Title: A Systems Based Investigation into Vitamin D and Skeletal Muscle Repair, Regeneration and Hypertrophy

Running head: Vitamin D in Skeletal Muscle Repair and Adaptation

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Abstract

Skeletal muscle is a direct target for Vitamin D. Observational studies suggest that low 25(OH)D correlates with functional recovery of skeletal muscle following eccentric contractions in humans and crush injury in rats. However, a definitive association is yet to be established.

In order to address this gap in knowledge in relation to damage repair, a randomised, placebo-controlled trial was performed in twenty males with insufficient concentrations of serum 25(OH)D (45 ± 25 nmol.L⁻¹). Prior to and following 6-weeks of supplemental Vitamin D₃ (4,000 IU.day⁻¹) or placebo (50 mg cellulose), participants performed 20×10 damaging eccentric contractions of the knee extensors with peak torque measured over the following 7 days of recovery. Parallel experimentation using isolated human skeletal muscle derived myoblast cells from biopsies of 14 males with low serum 25(OH)D (37 ± 11 nmol.L⁻¹) were subjected to mechanical wound injury, which enabled corresponding in vitro studies of muscle repair, regeneration and hypertrophy in the presence and absence of 10 nmol or 100 nmol 1α,25(OH)₂D₃.

Supplemental Vitamin D₃ increased serum 25(OH)D and improved recovery of peak torque at 48 hours and 7 days post-exercise. In vitro, 10 nmol 1α,25(OH)₂D₃ improved muscle cell migration dynamics and resulted in improved myotube fusion/differentiation at the biochemical, morphological and molecular level together with increased myotube hypertrophy at 7 and 10 days post-damage. Together, these preliminary data are the first to characterise a role for Vitamin D in human skeletal muscle regeneration and suggest that maintaining serum 25(OH)D may be beneficial for enhancing reparative processes and potentially for facilitating subsequent hypertrophy.
Key Words

Muscle damage, regeneration, Vitamin D

Glossary

1α,25(OH)2D3 - 1α,25-dihydroxyvitamin D3
25(OH)D – 25-hydroxyvitamin D
Ct – cycle threshold
DM – differentiation media
GM – growth media
IKD – isokinetic dynamometry
MDC – muscle derived cell
MRF4 – myogenic regulatory factor 4
MYOG – myogenin
Nm – newton metres
nmol.L−1 – nanomoles per litre
PARQ – physical activity readiness questionnaire
QM – quiescent media
RCT – randomized controlled trial
TMT – tris-mes-triton buffer
UVB – ultraviolet B radiation
VDR – Vitamin D receptor
**Introduction**

Eccentric contraction, contusions and toxic insults cause skeletal muscle damage that leads to decrements in functional capacity. Yet skeletal muscle is uniquely equipped to regenerate via the activation, proliferation, migration and differentiation of resident muscle stem cells, known as satellite cells [43]; indeed, muscle regeneration is significantly compromised when satellite cells are ablated [43]. Effective repair usually leads to full functional recovery of the damaged tissue whilst ineffective repair leads to fibrosis and sub optimal rescue of function such as in severe muscular dystrophies [32].

Many intrinsic, local and systemic factors interact to orchestrate the muscle repair process and thus identifying and targeting modifiable risk factors that compromise any step of this process can augment functional recovery. Recent insights from human trials have reported that Vitamin D₃, a member of a group of pleiotropic pro-steroid hormones is implicated in numerous biological processes. Vitamin D is primarily synthesized in the skin's dermis following exposure to ultraviolet B radiation (sunlight exposure), which results in the conversion of 7-dehydrocholesterol to pre-Vitamin D₃ and subsequently to Vitamin D₃. Nutritionally, both Vitamin D₂ and D₃ can be obtained from dietary sources although in far less quantity than can be synthesized following sunlight exposure. It is important to consider that presently, little biological significance can be attributed to Vitamin D₂ and therefore Vitamin D₃ is thought to be the main contributor to Vitamin D status [Reviewed in 34]. Whether Vitamin D₃ is obtained from dietary sources of via UVB exposure, the metabolite will undergo hydroxylation at the liver to form 25-hydroxyvitamin D (25[OH]D), the main marker of Vitamin D status, and a further hydroxylation step in the kidney to form the biologically active 1α-dihydroxyvitamin D₃ (1α,25[OH]₂D₃). The bioactive metabolite can interact with the Vitamin D receptor (VDR) to modulate genomic and non-genomic processes that function to control biological processes in the cell [25]. Classically, Vitamin D is understood to be a major regulator of calcium and phosphate homeostasis, being required for normal bone mineralization. Indeed, the bone disorder rickets is caused by insufficient Vitamin D₃ exposure and
is reversible with supplemental Vitamin D [27]. Research evidence from the past decade however is now indicative that many tissues of the human body are responsive to Vitamin D and function sub-optimally when exposure to Vitamin D is limited [26]. Despite the understanding of the importance of Vitamin D in human health, low circulating Vitamin D concentrations (measured as total serum 25[OH]D) prevail in humans and are indeed associated with numerous preventable disease states. One such tissue that may be affected by low Vitamin D concentrations is skeletal muscle. Recently it has been suggested that the sterol is implicated in skeletal muscle regeneration and remodelling (reviewed in [34]) and indeed, sufficient 25OHD positively correlates with muscle force recovery from damaging eccentric exercise [6, 7]. These studies, while promising, are compromised by the observational nature of their design and raise the question of the potential cause-effect mechanisms between Vitamin D and muscle repair and remodelling.

Fragments of evidence from animal and cellular models have provided supporting preliminary data for the observational insights in humans. In-vivo crush injury rat models, Vitamin D sufficiency culminates in significant increases in BrdU positive proliferating myoblasts, decreased apoptosis via staining tissue for DNA damage and subsequent improvements in maximal force recovery in comparison to deficient rats [51]. In addition, Vitamin D supplementation, following BaCl2 damage of murine tibialis anterior, culminates in improved regeneration and increased expression of the Vitamin D Receptor (VDR) [25], highlighting the potential of the Vitamin D pathway in controlling aspects of the regeneration process [50]. Complementing these in vivo rodent studies, in vitro studies using C2C12 murine myoblasts also indicate that myoblast proliferation, differentiation, myotube hypertrophy and survival are mechanisms facilitated by Vitamin D, however, similar human muscle cell studies remain to be performed [21, 45, 50]. This is particularly relevant, given that circulating 25[OH]D and 1α,25[OH]2D are undetectable in many rodent species.

Taken together, a definitive cause-effect relationship between Vitamin D and skeletal muscle repair and remodelling is yet to be established in humans by well-controlled, translational
investigations. Therefore, the aim of the current work was to implement a systems based trial to
delineate the role of Vitamin D in humans using an *in-vivo* and *in-vitro* design and our aims were
with twofold objectives: 1) To investigate the effect of low serum 25[OH]D on functional recovery
from eccentric exercise, implementing a RCT 2) To identify aspects of muscle cell regeneration
that are responsive to supplemental Vitamin D, using human primary muscle derived cells, from
deficient male participants, in an *in vitro* model of muscle damage, repair and regeneration in the
presence or absence of Vitamin D₃. This *in vivo-in vitro* model allowed us to identify the impact of
Vitamin D on whole tissue muscle recovery of function and the cellular adaptations that Vitamin D
may modulate during functional repair of skeletal muscle following a damaging event.

