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Chapter 2

Effects of alfacalcidol on the contractile properties of the Gastrocnemius medialis muscle in adult and old rats

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Abstract

Background
Vitamin D deficiency is associated with muscle weakness. It is unknown, however, how supra-physiological levels of vitamin D affect skeletal muscle.

Methods
To investigate the effects of increased serum vitamin D (1,25(OH)₂D₃ or 1,25D) levels on the contractile properties of the medial gastrocnemius muscle, adult and old female Fischer 344 x Brown Norway F1 rats were orally treated with vehicle or the vitamin D analogue alfacalcidol for 1 or 6 weeks.

Results
Alfacalcidol treatment resulted in elevated 1,25D serum levels. This was accompanied by hypercalcaemia and a reduction in body mass, the latter largely attributable to a reduced food intake. However, kidney function, as reflected by normal creatinine serum levels, as well as heart mass were unaffected. The 17% reduction in maximal isometric force and power was explicable by a similar loss of muscle mass. The force-frequency relation of the 6-week-treated old rats was shifted to the left, but neither the shape of the force-velocity relation nor the fatigability of the muscle were altered.

Conclusion
Supra-physiological doses of vitamin D were accompanied by significant reductions in body and muscle mass, but no improvement in muscle functioning. Weight loss was largely due to a reduced food intake. Although undesirable, this and the maintained kidney function, heart mass and quality of the remaining muscle tissue suggests that quick normalisation of circulating vitamin D would reverse these effects on body and muscle mass. Furthermore, food intake and body mass should be carefully monitored when treating people with vitamin D analogues.

Key words: vitamin D, alfacalcidol, muscle contractile properties, skeletal muscle, fatigue
Introduction
The active form of vitamin D, 1α,25(OH)2D3 (1,25D) elicits cellular responses through both genomic and non-genomic actions (Ceglia 2008). Vitamin D has been shown to be important in phosphate and calcium homeostasis (DeLuca 1988) acting on the intestine (Van Cromphaut, Dewerchin et al. 2001), kidney (Liu, Yu et al. 1998), parathyroid gland (Fraser 2009) and bone (Panda, Miao et al. 2004). It exerts its effects through the vitamin D receptor (VDR) and the discovery of the VDR in muscle tissue (Zanello, Collins et al. 1997; Bischoff, Borchers et al. 2001) suggests that vitamin D will also act on muscle tissue. Indeed, the deregulated expression of myoregulatory transcription factors and abnormal muscle development in VDR knock-out mice demonstrates the importance of vitamin D in skeletal muscle development (Endo, Inoue et al. 2003). This suggests that vitamin D deficiency could have important consequences for muscle metabolism and functioning. Indeed, vitamin D supplementation during vitamin D deficiency has been shown to improve musculoskeletal function in institutionalized elderly by 4-11% within 12 weeks (Bischoff, Stahelin et al. 2003).

It has been found in human and mouse primary hepatocyte cultures that a concentration of 100 nmol·L⁻¹ vitamin D elicits maximal activation of the VDR (Reschly, Bainy et al. 2007). The serum vitamin D levels in rats, around 185 pmol·L⁻¹ (Anderson, Sawyer et al. 2007), are far below this level. Although it is known that vitamin D supplementation during vitamin D deficiency enhances muscle function, it remains to be established what the impact on muscle function is when circulating vitamin D levels are elevated in non-deficient adult and old rats. To do so, in this study alfacalcidol, which is a synthetic calcitriol analogue, is administered to adult and old rats. We used old rats, as many responses to e.g. electrical stimulation (Walters, Sweeney et al. 1991), overload (Degens and Alway 2003) and disuse (Alway, Degens et al. 2003; Degens and Alway 2003) are reduced. This might be due to reduced circulating levels of vitamin D. The aims of this study were to determine the effects of increased serum 1,25D on muscle contractile properties in old and adult rats. We hypothesized that old rats are vitamin D deficient and that alfacalcidol treatment would reverse the decline in contractile properties in old rats.
Material and Methods

