The irritative property of α-tricalcium phosphate to the rabbit skin

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Abstract. The aim of this study was to assess the irritant properties of a new developed calcium phosphate ceramic, α-tricalcium phosphate (α-TCP) after single application to intact skin of the rabbit. The test substance, α-TCP was produced by modified hydrothermal method and prepared in two different forms, as a solid material (disc 5 × 2 mm) and paste. Both, solid material and paste of α-TCP were evaluated for primary skin irritation to the ISO 10993-10:2002/Amd 1:2006 Biological Evaluation of Medical Devices – Part 10. At the end of the study macroscopic examination of the skin was performed. In this model, general and local tolerances were good. Score of primary irritation (SPI) and primary irritation index (PII) of α-TCP for both, solid material and paste, revealed that there was no significant toxicity/irritability (PII = 0.0) as compared to the negative control (PII = 0.0). Positive control did cause significant skin irritation in acute irritation test using Draize technique in rabbit model (PII = 2.11). Based on present results, it can be concluded that the the irritation potential of the tested material is negligible. However, other procedures for preclinical safety assessments of the α-TCP material are needed in order to completely elucidate its toxic potential.

Key words: Biocompatibility — Calcium phosphate ceramics — Skin irritation test

Introduction

A biomaterial is any material, natural or man-made, that comprises whole or part of a living structure, or biomedical device which performs, augments, or replaces a natural function (Williams 1986). Biomaterials are used in heart valves, blood vessel prostheses, joint replacements, bone plates, bone cement, artificial ligaments and tendons, dental implants for tooth fixation, skin repair devices, cochlear replacements, contact lenses etc. The clinical application of a biomaterial should not cause any adverse reaction in the organism and should not endanger the life of the patient (Bollen 2005). Any material to be used as part of a biomaterial device has to be biocompatible (Barile 2008).

The definition of biocompatibility includes that the material has to be nontoxic, nonallergenic, noncarcinogenic and nonmutagenic, and that it does not influence the fertility of a given patient (Williams 1986). Preclinical safety assessments of the toxic potential of the materials are needed in order to minimize the potential hazard to the patients. At present, safety assessments of medical devices are guided by the studies recommended in the International Organization for Standardization (ISO) 10993 standard. Since physiological responses of the body to foreign material are complex, it would seem odd that one can make one test to determine the biocompatibility of any given material. At present, tests that may be used in an evaluation of medical device biocompatibility include procedures for cytotoxicity, skin sensitization, dermal irritation and intracutaneous reactivity, acute systemic toxicity, subchronic toxicity, mutagenicity, implantation, hemocompatibility, chronic toxicity, and carcinogenicity (ISO 10993; Bollen 2000).

Having a chemical composition very close to the mineral phase of natural bone, calcium phosphate compounds have long been the subject of intensive investigation as bone substitutes. Several materials consisting of hydroxyapatite

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(HAP), α-tricalcium phosphate (α-TCP), or β-TCP have been clinically applied in orthopedic, reconstructive and maxillofacial surgery (Bohner 2000). α-TCP is known to be biocompatible, osteoconductive, osteoinductive and highly soluble at physiological pH (resorbable ceramic) and is expected to yield a porous structure capable of osseointegration i.e. promoting bone ingrowth (Durucan and Brown 2000). A number of animal studies indicated that application of α-TCP to bone defects was effective in promoting new bone formation (Kurashina et al. 1997; Wiltfang et al. 2002). However, some problems with these ceramics have been reported (Lu 2004; Fellah 2007). A major problem is that devices implanted within living tissue must interact with the physiological functions of the host with no development of the foreign body reaction around implant material (Williams 1986; Barile 2008). However, with many orthopedic devices, failure commonly occurs within 5–10 years of implantation. Better understanding of the interaction between ceramics and the adverse environment of the human body is important in improving and devising effective ceramic implants used in various treatment applications (Bollen and Harling 2002; Barile 2008).

Recently, in our laboratory it has been developed calcium phosphate cement – α-TCP (Janackovic et al. 2001, 2003). The cement obtained by our, modified hydrothermal method, beside α-TCP, contains a small quantity of calcium-HAP as residual phase, which could act as a centre for nucleation of newly formed HAP (Jokic et al. 2007). As a consequence, faster transformation into the HAP phase is observed during the setting of cement paste in simulated body fluid. Further, the HAP obtained by the hydrolysis of this cement has a more compact and denser microstructure with the finer nanostructure and crystal size of newly formed calcium deficient hydroxyapatite (CDHAP) below 100 nm, and compressive strength up to 80 MPa after 3 days of immersion in simulated body fluid (Jokic et al. 2006). A precise and detailed physical and chemical characterization of developed α-TCP has already been done (Janackovic et al. 2001, 2003; Jokic et al. 2006, 2007), but its biological evaluation was not examined.