It was hypothesized that, 1) raising serum 25[OH]D from a low level with supplemental Vitamin
D₃, could improve the functional recovery from eccentric exercise and 2) treatment of isolated
muscle cells from humans with the active Vitamin D metabolite, 1α,25[OH]₂D₃, would improve
migration, the capacity for differentiation/fusion and myotube hypertrophy following damage *in
vitro*.

**Materials and Methods**

*Inclusion Criteria and Ethical Approval*

Strict inclusion criteria were implemented for both trials. Inclusion was limited to males aged 18-
30 with no underlying medical ailments as identified by a medical history questionnaire, PARQ
and screening by a trained physician. Those taking fish oils, multivitamins, Vitamin D supplements,
or using sun beds were excluded from the trials. Finally, only individuals undertaking ≥ 3 hours of
physical activity per week above daily tasks were included in the trial. Following informed consent
and meeting the initial inclusion criteria, participants provided a venous blood sample which was
analysed for total serum 25[OH]D (nmol.L⁻¹) and were excluded if serum concentration was ≥75
nmol.L⁻¹, suggestive of adequate Vitamin D concentration [26, 31].

Ethical approval for study 1 (*in vivo*) was granted by Liverpool John Moores University Research
Ethics Committee and for study 2 (*in vitro*) by the NHS West Midlands National Research Ethics
Committee (NREC approval number: WM/09/13). All data were collected and stored in line with the declaration of Helsinki and the Human Tissues Act.

Blood Sampling and Analysis of Vitamin D Metabolites

For the analysis of Vitamin D metabolites (D$_2$ and D$_3$) in both study 1 and 2, following informed consent, serum was harvested from fasted venous blood samples collected from the antecubital vein. Serum was stored at -80°C until required for analysis.

For the analysis of total serum 25(OH)D concentration (sum of D$_2$ and D$_3$ metabolites) high-pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) was implemented. The LC-MS/MS method of analysis has been validated against other commercially available assays and is regarded as the gold standard for the assessment of Vitamin D metabolites [48]. Analyses were performed in a Vitamin D External Quality Assurance Scheme (DEQAS) accredited laboratory. Assay procedures were conducted as previously described [35].

Randomized Controlled Trial Methods

Twenty volunteers (21 ± 1 years, 179 ± 4 cm, 84 ± 13 kg) met the inclusion criteria for study 1 and were allocated to the RCT. Participants were first block randomized based on their basal serum 25(OH)D and maximal isokinetic torque of the right knee extensors at 60 deg.sec$^{-1}$ (1.05 rad.sec$^{-1}$). On the day of muscle damage, participants were instructed to produce a maximal voluntary contraction (MVC) prior to and following the bout of eccentric exercise. MVC torque was subsequently measured at 24, 48 hours and 7 days following the exercise bout to monitor functional recovery. Participants then received either an oral Vitamin D$_3$ supplement (VITD; 4,000 IU.day$^{-1}$ European Food Safety standard safe upper limit [18]) or a visually identical placebo capsule (PLB; 50 mg cellulose) for six weeks. Following which, a second blood sample was drawn for analysis of Vitamin D metabolites and the eccentric exercise bout and MVC protocol were repeated (Figure 1).
MVC torque was assessed on a Biodex isokinetic dynamometer (Biodex Medical Systems Inc., Shirley, NY), previously validated for its use in reliable assessment of muscle function variables related to torque production [17]. Participants were seated as per the manufacturers’ guidelines with a 90 degree flexion of the hip and non-extendable straps crossing the chest and abdomen and across the quadriceps to maximise isolation of the target muscle group. The test protocol consisted of four consecutive maximal extension movements of the right quadriceps at two different fixed movement velocities, 60 deg.sec\(^{-1}\) (1.05 rad.sec\(^{-1}\)) and 180 deg.sec\(^{-1}\) (3.14 rad.sec\(^{-1}\)), separated by a five minute rest to allow full recovery of the high energy phosphate pool, from which peak torque (Nm) was calculated [49]. All participants were familiarized with the protocol until the co-efficient of variation for each participant was < 10% [5].

The eccentric exercise bout was a modified version of that previously described and known to cause muscle damage [36]. Furthermore, similar eccentric exercise protocols with a smaller volume of work have been shown to result in the activation and proliferation of satellite cells [30], an important consideration to allow the transfer of findings between our \textit{in vitro} and \textit{in vivo} models. Exercise was performed on a Cybex isokinetic dynamometer and consisted of 200 unilateral eccentric contractions at 30°.sec\(^{-1}\) (0.52 rad.sec\(^{-1}\)) executed as 20 sets of 10 contractions interspersed by 30-second rest intervals. Exercise was performed through the participants’ full range of motion (ROM), thus was specific to each participant. Muscle soreness was measured at the same time points as MVC measurements and was determined via pressure algometry. The distance from the inguinal crease to the tip of the patella was measured and muscle soreness readings were then obtained 5 cm from the patella and at the mid-point of the quadriceps. Participants indicated the point at which pressure from the algometer became painful and the reading indicated by the algometer was noted (in kg).

\(< \text{Figure 1.}>\)
Methods for the In Vitro Model of Muscle Regeneration

Reagents, Chemicals and Solvents

Growth media (GM) used for the expansion of human muscle derived cell populations consisted of Hams F-10 nutrient mix (Lonza, Basel, Switzerland) with added L-Glutamine (2.5 mM), 10% heat inactivated fetal bovine serum (hiFBS; Gibco, Thermo Fisher Scientific Inc. Altinchem, UK), 10% new born calf serum (NBCS; Gibco), 1% penicillin streptomycin (PS; 50 units penicillin/50 μg streptomycin; Life Technologies, Warrington, UK) and 1% amphotericin B (2.5 μg.ml⁻¹; Gibco). Differentiation media (DM) consisted of MEM-alpha (Lonza), 1% hiFBS, 1% NBCS, 1% PS (50 units penicillin/50 μg streptomycin) and 1% amphotericin B (2.5 μg.ml⁻¹). A quiescent media (QM) used in human skeletal muscle cell pre-treatments consisted of the same components as DM however hiFBS and NBCS were added at a concentration of 0.1% each (0.2% serum total). Phosphate buffered saline (PBS; Sigma-Aldrich) was used to wash cell monolayers. The active Vitamin D metabolite 1α,25-dihydroxyvitamin D₃ was purchased from Sigma-Aldrich and reconstituted as per the manufacturers guidelines in 100% ethanol (40 μl.well⁻¹ in DM final volume for highest 1α,25[OH]₂D₃ treatment). Desmin polyclonal rabbit anti-human antibody (Ab 15200) was purchased from Abcam (Abcam Plc. Cambridge, UK) and TE7 monoclonal mouse anti-human antibody (Ab CBL271) was purchased from Merck Millipore. Secondary fluorophore (TRITC goat anti-rabbit) and nuclear counterstain (DAPI) were purchased from Life Technologies.

