufficient women affects the change in TII muscle fiber cross-sectional area (CSA) over 4 months. In this randomized, double-blind, placebo-controlled pilot study, 16 healthy women (65-85 yr) were assigned to receive vitamin D₃, 4000 IU or placebo for 4 months. Serum 25-hydroxyvitamin D (25(OH)D) level and a muscle biopsy of the vastus lateralis were performed at baseline and 4 months. Sessions were probed with type I, Ila, and Ix myosin heavy chain isoform-specific monoclonal antibodies (mAbs). Mean baseline 25(OH)D was similar in the two groups (placebo [n=9]: 19.2 ± 3.3 ng/ml; vitamin D [n=7]: 17.3 ± 4.4 ng/ml). As expected, mean final 25(OH)D differed in the two groups (placebo: 21.7 ± 7.3 ng/ml; vitamin D: 32.3 ± 9.2 ng/ml; P=0.006). Mean baseline TII muscle fiber CSA did not differ significantly in the 2 groups (placebo: 2332 ± 460 μm²; vitamin D: 2263 ± 391 μm²). However, there was a trend toward a greater percent (%) change in TII muscle fiber CSA with vitamin D₃ supplementation (placebo: -1.18%; vitamin D: 11% ± 12%; P=0.173). Although not statistically significant, a positive linear association was observed between change in 25(OH)D and % change in TII muscle fiber CSA (r²=0.353, P=0.171). These findings are consistent with a potential beneficial effect of vitamin D₃ on TII muscle fiber size. A larger study sample is needed to definitely establish an increase in TII fiber area with vitamin D supplementation.

Introduction

There has been growing evidence that vitamin D plays an important role in skeletal muscle. Early clinical descriptions of a reversible myopathy associated with profound vitamin D deficiency recognized a potential association between vitamin D and muscle. More recent studies have reported reduced muscle mass, strength, and performance and an increased risk of falls in older individuals with milder decreases in serum 25-hydroxyvitamin D (25(OH)D) levels, termed vitamin D insufficiency. Histological analyses of muscle biopsies in adults with profound vitamin D deficiency, reveals a predominance of type II (TII)-fast twitch muscle fibers. This pattern of fiber loss may help explain the clinical reports of higher falling tendency in vitamin D deficient older individuals.

It has been hypothesized that individuals with low vitamin D status who are placed on supplementation with vitamin D improve muscle strength and performance in part by an increase in muscle mass. To date, there is inadequate evidence from well-designed trials to support this hypothesis.

Objectives

We sought to determine whether supplementation with vitamin D₃ in older vitamin D insufficient women affects the change in TII muscle fiber cross-sectional area (CSA) over 4 months.

Methods

Study Design and Subjects. • Double-blind, 4-month randomized controlled pilot study. • Sixteen women, age 65 and older. 25(OH)D levels <24 ng/ml • Randomized to vitamin D₃ 4000 IU or placebo daily. • Blood and 24 hour urine measured on day 1 and 113. • Muscle biopsies on day 1 and 113. • Safety random spot urine for calcium and creatinine on day 30. • Nutrient intake was assessed on day 1 and 113 with validated Fred Hutchinson food frequency questionnaire. • All 16 subjects completed the study.

Study pills. • Vitamin D₃ 4000 IU or placebo tablet daily with breakfast. • Study pills were made by Tishcon Corporation in Westbury, NY.

Muscle biopsy. • Obtained from the vastus lateralis at the level of the mid-thigh using a 5mm Duchenne biopsy needle (Bergstrom) and suction.

Immunohistochemistry. • 7 μm sections were cut at -23 °C with a cryostat microtome. • Samples were washed with PBS for 5 min, fixed in 3% neutral buffered formalin, and then blocked in 0.25% goat serum. • Slides were probed for 90min (37°C) with a primary mAb to identify type I (A483), type Ila (N261), and type Ix (121F) muscle fibers. • A rabbit polyclonal antibody raised against laminin was used to facilitate identifying individual muscle fibers. • Alexa Fluor® secondary antibodies were used for detection. • Digital imaging was performed through 100X and/or 400X magnification.

