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Research Article

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Influence of Calcium Supplements for the Biomimetic Apatite Formation on Titanium Implants-An *in vitro* study

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ABSTRACT
Background: Osteoporosis, the most frequent metabolic disease of the bone, represents an important cause of morbidity in the elderly. The major clinical manifestations of osteoporosis, diffuse disease of the skeleton, consists of fractures of the vertebral, of the limbs and the femoral neck. In this situation, the calcium supplements available in markets may help to meet our calcium requirements.
Methods: The alkaline-treated Ti samples were soaked in 24 ml of SBF and SBF modified with calcium supplements available in markets like- calcium lactate, calcium nitrate, calcium acetate, calcium carbonate respectively for 20 days. The influence of calcium supplements was evaluated by the apatite-forming ability in a simulated body fluid (SBF) by various characterization and evaluation techniques and found out the best calcium supplement that can be better absorbed during apatite formation.
Results: An increased apatite forming ability for the NaOH-heat treated Ti after immersion in SBF modified with calcium supplements than NaOH and heat-treated Ti, was confirmed by the Scanning Electron Microscope (SEM), Energy Dispersive X-ray (EDX), the X-Ray Diffraction (XRD), and the Fourier Transform Infrared Spectrometer (FTIR) analysis.
Conclusion: Calcium gluconate, calcium nitrate, and calcium carbonate are likely to be the most effective calcium supplements for osteoporosis prevention. Thus, even in the living organism, the treated Ti is predicted to produce bone-like apatite on its surface and connect to live bone.

Key-words: Alkaline-treated, Bone-like apatite, Calcium supplements, Modified SBF, Osteoporosis, Simulated body fluid

INTRODUCTION
Titanium (Ti) is often regarded as the best metal for *in vivo* applications, because of its exceptional bio compatibility [1]. Despite their high biocompatibility, titanium implants seldom create a direct chemical connection with bone tissue and are frequently referred to be inert material [2,3]. Hydroxyapatite also known as $Ca_{10}(PO_4)_6/(OH)_2$ is a biocompatible ceramic substance, can create a strong chemical connection with natural bone tissue [4,5] and may be utilized to cover metallic implants [6]. One of the potential implant materials for orthopaedic purposes is HA-coated Ti metal [7].

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The implant's underlying metal is ductile, and the bioactive surface encourages bone growth and improves implant-bone adhesion [8].
Coating HA onto metallic substrates has been done using a variety of methods; including physical vapour deposition, chemical vapour deposition, plasma spray, and laser deposition [9]. All of these methods have the drawback of being high-temperature procedures that result in the production of readily dissolvable calcium phosphate (CaP) phases such as amorphous HA, $Ca_3(PO_4)_2$, and $Ca_4P_2O_9$ [10,11]. Furthermore, these methods were unable to produce high crystallinity HA coatings, which are necessary for optimal biocompatibility.
The biomimetic deposition is a low-temperature method for generating bioactive CaP coatings on porous and non-porous surfaces [12-15]. The best technique to generate man-sized apatite crystals with regulated size on the surface of Ti is to use a biomimetic approach that entails generating nucleation sites on the metal surface by

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modifying its surface with functional groups and then mineralization in simulated bodily fluids (SBF) [16]. However, to achieve excellent biomimetic apatite deposition, two requirements must be met: (1) super-saturation of calcium and phosphate ions in the solution [17-21] and (2) activation of the Ti surface [22,23].

Children need calcium to build strong bones. Adults need calcium to maintain strong bones. Over time, inadequate calcium intake can cause osteoporosis, the brittle bone disease. People with osteoporosis are at high risk for broken bones, especially at the wrist, hip and spine. These fractures cause chronic pain and disability, loss of independence, decreased quality of life and a higher risk of death. In this study, commercially available calcium supplements were used, and their impact on biomimetic apatite development on titanium implants was investigated using an in vitro biomimetic technique, resulting in the identification of the optimum calcium supplement for improved absorption.

MATERIALS AND METHODS

Pre-treatment of Ti sample- Commercially pure Ti (CP-Ti), (Sigma Aldrich) of surface area (20x20x1mm3) was used. Each Ti metal sample was mechanically grounded with 60 grit sandpaper to achieve a mirror-finished surface. Subsequently, the samples were cleaned with ethanol, distilled water and dried. The samples were alkaline treated for 24 hours at 60°C with a 5 M NaOH aqueous solution. The samples were gently washed with ultrapure water before being dried at 40º degrees Celsius. They were then heated to 600°C at a rate of 50°C/min for 1 hour and then cooled naturally in an electrical furnace [24].

