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An *In vivo* Experimental Study on Investigation of the Osseointegration of Zeolites A and Silicalite in Rats

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ABSTRACT

Background: Zeolites are naturally occurring and can be artificially synthesized hydrated microporous crystallized aluminosilicates. Thus far, medical materials have comprised polycrystalline materials, glass, glass-ceramics and ceramic-filled composites for bone repair. The study aimed to investigate the potential *in vivo* osseointegration of two types of zeolites (A and silicalite) in rats by histologically presenting the repair process.

Methods: Bone cavities of 1 mm^3 were formed in rats and filled either with zeolite A or silicalite to investigate the possibility of using zeolites to repair bone defects. A comparative histological evaluation was performed regarding the interaction of zeolites with bone tissue and their osseointegration capacity for 15, 30 and 45-day intervals.

Result: According to the results obtained, the growth of both fibrous and bone tissues took place around the zeolites placed in the live organism. It was observed that the zeolites used in this study did not give rise to necrosis, local tissue reaction, allergic or and any other harmful response. In conclusion, histopathology revealed that zeolites A and silicalite were biocompatible with the bone and could integrate with it at certain time intervals.

Key words: Osseointegration, Rat, Silicalite, Zeolite A.

INTRODUCTION

The medical materials used so far for skeletal repair are bio-ceramics, including polycrystalline materials, glasses, glass ceramics and ceramic-filled composites (Best *et al.* 2008; da Rocha Barros *et al.* 2002; Gil-Albarova *et al.* 2004). Bio-ceramics may be categorized as either bio-inert or bioactive according to their interaction with the host tissue. They may be prepared in different forms, such as porous or dense bulk forms, as well as granules and coatings (Best *et al.* 2008; Gil-Albarova *et al.* 2004).

Bio-inert materials play a passive role in the biological environment and in their presence, local reactions are often characterized by their fibrous tissue encapsulation. On the other hand, bioactive materials exchange Ca and PO_4 ions and form new bone tissues by direct or close contact. Bio-glass ceramics are good examples of bioactive ceramics of common interest (Sopyan *et al.* 2007; Tampieri *et al.* 2001).

Zeolites are hydrated microporous crystalline aluminosilicates that may occur naturally or be synthesized in laboratory conditions (Best *et al.* 2008). The zeolite framework consists of an assemblage of SiO_4 and AlO_4 tetrahedra, joined together in various regular arrangements through shared oxygen atoms, to form an open crystal lattice (Gil-Albarova *et al.* 2004). The micropore structure is determined by the crystal lattice, which contains pores of molecular dimensions, into which guest molecules can penetrate (Gil-Albarova *et al.* 2004; Sopyan *et al.* 2007; Tampieri *et al.* 2001).

It has been reported that zeolites have unique properties suitable for selective adsorption/desorption of molecules, reaction catalysis, ion exchange, *etc.* (Parlat *et al.* 1999). These materials have a wide range of existing and potential applications. Their use in medicine is a recent but promising subject of research (Kyriakis *et al.* 2002; Parlat *et al.* 1999). Zeolites have been tested against the toxic effects of aflatoxins (Parlat *et al.* 1999) and mycotoxins (Kyriakis *et al.* 2002) to prevent diarrhea (Rodriguez-Fuentes *et al.* 1997) and in the treatment of various types of tumors (Pavelic *et al.* 2001). Several studies have been performed concerning the utilization of zeolites as antibacterial agents (Macedo *et al.* 2004), antacid agents (Linares *et al.* 2005) and controlled release materials (Zhang *et al.* 2006). They are also used as manure in agriculture (Triatmoko *et al.* 2020) and dietary supplements in animal breeding (Yazdani and Hajileri. 2009). Some investigations have also been performed regarding bone health. Supplementation of zeolite to dairy calves

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seemed to alter the rate of bone turnover without affecting bone strength (Turner *et al*. 2008). Supplementation of zeolite A to yearling horses resulted in increases in plasma Si concentrations (Lang *et al*. 2001). Additionally, there were indications that these horses had decreased bone resorption, providing more significant net-bone formation. However, knowledge of the experimental studies of using zeolites to repair bone defects in rats is limited.

