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Abstract: The presence of glycoside derivatives of 1□,25(OH)2D3 endows plants to gradual release of the free bioactive form of 1□,25(OH)2D3 from its glycoconjugates by endogenous animal tissue glycosidases. This results in increased half-life of the hormone in blood when purified plant fractions are administered for therapeutic purposes. In this work, we evaluated the role 1□,25(OH)2D3-glycosides enriched natural product (Solbone A) from Solanum glaucophyllum leaf extract compared with synthetic 1□,25(OH)2D3 on myogenic differentiation in C2C12 myoblasts. For these, differentiation markers and miogenic parameters were studied in C2C12 myoblasts. Results showed that Solbone A, likewise the synthetic hormone, increased creatine kinase activity at day 2 after differentiation induction (60%, $p < 0.05$). Solbone A and synthetic 1□,25(OH)2D3 increased vitamin D3 receptor protein expression at 10 nM (50 and 30 %, respectively) and the transcription factor myogenin (80%, $p<0.05$). However, tropomyosin expression by both compounds was induced at 1 nM (20%, $p<0.05$). In addition, myosin heavy chain (MHC) protein expression was increased 50 % at day 4 of differentiation. Solbone A or synthetic 1□,25(OH)2D3 had no effects on myogenin nor MHC cell localization. Cellular mass increased with myogenesis progression, being Solbone A more effective than synthetic 1□,25(OH)2D3. Finally, Solbone A, as well as synthetic 1□,25(OH)2D3, augmented the index fusion of cultured muscle fibers. In conclusion, these results demonstrated that Solbone A exhibit at least equal or greater effects on early myoblast differentiation as synthetic hormone, suggesting that plant glycosides could be an effective, accessible and cheaper substitute for synthetic 1□,25(OH)2D3 to promote muscle growth

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## Highlights

- Solbone and 1α,25$(OH)_2D_3$ enhanced CK activity at the onset of C2C12 cell myogenesis
- Solbone, similary to synthetic 1α,25$(OH)_2D_3$, induced VDR and myogenin expression.
- Solbone and synthetic 1α,25$(OH)_2D_3$ increased tropomyosin and MHC expression.
- Solbone stimulated myotube formation at the onset of C2C12 cell myogenesis

# *In vitro* effects of 1α,25(OH)$_2$D$_3$-glycosides from Solbone A (*Solanum glaucophyllum* leaves extract; Herbonis AG) compared to synthetic 1α,25(OH)$_2$D$_3$ on myogenesis.

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[#]In memory of Ricardo Boland (deceased Oct, 2014).

**Abstract**

The presence of glycoside derivatives of $1\alpha,25(OH)_2D_3$ endows plants to gradual release of the free bioactive form of $1\alpha,25(OH)_2D_3$ from its glycoconjugates by endogenous animal tissue glycosidases. This results in increased half-life of the hormone in blood when purified plant fractions are administered for therapeutic purposes. In this work, we evaluated the role $1\alpha,25(OH)_2D_3$-glycosides enriched natural product (Solbone A) from *Solanum glaucophyllum* leaf extract compared with synthetic $1\alpha,25(OH)_2D_3$ on myogenic differentiation in C2C12 myoblasts. For these, differentiation markers and miogenic parameters were studied in C2C12 myoblasts. Results showed that Solbone A, likewise the synthetic hormone, increased creatine kinase activity at day 2 after differentiation induction (60%, $p < 0.05$). Solbone A and synthetic $1\alpha,25(OH)_2D_3$ increased vitamin $D_3$ receptor protein expression at 10 nM (50 and 30 %, respectively) and the transcription factor myogenin (80%, $p<0.05$). However, tropomyosin expression by both compounds was induced at 1 nM (20%, $p<0.05$). In addition, myosin heavy chain (MHC) protein expression was increased 50 % at day 4 of differentiation. Solbone A or synthetic $1\alpha,25(OH)_2D_3$ had no effects on myogenin nor MHC cell localization. Cellular mass increased with myogenesis progression, being Solbone A more effective than synthetic $1\alpha,25(OH)_2D_3$. Finally, Solbone A, as well as synthetic $1\alpha,25(OH)_2D_3$, augmented the index fusion of cultured muscle fibers. In conclusion, these results demonstrated that Solbone A exhibit at least equal or greater effects on early myoblast differentiation as synthetic hormone, suggesting that plant glycosides could be an effective, accessible and cheaper substitute for synthetic $1\alpha,25(OH)_2D_3$ to promote muscle growth.

***Keywords**: Solbone A, synthetic 1$\alpha$,25(OH)$_2$D$_3$, C2C12 cells, differentiation*

## Introduction

Intensive poultry industry for producing both, broilers and laying hens, as a source of food is a steadily increasing animal husbandry activity. Nevertheless, due to the need of faster development rates, industry performs maneuvers that may cause the appearance of various health problems with the consequent economic loss. A balanced diet turns out to be essential for good health and vigor of domesticated birds. Among dietary supplements, the bioactive derivate of vitamin $D_3$, 1α,25-dihydroxyvitamin $D_3$ (1α,25$(OH)_2D_3$), improves both health and muscle strength of broilers correcting frequent myopathies. Supporting the action on muscle physiology, work derived from our laboratory demonstrated the presence of the vitamin D receptor (VDR) in monolayers of chick myoblasts [1] and myotubes (multinucleated cells) as well as in chicken skeletal muscle matured in vivo [2]. Unfortunately elevated production costs of synthetic 1α,25$(OH)_2D_3$ makes unfeasible to use it as supplement for poultry diets. Of relevance, various flowering plants have been shown to contain 1α,25$(OH)_2D_3$ and related vitamin $D_3$ metabolites as detected by highly specific and sensitive biological and chemical procedures [3]. Among the plants in which vitamin $D_3$ metabolites have been found, *Solanum glaucophyllum* is the one which accumulates 1α,25$(OH)_2D_3$ to the greatest extent.

In addition, 1α,25$(OH)_2D_3$ and other vitamin $D_3$ related sterols are present as glycoconjugates as well as in free forms in variable proportions [4]. The presence of glycoside derivatives of 1α,25$(OH)_2D_3$ is a conspicuous feature which endows plants with special pharmacokinetic properties, due to gradual release of the free bioactive form of 1α,25$(OH)_2D_3$ from its glycoconjugates by endogenous animal tissue glycosidases. This results in increased half-life of the hormone in blood when purified plant fractions are administered for therapeutic

purposes [5;6]. Taken altogether, the plant kingdom represents a cheaper and non-polluting source of natural products of commercial interest to cover the widespread nutritional and veterinary medical application of 1α,25$(OH)_2D_3$. Before exerting its actions, vitamin $D_3$ must be converted to a metabolically active form by hydroxylation reactions to render 1α,25-dihydroxyvitamin $D_3$ (1α,25$(OH)_2D_3$). This bioactive derivative plays a critical role in mineral homeostasis, regulation of cell growth and differentiation, muscle intracellular $Ca^{2+}$ levels and contractility; vascular, endocrine and reproductive functions and immunomodulation [7;8].

It is widely known that vitamin D deficiency, like osteomalacia, or metabolic abnormalities such as hypocalcemia, hypophosphatemia, and hyperparathyroidism, are associated with myopathies characterized by muscle weakness, hypotonia, and atrophy in addition to weak bones. This has been confirmed by electrophysiological studies revealing in vitamin D-deficient chicks muscle weakness due to abnormal kinetics of muscle contraction [9] and histological observations of muscle [10], Also, Rodman et al. [11] showed alterations in skeletal muscle calcium metabolism not secondary to variations in plasma calcium and phosphate and atrophy predominantly of type II fibers, these processes being exacerbated by ageing. Accordingly, there is evidence that the administration of vitamin D bioactive derivatives reverse impaired muscle function and structure as well as improve normal tissue performance. Scarce information is available regarding the mechanism by which 1α,25$(OH)_2D_3$ exerts myogenic differentiation at the cellular and/or molecular level. The objective of this work was to evaluate the role of the natural product Solbone composed of 1α,25$(OH)_2D_3$-glycosides compared with synthetic 1α,25$(OH)_2D_3$ on myogenic differentiation in C2C12 myoblasts.

