

Grade: 1,3

Protocol for the experiment  
**Bone Tissue Engineering (MKG Lab)**

Master Biomedical Technologies, Labwork Implantology

June 09, 2022

# 1 Introduction

The use of cell-based therapies is nowadays a promising approach for the repair of bone defects in oral and maxillofacial surgery. Multipotent bone marrow mesenchymal stromal cells (bmMSCs), which have the ability to differentiate into bone tissue-forming osteoblasts, are often used to generate new bone tissue [1]. However, their availability is limited and their osteogenic potential decreases with increasing age. An alternative is provided by human jaw periosteal cells (JPCs), which exhibit age-independent osteogenic differentiation and strong multipotency, while being easier to access and having higher proliferation and self-renewal rates compared to bmMSCs [1] [2].

To enhance the formation of bone tissue, cells are cultured in osteogenic medium containing different activation factors before seeding into a scaffold [3]. To study cell osteogenesis, the activity of the enzyme alkaline phosphatase (ALP) is used as a marker, since it promotes osteoblast mineralization and thus bone formation through the generation of phosphates [4]. For the creation of three-dimensional bone tissue, after cultivation in the osteogenic medium, the cells are seeded into scaffolds made of biomaterials such as polylactic acid. It is of interest to determine a scaffold, that serves to attach the cells and which should be, at the same time, biocompatible and stable, as well as have osteoconductive properties.  $\beta$ -tricalcium-phosphate ( $\beta$ -TCP) is a promising material for this purpose due to its stability and biocompatibility. Moreover, the material is resorbed by the body after some time, releasing calcium and phosphate ions, which are essential components in the formation of new bone tissue [1].

In this experiment, different influences on the osteogenesis of JPCs were analysed. For this purpose, the effect of the surrounding structure on the cells was investigated by seeding the cells two-dimensionally in wells and three-dimensionally in  $\beta$ -TCP scaffolds. Secondly, the influence of an osteogenic medium on the JPCs was studied by culturing one group in the osteogenic medium and leading the control group untreated. To measure osteogenesis, ALP activity was measured using an ALP coulometric kit [5].

# 2 Methods

The experiment was performed as described in the handout [5]. Before, JPCs were cultured under four different conditions. In two samples, cells were seeded two-dimensionally in wells and in the other two in three-dimensional  $\beta$ -TCP scaffolds. In both cases one of each sample was previously placed in osteogenic medium before seeding. During the

labwork, one triplicate of each condition was prepared, however, for the three-dimensional samples, the triplicates were mixed and then divided back into triplicates afterwards. The prepared cell suspensions of the four conditions were placed in wells for further analysis [5].

The ALP activity of the cell suspensions was measured using the Sensolyte® p-nitrophenyl phosphate (pNPP) ALP assay kit. The principle of the assay is based on the dephosphorylation of pNPP substrate by the ALP present in the samples. When the colourless pNPP was added to the individual samples, it was converted to 4-methoxyphenol and phosphoric acid by the ALP in the presence of water. The absorbance of the samples, which occurred due to the resulting yellow coloration, could be determined by photometric measurement with light of wavelength 405 nm and was proportional to the ALP activity [6].

Since each sample had a different number of cells, the total protein amount was measured using the Pierce™ bicinchoninic acid (BCA) protein assay for subsequent normalization of the ALP activity. A mixture of BCA and cupric sulphate solution was added to the samples. After the addition, the  $\text{Cu}^{+2}$  in the solution was reduced to  $\text{Cu}^{+1}$  by the proteins contained in the samples. The reduced copper could then be detected by BCA, since the binding of two BCA molecules to a copper ion resulted in a purple coloration of the samples. The resulting absorbance, proportional to the total protein amount, was measured photometrically at a wavelength of 550 nm [7]. In this experiment, the photometric measurement was delayed by 15 minutes, causing the incubation of the samples to last 45 minutes instead of 30 minutes [5].

