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Characterization of Titanium Micro Screws Using Hafnium Oxide Nanoparticles and In Vitro Evaluation of Their Osteogenic Potential Using the MG-63 Osteoblast Cell Line

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1. Abstract

Aim: This study aims to evaluate the osteogenic activity of hafnium oxide-coated titanium micro screws using the MG-63 osteoblast cell line in an in vitro environment. The focus is on assessing cell viability, proliferation, differentiation, and the impact of hafnium coating on implant integration.

Materials & Methods: The study utilized titanium micro screws as the control group and hafnium oxide-coated titanium micro screws as the test group. MG-63 osteoblast cells were cultured in both groups to assess cell viability and proliferation using MTT assays, and osteogenic differentiation was evaluated through qPCR gene expression, focusing on markers such as BMP-2, ALP, and Runx2.

Results: Hafnium-coated titanium micro screws demonstrated higher levels of osteogenic markers, including BMP-2, ALP, and Runx2, compared to uncoated screws. Both groups exhibited similar biocompatibility using the MTT assay, with no cytotoxic effects on the osteoblast cells. However, the hafnium coating significantly enhanced cell proliferation and differentiation, suggesting improved osteogenic potential.

Conclusion: Hafnium oxide-coated titanium micro screws significantly improve osteogenic activity in vitro, showing potential for enhanced osseointegration in clinical applications. The results indicate that the hafnium coating enhances osteoblast differentiation and bone formation, making it a promising material for orthopedic and dental implants.

Keywords: hafnium oxide, titanium, micro screws, nanoparticles, osteogenic activity, MG-63 cell line, osseointegration, surface modification, MTT assay, BMP-2, ALP, Runx2

2. Introduction

Titanium implants are extensively utilized in both orthopedic and dental applications due to their exceptional mechanical properties, remarkable corrosion resistance, and high biocompatibility[12]. These attributes make titanium a preferred choice for implants[3]. However, promoting osteogenic activity on titanium surfaces is essential for enhancing osteointegration, the process through which the implant becomes securely integrated with the bone tissue, and improving overall clinical outcomes[4]. Recent advances in the field have focused on

surface modifications aimed at augmenting the bioactivity of titanium implants[6]. Among the promising materials, hafnium, a biocompatible transition metal, has emerged as an innovative coating option because of its stability and potential to influence cellular behavior favorably [6, 7].

Surface modifications of titanium implants play a pivotal role in influencing the biological responses that occur at the implant-tissue interface. A variety of techniques, including anodization, plasma spraying, and ion implantation, have been employed to improve the surface characteristics of titanium implants (8, 9). These modifications aim to enhance important factors such as surface roughness, wettability, and chemical composition, all of which are critical for promoting the adhesion and activity of osteoblasts[10]. Hafnium coating stands out as particularly promising because of its chemical similarity to titanium as well as its potential to create a more osteoconductive surface, which could enhance the integration between the bone and the implant [7,11].

Hafnium's biocompatibility and corrosion resistance make it an attractive candidate for various biomedical applications, including its role as a coating material for titanium implants[12]. Previous studies have indicated that hafnium exhibits excellent cytocompatibility and supports the proliferation of various cell types, which is a positive indicator of its potential effectiveness in medical applications[12–14]. However, the specific effects of hafnium on osteoblast activity and its role in bone tissue formation remain relatively underexplored in the current literature. This study seeks to bridge that gap by systematically investigating the influence of hafnium-coated titanium implants on osteoblast functions, including cell attachment, proliferation, differentiation, and mineralization. These cellular responses serve as critical indicators of the implant's potential to support bone regeneration and ensure long-term stability. Understanding the interactions between hafnium coatings and osteoblasts is crucial for the development of next-generation implants that not only accelerate bone formation but also significantly enhance the success rates of these implants in clinical settings.

The overall success of titanium implants is highly contingent upon the ability of their surfaces to support the formation of new bone tissue. By enhancing these surface properties through hafnium coating, it is hypothesized that the osteogenic potential of titanium implants can be significantly improved. This study aims to evaluate the osteogenic activity of hafnium-coated titanium implants specifically in osteoblast cells. It will focus on assessing how hafnium affects key processes such as cell proliferation, differentiation, and mineralization. This study will employ in vitro models of osteoblast cells to comprehensively assess the biological activity associated with hafnium-coated surfaces. The outcomes of this research could provide valuable insights into the development of advanced biomaterials specifically designed for orthopedic and dental implants. Ultimately, this could lead to better patient outcomes, reduced rates of implant failure, and an overall enhancement in the quality of care provided in clinical settings.