## Experimental

### *Chemicals*

1α,25$(OH)_2D_3$ and Solbone® were kindly provided by Herbonis AG (Basel, Switzerland). Dulbecco's modified Eagle's medium (DMEM) low glucose, with l-glutamine and HEPES, without phenol red, was from US Biological (Swampscott, MA, USA). Fetal bovine serum (FBS) was from Sigma–Aldrich Co. (St. Louis, MO, USA). Sterile horse serum (HS) was from Natocor (Córdoba, Argentina). Primary antibodies anti-myogenin, anti-tropomyosin, anti-myosin heavy chain, anti-tubulin, anti-VDR and secondary antibodies goat anti-rabbit and rabbit anti-mouse horse radish peroxidase-conjugated IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) and MitoTracker Red (MitoTracker Red CMXRos) dyes and Alexa Fluor 488-conjugated anti-mouse secondary antibody were purchased from Molecular Probes (Eugene,OR, USA). The Western Lighting Plus enhanced chemiluminiscence substrate (ECL) was from PerkinElmer, Inc. (Boston, USA). The creatine kinase (CK) assay kit monotest CK NAC was from Wiener Lab. (Rosario, Argentina). The C2C12 murine skeletal muscle cell line was from American Type Culture Collection (Manassas, VA). All other reagents were of analytical grade.

### *Vegetal material*

Solbone A (Herbonis AG) is a water soluble dried leaves extract of the plant *Solanum glaucophyllum* standardized to 50 ppm active vitamin $D_3$ present as 1α,25$(OH)_2D_3$–glycosid (1-[ß-D-glucopyranosyl]-1α,25-dihydroxycholecalciferol) [12].

### *Cell Culture and treatment*

The murine skeletal muscle cell line C2C12 is a good model for studying myogenesis. C2C12 cells were seeded at an appropriate density (120,000 cells/$cm^2$) in Petri dishes (100 mm diameter) and cultured with DMEM supplemented with 10% heat-inactivated (30 min, 56ºC) fetal bovine serum (FBS), 1% nystatine, and 2% streptomycin, without phenol red (GM, growth medium). Cells were incubated at 37ºC under a humidified atmosphere of 5% $CO_2$ in air. Under these conditions, C2C12 myoblasts resemble the activated satellite cells that surround the mature myofibers and proliferate and differentiate participating in the repair of the tissue when a cellular injury exists [13].

To promote myoblast differentiation, C2C12 cells in GM grown up to 70-80 % of confluence were replaced by DMEM without phenol red, supplemented with 1% horse serum (HS, DM, differentiation medium) and cultured up to four days. DM was changed every day.

To study the effects of Solbone A, active vitamin $D_3$ present as 1α,25-Dihydroxyvitamin $D_3$-glycosides, compared to the synthetic $1\alpha,25(OH)_2D_3$, cells were incubated with water dissolved Solbone A and diluted to 1 or 10 nM (calculated as total $1\alpha,25(OH)_2D_3$) in DMEM + HS from the beginning of the differentiation induction and during the experimental period. Another groups of cells were incubated with 1 or 10 nM of synthetic hormone $1\alpha,25(OH)_2D_3$ dissolved in less than 0.01% ethanol as vehicle. Simultaneously, control cells were incubated with the respective vehicles, water or ethanol. The concentrations of $1\alpha,25(OH)_2D_3$ employed in the experimental design were based on prior dose-response studies performed in our laboratory [14]. Because of the short half-life of $1\alpha,25(OH)_2D_3$, the cell culture media were replaced daily.

***Phase- contrast and Fluorescence Microscopy***

Cells were analyzed with phase-contrast microscope Olympus CK2-TR with photographic system using 10X, 20X and 40X objectives.

For fluorescence studies, cells were stained with MitoTracker Red prepared in DMSO and then added to culture medium at a final concentration of 1 mmol/l. After 15-30 min incubation at 37ºC, the cells were washed with PBS (pH 7.4, 8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l $KH_2PO_4$, and 1.44 g/l $Na_2HPO_4$) and fixed with methanol at - 20 ºC for 30 min. For DAPI staining, fixed cells were incubated for 30 min at room temperature in darkness with 1:500 of a stock solution of DAPI (5 mg/ml) and washed with PBS. For immunocytochemistry,cells were fixed as before. After fixation, cells were rinsed three times with PBS, and nonspecific sites were blocked for 1 h in 2 % BSA. Then, cells were incubated with appropriate primary antibodies dilution overnight at 4 ºC. The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. In all cases, cells were examined using a fluorescence microscope (NIKON Eclipse Ti-S) equipped with standard filter sets to capture fluorescent signals, and images were collected using a digital camera.

***Cell lysates protein content determination and cellular mass calculation***

Cells were lysed using a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2 mM $Na_2VO_4$, 2 mM EDTA, 25 mM NaF, 1 mM PMSF, 1% NP40, leupeptin 20 µg/ml and aprotinin 20 µg/ml. Whole cell lysates were collected by aspiration, weighed on an analytical balance and centrifuged at 12,000 *g* during 15 min. The protein content of the supernatant was quantified by the Bradford procedure.Cellular mass was calculated multiplying the protein content by the weight of the sample and data are expressed as percent variation with respect to the day 0 of differentiation.

***Creatine kinase assay***

Creatine kinase (CK) activity was measured in whole cell lysates by coupling the hexokinase and glucose-6-phosphate dehydrogenase reactions according to manufactur instructions (kit monotest CK NAC from Wiener lab., Argentina). One unit of activity represents 1 µmol of NADPH generated per min per mg protein.

***Western Blot analysis***

Equal amounts of protein (15-20 µg) from each sample were dissolved in Laemmli sample buffer [15] and separated on 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Unspecific sites on the membrane were blocked with TBST buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1% Tween-20) containing 5% dry milk for 1 h at room temperature and then, they were subject to immunoblotting using different primary antibodies [anti-myogenin (1:1000), anti-tropomyosin (1:1000), anti- myosin heavy chain (1:500) and anti-tubulin (1:5000), Anti-VDR (1:4000)] overnight at 4ºC. Membranes were then washed three times in TBST, incubated in TBST containing 1% dry milk with peroxidase-conjugated secondary antibody for 1 h at room temperature and washed again three times with TBST. Next, membranes were visualized using an enhanced chemiluminescence technique (ECL) according to the manufacturer's instructions. Blots were quantified using image J software, a public domain program, developed at the National Institutes of Health. To strip the membranes for reprobing with other antibodies, the membranes were washed 10 min in TBST, incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 50 mM mercaptoethanol) for 30 min at 55$^{o}$C, washed 10 min in TBST and then blocked and blotted as described above.

***Index fusion determination***

Culture cells were stained with MitoTracker Red and DAPI as described in "Fluorescence Microscopy". Images with both dyes were collected using a digital camera and were merged. Nuclei were counted in ten randomly chosen microscope fields (2 culture dishes, 5 fields in each dish) at an objective magnification of 40 X. One microscope field usually contained between 150 and 250 nuclei. The fusion index is defined as the number of nuclei in myotubes divided by the total number of nuclei.

***Statistical Analysis***

All experiments were performed at least three times. Quantitative results were expressed as means ± SD. Statistical analysis was carried out using InfoStat software. Analysis of variance (ANOVA) was used to determine differences among mean values between control and treated conditions, which were then, compared using the *post hoc* tests of multiple comparisons Bonferroni or Fisher's Least Significant Difference (LSD). Differences were considered significant at $p<0.05$.