Muscle Biopsy Procedure

Fourteen volunteers (age = 25 ± 3 years; height = 181 ± 5 cm; weight = 81 ± 10 kg) were included in study 2 and provided a skeletal muscle biopsy. The average total serum 25[OH]D concentration for the group was 37 ± 11 nmol.L⁻¹. Participants were instructed to avoid exercise training 48 hours preceding the biopsy procedure. On arrival at the laboratory, participants were asked to relax in a supine position on a hospital bed whilst the biopsy site was prepared. Briefly, the incision site
(Vastus Lateralis) was shaved to maximise sterility and washed with an alcohol swab and Hydrex surgical scrub (ECOLAB Ltd. Leeds, UK) following which a sterile sheet was used to maintain sterility. To anaesthetise the biopsy site, 1.5 ml of bupivacaine hydrochloride (Astra Zenica. Luton, UK) was administered at a concentration of 5 mg.ml\(^{-1}\). A sterile single use scalpel was used to penetrate the skin and deep muscle fascia and a Bard disposable core biopsy instrument (12 g x 10 cm. CR Bard Ltd. Crawley, UK) to retrieve a biopsy of muscle (approximately 20-30 mg tissue).

Isolation and Characterization of Human Muscle Derived Cells

Mixed populations of myoblasts and fibroblasts were harvested from all 14 biopsy specimens by implementing a modified method of that previously described [9] and characterized by immunofluorescent staining (described in detail below) for the detection of specific proteins expressed myoblasts (desmin) and fibroblasts (TE7). Myogenic proportion ranged from 25 – 65 % (median = 45 %) and all populations were included for analysis. Pilot data revealed that the proportion of myoblasts to fibroblasts does not affect the ability of either cell type to migrate or the ability of myoblasts to fuse (data not shown). Thus, cell populations were not sorted into pure populations due to the importance of the presence of fibroblasts in myogenesis [33, 41]. Biopsy samples were transferred in pre-cooled transfer media (TM; containing Hams F-10, 2 % hiFBS, 1 % pen-strep and 1 % amphotericin-B) to the laboratory (maximum 30 min). To dissociate the tissue, biopsy samples were carefully dissected with a sterile scalpel in petri dishes to remove visible connective and adipose tissue whilst still in Hams F-10 TM. Following three washes with ice cold phosphate buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4 in dH\(_2\)O) and antibiotics (1 % pen-strep and 1 % amphotericin-B), 5ml Trypsin EDTA was added and the samples were scissor minced to fragments <1 mm\(^3\). The dissected sample was triturated on a magnetic stirring platform at 37 °C. The trypsinization process was repeated 2 times in succession and supernatant derived following each treatment was collected and pooled with horse serum (HS) at a concentration of 10% of the total volume to inhibit further
protease activity. Once Trypsin treatments were complete, pooled cell supernatant was centrifuged at 1300 rpm for 5 minutes to produce a cell pellet. The supernatant was discarded and the cell pellet re-suspended in GM and plated on a T25 cm² culture flask for cell population expansion. Following approximately 10 days in culture, T25 cm² culture flasks reached ~80 % confluence and were passaged via trypsinization. Cells were counted using Trypan Blue exclusion and frozen in GM with 10 % dimethyl sulfoxide (DMSO) as a cryopreservant or re-plated to expand the population. All experiments were performed on cells between passage 3-5 to avoid issues of senescence [3].

Cell Culture

All cell culture experiments were performed under a Kojair Biowizard Silverline class II hood (Kojair. Vippula, Finland). Cells were incubated in a HERAcell 150i CO₂ Incubator (Thermo Scientific Inc. Cheshire, UK). Cell populations were cultured on T75 cm² (Nunc. Roskilde, Denmark) and T25 cm² (Corning Inc., Life Sciences. Massachusetts, US) culture flasks and experiments were performed in sterile six well plates (Nunc. Roskilde, Denmark). Culture flasks and six well plates were coated with a 2 mg.L⁻¹ porcine gelatin solution (~90-110 g Bloom; Sigma-Aldrich Company Ltd. Dorset, UK) to allow cell adhesion.

Cell Treatments

For the expansion of cell populations, cells were grown in GM, which was changed every 48 hours following two brief washes with 1x PBS. Once cell monolayers reached a confluent state, GM was removed, monolayers were washed twice with PBS and GM was replaced with QM for 20 hours, following which QM was exchanged for QM + mitomycin-C (10 µg.ml⁻¹) for 3 hours to allow replication arrest as determined by pilot experimentation and by previous work [15]. This method was implemented to study migration in the absence of interfering proliferation. Subsequent to 3 hours treatment with QM + mitomycin-C, cells were damaged by a vertical scrape with a 1 ml
pipette tip. The mitomycin-C pre-treatment media was aspirated and damaged cell monolayers were washed three times with 1x PBS to remove cell debris and residual pre-treatment media. Each six well culture plate was subjected to a low dose of exogenous 1α,25(OH)2D3 (10 nmol in DM, Lo), a high dose (100 nmol in DM, Hi) or control vehicle (20 μl.ml−1 of 100% EtOH, Veh) in DM; n=2 wells per dose/experiment (experiments performed on n = 14). The doses of Vitamin D selected were based on previously published research [11, 22, 23, 50] and were used to determine whether potential responses in cell migration, fusion and hypertrophy were dose dependent. Immediately following the addition of treatments, monolayers were placed in a controlled live imaging microscopy environment (Leica DMB 6000 equipped with PeCon incubation system and gas control system) of 37 °C, 5 % CO2 and images were captured every 30 minutes for 48 hours for the analysis of cell migration dynamics (migration distance, velocity, and directionality, see below).

Wound Healing Assay and Migration Analysis

TIF files captured over the 48-hour filming period were exported from Leica Application Suite and loaded as TIF image stacks in ImageJ with a Cell Counter plug in. Cells in the outer (segment 1) and inner (segment 2) wound spaces were counted (see Figure 2). TIF files were also exported as TIF image stacks into ImageJ with a Manual Tracking Tool plug-in (ibidi GmbH: München, Germany). The individual trajectory of each cell was tracked in the x and y axis and derived raw co-ordinate data exported in an ImageJ Chemotaxis and Migration Tool plug-in (ibidi GmbH: München, Germany) for analysis. The chemotaxis tool analysed raw data from the manual tracking tool and provided quantitative data on the migration of individual and grouped cell trajectories including; migration velocity (V as μm.min−1), accumulated migration distance (DAcc as μm), Euclidean migration distance (Deuc as μm) and directionality (Dir as arbitrary units from 0 being random migration and 1 being a straight line).