Animals

Female Fischer x Brown Norway F1 rats were obtained from Harlan (USA) (n = 52). This strain of rats is recommended by the National Institute of Ageing as the strain of choice for the study of ageing processes as it suffers less than other strains from co-morbidities (Lipman, Chrisp et al. 1996). Rats were housed one to a cage at a 12:12 light dark cycle with food and standard laboratory chow provided ad libitum. The rats were 7-, 27.5- or 29-month old at the end of the treatment period and divided randomly in 1-week, 6-week, or vehicle treated groups (Table 2.1). Rats were orally administered either vehicle or Alfacalcidol (0.1 g·kg⁻¹) (Chugai Pharmaceutical, Japan) daily for 1 week, to study short term effects, or for 5 days during 6 weeks to study long term effects. This dose has been shown to inhibit bone resorption and enhance bone formation in ovariectomized rats treated for 5 weeks (Shiraishi, Takeda et al. 2000). Rats were weighed before vehicle and alfacalcidol administration to determine the dose. Food and water consumption were monitored in the 6-week treatment groups. At the end of the treatment period the contractile properties of the medial gastrocnemius muscle (Gm) were determined. All experiments were approved by the local ethic committee of the VU University Amsterdam and conform to the Dutch Research Council’s guide for care and use of laboratory animals. Rats were anaesthetized by an initial dose of urethane (0.75 g·kg⁻¹ i.p). After 10 minutes an additional dose of 0.75 g·kg⁻¹ urethane was given. If the rats still responded to nociceptive stimuli, supplementary injections of 0.63 g·kg⁻¹ were applied during the experiment. The Gm of the right leg was dissected while keeping the proximal origin and the blood supply intact. The femur was fixed and the distal tendon, with a small part of the calcaneus, connected to a force transducer. Length changes of the Gm were controlled by a servomotor connected to the lever arm to which the force transducer was mounted. The sciatic nerve was cut and contractions induced by supramaximal electrical stimulation (1 mA, pulse width 200 μs), defined as the current above which the twitch force did not increase further. Subsequently, the muscle was set at optimal length (Lₒ), defined as the length at which the active twitch force was maximal, with a series of twitch contractions (1 per minute). Then Lₒ was fine-adjusted with several tetanic contractions (150 Hz, 150 ms). Muscle temperature was maintained at 34-36 °C with a water-saturated airflow around the muscle, which also kept the muscle moistened. Stimulation and length changes were computer controlled. Force and length signals were digitized using an AD-converter at a sampling rate of 10 kHz. At the end of the measurements the Gm was excised, weighed, stretched to Lₒ on cork and frozen in liquid nitrogen.
Table 2.1: group arrangement, number of animals in each group, treatment and age of the rats used in this experiment.

<table>
<thead>
<tr>
<th>group</th>
<th># animals</th>
<th>treatment</th>
<th>Age (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>9</td>
<td>none</td>
<td>7</td>
</tr>
<tr>
<td>A1WA</td>
<td>6</td>
<td>1 week Alfacalcidol (0.1 g.kg(^{-1}) BW)</td>
<td>7</td>
</tr>
<tr>
<td>A6WO</td>
<td>9</td>
<td>6 weeks Alfacalcidol (0.1 g (in 1 ml).kg(^{-1}) BW)</td>
<td>27.5</td>
</tr>
<tr>
<td>V6WO</td>
<td>9</td>
<td>6 weeks Vehicle (1 ml. kg(^{-1}) BW)</td>
<td>27.5</td>
</tr>
<tr>
<td>A1WO</td>
<td>9</td>
<td>1 week Alfacalcidol (0.1 g.kg(^{-1}) BW)</td>
<td>29</td>
</tr>
<tr>
<td>V1WO</td>
<td>9</td>
<td>1 week Vehicle (1 ml. kg(^{-1}) BW)</td>
<td>29</td>
</tr>
</tbody>
</table>