**Results**

To investigate the effects of 1α,25$(OH)_2D_3$-glycosides enriched natural product Solbone A on the differentiation of muscle cells, the murine skeletal muscle cell line C2C12 in differentiation state was chosen as experimental model. In previous studies, we have characterized a differentiation pattern typical of the onset of myogenesis (data not shown). In the proliferative state, non confluent myoblasts were polygonal and had only one nucleus in growth medium (GM). After switching culture conditions from GM to differentiation medium (DM), myoblasts differentiation was revealed showing tipicaly morphological changes

such as alignment, elongation and fusion of mononucleated cells to multinucleated myotubes whereas a subpopulation of cells remained undifferentiated as reserve cells. In parallel to these morphological changes, the total protein content and specific biochemical markers of muscle differentiation such as creatine kinase, myogenin, tropomiosin and myosin were raised during the first seven days of the progression of differentiation as reported before (data not shown) [13;16;17]. Under these conditions, the role of the natural product Solbone A on myogenesis was studied and compared to syntethic $1\alpha,25(OH)_2D_3$.

First, the activity of the muscle differentiation marker enzyme, creatine kinase (CK), was evaluated in C2C12 myoblasts. Cells were cultured in DM in the presence or absence of 1 nM or 10 nM of Solbone A or synthetic $1\alpha,25(OH)_2D_3$ or in their abscence from 1 to 4 days. CK activity was measured in whole cell lysates as described in Experimental. As expected, CK activity increased according with myogenesis progression. The first day after differentiation induction, 1 nM of synthetic $1\alpha,25(OH)_2D_3$ increased CK activity whereas Solbone A reached the highest levels at the second day showing an increase of 60% above control ($p < 0.05$) (Fig. 1 A). Although similar changes in CK activity were observed in both conditions at 10 nM, the effects of synthetic $1\alpha,25(OH)_2D_3$-were more attenuated (30 %; $p < 0.05$) (Fig. 1 B). In view of these results, we evaluated the effects of Solbone A on distinct parameters paying particular attention at the second day of differentiation. Next, to further explore the effects of Solbone A on miogenesis, vitamin $D_3$ receptor (VDR) expression as well as the myogenic transcription factor myogenin and the early and late muscle specific proteins, tropomyosin and myosin heavy chain (MHC) were evaluated by Western blot analysis. C2C12 cells were cultured in DM and

treated with Solbone A or synthetic 1α,25$(OH)_2D_3$ at 1 or 10 nM or in their absence for 2 days. The results shown in Fig. 2 revealed that both compounds statistically increased VDR expression by 30 % and 50 %, respectively ($p<0.05$). In accordance, cells exposed to 10 nM of Solbone A or synthetic hormone showed a marked rise of the key myogenic regulatory factor myogenin in comparison with control cells (100 % and 70 % respectively, $p<0.05$) (Fig. 3). However, Solbone A likewise the synthetic hormone statistically increased by 20% ($p<0.05$) the early myogenesis protein marker expression tropomyosin at 1 nM at day 2 of differentiation (Fig. 4). Myosin heavy chain (MHC) is a mature muscle cell marker. Thus, the effect of Solbone A on the expression of MHC was measured at days 2 and 4 after differentiation induction. The results shown in Fig 5 revealed that cells exposed to Solbone A 1nM (Fig. 5 A) and 10 nM (Fig 5 B) increased MHC expression (30 % and 60 %, respectively; $p<0.05$) in comparison with control cells at day 2 of myoblast differentiation. Enhanced MHC expression induced by 1 nM of synthetic or natural hormone persisted at day 4 of differentiation (Fig. 5 A) although no statistically significant differences were found at 10 nM of Solbone A (Fig 5 B). Considering the above results, we examined the cellular localization of myogenin and MHC treated with 1α,25$(OH)_2D_3$-glycosides from Solbone A and synthetic 1α,25$(OH)_2D_3$ and compared the effects of both compounds on myotubes formation. After the experimental period cells were fixed, unspecific sites were blocked, and were incubated with appropriate primary antibodies that were then recognized by fluorophore-conjugated secondary antibodies as was described in Experimental. In control condition, myogenin localized mainly to the nucleus while MHC was located in the cytoplasm (Fig. 6). Nevertheless, natural

1α,25$(OH)_2D_3$-glycosides from Solbone A or synthetic 1α,25$(OH)_2D_3$ (1 nM or 10 nM) had no effects on the subcellular localization of myogenin nor MHC. Next, we evaluated if the natural 1α,25$(OH)_2D_3$-glycosides enriched Solbone A induces changes on C2C12 cells morphology under the differentiation state. To that end, C2C12 cells were treated in the presence or absence of 1 nM or 10 nM of Solbone A or synthetic 1α,25$(OH)_2D_3$ and cellular mass and index fusion were calculated as described in Experimental. The results shown in Fig. 7 demonstrated that muscle growth measured by cellular mass increased according with myogenesis progression. Solbone A (1 nM or 10 nM) treated cells showed the greatest increase after 4 days of initiated the differentiation (Fig. 7, A and B). Micrographs of 4 days differentiation cultures support this enhancement in the cellular mass which is reflected in the morphology of cultured muscle fibers (Fig. 7, lower panel). As shown in Fig. 8, muscle growth measured by index fusion in 4 days differentiation cultures increased with 1 nM or 10 nM Solbone A treatments as well as with synthetic 1α,25$(OH)_2D_3$. Accordingly, morphological changes in Solbone A treated cells were much more evident than those of synthetic 1α,25$(OH)_2D_3$ reflecting the augment in cell mass. As revealed in the micrographs of 4 days differentiation muscle cell cultures, both concentrations of Solbone A produced a more prominent alignment, elongation and fusion of mononucleated cells to multinucleated myotubes than the control and synthetic 1α,25$(OH)_2D_3$.

## Discussion

Intensive poultry industry for producing both, broilers and laying hens, as a source of food is a steadily increasing animal husbandry activity since it can produce meat in a faster and cheaper manner than ruminants in similar

systems. Nevertheless, due to the need of faster development rates, industry turns either into genetic manipulation whenever possible in order to obtain races that grow quickly, or more frequently, birds are kept under raising conditions that maximize their growth. However, these maneuvers may cause the appearance of various health problems such as heart failure, injuries, lameness and death. Also, considerable controversy persists regarding the use of human antibiotics to promote growth in animals raised for food. The World Health Organization, the American Medical Association, and the American Public Health Association claim that the use of growth-promoting antibiotics leads to increased antibiotic-resistant infections in humans [18]. Consequently, to minimize these problems, a balanced diet turns out to be essential for good health and vigor of domesticated birds. Among dietary supplements, the use of vitamin $D_3$ improves both bone health and muscle strength of broilers correcting frequent myopathies [19-21], feedlot steers good health [22] and meat tenderness [23].

As we already mentioned, *Solanum glaucophyllum* has been shown to contain the greatest amounts of 1α,25$(OH)_2D_3$ glycoconjugates and related vitamin $D_3$ metabolites among various flowering plants [4]. The natural product Solbone A is a dried leaves extract of this plant [12] and represent the natural source of the active form of vitamin D first available in a cold water soluble form. Among other positive aspects, Solbone A effect starts earlier than regular vitamin $D_3$ which needs two metabolic steps to become active [24]. The active component of Solbone A is available at the point of entry to the body, where it induces the transport of calcium and phosphorus and therefore is recommended when liver and/or kidney function is impaired, in older animals and in conditions induced by stress [12;25;26].

In order to study the role of the natural product Solbone A compared to synthetic 1α,25$(OH)_2D_3$ on myogenesis, CK activity, VDR and myogenic markers expression, morphological characteristics, cellular mass and index fusion, were evaluated in C2C12 myoblasts. Similarly to synthetic hormone, 1 and 10 nM of Solbone A enhanced CK activity (60% and 30 %, respectively) at day 2 of differentiation induction (Fig. 1). Supporting this observation, it have been reported that, the synthetic hormone increased creatine kinase activity in primary cultured chick myoblasts under differentiated conditions indicating stimulation of myogenesis [27].

1α,25$(OH)_2D_3$, as other steroid hormones, exerts its action binding to a specific receptor: the vitamin D receptor (VDR), localized commonly in the nucleus [28-31]. We have known for more than 30 years that vitamin D exerts effects on muscle cells at a molecular level [32-35]. However, recent reports suggest that VDR is not detectable in muscle and therefore propose the effects of vitamin D on muscle via an indirect route of action [36;37]. This controversy in the field could be due to differences in the expression of VDR in muscle of different species and throughout the various stages of muscle differentiation [7]. In addition, as we have mention before, we previously reported the presence of the VDR in chick myoblasts [1] and myotubes and in chicken skeletal muscle *in vivo* [2]. Thus, the expression of VDR in muscle tissue further supports a direct role of vitamin D in muscle physiology.

