This work presents an *in vitro* study about the ability to stimulate the osteogenesis of DPSCs by Myth implant texture.

Surface structure was viewed by SEM (Scanning Electron Microscope)and reported in figure 1, which highlights its roughness.

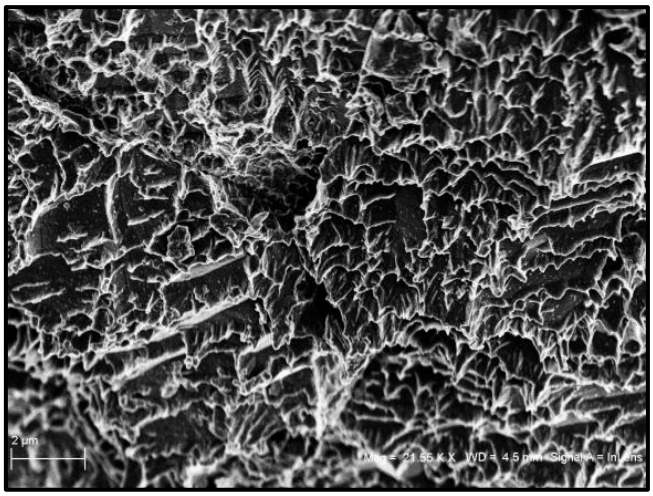


***Fig. 1*** *Myth implant texture observed by SEM.*

To conduct this studies, a complex cells/implant was realized: in particular,as Myth is aimed at a dental use, Dental Pulp Stem Cells (DPSCs), a lineage of mesenchymal stem cells extracted by dental pulp,was chosen.

**Cytotoxicity test: conditioned medium**

Firstly was evaluatedthe biocompatibility of the tested implant, by MTT assays. The conditioned culture medium was prepared by incubating the materials of interest at 37 °C, for 24 and 48 hours in DMEM without phenol red and without serum (1 ml of DMEM every 0.2g of material), supplemented with antibiotics (penicillin, streptomycin), amino acids and glutamine. The viability of the cells was determined by MTT colorimetric assay and expressed as  percentage of cell viability compared to the control (cells incubated in culture medium not conditioned). The measurements were performed in triplicate.

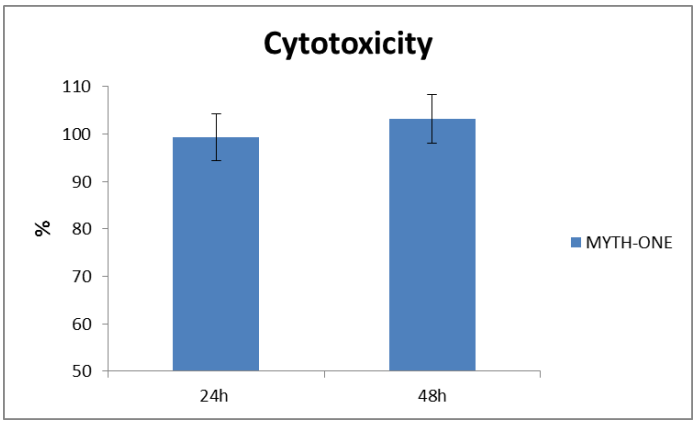


***Fig.2*** *Cytotoxicity test on myth implant by conditioned medium after 24 and 48 hours of incubation.*

The high values of percentage showed in the graph (Fig. 2) prove a total biocompatibility of the implants, suggesting that no particles damaging for cells were released by them. So Myth implant can be considered biologically safe.

**Cell proliferation assay: MTT tests**

Once plated cells on implants, cellular viability was evaluated by MTT test. Approximately 100,000 cells were plated on materials and left in rotating culture. Each sample was prepared in triplicate. The proliferation was assessed 3 and 5 days after seeding, by MTT colorimetric assay. Cells plated in flasks were used as control. Cellular activity was evaluated by spectrophotometer reading the quantity of formazan salts produced by the enzymatic activity of live cells. The amount is expressed in percentage versus the control cultured in the plate.