CA: control adult; A1WA: (alfacalcidol, 1 week, adult), adult rats treated with alfacalcidol for 1 week; A6WO: (alfacalcidol, 6 weeks, old), old rats treated with alfacalcidol for 6 weeks; V6WO: (vehicle, 6 weeks, old), old rats treated with vehicle for 6 weeks; A1WO: (alfacalcidol, 1 week, old), old rats treated with alfacalcidol for 1 week V1WO: (vehicle, 1 week, old) old rats treated with vehicle for 1 week

Protocols

**Frequency – force relation**
To determine the frequency-force relation the muscle was stimulated at the following frequencies in random order: 20, 40, 60, 100, 150 and 250 Hz. The stimulation duration was 150 ms. The time between each contraction was 3 minutes to prevent the development of fatigue and minimize potentiation.

**Force – velocity relation**
To determine the force-velocity relation, the muscles were maximally stimulated with a 400-Hz 150-ms trains (de Haan 1998). During the contractions the muscles were allowed to shorten at a constant velocity (10, 20, 30, 50, 75, 100 and 125 mm·s\(^{-1}\)). Just before the contraction started, the muscle was passively stretched to a length 0.5-1 mm above \(L_o\). Each contraction started with a short isometric phase during which the force increased to the level that could be sustained during the subsequent shortening at the specific imposed velocity. This ensured that the force was constant when the muscle passed \(L_o\) during shortening (De Haan, de Ruiter et al. 1993). Rest between contractions was 3 minutes.

**Fatigue**
The fatigue protocol consisted of a series of 20 isometric contractions (150 Hz; 150 ms; 1 contraction every 500 ms).

**Data analysis**
For all isometric contractions, net peak force was calculated. Subsequently, the maximal tetanic force was normalized to muscle mass. The decrease during the fatigue protocol was expressed relative to the force of the first tetanic contraction and for every tetanus during the fatigue protocol, the half relaxation time was calculated. Half relaxation time was calculated as the time for force to decrease from the maximum to 50% of the maximum at the end of the stimulation.
Blood serum values
Blood was collected from the vena cava after the contractile properties of the Gm had been determined. Serum values for 1,25D were measured using a 1,25(OH)₂D ELISA kit (Immunodiagnostic Systems Ltd., Boldon, England). Albumin, calcium (Ca), creatinine, and inorganic phosphate (Pi) were determined with a Hitachi Biochemical Automatic Analyzer 7070 (Hitachi Co., Ltd., Tokyo, Japan).

Statistics
To determine whether there were any statistical differences a three way ANOVA with as factors age (three levels: 7, 27.5 and 29 months) and duration of alfacalcidol treatment (control, 1 and 6 weeks) was performed on the treatment group and the corresponding control group. A bonferroni post hoc test was performed if a significant effect was found. For the force-frequency relation and force-velocity relation a two-way ANOVA with repeated measures on one factor was performed to test for differences in the whole curve. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SD.
Results

Body and muscle mass
Table 2 shows the body and muscle mass data for the different groups before and after treatment. The old animals were heavier than the adult animals ($P \leq 0.001$). The vehicle treatment did not significantly affect body mass. Treatment with alfacalcidol, on the other hand, caused a 6% reduction in body mass after one week of treatment in adult and old rats ($P < 0.03$), which progressed to a 22% reduction after 6 weeks of treatment in the old rats ($P = 0.001$) (Fig 2.1).

![Figure 2.1](image)

**Figure 2.1**: Six week Alfacalcidol treatment reduces the body mass old rats. Adult rats were orally treated with vehicle or alfacalcidol for 1 week, and old rats for 1 and 6 weeks while, the adult control group received no treatment. A) Effects of 1 week treatment with alfacalcidol. The adult and old alfacalcidol treated animals had a significant decrease in body mass compared to the old rats receiving vehicle ($P < 0.03$). B) Effects of 6 week alfacalcidol and vehicle treatment on old rats. After 6 weeks alfacalcidol treatment rats showed a substantial loss of body mass compared to initial body mass of the 6 weeks vehicle treated rats ($P = 0.001$, ANOVA, bonferroni post hoc).

