



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

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1 Interpretive summary: Application of whey protein isolate in bone regeneration: promotion of  
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3 Whey protein isolate (WPI) consists of 2 main components, namely  $\beta$ -lactoglobulin (bLG)  
4 and  $\alpha$ -lactalbumin, WPI can modulate immunity and acts as an antioxidant, antitumor,  
5 antiviral and antibacterial agent. However, its effects on bone-forming cells remain unknown.  
6 The aim of this study was to test differentiation-stimulating potential of WPI rich in bLG on  
7 osteoblast-like cells and adipose-derived stem cells with emphasis on its potential use in tissue  
8 engineering. We observed positive effects on cell proliferation, expression of markers of cell  
9 differentiation and calcium deposition. Thus, WPI has potential in tissue engineering.

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11 WPI PROMOTES DIFFERENTIATION OF BONE-FORMING CELLS

12

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

15

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31

**ABSTRACT**

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

48

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

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## INTRODUCTION

52

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

62 Despite the controversy of milk consumption in adulthood (Pereira, 2014), milk is a  
63 cheap source of compounds needed for bone development and regeneration. Milk contains 2  
64 main groups of proteins: caseins (which represent 80% of all proteins in ruminants' milk and  
65 consists of 4 major proteins) and whey protein (which represents 20% of all proteins in  
66 ruminant's milk and consists of 2 main components, namely  $\beta$ -lactoglobulin (bLG) and  $\alpha$ -  
67 lactalbumin, and smaller amounts of serum albumin, lactoferrin, and other proteins (Do et al.,  
68 2016). Whey protein was considered to be a waste product in the dairy industry. It contains  
69 the aforementioned compounds in different ratios depending on the method of cheese  
70 manufacture. Various types of whey protein exist, such as reduced-lactose whey,  
71 demineralized whey, whey protein concentrates, and whey protein isolates (WPI) (Walzem et  
72 al., 2002). Whey proteins contain a higher amount of amino acids rich in sulfur in comparison  
73 to caseins. It is believed that proteins rich in sulfur provide a higher protein efficiency ratio  
74 (i.e. weight gain to intake of protein during the tested period). Moreover, these types of  
75 proteins are important in immune modulation (Bounous and Gold, 1991). Whey proteins also

3

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

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

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

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

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

111

## 112 MATERIALS AND METHODS

### 113 *WPI Preparation, Composition and Sterilisation*

114 WPI (Whey protein isolate, BiPRO, Davisco Foods International, Inc., Eden Prairie, US) with  
115 97.7% protein and 75% bLG in dry matter (according to the manufacturer's specification) was  
116 used. Our HPLC analysis according to Keppler et al., (2014) (see supplementary Figure 1)  
117 confirms that the WPI dissolved in cell culture medium is dominated by approximately 80 %  
118 bLG genetic variants b and a (elution time 19.2 and 20.1 min) and some alpha lactalbumin  
119 (elution time 10.7 min.) This is in accordance with previous analyses of the same WPI  
120 dissolved in water (Keppler et al. 2017a; Keppler et al. 2017b). As expected, there was no  
121 protein loss due to filtration.

122

### 123 *Adipose-Derived Stem Cells Isolation and Characterization*

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

148

**149 *Real-Time Monitoring of Cell Adhesion and Proliferation***

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

167

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

169

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

171 Real-time quantitative PCR (Q-PCR) was used to determine the effect of WPI content on the  
172 level of expression of genes for *COL 1* (Saos-2, ASC, FIB), *ALP* (Saos-2, ASC), and *OC*  
173 (Saos-2, ASC). The expression of transcription factor *RUNX2*, also involved in osteogenic  
174 cell differentiation, was evaluated in Saos-2 and ASC. Cells were grown in the tested  
175 solutions for 7 days. Total RNA was extracted from Saos-2, ASC and FIB using Total RNA  
176 purification Micro Kit (NORGENE Biotek Corp, Cat. No. 35300) according to the  
177 manufacturer's instructions. The mRNA concentration was measured using  
178 NanoPhotometer™ S/N (IMPLEN). cDNA was synthesized with the ProtoScript®M-MuLV  
179 First Strand cDNA Synthesis kit (New England BioLabs, Cat. No. E6300S) using 250 ng of  
180 total RNA and oligo-dT primers. The reaction was performed in T-Personal Thermocycler  
181 (Biometra). Q-PCR primers were purchased from Generi Biotech Ltd. and are listed in Table  
182 1. The primers were designed according to the literature (Reseland et al., 2006; Franke et al.,  
183 2007; Zhang et al., 2010; Frank et al., 2002). Real-time quantitative PCR was performed  
184 using SYBR Green (Roche) in the total reaction volume to 20 µL and iCycler detection  
185 system (iQ™ 5 Multicolor Real-Time PCR Detection System, Bio-Rad) with cycling  
186 parameters of 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by  
187 a melt curve. Assays were conducted in quadruplicates. Data were analysed by the  $2^{-\Delta\Delta C_q}$   
188 method. The point at which the PCR product was first detected above a fixed threshold  
189 (termed cycle threshold,  $C_q$ ), was determined for each sample. Changes in the expression of  
190 target genes were calculated using the equation:

191

$$192 \Delta\Delta C_q = (C_q^{\text{target}} - C_q^{\text{GAPDH}})_{\text{sample}} - (C_q^{\text{target}} - C_q^{\text{GAPDH}})_{\text{calibrator}}$$

193

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

197

### 198 *Alkaline Phosphatase (ALP) Activity*

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

217

### 218 *Calcium Deposition*

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

230

### 231 *Statistical Evaluation*

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

237

238

## RESULTS AND DISCUSSION

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

10

242 Nevertheless, the values at 800  $\mu\text{g/ml}$  were not significantly different from those at lower  
243 WPI concentrations. An earlier study (Xu, 2009) investigated the proliferative effect of two  
244 concentrations of whey protein (0.02 and 0.1  $\text{mg/ml}$ ) on rat osteoblasts. The author found a  
245 positive effect of whey protein on the cell proliferation, which was dose-dependent, similarly  
246 as in our present study. In another study it was reported that bLG (a major part of WPI)  
247 improved the proliferation of enteroendocrine cells (Gillespie et al., 2015). These authors  
248 reported proliferation-stimulating effects of bLG only in a concentration window of 312.5-  
249 2500  $\mu\text{g/ml}$ . In another study on mouse spleen resting cells, bLG stimulated proliferation in  
250 the concentration range 50-500  $\mu\text{g/ml}$  in the time range 12-96 h (Mahmud et al., 2004). These  
251 bLG concentration ranges are similar to the range investigated in the present study, where 50-  
252 800  $\mu\text{g/ml}$  of WPI represented 40-640  $\mu\text{g/ml}$  of bLG, and within this range, the proliferation  
253 of Saos-2 cell and fibroblasts was promoted. In another study using hybridomas, bLG  
254 concentrations between 750 and 3,000  $\mu\text{g/ml}$  stimulated proliferation after 48 h (Moulti-Mati  
255 et al., 1991). The positive effect of bLG on osteogenic differentiation needs to be elucidated  
256 in further studies. Last but not least, lactoferrin, another milk-derived protein, stimulated the  
257 adhesion, growth and osteogenic differentiation of Saos-2 cells in our earlier study  
258 (Vandrovcova et al., 2015).

259 Q-PCR was performed on day 7 (Fig. 2). Markers of osteogenic differentiation were  
260 measured in Saos-2 cells and ASC. The transcription factor *RUNX2*, an early marker of  
261 osteogenic differentiation, was evaluated in Saos-2 cells and ASC. Despite the apparent  
262 tendency in ASC, only the highest concentration of WPI promoted expression of *RUNX2* in  
263 Saos-2 cells significantly. *RUNX2* is a potent osteoblast transcription factor, which promotes  
264 expression of type-I collagen in the early differentiation stage (Fakhry et al., 2013). However,  
265 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  $TNF-\alpha$  (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  $\mu$ l/ml of WPI in comparison to the media with other tested  
277 concentrations, and in FIB in media with 50, 300 and 800  $\mu$ g/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

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

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

304 In view of the fact that ASC and FIB showed almost no activity of ALP (i.e., an  
305 enzyme involved in the bone matrix mineralization), calcium deposition was evaluated only in  
306 Saos-2 cells after 14 and 21 days (Fig. 4). After 7 days of cultivation the calcium deposition  
307 was low, under the limit of detection. Moreover, the expression of early and medium-term  
308 markers of osteogenic cell differentiation, i.e. *COL I* and *ALP*, did not differ significantly in  
309 Saos-2 after 7 days of cultivation in media with various WPI concentrations. However, on day  
310 14 after seeding, the influence of the presence of WPI was evident. On day 21 after seeding,  
311 the results were even more apparent. Nevertheless, in that time interval, the supportive effect  
312 was rather negatively correlated with increasing WPI concentration (Fig. 4).

