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Newly Synthesized Polymer Hydrogels and Hydroxyapatite Nanoparticles (nHAP) for Biomedical Application: Histological and Biochemical Studies in Rats

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Abstract

The development of biocompatible zwitterionicpolymers and polymer-reinforced calcium phosphate pastes and cements in combination with specific drugs, has been considered as a promising strategy in bone tissue engineering and dental medicine. The main purpose of this work was to evaluate the relationship between physicochemical and mechanical properties of newly synthesized polymer hydrogels and hydroxyapatite nanoparticles (nHAP) and their biocompatibility *in vivo*. Standard hematological, biochemical and histological laboratory tests with Wistar rats and statistical analysis of the data obtained were performed. The results from the histological, hematological and biochemical analyses revealed that all tested materials are characterized by good biocompatibility and biodegradation. No hard inflammatory effects were noticed, only slight foreign body reaction responses were observed. The histological findings made by us confirmed the acceptance of the implanted materials and the good tolerance to their componential compounds.

Key words: zwitterionic polymers, histological, hematological and biochemical tests

Introduction

Application and development of biocompatible zwitterionic polymers and composite polymer-reinforced calcium phosphate pastes and cements in combination with specific

drugs, have been considered as a promising strategy in biomedicine. In bone tissue engineering and dental medicine with beneficial effects are used calcium-phosphate (Ca-P) cements and pastes (hydroxyapatite (HA) (self-setting HA or brushite (dicalcium phosphate dihydrate (DCPD)) and collagen [2, 6]. Due to their good biocompatibility and extensive bone conductivity they are applied as substitutes in orthopedic and reconstructive surgery. These products possess certain limitations and studies investigate the development and the incorporation mainly in composed of polymer reinforced synthetic materials as an alternative strategy for biomaterials which mimic the extracellular matrix of bone tissue. Compounds with promising usage in surgery especially as an effective wound regeneration system are zwitterionic polymers due to their chemical structure resemble many naturally occurring substances. Polycarboxybetaines (PCB) are analogues to the betaine form of α-amino acids and have the lowest protein absorption found. Polysulfobetaines (PSB) have also a zwitterionic moiety but they do not have a naturally existing analogue. Zwitterionic polymers are able to absorb the excess of exudate and prevent bacteria adhesion and the formation of biofilms [3], useful in biomedical application.

The main purpose of this work was to study and evaluate the relationship between synthesis, boundary phase and mechanical properties of newly synthesized polymer hydrogels and hydroxyapatite nanoparticles (nHAP) on one hand and the biological response on the other hand, by applying modern methodologies, the results of which to serve as a scientific base for the design of a broad range of biomaterials. We focused in our survey on composite polymer-reinforced calcium phosphate pastes and poly (sulfobetaine and carboxybetainemethacrylate) (PSB, PCB) networks with promising addendum in wound dressing procedures,surgery and dental medicine.

Materials and Methods

Study on synthesis and properties

Synthesis of polymer-reinforced nanometric calcium phosphates /hydroxyapatite nanoparticles/ was performed for molar pulp coverage. For the synthesis of poly (sulfobetaine methacrylate) (PSB) and poly(carboxybetaine methacrylate) (PCB) networks substances (PSB in aqueous solution; PCB dissolved in ethylene glycol / ethanol / distilled water in a 3:1:1 ratio) were mixed with initiators $K_2S_2O_8$ or $Na_2S_2O_5$, $(NH_4)\ S_2O_8$ and the cross-linking agent PEGDA or N,N'-Methylenebisacrylamide with varying concentrations. The compound was homogenized and then allowed to cross-linking polymerization carried out at 60° for 6 – 15 hours. The resulting hydrogels were purified from the unreacted reagents by dipping them into a large amount of distilled water. The water was changed every day within 2 weeks as the waters were monitored with UV every day. Some of the prepared networks after drying, in order to obtain PSB + CaP, were immersed in a 0.6M $CaCl_2$ solution, where they stayed for 48 hours. The samples were washed with water and immersed in a 0.3M solution of K_2HPO_4. There the samples sank for a week, removing the crust from the precipitated calcium phosphates.

Physical, chemical and biochemical characterization of biomaterials was performed, mainly the prepared polymer networkswere studied, due to their specificity as swelling kinetics; rheological behavior; salt effect on the swelling behavior; their temperature sensitivity; swelling capacity in simulated body fluid and enzyme absorption concerning their in vitro collagenase and myeloperoxidase inhibitory activity and *ex vivo* inhibitory activity of hydrogels against collagenase in wound exudates. Standard investigation on calcium and zinc binding ability, bacterial growth inhibition and cytotoxicity were also evaluated, before applying in live organisms, in order to validate the stability and safety.

