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Surface-functionalized Titanium Implant with Gold Nanoparticles on Osteogenic and Antimicrobial Behavior: A Novel Preliminary Research on mg 63 Cell Line

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The enhancement of implant surfaces to promote better osseointegration and reduce infection risks is critical in biomedical applications. Gold nanoparticles (GNP) have shown promise in improving the biological performance of implant materials. This study explores the effects of GNP-coated titanium surfaces on the proliferation, differentiation, and mineralization of MG63 osteoblast-like cells. Titanium discs were coated with gold nanoparticles synthesized using a green, environmentally friendly method. The coated surfaces were characterized using SEM, EDAX, and SPR techniques to confirm the presence and stability of the GNP. MG63 cells were cultured on these GNP-coated titanium discs, and their growth, proliferation, and differentiation were assessed using fluorescence microscopy, Von Kossa staining, vinculin focal adhesion proteins, and alkaline phosphatase (ALP) activity measurements. The antimicrobial efficacy of the GNP-coated surfaces was also evaluated against Staphylococcus aureus and Escherichia coli. Titanium surfaces coated with (GNP) at 5 ppm significantly enhanced the growth and differentiation of MG63 cells, with no evidence of acute cytotoxicity. The GNP-coated surfaces facilitated improved cell attachment, proliferation, and mineralization, indicating strong osteogenic potential. Additionally, the GNP-coated surfaces exhibited notable antimicrobial efficacy, MICrecorded at 0.3135 mg/cm³ for Staphylococcus aureus and 0.2915 mg/cm³ for Escherichia coli. Gold nanoparticle coatings at a concentration of 5 ppm significantly improve the osteogenic potential of titanium surfaces while also providing antimicrobial protection. These findings suggest that GNP-coated titanium implants could offer a dual benefit of enhanced osseointegration and infection prevention, making them a promising option for future clinical applications. Further in vivo studies are necessary to validate these results and understand the broader implications of GNP in biomedical implants.

Keywords: Antimicrobial nanoparticles; Gold nanoparticles; Implant surface treatment; Osteogenic activity; Osseointegration.

Implant dentistry has emerged as a widely accepted fixed prosthetic solution for replacing missing teeth, where achieving direct contact between the implant surface and bone is crucial for long-term success [1]. Studies have shown that the topographical features of implant surfaces significantly influence bone cell behaviour. Consequently, various modification technologies,

have been developed to enhance the rate of bone-to-implant integration [2, 3].

A major challenge in implant dentistry Peri-implantitis is a primary factor contributing to implant failure. Roughly 30.7%. of implants are affected by peri-implant mucositis, while 9.6% are impacted by peri-implantitis [4]. To address this issue, bioactive antimicrobial molecules are being reintroduced into implant biomaterials to influence cell behaviour and reduce the risk of infection (5). Additionally, nanoparticle coatings on implant surfaces have shown the potential to lower implant failure rates by providing antimicrobial and osteoconductive properties [5].

GNP have garnered significant attention due to their diverse applications in drug delivery, biological imaging, and therapeutic interventions. Evidence suggests that GNP exhibit both osteogenic and antibacterial properties, making them promising candidates for enhancing implant surfaces[6-25]. This research assesses the osteogenic differentiation and antimicrobial effects of titanium surfaces modified with GNP, using the human osteosarcoma (MG-63) cell line as a model. The GNP in this study were synthesized using green chemistry methods, which involve plant extracts as reducing agents and stabilizers. This approach is both cost-effective and environmentally friendly, producing biocompatible nanoparticles without harmful byproducts [26-30].

The functionalization of titanium surfaces with GNP is intended to improve osseointegration, leading to faster and more predictable bone formation, enhanced implant stability, and bactericidal properties [5].

As far as we are aware, this work represents the initial exploration into the impact of GNP-functionalized implant surfaces prepared through green synthesis. Utilising botanical extracts for nanoparticle, minimizes the use of hazardous chemicals and waste, offering a sustainable alternative within green chemistry [31-37].