313 Besides the direct positive effects of WPI on proliferation of Saos-2 and FIB, and  
314 osteogenic differentiation of ASC suggested by the results of this study, WPI has several other  
315 properties that may be advantageous in bone regeneration. As mentioned in the introduction,  
316 its main component bLG has an affinity for hydrophobic molecules which are poorly soluble  
317 in water and can be employed as a carrier protein to improve their solubility and  
318 bioavailability. One can speculate that bLG could be employed as a carrier or delivery protein  
319 for certain molecules, which promote osteogenic differentiation, such as purmorphamine  
320 (Rezia Rad et al. 2016) or which are suspected to promote bone healing, such as vitamin D  
321 (Gorter et al., 2014). In addition, it is possible to use WPI to fabricate hydrogels (Puyol et al.,  
322 2001). Hydrogels are gaining interest as biomaterials for bone regeneration (Gkioni et al.,  
323 2010). Furthermore, bLG is inexpensive, as whey protein isolate is a commonly used food  
324 supplement, e.g. in bodybuilding (Marshall, 2004), and is thus produced in large quantities.  
325 Hence, we believe that applications of WPI in bone regeneration, both in solution and as a  
326 biomaterial component, are worthy of further investigation.

327

328

## CONCLUSION

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

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

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340

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For Peer Review

## CAPTIONS

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

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

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

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501 tested groups; 0, 50: in comparison with the cells grown in media without WPI and with 50  
 502  $\mu\text{g/ml}$  of WPI, respectively.

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

509

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

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

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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  $\mu\text{m}$  acetate cellulose filter. ALA, alpha-  
517 Lactalbumin; BLG, beta-Lactoglobulin genetic variants A and B.

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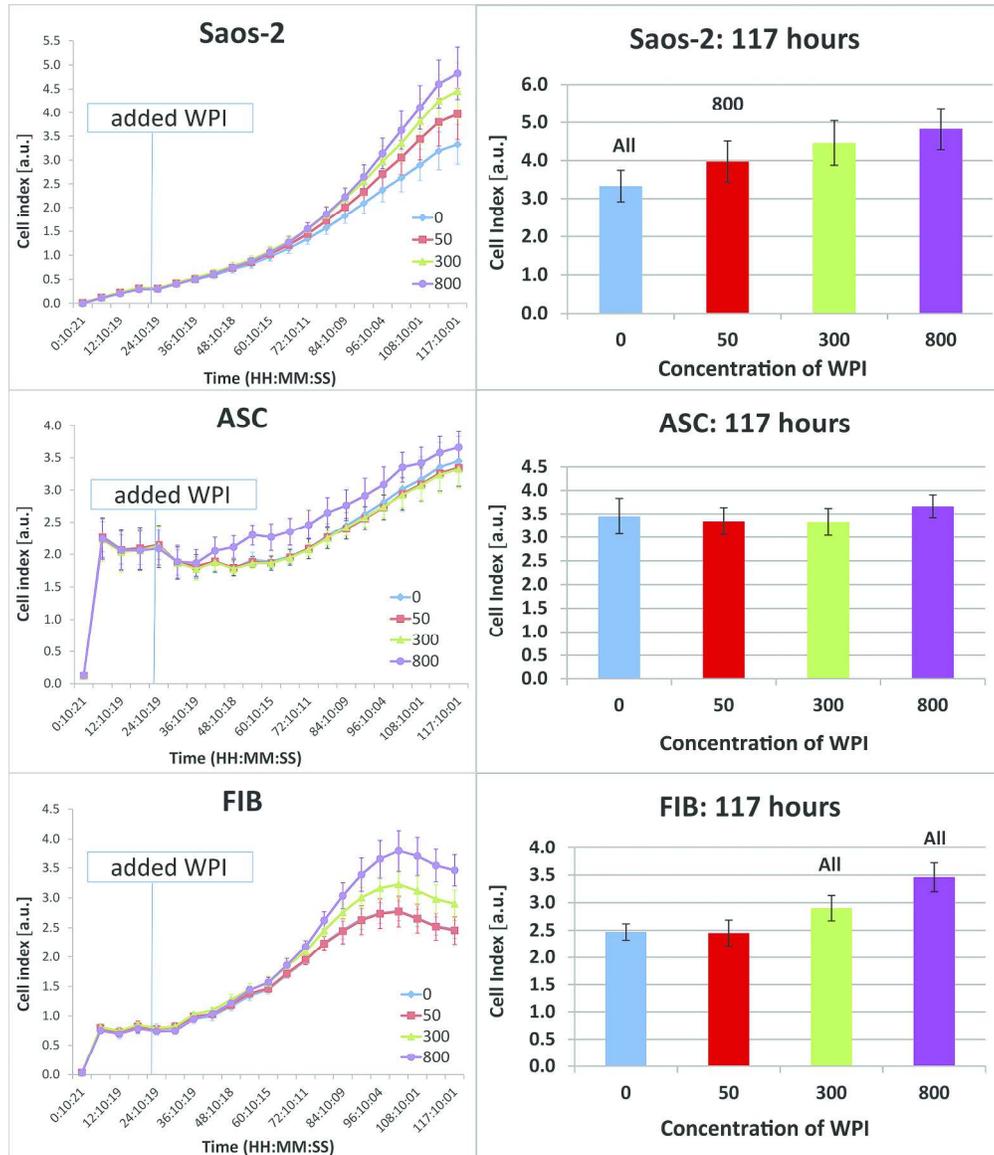
519 Supplementary Figure 2. Cell surface antigens of human ASCs were detected by flow  
520 cytometry. The flow cytometry analysis showed a positive expression of standard surface  
521 markers of ASCs, namely CD 29, CD 73, CD 90, and CD 105. However, no expression of the  
522 markers of CD 31, CD 34,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