Ageing did not significantly affect muscle mass. This applied not only to the gastrocnemius muscle (Table 2), but also to the soleus, plantaris, extensor digitorum longus and tibialis anterior muscles (data not shown). While 1 week of alfacalcidol treatment did not cause a significant reduction in Gm mass, it was reduced by about 17% after 6 weeks of alfacalcidol treatment ($P < 0.001$). The same was found for the plantaris muscle ($P < 0.05$), but in the other muscles it did not reach significance. The Gm mass normalized to body mass was lower in the old than the adult rats. Treatment with alfacalcidol did not reduce this ratio, indicating that the decrease in body mass is not only due to a decrease in muscle mass, but also due to a decrease in other tissue, e.g. fat tissue, to a similar extent (data not shown). The heart mass increased with age, but when expressed per unit body mass it decreased. The heart mass was not significantly affected by alfacalcidol, while as a consequence of the loss of body mass the heart:body mass ratio was increased after alfacalcidol treatment ($P < 0.002$).
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Muscle Functional characteristics

Maximal isometric force

Fmax was lower in the old vehicle treated rats than the young control group (P < 0.05) (Table 2). The specific tension was 12% lower in the old (V1WO) than the adult group (P < 0.05). Treatment with alfacalcidol for 1 week did not affect Fmax, but after 6 weeks of treatment Fmax was reduced by 17% in the old rats. These age- and treatment related reductions in Fmax were explicable by the decrease in muscle mass, as the specific force (Fmax normalized by muscle mass) was similar in all groups (Fig.2).

<table>
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<tbody>
<tr>
<td>Body mass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before (g)</td>
<td>-</td>
<td>203 (78)</td>
<td>296 (31)</td>
<td>282 (22)</td>
<td>273 (35)</td>
<td>286 (26)</td>
</tr>
<tr>
<td>Body mass end</td>
<td>221 (16)</td>
<td>190 (73)*/</td>
<td>294 (30)*</td>
<td>265 (21)~</td>
<td>276 (36)*</td>
<td>224 (19)~</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gm muscle mass</td>
<td>669 (47)</td>
<td>638 (44)</td>
<td>627 (46)</td>
<td>603 (50)</td>
<td>622 (54)</td>
<td>515 (37)#</td>
</tr>
<tr>
<td>Fmax (N)</td>
<td>12.1 (1.3)</td>
<td>11.2 (1.0)*</td>
<td>9.8 (1.0)*</td>
<td>10.0 (0.6)</td>
<td>10.7 (0.7)*</td>
<td>8.8 (0.8)**</td>
</tr>
</tbody>
</table>

Table 2.2: Mean (±SD) of body mass, Gastrocnemius muscle (Gm) mass, contractile properties of the Gm (Fmax: maximal tetanic force, Fmax/muscle mass: Maximal tetanic force per unit muscle mass, HRT: half relaxation time (ms), and Pmax: maximal power) and vitamin D blood serum concentrations of the different groups. * significantly different from CA; # significantly different from other groups; ** significantly different from V6WO (P < 0.05, ANOVA, bonferroni posthoc).
Figure 2.2: Specific force is similar in all groups. A) Adult rats were orally treated with alfacalcidol for 1 week, the control adult group received no treatment B) old rats were treated for 1 and 6 weeks with vehicle or alfacalcidol. No significant differences were found in the specific force between the groups, except a lower specific force was seen for the V1WO group compared to the CA group (P < 0.05, ANOVA, bonferroni posthoc).

Force-Frequency Characteristics
The force-frequency curve of the old rats treated with vehicle for 1 and 6 weeks was shifted to the left compared to the control adult group, indicating an age effect (P < 0.001) (Fig 3A). Only alfacalcidol treatment for 6 weeks induced a shift in force frequency curve in to the left compared to their corresponding control group (P < 0.05) (Fig. 3B and C).
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Figure 2.3: Influence of age and alfacalcidol treatment on the force-frequency relation. A) Force frequency relation is shifted to the left in old animals, indicating an age effect. Old rats were treated with vehicle for 1 or 6 weeks, the control adult group received no treatment. * whole curve of V6WO and V1WO different from CA (P < 0.001) B) 1 week treatment with alfacalcidol in old animals did not effect the force frequency relation. Old rats were treated for 1 week with vehicle or alfacalcidol. C) 6 weeks alfacalcidol treatment in old rats resulted in a left-shift in the force frequency relation. Old rats were treated with alfacalcidol or vehicle for 6 weeks. Lower frequencies resulted in a higher percentage of the Fmax compared to the old rats treated with vehicle during 6 weeks.