### 3 Results

After the two measurements, the absorbance values of the individual wells, which were stored in two Excel files, were corrected and, using the standards, a regression line was created for the ALP and BCA measurements each, which describes the relationship between absorbance and concentration of the end products of the chemical reactions. Since the absorbance values of the standards in the ALP assay were unusually low, those of a previous measurement were taken for the calculation of the regression line. The slopes of the two lines were used to determine the concentrations in all samples (see table 1). In the upper part of table 1, the concentration values of the samples seeded in the two-dimensional wells (2D) are plotted, and in the lower part, the values of the samples introduced in the scaffolds (3D) can be seen. The values of the ALP activity (ALP) and the

total protein concentration (BCA) of the control group (CO) and the group that was previously cultured in an osteogenic medium (OB) are shown, respectively. For the values of the ALP activity, samples diluted with assay buffer at a ratio of 1:5 were used because the absorbance values of the undiluted values were too high to measure with the photometer. In the evaluation, the ALP values were multiplied by five so that they were comparable to the other measured values.

Since each well contained a different cell concentration, the values were normalized by dividing the ALP concentration by the respective total protein concentration (ratio ALP/BCA). Finally, the means and standard deviations (SD) of all triplicates were calculated.

*Table 1: Resulting concentrations and calculated normalized ALP activity of the triplicates*

|           | <b>Sample</b>               | <b>Trial 1</b> | <b>Trial 2</b> | <b>Trial 3</b> | <b>Mean</b> | <b>SD</b> |
|-----------|-----------------------------|----------------|----------------|----------------|-------------|-----------|
| <b>2D</b> | <b>ALP_CO [ng/ml]</b>       | 941.780        | 937.210        | 975.860        | 951.617     | 21.119    |
|           | <b>BCA_CO [mg/ml]</b>       | 0.434          | 0.461          | 0.472          | 0.456       | 0.020     |
|           | <b>Ratio ALP/BCA_CO [%]</b> | 0.217          | 0.203          | 0.207          | 0.209       | 0.007     |
|           | <b>ALP_OB [ng/ml]</b>       | 1390.650       | 1605.525       | 1118.835       | 1371.670    | 243.900   |
|           | <b>BCA_OB [mg/ml]</b>       | 0.423          | 0.625          | 0.374          | 0.474       | 0.133     |
|           | <b>Ratio ALP/BCA_OB [%]</b> | 0.329          | 0.257          | 0.299          | 0.295       | 0.036     |
| <b>3D</b> | <b>ALP_CO [ng/ml]</b>       | 93.080         | 118.850        | 156.255        | 122.728     | 31.7656   |
|           | <b>BCA_CO [mg/ml]</b>       | 0.381          | 0.389          | 0.386          | 0.385       | 0.004     |
|           | <b>Ratio ALP/BCA_CO [%]</b> | 0.024          | 0.031          | 0.040          | 0.032       | 0.008     |
|           | <b>ALP_OB [ng/ml]</b>       | 21.595         | 29.490         | 49.855         | 33.647      | 14.581    |
|           | <b>BCA_OB [mg/ml]</b>       | 0.373          | 0.410          | 0.403          | 0.400       | 0.020     |
|           | <b>Ratio ALP/BCA_OB [%]</b> | 0.006          | 0.007          | 0.012          | 0.008       | 0.003     |

The means of the normalized ALP activity of each condition were plotted in a graph (see figure 1). In addition, an F-test was performed to compare the standard deviations of the samples, as well as a two-tailed t-test with a significance interval of 5 %. The results of the t-test were added to the graph.

It can be seen that the two samples cultured two-dimensionally each had significantly higher values with 0.209 % (CO) and 0.385 % (OB) in ALP activity than the correspondent samples with 0.032 % (CO) and 0.008 % (OB) ALP activity cultured in three-dimensional

scaffolds. No significant difference was observed between the two two-dimensional samples, whereas in the three-dimensional samples, the control sample had significantly higher ALP activity than the sample cultured in osteogenic medium.