< Figure 2. >
Creatine Kinase Activity

At 0 and 48 hours, 7- and 10 days following the mechanical scrape insult, creatine kinase (CK) activity was analysed as a marker of muscle cell differentiation/fusion into myotubes as previously described [2, 20, 44, 47]. Cell monolayers were first lysed with 300 µl per well of 50 µM tris-mes, 1% triton-X 100, pH 7.8 (TMT). Ten µl of TMT cell lysate was loaded in duplicate wells on a 96 well UV plate and used for quantification of CK activity. The creatine kinase reaction reagent and diluent (Cat cachem Inc. Connecticut, US) were prepared as per the manufacturer’s instructions and heated for 2 minutes at 37°C. When reconstituted, the reagent contained the following active ingredients: 30 mmol.L⁻¹ PCr, 2 mmol.L⁻¹ ADP, 5 mmol.L⁻¹ AMP, 2 mmol.L⁻¹ NAD, 20 mmol.L⁻¹ N-Acetyl-L-Cystine, 3000 U.L⁻¹ HK, 2000 U.L⁻¹ G-6-PDH, 10 mmol.L⁻¹ Mg²⁺, 20 mmol.L⁻¹ D-Glucose, 10 µmol.L⁻¹ di(adenosine 5’) pentaphosphate and 2 mmol.L⁻¹ EDTA. The reagent mixture was then added to the samples and the change in absorbance monitored continuously over 10 minutes in a spectrophotometer (Thermo Multiskan Spectrum plate reader) at a wavelength of 340 nm. Final concentrations are relativized to total protein are reported as mU.mg.ml⁻¹.

For the quantification of total protein, a bicinechonic acid assay (BCA assay) was also performed on lysed samples. Bovine serum albumin protein standards were prepared at 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 mg.ml⁻¹. The BCA reaction materials were purchased as part of a Pierce BCA protein assay kit (Rockford, IL, USA) and prepared as per the manufacturers guidelines. Two hundred µl of the working reagent was added to all wells containing 10 µl of sample using a multichannel pipette (excluding the blank which was TMT alone), and the plate was incubated at 37°C for 60 mins. Following background subtraction, the absorbance was recorded at 30 and 60 minutes at 595 nM using a Thermo Multiskan Spectrum plate reader. The standard curve was generated by plotting the average blank-corrected 595 nM measurement of each BSA standard
against its pre-programmed known concentration in mg.ml$^{-1}$. Sample concentrations were calculated from the standard curve.

**Morphology and Immunocytochemistry**

In order to determine myotube formation at 7 and 10 days, damaged monolayers were imaged at 4 sites per well in the wound site immediately post damage (0 hours). These image co-ordinates were then saved to allow monitoring of a consistent wound site to avoid experimental bias. Images were captured at 7 and 10 days, exported as TIFF image files and analysed in ImageJ. Morphology for assessment of muscle cell fusion/differentiation was assessed by myotubes per field of view, and myotube hypertrophy via the assessment of myotube diameter and myotube area. Myotubes were counted via ImageJ *cell counter* plug-in and only myotubes for which the entire length of the tube was visible in the field of view were considered. Myotubes were determined as cells containing 3 or more nuclei. Myotube area was determined by manually drawing a line around the sarcolemma of each myotube. By normalizing the pixel scale to the micron scale of each image a value expressed as μm$^2$ is obtained. To calculate myotube diameter, three equidistant diameters along the length of the myotube (left centre, centre and right centre) were measured and averaged. A total of 3 image per well were analysed per treatment, treatments were performed in duplicate and experiments performed on 14 cell populations from 14 different individuals. Therefore 84 images per condition were assessed.

Experiments above were repeated for immunocytochemical staining in order to visualise nuclei for the accurate determination of myonuclear fusion index and myonuclear domain size. Monolayers were fixed at 10 days post insult via 5 minute graded methanol incubations (25 %, 50 % and 100 % v/v methanol in 1x tris buffered saline (TBS)) and stored wet in 1x TBS until required for staining. Monolayers were permabilized and blocked for 2 hours prior to staining with 5% goat serum and 0.2% Triton X100 in 1x TBS. Cells were incubated overnight at 5°C with 1° Desmin antibody (1:200). After overnight incubation, 1° antibody was removed and the cells washed three times
with 1x TBS. Secondary antibody TRITC antibody (1:200) was then applied and left for two hours at 5°C. Finally, following removal of 2° antibody and three TBS washes, a nuclear counterstain (Sytox-Green; 1:5000) was applied and monolayers incubated for 1 hour before a final three TBS washes. Fluorescent images were then captured at 10 days post damage and nuclei counted via ImageJ cell counter plug-in. A total of 3 image per well were analysed per treatment and treatments were performed in duplicate (n = 2/condition/sample). This immunostaining procedure was also used in the characterization of cell populations described above in which cells were stained with desmin (1:200) and TE7 (1:200) to determine the relative proportion of myoblasts to fibroblasts.

Gene Expression: RNA isolation, primer design and RT-qPCR

Total RNA was isolated at 0 hours post mechanical damage via wound infliction and then at 48 hours, 7 and 10 days post-damage. Monolayers were washed with 1x PBS (1 ml per well) and RNA was extracted using 300 µl per well TRI-reagent (Sigma-Aldrich, Dorset, UK). RNA was isolated via guanidium thyocyanate phenol chloroform extraction. Concentration and purity were assessed via UV spectroscopy using Nanodrop spectrophotometer 3000 (Fisher Scientific, Rosklide, Denmark). Only samples with a purity ratio between 1.9 and 2.2 were used for the downstream application of RT-qPCR.

Purified RNA was diluted to 7.3 ng.ml⁻¹ in 9.5 µl DNase RNase free H₂O (Sigma-Aldrich Company Ltd. Dorset, UK) to create a final reaction concentration of 70 ng. RNA was reverse transcribed and amplified with specific primer sequences in a rotor-gene Q (Qiagen, Manchester, UK) PCR machine using a one-step SYBR Green I RT-qPCR kit (Qiagen). Briefly, double stranded cDNA was first synthesized at 50°C for 10 minutes with the use of dTP oligonucleotides and reverse transcriptase. c-DNA was denatured to single stranded DNA at 95°C for 10 seconds and combined primer annealing and extension was initiated at 60°C for 30 seconds. Primers sequences were designed using National Centre of Biotechnology Primer-BLAST software and RTprimerDB (http://medgen.ugent.be/rtprimerdb/) and highly purified salt free primer for each
sequence was purchased from Sigma-Aldrich. Detailed primer information can be found in Table 1. A relative method of mRNA expression was used as previously described with a stable reference gene (RPL13a; CV % = 1.8 %) and 0 hours untreated control sample (ΔΔCt method; [38]). Melt curve analysis was performed to confirm all PCR products demonstrated a clear single peak (same) melt temperature showing that only one gene target had been amplified and that primer-dimer issues were not present.