* A6WO curve different from corresponding control group (V6WO) (P < 0.05) (Two-way repeated ANOVA)
Force-Velocity Characteristics
No significant differences were found in the force-velocity relations between the groups (data not shown). Figure 2.4 shows a typical example of a force-velocity relation of a control adult animal. The Gm of the oldest animals (V1WO) had lower maximal power outputs than the adult control group (P < 0.001) (data not shown). As a consequence of the reduction in Fmax after 6 weeks of treatment with alfacalcidol the max power was similarly reduced after 6 weeks of alfacalcidol treatment (P < 0.007).

Figure 2.4: A typical example of a force-velocity relation from an adult animal and an old alfacalcidol treated animal.

Fatigue
During 20 repeated isometric contractions a linear decrease to 74% of the initial force was seen in all groups. Concomitantly, the half relaxation (HRT) time increased during the isometric repetitions in all groups. The HRT of the non-fatigued non-treated adult muscles was shorter than that of both old control groups (P < 0.02). There was no significant effect of alfacalcidol in the HRT (data not shown).

Blood serum levels
Figure 2.5 shows that the 1,25D blood serum level was 3-4-fold higher in both adult (P = 0.004) and old rats (P = 0.002) treated for 1 week with alfacalcidol compared with vehicle treated rats. 1,25D serum levels after 6 week treatment in the old rats with alfacalcidol was not different from the 1 week alfacalcidol group. The blood serum levels of 1,25D in the V1WO and V6WO were not significantly different from those of the CA group,
indicating that the vehicle itself had no significant effect on the 1,25D concentration in the blood. Albumin and creatinine serum levels were similar in all groups (data not shown). Ca\(^{2+}\) levels were 1.1-1.3 fold elevated in the alfacalcidol treated groups compared to the corresponding control groups (P < 0.02, Fig 2.6). Pi serum levels of the old animals were lower than that in the adult control animals (P < 0.001). Alfacalcidol treatment did not induce significant changes in the circulating levels of Pi in either the adult or old rats, irrespective of duration of treatment.

**Figure 2.5**: 1,25D concentration in blood serum is increased after alfacalcidol treatment. Furthermore, old rats are not vitamin D deficient. A) Adult rats orally treated with alfacalcidol for 1 week, the control adult group received no treatment B) old rats were treated for 1 and 6 weeks with vehicle or alfacalcidol. 1,25D blood serum levels were in the control group 46 pmol/L, the adult animals treated with alfacalcidol for 1 week mean 1,25D level was 226 pmol/L. In the old animals treated with vehicle the 1,25D blood serum was not changed compared to the control group. After 1 and 6 weeks alfacalcidol treatment the 1,25 level was similar to the 1,25D blood serum level of the adult animals treated with alfacalcidol for 1 week. *different from corresponding control group (P < 0.05) (ANOVA, bonferroni posthoc)
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Figure 2.6: Ca$^{2+}$ concentration in blood serum is increased after alfacalcidol treatment. A) Adult rats orally treated with alfacalcidol for 1 week, the control adult group received no treatment B) old rats were treated for 1 and 6 weeks with vehicle or alfacalcidol. *different from corresponding control group (P < 0.02) (ANOVA, bonferroni posthoc).

Food and water consumption
Food and water intake were monitored daily. The food intake was 38% lower (P < 0.001) in the alfacalcidol than the vehicle groups. The water intake, on the other hand, was increased by 12.4% (P = 0.002) (Fig. 2.7).