MATERIALS AND METHODS

GNP Preparation and Analysis

Genuine Pterocarpus marsupium wood(PM) (sample NO: RMRC922 - ICMR) and Sigma-Aldrich, India gold(III) chloride were bought to synthesize (GNPs), 1 mL of an aqueous extract of PM heartwood was introduced into a 10 mL gold solution (III) chloride (0.5 mM) at 40ºC. A noticeable colour shift suggested GNP production.

The GNPs were then centrifuged at 17,000 RPM and the sample was centrifuged at a temperature of 4ºC for 20 minutes using a Kubota 6500 centrifuge.

The resultant GNP were cleaned and suspended. The GNP size, polydispersity index, and zeta potential were determined using a Malvern Instruments Zeta Seizer. Additionally, the samples were analysed using Transmission Electron Microscopy (TEM) with a Hitachi H-7500 microscope. The GNPs seemed uniform, and colloidal and had a diameter of approximately 20 nm. They showed polydispersity but did not experience any sedimentation .[38]

Titanium disc prep

Titanium alloy sample preparation for experimentation

Titanium alloy (Type IV Ti) was obtained from Munani Metals. Standardized discs were made that are 10 mm across and 3 mm thick. These discs were divided into two subgroups (n=20): Group A, consisting of plain titanium discs, and Group B, consisting of titanium discs treated with a 5 ppm concentration of gold nanoparticles. Every aspect of the sample and technique was assessed by an impartial statistician

Methodology for Assessing Surface Roughness

First, a steam cleaner with 0.3 MPa pressure was used to clean the representatives from every group. Next, they were cleaned with ultrasonic waves to remove any residual particles on the surface. After drying, The texture of the outer layer of all specimens was assessed both quantitatively and qualitatively. To do quantitative analysis, we utilised (Surtronic S-128 – Taylor Hobson) to assess the average roughness character (Ra) for every sample.

Green-synthesized GNP was applied layer by layer to Group B (Test). Group A (Control) and Group B (Test) The discs were subsequently analysed using scanning electron microscopy (SEM) [39].

Cell culture

The osteosarcoma MG63 cell strain (CRL-1427 - ATCC), sourced from NCCS Pune, India, was cultured in DMEM, and bought from Sigma-Aldrich Co.

The medium was enriched with essential growth factors and nutrients to promote cells with Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich Co.) with 5% CO2, 95% air, 100% relative humidity, 10% foetal bovine serum (FBS Gibco), and 2 mN glutamine.

For the experimental methods in both Group B (test) and Group A (control), a precise concentration of 1 × 10u cells per well of titanium discs containing MG63 cells was meticulously planted. The injected cells were nurtured for 72 hours at a constant temperature of 37°C, with 5% CO2 and 95% air,100% relative humidity .

MTT test assay

The MTT assay was employed to assess the growth of MG63 cells on titanium surfaces in group A and group B MG63 cells, which were cultured in three separate samples and kept in an incubator for 72 hours. Each well was filled with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for evaluating cellular biochemical assignment with 50 ìL of reagent and cultured for another four hours following the incubation time. The obtained formazan crystals were dissolved in isopropanol and kept for 30 minutes. The OD was measured by using spectrophotometry at a wavelength of 492 nm[40] .

The ALP Assay

The ALP Assay was conducted using MG63 cells growing in triplicate. In the culture process, the activity of ALP was evaluated by introducing p-nitrophenyl phosphate. The concentration of resultant p-nitrophenol was then quantified using Bio-Tek equipment at a wavelength of 405 nm by spectrophotometry.

Antimicrobial Activity

The antimicrobial efficacy of green-synthesized GNP was assessed using the MIC method. Serial dilutions of the GNP were prepared to determine the MIC against (*Staphylococcus aureus*,) and (*Escherichia coli*) bacteria. The bacterial cultures were maintained at 10u -10v CFU/ml and incubated. The MIC of GNP against (S. aureus) and (E. coli) was measured using a spectrophotometer at different GNP concentrations.

Evaluation of MG63 cellular proliferation

The proliferation and activity of MG63 cells on both uncoated titanium discs (GROUP A) and titanium discs functionalised with gold nanoparticles (GROUP B) were assessed using acridine orange (AO) staining. An acridine orange treatment was performed after the cells were immobilised. The stained samples were examined and analysed using a FluoView Confocal Microscope (FV1000; Olympus) to evaluate the shape and arrangement of cells on the titanium surfaces.