Evaluation of the bioactivity of the alkaline-treated Ti sample by surface potential measurement- The apatite-forming ability in simulated bodily fluid (SBF) proposed by Fer *et al.* [25] with ion concentrations approximately comparable to those of human blood plasma, was used to assess the bioactivity. The alkaline-treated Ti samples were soaked in 24 ml of SBF at 36.5°C. Another set of samples were immersed in modified SBF solution by adding 10 mg of a calcium supplement like calcium gluconate to 100 mL of the solution. The same was repeated for another set of alkaline treated Ti samples for other calcium supplements like- calcium lactate, calcium nitrate, calcium acetate, calcium carbonate respectively. The samples were removed after soaking in SBF and modified SBF for 20 days, washed with ultrapure water, and dried at 40°C.

Evaluation of the bioactivity of the alkaline-treated Ti sample by chemical evaluation techniques- The surface potential change of the alkaline treated substrates in modified SBF solutions were carried out using by measuring the open circuit potential (OCP). FTIR analyses of the samples after the biomimetic study were carried out to find the apatite growth. A few micrograms of the coatings were scraped off and mixed with KBr and pressed into pellets for FTIR analysis using Shimadzu FTIR spectrophotometer, Model: IR Prestige-21. An X-ray diffractometer, model: X-Pert Pro was used to examine the phases and crystallinity of the samples. CuK radiation at40 kV and 30 mA were utilized as the X-ray source in the XRD experiments and the samples were scanned from 10-70° 2θ at a scan rate of 1.2°/min. The crystalline size of the particles D_{XRD} was calculated according to the Scherer equation:

$$D_{XRD} = 0.9\lambda / B\cos\theta$$

Where,

λ- The wavelength of the radiation

θ- The diffraction angle

B- The corrected half-width of the diffraction peak [26-28]

Evaluation of the bioactivity of the alkaline-treated Ti sample by surface morphological analysis- The surfaces of the samples subjected to the chemical, heat treatments and soaked in modified SBF were analyzed by field emission scanning electron microscopy (FE-SEM: S-4300, Hitachi Co., Tokyo, Japan) equipped with energy dispersive X-ray (EDX: EMAX-7000, HORIBA Ltd., Kyoto, Field emission scanning electron microscopy (FE-SEM: S-4300, Hitachi Co., Tokyo, Japan) equipped with energy dispersive X-ray (EDX: EMAX-7000, HORIBA Ltd., Kyoto, Japan) spectroscopy was used to examine the surfaces of the samples that had been subjected to chemical, heat, and modified SBF treatments.

RESULTS

Fig. 1 shows the change in surface potential of Ti samples following NaOH-heat treatments and 20 days in modified SBF. The surface potential, which was initially highly negative, shifted towards the anodic direction, reached a maximum value within 5 days, and then shifted back to the cathodic direction.

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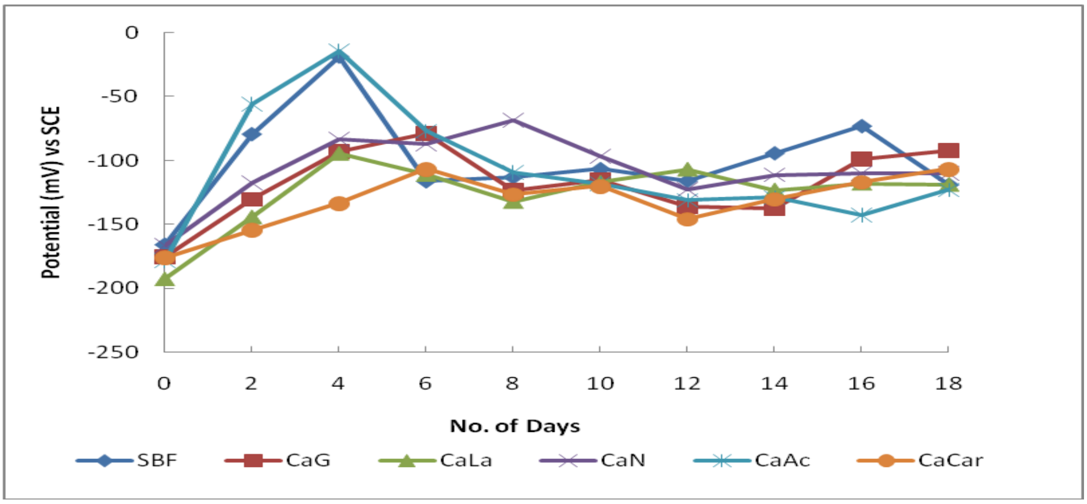