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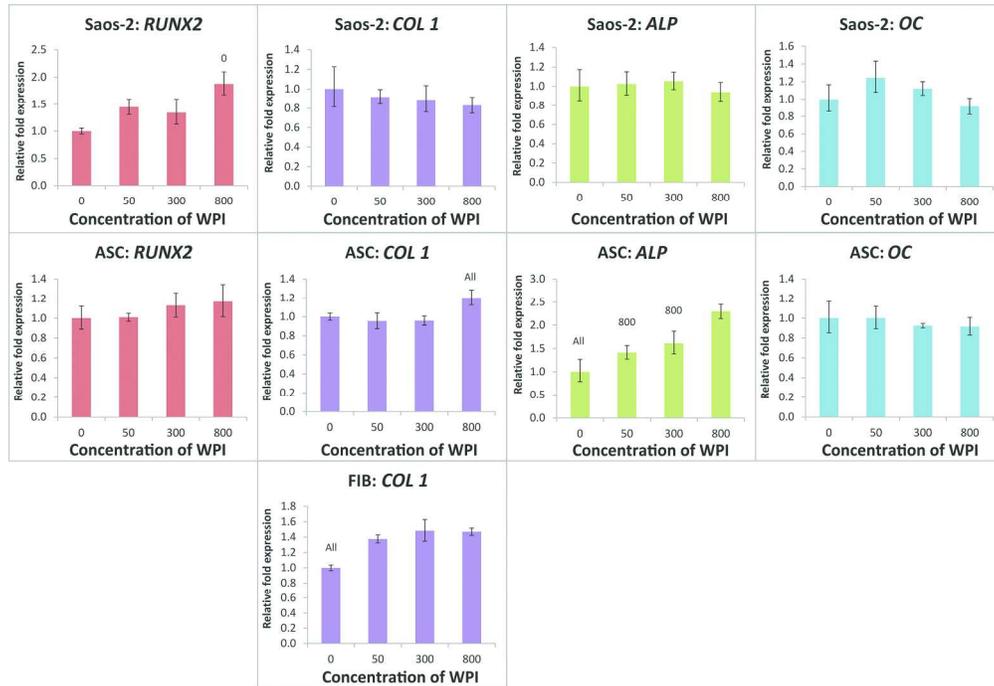
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Before cell experiments, the WPI was dissolved in the appropriate cell culture medium and sterilised using a 0.2 µm acetate cellulose filter (Sartorius). The medium or 800 µg/ml WPI dissolved in the medium were either injected directly or filtered 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% trifluoroacetic 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.