Figure 2.7: Food intake is lower and water intake is higher in alfacalcidol treated rats. A) food intake during vehicle and alfacalcidol treatment in old rats. B) Water intake during vehicle and alfacalcidol treatment in old rats. Food intake was 38% lower and water intake was 12.4% higher in alfacalcidol treated animals compared to vehicle treated rats. *different from corresponding control group (P < 0.05) (ANOVA, bonferroni posthoc).
Discussion

The aims of this study were to determine the effects of an increase in serum 1,25D on the Gm muscle contractile properties in old and adult rats. We observed that ageing resulted in a reduction in force and power generating capacity of the muscle. Administration of the vitamin D analogue alfacalcidol did increase the 1,25D serum levels but in contrast to our hypothesis the old rats showed no vitamin D deficiency. Furthermore, the increased 1,25D serum levels did not improve the contractile characteristics of the Gm. Rather, a decrease in maximal force and power generating capacity and a left-shift of the force frequency relation of the Gm in old rats was found. Thus, while administration of alfacalcidol or vitamin D analogues has been proven beneficial for muscle function in conditions of vitamin D deficiency (Pfeifer, Begerow et al. 2000; Bischoff, Stahelin et al. 2003; Bischoff-Ferrari, Dietrich et al. 2004), our data indicate that elevated vitamin D levels may negatively affect muscle function. This suggest that the effect of vitamin D on muscle and the whole body may have a U-shape where below and above normal physiological levels circulating vitamin D or their analogues have a negative, rather than a beneficial effect.

Ageing and blood serum levels

Albumin, calcium and creatinine levels were not affected by ageing in our study, but the inorganic phosphate concentrations in serum were significantly lower in the old than the adult animals.

During ageing in humans levels of 1,25D in blood serum decrease (Corless, Boucher et al. 1975; Lips 2001), but we did not find lower 1,25D levels in our old rats. It is possible that part of the discrepancy between the decrease in vitamin D during ageing in men and the unaltered levels we found in rats is explicable by an age-related reduction in sun-exposure and dietary intake in humans (Holick, Matsuoka et al. 1989), whereas neither the adult nor the old rats were exposed to sunshine and rats were fed the same diet at all ages.

Alfacalcidol and VDR activity

The levels of circulating vitamin D we observed in the control adult and old rats were between 45 and 100 pmol·L⁻¹. This concentration has been found to activate approximately 3% of the VDR in mouse and human (Reschly, Bainy et al. 2007). Although the dose response curve for rat is not known it is likely that the activation of the VDR would be in the same range in the rat. By elevating the vitamin D levels with alfacalcidol treatment one would expect, based on the dose response curve of Reschly et al. (Reschly, Bainy et al. 2007), that the activation of the VDR would increase to approximately 12 - 20% at the levels of vitamin D we observed. Such an increase may have a significant impact on skeletal muscle structure and function as VDR knock-out mice, for instance, show abnormal muscle development (Endo, Inoue et al. 2003).

Muscle contractile properties

In line with previous observations, we observed a significant slowing of relaxation, a decline in force (Degens and Alway 2003) and power generating capacity, as observed in old mice (Brooks and Faulkner 1991) with ageing. These changes were largely due to a reduction in specific tension as muscle mass was maintained, similar to what has been observed previously (Degens, Hoofd et al. 1995; Degens and Alway 2003).
Six weeks of alfacalcidol treatment did cause a left shift in the force frequency relation in old rats. Such a phenomenon would be explicable by an increased relaxation time which we did not observe, however, after alfacalcidol treatment. Further support for the absence of changes in the rate in relaxation comes from the unaltered force velocity relation, suggesting that there is no major change in cross-bridge kinetics, and hence fibre type composition, after alfacalcidol treatment. Another possibility is that the Ca\textsuperscript{2+} sensitivity of the regulatory proteins on the thin filaments is reduced, a situation which has for instance been observed in diaphragm fibres from patients with chronic obstructive pulmonary disease (Ottenheijm, Heunks et al. 2005). Finally, the release of Ca\textsuperscript{2+} by the sarcoplasmic reticulum might be enhanced at low stimulation frequencies and/or the intracellular Ca\textsuperscript{2+} concentration might already be slightly elevated after treatment with alfacalcidol. Indeed, vitamin D has been shown to change the intracellular Ca\textsuperscript{2+} concentration via both non-genomic (Vazquez, Selles et al. 1999) and genomic actions (Boland 1986).