Biochemical Measurements. Serum 25(OH)D was measured with Diasorin RIA kits and serum PTH by immunoradiometric assay (Nichols Institute Diagnostics). Coefficients of variation (CV) of these assays ranged from 5.6-7.7%. Serum calcium was measured by Cobas Mira centrifugal analyzer (Roche Instruments) and 24-hr urinary calcium on a Nova Calibration chemistry analyzer (Nova Biochemical) with CV of <3%. Urinary creatinine was measured on an automated clinical chemistry analyzer of Olympus AU4000, Olympus America Inc.) with CVs of 3.0-6.5%. Urinary nitrogen was measured with a model FP-2000 nitrogen/protein determinator (LECO), which employs a Dumas combustion method and detection using a thermal conductivity cell (precision of 15 ppm).

Results

• Mean baseline clinical characteristics did not differ significantly in the two groups (Table 1).
• Mean baseline 25(OH)D was similar in the two groups (Table 2). • As expected, mean final 25(OH)D differed in the two groups (P=0.006; Table 2). • Mean baseline TII muscle fiber CSA did not differ significantly in the 2 groups (Table 3).
• There was a trend toward a greater percent (%) change in TII muscle fiber CSA with vitamin D₃ supplementation (P=0.173; Table 3).
• Although not statistically significant, a positive linear association was noted between change in 25(OH)D and % change in TII muscle fiber CSA (r²=0.353, P=0.171; Figure 1).

Table 1. Baseline clinical characteristics by group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>Vitamin D</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.1 ± 2.6</td>
<td>76.9 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.0 ± 3.2</td>
<td>29.4 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>875 ± 246</td>
<td>834 ± 688</td>
<td></td>
</tr>
<tr>
<td>Protein intake (g/d)</td>
<td>52.4 ± 24.0</td>
<td>62.9 ± 34.2</td>
<td></td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>58.1 ± 15.2</td>
<td>53.7 ± 28.9</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg/d)</td>
<td>9.5 ± 0.5</td>
<td>9.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>24hr Urine Ca/Cr (mg/g)</td>
<td>117 ± 40</td>
<td>138 ± 85</td>
<td></td>
</tr>
<tr>
<td>24hr Urine Nitrogen (g)</td>
<td>8.0 ± 1.5</td>
<td>8.1 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>GFR</td>
<td>8.4 ± 0.9</td>
<td>8.3 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Serum 25(OH)D levels at baseline, final, and percent change (final – baseline)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo Mean ± SD</th>
<th>Vitamin D Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline 25OHD</td>
<td>19.2 ± 3.3 ng/ml</td>
<td>17.3 ± 4.4 ng/ml</td>
<td>0.323</td>
</tr>
<tr>
<td>Final 25OHD</td>
<td>21.7 ± 7.3 ng/ml</td>
<td>32.3 ± 5.2 ng/ml</td>
<td>0.006</td>
</tr>
<tr>
<td>A 25OHD(final – baseline)</td>
<td>2.4 ± 5.0 ng/ml</td>
<td>15.0 ± 6.1 ng/ml</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3. Muscle fiber CSA at baseline, final, and percent change ([final-baseline]/baseline x 100) in the two groups

<table>
<thead>
<tr>
<th>Type II muscle fiber CSA (μm²)</th>
<th>Baseline</th>
<th>Final</th>
<th>Percent Change (%)</th>
<th>Placebo Mean ± SD</th>
<th>Vitamin D Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2323 ± 460</td>
<td>2263 ± 391</td>
<td>1.6%</td>
<td>0.765</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2220 ± 529</td>
<td>2166 ± 515</td>
<td>-2.2%</td>
<td>0.210</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1 ± 18</td>
<td>-11 ± 19</td>
<td>-10%</td>
<td>0.173</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conclusions

• On average, TII muscle fiber cross-sectional area increased by 11% with vitamin D₃ supplementation over 4 months; however, this was not statistically significant at the 0.05 level. • In the sample as a whole, change in 25(OH)D predicted the percent change in TII muscle fiber cross-sectional area, though this was not statistically significant at the 0.05 level. • Those findings are consistent with a potential beneficial effect of vitamin D on TII muscle fiber size. • A larger study sample is needed to definitively establish an increase in TII fiber CSA area with vitamin D supplementation.

