Vitamin K2 improves proliferation of bovine skeletal muscle cells *in vitro*

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Abstract – Skeletal muscle function is highly dependent on the ability to regenerate, however during ageing or pathology, the proliferative capacity is reduced, leading to loss of muscle function. Dietary components have an important role in skeletal muscle function, and it is therefore important to identify components that can be included in a balanced diet in order to stimulate muscle growth. In this study, we used primary bovine skeletal muscle cells, cultured in monolayers *in vitro*, to assess a potential positive effect of vitamin K2 in muscle cells. Our experiments demonstrate that the muscle cells were metabolically active, independent of supplied MK-4, and that the added MK-4 was not toxic to the muscle cells. Furthermore, adding MK-4 to the cells lead to an increased muscle proliferation and increased expression of the myogenic transcription factors myod and myogenin. The observed effect on muscle proliferation most likely involved specific lipoprotein density receptors and a different production of extracellular matrix components. Meat and animal by-products are good nutritional sources of vitamin K2, and can be included in a balanced diet that stimulates muscle growth, improve muscle maintenance and attenuate muscle function.

Key Words – Dietary components, Muscle cell growth, Vitamin K2, Menaquinone.

I. INTRODUCTION

Vitamin K was first recognized for its vital function in coagulation of blood, and is demonstrated to be important for bone formation, soft-tissue calcification, cell growth and apoptosis [1]. Vitamin K2 has also been shown to have antiproliferative effect on several types of cancer cells [2]. Dietary intake of vitamin K is obtained from green plants in the form of phylloquinone (vitamin K1), and animal foods like meat and cheese in the form of menaquinones (vitamin K2). There are several different vitamers in the "menaquinone-family", in which MK-4 is the smallest. We have previously shown that MK-4 concentration varies between different bovine muscles, but also between breeds [3]. The longer vitamers (MK-7 – MK-13) are produced by bacteria and gut microflora in mammals. These longer vitamin K2-members are mainly stored in the liver. Therefore meat and animal by-products are good nutritional sources of vitamin K2. Although vitamin K2 seems to be present in most muscles and organs the mechanisms for how it is transported from the intestine to the individual muscles and organs are not fully understood.

The skeletal muscles account for a large part of the human body mass and are mainly composed of post-mitotic, multinucleated muscle fibers. The skeletal muscle comprises more than 600 individual muscles, and is not only important for movement, but is also a major metabolic organ. Skeletal muscle function is highly dependent on the ability to regenerate. Fifty years ago Mauro first suggested that satellite cells were involved in the skeletal muscle regeneration [4]. Since the first discovery of these cells, numerous reports have identified these stem cells as primary contributors to the postnatal growth, maintenance and repair of skeletal muscles. The satellite cells are located between the basal lamina and sarcolemma (plasma membrane) of skeletal muscle fibers and are normally quiescent in the adult muscle before they become activated upon exercise, injury or disease. Then the cells have a remarkable ability to self-renew, expand, grow or undergo myogenic differentiation to fuse and restore damaged muscle [5]. During ageing or pathology, the proliferative capacity is reduced, leading to loss of muscle function. Dietary components have an important role in normal skeletal muscle function, and therefore it is important to identify components that can be included in a balanced diet in order to stimulate muscle growth, and as such improve muscle maintenance and attenuate muscle function.

In this study we aimed to assess the potential positive effects of vitamin K2 using an *in vitro* model of bovine skeletal muscle cells.

II. MATERIALS AND METHODS

Vitamin K2: Menaquinone, MK-4 (Sigma Aldrich) dissolved in EtOH. *Skeletal muscle cells:* Bovine primary skeletal muscle cells were isolated as previously described [6]. 4 hours after seeding (day 1), the cells were treated with 1, 10, 20 or 50 µM MK-4 or control (0.05% EtOH). Then the cells were grown for 3 days before analysis. *Cell viability assay:* Measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega). *Cytotoxicity assay:* Toxicity was measured as LDH release into the cell media and was performed according to protocol (cat. no. 11 644 793 001, Roche Applied Science, Mannheim, Germany). Incubation with 2% Triton X-100 for 2 h was used as positive control. *Cell proliferation*: Cell proliferation was measured using CyQUANT cell proliferation assay (Invitrogen). *RNA extraction and real-time PCR*: Cells were lysed and further purified using RNeasy minikit including DNase treatment. Real-time PCR was performed on a ABI Prism 7700 Sequencing detection system. Gene expression was normalized to TATA and ΔCt values calculated. Comparison of gene expression between two samples was derived from subtraction of ΔCt values between the two samples to give a ΔΔCt value, and relative gene expression calculated as $2^{-\Delta\Delta Ct}$. *Statistical analysis*: ANOVA was performed using the Minitab 17 software. Tukey's test was used to identify difference between samples. Means that do not share a letter are significantly different. Bars presented are mean of minimum three independent experiments ±SEM.

