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Application of whey protein isolate in bone regeneration: Effects 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, 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, we tested the influence of different concentrations of WPI on behavior of human osteoblast-like Saos-2 cells, human adipose tissue-derived stem cells (ASC), and human neonatal dermal fibroblasts (FIB). 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 [type-I collagen (*COL1*) and alkaline phosphatase (*ALP*)] as well as ALP activity, increased dose-dependently in ASC. Importantly, Saos-2 cells were able to deposit calcium in the presence of WPI, even in a proliferation medium without other supplements that support osteogenic cell differentiation. The results indicate that, depending on the cell type, WPI can act as an enhancer of cell proliferation and osteogenic differentiation. Therefore, 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

The human population, especially in developed countries, is aging, which makes age-related health issues more important. 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). Many growth factors and hormones are known for their supportive effect on bone growth (e.g., bone morphogenetic protein 2, BMP-2); nevertheless, their isolation and 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., 2006). Hence, the need exists 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 (80% of all proteins in ruminant milk), consisting of 4 major proteins, and whey protein (20% of all proteins in ruminant milk), consisting of 2 main components, β-LG and α-LA, and smaller amounts of serum albumin, lactoferrin, and other proteins (Do et al., 2016). Whey protein was considered 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 AA rich in sulfur compared with 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

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

Many studies have focused on the effect of consumption of milk or its derivatives in vivo (Parodi, 2007; Yamaguchi et al., 2014); however, a relatively small number of studies have focused on the performance of specific compounds in vitro (Gillespie et al., 2015; Pyo et al., 2016). In particular, β-LG 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, we tested the influence of different concentrations of WPI rich in β-LG (80% wt) on cell growth and differentiation. Three 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 behavior, were chosen as being representative of bone cells (Czekanska et al., 2012). The human ASC were chosen as being 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 (Levi and Longaker, 2011; Bhattacharya et al., 2016). 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 to 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 requires a painful procedure, and a relatively small percentage of stem cells is present in the bone marrow aspirate (500 times less than ASC; Mizuno, 2009). Fibroblasts represent a primoculture of cells, which are considered 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 evaluations: (1) cell proliferation by a real-time detecting system, (2) expression of cell differentiation markers by real-time quantitative (**q**)PCR, (3) activity of alkaline phosphatase (**ALP**), and (4) deposition of calcium.

MATERIALS AND METHODS

Preparation, Composition, and Sterilization of WPI

Whey protein isolate (BiPRO, Davisco Foods International Inc., Eden Prairie, MN) with 97.7% protein and 75% β-LG in DM (according to the manufacturer's specification) was used. Our HPLC analysis according to Keppler et al. (2014; see Supplemental Figure S1; https://doi.org/10.3168/jds.2017-13119) confirmed that the WPI dissolved in cell culture medium comprised approximately 80% β-LG genetic variants b and a (elution time 19.2 and 20.1 min, respectively) and some α-LA (elution time 10.7 min). This is in accordance with previous analyses of the same WPI dissolved in water (Keppler et al., 2017; Keppler and Schwarz, 2017). As expected, there was no protein loss due to filtration.

Isolation and Characterization of ASC

The 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, Czech Republic (August 21, 2014) and by the Institute of Physiology CAS in Prague, Czech Republic (August 18, 2014). Informed consent was obtained from the patient before the liposuction procedure. Lipoaspirate (50 mL) was collected from the belly area of a 40-yr-old female patient. Liposuction was performed under negative pressure (−700 mmHg), and the ASC were isolated by a procedure described previously (Estes et al., 2010). The fat was washed several times with PBS (Sigma-Aldrich, St. Louis, MO) to remove remaining blood, and then digested with 0.1% collagenase type-I (Worthington, Lakewood, NJ) 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 was obtained and remaining supernatant (fat) removed. Then, Dulbecco's modified Eagle's medium (**DMEM**; Gibco/Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Gibco), 10 ng/mL human fibroblast growth factor-2 (**FGF-2**, Cat. No. Z03116-1, GenScript, Piscataway, NJ), 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, Merck, Darmstadt, Germany; Estes et al., 2010). Finally, the cells were seeded at an density equivalent to 0.16 mL of the original liposuction aspirate per cm^2. The successful isolation of ASC was confirmed by flow cytometry. The population of ASC was positive for CD73 (ecto-5′-nucleotidase), CD90 (immunoglobulin Thy-1), CD105 (endoglin), CD29 (fibronectin receptor), and CD146 (receptor for laminin) and negative for CD31 (platelet endothelial cell adhesion molecule), CD34 (hematopoietic progenitor cell antigen), and CD45 (leucocytes) surface markers (Supplemental Figure S2; https://doi.org/10.3168/jds.2017-13119).