Additionally, amplified PCR products were electrophoretically separated to ensure correct end product length of amplified genes. Eight µl of Norgen FullRange 100 bp DNA ladder and 8 µl of sample were loaded after preparation with a DNA loading dye (Geneflow Ltd. Staffordshire, UK.) into an agarose gel (2% Agarose (Bioline Reagents Ltd. London, UK) in 1X Tris-acetate-EDTA buffer (Invitrogen, Life Technologies Ltd. Paisley, UK)) prepared with Midori Green nucleic acid stain (Nippon Genetics Europe GmbH. Düren, Germany) at a dilution of 1:200. Samples were electrophoretically separated at 50V for 20 minutes followed by 70V or a further 20 minutes. Following electrophoretic separation, gels were placed on a UV trans-illuminator for visualization and analysis.

<Table 1.>

Statistical Analysis

All statistical analyses were performed using SPSS Predictive Analytics Software (v.20, IBM Corporation. New York, US). For the comparison of two group means, a T-test was used and where comparison of multiple groups of means was required an analysis of variance (ANOVA) was used. Data sets were first checked for normal distribution and where data violated the assumption of normality, an appropriate correction factor was used. If data violated the assumption of sphericity Greenhouse-Geiser or Huyn-Feldt correction factors were used. Where significant main effect and interactions were present, the Bonferroni post-hoc pairwise comparisons test was used to detect where significances lay between paired comparisons, an analysis that includes
correction for an ANOVA’s multiple comparisons. Significance was assumed when \( \alpha \) reached \( \leq 0.05 \). All data are presented as mean ± standard deviation (SD).

**Results**

*Randomized Controlled Trial*

At baseline, no significant differences were detected between experimental groups for total serum 25\(\text{[OH]}\)D \((P > 0.05)\), with mean serum concentrations of \(45 \pm 15\) and \(45 \pm 25\) nmol.L\(^{-1}\) for PLB and VITD, respectively. Following 6 weeks of supplementation with 4,000 IU.day\(^{-1}\) Vitamin D\(_3\), a significant interaction effect was observed between treatment group and time \((P < 0.005)\). Post-hoc analysis revealed that the VITD group showed a significant increase in total serum 25\(\text{[OH]}\)D at week 6 compared with pre supplementation \((\text{pre} = 45 \pm 25\) vs. \text{post} = 115 \pm 31\) nmol.L\(^{-1}\), \(P < 0.005\)\) and a significant difference when compared with PLB \((P < 0.005)\). Conversely, the PLB group demonstrated a significant decline in serum 25\(\text{[OH]}\)D at week 6 compared with pre supplementation \((\text{pre} = 45 \pm 25\) vs. \text{post} = 33 \pm 13\) nmol.L\(^{-1}\), \(P = 0.013\)\).

At both pre and post supplementation test points, VITD and PLB demonstrated comparable, significant losses of peak torque immediately post exercise at both 60 and 180 deg.sec\(^{-1}\) \((\text{all at } P < 0.005)\), indicative that the eccentric exercise protocol effective caused skeletal muscle damage. At the pre supplementation time point, no interaction effect detected between treatment group and maximal torque recovery over 7 days for either 60 deg.sec\(^{-1}\) \((P = 0.281)\) or 180 deg.sec\(^{-1}\) \((P = 0.310)\). However, following supplementation, a significant interaction effect between recovery time point, test week (pre-post supplementation) and treatment group at 60 deg.sec\(^{-1}\) \((P = 0.049)\) was detected. Exploration of this interaction identified a significant improvement in torque recovery in the supplemental VITD group following supplementation at 48 hours \((14\% \text{ improvement, } P = 0.042)\) and 7 days post eccentric exercise \((13.7\% \text{ improvement, } P = 0.001)\) compared with pre supplementation. Although a slight improvement was observed for VITD post supplementation at 180 deg.sec\(^{-1}\) \((48 \text{ hours } = 5.6\% \text{ and 7 days } = 9.9\% \text{ improvement})\) this result failed to meet...
statistical significance (Figure 3). We then also plotted the change in peak torque recovery at 7 days against the change in total serum 25(OH)D as a linear regression to determine whether a relationship existed (Figure 3F). Interestingly, 88% of the variation in peak torque recovery could be explained by the change in serum 25(OH)D ($r^2 = 0.88$). Despite improvements in functional capacity, there was no interaction between treatment group and time for measures of muscle soreness at either mid quadriceps ($P = 0.71$) or 5 cm patella ($P = 0.418$) possibly due to the highly subjective nature of the sensation of pain and a disconnect between the mechanisms regulated by Vitamin D in the repair process and those that regulate the sensation of pain.

< Figure 3. >
In Vitro Model of Muscle Damage to Assess Repair and Regeneration

Having ascertained a beneficial impact on strength recovery post-eccentric exercise induced damage in the presence of Vitamin D, we next wished to establish the influence of Vitamin D on muscle derived cell migration, a key initial event in muscle repair. Cell monolayers were treated with 10 and 100 nmol 1α,25(OH)2D3 or vehicle (EtOH) following a damaging mechanical scrape insult and live imaged for 48 hours. A significant treatment group effect was detected for V, Dacc, Desc and Di (P < 0.005). Both Hi and Lo dose 1α,25(OH)2D3 significantly enhanced cell migration V compared to vehicle with 1.37 and 1.43 fold increases observed, respectively (P < 0.0005), versus control. Hi dose was also superior to Lo dose (0.331 ± 0.11 vs. 0.318 ± 0.1 μm.min⁻¹ respectively, P = 0.033). A similar observation was made in Dacc as Hi treatment promoted greater migration (953 ± 305 μm) distances than Lo ((909 ± 281 μm) P = 0.009) whilst both Hi and Lo were superior to Veh ((666 ± 288 μm) P < 0.0005). However, although both Lo and Hi treatment improved Desc when compared with Veh, there were no differences between the two doses (P = 0.193), which would imply a loss of directionality in the Hi dose treatment. Indeed, analysis revealed that Hi dose treated skeletal muscle cells demonstrated a loss of directionality (0.498 ± 0.21 AU) compared to Lo (0.546 ± 0.2 AU) and Veh (0.547 ± 0.23 AU) treated cells (P < 0.005) whereas Lo and Veh showed no differences. Taken together, both Lo and Hi resulted in more cells at the inner wound space (Lo = 20 ± 9 and Hi = 21 ± 8 cells) versus Veh at 48 hours (16 ± 7 cells) as a consequence of improved migration speed and distance. No significant differences were evident in wound size between conditions indicating that this is unlikely to have affected results.
(Lo = 949 ± 146 vs Hi = 927 ± 103 vs. Veh = 925 ± 108 μm (P = 0.810)). Data are presented in Figure 4.
Treatment of cultures with 10 or 100 nmol 1α,25[OH]₂D₃ resulted in superior migration dynamics in comparison to vehicle alone, however the same dosing strategy led to differential effects on myoblast fusion at 7 and 10 days following the damaging event in the current investigation. Morphological data analysis revealed that myoblast fusion was significantly inhibited as fewer myotubes were observed per field with 100 nmol 1α,25[OH]₂D₃ versus 10 nmol and vehicle at 7 days post damage (Hi = 2 ± 2 vs. Lo = 6 ± 4 vs. Veh = 3 ± 1 myotubes per field (P < 0.0005). In contrast, Lo treatment led to significant improvements in myotube number compared with 100 nmol and Veh at both 7 (as above) and 10 days (Lo =10 ± 3 vs. Hi = 6 ± 2 vs. Veh = 6 ± 2 myotubes per field (P < 0.005)). This observation was also similar for myotube area with Lo treatment resulting in significantly greater myotube area versus Hi and Veh at 7 (Lo = 4984 ± 2776 vs Hi = 4603 ± 1697 vs 4227 ± 1768 μm² (P = 0.003)) and 10 days (Lo = 5488 ± 2853 vs Hi = 4671 ± 2932 vs Veh = 4388 ± 2312 μm² (P < 0.0005)). Myotube diameter was significantly greater at 7 days in both Lo and Hi dose treatments versus Veh (Lo = 14.13 ± 4 vs Hi = 13.7 ± 4.5 vs Veh = 12.12 ± 3.8 μm (P = 0.005)), however this effect was lost at 10 days (Lo = 13.6 ± 4 vs Hi = 12.6 ± 4.1 vs Veh = 12.1 ± 3.7 μm (P = 0.256)).