Von Kossa staining

Von Kossa staining was conducted on MG63 cells after they were cultured for fourteen days. The cells were initially fixed for ten minutes, followed by dehydration through a graded series of ethanol concentrations, ranging from 70% to 100%.

After dehydration, the samples were rehydrated and treated with 2% silver nitrate. They were then exposed to direct sunlight for 20 minutes to allow for the precipitation of calcium salts. Following this, the samples were treated with 5% sodium thiosulfate for 3 minutes to remove unreacted silver, and an acid fuchsine counterstain was applied for 5 minutes to enhance contrast. The samples were then dried and prepared for image analysis.

Vinculin immunofluorescence staining

Vinculin immunofluorescence staining, enhanced by GNP, is employed to investigate cell adhesion and cytoskeletal organization. Vinculin plays a critical role in cell adhesion, securing cells to the cellular scaffold To assess the influence of GNP on attachment factors., MG-63 cells were subjected to an overnight incubation. Subsequently, The cells were treated with GNP and incubated for five days.

Post-treatment, the cells were fixed, cells were fixed and then cleaned with PBS and made permeable. Following permeabilization, the cells were incubated for 30 minutes with 1% bovine serum albumin (BSA) in PBS. The cells were then exposed overnight at 4–8°C to primary antibodies against diluted vinculin. The cells were treated with fluorescently marked antibodies for 1 hour upon cleaning with PBS containing 0.05% Tween. Vinculin expression was then assessed using fluorescence microscopy.

Analysis biostatistics

An impartial statistician examined the data and provided the M ± SD. The assessment was carried out with SPSS 22.0. ANOVA was used to find significant differences in all measured indices,

and the Dunnett test (for multiple comparisons) compared GROUP A and GROUP B. A p-value less than 0.001 indicates the significance of the statistic.

RESULTS AND DISCUSSION

Due to their biocompatibility, corrosion resistance and desirable mechanical properties, titanium and its alloys have been the commonly used material for orthopaedic and dental implants over several decades[5]. However, these surface characteristics making titanium implants biocompatible also lead to bacterial colonisation and biofilm formation, increasing the risk of implant-associated infections. These injuries represent a significant source of infection, which most commonly ultimately leads to implant failure and costly patient procedures[6]. Conventional surface treatments for titanium implants emphasise passivation or re-passivation of the material to facilitate biocompatibility through the induction of a stable, adhering titanium oxide layer. Whilst this layer is inadequate in its own right to prevent bacterial adhesion and subsequent biofilm formation that could lead to a loss of long-term implant efficacy, this protective layer is used, in conjunction with other treatments that reduce reactive oxygen species and enhance fluid overload protection [7-9,37].

The surface attributes of implants can be enhanced by nanotechnology, especially the use of nanoparticles[37], GNP are distinguished because of their biological compatibility. Gold nanoparticles (GNP) were synthesized using a green synthesis method, which is recognized for its simplicity, cost-effectiveness, and environmentally friendly nature as it avoids producing harmful by-products commonly associated with synthetic approaches [33]. Phytochemical analysis of the plant extract indicated the successful synthesis of GNP, with characteristic peaks observed at 2.10 and 9.50, confirming the presence of pure gold.

The Surface Plasmon Resonance (SPR) observed at 538 nm signalled the formation of primarily spherical GNP. These particles exhibited a negative charge with a zeta potential of -27.8 ± 1.23 mV, which suggests good stability due to electrostatic repulsion, thereby preventing aggregation [37].

Scanning Electron Microscopy (SEM) of the uncoated titanium discs revealed the existence of several concentric ridges with smooth and randomly distributed edges (Fig-1).

