EXPERIMENTAL STUDY

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In vivo investigation of calcium phosphate coatings on Ti6-Al-4V alloy substrates using lactic acid - sodium lactate buffered synthetic body fluid

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Objective: The aim of this study was to evaluate the mode of failure and biomechanical characteristics of Ti-6Al-4V anchors biomimetically coated with calcium phosphate (CaP) for soft tissue fixation to bone in an animal model.

Methods: The current study included 14 adult New Zealand white rabbits equally divided into two groups. Calcium phosphate-coated Ti-6Al-4V anchors were used in the test group and non-coated Ti-6Al-4V anchors in the control group. A new approach was applied to synthesize the CaP coatings via the biomimetic growth in the *Lac*-SBF containing Ca^{2+} and PO_4^{3-} ions, Na-lactate and lactic acid (HL). Titanium anchors were implanted into the right tibia, followed by biomechanical tensile strength tests. Histological studies were carried out after removal of anchors (bone-implant surface).

Results: The CaP-coated Ti-6Al-4V anchors had significantly higher tensile strength (p=0.003) and displacement values (p=0.004) than the non-coated anchors. Control group scores were higher than those of the test group (14 and 9, respectively) in tensile strength tests.

Conclusion: The new CaP coating can be used in orthopedic surgery as catalyzer to improve bone ingrowth. We believe that our research will form a model for further research on biomimetic coatings on Ti-6Al-4V substrates.

Key words: Anchor; biomimetic method; calcium phosphate; *in vivo*; lactic acid.

Suture anchors are of considerable interest to orthopedic surgeons due to their ability to solve major problems associated with soft tissue fixation to bone.[1] Despite increasingly widespread use of these implants, little is known about their biomechanical characteristics. Biomechanical assessment involves fatigue testing of materials for a given number of cycles under submaximal load.[2] The biocompatibility of metallic materials is an important consideration in orthopedic applications.[3] Metallic biomaterials, such as Ti and its alloys, stainless steel, and CoCrMo alloys are widely used in many load-bearing biomedical applications.[3,4] Hydroxyapatite (HA), also known as $Ca_5(PO_4)_3(OH)$, is a potential bioceramic candidate for load transferring between the bone

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and prosthesis due to its superior biocompatibility.[5] Hydroxyapatite is also a good bone substitute candidate as it is chemically similar to bone minerals.[6] Hydroxyapatite possesses several interesting properties, including the adsorption of proteins and viruses and exchange of ionic species in its crystal lattice.[7] Hydroxyapatite prevents metallic ion release in the physiological environment and provides good mechanical stability for Ti-6Al-4V alloy substrates. In addition, HA coating acts as a barrier between the body and metallic implants, and provides a surface on which bone can easily grow, generating mechanical interlocking and chemical bonding at the bone-implant interface.[8]

Various coating methods have been applied to commercially pure Ti and Ti-6Al-4V because of the excellent mechanical properties of these metals combined with the osteoconductivity of bioceramic-plasma spraying, ion-enhanced coating, sol-gel deposition, electrochemical deposition and biomimetic deposition. The development of biomimetic bone replacement materials for use in medicine is an expanding field of research.[9,10]

The present study aimed to evaluate the mode of failure and biomechanical characteristics of Ti-6Al-4V anchors biomimetically coated with calcium phosphate (CaP) and implanted in the right tibia of rabbits for soft tissue fixation to bone. This study also sought to improve the coating properties of the material in order to increase anchor stability. We developed a new synthetic body fluid (*Lac*-SBF) via the biomimetic method, which we hypothesize would contribute novel data to the literature on anchor coating. Calcium phosphate can be used during orthopedic surgery to facilitate and improve bone growth. This study describes a novel method to simplify bone-tissue reconstruction. Conventional SBF is produced without the need for 37°C of heat and converts into hydroxyapatite at 37°C of body temperature. As *Lac*-SBF degrades quickly, it is produced to be resorbed with more difficulty at 37°C.[11]

In the present study, the formation of CaP on Ti-6Al-4V substrates via a newly developed *Lac*-SBF was evaluated.[11,12] In addition, the types of interactions between CaP and Ti-6Al-4V systems were examined. The primary goal of the study was to determine the biomechanical properties of Ti-6Al-4V anchors coated with CaP based on tensile testing and to determine the stability of the anchors via biomechanical testing and histopathological examination. *Lac*-SBF was developed to prove that better osteointegrating HA anchors have better biomechanical properties.