Acknowledgement

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Appendix D – Published Manuscript on the Vitamin D Receptor


Multi-step immunofluorescent analysis of vitamin D receptor loci and myosin heavy chain isoforms in human skeletal muscle

Short title: VDR localization by immunohistochemistry

Lisa Ceglia¹,², Mauricio da S. Morais³, Lara K. Park², Evan Morris³, Susan S. Harris², Heike Bischoff-Ferrari¹,², Roger A. Fielding³, Bess Dawson-Hughes¹,²

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ABSTRACT

Vitamin D receptors have been shown to be present in human skeletal muscle using different techniques. We developed a multi-staining immunofluorescent method to detect vitamin D receptor expression and co-localize it with myosin heavy chain isoform expression in skeletal muscle biopsies in older female subjects. Serial sections were cut from frozen samples obtained by needle biopsy of the vastus lateralis. Samples were probed with a primary vitamin D receptor monoclonal antibody and then re-probed with a type IIa myosin heavy chain isoform-specific antibody. Independent unfixed sections followed a similar protocol and were probed with type IIx and type I myosin heavy chain isoform-specific antibodies. Immunohistochemistry and fluorescent microscopy co-localized vitamin D receptor loci and myosin heavy chain isoforms in whole skeletal muscle sections. We quantified intranuclear vitamin D receptor staining patterns and number of individual muscle fiber subtypes within a muscle section. Immunohistochemical staining of the vitamin D receptor was confirmed by Western blot using the same monoclonal antibody. This multi-staining immunofluorescent technique allows for measurement of intranuclear vitamin D receptor expression in the context of the specific muscle fiber type profile in a single section. This method can thus be a useful approach to study potential relationships between muscle fiber subtypes and vitamin D receptor expression.
INTRODUCTION

Recent data suggest that vitamin D supplementation reduces the risk of falls (Bischoff-Ferrari et al. 2009) and improves measures of muscle performance (Pfeifer et al. 2000; Bischoff et al. 2003; Pfeifer et al. 2008) in older adults with low vitamin D status. Muscle biopsies in adults with profound vitamin D deficiency reveal atrophy of type II (fast-twitch) muscle fibers, which are the first fibers to be recruited when preventing a fall (McComas 1996). While the actions of vitamin D on skeletal muscle are not well-understood, current research suggests that the effects of vitamin D may, at least in part, be mediated through the vitamin D receptor (VDR). The VDR is a ligand-activated transcription factor and a member of the superfamily of nuclear receptors for steroid hormones (DeLuca 1988). The active form of vitamin D, 1α,25-dihydroxyvitamin D [1,25(OH)₂D], is believed to bind to a nuclear VDR which, in turn, modulates the expression of genes related to the regulation of calcium transport and cell proliferation and differentiation (Boland et al. 1985; Simpson et al. 1985). The nuclear VDR has been identified in human skeletal muscle tissue using different techniques (Costa et al. 1986; Bischoff et al. 2001). Prior published localization techniques, however, have not had the capability of studying potential relationships between degree of intranuclear receptor expression and muscle fiber subtype.

The aim of this study was to co-localize VDR-positive myonuclei and specific skeletal muscle fiber subtypes within a human skeletal muscle section. To accomplish this aim, we developed a multiple immunofluorescent staining technique that identifies the VDR and muscle fiber subtypes by incubating a muscle section with a monoclonal antibody to the VDR and then myosin heavy chain (MHC) isof orm-specific antibodies directly labeled with distinct fluorophores. The intranuclear VDR signal detected using a monoclonal antibody was verified by Western blot and compared to two alternative primary VDR antibodies.

METHODS

Subjects
Eight muscle biopsy specimens used to develop the method were obtained from four healthy postmenopausal female participants, age 65 to 85. The Tufts Medical Center-Tufts University Health Sciences Campus Institutional Review Board approved the study, and written informed consent was obtained from each participant.

