Application of whey protein isolate in bone regeneration: effect on growth and osteogenic differentiation of bone-forming cells

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Whey protein isolate (WPI) consists of 2 main components, namely β-lactoglobulin (bLG) and α-lactalbumin, WPI can modulate immunity and acts as an antioxidant, antitumor, antiviral and antibacterial agent. However, its effects on bone-forming cells remain unknown. The aim of this study was to test differentiation-stimulating potential of WPI rich in bLG on osteoblast-like cells and adipose-derived stem cells with emphasis on its potential use in tissue engineering. We observed positive effects on cell proliferation, expression of markers of cell differentiation and calcium deposition. Thus, WPI has potential in tissue engineering.

WPI PROMOTES DIFFERENTIATION OF BONE-FORMING CELLS

Application of whey protein isolate in bone regeneration: effect on growth and osteogenic differentiation of bone-forming cells

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ABSTRACT

Recently, milk-derived proteins have attracted attention for applications in the biomedical field, such as tissue regeneration. Whey protein isolate (WPI), especially its main component β-lactoglobulin (bLG), can modulate immunity and acts as an antioxidant, antitumor, antiviral and antibacterial agent. There are very few reports of the application of WPI in tissue engineering, especially in bone tissue engineering. In this study, the influence of different concentrations of WPI on behaviour of human osteoblast-like Saos-2 cells, human adipose tissue-derived stem cells (ASC), and human neonatal dermal fibroblasts (FIB) was tested. The positive effect on growth was apparent for Saos-2 cells and FIB but not for ASC. However, the expression of markers characteristic for early osteogenic cell differentiation i.e. type-I collagen (*COL 1*) and alkaline phosphatase (*ALP*), and also the activity of ALP, increased dose-dependently in ASC cells. Importantly, Saos-2 cells were able to deposit calcium in the presence of WPI even in a proliferation medium without other supplements supporting osteogenic cell differentiation. The results indicate that, depending on the cell type, WPI can act as an enhancer of cell proliferation and osteogenic differentiation. For these reasons, enrichment of biomaterials for bone regeneration with WPI seems a promising approach, especially due to the low cost of WPI.

Key words: whey protein isolate, cell proliferation, osteogenic differentiation, adipose-derived stem cell

INTRODUCTION

Human society, especially in developed countries, is aging, which makes age-related health issues more important. The diseases of the locomotive system, such as fractures, osteoporosis, rheumatoid arthritis and others, are the second most frequent diseases after the cardiovascular diseases (Schliemann et al., 2015). There are many growth factors and hormones known for their supportive effect on bone growth (e.g. bone morphogenetic protein 2 (BMP-2)), nevertheless, isolation and/or production can be complicated and expensive (Bhattacharya et al., 2016). Moreover, there are indications that the application of BMP-2 may lead to negative side effects (Shields et al. 2016). Hence, there is a need for effective and inexpensive alternatives.

Despite the controversy of milk consumption in adulthood (Pereira, 2014), milk is a cheap source of compounds needed for bone development and regeneration. Milk contains 2 main groups of proteins: caseins (which represent 80% of all proteins in ruminants' milk and consists of 4 major proteins) and whey protein (which represents 20% of all proteins in ruminant's milk and consists of 2 main components, namely β-lactoglobulin (bLG) and α-lactalbumin, and smaller amounts of serum albumin, lactoferrin, and other proteins (Do et al., 2016). Whey protein was considered to be a waste product in the dairy industry. It contains the aforementioned compounds in different ratios depending on the method of cheese manufacture. Various types of whey protein exist, such as reduced-lactose whey, demineralized whey, whey protein concentrates, and whey protein isolates (WPI) (Walzem et al., 2002). Whey proteins contain a higher amount of amino acids rich in sulfur in comparison to caseins. It is believed that proteins rich in sulfur provide a higher protein efficiency ratio (i.e. weight gain to intake of protein during the tested period). Moreover, these types of proteins are important in immune modulation (Bounous and Gold, 1991). Whey proteins also

consist of branched-chain amino acids, which promote protein synthesis in muscle cells (Walzem et al., 2002). As mentioned above, the major component of WPI is bLG. It is an interesting protein from a biological point of view. It is a major whey protein of ruminants. It belongs to the lipocalin protein family, which is responsible for a wide variety of functions, especially ligand-binding functions (Flower et al., 2000). The beta-barrel (calyx) within the bLG molecule exhibits ligand-binding properties and it can accommodate hydrophobic molecules, such as vitamins A, D and cholesterol (Kontopidis et al., 2004). Due to this affinity of bLG for hydrophobic molecules, it is used as a carrier protein to improve their uptake and solubility (Diarrassouba et al., 2015; Lee et al., 2013; Ha et al., 2013).

Many studies have been focused on the effect of consumption of milk or its derivatives *in vivo* (Yamaguchi et al., 2015, Parodi, 2007), however a relatively small number of studies have focused on the performance of specific compounds *in vitro* (Pyo et al., 2016, Gillespie et al., 2015). In particular, bLG has been used to influence intestinal and cancer cells. However, applications outside the digestive system have not been studied. For example, the effect of WPI on bone-forming cells remains unexplored.

In this study, the influence of different concentrations of WPI rich in bLG (80% wt) on cell growth and differentiation was tested. Three different cell types were studied: human osteoblast-like Saos-2 cells, human adipose-derived stem cells (ASC), and human neonatal dermal fibroblasts (FIB). Saos-2 cells, a cell line of relatively mature cells with standardized behaviour, were chosen as a representative of bone cells (Czekanska et al., 2012). ASC were chosen as a representative of mesenchymal stem cells. Recently, it was proven that ASC have comparable morphology, phenotype and potential differentiation ability to bone marrow mesenchymal stem cells (Bhattacharya et al., 2016; Levi and Longaker, 2011). Additionally, due to their subcutaneous localization, these cells are easily accessible by liposuction in

relatively high amounts. The yield of ASC in the stromal vascular fraction of a lipoaspirate can reach 1-5%; this percentage differs depending on the harvesting site (Jurgens et al., 2008; Kolaparthy et al., 2015). For comparison, the isolation of bone marrow mesenchymal stem cells is connected with a painful procedure, and a relatively small percentage of stem cells is present in the bone marrow aspirate (500 times smaller compared to ASC) (Mizuno, 2009). FIB represent a primoculture of cells, which are considered as an excellent cell model to study many aspects of cell physiology (Tschumperlin, 2013).

In this study, the effect of WPI on Saos-2 cells, ASC and FIB were compared using following tests: (i) cell proliferation by a real-time detecting system, (ii) expression of cell differentiation markers by real-time qPCR, (iii) activity of alkaline phosphatase and (iv) deposition of calcium.