Real-Time Monitoring of Cell Adhesion and Proliferation

The cellular response of osteoblast-like Saos-2 cells (purchased from European Collection of Cell Cultures, Salisbury, UK), ASC (in passage 2), and FIB (in passage 2; purchased from Lonza, Basel, Switzerland) to different WPI concentrations was studied at 37°C in a humidified air atmosphere containing 5% CO_2 for 117 h. The Saos-2 cells, FIB, and ASC were cultured in McCoy's 5A medium, DMEM, and DMEM supplemented with FGF-2, respectively. All of the media contained fetal bovine serum (15% for Saos-2 cells, 10% for FIB and ACS) and gentamicin (40 μg/mL). A real-time cell analyzer (xCelligence, Roche Applied Science, Mannheim, Germany) was used to evaluate the growth of cells in the prepared solutions continuously over a 5-d time span. The cells were seeded into 96-well sensory E plates (E-Plate 96, Cat. No. 05232368001; BioTech a.s., Prague, Czech Republic), and background impedance was measured in each well. The cell densities were 3,500 cells/well (~10,300 cells/cm^2) for Saos-2 and FIB, and 7,000 cells/well (~20,600 cells/cm^2) for ASC. The final volume was 200 μL. After 24 h, cultivation medium was exchanged for appropriate media containing specific concentrations of WPI (0, 50, 300, or 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

$$\text{Cell index} = (\text{impedance at individual time interval} - \text{background impedance})/15\ \Omega.$$

Real-Time Quantitative PCR of Markers of Osteogenic Cell Differentiation

Real-time quantitative PCR was used to determine the effect of WPI content on the level of expression of the genes *COL1* (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 d. Total RNA was extracted from Saos-2, ASC, and FIB using Total RNA purification Micro Kit (Cat. No. 35300; Norgene Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. The mRNA concentration was measured using NanoPhotometer S/N (Implen GmbH, Munich, Germany), and cDNA was synthesized with the ProtoScript M-MuLV First Strand cDNA Synthesis kit (Cat. No. E6300S; New England BioLabs, Ipswich, MA) using 250 ng of total RNA and oligo-dT primers. The reaction was performed in T-Personal Thermocycler (Biometra GmbH, Göttingen, Germany). The qPCR primers were purchased from Generi Biotech Ltd. (Hradec Králové, Czech Republic) and are listed in Table 1. The primers were designed according to the literature (Frank et al., 2002; Reseland et al., 2006; Franke et al., 2007; Zhang et al., 2010). The qPCR was performed using SYBR Green (Roche, Mannheim, Germany) in a total reaction volume of 20 μL and iCycler detection system (iQ 5 Multicolor Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA) with cycling parameters of 10 min at 95°C, 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 analyzed by the $2^{-\Delta\Delta Cq}$ method. The point at which the PCR product was first detected above a fixed threshold (termed cycle threshold, $\mathbf{C_q}$) was determined for each sample. Changes in the expression of target genes were calculated using the equation:

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

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

Alkaline Phosphatase Activity

We studied the influence of different concentrations of WPI on activity of ALP in Saos-2 cells, FIB, and ASC. Cells were cultured in McCoy's 5A medium, DMEM, and DMEM supplemented with FGF-2, respectively. All of the media contained fetal bovine serum (15% for Saos-2 cells, 10% for ACS and FIB) and gentamicin (40 μg/mL). The cells were seeded into 24-well cell culture plates (TPP, Trasadingen, Switzerland). The cell densities were 28,000 cells/well (~15,000 cells/cm^2) for Saos-

2 cells and FIB, and 10,000 cells/well (~5,400 cells/cm^2) for ASC. The final volume was 1 mL. After 24 h, the cultivation medium was exchanged for appropriate media containing specific concentrations of WPI (0, 50, 300, or 800 μg/mL). After 7 d of cultivation, the cell layers were washed twice with PBS; then, 1 mL of the substrate solution [1 mg/mL *p*-nitrophenyl phosphate in substrate buffer (50 m*M* glycine, 1 m*M* $MgCl_2$, pH 10.5; Sigma-Aldrich)] 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 μ*M*; Sigma-Aldrich). The results were normalized by the cell index. The experiments were performed in quadruplicate and repeated 3 times.

Calcium Deposition

The influence of different concentrations of WPI on calcium deposition of Saos-2 cells was studied. Cells were cultured in McCoy's 5A supplemented with 15% fetal bovine serum and 40 μg/mL gentamicin. The cells were seeded into 24-well cell culture plates (TPP). The cell density was 28,000 cells/well (~15,000 cells/cm^2). The final volume was 1 mL. After 24 h, cultivation medium was exchanged for appropriate medium containing specific concentrations of WPI (0, 50, 300, or 800 μg/mL). After 7, 14, and 21 d of cultivation, the cell layers were rinsed with PBS, dried, and lysed in 0.5 *M* HCl for 24 h at 4°C. The calcium in the cell lysates and standards was directly determined using the Calcium Colorimetric Assay Kit (Biovision Inc., Milpitas, CA) according to the manufacturer's protocol. The experiments were performed in quadruplicate and were repeated 3 times. The results were normalized to the cell index.

Statistical Evaluation

The quantitative data of cell proliferation were presented as mean ± standard deviation from 7 measurements. The PCR, ALP activity, and Ca deposition data were presented as mean ± standard deviation of 4 measurements. The statistical analyses were performed using SigmaStat (Jandel Corp./Systat Software Inc., San Jose, CA) by one-way ANOVA and the 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 (Figure 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 WPI were not significantly different from those at lower WPI concentrations. An earlier study (Xu, 2009) investigated the proliferative effect of 2 concentrations of whey protein (0.02 and 0.1 mg/mL) on rat osteoblasts. That author found a positive effect of whey protein on the cell proliferation, which was dose-dependent, similar to our results. In another study, it was reported that β-LG (a major part of WPI) improved the proliferation of enteroendocrine cells (Gillespie et al., 2015). Those authors reported proliferation-stimulating effects of β-LG only within a concentration window of 312.5 to 2,500 μg/mL. In another study on mouse spleen resting cells, β-LG stimulated proliferation in a concentration range of 50 to 500 μg/mL in the time range 12 to 96 h (Mahmud et al., 2004). These β-LG concentration ranges are similar to those investigated in the present study, where 50 to 800 μg/mL WPI represented 40 to 640 μg/mL β-LG; within this range, the proliferation of Saos-2 cell and fibroblasts was promoted. In another study using hybridomas, β-LG concentrations between 750 and 3,000

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

Gene	Primer sequence	Product size (bp)
RUNX2	Forward: 5′-GCCTTCAAGGTGGTAGCCC-3′ Reverse: 5′-CGTTACCCGCCATGACAGTA-3′	100
COL1	Forward: 5′-CAGCCGCTTCACCTACAGC-3′ Reverse: 5′-TTTTGTATTCAATCACTGTCTTGCC-3′	83
ALP	Forward: 5′-GACCCTTGACCCCCACAAT-3′ Reverse: 5′-GCTCGTACTGCATGTCCCCT-3′	68
OC	Forward: 5′-GAAGCCCAGCGGTGCA-3′ Reverse: 5′-CACTACCTCGCTGCCCTCC-3′	70
GAPDH	Forward: 5′-TGCACCACCAACTGCTTAGC-3′ Reverse: 5′-GGCATGGACTGTGGTCATGAG-3′	87