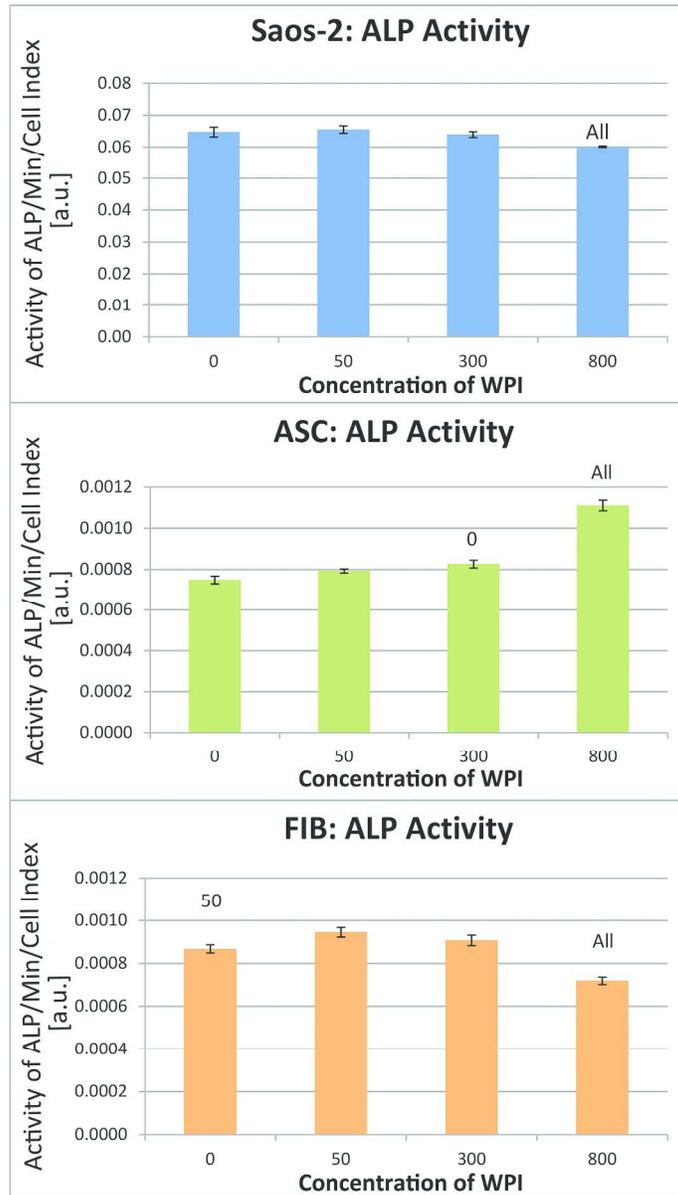
The effect of the filtration on the WPI composition would be common for all three media. However, it is important to know if the protein composition is altered by filtration, as this would influence the experimental setup (to a similar degree in all cases, but nonetheless this is important in case of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



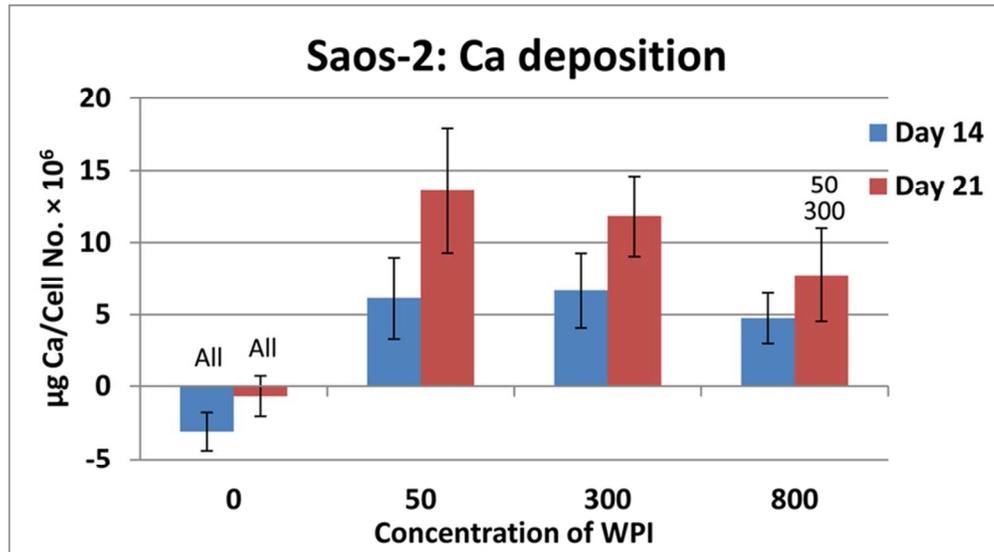
162x188mm (600 x 600 DPI)