Fig. 1: Surface potential change for the Ti samples after NaOH-heat treatment and soaked in modified SBF for 20 days

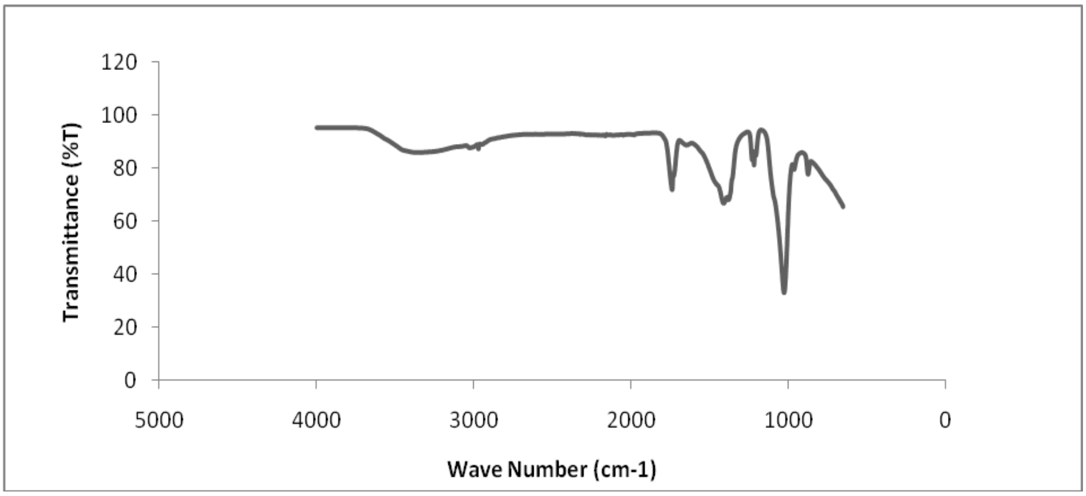

Fig. 2: FTIR spectrum of the Ti samples after NaOH-heat treatment soaked in modified SBF for 20 days

Fig. 2 shows the FTIR spectrum of the Ti samples after NaOH-heat treatments and soaked in modified SBF for 20 days. A spectral region from 4000-5000 cm^{-1} was analyzed. The frequency corresponding to 3444.74 cm^{-1} was due to the OH stretching vibration of the HA coating. The peaks at 1033 cm^{-1} correspond to that of PO_4^{3-} and the peak at 879 cm^{-1} was due to the presence of HPO_4^{2-} ions. The peaks at 1432 cm^{-1} were due to carbonate ions.

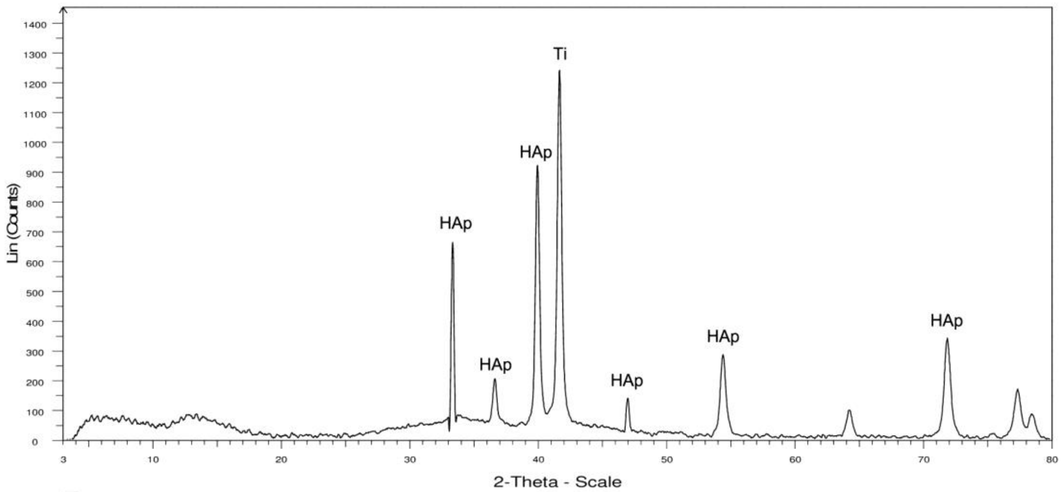

Fig. 3: XRD patterns of the surfaces of Ti after NaOH-heat treatments and soaked in SBF modified with calcium gluconate 20 days