μg/mL stimulated proliferation after 48 h (Moulti-Mati et al., 1991). The positive effect of β-LG on osteogenic differentiation needs to be elucidated in further studies. Finally, lactoferrin, another milk-derived protein, stimulated the adhesion, growth, and osteogenic differentiation of Saos-2 cells in our earlier study (Vandrovcova at al., 2015).

Quantitative PCR was performed on d 7 (Figure 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 significantly promoted expression of *RUNX2* in Saos-2 cells. The RUNX2 protein 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

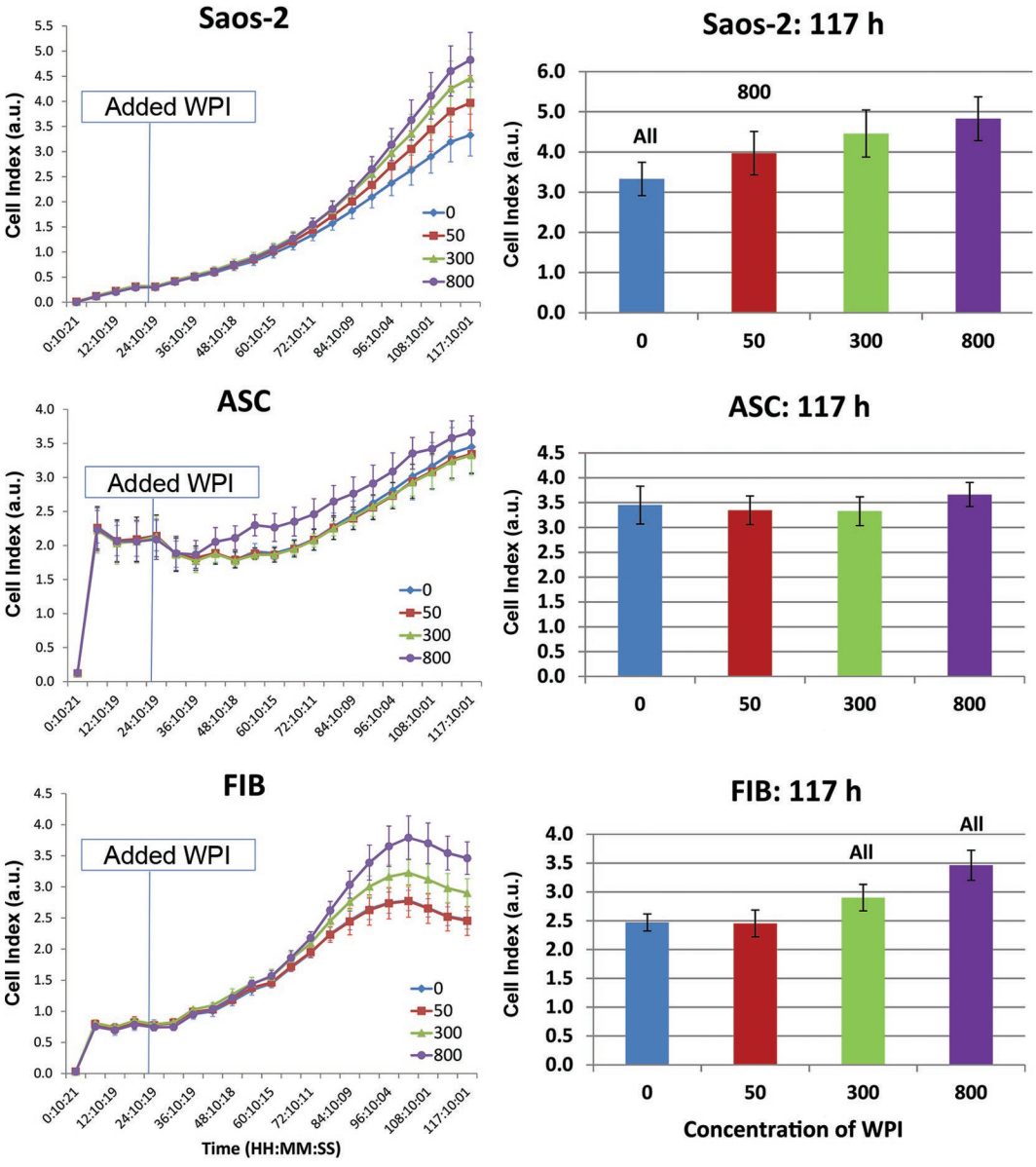

Figure 1. The growth curves represent proliferation of 3 types of cells measured with the xCELLigence system (Roche Applied Science, Mannheim, Germany): osteoblast-like cells (Saos-2), adipose-derived stem cells (ASC), and human neonatal dermal fibroblasts (FIB). After 24 h, proliferative medium was replaced by medium containing different concentrations of whey protein isolate (WPI; 0, 50, 300, or 800 μg/mL). Graphs represent cell number (estimated as a cell index in arbitrary units, a.u.) at the last point of the measurement. For the statistical evaluation, the ANOVA Student-Newman-Keuls method was used. Statistical significance ($P \leq 0.05$): All = compared with all other tested groups, 800 = compared with the cells grown in medium with 800 μg/mL WPI. Color version available online.