In vivo biocompatibility testing

Animal design and implantation of biomaterials

In vivo studies were performed in order to test the biological response of the polymer hydrogels and bruschite cements (HA). The tests were performed on experimental rats and the defects were introduced on soft tissues (subcutaneous, near the muscles application) and hard tissues (teeth).

All procedures were consistent with the definitions and recommendations for in vivo animal studies, designed to provide initial evidence of medical device safety, potential performance when used in a living system, and/or the biologic response to the device, in accordance with International Standard ISO 10993 -1:2009 for biological evaluation of medical devices and criteria for evaluation set out in ISO 7405:1997 conserving tissue and inflammatory reactions, and ISO 10003-6, 2007 Part 6- Tests for local effects after implantation. The animal usage was in accordance with the requirements and Regulation № 15/ 03.02.2006 regarding laboratory animals and animal welfare.

I A and I B *in vivo* experiments: 48 Wistar male rats were used in both experiments (24 animals in I A experiment and 24 – in I B experiment). The animals were divided into 6 groups (4 animals in each group in IA and the same scheme in IB experiment):

I A experiment – the 1st group consisted of animals without implantation, from the 2nd tothe 6th group – implantation of PSB networks with cross-linking agent PEGDA mol. % from 0,1 to 3 was performed.

I B experiment – animals from the 1st to the 3th group were implantated with PSB CaP networks, additionally immersed with $CaCl_2$ and K_2HPO_4.(PSP CaP), those from the 4th to the 6th –implantation with PCB networks was done.

An aseptic surgical procedure was used after standard ketamine &xylazine anesthesia in appropriate doses. Sterilized implants were inserted in the fossa poplitea near to the fascial superficies of biceps femoral muscle and m. semitendinosus. After 12 weeks of evaluation period all animals were humanely euthanized. To enable access to the subcutaneous space and implants were made incisions full thickness through the skin and surrounding muscle tissues.

In the II *in vivo* experiment: 4 Wistar male rats were used with molar implantation of hydroxyapatite nanoparticles according to standard dentistry procedures.

A new procedure was developed for creating initial artificial caries of intact teeth (incisors and molars) in a gel medium with lactic acid and in a collagen gel with acrylic and metacrylic acids, resulting in enamel defects. The created initial artificial caries is subjected to subsequent remineralization with the hybrid hydroxyapatite materials obtained. A model for direct pulp coverage on test animals was created by inducing tertiary dentinogenesis. An aseptically procedure was used after standard ketamine and xylazine anesthesia of rats in appropriate doses. Sterilized implants were inserted in the pulp cavity. After 8 weeks of evaluation period all animals were humanely euthanized. Materials from teeth were processed for histology.

Standard hematological and biochemical laboratory tests were performed on blood and serum parameters. The markers of bone metabolism (total alkaline phosphatase (TAP) activity and bone alkaline phosphatase (BAP), calcium (Ca), phosphorus (P), as well as markers of potential hepatotoxicity (aspartate aminotransferase and alanine aminotransferase activity (ASAT, ALAT) etc. were measured on semi-automated biochemical analyser BC-88A (MINDRAY, China), hematological indicators were determined on hematological analyzer BC 2800 (MINDRAY, China).

Ca and P content in soft tissues was determined by a standard atomic absorption method.

The element content changes in soft tissues around the implants are an important early indicator of biochemical homeostasis and some parameters of the body's reactivity to the implants.

Statistical analysis of the results

The statistical analysis of the data were be made by GraphPADInStat, Software, USA and using one-way ANOVA, followed by Bonferroni's post hoc test. The results were presented as $x\sim \pm$ S.E.M. Statistical significance was marked as follows: *** $P<0.001$.

Histological procedures for light microscopy.

After surgical excision tissue samples (10/10/10mm in size) with introduced implants were routinely fixed in 10% buffered formalin, rinsed in water and placed in 8% formic acid for demineralization. After dehydration in graded ethanol and xylene clearing, materials were embedded in paraffin. Tissue sections (3-5 μm thick) were stained in hematoxylin and eosin (H&E) and examined by a light microscope (Leica DM 5000B, Wetzlar, Germany).

The sections were examined standardly for the presence of a fibrous capsule and its thickness, newly formed vessels, foreign body reaction and occurrence of various types of inflammatory cells. Evaluation system of three categories was used to measure the microscopic observations. The inflammatory reactions were scored according to the following criteria (ISO_7405):

No reaction or slight reaction: Fibrous capsule formation and absence of inflammatory cells or presence of a fibrous capsule formation with few lymphocytes and plasmocytes.

