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Local Tissue Reaction and Biodegradation of Hydroxyapatite/ Tricalcium Phosphate Composites

[1]Aidin Sadeghilar, [2]Angela Ng Min Hwei, [1]Nor Hazla Mohamed Haflah,
[3]Isa Mohamed Rose and [4]Somayyeh Firouzi

[1]Department of Orthopedics, Faculty of Medicine,
Universiti Kebangsaan Malaysia Medical Center, 56000, Kuala Lumpur, Malaysia
[2] Department of tissue engineering, Faculty of Medicine,
Universiti Kebangsaan Malaysia Medical Center, 56000, Kuala Lumpur, Malaysia
[3]Department of Pathology, Faculty of Medicine,
Universiti Kebangsaan Malaysia Medical Center, 56000, Kuala Lumpur, Malaysia
[4]Department of Nutrition and Dietetic, Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia, 43300, Serdang, Selangor, Malaysia

Abstract: *Background:* Different ratios of Hydroxyapatite (HA) / Tricalcium phosphate (TCP) composite ceramics show different characteristics in in-vivo applications. The aim of this study was to evaluate early local tissue reaction and biodegradation rate of different ratios of HA/TCP in animal model. *Methods and Materials:* Six different ratios of HA/TCP scaffold was prepared using combined technique of gel-casting and polymer sponge methods. The scaffolds were implanted in tramuscular of 12 white New Zealand rabbits. After three months, animals were sacrificed and the scaffolds examined histologically and radiologically in order to determine local tissue reaction and biodegradation. *Results:* There was no significant differences in local tissue reaction among different ratios of HA/TCP composition. However, data showed that except in models with 100% TCP, increasing TCP shows higher degradation rate. *Conclusion:* Our study indicates that biodegradation rate depends on the higher TCP content in the ceramic composite. Different ratios of HA/TCP are perfectly compatible for in vivo applications in animal models.

Key words: Hydroxyapatite · Tricalcium phosphate · Ceramics · Artificial bone · Scaffolds

INTRODUCTION

Artificial bone substitutes are frequently used to fill bone defects as an alternative modality for autografts and allografts due to limitations of their availability [1, 2]. Calcium phosphate ceramics, including Hydroxyapatite (HA) and Tricalciumphosphate (TCP) are applied as artificial bone substitutes due to their similar chemical properties to bone matrix. The Degradation of HA and TCP ranges from highly stable and steady soluble scaffolds with 100% HA to fast degradation and loss of structure in scaffolds with 100% TCP. Several studies on different ratios of HA and TCP have been performed to determine the most desirable properties of the composite ceramics for the purpose of tissue engineering applications [1-3]. The optimum ratio of these two ceramics should lead to the formation of the gradually degradable bone substitute which eventually be replaced by bone while preserving adequate mechanical strength. In-vivo characteristics of biphasic ceramics including biodegradation and biocompatibility of calcium phosphate ceramics have been evaluated in different studies [4-6]. However, data regarding biodegradation and local tissue reaction in different ratios of HA/ TCP are limited and inconclusive. Hence, aim of this study was to evaluate early local tissue reactions and biodegradation of these ceramic composites in wide range of HA/TCP ratios in animal model.

Corresponding Author: Aidin Sadeghilar, Department of Orthopedics, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Center, Kuala Lumpur, Malaysia Tel: +61-475016422.

MATERIALS AND METHODS

Composite Design: Six different ratios of biphasic calcium phosphate (BCP) ceramics were designed. Ratio compromised 100% HA, 80/20 HA/TCP, 60/40 HA/TCP, 40/60 HA/TCP, 20/80 HA/TCP and 100% TCP.

Preparation of Implant Materials: β-TCP (β -$Ca_3(PO_4)_2$) and HA ($Ca_{10}(OH)_2(PO_4)_6$) powders were utilized to prepare slurry (both of them were nano-powder with particle size of <200 nm). Darvan C, a 25% aqueous solution of ammonium polymethacrylate, was used as dispersant. The compositions of the gel casting method were organic monomer and cross-linker: monofunctional acrylamide, $C_2H_3CONH_2$ (monomer) and difunctionalmethylenebisacrylamide $(C_2H_3CONH)_2CH_2$ (cross-linker). Ammonium persulphate $(NH_4)_2S_2O_8$ and N,N,N',N'tetramethylethylenediamine (TEMED) were applied as initiator and catalyst; respectively [7]. All powders purchased from Sigma Aldrich Company.