3. Materials and Methods

Study Design

The research proposal was submitted to the Institutional Review Board at Saveetha University and received formal approval from the board members, as indicated by the reference number (SRB/SDC/UG-2024/24/PROSTHO/211). This study was designed as an in vitro investigation, which included a comparison group to evaluate the effectiveness of the different treatments. The comparison group consisted of titanium micro screws, designated as Group A, which were uncoated. In contrast, the intervention group was comprised of hafnium oxide nanoparticle-coated titanium implant micro screws, referred to as Group B (Hf-coated). This design allowed for a systematic evaluation of the effects of the hafnium coating on the performance of the titanium micro screws compared to their uncoated counterparts, providing valuable insights into the potential benefits of the coating in enhancing the properties of the implants.

Sample Preparation

The titanium micro screws used in this study were sourced from the Le Forte System by Jeil Medical Corporation, located in the Republic of Korea. These micro screws featured a head diameter of 2 mm, an outer thread diameter of 1.5 mm, and an overall length of 6 mm. To analyze their surface characteristics, the screws were examined under a scanning electron microscope (Figure 1a). The screws were polished using graded thickness silicon carbide emery papers, specifically 400, 600, 800, and 1000 grit, which were employed to achieve a smooth and uniform surface finish[15]. Once the polishing process was completed, the titanium micro

screws were thoroughly cleaned using deionized water in a bath sonicator to remove any residual particles or contaminants.

Following this cleaning procedure, the screws were treated with a 2% hafnium sol, which was prepared using hafnium oxide nanoparticle powder sourced from Nano Research Elements™ in Haryana, India. After being treated with the hafnium sol, the titanium discs were rinsed two or three times to ensure optimal coating. They were then dried in a hot air oven set to a temperature of 50 °C to facilitate the adherence of the hafnium coating. Simultaneously, to ensure adequate dispersion of the hafnium oxide nanoparticles, a separate preparation was conducted where 200 mg of hafnium oxide nanoparticle powder was mixed with double-distilled water and sonicated.

This step was crucial for achieving a uniform distribution of the nanoparticles[16]. Subsequently, a direct current power source was applied to the resulting dispersion of hafnium oxide nanoparticles, facilitating the coating process on the titanium screws. The hafnium oxide nanoparticle-coated titanium screws that were obtained through this meticulous procedure were then subjected to further analysis under a scanning electron microscope (Figure 1b). This thorough methodology highlights the detailed approach taken in preparing and characterizing the titanium micro screws for the study.

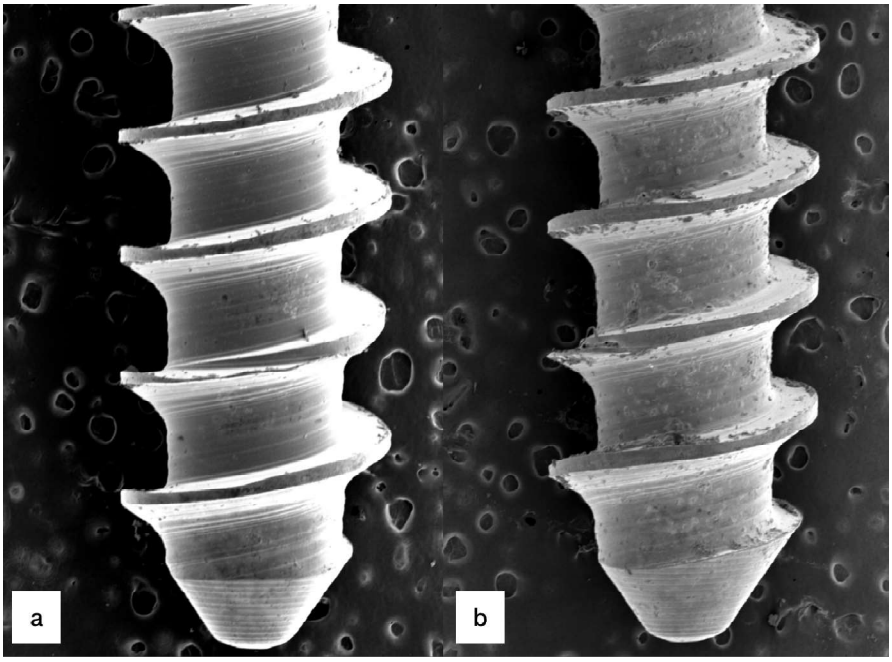

Figure 1: The figure presents scanning electron microscope images showcasing samples from both groups involved in the study. Panel (a) illustrates the uncoated titanium micro screws, providing detailed insights into their surface morphology and structural characteristics. In contrast, panel (b) displays the hafnium oxide nanoparticle-coated titanium micro screws, highlighting the effects of the hafnium coating on their surface features.