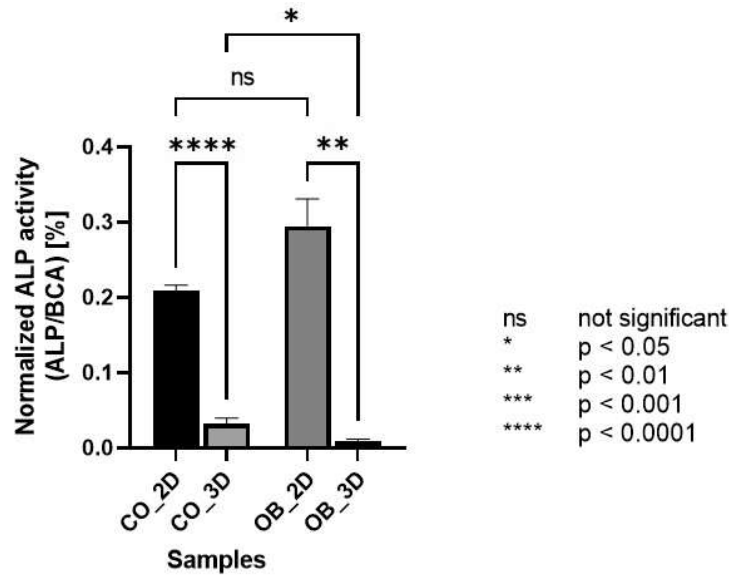


Figure 1: Normalized ALP activity of the four conditions with added results of the t-test

## 4 Discussion

In this experiment, it was shown that, contrary to expectations,  $\beta$ -TCP scaffolds were not suitable for colonization by JPCs, as this resulted in significantly lower ALP activity and thus osteogenesis of the cells compared to cells cultured two-dimensionally. Reasons for this could be that the attachment of the cells to the scaffolds and the cell-matrix interaction had not worked properly, limiting cell proliferation. Coating the scaffold surface with substances that improve matrix-cell interaction could solve this problem. In addition, it is likely that statistical fluctuations affected the results because only triplicates of the samples were examined and, moreover, replicates of the three-dimensional samples were mixed during the experiment. Furthermore, it is suspected that impurities and measurement errors during pipetting, as well as the 15-minute delayed absorbance measurement of the BCA assay influenced the results.

When the two samples that had been grown in two dimensions were examined, it was found that there was no significant difference in the ALP activity. In the two three-dimensional samples, however, there was significance, although the p-value was only less than 0.05. It is suggested that the significant higher value of the control group may have occurred due to statistical fluctuations and measurement error, as in both two-dimensional and three-dimensional the normalized ALP activity of the samples had similar values and

thus the osteogenesis was almost identical, contrary to expectations. It was expected that cells cultured in the osteogenic medium would exhibit higher osteogenesis [1]. However, since the response to different osteogenic media varies depending on the donor cells, this result may have occurred [8].

To exactly describe the influence of the osteogenic medium on the JPCs, further studies using more samples than in this experiment are necessary.

## 5 References

- [1] M. Weber et al., "Influence of Human Jaw Periosteal Cells Seeded  $\beta$ -Tricalcium Phosphate Scaffolds on Blood Coagulation," *International Journal of Molecular Sciences* 22(18), 2021.
- [2] D. Alexander et al., "LNGFR Induction During Osteogenesis of Human," *Cellular Physiology and Biochemistry* 24, pp. 283-290, 2009.
- [3] X. Fu et al., "Improved osteogenesis and upregulated immunogenicity in human placenta-derived mesenchymal stem cells primed with osteogenic induction medium," *Stem Cell Research & Therapy* 7, 2016.
- [4] T. Osathanon et al., "Immobilization of alkaline phosphatase on microporous nanofibrous fibrin scaffolds for bone tissue engineering," *Biomaterials* 30(27), pp. 4513-4521, 2009.
- [5] D. Alexander-Friedrich et al., "Handout practical course in medical technology, Master module "Implantology"".
- [6] ANASPEC, *Manual "SensoLyte®pNPP Alkaline Phosphatase Assay Kit \*Colorimetric\*"*.
- [7] "Thermo Scientific™," Pierce™ BCA Protein Assay Kit, [Online]. Available: <https://www.thermofisher.com/order/catalog/product/23225>. [Accessed 09 June 2022].
- [8] F. Umrath et al., "iPSC-Derived MSCs Versus Originating Jaw Periosteal Cells: Comparison of Resulting Phenotype," *International Journal of Molecular Sciences*, 2020.