MATERIALS AND METHODS

WPI Preparation, Composition and Sterilisation

WPI (Whey protein isolate, BiPRO, Davisco Foods International, Inc., Eden Prairie, US) with 97.7% protein and 75% bLG in dry matter (according to the manufacturer's specification) was used. Our HPLC analysis according to Keppler et al., (2014) (see supplementary Figure 1) confirms that the WPI dissolved in cell culture medium is dominated by approximately 80 % bLG genetic variants b and a (elution time 19.2 and 20.1 min) and some alpha lactalbumin (elution time 10.7 min.) This is in accordance with previous analyses of the same WPI dissolved in water (Keppler et al. 2017a; Keppler et al. 2017b). As expected, there was no protein loss due to filtration.

Adipose-Derived Stem Cells Isolation and Characterization

ASC were obtained in compliance with the tenets of the Declaration of Helsinki for experiments with human tissues and under an ethical approval issued by the Ethical Committee in the Bulovka Hospital in Prague, the Czech Republic (August 21, 2014) and by the Institute of Physiology CAS in Prague, the Czech Republic (August 18, 2014). Informed consent was obtained from the patient before the liposuction procedure. Lipoaspirate of volume 50 ml was collected from the belly area of a 40-year-old female patient. Liposuction was performed under negative pressure (-700 mmHg), and the ASC were isolated by a procedure described earlier (Estes et al., 2010). The fat was washed several times with phosphate buffered saline (PBS, Sigma-Aldrich, USA) to remove remaining blood, and then digested with 0.1% collagenase type-I (Worthington, USA) for 1 h at 37 °C. The sample was then centrifuged (300 g) for 5 min at 21 °C. The tube was shaken vigorously for 10 s and centrifuged under the same conditions one more time. The pellet of stromal vascular fraction (SVF) was obtained and remaining supernatant (fat) removed. Then, a DMEM medium (GIBCO) supplemented with 10% foetal bovine serum (GIBCO) 10 ng/ml human fibroblast growth factor-2 (FGF-2, GenScript, Cat. No. Z03116-1), and gentamicin (40 µg/ml, LEK, Ljubljana, Slovenia) was added, and the pellet was filtered through a cell filter with 100 µm pores (Millex Syringe-driven Filter Unit, Germany) (Estes et al., 2010). Finally, the cells were seeded at an equivalent density to 0.16 ml of the original liposuction aspirate per cm^2. The successful isolation of the adipose-derived stem cells was confirmed by flow cytometry. The population of ASC was positive for CD 73 (73 (ecto-5'-nucleotidase), CD 90 (immunoglobulin Thy-1), CD 105 (endoglin), CD 29 (fibronectin receptor) and CD 146 (receptor for laminin) and negative for CD 31 (platelet endothelial cell adhesion molecule), CD 34 (hematopoietic progenitor cell antigen) and CD 45 (leucocytes) surface markers (supplementary Fig. 2).

Real-Time Monitoring of Cell Adhesion and Proliferation

Cellular response of osteoblast-like Saos-2 cells (purchased from European Collection of Cell Cultures, Salisbury, UK), adipose-derived stem cells (ASC, in passage 2) and human neonatal dermal fibroblasts (FIB, purchased from Lonza, Basel, Switzerland, in passage 2) to different WPI concentrations was studied at 37°C in a humidified air atmosphere containing 5% of CO_2 for 117 hours. The Saos-2 cells, FIB and ASC were cultured in McCoy' 5A medium, DMEM medium, and DMEM supplemented with FGF-2, respectively. All of the media contained foetal bovine serum (15% for Saos-2 cells, 10% for FIB and ACS) and gentamicin (40 µg/ml). A real-time cell analyser (xCelligence, Roche Applied Science, Mannheim, Germany) was used to evaluate the growth of cells in the prepared solutions continuously, during a 5 day time span. The cells were seeded into 96-well sensory E plates (E-Plate 96, BioTech a.s., Prague, CR, Cat. No. 05232368001), and background impedance was measured in each well. The cell densities were: 3,500 cells/well (approximately 10,300 cells/cm^2) for Saos-2 and FIB, and 7,000 cells/well (approximately 20,600 cells/cm^2) for ASC. The final volume was 200 µl. After 24 hours, cultivation medium was exchanged for appropriate media containing specific concentrations of WPI (0, 50, 300 and 800 µg/ml). Each concentration was added to the wells in heptaplicates. Cell index values (reflecting cell attachment, spreading and proliferation) were calculated automatically by the instrument according to the formula:

Cell index = (impedance at individual time interval - background impedance) / 15Ω

Real-Time Q-PCR of Markers of Osteogenic Cell Differentiation

Real-time quantitative PCR (Q-PCR) was used to determine the effect of WPI content on the level of expression of genes for *COL 1* (Saos-2, ASC, FIB), *ALP* (Saos-2, ASC), and *OC* (Saos-2, ASC). The expression of transcription factor *RUNX2*, also involved in osteogenic cell differentiation, was evaluated in Saos-2 and ASC. Cells were grown in the tested solutions for 7 days. Total RNA was extracted from Saos-2, ASC and FIB using Total RNA purification Micro Kit (NORGENE Biotek Corp, Cat. No. 35300) according to the manufacturer's instructions. The mRNA concentration was measured using NanoPhotometer^{TM} S/N (IMPLEN). cDNA was synthesized with the ProtoScript®M-MuLV First Strand cDNA Synthesis kit (New England BioLabs, Cat. No. E6300S) using 250 ng of total RNA and oligo-dT primers. The reaction was performed in T-Personal Thermocycler (Biometra). Q-PCR primers were purchased from Generi Biotech Ltd. and are listed in Table 1. The primers were designed according to the literature (Reseland et al., 2006; Franke et al., 2007; Zhang et al., 2010; Frank et al., 2002). Real-time quantitative PCR was performed using SYBR Green (Roche) in the total reaction volume to 20 µL and iCycler detection system (iQ™ 5 Multicolor Real-Time PCR Detection System, Bio-Rad) with cycling parameters of 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by a melt curve. Assays were conducted in quadruplicates. Data were analysed by the $2^{-\Delta\Delta Cq}$ method. The point at which the PCR product was first detected above a fixed threshold (termed cycle threshold, C_q), was determined for each sample. Changes in the expression of target genes were calculated using the equation:

$$\Delta\Delta C_q = (C_q^{target} - C_q^{GAPDH})_{sample} - (C_q^{target} - C_q^{GAPDH})_{calibrator}$$

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and data was normalized to the expression levels of cells grown in medium without WPI (0, calibrator).

