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ENGINEERING A VASCULARIZED BONE TISSUE: METHODS, EVALUATION AND DISCUSSION

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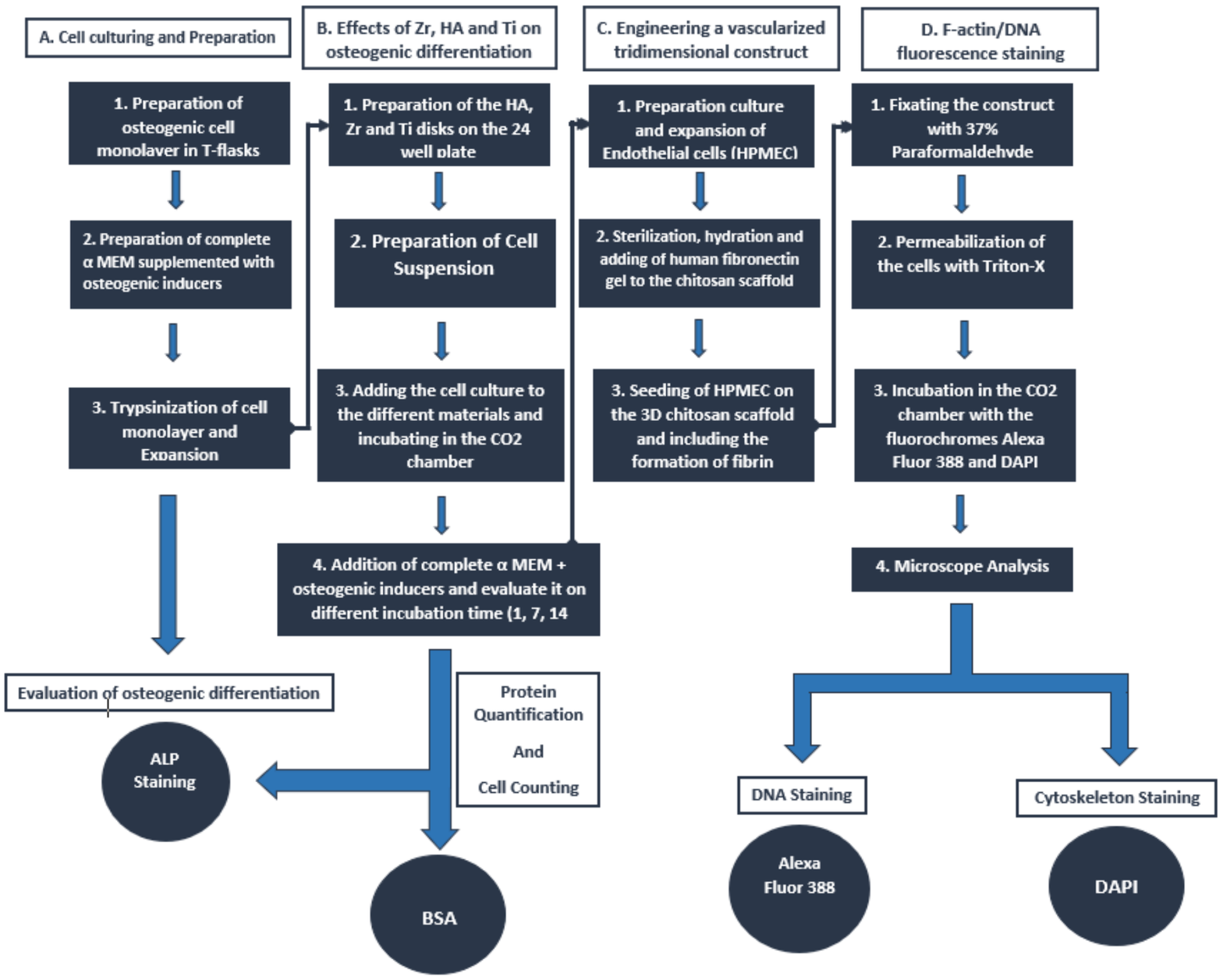
THEORETICAL BACKGROUND

The main objective of the research was to study the effect of various materials (zirconia, hydroxyapatite and titanium) in osteogenic differentiation and proliferation of MC3T3-E1 cells. Later we seed the bone cells in a chitosan scaffold in order to induce in vitro vascularization with HPMEC endothelial cells. This step was evaluated through microscopic analysis.