Moderate reaction: Fibrous capsule formation with the presence of macrophages (MA), polymorphonuclear leukocytes (PMNL), lymphocytes, and plasmocytes.

Severe reaction: Presence of large accumulations of PMNL, lymphocytes, plasmocytes, macrophages, foreign-body giant cells, and congested capillaries.

Results and Discussion

The results from the histological, hematological and biochemical analyses revealed that all tested materials are characterized by good biocompatibility andgood biodegradation; they do not cause hard inflammatory effects, and induce only slight foreign body reaction responses.

Ca and ALP levels (**Figs. 1, 2**) were slightly elevated in PSB CaP group (**Fig. 3**) compared to the controls and the other experimental groups, while that of P (**Figs. 1, 3**) remained similar to that of other groups. It can be assumed that the increased activity of ALP was associated with upcoming processes of demineralization of the implant, containing Ca and the ability these networks to serve as donation capacity to the surrounding tissues, if used in bone defects implementation surgery. The values of the parameters in the other groups were similar and there was no evidence of deviations from the norm. Biochemical parameters suggest good biocompatibility of the new materials with the experimental model tissues.

The local mini-environment in soft tissues reflects and responds to implants. Immune environmental reactivity studies are the initial step to investigate the biocompatibility of materials as tissue substitutes.

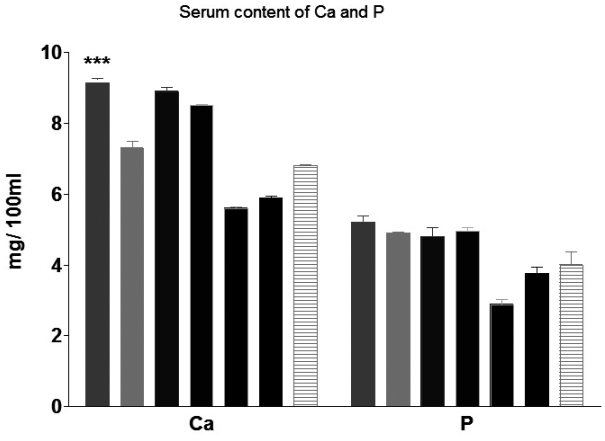


Fig. 1. Serum content of calcium (Ca) and phosphorus (P).

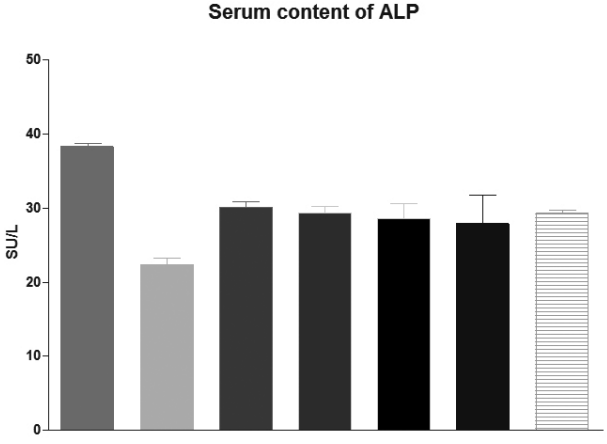


Fig.2. Serum content of alkaline phosphatase (ALP).

