In vitro and in vivo tests of a novel alkali-free bioactive glass for bone regeneration

VIEIRA, F¹; CARAMÊS, G²; BRITO, A³; CARVALHO, L³; FERREIRA M.,M³; CARAMÊS, J⁴.

1. Caramês Advanced Education Center. Implantology Institute (Lisbon, Portugal)
2. Herman Ostrow School of Dentistry, University of Southern California. Implantology Institute (Lisbon, Portugal)
3. Faculty of Medicine, University of Coimbra, Portugal.
4. UICOB, Faculty of Dental Medicine, University of Lisbon, Portugal. Implantology Institute (Lisbon, Portugal)

Abstract

Commercially available bioactive glasses compositions present several shortcomings mostly related to its mechanical properties and high alkali content. Ceragraft[®], an alkali-free bioactive glass, has been developed for bone regeneration applications. The aim of this study is to perform a biological evaluation of Ceragraft[®] in order to verify its safety and efficacy, as per the International Standards and guidelines. Thus, an *in vitro* cytotoxicity evaluation in a mouse embryo fibroblast cell line and an *in vivo* biocompatibility evaluation was performed. The latter included an acute systemic toxicity test, a delayed hypersensitivity test, an intracutaneous reactivity test and an implantation study in bone defects. Statistical analysis was performed, SPSS soft. v.23. Normal distribution was assessed by Shapiro-Wilk test and variance of quantitative variables was ascertained by Levene test. Statistical differences were then determined by ANOVA with post-hoc comparison using Games-Howel test ($p < .05$). In *in vitro* cytotoxicity test no morphological abnormalities were found when Ceragraft[®] extract was employed in the assay. RCV was 98,4% in the Ceragraft[®] extract group (SD=4,7) and no differences to the vehicle control (VG) and the negative control group was found. In the acute systemic toxicity test, all the animals were healthy and showed an increase in body weight. During the time testing, in none of the treated and control animals was observed mortality or toxic signs or symptoms. In the delayed hypersensitivity test, no abnormalities were observed in control and treated animals. Magnusson - Kligman scale grading scale - 0 - was observed in all cases. In the intracutaneous reactivity test, no abnormalities immediately after intradermal injection of test group was observed. After long-term bone implantation test, no toxic symptoms have been detected in both groups. Macroscopically and histologically, no abnormality has been detected in harvest femurs or other organs in all animals. Regarding hematological and blood chemistry parameters all values were considered physiological in the test group, with exception to albumin in two rabbits which were slightly higher. Mean percentage of NFB was 65,6% in test group and 29,7% in control group. This difference was statistically significant ($p=.001$). Considering the biological evaluation of Ceragraft[®], this new alkali-free bioactive glass seems to be safe in contact with animal cells and tissues. Furthermore, after a long-term implantation test in bone defects a favorable regenerative behavior was found.

Fig 1. Ossmed[®] is located in Biocant Park, Cantanhede, Portugal.

Background and Aim

Aiming for new biomaterials in bone regeneration, researchers have been improving bioactive glasses (BGs) with optimized characteristics. (1-4). Until now, the commercially available bioactive glasses compositions present several shortcomings mostly related to its mechanical properties and high alkali content. (5-7). 45S5 Bioglass[®] is associated with fast dissolution and resorption rates that negatively affect the balance of natural bone remodeling. (8) High pH value caused by sodium leaching also increased *in vitro* cytotoxicity (9), delayed hydroxyl carbonated apatite (HCA) formation (10) and a slight depolymerization trend of the silicate glass network. (6,9). Proximity between glass transition temperature (550°C) and the onset of crystallisation (610 °C) resulted in a weak mechanical strength. (11) These limitations outlined the pertinency of developing new BG's compositions. (10) Ceragraft[®] (commercial name of FastOs, from Ossmed[®] is an alkali-free BG presented under the form of granules (40-500 µm). (12)

Aim: This poster describes a methodological approach to evaluate the safety and efficacy of Ceragraft[®] in accordance with international standards and guidelines.

Methods and Materials

ISO 10993 (Biological evaluation of medical devices)

Ceragraft[®]

composition in wt %

- 39% SiO₂
- 34% CaO
- 13% P₂O₅
- 13% MgO
- 1% CaF₂

Fig 2. Scanning electron microscopy (SEM) images of Ceragraft[®] granules.

in vitro cytotoxicity (I)

Mouse embryo fibroblast cells (BALB\3T3 Clone A31)

Qualitatively (1): Morphological abnormalities shown by the cells under a phase contrast microscope, were summarized following numerical code: 0 - none, 1 -slight, 2 - mild, 3 - moderate, 4 - severe, (**ISO 10993-5**); **Quantitatively (2):** Relative cell viability (RCV) through the neutral red uptake (NRU) assay.

in vivo - acute systemic toxicity test (II)

Mice (ICR (CD-1[®]) strain)

Ceragraft[®] extracts and controls (sodium chloride and cottonseed oil) were injected in 20 male mice, following an intravenous route for polar vehicle and an intraperitoneal route for the non-polar vehicle, at a dose of 50 ml/Kg (**ISO 10993-12**);

Monitoring before and 24, 48 and 72 hours after the injections for evidence of any abnormalities.

in vivo - a delayed hypersensitivity / intracutaneous test (III)

Hipersensitivity guinea pig maximization was tested (**ISO 10993-10**) in 30 animals;

Intracutaneous reactivity test (**ISO 10993-10**) in 3 albino rabbits.

in vivo- implantation test in bone defects (IV)

Fig 3. Surgical bone in the right femur of a NZWR.

3 bone defects with a depth of 6 mm and 2 mm diameter were created in each femur;

52 weeks : blood sampling (hematological and blood chemistry parameters), sacrifice and full necropsy.

Results

(I)

Concentration %	Microscope obs.	Corrected OD		Relative viability	
		Mean	SD	Mean	SD
Mean VC (vehicle control)	0	0,908	0,048	100	5,2
NE (Ceragraft [®] neat extraction)	0	0,893	0,043	98,4	4,7
PC (positive control)	4	0,000	0,002	0,0	0,2
NC (negative control)	0	0,938	0,022	103,3	2,4

After 24h, discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth - grade 0.

(II)

During the 72 hours of testing, in none of the treated and control animals was observed mortality or toxic signs or symptoms.

(III)

At 48 and 72 hours, no abnormalities were observed in all animals (control and treated) (i.e. 0 in Magnusson - Kligman scale grading scale). None hypersensitivity was elicited under the laboratory conditions simulated; No abnormalities immediately after intradermal injection.

(IV)

Fig 4. Histologic sections of test and negative control group and NFB graphic comparison.

All hematological parameters - normal; Necropsy normal

Conclusion

Considering the biological evaluation of Ceragraft[®] according with the International Standards and guidelines, this new alkali-free bioactive glass seems to be safe in contact with animal cells and tissues. Furthermore, after a long-term implantation test in bone defects a regenerative behavior was found. Similar and positive results were found when testing the same biomaterial *in vitro* in contact with human mesenchymal stem cells (13) and in different animal models. (11, 14) Aiming the future application of Ceragraft[®] in bone regeneration, clinical studies designed to properly answer this issue (e.g. randomised controlled trials) are ethical and necessary-

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