Therefore, we investigated the possibility of using zeolites for the repair of bone defects. In this study, bone cavities formed in rats were filled either with zeolite A or silicalite and a comparative histological evaluation was performed regarding their interaction with bone tissue and capacity for osseointegration for 15, 30 and 45-day intervals.

MATERIALS AND METHODS

Animals

The study was conducted at the Istanbul University, Cerrahpasa Medical Faculty, Research Center for Experimental Animals in 2011. Nine male adult Wistar albino rats supplied by the Laboratory Animals' Production Center of Cerrahpasa Medical Faculty, Istanbul University, were used in this study. The animals were allocated into two main groups (one experimental and one control group). The rats were transported to the laboratory one week before the study, kept under a 12:12 h light-dark cycle fed on a commercial pelleted diet and received tap water ad libitum. The study was approved by the Ethics Committee of Istanbul University.

Material properties

Two different types of commercial zeolites, namely, zeolite A and silicalite (Aldrich), were utilized in the experiments carried out. The Sodium form of Zeolite A was used in this study. Zeolite A and silicalite have quite different characteristics. The former has the lowest possible silicalite (Si)/ aluminum (Al) ratio for zeolites, which is 1 and is very hydrophilic in nature. On the other hand, silicalite has a very high Si/Al ratio close to ∞ and is hydrophobic in nature, preferring to adsorb organic molecules rather than water.

In-vivo experiment

Bilateral tibial regions of the rats were shaved and disinfected under anesthesia with diethyl ether. Then, the animals were subjected to inhalation anesthesia with isoflurane at an initial concentration of 5%, followed by a concentration of 2%. The epidermal incision was made starting from the tibia up to the medial end. The bone was attained by dissecting the epidermis and dermis and by stripping the periosteum. Under sterile conditions, using fine pointed drills, cavities of 1 mm^3 were carved at tibial metaphysic and zeolite A was placed at the right metaphysis while silicalite was inserted in the left hind metaphysis. The same procedure has been carried out for the control group except for either applying zeolite A or zeolite silicate. In all animals, the covering muscles and the skin were closed using 4/0 polyglycolic acid suture and 4/0 silk surgical suture, respectively. Cephalexin monohydrate (Sef®, Mustafa Nevzat *Pharmaceutical* Industry, Turkey) was used as the antibiotic during the post-surgery period. The antibiotic dose determined as 6-15 mg/kg was applied twice a day *via* intramuscular injection. Neither death nor infection occurred in the animals tested. During the observation period, animals were fed on a commercial feed containing min.1 g, max.1.80 g Ca and min. 0.90% P.

The zeolite in powder form was kept in live rat bone tissue for different periods. The animals of the experimental group were sacrificed on days 15, 30 and 45 (each subgroup contained two rats) and the control group (one rat for each time point) was sacrificed by ether anesthesia on the same days. Then, the left and right tibial bones of the animals were removed.

Histopathology

Tibial bone portions treated with zeolites A and silicalite and the tibial bones of the control group were initially subjected to 24 h fixation by 10% buffered formalin solution. Then, they were decalcified for 16 h to 24 h by 5% nitric acid under automatic follow-up. After the decalcification procedure, the tissue specimens were routinely processed, embedded in paraffin, cut at about 3-5 mm thickness and then stained with Hematoxylin and Eosin (H&E) to be evaluated by light microscopy.

RESULTS AND DISCUSSION

Histopathology revealed abundant zeolite particles phagocytized by macrophages in the tissue sections collected from the medullary cavity of the right tibias of the animals sacrificed 15 days after zeolite A application. Numerous fibrocytes and fibroblasts accompanied by collagen fibers arranged in a pattern of multidirectional sheets were observed among these particles, as well (Fig 1). Abundant collagen bundles, fibrocytes, fibroblasts and bone spicules filled with hydroxyapatite were noted among the silicalite particles in the same animals' tissue specimens sampled from the left tibial medullary regions (Fig 2). In the control group, on day 15, granulation tissue consisting of fibrocytes, fibroblasts and collagen bundles was observed in the cavitation site.