Findings from biochemical and fluorescent imaging were in agreement with morphological findings. Creatine kinase activity data were analysed via a mixed design ANOVA. Results show CK activity was elevated above Veh with Lo treatment and repressed with Hi compared with both Lo and Veh at 7 days (Lo = 302.5 ± 173 vs Hi = 186.7 ± 132 vs Veh = 290 ± 160.5 mU.mg.ml⁻¹), although this effect did not reach statistical significance. At 10 days, Lo treatment cells demonstrated significantly higher CK activity compared with both Hi and Veh (Lo = 340.4 ± 183 vs Hi = 258.4 ± 188 vs Veh = 229.4 ± 139.5 mU.mg.ml⁻¹ (P = 0.017)). Myonuclei and myonuclear domain size were analysed by one-way ANOVA. The CK observations correlated with a significantly greater accretion of myonuclei in Lo treated cells versus Hi and Veh at 10 days post damage (Lo = 5.9 ± 2.3 vs Hi = 3.5 ± 1.4 vs Veh = 3.4 ± 1.2 nuclei per myotube per field (P <
however myonuclear domain size was smaller at 10 days with Lo treatment (Lo = 986.5 ± 439.4 vs Hi = 1460 ± 726.3 vs Veh 1257.3 ± 584 μm² (P < 0.0005)). See Figures 5 and 6.

< Figure 5. >
To determine the impact of treatment on myogenic gene expression, ΔΔCt analyses were performed by comparing the fold change in target gene expression against both a non-treated 0 hours control and a stable reference gene (RPL13a). Results demonstrated that on average Lo treated cells up regulated MRF4 expression to a greater extent at both 7 and 10 days (3.2 ± 2.7 and 3.7 ± 1.5 fold) than Hi (-0.5 ± 0.3 fold and 0 ± 0.2) and Veh treated cells (2.4 ± 2.8 and 2.8 ± 2.4 fold). Similarly, Lo treated cells showed an increased myogenin expression at 10 days (84 ± 95 fold) compared with Hi (64 fold ± 79) and Veh (62 fold ± 70 fold), however these data failed to meet statistical significance ($P > 0.05$), likely due to very large variation in basal expression of the MRFs. VDR expression showed no discernible difference between groups at any time point, however all significantly increased expression in a similar trend with myogenin between 48 hours and seven days as a main effect for time was detected ($P = 0.022$). Interestingly, Hi treated cells also showed an impairment (although no statistically significant) in the ability induce both MRFs at 7 days post insult versus control and Lo treated cells which correlates with biochemical observations demonstrating lower CK activity in Hi treated cells at 7 days (Figure 7).
Discussion

The aims of the current investigation were twofold. First we looked to identify the effect of increasing serum 25(OH)D from a level of insufficiency on the functional recovery of skeletal muscle following eccentric work. Second we aimed to establish cellular aspects of muscle regeneration and remodelling that may be responsive to Vitamin D, providing a novel mechanistic underpinning for in vivo observations. It was hypothesized that increasing total serum 25(OH)D with supplemental Vitamin D₃ would lead to an improvement in peak torque recovery following eccentric exercise. Furthermore, we postulated that myogenic progenitor migration, fusion and myotube hypertrophy would be improved in the presence of exogenous 1α,25(OH)₂D₃.

The data presented provide novel insights that point toward an important role of Vitamin D in muscle recovery in vivo and repair, regeneration and hypertrophy in-vitro. The main findings from this work demonstrate that elevating serum 25(OH)D from ~40 nmol.L⁻¹ to >75 nmol.L⁻¹ with supplemental Vitamin D₃ (4,000 IU.day⁻¹) results in improved functional recovery from eccentric exercise at 48 hours and 7 days post exercise versus a placebo control group, which showed no changes in recovery rate. In an attempt to provide initial insights into the mechanisms responsible, we also uncovered novel roles for Vitamin D₃ in muscle progenitor migration, fusion and myotube hypertrophy following an artificial wound injury in vitro. Both the migration velocity and distance travelled into the wound site were significantly enhanced with both 10 and 100 nmol 1α,25(OH)₂D₃ treatments implying that Vitamin D₃ may function to stimulate cell migration in a positive manner when considered in the context of muscle repair. Furthermore, the 10 nmol treatment also led to elevated CK activity above that of 100 nmol and vehicle at all time points, reaching significance at 10 days post insult. In accordance with these data, myotube number and size were both significantly elevated in the 10 nmol treatment at 10 days versus 100 nmol and vehicle, likely attributable to an increased ability to accrete myonuclei, further supported by a reduction in myonuclear domain that suggests there were more nuclei in longer/larger myotubes with less domain to serve per myotube. Interestingly, the 100 nmol dose of Vitamin D₃ suppressed
this effect and fusion capability only reached the level of the vehicle control by day ten. Together, these data imply that enhanced cellular characteristics of the muscle regeneration process may partly explain improved functional recovery of skeletal muscle with higher serum 25\([\text{OH}]\)D \textit{in vivo} and may also point towards a positive role for Vitamin D in muscle remodelling given the increases in myotube size and nuclear accretion.