METHODOLOGY



RESULTS AND DISCUSSION

ALP STAINING

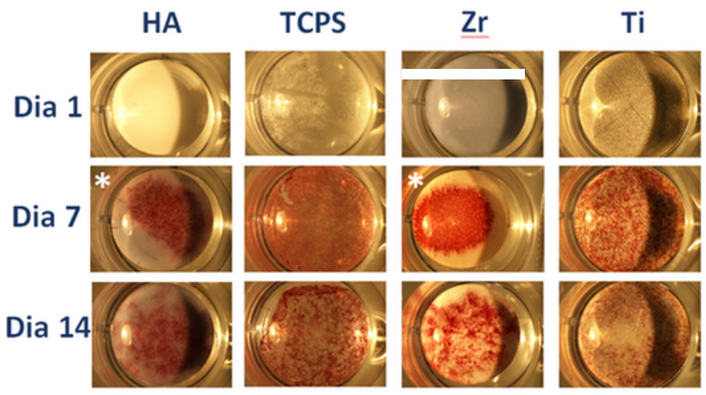


Figure 1. Comparison between HA, TCPS, Zr and Ti, using ALP activty histochemichal staining (* photos from class A).

With Sigma-Aldrich method a visual distinction of the proliferation of the MC3T3-E1 cells throughout time can be seen and how it is affected by the type of material the cell is seeded on. In day one, none of the materials had differentiated cells so the red speckles don't appear. However in day 7, the cells are in exponential phase, their activity is high, showing a bright red stain. We can see that, independently of the material, the cells are growing and nesting. Finally on day 14 we see that some of the red has faded, due to fully differentiated tissue that has grown and leading to a lesser phosphatase activity. However, we can clearly check that the whole top surface of the disk is covered, especially HA, which means that material is biocompatible and a good inducer of osteodifferentiation.

PROTEIN QUANTIFICATION

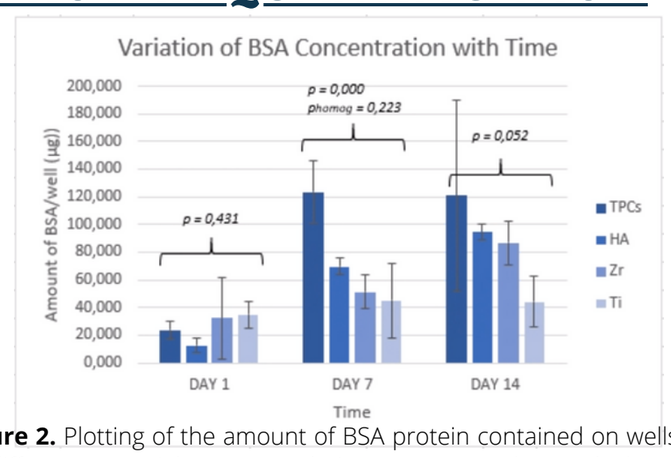


Figure 2. Plotting of the amount of BSA protein contained on wells of different materials at several time stamps. Values calculated through calibration curves.

The analytical results show significant differences between groups regarding protein levels only at the 7th day. With a $p<0,05$, the group proceeded to test the sample Homogeneity and obtained a $p=0,223$, classifying it as having an Homogeneous Standard Deviation. Considering that, a Tukey HSD test was done to study the real differences between the materials.

Table I: Tukey HSD test results for day 7.