In the tissue specimens of the animals sacrificed 30 days after Zeolite A administration, phagocytes or free zeolite particles were observed in their right tibias. These particles were replaced by hyalinized collagen fibers in the majority of microscopic fields examined and fibrous tissue was evident with numerous fibrocytes, fibroblasts and bone spicules (Fig 3).

On day 30, phagocytized silicalite particles were distinctively replaced by collagen fibers and fibrous tissue in the animals treated with silicalite. Furthermore, silicalite particles were observed in the medulla of the novel woven bone formed by the hyalinization of collagen fibers in a microscopic field (Fig 4). On day 30, there was distinct fibrous

tissue mainly consisting of thick hyalinized collagen bundles and primary osseous formations in the control group.

On day 45, zeolite particles phagocytized by macrophages, collagen fibers, fibrous connective tissue elements and collagen hyalinization, indicating transformation to primary bone could be observed. Fibrosis was initiated at the periosteum and spread to muscle tissues nearby. Hyalinized collagen bundles, epithelioid cells, histiocytes, fibrocytes, fibroblasts and granulation tissue consisting of inflammation cells as well as suture materials within these tissues were also detected (Fig 5).

Fig 6 represents the microscopic image obtained after 45 days of the experiment when zeolite A was utilized. After this period, bone marrow existed in the medullar cavity. No foreign substance reaction took place against the zeolite particles. On the other hand, formations of thin fibrous strips, collagen fibers, fibrocytes, fibroblasts, small capillary veins and standard trabecular bone pieces were determined to have occurred. Trabecular particles pertaining to normal bones were also observed. In the medulla, zeolite A particles and fibrous connective tissue cells replacing the collagen fibers also existed.

After 45 days of the experiment, formations of thin fibrous strips and bone lamella were detected in the medullar cavity when silicalite was utilized. Collagen fibers, fibrocytes, fibroblasts and woven bone trabeculae existed in the regions where zeolite particles were placed. The microscopic image depicting these results was shown in Fig 6. On day 45, histopathology revealed dense hyalinization in collagen bundles accompanied by thin fibrous bundles and woven osseous lamellae. In the control group for the same time point, histopathology revealed the formation of primary bone (Fig 7) through dense hyalinization in collagen bundles

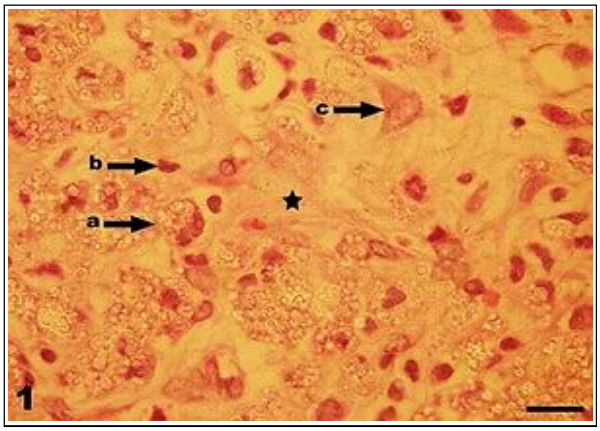


Fig 1: Microscopic image of the defected bone filled with zeolite A, taken after 15 days. a: Zeolite particles phagocytized by macrophages, b: Fibrocyte, c: Fibroblast, star: Collagen fibers. H&E, Bar: 30 µm.