The implants promote cell proliferation approximatelywith the same values of the cells culture in standard conditions (Fig. 3).

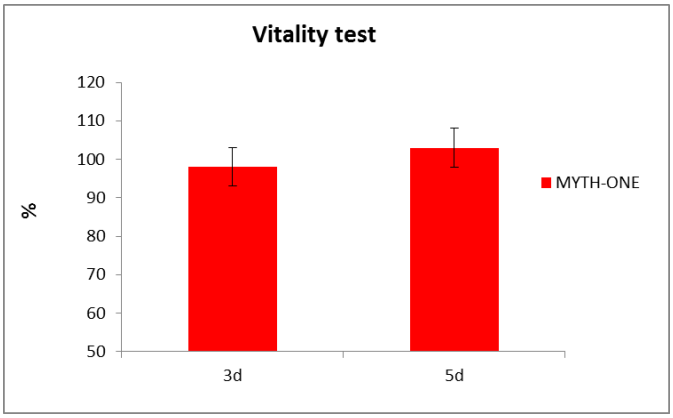


***Fig. 3*** *Proliferation assays on construct DPSCs/Myth at 3 and 5 days of culture.*

**Cell adhesion: immunofluorescence**

About 250,000 cells were plated on 2 implants and incubated in rotating culture at 37 °C in 5% $CO_2$. After 3 days of culture, the medium was removed and the implants were washed with PBS and fixed in 4% PFA. Then it was performed a fluorescence by labelingwith Hoechst, a intercalating-DNA dye that displays cells nuclei. The images show the nuclei of adhered cells, evenly distributed on the implants surfaces (Fig. 4).

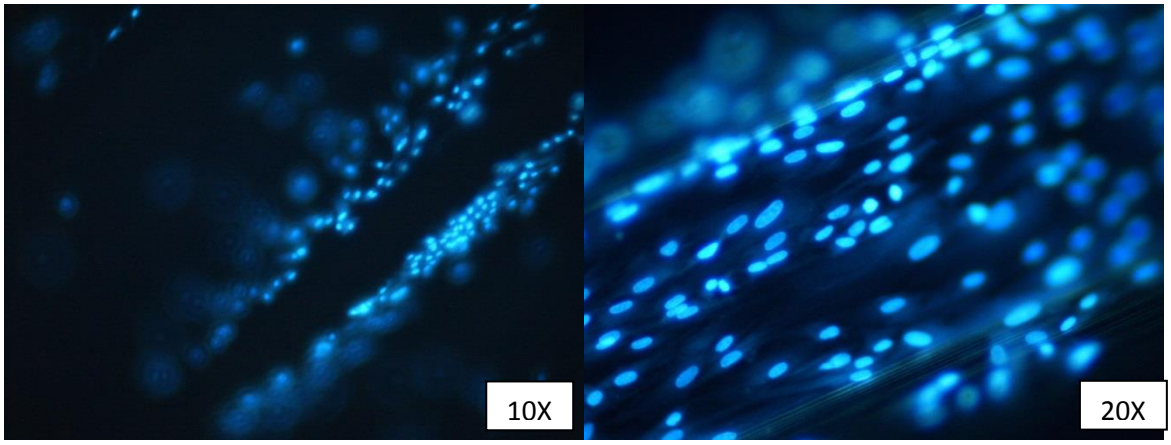


***Fig. 4*** *Immunofluorescence by Hoechst on device DPSCs/Myth at 3 days of culture.*

**Cell adhesion: Scanning Electron Microscopy (SEM)**

DPSCs/implants complexes, cultured for 3 days in the same conditions described above, after fixation were processed for SEM analyses, to obtain a clearer view of cells adhesion. As the collected photos showed, adhered cells tended to spread onto Myth surfaces  acquiring an osteoblastic morphology (Fig. 5).