Preparation of Scaffolds: The scaffolds were prepared by polymeric sponges which were soaked in ceramic slurry containing monomers and cross-linker. A combination of the gel-casting method and polymer sponge method was applied to produce the scaffolds [7, 8]. To prepare slurry, different ratios of HA/TCP composites solved in a solution containing monomer, cross-linker and dispersant hydrated with DI water (Table 1).

To make porous scaffolds, polyurethane foam cut into defined shapes and sizes (10mm long tubular shaped with a diameter of about 9mm), then were soaked in the slurries. The polymerized scaffolds were dried in air for 24 hour and then underwent sintering process. The scaffolds were heated at a gradually increasing rate of 1°C/min to 600°C and kept at this temperature for 1 hour to burn out the polyurethane foams. The possibility of presence of residues was excluded using microanalyses EDS then were sintered with increasing temperature at a rate of 3°C/ min to 1150°C and maintained in the mentioned temperature for 2 hours [7, 8].

Surgical Protocol: A total number of 72 implants were used in six HA/TCP groups (12 scaffolds per each group). The surgery and animal care were conducted according to regulation of animal rights and all the procedure approved by "Animal ethics committee of National University of Malaysia". One sample from each group was chosen to be implanted on a white New Zealand rabbit, intramuscularly, (total number of six scaffolds for each rabbit). Allrabbits were four months old female with the mean weight of 1950±230 gram.

To ease the determination of groups throughout the experimental period, the 100% HA ceramic group was implanted into the right forelimb, 100%TCP group into the left forelimb, 60/40 ratio into right hind limb, 40/60 ratio into left hind limb, 80/20 ratio into the right dorsum and 20/80 ratio in left dorsum. For pain management subcutaneous injection of Meloxicam was used (0.3 mg/kg daily for five days). Intramuscular injection of Endrofloxacin 5% was given to prevent infection (5mg/kg daily for five days).

Rabbits were sacrificed with an overdose injection of pentobarbital sodium (50mg/kg) after three months and all the samples with surrounding fibrous tissue were collected for analysis.

Histologic Study: After gross examination, the surrounding fibrous tissues were separated from harvested implants and fixed in 10% buffered formalin. Then, dehydrated with alcohol solution and embedded in paraffin. Sections were prepared with a rotatory microtome(thickness: 5-7 μm). These slices were stained with 5% Hematoxylin and Eosin and graded by a blinded pathologist with a light microscope. Tissue response to the implant was scored according to the scoring system to evaluate 8histologic parameters as indicators of tissue reaction. The scoring system was modified based on Ellman and colleagues [9] by assessing local tissue reaction to Foreign Bodies (FB) in which "0" account for entity not present,"1" for entity present to a mild degree; 2 for entity present to a moderate degree; and 3 for entity present to a marked degree.

Table 1: Composition of slurry for scaffold preparation

Component	Amount added
Hydroxyapatite or Tricalciumphosphate or their composite with different ratios	15gr
Dispersant (25%)	0.7-1.6 ml (depending on viscosity)
Acrylamide (in 100ml solution)	14.17 gr
Methylenebisacrylamide (in 100ml solution)	2.83 gr
Ammonium presulphate (10%)	0.3 ml
N,N,N,'N' tetramethylethylenediamine	0.2 ml

Radiological Study: The implant then were undergone 3D reconstruction imaging using quantitative computerized in-vivo Micro Tomography to obtain their objective volume. All implants were scanned before and after implantation to determine post implantation degradation.

Statistical Analysis: Data from different variables of histological findings as well as volume of the scaffolds before and after implantation were expressed as Mean ± SD. One Way Anova was used to determine the mean difference of different histological findings. Paired t test was used to determine the mean difference between objective volume of scaffolds before and after implantation. All statistical tests were performed using Statistical Package for Social Sciences (SPSS, Inc. Chicago, IL) version 19software and p value of less than 0.05 was considered significant.