Chemical Reagents Used

The primary osteoblast cells utilized in this study were derived from the MG-63 cell line, which was procured from the National Center for Cell Sciences (NCCS) located in Pune, India. These MG-63 cells are widely recognized for their osteogenic properties and were specifically selected to evaluate various aspects of osteogenic activity, including cell viability, proliferation, and the differentiation processes associated with osteoblastic cells[17]. For the experimental setup, the osteoblasts from the MG-63 cell line were cultured on both the uncoated and hafnium-coated titanium micro screws, allowing for a comparative analysis of their behavior over a period of 24 hours.

The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), a nutrient-rich culture medium that supports cell growth, supplemented with 10% heat-inactivated fetal bovine serum. This serum provides

essential growth factors and hormones necessary for optimal cell function and proliferation. Additionally, antibiotics, specifically penicillin-streptomycin, were included in the medium to prevent bacterial contamination, ensuring a sterile environment conducive to cell culture. The cultures were incubated at a controlled temperature of 37°C in a 5% CO2 atmosphere with 95% air, conditions that mimic physiological environments and promote healthy cell growth.

To assess various parameters related to cell viability, differentiation, and mineralization, Trypsin-EDTA was employed for cell detachment during subculturing or harvesting processes. Moreover, specific assay kits designed for measuring cell viability, differentiation, and mineralization were utilized throughout the study. These reagents and materials, critical for the successful execution of the experiments, are detailed in Table 1. This comprehensive approach allows for a thorough evaluation of the osteogenic activity of the osteoblasts in response to the different titanium screw coatings.

Table 1: Details of the chemical reagents used in the experiments carried out in the current research

Chemical Reagents	**Manufacturer's details and location**
DMEM	GIBCO Enterprises Canada
FBS	GIBCO Enterprises , Canada
Antibiotics: Penicillin-streptomycin	EnterpEnterprisesnterprises, Canada
Trypsin-EDTA	GIBCO Enterprises Canada
MTT	Sigma-Aldrich Company St. Louis, MO, USA
Oligonucleotide primers for BMP-2	Sigma-Aldrich Company St. Louis, MO, USA
ALP	Sigma-Aldrich Company St. Louis, MO, USA
Runx2	Sigma-Aldrich Company St. Louis, MO, USA
iScriptcDNA synthesis kit	Bio-Rad, USA
KAPA SYBR® FAST PCR master mix kit	Kapa Biosystems, USA

DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum, trypsin-EDTA: Trypsin Ethylene Diamine Tetra Acetic Acid, MTT: (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide); BMP-2: Bone Morphogenic Protein -2; ALP: Alkaline Phosphate; Runx2: Runt-Related Transcription Factor 2; PCR: polymerase chain reaction

Cell viability and proliferation were measured using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assays over specific time points. Osteogenic differentiation by qPCR gene expression was conducted using alkaline phosphatase (ALP), bone morphogenic protein-2 (BMP-2) and runt-related transcription factor 2 (Runx2) as osteogenic markers.

Statistical analysis

Using the IBM SPSS Statistics for Windows, Version 23.0 (released 2015; IBM Corp., Armonk, New York, United States), a one-way ANOVA was used for the statistical analysis in this study. Mean ± SD was used to report the results in the form of bar graphs.

4. Results

Cell Viability and Proliferation

The percentage of cell viability of osteoblast cells cultured on two different groups of implant screws: uncoated titanium micro screws (uncoated screws) -98% and hafnium oxide nanoparticle-coated (Hf-coated) titanium micro screws -100% cell viability after 24 hours. The bars show that both uncoated and hafnium-coated screws have similar levels of cell viability, around 100% (Figure 2).