Alkaline Phosphatase (ALP) Activity

The influence of different concentrations of WPI on activity of alkaline phosphatase (ALP) of Saos-2 cells, FIB and ASC was studied. Cells were cultured in McCoy' 5A medium, DMEM medium, and DMEM supplemented with FGF-2, respectively. All of the media contained foetal bovine serum (15% for Saos-2 cells, 10% for ACSs and FIB) and gentamicin (40 µg/ml). The cells were seeded into 24-well cell culture plates (TPP, Switzerland). The cell densities were: 28,000 cells/well (approximately 15,000 cells/cm^2) for Saos-2 cells and FIB, and 10,000 cells/well (approximately 5,400 cells/cm^2) for ASC. The final volume was 1 ml. After 24 hours, the cultivation medium was exchanged for appropriate media containing specific concentrations of WPI (0, 50, 300 and 800 µg/ml). After 7 days of cultivation, the cell layers were twice washed with PBS; then, 1 ml of the substrate solution (1 mg/ml p-nitrophenyl phosphate in substrate buffer [50 mM glycine, 1 mM $MgCl_2$, pH 10.5]) (Sigma-Aldrich, USA) was added directly to the cells. The reaction was performed for 5 min (Saos-2 cells), 15 min (ASC), or 21 min (FIB) at room temperature; the substrate solution was then removed and mixed with the same volume of 1 M NaOH solution. The absorbance (405 nm) of the samples was measured together with the absorbance of the known concentrations of p-nitrophenol diluted in 0.02 M NaOH (9–90 µM) (Sigma-Aldrich, USA). The results were normalized by the cell index. The experiments were performed in triplicate and were repeated three times.

Calcium Deposition

The influence of different concentrations of WPI on calcium deposition of Saos-2 cells was studied. Cells were cultured in McCoy' 5A supplemented with 15 % of foetal bovine serum and gentamicin (40 µg/ml). The cells were seeded into 24-well cell culture plates (TPP, Switzerland). The cell density was 28,000 cells/well (approximately 15,000 cells/cm^2). The final volume was 1 ml. After 24 hours, cultivation medium was exchanged for appropriate medium containing specific concentrations of WPI (0, 50, 300 and 800 µg/ml). After 7, 14 and 21 days of cultivation, the cell layers were rinsed with PBS, dried, and lysed in 0.5 M HCl for 24 hours at 4°C. The calcium in the cell lysates and standards was directly determined using the Calcium Colorimetric Assay Kit (Biovision Inc., Milpitas, CA, USA) according to the manufacturer's protocol. The experiments were performed in triplicate and were repeated three times. The results were normalized to the cell index.

Statistical Evaluation

The quantitative data of cell proliferation were presented as mean ± standard deviation (S.D.) from 7 measurements. PCR, ALP activity and Ca deposition data were presented as mean ± S.D. from 4 measurements. The statistical analyses were performed using SigmaStat (Jandel Corporation, USA) by the One-Way Analysis of Variance (ANOVA), Student-Newman-Keuls method. The value $p \leq 0.05$ was considered significant ($p \leq 0.01$ for PCR data).

RESULTS AND DISCUSSION

This study established a positive effect of WPI on the proliferation of Saos-2 cells and FIB (Fig. 1). The growth of ASC was less sensitive. In these cells, a slightly positive influence of WPI was demonstrated only at the highest concentration (800 µg/ml).

Nevertheless, the values at 800 µg/ml were not significantly different from those at lower WPI concentrations. An earlier study (Xu, 2009) investigated the proliferative effect of two concentrations of whey protein (0.02 and 0.1 mg/ml) on rat osteoblasts. The author found a positive effect of whey protein on the cell proliferation, which was dose-dependent, similarly as in our present study. In another study it was reported that bLG (a major part of WPI) improved the proliferation of enteroendocrine cells (Gillespie et al., 2015). These authors reported proliferation-stimulating effects of bLG only in a concentration window of 312.5-2500 µg/ml. In another study on mouse spleen resting cells, bLG stimulated proliferation in the concentration range 50-500 µg/ml in the time range 12-96 h (Mahmud et al., 2004). These bLG concentration ranges are similar to the range investigated in the present study, where 50-800 µg/ml of WPI represented 40-640 µg/ml of bLG, and within this range, the proliferation of Saos-2 cell and fibroblasts was promoted. In another study using hybridomas, bLG concentrations between 750 and 3,000 µg/ml stimulated proliferation after 48 h (Moulti-Mati et al., 1991). The positive effect of bLG on osteogenic differentiation needs to be elucidated in further studies. Last but not least, lactoferrin, another milk-derived protein, stimulated the adhesion, growth and osteogenic differentiation of Saos-2 cells in our earlier study (Vandrovcova at al., 2015).

Q-PCR was performed on day 7 (Fig. 2). Markers of osteogenic differentiation were measured in Saos-2 cells and ASC. The transcription factor *RUNX2*, an early marker of osteogenic differentiation, was evaluated in Saos-2 cells and ASC. Despite the apparent tendency in ASC, only the highest concentration of WPI promoted expression of *RUNX2* in Saos-2 cells significantly. *RUNX2* is a potent osteoblast transcription factor, which promotes expression of type-I collagen in the early differentiation stage (Fakhry et al., 2013). However, overexpression of *RUNX2* leads to suppression of osteoblast maturation and inhibits

266 expression of late osteogenic markers (Liu et al., 2001). It has been reported that the
267 expression of the *RUNX2* protected Saos-2 from the antiproliferative and apoptotic effects of
268 TNF-α (Olfa et al., 2010). It is also important if the cells are of osteosarcoma or osteoblast
269 origin. *RUNX2* is expressed at a lower level in several osteosarcoma cell lines; however, in
270 Saos-2 cells (which also are of osteosarcoma origin), the expression of *RUNX2* is increased
271 (Cameron et al., 2003). *RUNX* genes can act either as oncogenes or tumor suppressors (Blyth
272 et al., 2005). It is in accordance with our findings, where a higher *RUNX2* expression
273 supported proliferation rather than differentiation of Saos-2 cells, but tended to have an
274 opposite effect in ASC. Expression of *COL 1* was evaluated in all types of cells. Saos-2 cells
275 did not respond to WPI. The expression was, however, significantly increased in ASC
276 cultured in the medium with 800 µl/ml of WPI in comparison to the media with other tested
277 concentrations, and in FIB in media with 50, 300 and 800 µg/ml of WPI in comparison to
278 WPI-free medium. The expression of alkaline phosphatase (*ALP)*, which is considered as an
279 early or medium-term marker of cell differentiation, was improved in ASC by increasing the
280 concentration of WPI. No effect was proven in Saos-2 cells. Expression of osteocalcin (*OC*), a
281 late marker of osteogenic differentiation, was not influenced by increasing concentrations of
282 WPI, neither in Saos-2 cells nor in ASC. An explanation is the relatively short culture interval
283 of 7 days in our study, which might not be sufficient for expression of late markers of
284 osteogenic cell differentiation. On the other hand, *OC* expression can be enhanced even in a
285 shorter time interval, if the culture conditions strongly promote the osteogenic cell
286 differentiation. For example, in our earlier study focussing on the effects of lactoferrin on the
287 behaviour of Saos-2 cells, the cells on collagen-lactoferrin coatings produced significantly
288 higher levels of osteocalcin than cells on control polystyrene cell culture dishes (Vandrovcova
289 et al., 2015). In our present study, where the effect of WPI on osteogenic cell differentiation

appeared to be weaker, only early markers were detected after 7 days of cultivation. Thus, the osteogenic differentiation of cells under influence of WPI was in its early stage after 7 days but it can be expected that *OC* would be increased in later culture intervals.