RESULTS

No superficial or deep infection has been detected in either early or late phase of post implantation period.

Histopathological Findings: Findings showed that there was no significantdifference in overall tissue reaction among 6 groups of scaffolds in 7 parameters of local tissue reaction. However, fatty infiltration was significantly higher in 100% TCP group (1.92±0.669, $p<0.001$). 100% HA group showed the least fibrosis among all ceramic composites (0.831±0.251). Generally all HA/TCP composite ceramics were biocompatible as no statistically significant tissue reaction was observed (Table 2).

Radiological Findings: Objective volume reduction after three months in all six types of ceramics was examined. Scaffolds were degraded significantly after three months (Figure 1). All samples showed significant reduction in objective volume in paired t test (Mean difference: 82.1± 73.4, $p<0.001$).The rate of degradation among different ratios of HA to TCP composites were also statistically significant ($p<0.05$). The higher content of TCP in a composite, the higher degradation rate observed; with exception of 100% TCP group (Figure 2). The most degradable composite was 80/20 HA/TCP and the most insoluble composite was 100% HA group.

DISCUSSION

Radiological grade of ceramic absorption was relatively higher in lower ratio of HA/TCP composites at 12 weeks except 100% TCP, owing to the faster degradation of TCP and creeping substitution. The reason for considerable lower degradation rate in pure TCP composites is not clearly understood. However, it might be due to smaller porous structure formation in pure TCP ceramics than composite ceramics as bigger porous structures are formed due to the presence of bigger HA particles, which may allow better blood penetration to the scaffold and more degradation.

In three months, the outer parts of the scaffolds were replaced completely by fibrous tissue leaving the core of the scaffolds intact which might be due to less accessibility of blood supply towards center of the structures.

Some studies reported higher rate of infection in porous calcium phosphate structures compared to dense ceramic implants [6]. However we did not observe any implant related infection in our animals due to appropriate prophylactic antibiotic coverage.

In this study, biphasic calcium phosphatescaffolds were produced by combination of gel-casting and polymer sponge methods. The gel casting is a method facilitates faster formation of ceramics by in situ polymerization in which a macromolecular network is formed to retain the

Table 2:Histological scores (expressed as means ± standard deviations)

	Hemorrhage	Necrosis	Acute Inflammation	Chronic inflammation	Fibro vascular prolifiration	Fibrosis	foreign body debris	giant cell	Fatty infiltration [a]
100% HA	0.27±0.047	0.00±0.000	0.00±0.000	1.55±0.688	1.36±0.809	0.831±0.251	1.09±1.044	1.09±1.044	1.45±0.688
80/20	0.18±0.005	0.00±0.000	0.00±0.000	1.82±0.751	1.82±0.809	2.36±0.505	1.27±1.191	1.27±1.191	1.27±0.468
60/40	0.18±0.003	0.00±0.000	0.00±0.000	1.55±0.688	1.55±0.688	2.55±0.688	1.30±1.090	1.09±1.000	0.82±0.405
40/60	0.17±0.089	0.00±0.000	0.00±0.000	1.58±0.669	1.42±0.515	2.55±0.669	0.92±0.800	0.83±0.737	0.67±0.551
20/80	0.00±0.000	0.00±0.000	0.00±0.000	1.36±0.674	1.36±0.674	2.18±0.603	1.09±1.000	1.00±1.095	1.27±0.467
100% TCP	0.17±0.077	0.00±0.000	0.00±0.000	1.33±0.778	1.17±0.718	2.08±0.900	0.99±0.56	0.58±0.496	1.92±0.669