suppression of osteoblast maturation and inhibits expression of late osteogenic markers (Liu et al., 2001). It has been reported that expression of *RUNX2* protected Saos-2 from the antiproliferative and apoptotic effects of tumor necrosis factor-α (Olfa et al., 2010). Whether the cells are of osteosarcoma or osteoblast origin is also important. The *RUNX2* gene is expressed at a lower level in several osteosarcoma cell lines; however, in Saos-2 cells (which also are of osteosarcoma origin), expression of *RUNX2* is increased (Cameron et al., 2003). The *RUNX* genes can act either as oncogenes or as tumor suppressors (Blyth et al., 2005). These reports are in accordance with our findings, where higher *RUNX2* expression supported proliferation rather than differentiation of Saos-2 cells, but tended to have the opposite effect in ASC. Expression of *COL1* was evaluated in all types of cells. Saos-2 cells did not respond to WPI, whereas expression of *COL1* was significantly increased in ASC cultured in medium with 800 μg/mL WPI compared with other tested concentrations, and in FIB cultured in medium with 50, 300, or 800 μg/mL of WPI compared with WPI-free medium. The expression of *ALP*, which is considered an early or medium-term marker of cell differentiation, was improved in ASC with increasing concentrations of WPI. No effect was shown in Saos-2 cells. Expression of osteocalcin (*OC*), a late marker of osteogenic differentiation, was not influenced by increasing concentrations of WPI, in either Saos-2 cells or ASC. One explanation might be the relatively short culture interval of 7 d in our study, which might not be sufficient for expression of late markers of osteogenic cell differentiation. On the other hand, *OC* expression can be enhanced in a shorter time interval if the culture conditions strongly promote osteogenic cell