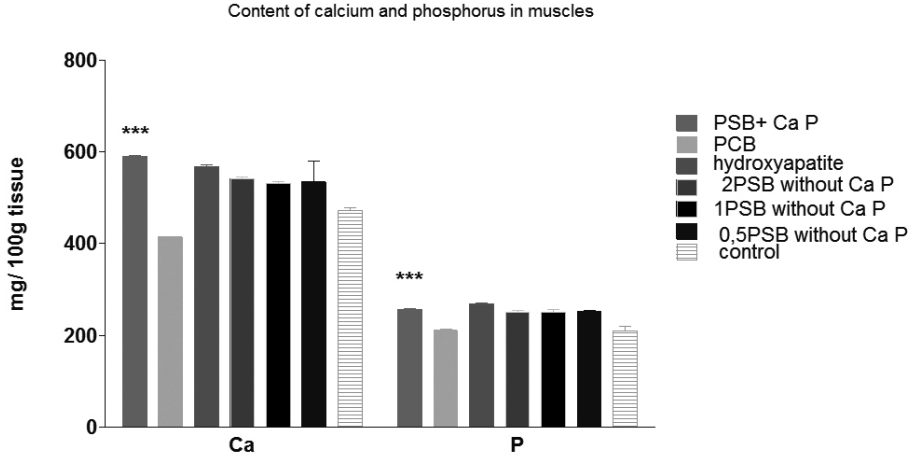
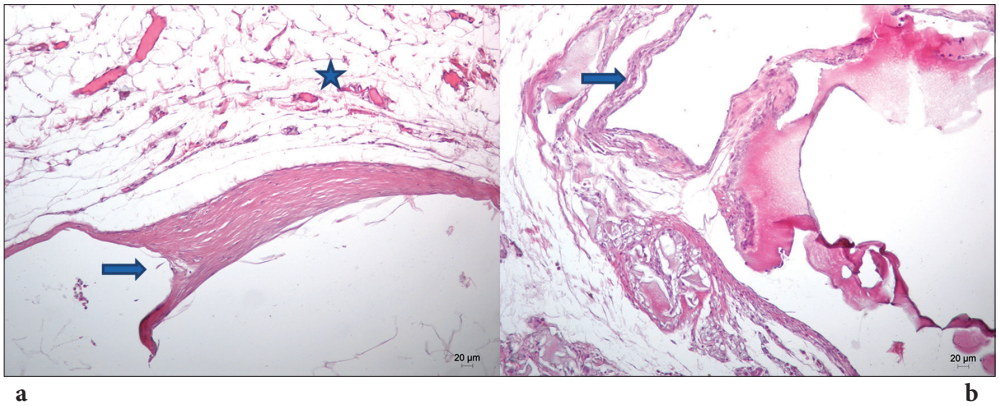


Fig. 3 Content of calcium (Ca) and phosphorus (P) in muscles

The histological aspects revealed regeneration- reparation processes around the introduced implants in soft tissues and in teeth. After the application in the muscle fascial space, a recovery of the surroundings was observed (**Fig.4 a,b**). Signs of a slight non-cellular edema were presented between loose fascial strands and hyalinization of some collagenous fibers was noticed. Newly formed capillaries perforating the nearby tissues and closely attached to the neoformative peripheral fibrous capsule were also found. Progressive fibroblast population and endothelial cells forming the intracapsularneovasculature were presented. As dominant early responders to biomaterial implantation tissue macrophages composing foreign body reactions were noticed. The lymphoplasmocytic reaction was negligible.



a **b**

Fig.4 a, b. Formation of a thin fibrous capsule (arrow) and newly formed capillaries (5-point star) peripherally to the neoformative fibrous tissues. H&E.

In terms of dental implants, a good general feeling was observed, and after feeding with soft food at the beginning, the animals moved seamlessly to solid, regular granular food. Histologically, filling of the pulp cavity with nanometric hydroxyapatite material, reflected in proliferation reaction of cementoblasts and cementocytes and production of non-cellular cement. This finding is connected with the response of the organism and is a healing reaction to the trauma processes during dental procedures. The main affirmative finding for positive tissue acceptance of the materials implanted was the intended proliferation of dentin producing odontoblasts, due to the hydroxyapatite stimulation, under the artificial defects created (**Fig. 5**). No signs of inflammatory dental granuloma were observed.

This biomedical evaluation assessed the used materials as not producing any adverse local or systemic effects. Histopathological signs of necrosis, calcification or residual effect or material intolerance were not evidential in selected areas of implantation. No signs of acute inflammation were noticed, only occasional lymphocytes and MA in some specimens. All materials had shown good biocompatibility and were assessed in first and second criteria of ISO_7405. The objective to validate the organism tolerance towards the used biomaterials as substitutional therapeutic agents was clearly resumed via histological and biochemical aspects of this study.

The histological findings made by us validated the acceptance of the polymer materials and the good tolerance to their componential compounds. Observation of biomaterial-tissue interfaces are important markers for the lifetime of the medical devices tested. Biomaterial surface properties play a crucial role in modulating the foreign body