[a]One way Anova, Benferoni post hoc, sig at $p<0.001$

Abbreviations: HA: Hydroxyapatite; TCP: Tricalcium Phosphate

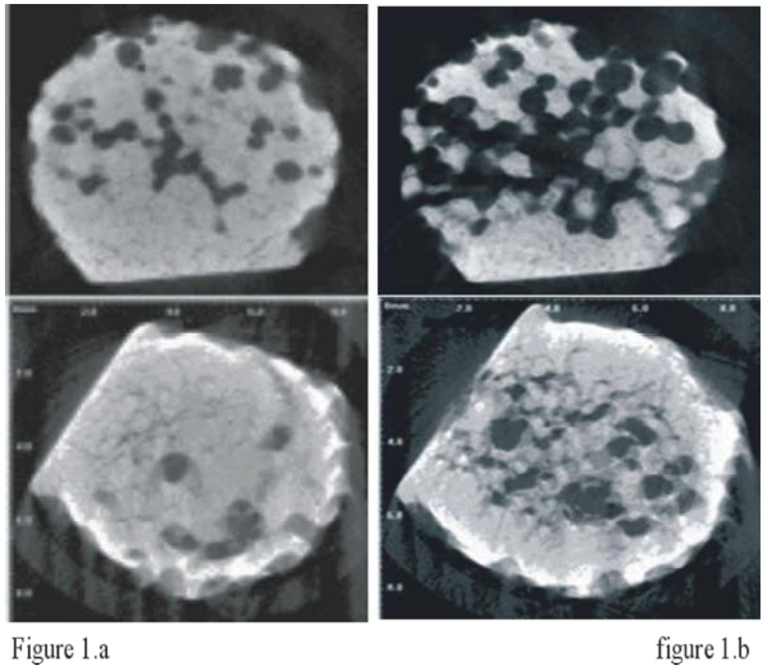

Fig 1: CT scan of scaffolds cross section for two different ratios. a: before implantation. b: after implantation

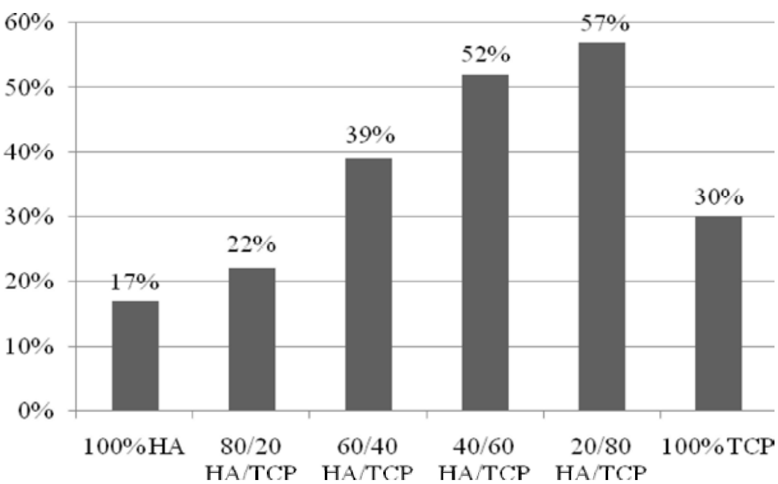

Fig 2: Reduction of objective volume in different ratios of composites three months after implantation

ceramic particles together, leading to a structure with better mechanical strength [10, 11]. A technique combining polymer sponge and gel casting methods results in formation of homogenous porous scaffolds with sufficient mechanical properties.

Burning out of a polyurethane is a considerable issue during sintering in the polymer sponge method to eliminate the effect of polymers in tissue reaction[12]. Adequate time should be given for the polymer pyrolysis during a certain stage of ceramic sintering. In a study conducted by Ramay and Zhang [7], weight measuring methodwas appliedto determine the temperature in which the complete burnout of the polyurethane foam occur. They conclude that the complete polyurethane foam burnout occursat 550°C. Therefore, to allow sufficient time for the complete pyrolisis of the foam in scaffolds before the sintering starts, the heating rate was set to 1°C/min up to 600°C, therefore polymers which have been utilized in the scaffold will not interfere with tissue reaction.

CONCLUSION

There is no significant differences in local tissue reaction among different ratios of HA to TCP and all of them are perfectly compatible for in vivo applications. The degree of degradation is reversely affected with higher ratio of HA/TCP, in other words, the ceramic with higher content of TCP will resorb faster.

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Conflict of Interest: The authors declare no conflict of interest.

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