Materials and methods

The study protocol was approved by the DEU Ethics Committee for Animal Experiments. Fourteen adult New Zealand white rabbits with a preoperative weight of 2500 g were equally divided into two groups. CaP-coated Ti-6Al-4V anchors were used in the test group and non-coated Ti-6Al-4V anchors in the control group.

Ti-6Al-4V anchors (Tipmed Ltd. Sti., İzmir, Turkey) measured 4×11.45 mm (Fig. 1). Titanium alloy anchors were coated via a biomimetic technique. NaOH and H_2O_2+NaOH were used for chemical etching in order to compare their merits after the coating process. Substrates were chemically etched in 5M NaOH aqueous solution at 60°C for 24 hours. After the addition of H_2O_2 to the 5M NaOH aqueous solution, other substrates were chemically treated at 60°C for 24 hours in an electric furnace. Substrates were then gently washed with distilled water and dried at 40°C for 24 hours in an electric furnace. After they were thoroughly rinsed with distilled water, the strips were finally heat-treated at 600°C in the furnace.

Next, 2.5× *Lac*-SBF solution was prepared in a total volume of 2500 mL. Ca^{2+} and HPO_4^{2-} ion concentrations were multiplied by a factor of 2.5 with respect to those of human blood plasma. Following the addition of $CaCl_2{\cdot}2H_2O$ and $MgCl_2{\cdot}6H_2O$ into the solution, a total volume of 40 ml of 1M HL was periodically added into the solution to prevent precipitation and achieve the target pH, as shown in Table 1. Ion concentrations of the *Lac*-SBF solutions were compared with those of Tris-buffered SBF. The ion concentrations of the 1× *Lac*-SBF solutions were identical to those of human blood plasma.[11,12]

Rabbits were maintained under standardized living conditions. The rabbits were anesthetized using keta-

Fig. 1. Photograph of an Ti-6Al-4V anchor. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Table 1. Comparative ion concentrations of Tris-SBF and the *Lac*-SBF in this study.

Ion	Kokubo-SBF (mM)	*Lac*-SBF ×1 (mM)	*Lac*-SBF ×2.5 (mM)	Blood plasma (mM)	Blood plasma meq/l
Na^+	142.0	142.0	142.0	142.0	142.0
Cl^-	147.8	103.0	103.0	103.0	103.0
HCO_3^-	4.2	27.0	27.0	27.0	27.0
K^+	5.0	5.0	5.0	5.0	5.0
Mg^{2+}	1.5	1.5	1.5	1.5	3.0
Ca^{2+}	2.5	2.5	6.25	2.5	5.0
HPO_4^{2-}	1.0	1.0	2.5	1.0	2.0
SO_4^{2-}	0.5	0.5	0.5	0.5	1.0
Lactate	-	22	26.5	(+) ion: (155 meq/l) (-) ion: (133 meq/l + 22 meq/l organic anions)	
Lactic acid (1M)	-	36 mL	40 mL		
Tris	50	-	-	-	-

mine (80 mg/kg) and xylazine (5 mg/kg). Postoperative analgesia was achieved using buprenorphine 0.1 mg/kg. Under sterile conditions, the proximal part of the right tibia was bilaterally opened via a medial approach. The metaphyses of the rabbit was drilled anteroposteriorly with the same torque force until the entire Ti anchors was inserted. Anchors were removed from the right tibia of each rabbit after 6 weeks and the animals were sacrificed.

Histopathological examinations were performed on bone specimens after removal of the anchors (bone-implant surface). Bone-tissue samples were kept in 10% formalin solution for 48 to 72 hours. Afterwards, samples were decalcified in 10% formic acid for 48 to 72 hours and then prepared as blocks embedded in paraffin. Cross-sections 5-µm-thick were prepared from the blocks. One of the preparations was stained with hematoxylin & eosin and the other was stained with Masson's trichrome. Fibrous histogenesis, inflammation, and granulation tissue were evaluated as follows: (0) no staining; (1) light staining; (2) moderate staining; (3) strong staining.[13,14]

Immediately after sacrifice, tibias were removed and subjected to tensile testing using a Shimadzu Autograph AG-I Series universal testing machine and Shimadzu Non-Contact Video Extensometer DVE-101/201 (Shimadzu Corp., Kyoto, Japan). Trapezium software was used for machine control and data acquisition (Fig. 2). Bones were attached to the test machine for stabilization and anchors pulled-out perpendicularly to the bone's surface. Tensile tests were performed at a constant crosshead speed of 5 mm/min at room temperature. Each anchor eyelet was pulled straight in line with the suture anchor axis and the force at failure was recorded. In all cases, fixation of the anchors in the test bone was stable and anchor fixation was fairly powerful in static loading.[15,16]

The differences in tensile test results and force data between the test and control groups were evaluated using the Mann-Whitney U test and SPSS v11.0 for Windows (SPSS Inc., Chicago, IL, USA).