In contrast, the SEM analysis of GNP-coated titanium discs displayed a uniform

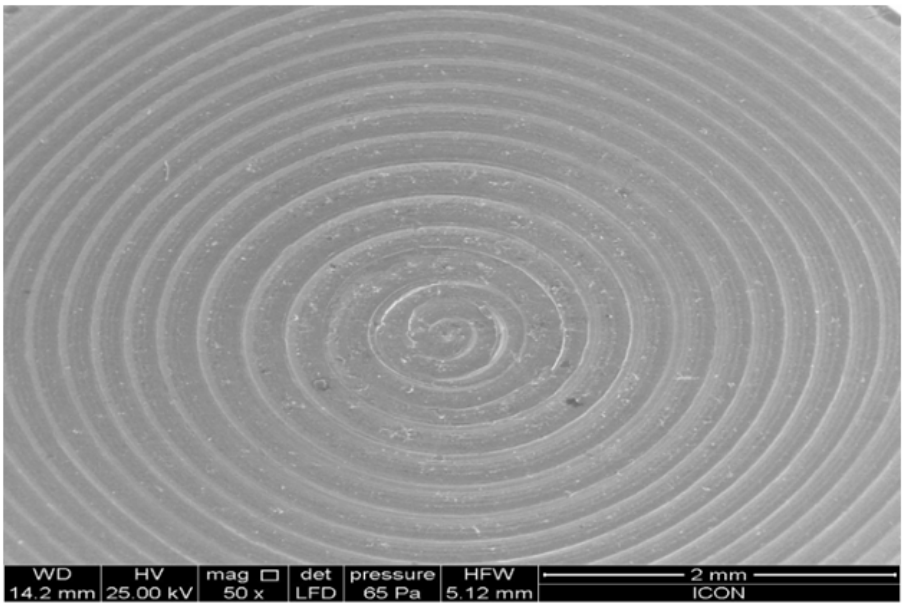

Fig. 1. SEM image of the uncoated commercially pure titanium disc

distribution of nanoparticle clusters across the surface, signifying successful GNP deposition (Fig 2 and 3).

The elemental analysis further confirmed the integration of GNPs into the titanium surface (Figure 4). The GNP were spherical with an approximate diameter of 20 nm. Prior studies by C.J. Murphy have shown that spherical GNPs of this size are non-cytotoxic to human cells [41].

In this investigation, we employed GNP with a diameter of 20 nm, characterized by a spherical shape, negative surface charge, and a concentration of 5 ppm. This choice was guided by prior studies evaluating the impact of particle size, surface properties, and modifications on GNP [41, 42]. The primary objectives were to examine how titanium discs coated with these GNP affect the proliferation of MG63 cells and to evaluate the antimicrobial effectiveness of the GNP.

Assessment of Gold Nanoparticle-Enhanced Titanium Discs on Bone Cell

The MG63 cell line was employed to evaluate the osteogenic activity of GNP. Using acridine orange staining, we observed that MG63 cells on GNP-coated titanium discs GROUP B (5 ppm concentration) exhibited increased attachment, proliferation, and density, alongside improved spreading and enhanced cell-to-substrate contact (Fig-5). Conversely, the control group GROUP A showed more spherical cells on the uncoated titanium discs (Fig-6).

A comparative study of MG63 cell proliferation revealed a significant increase in cell viability in the GNP-treated group, reaching 104.4% at 24 hours, compared to 41% viability