The activity of ALP was evaluated on day 7 (Fig. 3). Saos-2 cells are known to contain higher amounts of ALP and the reaction needed to be stopped after 5 min of incubation. Due to a relatively high content of ALP even under standard cultivation conditions, no increase in ALP activity was found in Saos-2 cells in media with WPI. ASC showed slight activity of ALP on the edge of the detection limit after 15 min. The highest concentration of WPI stimulated cells to produce a detectable ALP signal, which was in accordance with our PCR results. Similarly the study by Xu mentioned above (Xu, 2009), performed on rat osteoblasts, revealed that the whey protein added in the culture medium stimulated the production of ALP in a dose-dependent manner. Fibroblasts are known not to contain ALP. It was decided to measure the ALP activity in fibroblasts as well as a negative control. The cells were exposed to the ALP substrate for 21 min and the values did not reach the limit of detection.

In view of the fact that ASC and FIB showed almost no activity of ALP (i.e., an enzyme involved in the bone matrix mineralization), calcium deposition was evaluated only in Saos-2 cells after 14 and 21 days (Fig. 4). After 7 days of cultivation the calcium deposition was low, under the limit of detection. Moreover, the expression of early and medium-term markers of osteogenic cell differentiation, i.e. *COL I* and *ALP*, did not differ significantly in Saos-2 after 7 days of cultivation in media with various WPI concentrations. However, on day 14 after seeding, the influence of the presence of WPI was evident. On day 21 after seeding, the results were even more apparent. Nevertheless, in that time interval, the supportive effect was rather negatively correlated with increasing WPI concentration (Fig. 4).

Besides the direct positive effects of WPI on proliferation of Saos-2 and FIB, and osteogenic differentiation of ASC suggested by the results of this study, WPI has several other properties that may be advantageous in bone regeneration. As mentioned in the introduction, its main component bLG has an affinity for hydrophobic molecules which are poorly soluble in water and can be employed as a carrier protein to improve their solubility and bioavailablity. One can speculate that bLG could be employed as a carrier or delivery protein for certain molecules, which promote osteogenic differentiation, such as purmorphamine (Rezia Rad et al. 2016) or which are suspected to promote bone healing, such as vitamin D (Gorter et al., 2014). In addition, it is possible to use WPI to fabricate hydrogels (Puyol et al., 2001). Hydrogels are gaining interest as biomaterials for bone regeneration (Gkioni et al., 2010). Furthermore, bLG is inexpensive, as whey protein isolate is a commonly used food supplement, e.g. in bodybuilding (Marshall, 2004), and is thus produced in large quantities. Hence, we believe that applications of WPI in bone regeneration, both in solution and as a biomaterial component, are worthy of further investigation.

CONCLUSION

The growth of Saos-2 cells and FIB was supported in an apparently dose-dependent manner by WPI. The expressions of markers of osteogenic differentiation by ASC, such as *COL 1* and *ALP*, were improved by WPI in a concentration-dependent manner (the best results were found for 800 µl/ml of WPI). FIB also increased the expression of *COL 1* in the presence of WPI in comparison with no WPI. The presence of WPI stimulated Saos-2 cells to deposit calcium even in the standard culture medium without osteogenic supplements. It can be concluded that WPI has a positive effect on the growth of Saos-2 cells and deposition of calcium by Saos-2, on the growth of FIB and their expression of type-I collagen, and on the

osteogenic differentiation of ASC, manifested by the expression of *COL 1* and *ALP,* and the activity of ALP.

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REFERENCES

Bhattacharya I., C. Ghayor, and F. E. Weber. 2016. The use of adipose tissue-derived progenitors in bone tissue engineering - a review. Transfus Med Hemother. 43:336-343.

Blyth K., E. R. Cameron, and J. C. Neil. 2005. The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer. 5:376-387.

Bounous G, and P. Gold. 1991. The biological activity of undenatured dietary whey proteins: role of glutathione. Clin Invest Med. 14:296-309.

Cameron E. R., K. Blyth, L. Hanlon, A. Kilbey, N. Mackay, M. Stewart, A. Terry, F. Vaillant, S. Wotton, and J. C. Neil. 2003. The runx genes as dominant oncogenes. Blood Cells Mol Dis. 30:194-200.

Czekanska E. M., M. J. Stoddart, R. G. Richards, and J. S. Hayes. 2012. In search of an osteoblast cell model for in vitro research. Eur Cells Mater. 24:1-17.

Diarrasouba F., G. Garrait, G. Remondetto, P. Alvarez, E. Beyssac, and M. Subirade. 2015. Food protein-based microspheres for increased uptake of vitamin D3. Food Chem. 173:1066-1072.

Do A. B., K. Williams, and O. T. Toomer. 2016. In vitro digestibility and immunoreactivity of bovine milk proteins. Food Chem. 190:581-587.

Estes B. T., B. O. Diekman, J. M. Gimble, and F. Guilak. 2010. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc. 5:1294-1311.

Fakhry M., E. Hamade, B. Badran, R. Buchet, and D. Magne. 2013. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World J Stem Cells 5:136-148.

Flower D. R., A. C. T. North, and C.E. Sansom CE. 2000. The lipocalin protein family: structural and sequence overview. Biochim Biophys Acta. 1482:9-24.

Frank O., M. Heim, M. Jakob, A. Barbero, D. Schafer, I. Bendik I, D. Walter, M. Heberer, and I. Martin. 2002. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem. 85:737-746.

Franke S., H. Siggelkow, G. Wolf, and G. Hein. 2007. Advanced glycation endproducts influence the mRNA expression of RAGE, RANKL and various osteoblastic genes in human osteoblasts. Arch Physiol Biochem. 113:154-161.