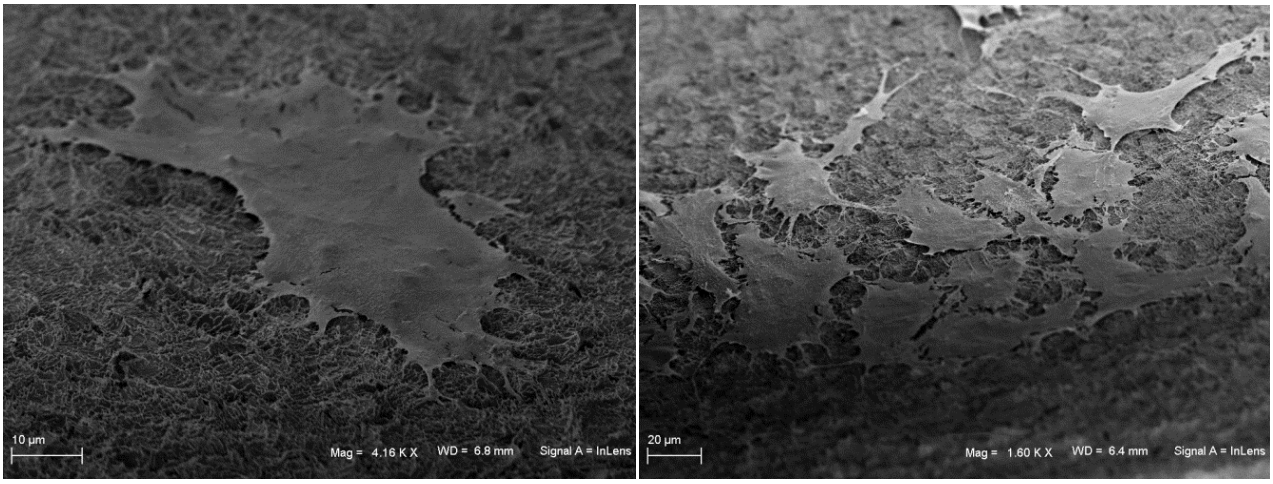


***Fig. 5*** *SEM photos of cells adhered on Myth surfaces after 3 days of culture*

**Bone matrix formation: histological analysis**

Cells seeded on Myth surfaces were cultured for three weeks in osteogenic medium in rotating culture. After PBS washing, the complexes were fixed and kept in a solution of Alizarin Red S 1% for 10 min. Alizarin is a red staining that binds calcium deposition by cells of an osteogenic lineage. Free calcium forms precipitates with alizarin, and tissue containing calcium stain red immediately, when immersed in a solution contained it.