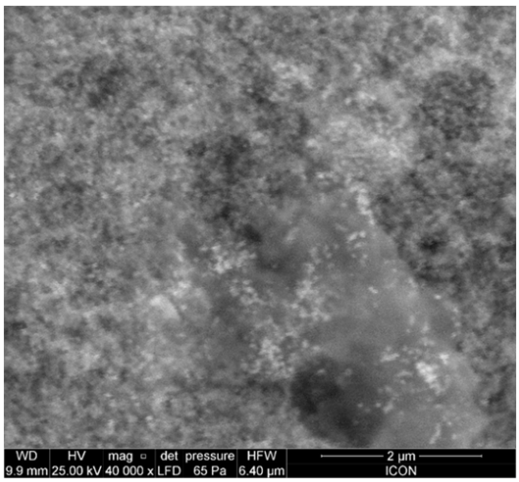

Fig. 2. SEM image of gold nanoparticles coated titanium disc surfaces

Fig. 3. SEM image of gold nanoparticles

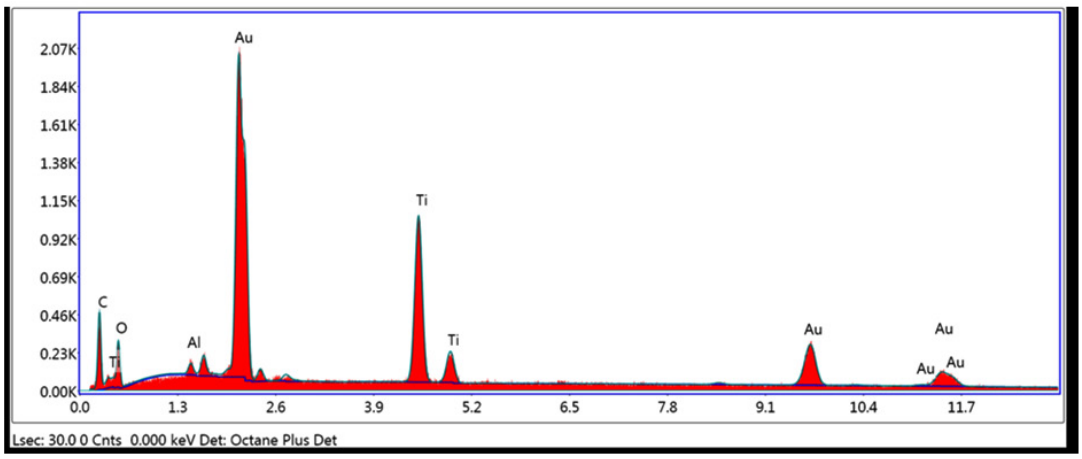

Fig. 4. EDAX of surface-treated titanium disc

in the GROUP A control group (Table I). This trend persisted, with the GROUP B GNP-treated group maintaining a viability of 107.4% after 72 hours (Table II and Graph Fig-7). These results highlight the osteogenic potential of GNP, enabling sustained cell viability and proliferation over extended periods. The specific cellular response to this concentration of GNP has not been widely documented, making it a notable finding in this research.

ALP activity and Von Kossa staining were used to detect early markers of osteoblastic activity, bone remodelling, and turnover. After 14 days of treatment with 5 ppm GNP, Von Kossa staining confirmed the presence of mineral deposits in MG63 cells (Fig- 8,9).

ALP activity in the GROUP B GNP-treated cells proliferation was higher (0.23 ± 0.005) compared to the GROUP A control group (0.22 ± 0.007), with a P-value of < 0.001 (Table III). These results corroborate previous studies on osteoblast differentiation and mineralization, reaffirming the osteogenic potential of GNPs [31-35].

Further analysis focused on the role of GNPs in altering the composition and organisation of focal attachment proteins, particularly vinculin.

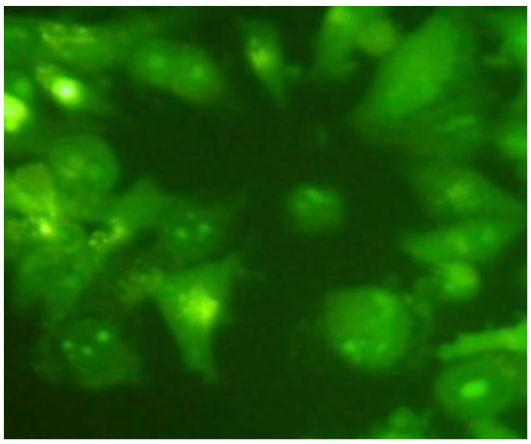

Fig. 5. Acridine orange image of MG63cells at 5 ppm concentration of gold nanoparticles

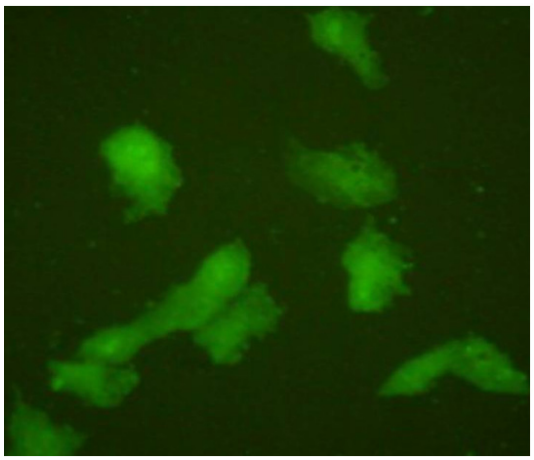

Fig. 6. Acridine orange image of MG63cells (control group).