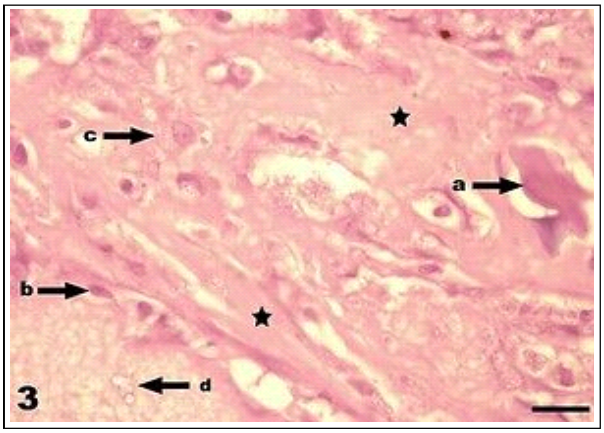


Fig 3: Microscopic image of the defected bone filled with zeolite A, taken after 30 days. a: Bone spicules, b: Fibroblast, c: Zeolite. A particles phagocytized by macrophages, d: Free zeolite A particles, star: Osteoid matrix. H&E, Bar: 30 µm.

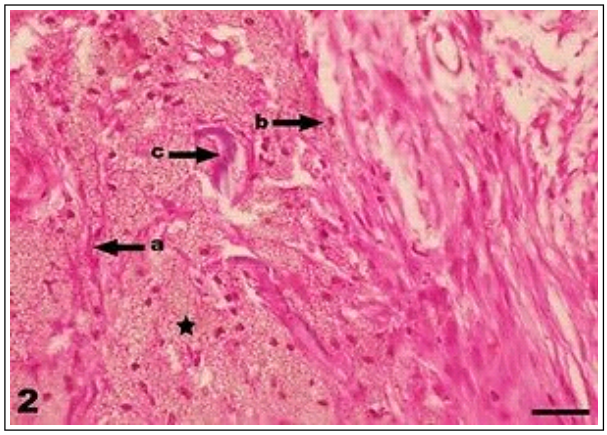


Fig 2: Microscopic image of the defected bone filled with silicalite, taken after 15 days. a: Collagen fibers, b: Fibrocyte, c: Bone spicules, star: Silicalite particles. H&E, Bar: 30 µm.

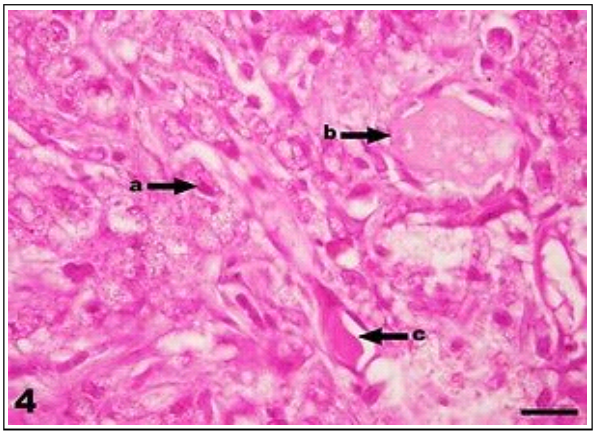


Fig 4: Microscopic image of the defected bone filled with silicalite, taken after 30 days. a: Silicalite particles phagocytized by macrophages, b: Hyalinized collagen bundles with silicalite particles in it c: Bone spicules. H&E, Bar: 30 µm.

accompanied by thin fibrous bundles and secondary lamellar bone (Fig 8).

Various studies are available concerning the use of zeolites in medicine (Kyriakis *et al.* 2002; Lang *et al.* 2001; Linares *et al*. 2005; Parlat *et al.* 1999; Pavelic *et al.* 2001; Rodriguez-Fuentes *et al.* 1997; Turner *et al*. 2008; Zhang *et al.* 2006). This study was the first to examine in vivo zeolite-bone interactions. The osseointegration abilities of two different types of zeolites were investigated. Our previous study showed that the crystal structures of different types of zeolites were not affected by being kept in simulated body fluid for up to 14 days (Ceyhan *et al.* 2007). Notable amounts of silicate were detected in simulated body fluid samples after their treatment with all types of zeolites investigated. Zeolite A and silicalite allowed the lowest and highest silicon transfer into the simulated body fluid, respectively. These zeolites did not have any significant unfavorable *in vitro* biological effect on two different cell generations, namely, chronic myelogenous leukemia and Swiss Albino fibroblast, under the conditions used in that study (Ceyhan *et al.* 2007). Therefore, based on the data proposing that these substances had no adverse effect concerning cell injury, we designed the presented study to investigate the effects of zeolite and silicalite on osseointegration.