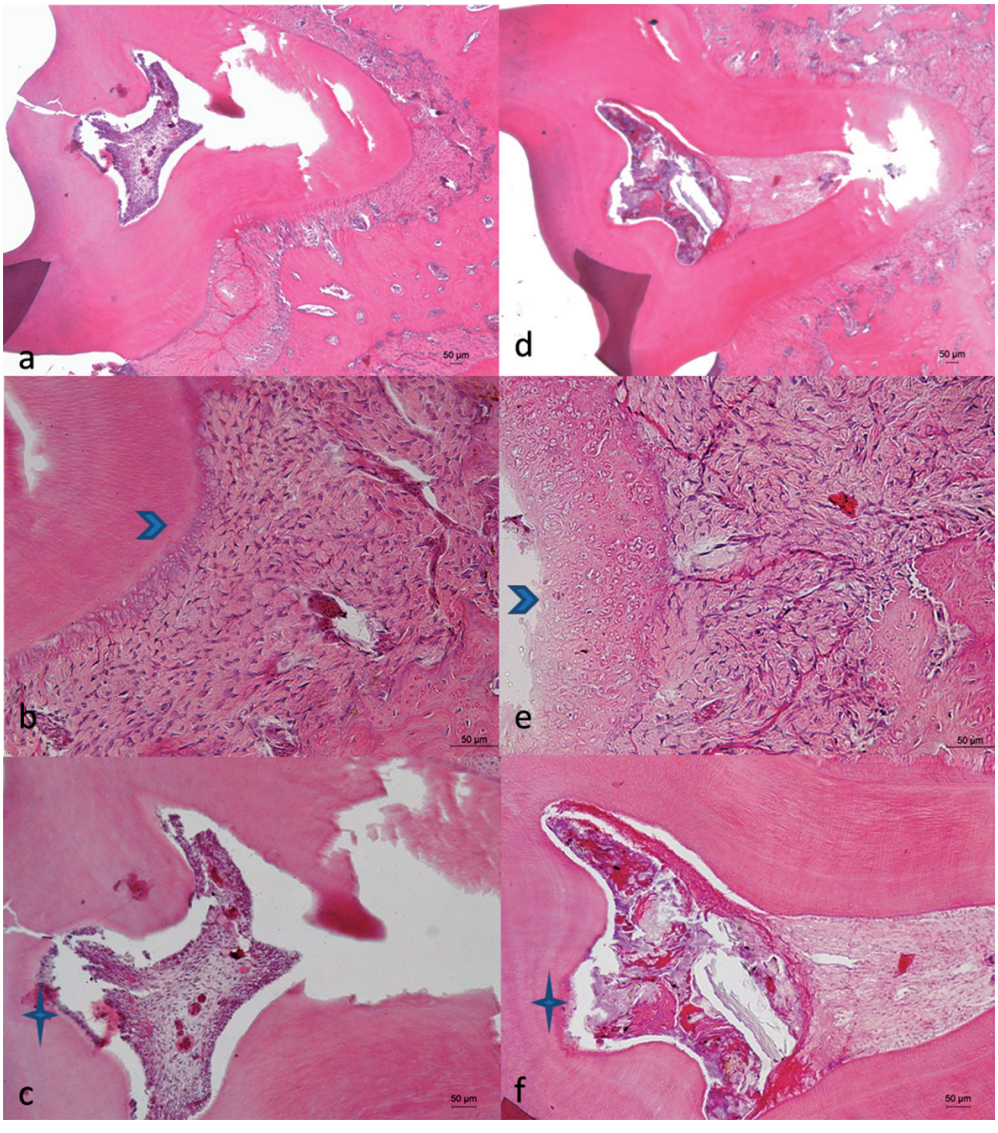


Fig. 5a, b, c. Intact tooth: odontoblasts (4-point star), layer of cellular and cell-free cement) (arrow head); d,e,f -Treated tooth, deposited material in the pulp, a small number of visible odontoblasts (4-point star), a proliferative reaction of the top layer of cellular and cell-free cement, protective response of root to trauma (arrow head). H&E.

reaction in the first several weeks following implantation of medical devices. Macrophage function in the body is to mediate degradation and phagocytosis of bioresorbable materials including foreign body reaction. These chronic inflammatory processes are essential in wound healing responses and may impact biocompatibility of implanted materials, their short- and long-term success in tissue engineering and regenerative medicine. Implantation *in vivo* always provokes cellular and tissue responses as stages in inflammatory and wound healing process, following application of biomaterials.

Moreover the formation of fibrous connective tissue around the implants is indicative of good tolerance by the surrounding tissues, according to [5] and [4] as well as the well-developed new vascularization.

Conclusions

- The biochemical and histological findings made by us confirm the acceptance of the implanted materials and the good tolerance to their component compounds.
- The observed processes of inflammation, proliferation and remodeling in the healing of wounds after implantation have their significance in studding biocompatibility and have a specific place in implantology.
- Surface properties of biomaterials play a crucial role in modulating the foreign reaction during the first few weeks after the implantation of medical devices, and are often an integral part of these processes [1, 7].
- The formation of a fibrous capsule and well-developed neoangiogenesis around the implants is indicative of good tolerance from the surrounding tissues [4, 5].

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