The purpose of the present investigation was to determine the irritant properties of this newly developed α-TCP material after single application to intact skin of the rabbit.

Materials and Methods

Synthesis of α-TCP

α-TCP cement was obtained by heating of CDHAP particles synthesized by the modification of our method described earlier (Janackovic et al. 2001, 2003; Jokic et al. 2006, 2007). The various amounts of $Ca(NO_3)_2$, $Na_2H_2EDTA{\cdot}2H_2O$, NaH_2PO_4 and urea (reagents were Merck p.a. grade) were dissolved in 2000 ml of distilled water (Table 1).

The solution was heated at 160°C during 3 h in a sealed tube. The precipitated CDHAP particles were further washed with distilled water and dried at 105°C for 2 h. Afterward the particles are heated at 1500°C during 120 min, with the heating rate of 10°C/min. The heated samples were milled planetary mill during 30 min. Obtained cement powder was further mixed with phosphate solution (2.5 wt.% solution of Na_2HPO_4) at a liquid to powder ratio of 0.32 ml/g in order to obtain the cement paste. These pastes were molded in cylindrical shaped in cylindrical pills (diameter 5 mm, height 1 mm) by pressing at 22 and 45 MPa.

Experimental Animals

A test material α-TCP was evaluated for primary skin irritation to the international standard for testing biocompatibility (ISO 10993-10:2002/Amd 1:2006 – see in References).

Experiments were performed on twelve rabbits of either sex (New Zealand White, weight 4.2 ± 0.4 kg) bred in the Institute of Physiology, and raised under controlled environmental conditions (temperature 22 ± 2°C; 14 h light/10 h dark). Animals were provided *ad libitum* access to a commercial rabbit-diet and drinking water was supplied to each cage. Experiments were approved by the Local Animal Ethical Committee of the School of Medicine, the University of Belgrade and were conducted in accordance with the principles and procedures of the NIH Guide for Care and Use of Laboratory Animals and ISO 10993-2: 2006 – see in References).

Table 1. Synthesis of α-tricalcium phosphate cement

Sample	$Ca(NO_3)_2{\cdot}4H_2O$ (g)	EDTA (g)	$NaH_2PO_4{\cdot}2H_2O$ (g)	$CO(NH_2)_2$ (g)
HAP (Ca/P = 1.50)	13.73	7.4	6.08	12.0

HAP, hydroxyapatite; Ca/P, calcium/phosphor ratio; $Ca(NO_3)_2{\cdot}4H_2O$, calcium nitrate; EDTA, ethylenediaminetetraacetic acid; $NaH_2PO_4{\cdot}2H_2O$, monosodium phosphate dehydrate; $CO(NH_2)_2$, urea (carbamide).

Experimental protocol

The area on the back of each rabbits was clipped free of fur with an electric clipper 24 h before the application of the sample. For the experiment, the clipped areas of skin of each rabbit were divided into four sites with the same area (20 × 20 mm). At each rabbit the test material α-TCP, either as a solid material (disc 5 × 2 mm) or as paste was applied to only two sites; the other two were used as negative controls (no material present, hypoallergic adhesive strip only). The positive control of this experiment was 98% lactic acid. The special control–blank liquid was the patch with H_2PO_4 (2.5%) (blank liquid: solvent portion treated in the same manner as the identical solvent used for the preparation of test samples-pasta, but without test material, and which is intended for the determination of background response of the solvent). Three rabbits were used per group; each rabbit had two patches with the same test material.

All the sites were covered by gauze and the back of the rabbit was wrapped with a non-occlusive bandage. After 4 h, the bandage and the test material were removed; 1 h later the sites were macro-pathologically examined for skin irritation and the observation was repeated after 24, 48 and 72 h (Draize et al. 1944). Skin reactions are graded separately for erythema and edema, each on a 0–4 grading scale. For erythema: 0 – no erythema; 1 – very slight erythema, barely perceptible; 2 – well-defined erythema; 3 – moderate to severe erythema; 4 – severe erythema (beet redness) to slight eschar formation (injuries in depth). For edema: 0 – no edema; 1 – very slight edema, barely perceptible; 2 – slight edema (edges of area well defined by raising); 3 – moderate edema (raised approximately 1 mm); 4 – severe edema (raised more than 1 mm and extending beyond the area of exposure).

The score of primary irritation (SPI) was calculated for each rabbit as the difference between the sum of the scores for erythema and edema at 24, 48 and 72 h divided by the number of the observations for the treated sites and the sum of the scores for erythema and edema at 24, 48 and 72 h divided by the number of the observations for the control sites (see formula below). The primary irritation index (PII) was calculated as the arithmetical mean of the SPI values of the three animals, e.g. of the six patches with the same test-material.