While the treatment with alfacalcidol did not significantly affect the fatigue resistance of the muscle, it did cause a significant reduction in the force generating capacity of both adult and old muscles. This muscle weakness was solely due to a concomitant loss of muscle mass, while the force generating capacity, or force per unit muscle mass, of the remaining muscle tissue was unaffected. Not only gastrocnemius muscle mass, but also the mass of the EDL, soleus, plantaris and TA was reduced, suggesting that muscle wasting occurred irrespective of the fibre type composition of the muscle. There was also a significant loss of body mass. The fact that the muscle:body mass ratios remained constant indicates that there was a proportional loss of fat and lean body mass. The heart mass, however, remained unaffected. The loss of body mass was largely due to a reduced food intake.

**Blood serum levels**

In our experiment we saw a progressive decrease in body mass caused by a reduced food intake during the alfacalcidol administration. Clearly, the decreased food intake and body mass indicates that starvation of the rats was a major problem. During starvation, however, also proteins are used in the oxidative metabolism and albumin is an abundant protein and therefore a possible target to metabolize during starvation. Yet, despite the significant decrease in body mass circulating albumin was not reduced (Krantz, Lee et al. 2005). It appears that the body maintains circulating albumin levels at the costs of other protein sources, predominantly muscle tissue (Millward 1979).

Alfacalcidol treatment resulted in elevated serum 1,25D, which facilitates the Ca\textsuperscript{2+} absorption from the intestine and reabsorption in the kidney (Pfeifer, Begerow et al. 2002). It is therefore not surprising that we found elevated Ca\textsuperscript{2+} concentrations in the serum of alfacalcidol-treated rats. The transport protein albumin has a high affinity for calcium and up to 40\% is bound and inactive, depending upon albumin concentration and pH (Margarson and Soni 1998). A decrease in albumin concentration can therefore lead to an increase in the free Ca\textsuperscript{2+} concentration in the blood. However, the albumin blood concentration was not decreased in the alfacalcidol groups compared to the vehicle and control groups. Furthermore, Ca was measured, which includes Ca\textsuperscript{2+}. A more plausible
explanation for the hypercalcemia is the increased free 1,25 D serum levels not bound to vitamin D-binding protein (BDP) (Vieth 1990; Pettifor, Bikle et al. 1995).

Further evidence for problems when vitamin D levels are elevated is the increased fluid intake in alfacalcidol-treated animals. Nevertheless, the blood analysis showed that the creatinine concentration was not significantly changed indicating that the kidney function was normal. Interestingly, also the heart was spared from the toxic effect, in contrast to the significant loss of skeletal muscle tissue.

Supplementation with active forms of vitamin D such as calcitriol and alfacalcidol have been associated with a higher risk of hypercalcaemia compared to native vitamin D (Avenell, Gillespie et al. 2009). Symptoms of vitamin D poisoning, which work via hypercalcaemia, include among other things diarrhoea, lethargy, weakness, polyuria, polydipsia and anorexia. In this study we observed polydipsia, hypercalcaemia and anorexia, which contributes to the idea that the animals which received alfacalcidol were treated with a too high dose of alfacalcidol leading to a deficit in food intake.

In conclusion, supraphysiological circulating levels of vitamin D are accompanied by significant reductions in body and muscle mass, largely due to a reduced food intake, with no apparent improvement in muscle functioning. Although undesirable, this and the maintained kidney function, heart mass and quality of the remaining muscle tissue suggests that quick normalisation of circulating vitamin D would reverse these effects on body and muscle mass. Furthermore, food intake and body mass should be carefully monitored when treating people with vitamin D analogues.

Acknowledgements
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