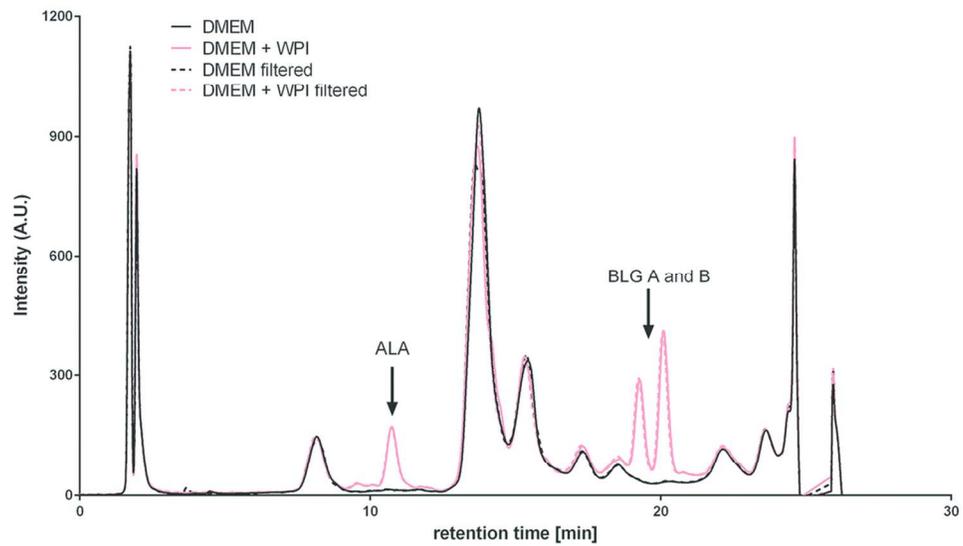
96x66mm (600 x 600 DPI)



155x272mm (600 x 600 DPI)

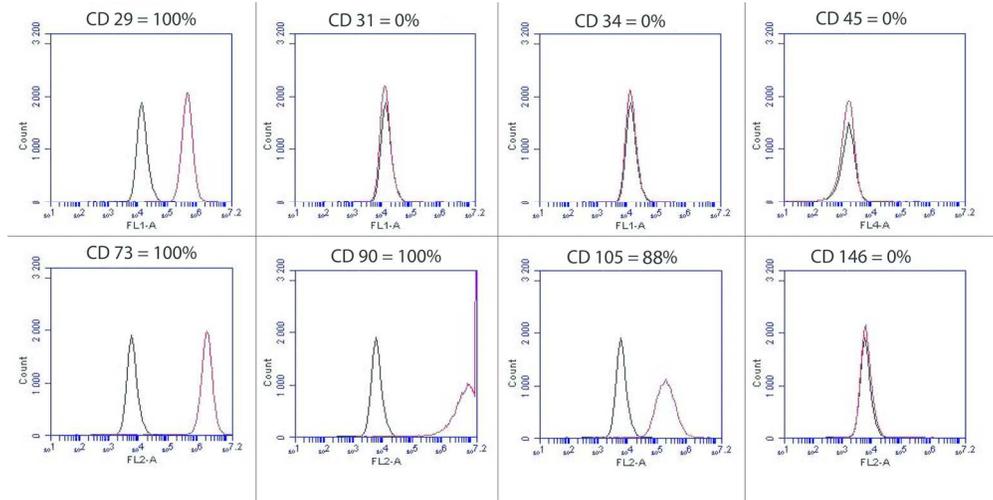


37x20mm (600 x 600 DPI)



50x28mm (600 x 600 DPI)

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70x35mm (600 x 600 DPI)