Our study showed that treatment with Solbone A of murine C2C12 cells enhanced VDR expression as does the synthetic hormone, after two days differentiation induction (Fig. 2). In accordance, same amounts of Solbone A showed a marked rise in the key myogenic regulatory factors myogenin like synthetic 1α,25$(OH)_2D_3$ (Fig. 3). In line with our results, it has been reported

that 1α,25$(OH)_2D_3$ increases VDR expression, induces its translocation to the nuclei [38] and triggers the synthesis of new proteins involved in muscle cell contractility, proliferation, and differentiation [39]. In addition, 1α,25$(OH)_2D_3$ promotes proliferation of C2C12 myoblasts through Akt activation by PI3K and p38 MAPK [35]. Moreover, mice VDR knockout models exhibit smaller muscle fibers, higher levels of Myogenic Regulatory Factors (family of transcription factors that modulate satellite cell differentiation –MRFs-) and persistence of immature muscle gene expression during adult life [34;40]. This phenotype is reversed upon vitamin D administration [34]. Also MyoD, another MRF, was reported to be modulated by 1α,25$(OH)_2D_3$ [38]. Further investigations on the regulatory mechanisms underlying 1α,25$(OH)_2D_3$-dependent myogenesis are still needed.

Solbone A likewise the synthetic hormone increased tropomyosin, the early myogenesis protein marker, expression at day 2 of differentiation (Fig. 4) and raised the expression of the specific mature myotube protein MHC even more than the synthetic hormone (Fig. 5). Of relevance, Src and $PI_3K$ are involved in Akt activation and in MHC and myogenin increased expression by 1α,25$(OH)_2D_3$ [35].

Once myogenic cells are committed, they must differentiate and fuse into multinucleated myotubes. Normal physiology of mature muscle fiber requires adequate vitamin $D_3$ levels, as shown in studies employing animals deprived of vitamin $D_3$, which were found to generate less than one-half the normal muscle tension in response to repetitive electrical stimulation. In addition, relaxation after muscle contraction is slowed in the vitamin D-deficient chickens [9] These alterations were reversed upon administration of vitamin $D_3$ and modifying dietary calcium concentration. Also, vitamin $D_3$ therapy increased calcium

content of skeletal muscle mitochondria in vitamin D-deficient chickens. The rise in mitochondrial calcium was correlated with increased force of skeletal muscle contraction [9]. This muscle weakness associated to deficiency of vitamin $D_3$ metabolites results in increased morbility and mortality of broilers and laying hens observed in large-scale poultry farming, causing thereby considerable economical losses.

Results obtained by cellular mass (Fig. 7) and index fusion (Fig. 8) as well as by image analysis from phase- contrast and fluorescence microscopy revealed that Solbone A improved growth and differentiation of C2C12 cells at the onset of myogenesis.

While Solbone A represents a natural source enriched in the active form of vitamin $D_3$, it should not be ignored that, like many other vegetable materials, contains other beneficial compounds such as flavonoids with antioxidant action. The presence of these active principles in the leaves extract could be affecting cell behavior and even exerting a synergistic action with respect to $1\alpha,25(OH)_2D_3$. Moreover, the gradual release of the bioactive form of its free glycoconjugates by endogenous glycosidases presents in animal tissues could result in an increase half-life of the hormone in blood when plants purified fractions are administered for therapeutic purposes representing another beneficial aspect of this natural product.

Further research is needed to explain whether the isolated $1\alpha,25(OH)_2D_3$-glycosides or its synergistic action with other compounds are responsible for the effects observed during the onset of C2C12 myoblast differentiation. In conclusion, our data suggest that Solbone A could represent an effective, accessible and economic source of natural $1\alpha,25(OH)_2D_3$-glycosides to stimulate myoblast growth and differentiation.

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## Figure Legends

**Fig. 1. Solbone A as synthetic 1α,25(OH)$_2$D$_3$ increased creatine kinase activity in C2C12 cell myogenesis as a function of culture time**. CK activity of cells exposed to 1 nM (**A**) or 10 nM (**B**) of synthetic (1α,25(OH)$_2$D$_3$) or natural 1α,25(OH)$_2$D$_3$-glycosides (Solbone A) or in their absence (control) is shown. CK activity represented in bar graphs is expressed as a radio between treated conditions (1α,25(OH)$_2$D$_3$ and Solbone A) versus control referred to day 0 of differentiation. Values are the mean ± S.D. of three independent experiments. Different letters indicate statistical differences among groups for each day (ANOVA - Bonferroni $p$<0.05).

**Fig. 2. Changes in VDR expression induced by synthetic 1α,25(OH)$_2$D$_3$ and Solbone A in C2C12 cell myogenesis at day two of differentiation**. Cells were exposed to 1 nM (**A**) or 10 nM (**B**) of synthetic (1α,25(OH)$_2$D$_3$) or natural 1α,25(OH)$_2$D$_3$ (Solbone A) or in their absence (control) for 2 days. Western blots were performed with anti-VDR and anti-tubulin antibodies. A representative blot from three independent experiments is shown. Protein bands quantification from three independent experiments was done using Image J program. The results (mean ± S.D.) were then represented in bar graphs as a radio of VDR protein normalized with tubulin from treated conditions (1α,25(OH)$_2$D$_3$ and Solbone A) versus the control. Different letters indicate statistical differences among groups for each concentration (ANOVA - Bonferroni $p$<0.05).

**Fig. 3. Synthetic 1α,25(OH)$_2$D$_3$ and Solbone A induced myogenin protein expression in C2C12 cell myogenesis at day two of differentiation**. Cells were exposed to 1 nM (**A**) or 10 nM (**B**) of synthetic (1α,25(OH)$_2$D$_3$) or natural

$1\alpha,25(OH)_2D_3$ (Solbone A) or in their absence (control) for 2 days. Western blots were performed with anti-myogenin and anti-tubulin antibodies. A representative blot from three independent experiments is shown. Protein bands from three independent experiments were quantified, normalized and expressed as described in legend of Fig. 2. Values represented in bar graphs are the mean ± S.D. Different letters indicate statistical differences among groups for each concentration (ANOVA - Bonferroni $p<0.05$).

**Fig. 4. Changes in tropomyosin expression induced by synthetic $1\alpha,25(OH)_2D_3$ and Solbone A in C2C12 cell myogenesis at day two of differentiation**. Cells were exposed to 1 nM (**A**) or 10 nM (**B**) of synthetic ($1\alpha,25(OH)_2D_3$) or natural $1\alpha,25(OH)_2D_3$ (Solbone A) or in their absence (control) for 2 days. Western blots were performed with anti- tropomyosin and anti-tubulin antibodies. A representative blot from three independent experiments is shown. Protein bands from three independent experiments were quantified, normalized and expressed as described in legend of Fig.2. Values represented in bar graphs are the mean ± S.D.. Different letters indicate statistical differences among groups for each concentration (ANOVA – LSD Fisher $p<0.05$).

**Fig. 5. Changes in myosin heavy chain (MHC) expression induced by synthetic $1\alpha,25(OH)_2D_3$ and Solbone A in C2C12 cell myogenesis as a function of culture time**. Cells were exposed to 1 nM (**A**) or 10 nM (**B**) of synthetic ($1\alpha,25(OH)_2D_3$) or natural $1\alpha,25(OH)_2D_3$ (Solbone A) or in their absence (control) for 2 and 4 days of differentiation. Western blots were performed with anti- tropomyosin and anti-tubulin antibodies. A representative blot from three independent experiments is shown. Protein bands from three

independent experiments were quantified, normalized and expressed as described in legend of Fig.2. Values represented in bar graphs are the mean ± S.D. Different letters indicate statistical differences among groups for each day (ANOVA – LSD Fisher $p<0.05$).