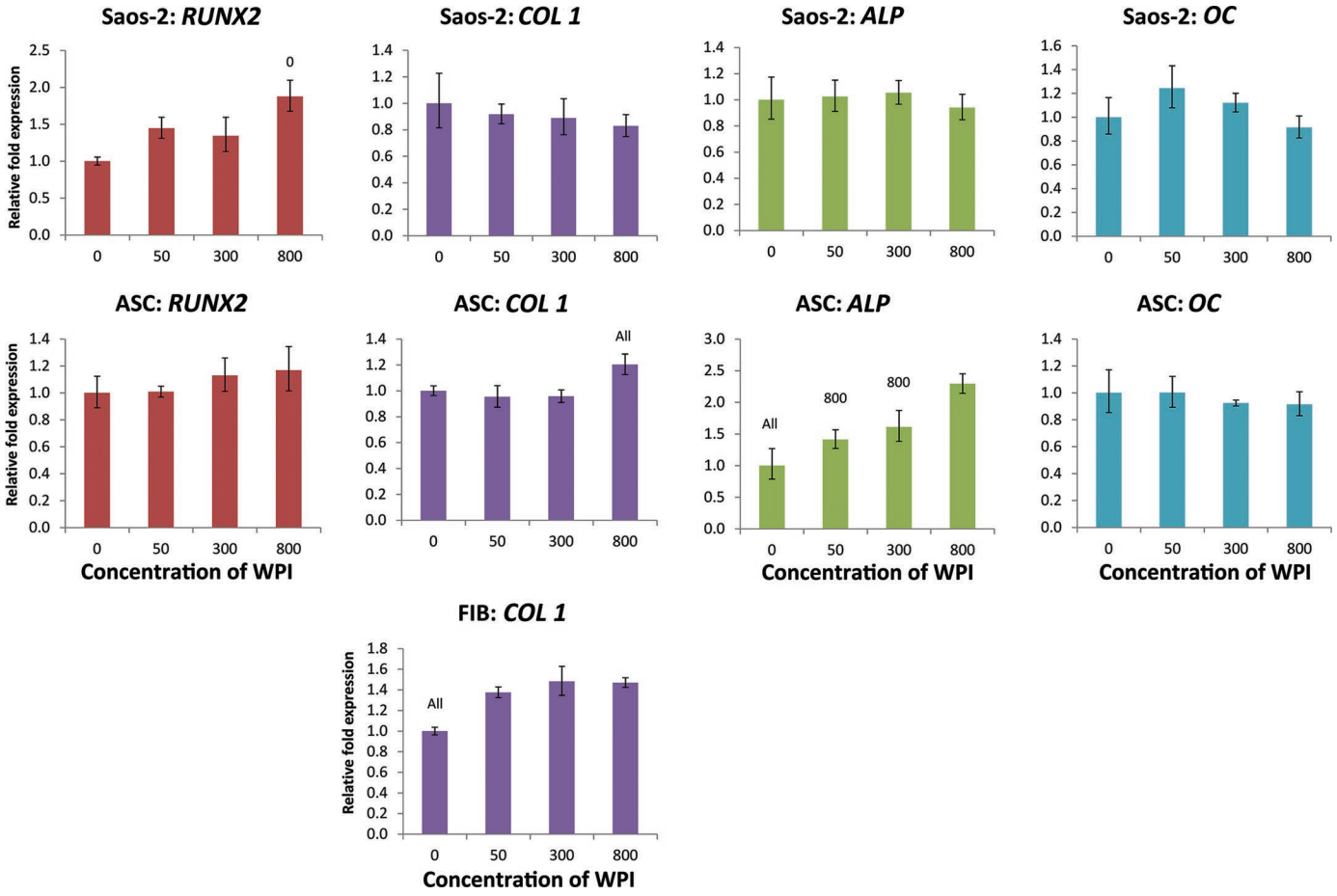

Figure 2. Real-time quantitative PCR results, with results expressed as relative fold expression compared with control values. Expression of transcription factor *RUNX2*, type I collagen (*COL1*), alkaline phosphatase (*ALP*), and osteocalcin (*OC*) were evaluated on d 7 after seeding in the presence of different concentrations of whey protein isolate (WPI; 0, 50, 300, or 800 μg/mL) in 3 cell types: osteoblast-like cells (Saos-2), adipose-derived stem cells (ASC), and human neonatal dermal fibroblasts (FIB). Data are presented as mean ± SD, n = 4. Statistical analysis was performed for the cycle threshold values (ΔCq). For the statistical evaluation, the ANOVA Student-Newman-Keuls method was used. Statistical significance ($P \leq 0.01$): All = compared with all other tested groups; 800 = compared with the cells grown in medium with 800 μg/mL WPI. Color version available online.

differentiation. For example, in our earlier study focusing on the effects of lactoferrin on the behavior of Saos-2 cells, cells on collagen-lactoferrin coatings produced significantly higher levels of osteocalcin than cells on control polystyrene cell culture dishes (Vandrovcova et al., 2015). In the present study, where the effect of WPI on osteogenic cell differentiation appeared to be weaker, only early markers were detected after 7 d of cultivation. Thus, the osteogenic differentiation of cells under influence of WPI was in its early stage after 7 d but we expect that *OC* would be increased after longer culture intervals.