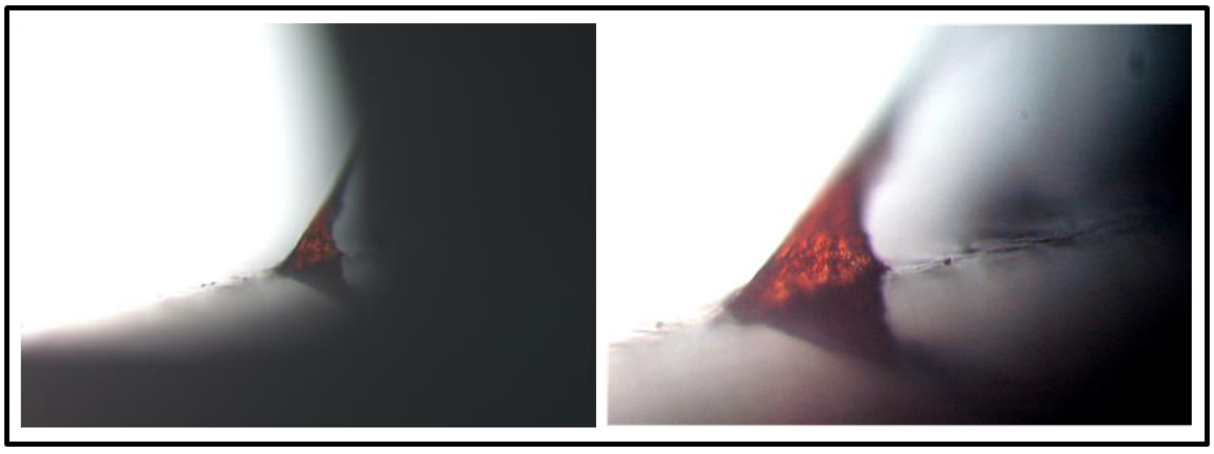

***Fig. 6*** *Alizarin red staining performed on complexes DPSCs/Myth after 3 weeks of culture.*

Images 6 show a layer of matrix positive for calcium deposition formed around the coils of implants. It provesthe activation of mineralization process.

**Osteoinduction: qRT-PCR**

The ability of implants texture to induce differentiation of DPSCs into osteoblast to activate bone matrix deposition, was evaluated by *Real Time Polymerase Chain Reaction.* The analyses were conducted on specimens collected after 7, 14 and 21 days of cell culture; in particular was examined the expression of genes encoding for molecules involved in matrix mineralization: *BAP*, *COLL 1*, *OPN*, *BSP* and *OC.* RNA extracted from pellets of cells cultured in 2D was used as control. Quantitative Real-Time PCR was performed using the SYBR Green method. The amount of cDNA of the gene of interest has been normalized to that of the cDNA of GAPDH.