Gillespie A. L., D. Calderwood, L. Hobson, and B. D. Green. 2015. Whey proteins have beneficial effects on intestinal enteroendorine cells stimulating cells growth and increasing the production and secretion of incretin hormones. Food Chem. 189:120-128.

Gkioni K., S. C. G. Leeuwenburgh, T. E. L. Douglas, A. G. Mikos, and J. A. Jansen. 2010. Mineralization of hydrogels for bone regeneration. Tissue Eng: Part B. 16:577-585.

Gorter E. A., N. A. T. Hamdy, N. M. Appelman-Dijkstra, and I. B. Schipper. 2014. The role of vitamin D in human fracture healing: a systematic review of the literature. Bone. 64:288-297.

384 Ha H. K., J. W. Kim, M. R. Lee, and W. J. Lee. 2013. Formation and characterization of
385 quercetin-loaded chitosan oligosaccharide/β-lactoglobulin nanoparticle. Food Res Int.
386 52:82-90.
387 Jurgens W. J., M. J. Oedayrajsingh-Varma, M. N. Helder, B. Zandiehdoulabi, T. E. Schouten,
388 D. J. Kuik, M. J. Ritt, and F. J. van Milligen. 2008. Effect of tissue-harvesting site on
389 yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell
390 and Tissue Res. 332:415-426.
391 Keppler J. K., F. D. Sönnichsen, P. C. Lorenzen, and K. Schwarz. 2014. Differences in heat
392 stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H
393 NMR and fluorescence quenching. Biochim Biophys Acta. 1844:1083-1093.
394 Keppler J. K., D. Martin, V. M. Garamus, C. Breton-Carabin, E. Nipoti, T. Coenye, and K.
395 Schwarz. 2017a, Functionality of whey proteins covalently modified by allyl
396 isothiocyanate. Part 1 physicochemical and antibacterial properties of native and
397 modified whey proteins at pH 2 to 7. Food Hydrocolloids. 65:130-143.
398 Keppler J. K., and K. Schwarz. 2017b. Increasing the emulsifying capacity of whey proteins
399 at acidic pH values through covalent modification with allyl isotiocyanate. Colloid Surf
400 A-Physicochem Eng Asp. 522:514-524.
401 Kolaparthy L. K., S. Sanivarapu, S. Moogla, and R. S. Kutcham. 2015. Adipose tissue -
402 adequate, accessible regenerative material. Int J Stem Cells. 8:121-127.
403 Kontopidis G., C. Holt, and L. Sawyer. 2004. Invited review: β-lactoglobulin: Binding
404 properties, structure, and function. J of Dairy Sci. 87:785-796.
405 Lee M. R., H. N. Choi, H. K. Ha, and W. J. Lee. 2013. Production and characterization of
406 beta-lactoglobulin/alginate nanoemulsion containing coenzyme Q10: Impact of heat
407 treatment and alginate concentrate. Korean J Food Sci Anim Resour. 33:67-74.

Levi B., and M. T. Longaker. 2011. Adipose derived stromal cells for skeletal regenerative medicine. Stem Cells. 29: 576-582.

Liu W., S. Toyosawa, T. Furuichi, N. Kanatani, C. Yoshida, Y. Liu, M. Himeno, S. Narai, A. Yamaguchi, and T. Komori. 2001. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol. 155:157-66.

Mahmud R., M. A. Matin, and H. Otani. 2004. Mitogenic effect of bovine β-lactoglobulin and its proteolytic digests on mouse spleen resting cells. Pak J Biol Sci. 7:2045-2050.

Marshall K. 2004. Therapeutic applications of whey protein. Altern Med Rev. 9:136-156.

Mizuno H. 2009. Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review. J Nippon Med Sch. 76:56-66.

Moulti-Mati F., A. Mati, J. Capiaumont, F. Belleville, G. Linden, and P. Nabet. 1991. Rôle de la β-lactoglobuline dans l'activité proliférative du lactosérum. Lait. 71:543-553.

Parodi P. W. 2007. A role for milk proteins and their peptides in cancer prevention. Curr Pharm Design. 13:813-828.

Pereira P. C. 2014. Milk nutritional composition and its role in human healt. Nutrition. 201430: 619-627.

Puyol P., M. D. Pérez, and D. S. Horne. 2001. Heat-induced gelation of whey protein isolates (WPI): effect of NaCl and protein concentration Food Hydrocolloids. 15:233-237.

Pyo M. C., S. Y. Yang, S. H. Chun, N. S. Oh, and K. W. Lee. 2016. Protective effects of maillard reaction products of whey protein concentrate against oxidative stress through an Nrf2-dependent pathway in HepG2 cells. Biol Pharm Bull. 39:1437-1447.

Reseland J. E., S. Reppe, A. M. Larsen, H. S. Berner, F. P. Reinholt, K. M. Gautvik, I. Slaby, and S. P. Lyngstadaas. 2006. The effect of enamel matrix derivative on gene expression in osteoblasts. Eur J Oral Sci. 11:205-211.

Rezia Rad M., M. Khojaste, M. Hasan Shahriari, S. Asgary, and A. Khojasteh. 2016. Purmorphamine increased adhesion, proliferation and expression of osteoblast phenotype markers of human dental pulp stem cells cultured on beta-tricalcium phosphate. Biomed Pharmacoter. 82:432-438.

Schliemann B., D. Wähnert, C. Theisen, M. Herbort, C. Kösters, M. J. Raschke, and A. Weimann. 2015. How to enhance the stability of locking plate fixation of proximal humerus fractures? An overview of current biomechanical and clinical data. Injury. 46: 1207-14.

Shields L. B., G. H. Raque, S. D. Glassman, M. Campbell, T. Vitaz, J. Harpring, and C. B. Shields. 2006. Adverse effects associated with high-dose recombinant human bone morphogenetic protein-2 use in anterior cervical spine fusion. SPINE. 31:542-547.

Tschumperlin D. J. 2013. Fibroblasts and the ground they walk on. Physiology. 28:380-390.

Vandrovcova M., T. E. L. Douglas, S. Heinemann, D. Scharnweber, P. Dubruel, and L. Bacakova. 2015. Collagen-lactoferrin fibrillar coatings enhance osteoblast proliferation and differentiation. J Biomed Mater Res A. 103:525-33.

Walzem R. L., C. J. Dillard, and J. B. German. 2002. Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. Crit Rev Food Sci Nutr. 42:353-75.

Xu R. 2009. Effect of whey protein on the proliferation and differentiation of osteoblasts. J Dairy Sci. 92:3014-3018.

Yamaguchi M., S. Takai, A. Hosono, and T. Seki. 2014. Bovine milk-derived α-lactoglobulin inhibits colon inflammation and carcinogenesis in azosymethane and dextran sodium sulfate-treated mice. Biosci Biotechnol Biochem. 78:672-679.