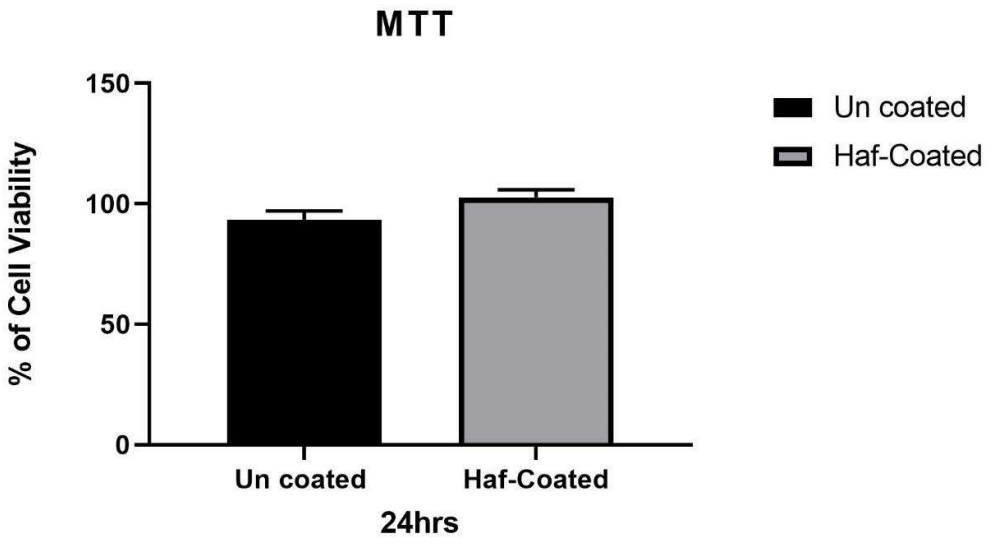

Figure 2: MTT Assay (Cell Viability) graph showing cytocompatibility of uncoated (black) and Hf-coated (Haf-coated) titanium micro screws (grey) on Osteoblast MG-63 cell line. Y-axis (Percentage of Cell Viability): This axis represents the viability of cells as a percentage, with 100% indicating normal cell viability. X-axis (implant micro screws): The graph compares uncoated and hafnium-coated implants.

Osteogenic Differentiation

The qPCR conducted with various osteogenic markers showed the relative mRNA expression for the uncoated and Hf-coated titanium micro screws. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control group for this testing.

BMP-2 (Bone Morphogenetic Protein 2): The hafnium-coated titanium micro screws show a significant increase in BMP-2 expression compared to the uncoated titanium screws.

ALP (Alkaline Phosphatase): ALP expression is slightly higher in the hafnium-coated micro screws than the uncoated titanium screws.

Runx2 (Runt-related transcription factor 2): Runx2 expression is also higher in the hafnium-coated group.

A graph comparing the expression levels of BMP-2, ALP, and Runx2 between uncoated and hafnium-coated titanium micro screws was plotted (Figure 3).

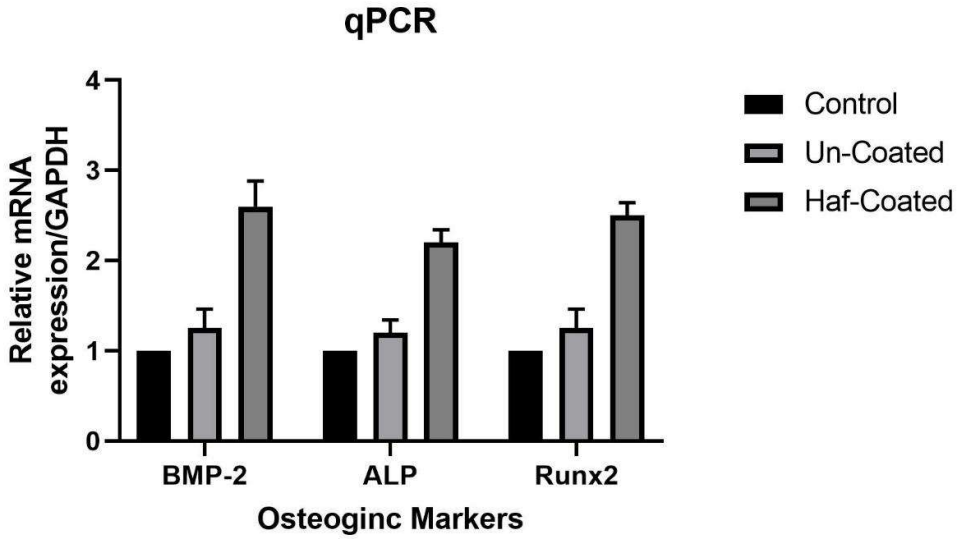

Figure 3: The above graph presents the

results from a quantitative PCR (qPCR) analysis, measuring the relative mRNA expression of three key osteogenic markers: BMP-2, ALP, and Runx2, normalized to GAPDH as a control (black) in uncoated titanium screws (light grey) and hafnium-coated titanium screws (dark grey). Y-axis (Relative mRNA Expression/GAPDH): This axis shows the level of mRNA expression relative to the housekeeping gene GAPDH, which serves as a control to normalize the data. X-axis (Osteogenic Markers: BMP-2, ALP, Runx2)