Muscle biopsy
Eight muscle biopsies were obtained from the vastus lateralis at the level of the mid-thigh under local anesthesia (xylocaine 1%) with a 5-mm Duchenne biopsy needle and suction (Bergström, 1975). The specimens were mounted in a vinyl cryomold (Tissue-Tek, USA) and secured using a viscous mounting medium (O.C.T., Tissue-Tek, USA) and then frozen in isopentane/liquid nitrogen slurry.

Immunohistochemistry
Eight muscle tissue specimens were cut in seven μm sections at -23°C with a cryostat microtome (Leica CM1850, Leica Microsystems, Germany), placed onto microscope slides, and left to air-dry at room temperature for at least 15 min. Samples were washed with phosphate buffered saline (PBS) for 5 min, fixed in 3% neutral buffered formalin for 10 min, and then blocked in PBS/2% goat serum for 20 min. Slides were probed overnight (4°C) with a primary mouse/anti-human VDR/NR1I1 monoclonal antibody
(clone H4537, Perseus Proteomics, Inc.; Table 1). Subsequently, sections were probed with goat/anti-mouse IgG 
2A Alexa Fluor-568® secondary antibody conjugate (Table 1). Samples that were probed with the VDR antibody, were then re-probed with a type Ila (N2.261 IgG1) MHC isoform-specific antibody (Table 1). Unfixed sections followed a similar protocol to identify other muscle fiber subtypes. Mouse antibodies against human type I (A4.951 IgG1; A4.840 IgM) and IIx (212F IgG1) MHC were used (Table 1). A rabbit anti-human antibody (IgG) raised against laminin was used to facilitate identifying individual muscle fibers. Specific goat anti-mouse and anti-rabbit Alexa Fluor® secondary antibodies were used for detection of MHC and laminin primary antibodies, respectively (Table 1). Slides were mounted with 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium to stain myonuclei (Vector Laboratories, Inc). Control sections were processed independently as described above, without primary, secondary or both antibodies (blank control). No staining signal higher than the natural self-fluorescence of the sections was observed (results not shown).

Digital imaging was performed through 100x and/or 400x final magnification. Adobe Photoshop® CS3, Nikon NIS-AR (3.01) and NIH Image J software (1.37v) were employed for data acquisition and data analysis.

**Immunoblotting**

To confirm VDR expression, three samples were prepared by homogenization in lysis buffer (50 mM Tris HCl pH 7.5, 1mM EDTA, 1mM EGTA, 10% glycerol (v/v), 1% Triton-X (v/v), 50mM NaF, 5mM Na Pyrophosphate) and then by centrifugation at 10,000rpm/4°C. Supernatants were stored at –80°C. Total protein concentrations of muscle extracts were measured using the Pierce 660nm protein assay (Thermo Fisher Scientific). To detect VDR, 30 µg of whole cell muscle lysates were loaded onto and resolved by SDS-PAGE using 10% mini-gels (150V/60 min with the BioRad Mini-Protean Tetra Cell System) and electrophoretically transferred (350 mA /120 min) to 0.45 µm nitrocellulose membranes. Five µg of 293T lysate (Santa Cruz Biotechnology, Inc.) were used as control. Membranes were blocked in 5% non-fat dry milk (NFM)/Tris-Buffered Saline-0.05% Tween 20 (TBS-T) solution, washed with 0.05% TBS-T, and probed with three different commercially-available primary antibodies to the VDR (VDR/NR1I1 monoclonal antibody, clone H4537 [Perseus Proteomics, Inc.]; VDR D-6 monoclonal antibody [sc-13133 Santa Cruz Biotechnology, Inc.] and VDR 333C6a [sc-81423 Santa Cruz Biotechnology, Inc.]) in 5% BSA/TBS-T (diluted 1:1000) overnight. Membranes were then washed with 0.05% TBS-T and incubated with different HRP-conjugated secondary antibodies in 1%NFM/TBS-T (diluted 1:2000) solution for detection using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