The activity of ALP was evaluated on d 7 (Figure 3). Saos-2 cells are known to contain high amounts of ALP and the reaction had to be stopped after 5 min of incubation. Because of a relatively high content of ALP 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 close to the detection limit after 15 min. The highest concentration of WPI (800 μg/mL) stimulated cells to produce a detectable ALP signal, which was in accordance with our PCR results. Similarly, the study by Xu (2009), performed on rat osteoblasts, showed that whey protein added in the culture medium stimulated the production of ALP in a dose-dependent manner. Fibroblasts are known not to contain ALP, so we decided to measure ALP activity in FIB 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 bone matrix mineralization), calcium deposition was evaluated only in Saos-2 cells after 14 and 21 d (Figure 4). After 7 d of cultivation, calcium deposition was low (below the limit of detection). Moreover, expression of early and medium-term markers of osteogenic cell differentiation (*COL1* and *ALP*) did not differ significantly in Saos-2 after 7 d of cultivation in media with various WPI concentrations. However, on d 14

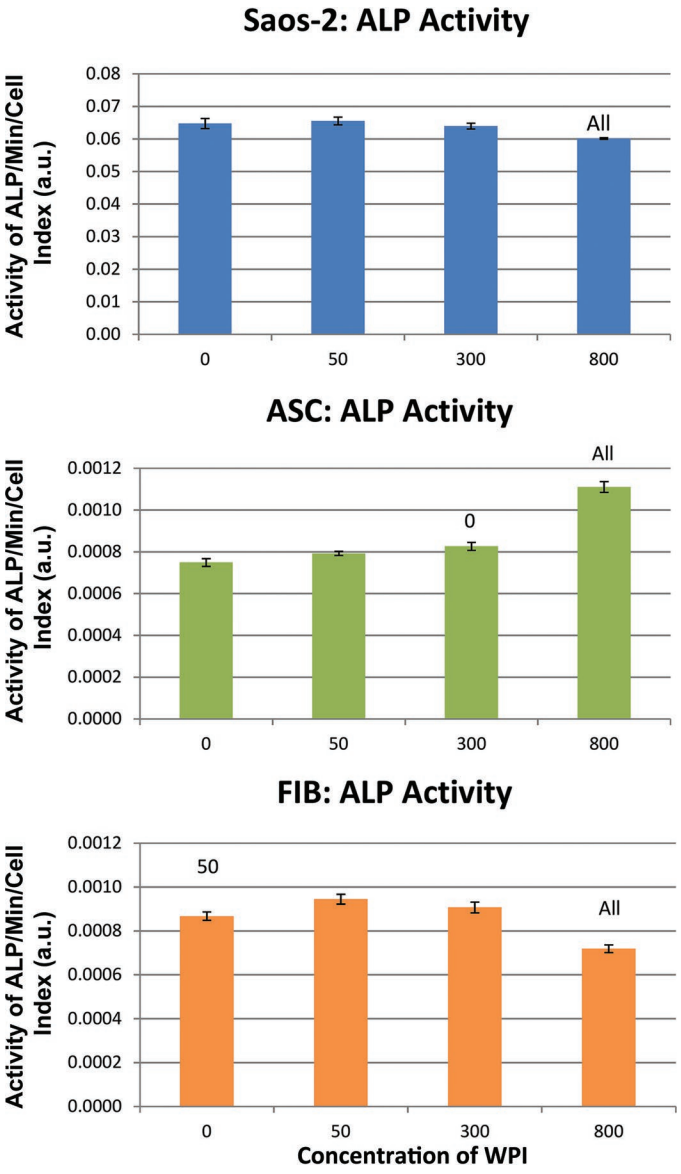

Figure 3. Activity of alkaline phosphatase (ALP) per minute and cell number (arbitrary units, a.u.) in 3 different cells types: osteoblast-like cells (Saos-2), adipose-derived stem cells (ASC), and human neonatal dermal fibroblasts (FIB) after seeding in the presence of different concentrations of whey protein isolate (WPI; 0, 50, 300, or 800 μg/mL). Data are presented as mean ± SD, n = 4. For the statistical evaluation, the ANOVA Student-Newman-Keuls method was used. Statistical significance ($P \leq 0.05$): All = compared with all other tested groups; 0, 50 = compared with the cells grown in medium without WPI and with 50 μg/mL WPI. Color version available online.

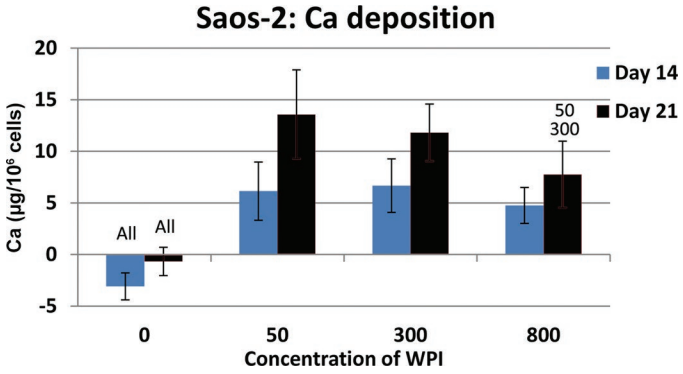