Formula used to calculate the SPI:

$$SPI = \left[\frac{\sum (Er+Ed)_{t1} + (Er+Ed)_{t2} + (Er+Ed)_{t3}}{n}\right]_T - \left[\frac{\sum (Er+Ed)_{t1} + (Er+Ed)_{t2} + (Er+Ed)_{t3}}{n}\right]_C$$

Er, erythema; Ed, edema, t1, 24 h; t2, 48 h; t3, 72 h; *n*, number of experiments; T, treated; C, control.

Test materials producing PII values 0 are considered no irritation, less than 2 are mildly irritating, 2 to 5 are moderately irritating, and greater than 5 are severely irritating.

Statistical analysis

Comparison between the mean values of SPI of the experimental groups was made by *t*-test. Statistical significance was considered at a probability $p < 0.05$.

Results

General tolerance

All rabbits recovered well and showed no signs of illness within the following 72 h.

Local tolerance

The effect of α-TCP, solid material

In all test animals, erythema or edema was not present after 24, 48 and 72 h in treated and control sites. Individual results of skin SPI for α-TCP solid material are reported in Table 2. There was no difference between treated and control sides ($p > 0.05$). The PII of the test material was 0 on a scale of 0.00 to 8.00.The PII of 0 is evaluated as no irritation.

Table 2. Score of erythema and edema after application of α-TCP solid phase

Animal (rabbit)		24 h		48 h		72 h		SPI	PII
		T	C	T	C	T	C		
1	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
2	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
3	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		

T, treated site; C, control site; SPI, score of primary irritation; PII, primary irritation index.

The effect of α-TCP paste

In all test animals, erythema or edema was not present after 24, 48 and 72 h in treated and control sites. Individual results of skin SPI for α-TCP paste are reported in Table 3. There was no difference between treated and control sides ($p > 0.05$). The PII of the test material was 0 on a scale of 0.00 to 8.00. The PII of 0 is evaluated as no irritation.

The effect of solvent Na_2HPO_4 (2.5%) – blank liquid

In all test animals, erythema or edema was not present after 24, 48 and 72 h in treated and control sites. Individual results of skin SPI for blank liquid, solvent Na_2HPO_4 (2.5%) used for preparation of α-TCP paste are reported in Table 4. There was no difference between treated and control sides ($p > 0.05$). The PII of the solvent–blank liquid was 0 on a scale of 0.00 to 8.00. The PII of 0 is evaluated as no irritation.

The effect of lactic acid (98%) – positive control

At the 24-h observation point, well defined erythema (score 2) was observed in one of the three test animals. In the other two animals moderate erythema (score 3) was observed. At the 48 h, moderate erythema remained on one of the three test animals (score 3); on the other two test animals there were well-defined (score 2) and very slight erythema (score 1). At 72-h observation points, the irrita-

Table 3. Score of erythema and edema after application of α-TCP paste

Animal (rabbit)		24 h		48 h		72 h		SPI	PII
		T	C	T	C	T	C		
1	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
2	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
3	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		

T, treated site; C, control site; SPI, score of primary irritation; PII, primary irritation index.

Table 4. Score of erythema and edema after application of blank liquid, Na_2HPO_4 (2.5%)

Animal (rabbit)		24 h		48 h		72 h		SPI	PII
		T	C	T	C	T	C		
1	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
2	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
3	Erythema	0–0	0–0	0–0	0–0	0–0	0–0	0/6 – 0/6 = 0	0
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		

T, treated site; C, control site; SPI, score of primary irritation; PII, primary irritation index.

Table 5. Score of erythema and edema after application of lactic acid 98% (positive control)

Animal (rabbit)		24 h		48 h		72 h		SPI	PII
		T	C	T	C	T	C		
1	Erythema	3–3	0–0	2–2	0–0	2–2	0–0	14/6 – 0/6 = 2.33	2.1
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
2	Erythema	2–2	0–0	1–1	0–0	1–1	0–0	8/6 – 0/6 = 1.33	
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		
3	Erythema	3–3	0–0	3–3	0–0	2–2	0–0	16/6 – 0/6 = 2.67	
	Edema	0–0	0–0	0–0	0–0	0–0	0–0		

T, treated site; C, control site; SPI, score of primary irritation; PII, primary irritation index.

tion was reversed, and well-defined erythema remained on two test animals and very slight erythema, barely perceptible remained on only one test animal. Edema was not observed at any time. Individual results of skin SPI for lactic acid (98%) are reported in Table 5. There was difference between SPI for treated and control sides ($p < 0.05$). The PII of the test material was 2.11 on a scale of 0.00 to 8.00. The PII of 2.11 is evaluated as moderately irritating.