Zhang L., X. Ren, E. Alt, X. Bai, S. Huang, Z, Xu, P. M. Lynch, M. P. Moyer, X.-F. Wen, and X.Wu. 2010. Chemoprevention of colorectal cancer by targeting APC-deficient cells for apoptosis. Nature. 464:1058-1061.

CAPTIONS

Figure 1. The growth curves represent proliferation of three types of cells measured with the xCELLigence system: osteoblast-like cells (Saos-2), adipose-derived stem cells (ASC) and human neonatal dermal fibroblasts (FIB). After 24 hours, proliferative medium was replaced by medium contained different concentrations of WPI. Graphs represent cell number (estimated as a cell index) at the last point of the measurement. ANOVA, Student–Newman–Keuls method. Statistical significance ($p \leq 0.05$): All: in comparison with all other tested groups, 800: in comparison with the cells grown in media with 800 µg/ml of WPI.

Figure 2. Q-PCR results. The fold ratios are values relative to the control value. The expression of transcription factor *RUNX2*, type I collagen (*COL 1*), alkaline phosphatase (*ALP*) and osteocalcin (*OC*) were evaluated on day 7 after seeding in the presence of different concentrations of WPI in 3 different cells types: osteoblast-like cells (Saos-2), adipose-derived stem cells (ASC) and human neonatal dermal fibroblasts (FIB). Data are presented as mean ± S.D. (standard deviation), n = 4. Statistical analysis was performed for the values ΔCq. ANOVA, Student–Newman–Keuls method. Statistical significance ($p \leq 0.01$): All: in comparison with all other tested groups; 0, 800: in comparison with the cells grown in media without WPI and with 800 µg/ml of WPI, respectively.

Figure 3. Activity of ALP per min and cell number (3 different cells types: osteoblast-like cells (Saos-2), adipose-derived stem cells (ASC) and human neonatal dermal fibroblasts (FIB). Data are presented as mean ± S.D. (standard deviation), n = 4. ANOVA, Student–Newman–Keuls method. Statistical significance ($p \leq 0.05$): All: in comparison with all other

tested groups; 0, 50: in comparison with the cells grown in media without WPI and with 50 µg/ml of WPI, respectively.

Figure 4. Calcium deposition by osteoblast-like cells (Saos-2) recalculated per cell number. Data are presented as mean ± S.D. (standard deviation), n = 4. ANOVA, Student–Newman–Keuls method. Statistical significance ($p \leq 0.05$): All: in comparison with all other tested groups; 50, 300: in comparison with the cells grown in media with 50 µg/ml and 300 µg/ml of WPI, respectively.

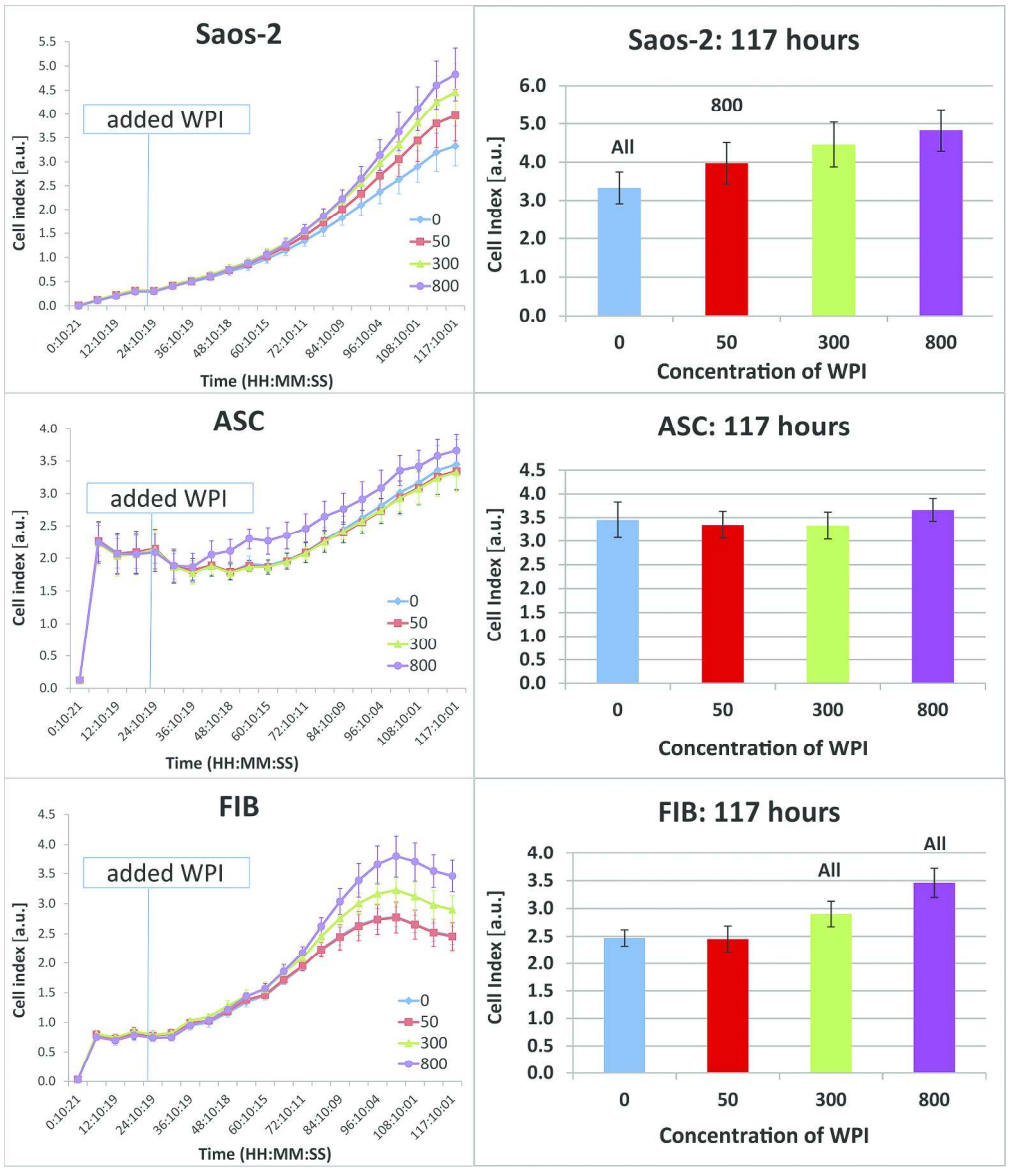
Table 1. Oligonucleotide primers for real-time q-PCR amplifications