The positive influence of 10 nmol 1α,25\([\text{OH}]\)\textsubscript{2}D\textsubscript{3} administration on cell migration is analogous to effects observed in other cell types. For example, exogenous treatment of vascular smooth muscle cells with 1α,25\([\text{OH}]\)\textsubscript{2}D\textsubscript{3} induced migration following activation of PI3 kinase, with observed effects being abolished by the addition of the PI3K inhibitor, LY294002 [42]. Indeed the importance of PI3K in myoblast migration has previously been characterized [15], and stimulation of this pathway by 1α,25\([\text{OH}]\)\textsubscript{2}D\textsubscript{3} has previously been reported in skeletal myoblasts [11] pointing towards stimulation of PI3K activity as a potential mediator of improved MDC migration dynamics observed in the current trial. Activation of PI3-kinases and their lipid product PI(3,4,5)P3 leads to increases in GTP bound Rac, which is an important small GTPase along with Rho involved in the control of downstream signalling that generates filamentous actin branching and lamellipodia formation [40]. It could therefore be postulated based on available evidence that Vitamin D may function to stimulate MDC migration through the activation of PI3K and increased downstream activity of small Rho GTPases resultanty altering actin cytoskeletal dynamics. Taken together, Vitamin D possesses the capability to improve the velocity of which skeletal muscle progenitors can reach a site of damage to permit repair and remodelling of the area. Future research should aim to investigate this pathway in the context of Vitamin D and examine cytoskeletal dynamics. Further, the relevance of enhanced muscle cell migration \textit{in vivo} should also be investigated.

The finding that 1α,25\([\text{OH}]\)\textsubscript{2}D\textsubscript{3} improved myoblast fusion is perhaps not surprising as Vitamin D is a closely related hormone system with some of the characteristics of true steroids as receptor ligands such as testosterone. Indeed testosterone has repeatedly been demonstrated to enhance myoblast differentiation \textit{in vitro} [14, 46]. In a similar observation to the current study, these
investigations also detected significant increases in hyperplasia and hypertrophy in the presence of the steroid. Interestingly, reports suggest that the significant myonuclear accretion observed during overload-induced hypertrophy is not lost during a 3-month period of severe disuse atrophy [10]. Furthermore, treatment of mice with testosterone propionate was demonstrated to induce nuclear accretion and hypertrophy that, following a period of testosterone withdrawal, led to atrophy but a sustained elevated myonuclear number that enhanced re-training induced fibre hypertrophy [19].

Together these data imply that steroid exposure stimulates adaptive remodelling that primes the muscle for adaptation in subsequent bouts of mechanical stimuli. The finding that Vitamin D increased nuclear accretion accounting for a hypertrophic effect in vitro is therefore particularly interesting and raises new questions as to whether the sterol may also function similarly to testosterone treatment. Mechanisms accounting for the hypertrophic response elicited by Vitamin D are yet to be established, although recent trials have provided evidence that Vitamin D treatment improves breast meat yield in male broiler chickens through the mTOR pathway [52], a known major regulator of mechanical overload induced muscle growth [24]. Furthermore, proteasomal enzyme activities, expression of the E2 ubiquitin conjugating enzyme, and ubiquitin conjugates are increased in Vitamin D deficient versus replete rats [8]. Recently, an in vivo report has also demonstrated that type IIa skeletal muscle fibres are increased in number and myostatin mRNA is down-regulated with Vitamin D supplementation in human males during a resistance training programme [1], providing preliminary support for the adaptive remodelling observations made in vitro in the current work.

Our in vitro findings also show similarities and disparities with other models of myogenesis in the context of Vitamin D. As a contrasting example, increases in myotube diameter and MHC type II have been detected following 100 nmol treatment of C2C12 myoblasts, indicative of a positive myogenic effect [22]. In another trial, following serum depletion 100 nmol 1α,25(OH)2D3 was shown to suppress myotube formation, analogous to the current study. However, the treatment led to a 1.8-fold increase in cross-sectional size of individual myotubes associated with slightly decreased myostatin expression [23]. The inconsistency between the current work and previous
findings may lie in fundamental differences in the metabolism of Vitamin D in humans and rodents. Although both species express the same components of the Vitamin D endocrine system, rodents typically obtain all Vitamin D from dietary sources, which are minimal amounts [29]. As a result, many rodent species show negligible quantities of the major circulating Vitamin D metabolites despite showing no typical symptoms of Vitamin D deficiency such as hypercalcaemia, hypercalciuria and elevated PTH concentration. Moreover, these species show discrimination of the D₃ metabolite over the D₂ form which is the reverse in humans [28]. An additional difference is that the current work implemented a co-culture model in which fibroblasts were also present with myoblasts. Indeed fibroblasts are known to positively regulate alignment and fusion of skeletal myoblasts [41] and thus it may therefore be argued that our data present a more physiologically relevant insight into the regulation of myogenesis by Vitamin D.

It is important to postulate whether our in vitro observations could be attributable for the improved resolution of MVC torque seen in vivo. Indeed, the activation and increased activity of satellite cells is evident within 24 hours following eccentric work in humans [12, 16, 30]. Moreover, increases in embryonic MHC content have been observed from 2- and peaking at 7 days following eccentric work indicative of incorporation of committed myoblasts into myofibres, which also shows a strong trend with functional recovery of peak torque that starts to recover at 24 hours and peaks at 7 days [37]. To confirm such a link, analysis of biopsy specimens in parallel to torque recovery are necessary.

It must be considered that myoblast migration, fusion and hypertrophy are only some of the cellular mechanisms that underlie the resolution of damaged skeletal muscle as a result of eccentric work. Satellite cell activation and expansion, an immune response, re-innervation, re-cappiluarization and extra cellular matrix remodelling are also important events that result in full function recovery of damage muscle tissue. It is well characterised that both innate and acquired immunity are potently regulated by Vitamin D [39], nerve recovery and re-myelination are improved in the presence of Vitamin D [13], pro-angiogenic growth factors are up-regulated in regenerating Vitamin D treated myoblasts in vitro [21] and increased secretion of extracellular
matrix components have been reported in Vitamin D deficient rats using a rotator cuff repair model [4]. Taken together, it is possible that Vitamin D had the profound effect observed *in vivo* as a result of interaction with a number of events that orchestrate muscle repair and remodelling. Thus work is now warranted to investigate these processes collectively in the presence and absence of Vitamin D in order to fully characterise how regenerating human skeletal muscle is affected by the availability of the sterol.

Although the present study provides a promising concept of a relationship between Vitamin D and muscle repair and remodelling, there are some limitations to be considered. Firstly, the sample size of the RCT is indeed small and larger scale studies will be needed to firmly establish the findings made in the current trial. This will also allow for sub group analysis and perhaps the detection of subtle functional changes that could not be detected in the supplemental Vitamin D group for peak torque recovery at 180 deg.sec\(^{-1}\). Moreover, the chosen model of exercise is highly specific with movements performed at two fixed velocities. Although this is ideal for experimental repeatability, such regimens are not common in everyday life and training. Thus, future experimental designs should expand on the current work and employ ‘real world’ protocols resulting in muscle damage such as downhill running or resistance training employing negative repetitions of common strength training exercises. Serial biopsies alongside functional measures of muscle recovery following eccentric work will also help to solidify the link between the *in vivo* and *in vitro* findings described in the current work. Finally, relating to our *in vitro* model, we utilised commonly implemented supra-physiological doses of 1α,25(OH)\(_2\)D\(_3\), however the authors now believe that estimation of the concentration of 1α,25(OH)\(_2\)D\(_3\) that skeletal muscle is exposed to is the next step to further optimising studies of Vitamin D and skeletal muscle *in vitro*.