Discussion

In this work we have reported the irritant properties of new developed α-TCP material. This *in vivo* investigation of the irritant properties of α-TCP paste and α-TCP solid material, tested after direct contact to rabbit skin, did not determine any cutaneous reaction such as erythema, edema, and necrosis. Moreover, the material did not give rise to allergic reactions.

A comprehensive, general guideline on the testing of biocompatibility of materials for medical applications is specified in the ISO 10993. Local skin compatibility is one of the crucial parameters for their possible clinical application. The irritation test is one of the estimates the local irritation potential of devices, materials or extracts, using sites such as skin or mucous membranes, usually in an animal model (ISO 10993-10:2002/Amd 1:2006).

The ISO 10993-10:2002/Amd 1:2006 standard describes skin-irritation tests for both single and cumulative exposure to a device. The preferred animal species is the albino rabbit, whose highly sensitive, light skin makes it possible to detect even very slight skin irritation caused by a substance.

The *in vivo* skin irritation/corrosion test in rabbits was introduced by Draize in the 1940s to predict hazardous effects of substances coming into contact with human skin (Draize 1944). This method gradually became the world standard, although several aspects of the test have been criticized. These include: the subjectivity of the method; the overestimation of human responses and the method's cruelty (McDouglas 1998; Patil et al. 1998). The European Centre for the Validation of Alternative Methods and the Interagency Coordinating Committee on the Validation of Alternative Methods were created in Europe and the United States respectively, in order to develop alternative methods. In Japan, the Japanese Society of Alternatives to Animals Experiments is responsible for carrying out validation assays. In recent years, different studies have been developed to validate alternatives to the skin irritation *in vivo* test (Human skin keratinocyte cytotoxicity/neutral red assay, Skin Squared^{TM} and Episkin^{TM}, Skintex assay). The application of tissue culture techniques, cellular and molecular biology, and analytic cytometric techniques can lead us closer to our goal of eliminating the need for animals in eye and skin irritation testing (Vinardell and Mitjans 2008). However, at present, despite the quantity and quality of the work carried out, with the exception of corrosion and phototoxicity, no alternative *in vitro* tests for skin irritation are available for regulatory purposes (reviewed in Vinardell and Mitjans, 2008). The *in vivo*, or Draize method has been the method of choice for determining the irritation/corrosion potential of chemicals (ISO 10993-10:2002/Amd 1:2006).

Since we tested a new developed material, no information was found in the available literature relative to the PII of this material. For this reason it is not possible to relate this investigation to similar relevant studies.

Previous biological evaluations of α-TCP material have been performed only in cell cultures using cytotoxicity tests. Our *in vitro* studies have suggested that there were significant differences ($p < 0.05$) between the tested concentrations of α-TCP. In the first experiment (qualitative and quantitative assay) α-TCP, in concentrations 200 mg/ml and 20 mg/ml, was clearly cytotoxic, while in concentration 2 mg/ml was mild cytotoxic. In experiment two (qualitative assay) α-TCP in concentration 100 mg/ml, 50 mg/ml and 10 mg/ml, showed clearly cytotoxic effect, in concentrations 10 mg/ml, 5 mg/ml and 1 mg/ml moderate cytotoxic effect, in concentration 0.5 mg/ml and 0.1 mg/ml mild cytotoxic effect while in concentrations 0.05 mg/ml and 0.01 mg/ml showed no cytotoxic effect. In experiment three, extract of α-TCP (50%, 75% and 100%) showed mild cytotoxic effect while in lower concentration (<50%) did not show any cytotoxic effect. We concluded that, in mouse fibroblast L929 cell culture system α-TCP showed concentration dependent cytototoxic effect which, predominantly, resulted from its inhibitory effect on cell growth (Jokanovic et al. 2008; Medovic et al. 2008; Stojanovic et al. 2008a,b).

Dos Santos et al. (2002) demonstrated that after 7 days of incubation the α-TCP-based cement has some degree of toxicity in cell cultures. The level of the α-TCP cement's cytotoxicity was determined, from which IC_{50} values ranging from 30% to 78%.

Otherwise, Ehara et al. (2003) showed that α-TCP is nontoxic even when tested in exaggerated culture conditions (i.e. prolonged exposure of cells to the materials). α-TCP showed no significant influence on cell proliferation, but the number of cells attached to the culture plates after incubation with/without α-TCP is the same at concentrations.

Any direct comparison or ranking between different biocompatibility tests would be inappropriate; therefore caution is warranted in attempting to correlate cell culture tests with animal experiments.

The similar materials to this that was tested in this investigation are already in clinical use. According to our data, PII of new developed α-TCP material (as a paste and solid material) is zero. The irritation potential of the testet material is negligible and cannot be considered as primary irritant to the skin, but further investigations are necessary to

demonstrate if in a range of physiological concentration, new developed calcium phosphate ceramic, α-TCP, demonstrates any health hazard potential.

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