Gene	Primer sequence	Product size (bp)
RUNX2	Forward: 5′-GCCTTCAAGGTGGTAGCCC-3′	100
	Reverse: 5′- CGTTACCCGCCATGACAGTA-3′	
COL 1	Forward: 5′-CAGCCGCTTCACCTACAGC-3′	83
	Reverse: 5′-TTTTGTATTCAATCACTGTCTTGCC-3′	
ALP	Forward: 5′-GACCCTTGACCCCCACAAT-3′	68
	Reverse: 5′-GCTCGTACTGCATGTCCCCT-3′	
OC	Forward: 5'-GAAGCCCAGCGGTGCA-3'	70
	Reverse: 5'-CACTACCTCGCTGCCCTCC-3'	
GAPDH	Forward: 5′-TGCACCACCAACTGCTTAGC-3′	87
	Reverse: 5′-GGCATGGACTGTGGTCATGAG-3′	

514 Supplementary Figure 1. RP-HPLC analysis (intensity versus retention time) of a pure
515 DMEM solution with or without addition of 0.8 mg/ml whey protein isolate before (solid line)
516 and after (dotted line) filtration with a 0.2 µm acetate cellulose filter. ALA, alpha-
517 Lactalbumin; BLG, beta-Lactoglobulin genetic variants A and B.
518
519 Supplementary Figure 2. Cell surface antigens of human ASCs were detected by flow
520 cytometry. The flow cytometry analysis showed a positive expression of standard surface
521 markers of ASCs, namely CD 29, CD 73, CD 90, and CD 105. However, no expression of the
522 markers of CD 31, CD 34, CD 45, and CD 146 was detected in isolated ASCs.
523
524

Before cell experiments, the WPI was dissolved in the appropriate cell culture medium and sterilised using a 0.2 µm acetate cellulose filter (Sartorius). The medium or 800 µg/ml WPI dissolved in the medium were either injected directly or filtered through a sterile filter and injected into high performance liquid chromatography (HPLC) apparatus and analysed as described in (Keppler et al., 2014). Briefly, a HPLC HP 1100 system (Agilent Technology, Germany) equipped with a diode array detector (DAD) at 205 nm wavelength was used with a PLRP-S 300 Å 8 µm, 150 × 4.6 mm column (Polymer Laboratories, Varian, Inc.). Eluent A was 0.1% trifluoracetic acid (TFA) in water and eluent B was 0.1% TFA in acetonitrile. The following gradient was used: 0 min — 35% B, 1 min — 35% B, 8 min — 38% B, 16 min — 42% B, 22 min — 46% B, 22.5 min — 100% B, 23 min — 100% B and 23.5–30 min — 35% B. The column temperature was set to 40 °C, the flow rate was 1 ml/min and the injection volume was 20 µl.

The effect of the filtration on the WPI composition would be common for all three media. However, it is important to know if the protein composition is altered by filtration, as this would influence the experimental setup (to a similar degree in all cases, but nonetheless this is important in case of reproduction).



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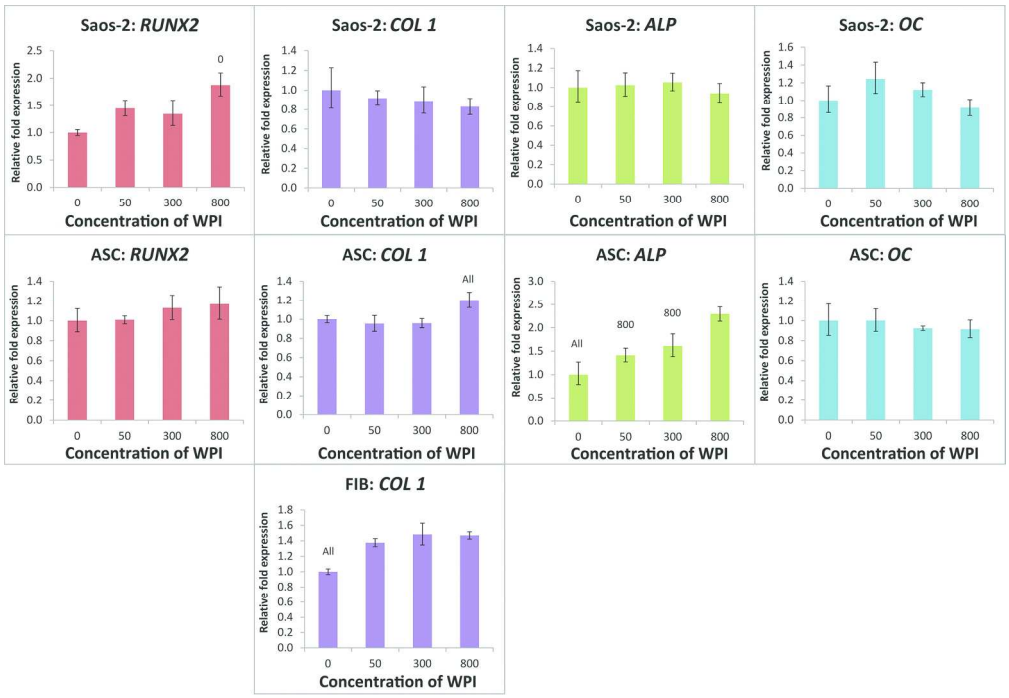

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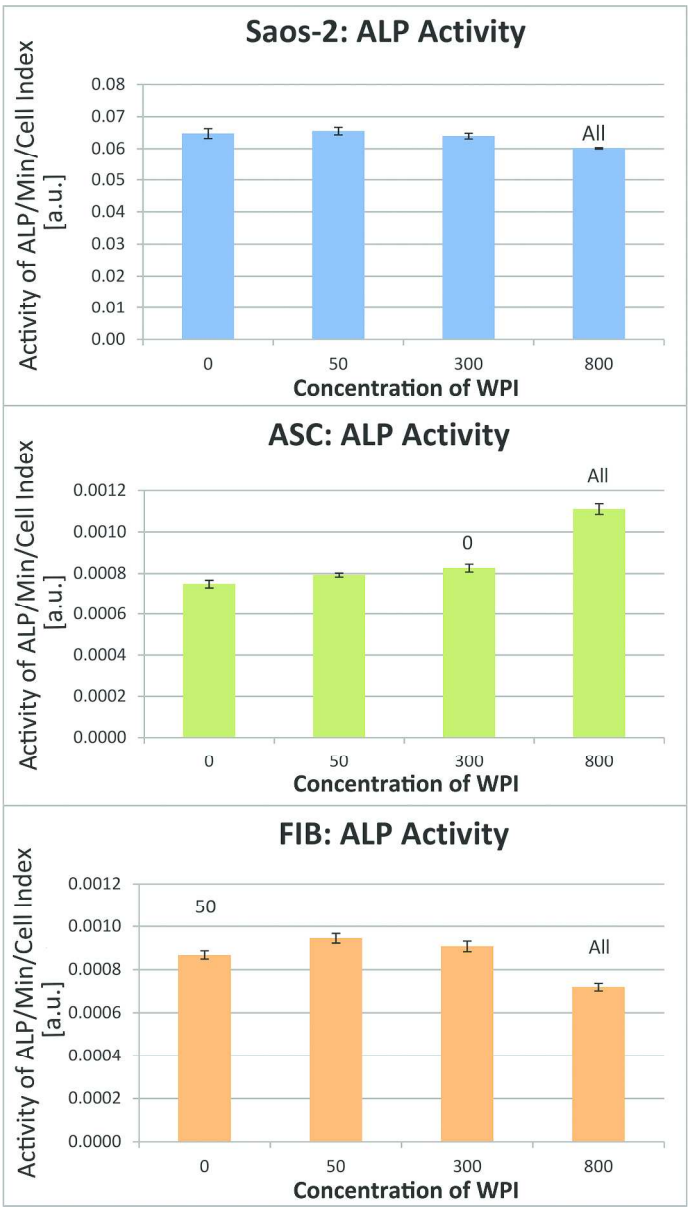