**Fig. 6. Effects of synthetic 1α,25(OH)$_2$D$_3$ and Solbone A on myogenin and myosin heavy chain (MHC) cellular localization in differentiated C2C12 cells**. Cells were exposed to 1 nM or 10 nM of synthetic (1α,25(OH)$_2$D$_3$) or natural 1α,25(OH)$_2$D$_3$ (Solbone A) or in their absence (control) for 4 days. Cells were fixed, unspecific sites were blocked and incubated with anti-myogenin and anti-MHC antibodies as was described in Experimental. The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Fluorescent micrographs, representatives from three independent experiments, reflect myogenin red stained, MHC green stained or both (merge). Magnification 400X.

**Fig. 7. Solbone A as the synthetic hormone increases cellular mass at the onset of myoblast differentiation**. Cells were exposed to 1 nM (**A**) or 10 nM (**B**) of synthetic or natural 1α,25(OH)$_2$D$_3$ or in their absence (control) for 2 and 4 days. Cell mass was calculates as described under Experimental. Data represented in bar graphs are expressed as percent of variation between treated conditions versus control referred to day 0 of differentiation. Values are the mean ± S.D. of three independent experiments. Different letters indicate statistical differences among groups for each day (ANOVA - Bonferroni $p<0.05$). Micrographs at phase-contrast microscopy represent effects on morphology of myoblast after 4 days of DMEM 2% HS differentiation induction. Images shown are representative from three independent experiments. Total magnification 400X. Bar = 50 µm.

**Fig. 8. Solbone A as the synthetic hormone increases the Index Fusion at the onset of myoblast differentiation**. Cells were exposed to 1 nM or 10 nM of synthetic or natural 1α,25$(OH)_2D_3$ (Solbone A) or in their absence (control) for 4days. Cells were stained with MitoTracker Red and DAPI and index fusion was calculated as was described in Experimental. Data represented in bar graphs are the mean ± S.D. of five independent experiments. Different letters indicate statistical differences among groups for each concentration (ANOVA - Bonferroni $p<0.05$). Right panel, merge images of mitochondria (red stained) and nuclei (blue stained) are shown. Fluorescent micrographs are representative from three independent experiments. Total magnification 400X. Bar = 50 µm.

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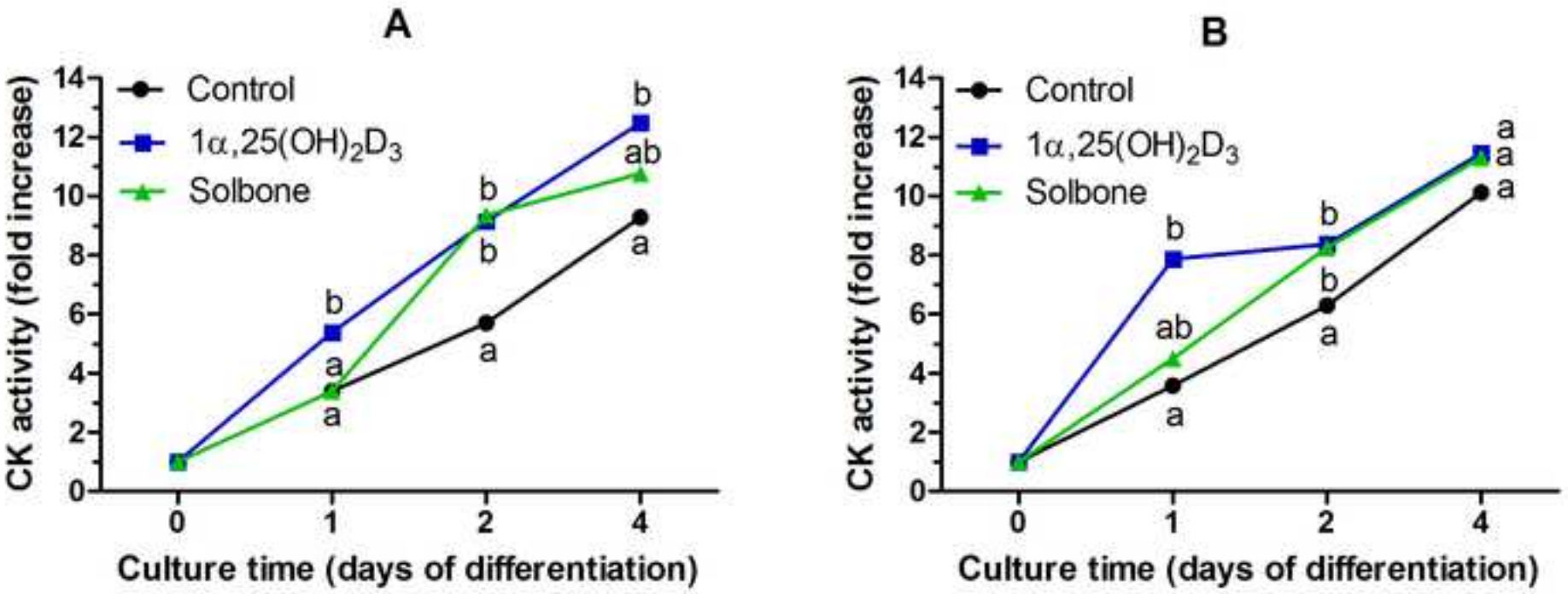


Fig. 1

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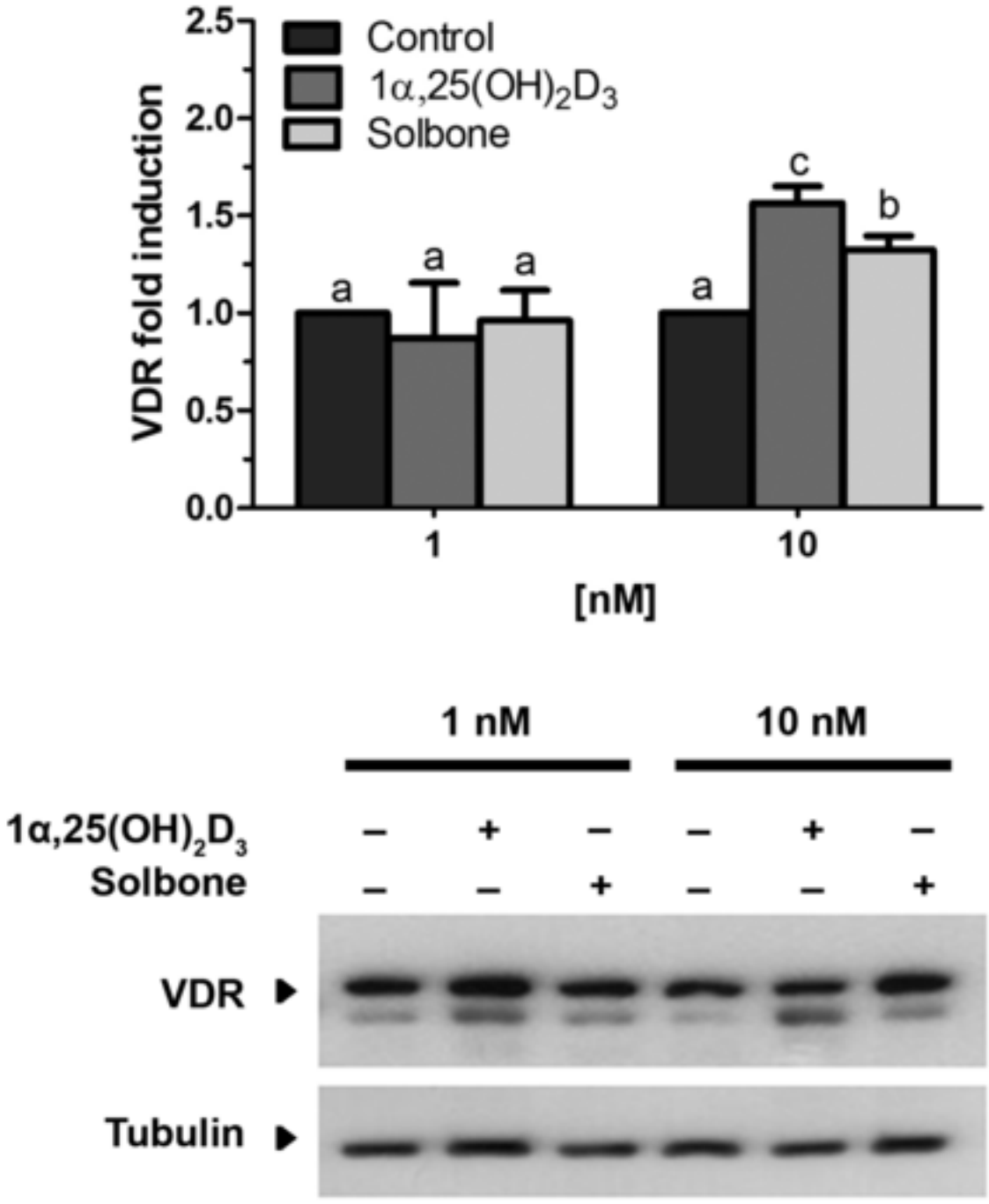