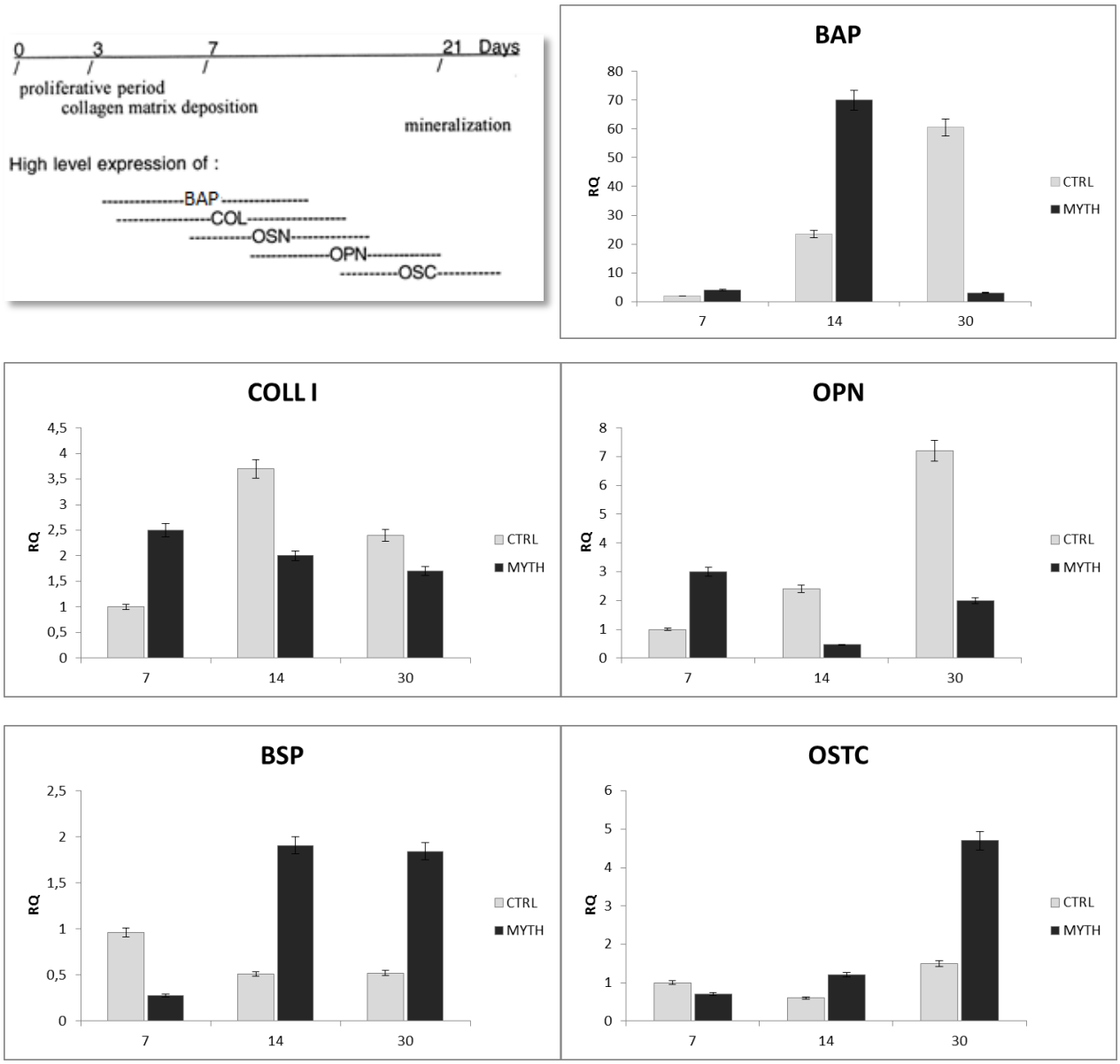


***Fig. 7*** *qRT-PCR of osteogenic genes BAP, COLL I, OPN, BSP and OC in cells seeded on Myth versus a 2D control (CTRL) at 7,14 and 30 days of culture.*

The image in the upper left shows the temporal expression of markers involved in osteogenic differentiation; histograms display the activation of genes *BAP* (*bone alkaline phosphatase*), *COLLI* (*collagen*), *OPN* (*osteopontin*), *BSP* (*bone sialo-protein*) and *OSTC* (*osteocalcin*)in cells seeded on Myth

implants versus a control 2D at 7, 14, 30 days of culture. The histograms show in cells-implants devices an up-regulation of genes *BSP* and *OSTC* compared to the 2D system. Moreover for the implants the deposition of the matrix is already carried outafter 7 days of culture (*COLL I*), compared to the control that, instead, presents the highest expression of *COLL I* just after 14 days of culture, a growing trend of *OPN* and lower expression of *BSP* and *OSTC* with respect to Myth specimens. Then the global analysis shows that the implant system enter early in a stage of matrix mineralization stimulating previously cells differentiation.

**Matrix mineralization: human-Osteocalcin ELISA test**

Osteocalcin is the latest marker of the mature osteoblasts. It is the most abundant non-collagenous protein of bone matrix. Once transcribed, osteocalcin undergoes post-translational modifications within the osteoblast before its secretion. Osteocalcin is released by osteoblasts during bone formation and is bounded with the mineralized bone matrix.

The concentration of osteocalcin released in culture medium by cells seeded on implants was evaluated by ELISA test. after 7, 14 and 30 days of culture, and as control was used culture medium of cells plated in flasks. The values of protein reported in the graph 8, show for the control (CTRL) a typical phasic trend, while the samples, collected by the implants, report an increasing in concentrations at 30 days of culture with a value higher than the relative control.