**RESULTS**

Immunohistochemistry and fluorescent microscopy co-localized VDR and MHC isoforms in whole skeletal muscle sections. Figure 1 illustrates incubation of muscle cryosections with directly labeled antibodies to types I, Ila, and IIx MHC isoforms and laminin. The emission wavelength of each different secondary antibody (Table 1) employed on the MHC isoform staining allows simultaneous identification of the muscle fiber subtypes in two muscle sections overlayed to create a single multi-colored image (Figure 1). We measured the relative number of individual muscle fiber subtypes within a cryosection, which in the Figure 1 section revealed a predominance of type II fibers (Table 2).
In each sample, we randomly selected ten higher magnification (400x) fields, which were representative of the whole section. In Figure 1, the ten fields comprised 46% of the muscle fibers in the whole section and had a similar type I to type II muscle fiber type ratio (32% to 68%) to that of the whole section (30% to 70%). The 400x images were captured using fluorescent (Figure 2a/b) and bright field microscopy (Figure 2c). Figure 2a shows DAPI staining. Figure 2b illustrates co-localization of VDR-positive nuclei with DAPI. Bright field images (Figure 2c) were used to assign the myonuclei to a particular fiber. We measured the relative number of VDR-positive myonuclei within each muscle fiber subtype in all 10 fields. Table 3 lists the ratio of VDR-positive myonuclei to total myonuclei by muscle fiber subtype from 10 fields from Figure 1. Of note, VDR immunohistochemical staining was not solely associated with clearly identified intranuclear staining as shown in Figure 2b (arrows).

To confirm identification of VDR by immunohistochemical staining in these cryosections, we performed Western blot analysis using the same VDR monoclonal antibody (VDR/NR1I1). The immunoblot was run alongside two other commercially-available primary antibodies to the VDR (D-6 and 333C6a) to confirm the accuracy of the VDR antibody used in the immunohistochemical stain (Figure 3). The molecular weight band at approximately 50 kDa is consistent across all three lanes incubated with a VDR antibody (Figure 3).

DISCUSSION

This study describes a multiple immunofluorescent staining technique which co-localizes the VDR and muscle fiber subtypes in a single human skeletal muscle section. Similar to a prior study in human skeletal muscle specimens (Bischoff et al. 2001), we identified VDR myonuclear staining using a human monoclonal antibody to the VDR. Unlike prior studies, however, our technique provides information on VDR expression in the context of muscle fiber subtype and its distribution. Using type I, IIa and IIx MHC isoform-specific antibodies directly labeled with distinct fluorophores, we were able to identify hybrid fibers which are of particular interest when studying factors such as vitamin D supplementation that may have effects on muscle morphology (Sorensen et al. 1979; Boland 2005; Sato et al. 2005). Our technique could be utilized in future studies to provide information on whether the VDR has differential expression based on muscle fiber subtype. Although we cannot generalize on a pattern of VDR expression by fiber subtype in this study, a more extensive sampling of muscle specimens in a larger study would allow for such an analysis. If VDR has a direct role on muscle as proposed, this knowledge may help to explain the morphological changes noted in vitamin D deficiency (Boland 2005) and in vitamin D repletion (Sorensen et al. 1979; Sato et al. 2005) demonstrating a potentially selective effect on type II muscle fibers.

Notably, our technique stained peripheral areas of the muscle fiber that did not appear connected with a myonucleus within these 7 µm sections. This preliminary finding will need to be explored further to determine whether this signal may be representative of the putative membrane-associated VDR believed to activate rapid, non-genomic, second messenger intracellular signaling cascades that influence muscle intracellular calcium regulation, muscle contractility and myogenesis (Boland 2005). Recent animal studies indentified VDR in isolated membrane fractions of both chick intestinal cells and chick embryonic skeletal muscle cells (Capiati et al. 2002; Huhtakangas et al. 2004).
The strength of this method is that, by incubating a single muscle section with different specific primary antibodies to different MHC isoforms, it successfully reduces the amount of muscle tissue, reagents, and time needed to perform these analyses. A limitation in this study was that we did not verify the VDR signal in muscle using an inhibitor or knockout model; however, we confirmed VDR expression in muscle sections by Western blot analysis using the same antibody and compared it to other antibodies to further corroborate our findings.