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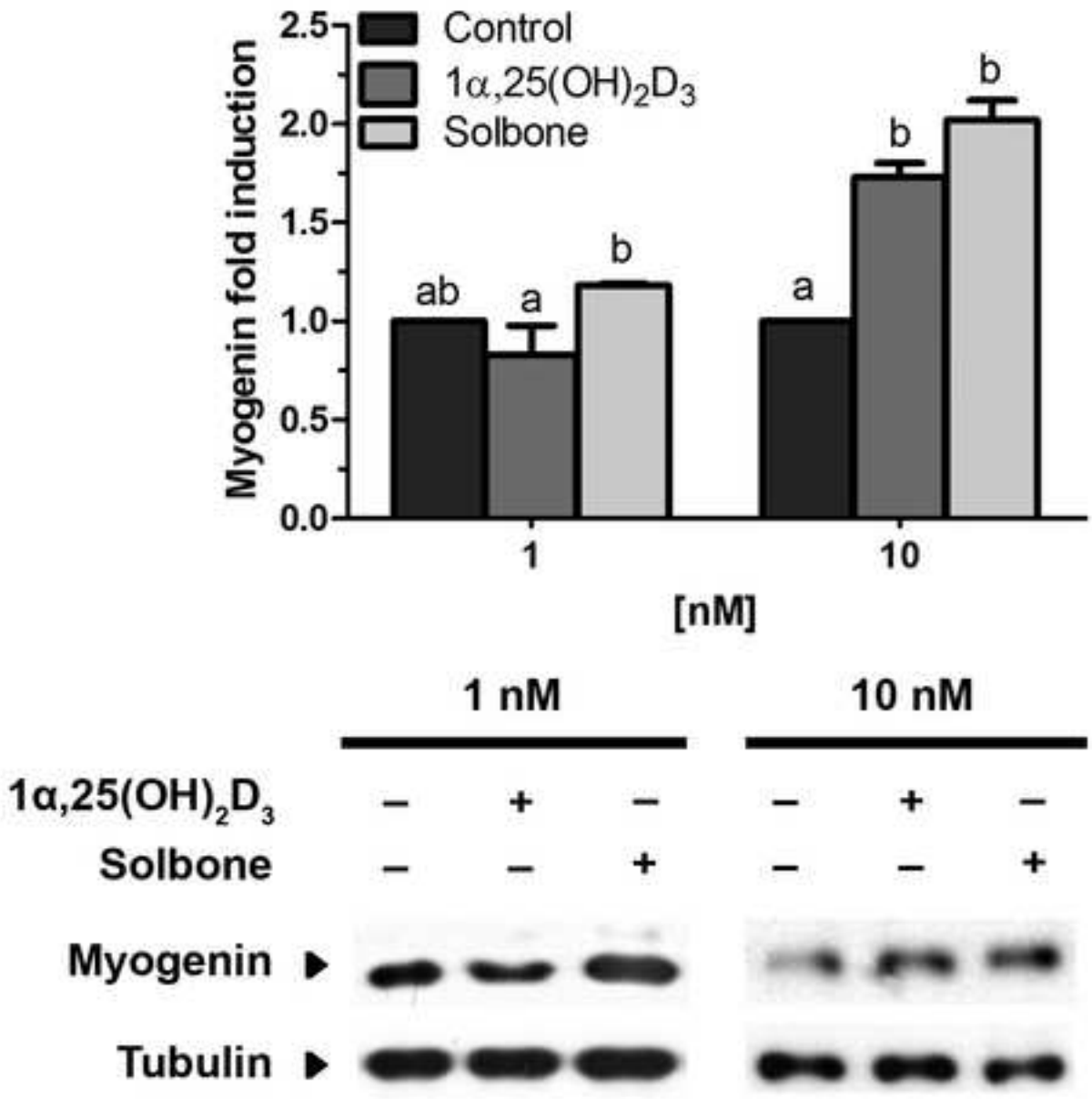


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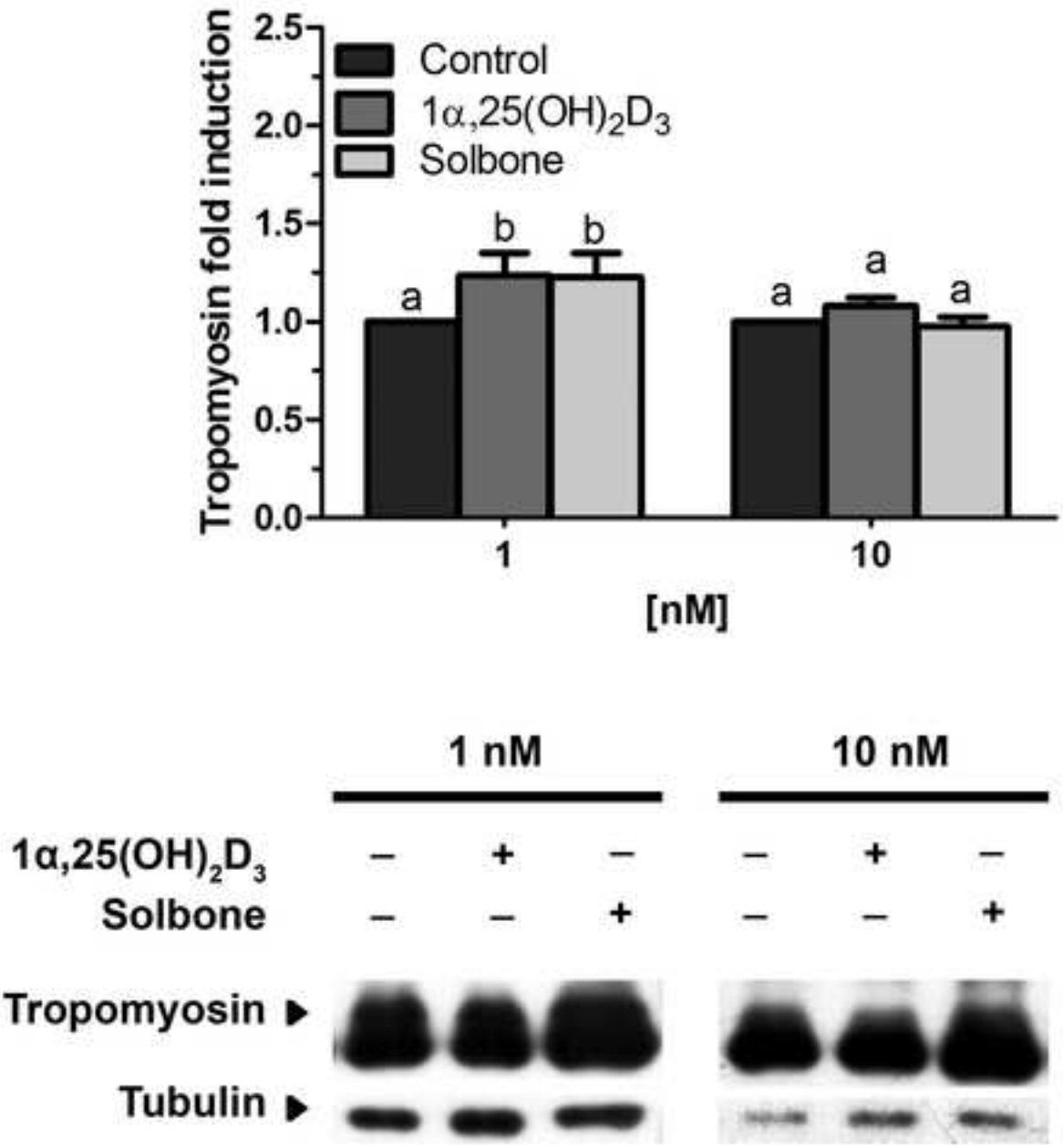