The effects of zeolites on bone health are still under investigation. On the contrary, dietary silicon appeared to be beneficial to bone and connective tissue health. Oral administration of zeolite A to 1-year-old horses was found to have increased bone mass density by reducing bone resorption (Frey *et al*. 1992). The exact role of silicon in bone health is still unclear; however, there are some suggestions of possible mechanisms, such as the synthesis of collagen and/or its stabilization and matrix calcification (mineralization) (Jugdaohsingh 2007). A few studies (Ceyhan

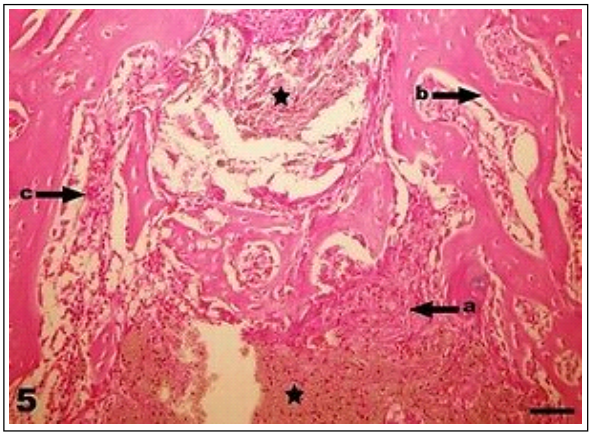


Fig 5: Microscopic image of the defected bone filled with zeolite A, taken after 45 days. a: Fibrous connective tissue, b: Bone trabecula, c: Medullar cells, star: Zeolite A particles. H&E, Bar: 50 µm.

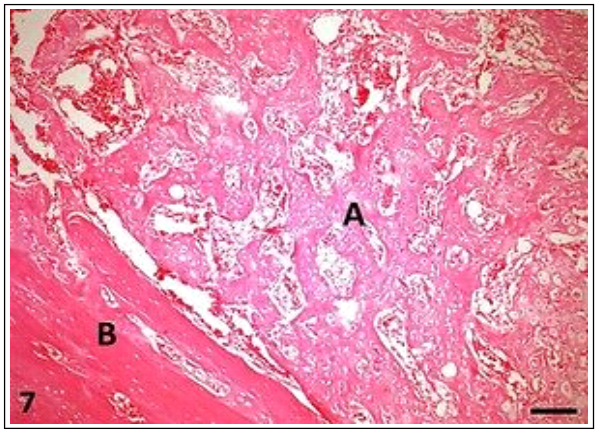


Fig 7: Control group on day 30. Highly vascular primary ossification with woven-nonlammelar bone pattern formed at the cavitation region (A). Mature bone tissue adjacent to the woven bone (B). H&E, Bar: 100 µm.

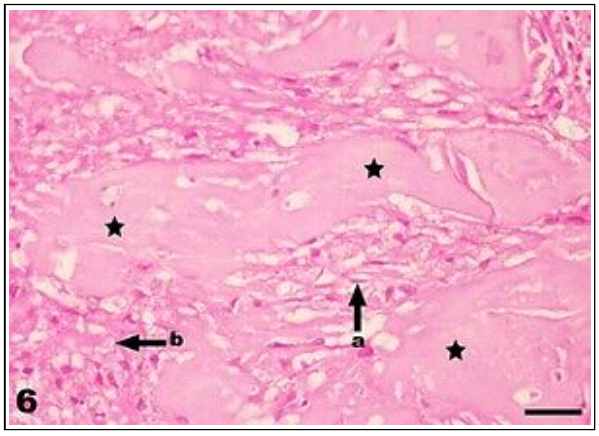


Fig 6: Microscopic image of the defected bone filled with silicalite, taken after 45 days. a: Fibrocyte, b: Silicalite particles, stars: Woven (immature-primary) bone trabecules. H&E, Bar: 30 µm.