5. Discussion

The results of cell viability and proliferation of this research indicate that both groups of uncoated and hafnium oxide-coated titanium screws are biocompatible using the MTT assay and do not cause significant cytotoxicity to the MG-63 osteoblast cells after 24 hours of exposure. The MTT assay is a widely used colorimetric method for assessing cell viability, toxicity, and proliferation in various biological studies[18]. Its popularity stems from its simplicity, sensitivity, and the ability to assess a large number of samples simultaneously[18,19]. Furthermore, its validity is supported by extensive use in drug development and toxicological research, making it a standard method for evaluating cell responses to various treatments and conditions. The MTT reducing assay is considered the 'gold standard' for cytotoxicity testing[20].

The hafnium-coated titanium microscrews show a significant increase in BMP-2 expression, which showed better bone formation and differentiation of osteoblasts as compared to the uncoated titanium screws. ALP expression and Runx2 expression are slightly higher in the hafnium-coated micro screws than the uncoated titanium screws, which also cemented the same facts in the current research. Key markers for mRNA expression in the qPCR method, such as alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2), and Runt-related transcription factor 2 (Runx2), play crucial roles in the osteogenic pathway[21,22]. ALP is often considered an early marker of osteoblast activity, reflecting the mineralization process[23], while BMP-2 is essential for promoting osteogenic differentiation and bone formation[23]. Runx2 is a master regulator that initiates osteoblast differentiation and is vital for the expression of other osteogenic genes[23–25]. Focusing on these specific markers allows researchers to gain insights into the molecular mechanisms driving osteogenesis.

Quantitative PCR (qPCR) is a powerful technique for studying gene expression, particularly in the context of osteoblastic differentiation[2627]. The benefits of qPCR include its high sensitivity and specificity, enabling the detection of low abundance transcripts and facilitating the quantification of gene expression changes over time[28–30]. This makes qPCR a valuable tool for studying osteoblastic differentiation and understanding the complexities of bone biology[23,26,28–31]. The markers used in the current study in qPCR for mRNA expression provide a comprehensive view of the osteogenic process, allowing researchers to evaluate the effectiveness of therapeutic interventions in bone-related diseases, study developmental biology, and advance tissue engineering strategies. Their importance lies not only in basic research but also in clinical applications, where they can guide the development of treatments for osteoporosis, fractures, and other bone disorders.

The study has several limitations. First, it was conducted in vitro, necessitating further validation in vivo to confirm the efficacy and safety of hafnium-coated implants in living organisms. Additionally, the research focused exclusively on osteoblasts, and including other cell types, such as mesenchymal stem cells or fibroblasts, would provide a more comprehensive understanding of the material's biocompatibility. Furthermore, the assessment primarily examined short-term outcomes, highlighting the need for long-term studies to evaluate the durability and stability of the hafnium coating over extended periods. Lastly, the controlled in vitro conditions may not accurately represent the complex physiological environment in vivo, indicating that further research is required to assess the coating's performance under varying biological conditions.

The improved osteogenic response observed with hafnium-coated implants indicates their potential to accelerate bone regeneration and enhance osseointegration, which are critical factors for the success of orthopedic and dental implants. These results support the continued development and clinical evaluation of hafnium coatings as a surface modification strategy for titanium implants, with the goal of improving patient outcomes by reducing implant failure rates and promoting faster recovery times. Further, in vivo studies and long-term assessments will be necessary to fully establish the clinical benefits of hafnium-coated titanium implants.

6. Conclusion

The findings of this study demonstrate that hafnium-coated titanium implants exhibit promising osteogenic activity in osteoblast cells. The specialised hafnium oxide nanoparticle coating enhanced surface roughness, which improved cell adhesion, proliferation, and differentiation. It not only maintains cell viability comparable to uncoated titanium but also significantly leads to an upregulation of key osteogenic markers (BMP-2, ALP, and Runx2). Consequently, this suggested that the coating enhances the osteogenic potential of the implants, potentially improving bone formation and implant integration.

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Conflict Of Interest

The authors declare no conflict of interest.

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