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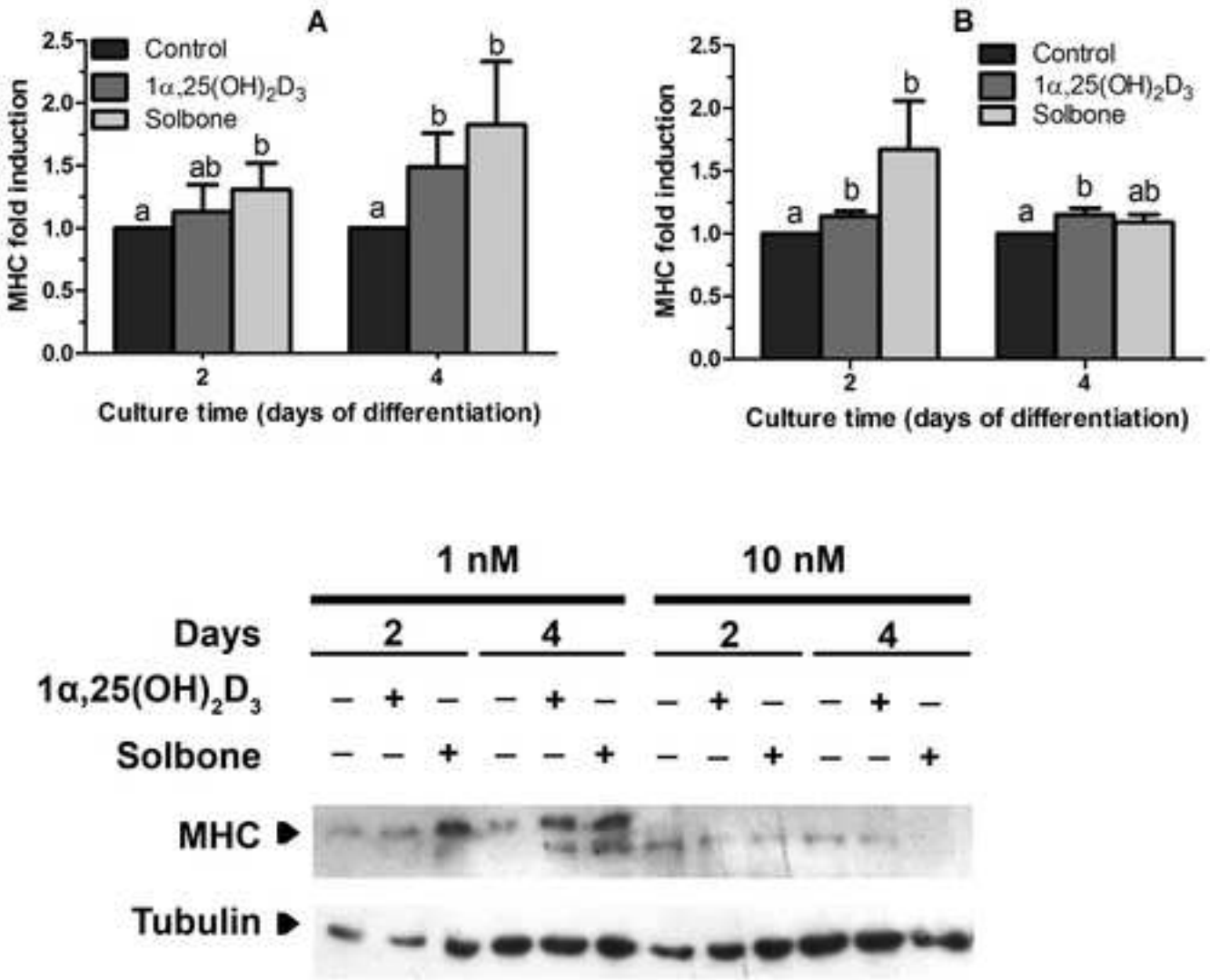


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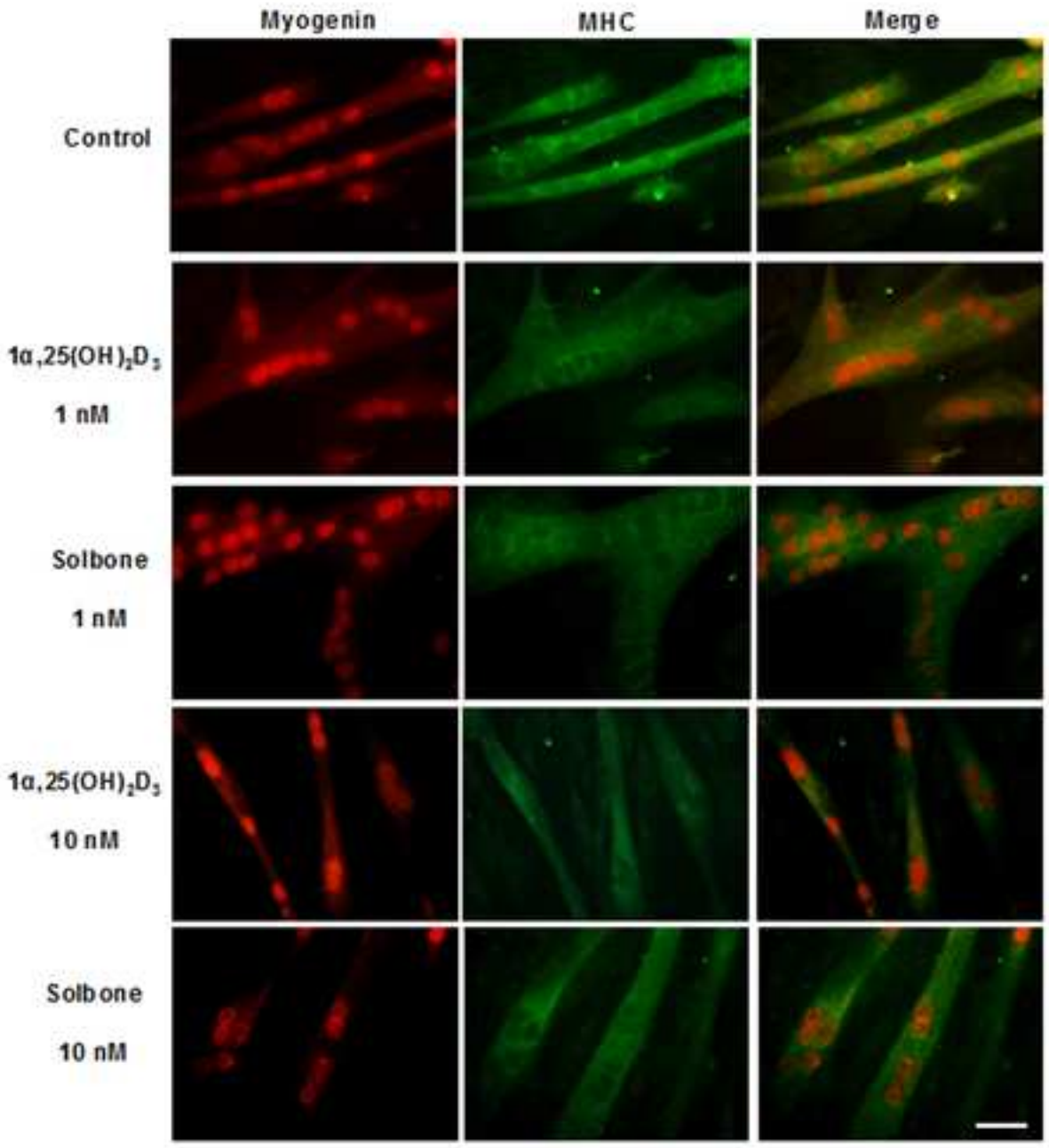