Figure 4. Calcium deposition by osteoblast-like cells (Saos-2) recalculated per cell number after seeding in the presence of different concentrations of whey protein isolate (WPI; 0, 50, 300, or 800 μg/mL). Data are presented as mean ± SD, n = 4. For the statistical evaluation, the ANOVA Student-Newman-Keuls method was used. Statistical significance ($P \leq 0.05$): All = compared with all other tested groups; 50, 300 = compared with the cells grown in medium with 50 and 300 μg/mL WPI. Color version available online.

after seeding, the influence of WPI was evident. On d 21 after seeding, the results were even more apparent. Nevertheless, in that time interval, the supportive effect was negatively correlated with increasing WPI concentration (Figure 4).

Besides the direct positive effects of WPI on proliferation of Saos-2 and FIB, and the osteogenic differentiation of ASC indicated by the results of this study, WPI has several other properties that may be advantageous in bone regeneration. Its main component, β-LG, has an affinity for hydrophobic molecules, which are poorly soluble in water, and β-LG can be used as a carrier protein to improve the solubility and bioavailability of hydrophobic molecules. Thus, β-LG could be used as a carrier or delivery protein for certain molecules that promote osteogenic differentiation, such as purmorphamine (Rezia Rad et al., 2016) or that are thought to promote bone healing, such as vitamin D (Gorter et al., 2014). In addition, WPI can be used to fabricate hydrogels (Puyol et al., 2001), which are gaining interest as biomaterials for bone regeneration (Gkioni et al., 2010). Furthermore, β-LG is inexpensive, as WPI 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.

CONCLUSIONS

The growth of Saos-2 cells and FIB was supported in an apparently dose-dependent manner by WPI. Expression of markers of osteogenic differentiation by ASC, such as *COL1* and *ALP*, were improved by WPI in a concentration-dependent manner (the greatest degree of expression was found for 800 μg/mL of WPI). The expression of *COL1* was also increased in FIB in the presence of WPI (compared with no WPI). The presence of WPI stimulated Saos-2 cells to deposit calcium even in standard culture medium without osteogenic supplements. We conclude 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, as manifested by the expression of *COL1* and *ALP* and the activity of ALP.

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