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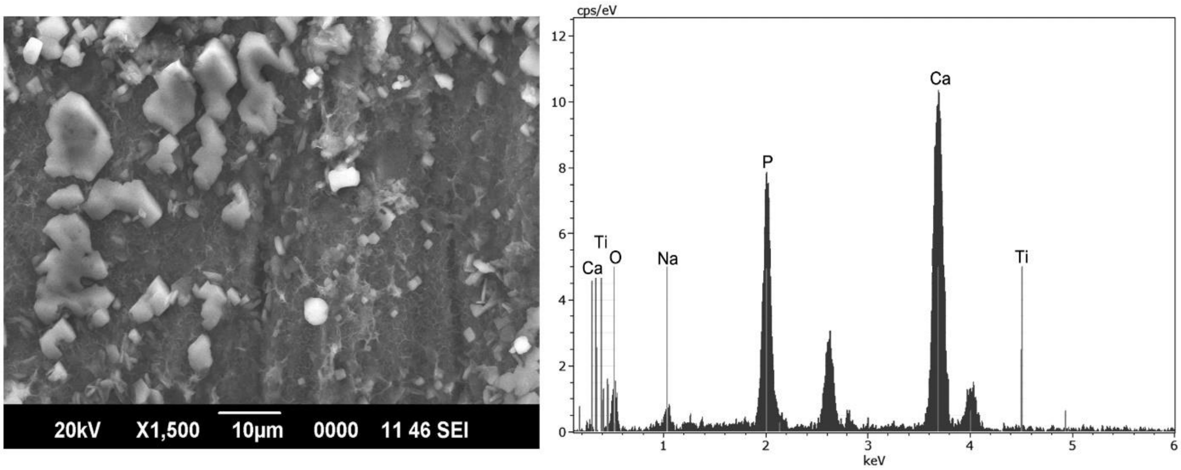
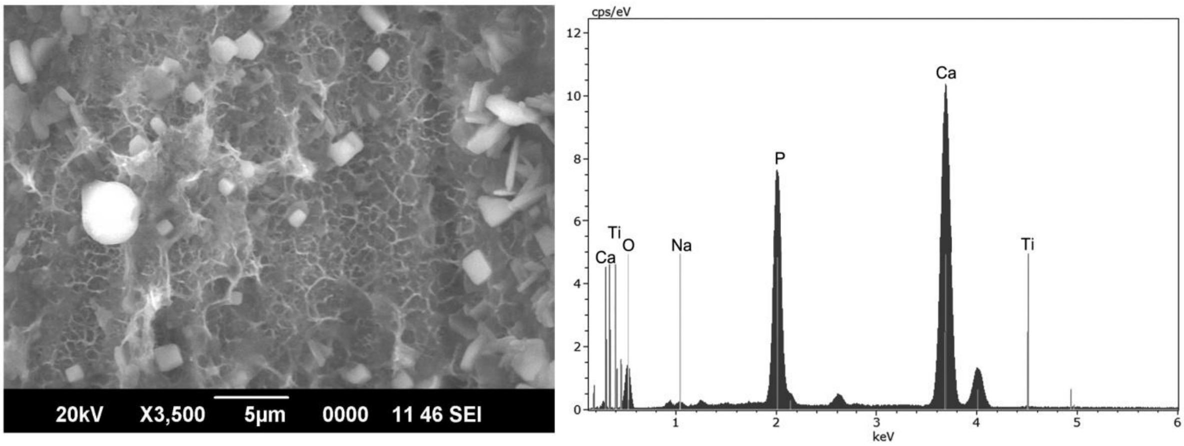
XRD patterns of the surfaces of Ti after (a) NaOH-heat treatments and (b) soaked in modified SBF for 20 days are shown in Fig. 3. The resultant values of 2θ are compared with the standard cards of JCPDS. XRD results indicate that the diffraction pattern of both the coatings matches with the XRD patterns of the HA of good quality and also with the JCPDS value of pure HA. No X-ray evidence was found for the existence of other CaP phases in the coatings. The peaks appeared at 2θ=25^o, 32^o, 39.2^o, 46.7^o, 49.46^o and 53.14^o obtained for both the coatings corresponds to that of crystalline apatite.