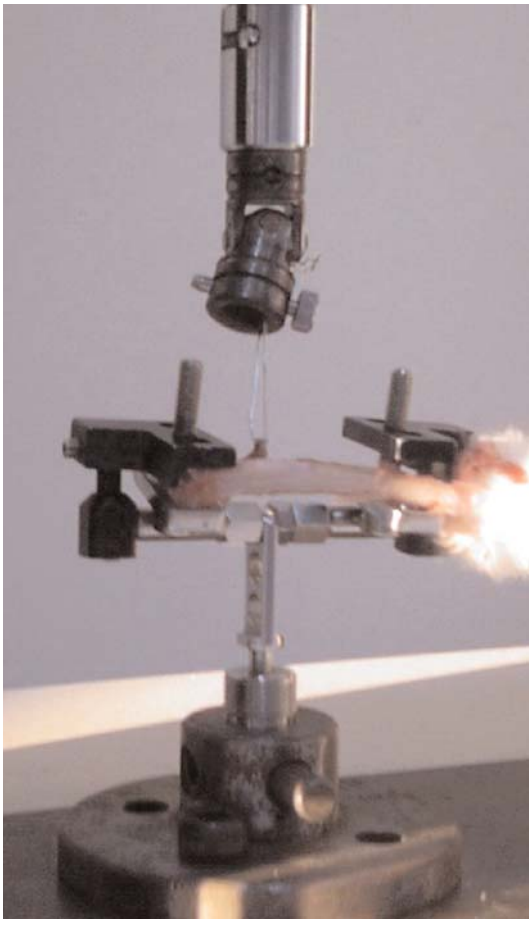

Fig. 2. Application of the tensile test. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

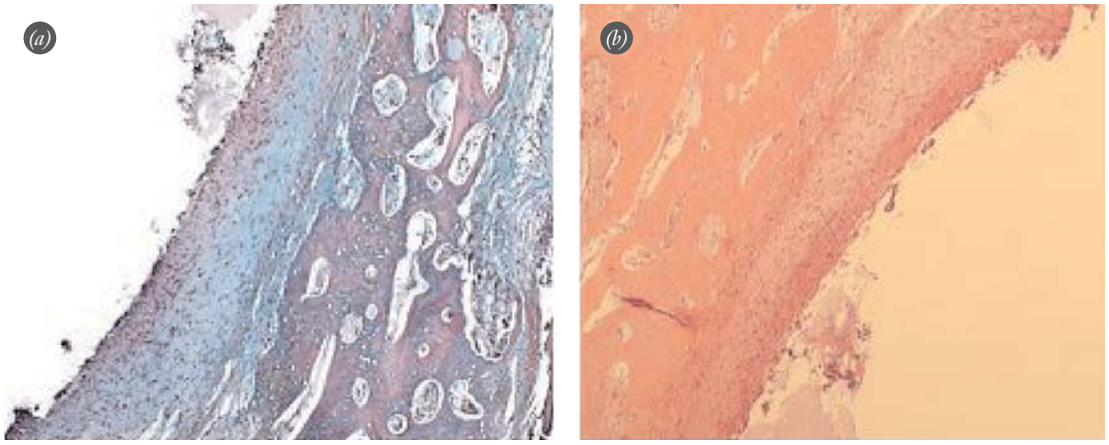

Fig. 3. Histological findings of the anchors. Evaluation of **(a)** fibrous histogenesis by Masson's trichrome stain (×100) and **(b)** inflammation by H&E stain (×100). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Results

No rabbits showed any signs of infection or inflammatory reaction during the 6 weeks in which the implants were in place. Masson's trichrome staining showed fibrous tissue around one anchor gap in the test group (Fig. 3a). Control group scores were higher than those of the test group (14 and 9, respectively) in tensile strength tests.[13,14] The appearance of fibrous tissue was similar in the test and control groups. Hematoxylin & eosin staining of decalcified sections in the control group is shown in Fig. 3b. No inflammatory or granulation reactions were noted in the decalcified specimens. Inflammation and granulation were evaluated by non (0) for CaP-coated Ti-6Al-4V anchors.

Biomechanical tensile test results for CaP-coated and uncoated anchors are given in Fig. 4. The CaP-coated Ti-6Al-4V anchors had significantly higher tensile strength (p=0.003) and displacement values (p=0.004) than the non-coated anchors.