III. RESULTS AND DISCUSSION

The prescence of vitamin K2 in skeletal muscle [3] implies a direct role in muscle regulation and function. Our experiments demonstrate that the amount of ATP was unchanged when MK-4 was added to the cells, indicating that the muscle cells were metabolically active (Fig.1A), independent of supplied MK-4. It has previously been shown that MK-4 plays a function in mitochondria by speeding up the transfere of electrons, which results in more efficient ATP production [7]. Also, MK-4 did not induce any LDH release into the media (Fig.1B), suggesting that the added concentrations of MK-4 were not toxic to the muscle cells.

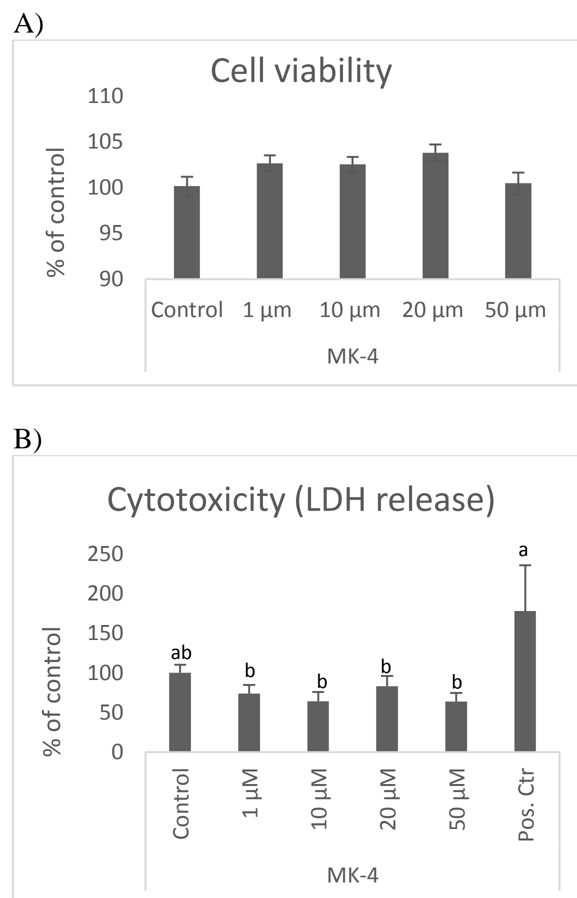

Fig. 1: A) The muscle cells were metabolically active during MK-4 incubation compared to control cells (0.05% EtOH). Primary muscle cells were incubated with MK-4 for three days, followed by measuring cell viability (CellTiter-Glo, Promega). B) MK-4 did not induce any lactate dehydrogenase release into the media, suggesting that MK-4 is not toxic to the cells (Cytotoxicity assay, Roche).

After 72 hours of MK-4 treatment, there were a significant increase in muscle cell proliferation, that was dose-dependent (Fig. 2A), compared to

control cells, demonstrating that 10 µM MK-4 increases muscle cell proliferation. Myogenic transcription factors (MRFs) such as MyoD, Myogenin, and MYH8 (myosin heavy chain 8), as well as the filament protein Desmin are important for muscle cell proliferation. MyoD and Myogenin are important during cell proliferation and early differentiation, while MYH8 is expressed in differentiated cell. We therefore investigated the gene expression of MRFs and desmin in cells incubated for 3 days with 10 µM MK-4 (Fig 2B). Our results show that MyoD and Myogenin increases during MK-4 treatment compared to control cell. We did not observe effect on either Desmin or MYH8.