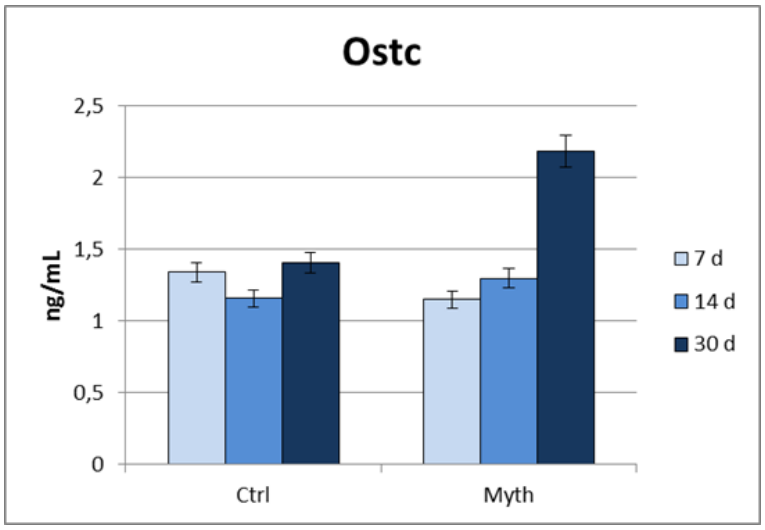


***Fig. 8*** *h-OC ELISA test of culture medium collected from 2D control (CTRL) and DPSCs/Myth devices after 7, 14 and 30 days of cells culture. The concentration was expressed in ng/mL.*

**Vasculogenesis: human-VEGF ELISA test**

Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis.

The same protocol used for h-OC ELISA test, was performed for the evaluation of the concentration of VEGF released into the culture medium from DPSC/implant versus a control 2D. The values relative to Myth show an increasing trend during the time, with a highest peak at 30 days of culture, but the concentration

is lower than that of the control for the respective times. The reason of that could be probably search in the greater number of cells that the flask surface is able to contain with respect to implants.

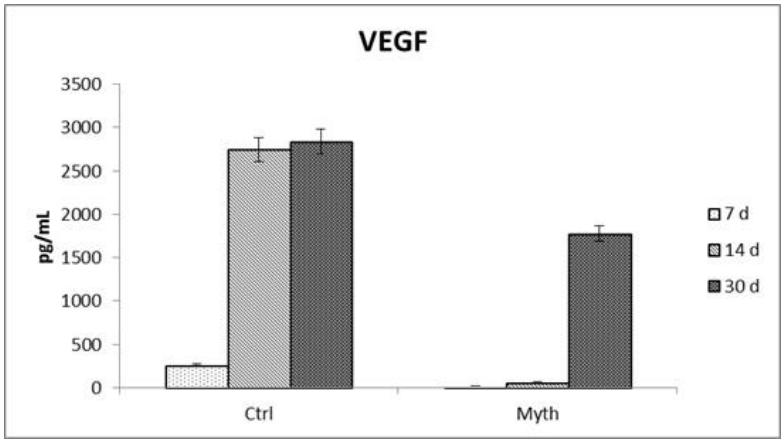


***Fig. 9*** *h-VEGF ELISA test of culture medium collected from 2D control (CTRL) and DPSCs/Myth devices after 7, 14 and 30 days of cells culture. The concentration was expressed in pg/mL.*

**Conclusions**

In this research project the capability of Myth texture to induce osteogenic process from DPSCs was been investigated; in particular were examined fundamental aspects that regulate a full and long-term osseointegration at the bone-implant interface.

Myth implants result completely biocompatible: they preserved the cells viability stimulating their proliferation. Immunofluorescence and SEM analyses allow a detailed view of cells onto implants surfaces and prove that implants texture enables cells adhesion and DPSCs differentiation into osteoblastsic morphology. After differentiation DPSCs growth on Myth surfaces implement extracellular matrix deposition and act mineralization process, as the positivity for alizarin red staining revealed. The cells differentiation into osteoblast and the activation of bone matrix formation were carried out in DPSCs seeded on Myth surfaces in a earlier stages with respect to the control. In particular the key protein for bone tissue formation, the Osteocalcin was already produced and released to be bound to ECM for mineralization. Also vasculogenesis process was carried out by cells-Myth devices, even if in a later stage with respect to the control.