Longitudinal *in vivo* analysis of nanostructured hydroxyapatite-functionalized gelatins osteoinductive properties in relation to endogenous or human mesenchymal stromal cells

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**Aims**  
Despite the high ability of bone tissue to regenerate itself, there are clinical situations in which using scaffolds becomes necessary (e.g. in cases of large defects caused by trauma, tumors or infection, but also in cases of spinal fusions or implant osteolysis).

Among scaffolds, relevant attention is addressed to gelatin, which is less antigenic than collagen\(^1\) and susceptible to degradation by proteases\(^2\). To improve the biological and mechanical properties of gelatin we have previously developed biomimetic porous gelatin-nanocrystalline hydroxyapatite (HA) scaffolds with tailored properties\(^3\). In the present study, we investigated the osteoinductive properties of these scaffolds (GEL-HA10), compared to scaffolds containing just gelatin (GEL) without the inorganic phase. The osteoinductive ability was studied in a heterotopic model (subcutaneous implant in nude mice). The influence of human mesenchymal stromal cells (hMSCs) within implants was examined as well.

**Method**  
The materials used for scaffolds preparation were Type A gelatin (280 Bloom, Italgelatine SpA, Cuneo, Italy) from pig skin, and hydroxyapatite nanocrystals synthesized in aqueous medium as reported in Panzavolta et.al\(^3\). Samples were 5 mm in diameter and 2 mm in thickness (volume ~40 mm\(^3\)), as shown in Fig.1.

![Figure 1: GEL and GEL-HA10 scaffolds and a detail of the porosity of GEL-HA10.](image-url)
In vivo experimentation was conducted in accordance with the European and Italian Law on animal experimentation. The protocol was approved by the Ethical Committee of the Rizzoli Orthopaedic Institute and authorized by the Italian Ministry of Health. Twenty-five athymic male mice (Harlan Laboratories Srl, Udine, Italy, aged 6 weeks, 25±5g b.w.) were used for the study. Commercial hMSCs were seeded on materials 24 hours before implantation at a density of $10^6$ cells/implant.

Non-seeded or hMSC-seeded GEL or GEL-HA10 samples were implanted into subcutaneous left and right side pockets formed in the dorsal surface of the mice. Experimental groups included: (1) GEL; (2) GEL + hMSCs; (3) GEL-HA10; (4) GEL-HA10 + hMSCs.

Surgery was performed under general anesthesia and mice were maintained in single cages in a confined room in the postoperative period until complete recovery. Animals were checked daily for evaluation of general clinical conditions. No complications or reactions in the implant sites were registered. At the end of experimental times (8 weeks), mice were euthanized under general anesthesia with i.c. injection of 0.5 ml Tanax (Hoechst Roussel Vet GmbH, Wiesbaden, Germany).

At 0 and 8 weeks the process of ectopic bone formation was monitored by in vivo microtomography (Skyscan 1176, Bruker microCT, Kontich, Belgium) applying a source voltage of 50 kV and a source current of 500 μA. The nominal resolution used for images was set at 9 μm (pixel size). The images (2672x4000 pixels) were then reconstructed with NRecon program (version 1.6.8.0, Bruker) to obtain the micro-CT sections (4000x4000 pixels, maintaining the relative pixel size). In addition to the specific alignment, beam hardening and a reduction of ring artifact were used as correction factors in the reconstruction process. The presence of mineralized tissue in the micro-CT sections was analyzed and expressed qualitatively and quantitatively using CTAn (Bruker microCT, Kontich Belgium) after applying a global threshold determined by the differences in pixel grey level between soft and hard tissues. The threshold of the rat vertebrae bone was taken as reference (Fig.2).

Figure 2: In-vivo micro-CT scan, reconstruction and a CTAn screenshot showing the threshold selected according to mouse vertebrae.
After euthanasia, the implants were retrieved and cut into halves. One half of the retrieved implants were fixed in 10% neutral buffered formalin for 24 hours and then decalcified and paraffin embedded. Five micrometer-thick sections were cut transversally and stained with hematoxylin and eosin for histological analysis. The other half of each implant was processed for gene expression analysis.

Relative expression was assessed differentially for the following human or mouse genes: $COL1A1$, $BGLAP$, and $ALPL$, with $GAPDH$ as reference. Statistical analysis was performed using the GraphPad Prism software. A two-way ANOVA with Holm-Sidak multiple comparisons was used to test the differences among samples and conditions. Unpaired t-tests were used to compare the expression of human genes between GEL and GEL-HA10 samples.

Results

The osteoinductive capability of the scaffolds was evaluated by the presence of mineralized tissue in the micro-CT sections. The mineralized areas in the correspondence of the implants presented lighter grey colored pixels (Fig.3). This ectopic bone formation was analyzed and expressed both as proportion (positive cases in total cases) and as volume in mm$^3$ (Fig.4). At 8 weeks, the results showed that both cell-free GEL and GEL-HA10 samples mineralized. On the other hand, with the presence of hMSC, the mineralized tissue was detected in only one in 10 cases (10%) in GEL samples, while GEL-HA10 continues to have a high percentage of samples with an ectopic bone formation (90%), even though the quantified volume of mineralized tissue was significantly lower than cell-free scaffolds (Fig.4).

Figure 3: In-vivo micro-CT sections of samples with subcutaneous GEL implantation just after the surgery (t=0) and after 8 weeks (t= 8 weeks). In the top yellow panels, detailed pictures corresponding to the implanted scaffolds are shown. At t=0 the implants are not yet mineralized while at t=8 a lighter grey pixels are detectable.
In positive samples, qPCR analysis showed that the expression of murine bone-specific genes was unvaried between scaffolds, with an upregulation of murine Col1a1 and Alpl enhanced by the presence of hMSCs. On the contrary, the different composition of scaffolds resulted in a different pattern of expression of human genes, revealing that the presence of nanocrystalline HA induced the upregulation of bone-specific genes.

In addition, results of Fig.4 showed that in GEL+hMSC samples nearly no mature mineralized tissue was found at 8 weeks, whereas mineralized areas were found in all other groups. The combination of those results (micro-CT and in vivo gene expression) suggests that the presence of mineralized tissue in GEL-HA10 could be entirely due to the osteogenic activity of hMSC and the presence of HA.

**Conclusion**

Both GEL and GEL-HA10 scaffolds mineralized when implanted without hMSCs. On the contrary, the presence of preimplanted hMSC abolished or reduced mineralization of GEL and GEL-HA10 scaffolds. However, we could observe a species-specific response to the presence of HA, which stimulated the osteogenic differentiation of human cells only.

**References:**