Discussion

Suture anchors are widely used for fixation of soft tissues to the bone.[17] Fixation failure has been documented and attributed to removal of anchors from bone, rupture of suture material and removal of sutures from soft tissue.[18-20] Eyelet design has a significant effect on suture abrasion and excess friction lowers load to failure and cycles to failure.[17,21,22] Anchor location in the bone varies with anchor type and clinical application. Load to failure varies with anchor placement in different bones and different regions of the same bone.[22-25] Suture anchor constructs are susceptible to failure at the bone-anchor interface, anchor-suture interface, suture-tissue interface and other abrasive areas of the suture, such as a bone edge, knot or crimp-clamp.[17,26,27]

In the present study, tensile testing was performed to investigate the ultimate holding strength of Ti anchors to soft tissue. The tensile strength of sutures attached to anchors implanted in tibias of an animal model was measured in order to evaluate the weakest point of the bone-anchor structure. Anchors failure was considered when the anchors completely pulled out from the bone surface. Based on the present biomechanical results, the CaP-coated Ti-6Al-4V anchors exhibited good osteoconductivity and high pull-out strength. The results of a novel H_2O_2+NaOH process described herein indicate that apatite coating on the metal surface appeared fast and dense. We modeled lactic acid, which exists in human metabolism, and used *Lac*-SBF instead of tris/HCl buffer. The ion concentration used in the present study was the same as for inorganic ions in human plasma, and to the best of our knowledge, this is the first report of such a sodium lac-

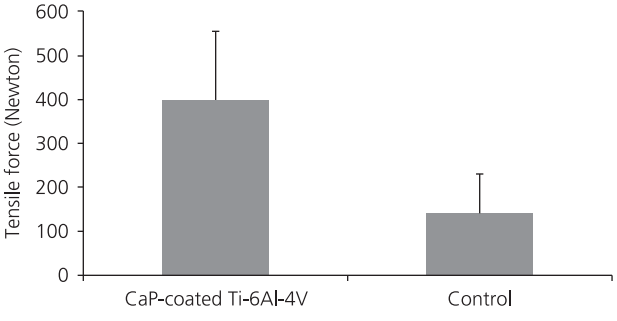

Fig. 4. Biomechanical test results of CaP-coated and uncoated anchor screws following *in vitro* tests.

tate and lactic acid-containing *Lac*-SBF solution. Although the Cl^- content was higher than 103 mM,[13,18,28] we prepared 103 mM Cl^- ion content, as in human plasma.[11,12]

Calcium phosphate coating of metal implants is an effective method for enhancing the bioactive properties of metal surfaces. It improves the bonding strength to bone tissue without inducing the growth of fiber tissue.[6] Herein, we described a biomimetic method of CaP coating Ti anchors in order to enhance bone growth and improve implant anchoring to host bone. This method, which employed lactic acid in the incubation medium as well as in the metal preparation before incubation in simulated body fluid, is novel. The presented model can also be used to evaluate and compare the histopathological properties of the attachment of anchor to bone, the attachment of sutures to anchors and whether the suture itself would fail after implantation *in vivo*. Histopathological examination of the decalcified CaP-coated Ti-6Al-4V anchor specimens showed that there was maintenance of biological properties, with no apparent inflammatory reactions. However, inflammation was seen in 3 cases from the control group. As such, the inflammation must be compared the first few days and after 6 weeks. According to the present histopathological findings, both the CaP-coated Ti-6Al-4V anchors and non-coated anchors exhibited similar fibrous tissue formation. According to Barros et al., there wasn't a significant difference between the suture anchor group and the transosseous suture group at 3, 6, or 12 weeks.[29]

The animal model design was a limitation of the present study. Additionally, the physiological loads borne by the suture anchor construct resulted in a decrease in load to failure as implantation time increased.[30] Therefore, anchors could be removed after 6 weeks in rabbits. The pull-out strength and histopathological response of the anchors were evaluated *in vivo* in a rabbit model. Based on the present findings, CaP-coated Ti-6Al-4V anchors could improve stability in the treatment of soft tissue fixation. We observed the same fibrous tissue and no inflammation or granulation reactions using the CaP-coated Ti-6Al-4V anchors in a rabbit model.

In conclusion, Ti-6Al-4V anchors biomimetically coated with CaP appear useful for soft tissue fixation to bone, improve fixation stability and fracture repair. In addition, we propose that the new CaP coating described in this study could be used during orthopedic surgery as a catalyzer to improve bone growth. Based on its unconventional nature, we think that the present findings will inform further research on biomimetic coatings on Ti-6Al-4V substrates.

Acknowledgement

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Conflicts of Interest: No conflicts declared.

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