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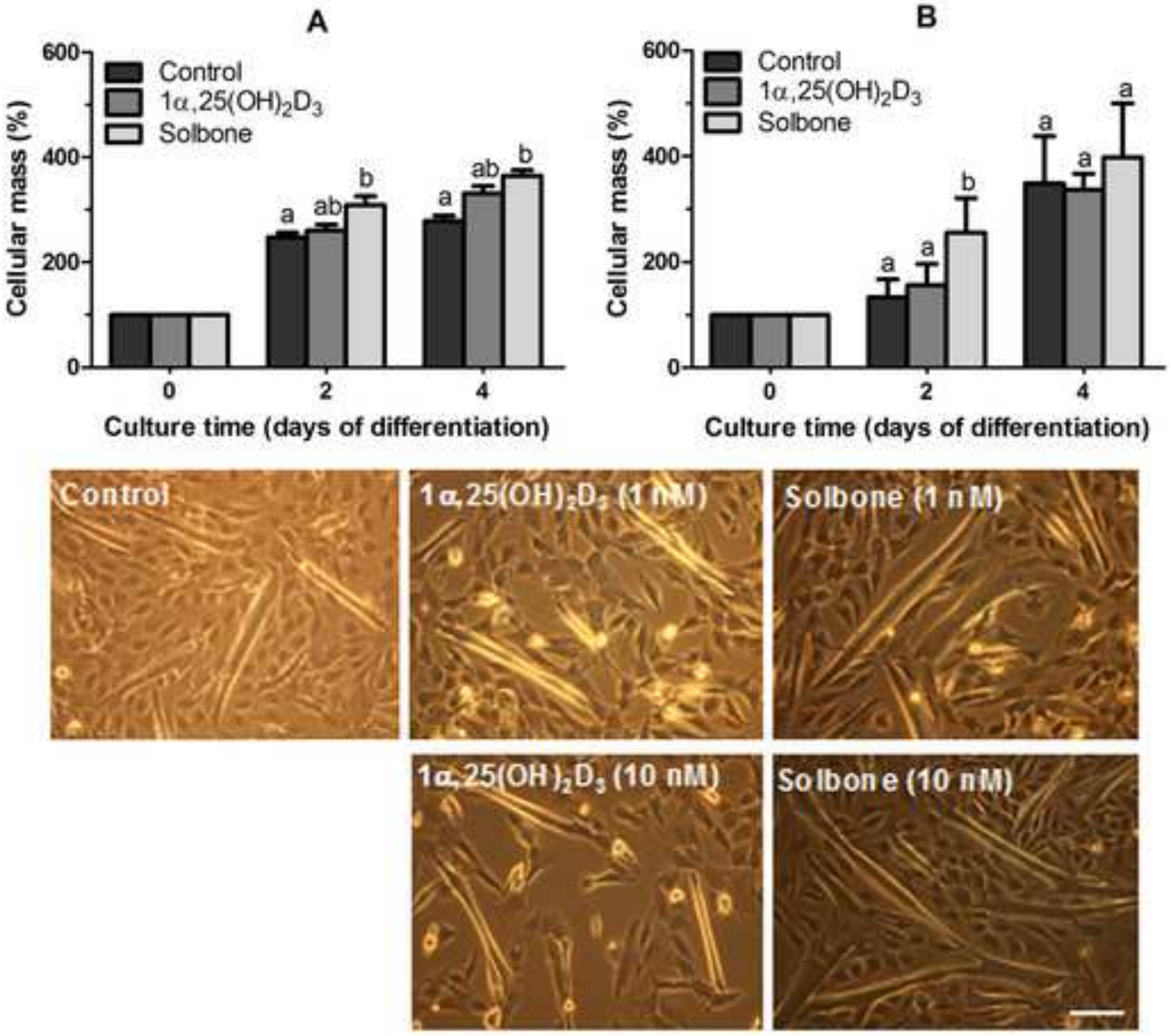


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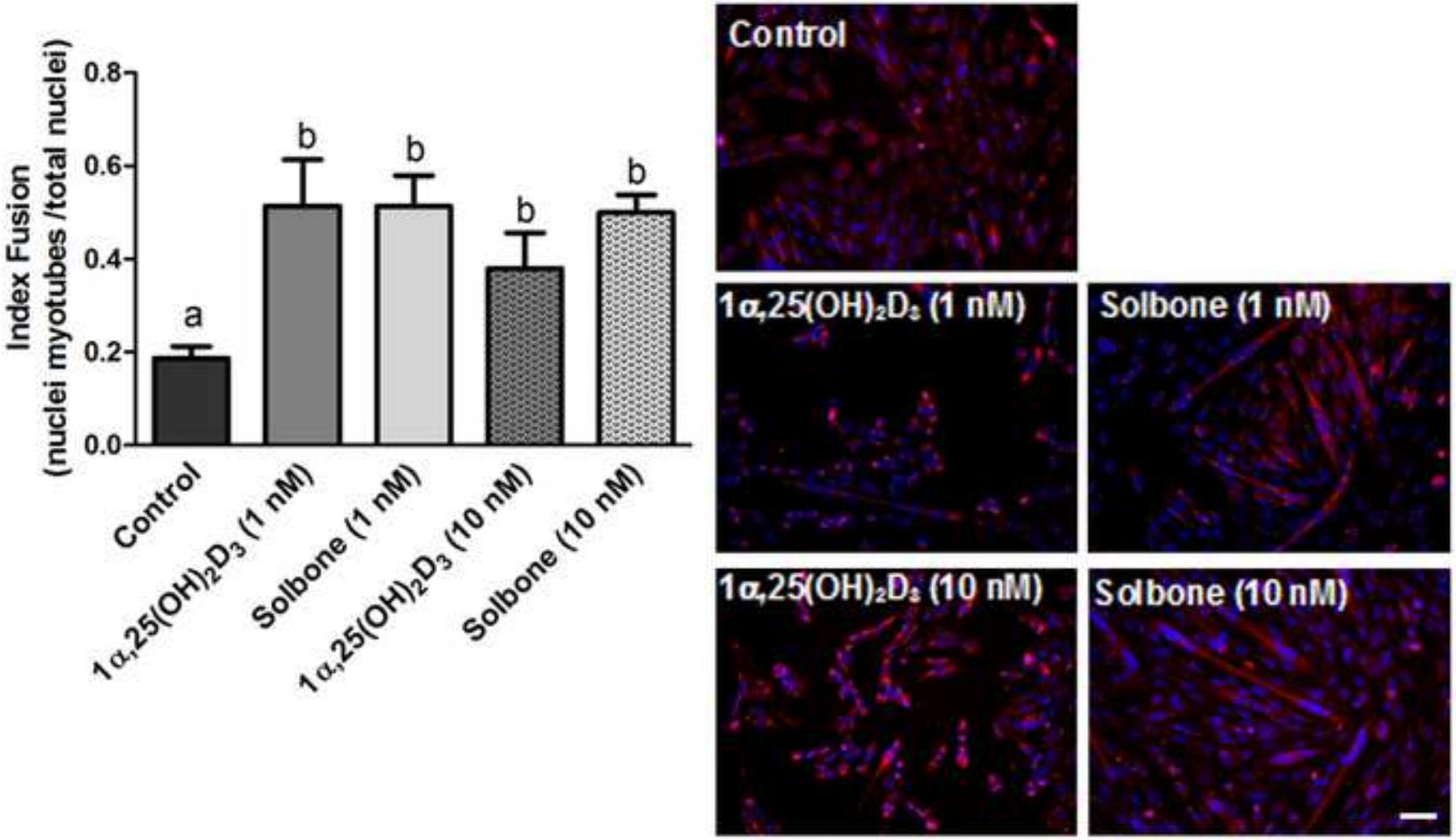


Fig. 8