In summary, this technique co-localizes VDR using a human monoclonal antibody and type I, IIa, and IIx muscle fibers using specific antibodies to MHC isoforms in a sample obtained from a human skeletal muscle biopsy. The method identifies and quantifies VDR-positive myonuclei in skeletal muscle, identifies specific MHC isoforms and VDR positive myonuclei within individual muscle fiber subtypes, and identifies peripheral VDR staining patterns that need further investigation. Use of this technique in a large sample of muscle specimens would provide patterns of VDR expression and its associations with MHC isoforms in human muscle.

ACKNOWLEDGEMENT

The A4.951, A4.840, and N2.261 monoclonal antibodies developed by H. M. Blau were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The 212F mAb was produced in-house from hybridomas generously donated by Dr. Peter Merrifield, University of Ontario, Canada. We are grateful to the staff of the Metabolic Research Unit at the Jean Mayer USDA HNRCA at Tufts University for assistance in carrying out on this study. LC is supported by NIH KL2 RR025751. The study was funded by the Jean Mayer USDA HNRCA at Tufts University and P30 AG031679. None of the authors had any conflicts of interest.
Legends for Figures

Figure 1. Composite image (100x) of multi-immunofluorescent staining of MHC isoforms in a human skeletal muscle tissue section. Colorization patterns are: *dark green* = type I, *green/blue* = hybrid I/IIa, *blue* = type IIa, *red* = type IIx, *purple* = hybrid IIax, and *light green* = laminin.

Figure 2. VDR-positive myonuclei in skeletal muscle section at 400x magnification showing: (A) DAPI staining myonuclei; (B) co-localization of VDR-positive myonuclei with DAPI; (C) bright field microscopy.

Figure 3. VDR expression in whole cell lysate of human skeletal muscle is shown using three commercially available primary antibodies. From left to right are VDR (D-6), VDR (333C6a) and VDR (NR1I1). VDR expression was verified by use of human VDR transfected 293T cell lysate from Santa Cruz Biotechnology, Inc.
REFERENCES


Table 1. List of primary and secondary antibodies used in immunohistochemistry and immunoblotting

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<th>Primaries</th>
<th>Antibody</th>
<th>Host</th>
<th>Reactivity</th>
<th>Clone</th>
<th>Dilution</th>
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<td>IgG2A</td>
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<td>(WB)</td>
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<td>1:333</td>
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<td>Produced in-house**</td>
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<td>IgG</td>
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Abbreviations in Table: VDR, Vitamin D receptor; IH, immunohistochemistry; WB, Western blot; MHC, myosin heavy chain; DBHB, Developmental Studies Hybridoma Bank

** From hybridomas generously donated by Dr. Peter Merrifield, University of Ontario, Canada.
Table 2. Muscle fiber type profile of Figure 1

<table>
<thead>
<tr>
<th>Muscle Fiber Type</th>
<th>Number of Fibers</th>
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<tr>
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<td>Type IIX</td>
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<tr>
<td>ALL TYPE I&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>ALL TYPE II&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>TOTAL</td>
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<td>100</td>
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<sup>a</sup> Type I fibers consist of type I and hybrid I-IIa.

<sup>b</sup> Type II fibers consist of type IIa, type IIx and hybrid IIa-IIx.
Table 3. *Relative number of VDR-positive myonuclei in 10 randomly selected 400x fields*

<table>
<thead>
<tr>
<th>Muscle Fiber Type</th>
<th>Number of VDR-Positive Nuclei</th>
<th>Total Number of Nuclei</th>
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<td><strong>ALL TYPE I</strong>a</td>
<td>179</td>
<td>192</td>
<td>0.93</td>
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<tr>
<td><strong>ALL TYPE II</strong>b</td>
<td>370</td>
<td>433</td>
<td>0.86</td>
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<tr>
<td><strong>TOTAL</strong></td>
<td>549</td>
<td>625</td>
<td>0.88</td>
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*a* Type I fibers consist of type I and hybrid I-IIa.

*b* Type II fibers consist of type IIa, type IIx and hybrid IIa-IIx.
50KDa →

Santa Cruz VDR (D-6)
Santa Cruz VDR (333C6a)
Perseus Proteomics VDR NR11

Whole Cell Human Muscle Lysate
Whole Cell Human Muscle Lysate
Whole Cell Human Muscle Lysate
Santa Cruz VDR 293T Lysate

VDR (50-55KDa)
Bibliography