To conclude, this study is the first to identify a novel role for Vitamin D in human skeletal muscle regeneration. *In vivo* observations made here demonstrate that low serum 25(OH)D is easily elevated with supplemental Vitamin D\(_3\) and may benefit skeletal muscle recovery, regeneration and hypertrophy. A challenge to the field is to now expand these preliminary findings by employing larger sample sizes and aiming to characterise all aspects of muscle regeneration that are
responsive to Vitamin D. Furthermore, new questions are raised as to whether ‘at risk’ populations
susceptible to muscle damage and/or Vitamin D inadequacy, such as the elderly who are known to
exhibit low serum 25[OH]D, experience aggravated declines in regenerative capacity and

Acknowledgements

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University) for their assistance with muscle biopsies.
References


Figure Captions

Figure 1. Schematic representation of experimental procedures. * denotes peak torque and muscle soreness measurement which were performed immediately prior to-, immediately post and then at 24 hours, 48 hours and 7 days following an eccentric exercise protocol consisting of 20 sets of 10 eccentric contractions performed at 30 deg.sec\(^{-1}\) and separated by 30 sec rest intervals between sets. Blood was collected pre supplementation and then again following the six week supplementation period for measurement of total serum 25[OH]D.

Figure 2. Representative image of a scrape wound inflicted with a 1ml pipette tip. The wound area is 900 μm in width and split into 3 x 300 μm segments. Magnification is 10.5x and scale bar is 100 μm. The out most segments are referred to as segment 1 and the single inner segment is referred to as segment 2.

Figure 3. a & b) Recovery profile of peak isokinetic torque at 60 and 180 deg.sec\(^{-1}\) following six weeks supplementation with 4,000 IU.day\(^{-1}\) Vitamin D\(_3\) or c & d) visually identical placebo. # denotes significance to pre supplementation torque at the highlighted time point. ≠ denotes significance for the loss of both pre and post supplementation torque immediately following eccentric exercise (post ex) when compared with pre exercise (pre ex) values. e) Total serum 25[OH]D response to either 4,000 IU.day\(^{-1}\) Vitamin D\(_3\) or placebo. ** denotes significance to pre supplementation and placebo. * denotes significance to baseline. f) Linear regression describing the relationship between Δ total serum 25[OH]D (nmol.L\(^{-1}\)) between pre- and post-supplementation and the Δ peak torque (Nm) recovery at 7 days post exercise between pre- and post-supplementation.

Figure 4. MDC migration dynamics following a mechanical scrape injury in the presence of Lo (10 nmol) or Hi (100 nmol) 1α,25[OH]\(_2\)D\(_3\) or vehicle solution (100% EtOH); a) representative phase contrast microscope images captured at 48 hours post wounding. Scale bar is 100 μm. n = 14; b) representative migration tracking plots produced via ImageJ chemotaxis tool. Trajectories mapped in μm; c) average number of cells migrated into segment 1 and 2 at 48 hours post damage; d) migration D\(_i\) (0-1); e) migration \(V\) (μm.min\(^{-1}\)); f) migration \(D_{Euc}\) and g) migration \(D_{Acc}\) (μm); * denotes significance to Veh and ** denotes significance to all other treatments.

Figure 5. Effect of Lo or Hi dose 1α,25[OH]\(_2\)D\(_3\) versus Veh on myotube morphology following 7 and 10 days following mechanical scrape wounding in DM; a) Average myotubes per field of view; b) average myotube diameter (μm); c) average myotube area (μm\(^2\)) and d) representative phase contrast microscope images captured at 10 days in the wound space of each condition. Scale bar is 100μm. * denotes significance to all other treatments at that time point.

Figure 6. Effect of Lo or Hi dose 1α,25[OH]\(_2\)D\(_3\) versus Veh on biochemical aspects of myotube formation and immunocytochemical analysis of nuclear accretion; a) Average CK activity (mU.mg.ml\(^{-1}\)) at 48 hours, 7 and 10 days following damage. * denotes significance to other conditions at a time point; b) average nuclei per myotube per field of
view and e) myonuclear domain size at 10 days following damage. * denotes significance to all other conditions; d)
Representative fluorescent microscopy images taken at 10 days following damage. Scale bar is 100 µm.

**Figure 7.** Time course effects of 1α,25(OH)2D3 on ΔΔCt mRNA expression levels of a) Myogenic regulatory factor 4; b) myogenin and c) VDR. Fold changes were calculated against a stable reference gene (RPL13a) and an internal calibrator (0 hours sample).
Tables

Table 1. Gene primer sequences for human MDC samples and amplicon lengths. All primers were used in the same PCR cycling conditions.

<table>
<thead>
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<th>Accession No.</th>
<th>Primer Sequence</th>
<th>Amplicon Length (bp)</th>
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<td>NM_001017536</td>
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<td>174</td>
<td>Reverse 1575/1576</td>
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<td></td>
<td></td>
<td>R: GGACGATCTGGGGAGACGA</td>
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<td></td>
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<td></td>
<td></td>
<td>R: AGGAAGGCGAGGTACTTCAACTT</td>
<td></td>
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</table>
Peak Isokinetic Torque (Nm)

PLB pre supp
PLB post supp

60 deg.sec\(^{-1}\)

180 deg.sec\(^{-1}\)

Total Serum 25[OH]D (nmol.L\(^{-1}\))

VITD pre supp
VITD post Supp

**

Δ 7-day peak torque recovery at 60 deg.sec\(^{-1}\) (Nm)

Δ total serum 25[OH]D (nmol.L\(^{-1}\))

y = 0.5351x - 3.7921

R\(^2\) = 0.8752
a

Lo (10 nmol)

Hi (100 nmol)

Veh (EtOH)

b

Y axis (µm)

X axis (µm)

b

Y axis (µm)

X axis (µm)

b

Y axis (µm)

X axis (µm)

b

Y axis (µm)

X axis (µm)

b

Number of Cells Migrated

Lo (10 nmol)

Hi (100 nmol)

Veh (100% EtOH)

c

d

Directionality (AU 0-1)

0.0 0.2 0.4 0.6 0.8 1.0

Migration Velocity (µm.min⁻¹)

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

Euclidean Distance (µm)

0 100 200 300 400 500 600 700 800 900 1000

Accumulated Distance (µm)

0 200 400 600 800 1000 1200 1400 1600 1800 2000

Legend:

Segment 1

- Lo (10 nmol)
- Hi (100 nmol)
- Veh (100% EtOH)
Lo (10 nmol)  
Hi (100 nmol)  
Veh (100% EtOH)

**a**

![Graph showing Myotubes per Field](image)

**b**

![Graph showing Myotube Diameter (μm)](image)

**c**

![Graph showing Myotube Area (μm²)](image)

**d**

Lo (10 nmol)  
Hi (100 nmol)  
Veh (EtOH)