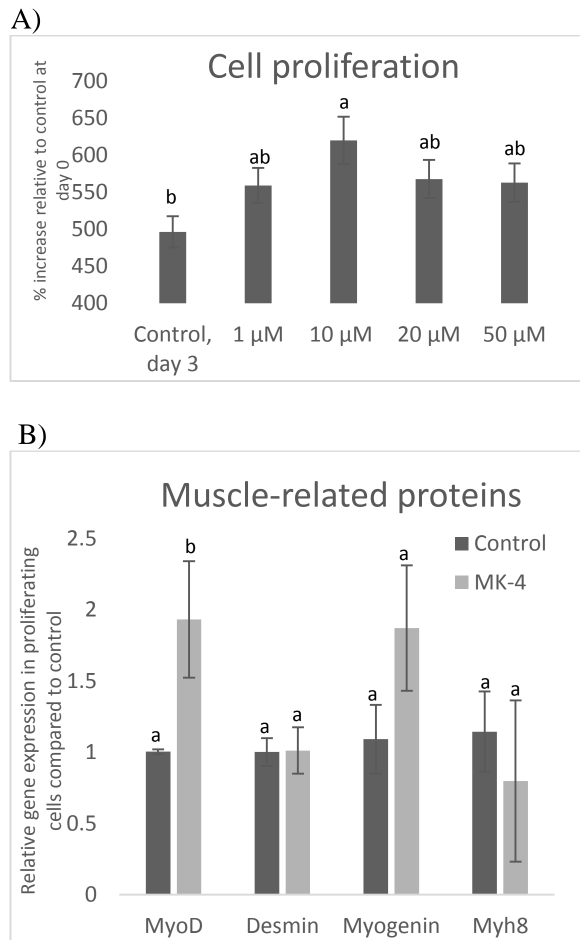

Fig 2: A) Increase in muscle cell proliferation by MK-4 treatment after 3 days, compared with control cells (0.05% EtOH) at day 0.
B) Relative gene expression during 10 µM MK-4 treatment. Bars show the relative mRNA expression of MyoD, Desmin, Myogenin and MyH8 in MK-4 cells compared to negative control cells (0.05% EtOH).

Previous experiments have shown that vitamin K2 is a transcptional regulator of extracellular matrix (ECM)-related genes that may contribute to collagen assembly in osteoblast cells [8]. Our experiments, although not significant, show an increase in the gene expression of syndecan-1, integrin β1 and collagen 1 in MK-4 treated cells (Fig 3A). The molceluar mechanism on how vitamin K is delivered to muscle cells is unknown, but previous experiments have suggested that receptor-mediated endocytosis of low-density lipoprotein receptor-related protein 1 (LRP1) and low-density lipoprotein receptor (LDLR) are important for delivery of vitamin K to bone cells [9]. The ECM gene Syndecan-1 has been demonstrated to be involved regulating low-density lipoprotein remnants. We show that both receptors may be involved in MK-4 uptake (Fig 3B) also in muscle cells.

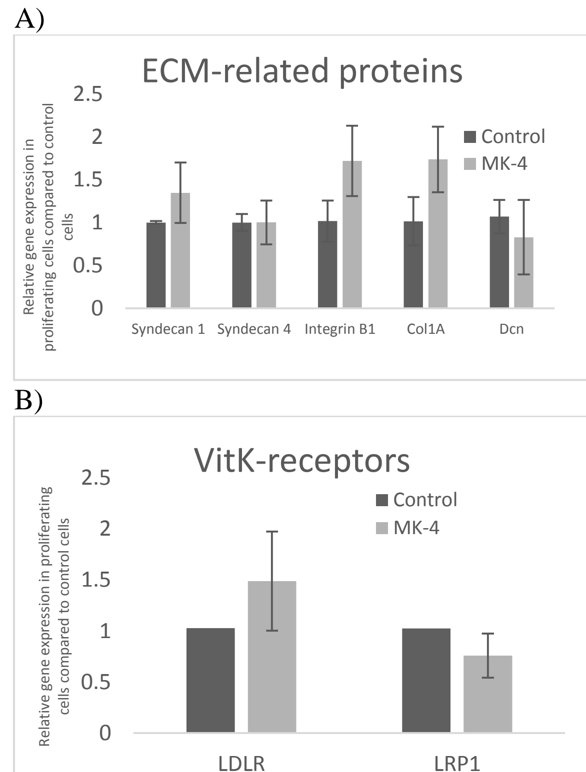

Fig 3: The relative differens in gene expression during 10 µM MK-4 treatment. Bars show the relative mRNA expression of ECM-related proteins in

A) and vitamin K receptors in B) in MK-4 cells compared to negative control cells (0.05% EtOH).

IV. CONCLUSION

Vitamin K is important for coagulation of blood, bone formation, soft-tissue calcification, cell growth and apoptosis, but the effect in skeletal muscle is less known. We show that MK-4 has a positive effect on skeletal muscle cell growth, involving specific lipoprotein density receptors and regulating extracellular matrix genes. Meat and animal by-products are good nutritional sources of vitamin K, and can be included in a balanced diet that stimulates muscle growth, improve muscle maintenance and attenuate muscle function.

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