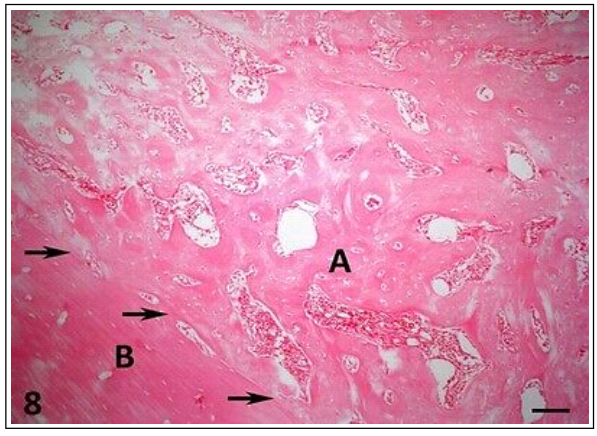


Fig 8: Control group on day 45. A: Intact bone tissue. B: Secondary lamellar bone tissue (stars) formed at the spontaneously healing cavitation region. Integration line (arrows) between newly formed secondary bone tissue and intact bone. H&E, Bar: 100 µm.

et al. 2007; Schainberg *et al.* 2005) investigating *in vitro* interaction of zeolites and bone were performed. In one example (Schainberg *et al.* 2005), zeolite appeared to be ineffective on cellular proliferation, alkaline phosphatase and collagen production. Silicates prominently induced collagen synthesis in our study.

In vitro studies showed that zeolite A induced the proliferation and differentiation of cells of the human osteoblast lineage (Keeting *et al.* 1992). The analysis of the effects of zeolite on the bone-resorbing activity of highly purified avian osteoclasts indicated that this material could inhibit bone resorption. It was also suggested that zeolite A or a partial substructure might have a potential positive activity on bone turnover (Schutze *et al.* 1995). In one of the latest studies, bone formation was reported to have been stimulated in ovariectomized rats treated with Panaceo Micro Activation (PMA)-zeolite-clinoptilolite, a cation exchange product of clinoptilolite. It was also shown to have markedly elevated osteocalcin (a specific marker for bone formation) levels, increased bone mineral density and improved quality of life in osteoporotic women by significantly attenuating pain (Pavelic *et al*. 2021). In the study, phagocytosis of the substances by macrophages, formation of collagen fibrils, hyalinization, replacement of immature (woven bone) tissue by novel bone tissue spicules in the implantation area were detected step-by-step on days 15, 30 and 45. These findings were all consistent with the previous studies (Keeting *et al.* 1992; Schutze *et al.* 1995; Pavelic *et al.* 2021); yet, the presented study is the first to histologically demonstrate the entire process of novel bone formation by the filler substances Zeolite A and silicalite, with the changes at different phases.

It was reported that foreign-body reactions, inflammation and massive tissue damages developed due to the application of bone-implant biomaterials (Macedo *et al.* 2004). In our study, histopathology revealed fibrous and bone tissue formation in the regions where zeolite particles existed. The utilization of zeolites A and silicalite as bone-implant applications did not lead to any undesired fatal effects on bone cells such as necrosis or local allergic tissue reactions. We consider that further studies, including statistical histological evaluations, should be performed to investigate the effects of zeolites on bone tissue.

CONCLUSION

In conclusion, the presented study is considered to have offered a new perspective into the issue of the biocompatibility of zeolites by investigating *in vivo* osseointegration of zeolites A and silicalite. The results indicated no significant difference in the bone-zeolite interaction when these two zeolites with quite different properties were utilized. Zeolite A is hydrophilic while silicalite is hydrophobic, with higher silicalite content and they represent extreme cases. These zeolites were observed to be biocompatible under the conditions investigated in this study. The results obtained were promising and promoted further investigations on this subject. Histological studies on animals involving statistical tests may more clearly reveal the effects of zeolites on bone formation.

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None.

Conflict of interest

The authors declare no conflicts of interest.

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