(a)



(b)

Fig. 4: SEM and EDX analysis of the surfaces of Ti after NaOH, heat treatments and immersion in SBF modified with (a) calcium gluconate and (b) calcium nitrate for 20 days

Fig. 4 shows that white crystalline precipitates of apatite were found to be more covered on the treated sample immersed in SBF modified with calcium gluconate. White crystalline precipitates of apatite were also found on the surfaces of other samples immersed in SBF modified with calcium lactate, calcium acetate, calcium carbonate. Peaks of varying intensities were also detected depending on the thickness of the film. Sharp peaks obtained for Ca and P during EDX analysis confirmed the formation of CaP phases on the surfaces were also found to have very intensive peaks of Ca and P than the SBF modified with calcium nitrate, calcium acetate, calcium lactate. Moreover, the intensity of the Ti substrate decreased due to interference from calcium phosphate deposits. Table 1 shows the elemental percentage of Ca and P on the surface of the surfaces of Ti after NaOH, heat treatments and immersion in SBF and modified SBF for 20 days which were analyzed by EDX. The table shows that NaOH-heat treated samples after immersion in modified SBF containing calcium gluconate, calcium nitrate and calcium carbonate, have a significant increase in the amount of Ca and P due to the formation of apatite.

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Table 1: Elemental percentage of Ca and P on the surface of the surfaces of Ti after NaOH, heat treatments and immersion in SBF and modified SBF for 20 days

Sample	Elemental (%)		
NaOH-heat treated Ti immersed	**O**	**P**	**Ca**
SBF	62.62	8.04	10.17
SBF modified with			
Calcium gluconate	65.06	20.97	22.58
Calcium lactate	29.49	8.47	10.58
Calcium nitrate	61.80	12.80	20.75
Calcium acetate	58.28	8.58	13.06
Calcium carbonate	48.15	13.03	13.48

DISCUSSION

The peak corresponds to that of PO_4^{3-}, $HPO_4^{2-}$and carbonate ions corresponds to the presence of crystalline apatite [28,29]. The peaks appeared at 2θ=25°, 32°, 39.2°, 46.7°, 49.46° and 53.14°obtained for both the coatings corresponds to that of crystalline apatite [29,30]. It has been found that the alkaline treated Ti forms a sodium titanate ($Na_2Ti_5O_{11}$) surface layer. This sodium titanate could form many Ti-OH groups in SBF. The mechanism of apatite formation onTiO_2 in SBF can be interpreted in terms of the electrostatic interaction of the surface oxide with the ions in the fluid. When exposed to SBF, the Na^+ ions in the surface layer of $Na_2Ti_5O_{11}$ are exchanged with H_3O^+ions present in the fluid facilitating the formation of Ti-OH groups. Because of the negatively charged ions at the surface, the initial surface potential during biomimetic development in SBF solution was extremely negative [30]. The positively charged calcium (Ca^{2+}) ions from the SBF solution are preferentially attracted to this negatively charged surface, and the potential is shifted to the anodic direction. The negatively charged phosphate (HPO_4^{2-}) ions are attracted to this positively charged surface, forming an amorphous CaP, and the potential shifts are negative. This CaP develops into crystalline apatite on its own. Because the SBF is significantly supersaturated about apatite even under normal circumstances, the apatite develops spontaneously by taking the calcium and phosphate ions in the fluid once created [31,32]. The NaOH treatment produced spherical precipitates on the Ti surface that were made up of nano-sized sodium hydrogen titanate with a layered structure. The sodium hydrogen titanate in SBF releases its Na^+ ions by exchanging them with H_3O^+ ions on its surface, forming Ti–OH groups. Thus, the formed Ti–OH groups induce apatite formation as earlier described for the NaOH-and heat-treated Ti metal [33,34]. The SBF utilized to study the effect of surface charge on the nucleation and development of calcium phosphate phases *in vitro* was a supersaturated solution containing calcium supplements. From SEM-EDX analysis results, calcium supplements, such as calcium gluconate, calcium nitrates, and calcium carbonate, have been shown to improve apatite formation. It is a component of various calcium supplements for the control of blood calcium levels due to its high re-sorbability. It is non-toxic and has high physiological compatibility.

CONCLUSIONS

The higher the calcium dose, the less it's absorbed. For maximum absorption, the calcium supplement added in the SBF was optimized. An increased apatite forming ability for the NaOH-heat treated Ti after immersion in SBF modified with calcium supplements than normal SBF was confirmed by the Scanning Electron Microscope (SEM), Energy Dispersive X-ray (EDX), the X-Ray Diffraction (XRD), and the Fourier Transform Infrared Spectrometer (FTIR) analysis. Thus, even in the living organism, the treated Ti is predicted to produce bone-like apatite on its surface and connect to live bone. Calcium gluconate, calcium nitrate, and calcium carbonate are likely to be the most effective calcium supplements for osteoporosis prevention.

To promote lifelong healthy bones and reduce calcium loss, it is recommended to intake vitamin D besides calcium supplements throughout your life.

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CONTRIBUTION OF AUTHORS

One author is only contributed in this article.

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