Tukey HSD Test*				
Materials	TCP	HA	Zr	Ti
TCP	-	0,015	0,002	0,001
HA	0,015	-	0,759	0,555
Zr	0,002	0,759	-	0,985
Ti	0,001	0,555	0,985	-

It is important to mention that 'p' value for the ANOVA test at the 14th day was 0,052, a value slightly above the threshold to decide if a statistical group have similar means or not and, therefore, it was considered as if the groups did not presented a notorious difference. This was against the prior belief since with a higher level of differentiation, was expected to indicate a higher distinction between materials.

MICROSCOPIC ANALYSIS

- **FLUORESCENCE MICROSCOPY**

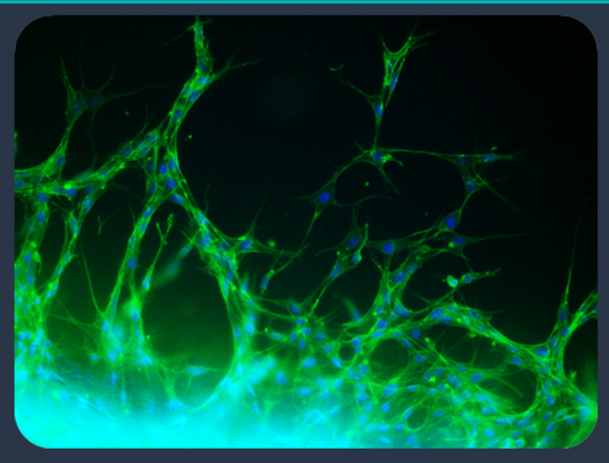


Figure 3. HPMEC cells stained with DAPI and Alexa Fluor 388 (x100 objective)

In this technique we visualize the sprouting of HPMEC cells that have migrated to the outskirts of the chitosan scaffold, initiating the process of vascularization. In blue, the DAPI colored the DNA, and a cluster of nuclei can be seen making a tube like structure . In green, the alexa Fluor colored the F-Actin filaments, so the filapodia is evident extending outwards and creating the vascular network. The different colors are achieved by applying different filters.

- **CONFOCAL LASER SCANNING MICROSCOPY (CLSM)**

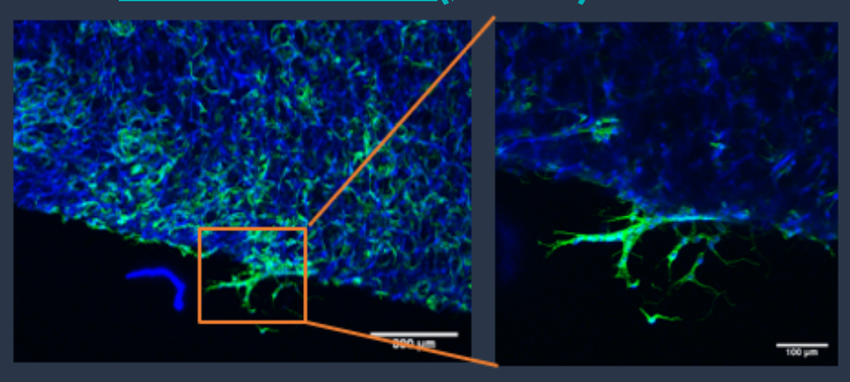


Figure 4. Scaffold with bone and endothelial cells. Left - confocal 10x objective. Right- digital zoom of 3x

The same sample was now observed in a CLSM and we can observe the lumen of the chitosan scaffold filled with the bone cells, but with the vessels starting to develop.

CONCLUSION

Thus, the statistical results reveal a lab error when preparing the different wells, since the expected outcome was to notice bigger differences between the time groups. By analyzing the images, the group concluded that it was possible to engineer a 3D vascularized tissue, however, further studies marking the different cells could improve the analysis.

REFERENCES

- Tissue regeneration and repair, 2020, Practical Class Protocol 1-5

ACKNOWLEDGMENTS

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