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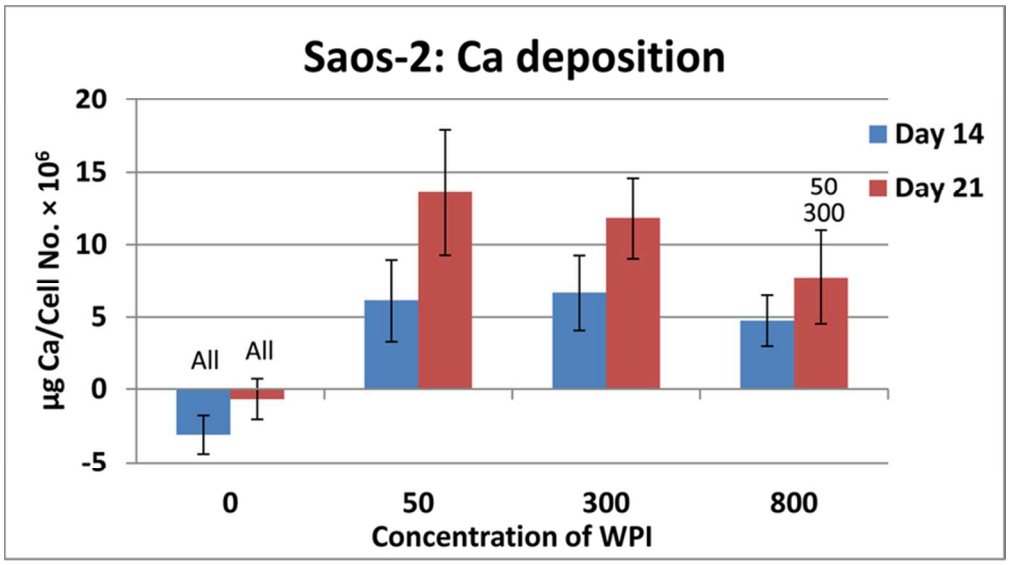

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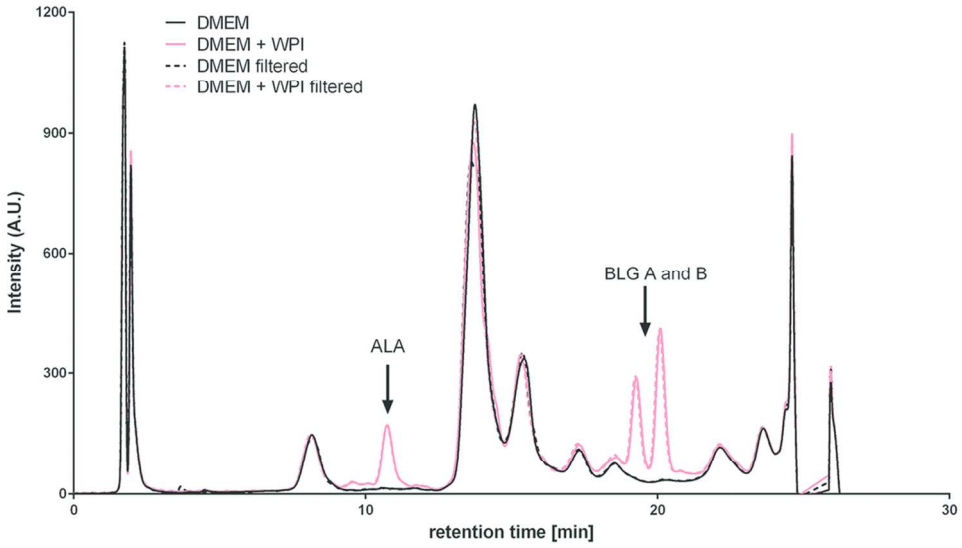

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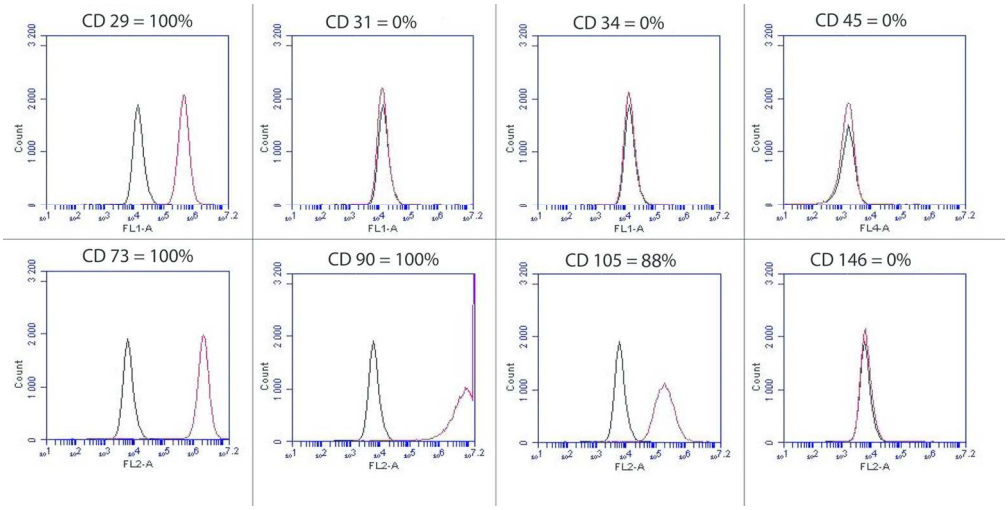

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