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

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

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

1
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 (Kontopoulos 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 cm². 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 CO₂ for 117 hours. The Saos-2 cells, FIB and ASC were cultured in McCoy's 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/cm²) for Saos-2 and FIB, and 7,000 cells/well (approximately 20,600 cells/cm²) 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:

\[ \text{Cell index} = \frac{\text{impedance at individual time interval} - \text{background impedance}}{15\Omega} \]

**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™ S/N (IMPLEN). cDNA was synthesized with the ProtoScript®MMuLV 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 $2^{-\Delta\Delta C_q}$ 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:

$$\Delta\Delta C_q = (C_q^{\text{target}} - C_q^{\text{GAPDH}})_{\text{sample}} - (C_q^{\text{target}} - C_q^{\text{GAPDH}})_{\text{calibrator}}$$
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and data 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's 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/cm²) for Saos-2 cells and FIB, and 10,000 cells/well (approximately 5,400 cells/cm²) 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 MgCl₂, 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.
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/cm²). 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 ≤ 0.05 was considered significant (p ≤ 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 (Mouli-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
expression of late osteogenic markers (Liu et al., 2001). It has been reported that the expression of the \textit{RUNX2} protected Saos-2 from the antiproliferative and apoptotic effects of TNF-\textalpha{} (Olfa et al., 2010). It is also important if the cells are of osteosarcoma or osteoblast origin. \textit{RUNX2} is expressed at a lower level in several osteosarcoma cell lines; however, in Saos-2 cells (which also are of osteosarcoma origin), the expression of \textit{RUNX2} is increased (Cameron et al., 2003). \textit{RUNX} genes can act either as oncogenes or tumor suppressors (Blyth et al., 2005). It is in accordance with our findings, where a higher \textit{RUNX2} expression supported proliferation rather than differentiation of Saos-2 cells, but tended to have an opposite effect in ASC. Expression of \textit{COL I} was evaluated in all types of cells. Saos-2 cells did not respond to WPI. The expression was, however, significantly increased in ASC cultured in the medium with 800 \(\mu\)l/ml of WPI in comparison to the media with other tested concentrations, and in FIB in media with 50, 300 and 800 \(\mu\)g/ml of WPI in comparison to WPI-free medium. The expression of alkaline phosphatase (\textit{ALP}), which is considered as an early or medium-term marker of cell differentiation, was improved in ASC by increasing the concentration of WPI. No effect was proven in Saos-2 cells. Expression of osteocalcin (\textit{OC}), a late marker of osteogenic differentiation, was not influenced by increasing concentrations of WPI, neither in Saos-2 cells nor in ASC. An explanation is the relatively short culture interval of 7 days in our study, which might not be sufficient for expression of late markers of osteogenic cell differentiation. On the other hand, \textit{OC} expression can be enhanced even in a shorter time interval, if the culture conditions strongly promote the osteogenic cell differentiation. For example, in our earlier study focussing on the effects of lactoferrin on the behaviour of Saos-2 cells, the cells on collagen-lactoferrin coatings produced significantly higher levels of osteocalcin than cells on control polystyrene cell culture dishes (Vandrovcova 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 bioavailability. 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 I 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 I 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 \textit{COL I} and \textit{ALP}, and the activity of ALP.

ACKNOWLEDGMENTS

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REFERENCES


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 $\Delta Cq$. 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 ≤ 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

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<th>Gene</th>
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<td>RUNX2</td>
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<td>Reverse: 5′- CGTTACC CGCCATGACAGTA-3′</td>
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<td>COL 1</td>
<td>Forward: 5′-CAGCCGCTTCACCTACAGC-3′</td>
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<td>Reverse: 5′-TTTTGTATTCAATCAGTCTTGCC-3′</td>
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Supplementary Figure 1. RP-HPLC analysis (intensity versus retention time) of a pure DMEM solution with or without addition of 0.8 mg/ml whey protein isolate before (solid line) and after (dotted line) filtration with a 0.2 µm acetate cellulose filter. ALA, alpha-Lactalbumin; BLG, beta-Lactoglobulin genetic variants A and B.

Supplementary Figure 2. Cell surface antigens of human ASCs were detected by flow cytometry. The flow cytometry analysis showed a positive expression of standard surface markers of ASCs, namely CD 29, CD 73, CD 90, and CD 105. However, no expression of the markers of CD 31, CD 34, CD 45, and CD 146 was detected in isolated ASCs.
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).
Saos-2: ALP Activity

ASC: ALP Activity

FIB: ALP Activity

155x272mm (600 x 600 DPI)
Saos-2: Ca deposition

![Graph showing Ca deposition over different concentrations of WPI for Day 14 and Day 21.](Image)

37x20mm (600 x 600 DPI)