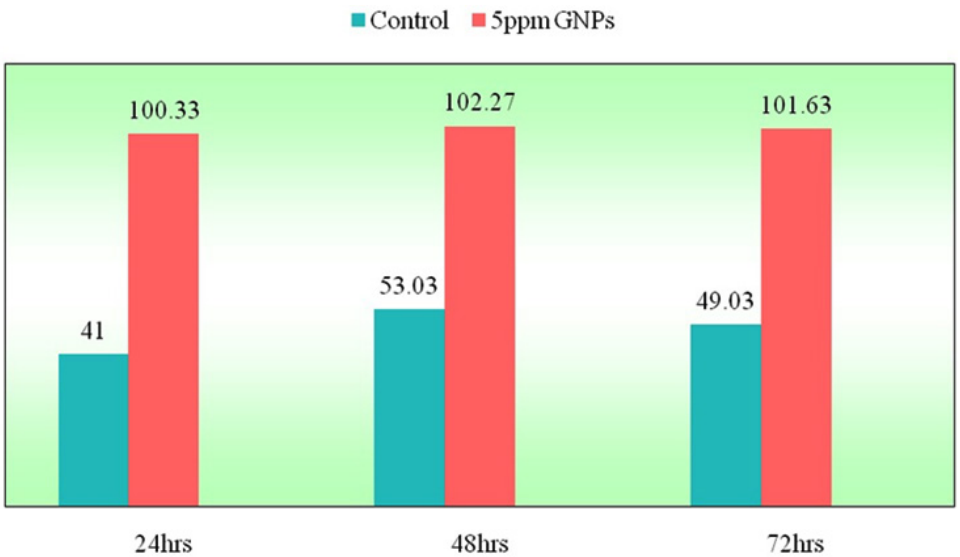

Fig. 7. Graph showing MG 63 cell proliferation comparison at different time intervals.

Vinculin is crucial for stabilizing focal adhesion complexes by binding to talin, which links integrins to actin filaments. The study demonstrated that cells treated with GNPs showed significantly enhanced vinculin expression and organisation compared to the control group, indicating that GNP may improve cell adhesion by stabilizing these complexes (Fig 10 and 11).

This enhancement in focal adhesion is closely linked to cellular differentiation, which can contribute to improved implant stability and integration. The results suggest that GNP not only elevate vinculin levels but also alters its arrangement, potentially affecting focal adhesion dynamics in MG-63 cells [43-46].

Minimum Inhibitory Concentration (MIC)

This research explored the antimicrobial efficacy of GNP-coated titanium discs against Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria while also examining their impact on osteoblast proliferation, differentiation, and mineralization. MIC testing demonstrated that 20 nm GNP possess significant antimicrobial properties, with MIC values determined at 0.3135 mg/cm³ for S. aureus and 0.2915 mg/cm³ for E. coli. These findings are consistent with previous research that has highlighted the bactericidal nature of GNP [17-19]. Furthermore, the study supports the osteogenic potential of GNP, as documented in earlier research [26-35]. This investigation confirms the

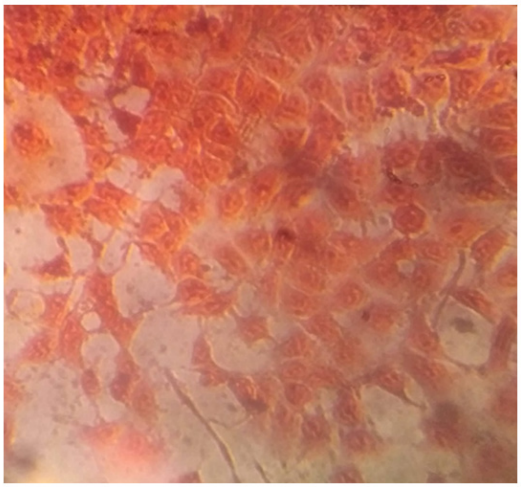

Fig. 8. Von Kossa staining of MG63cells (control group)

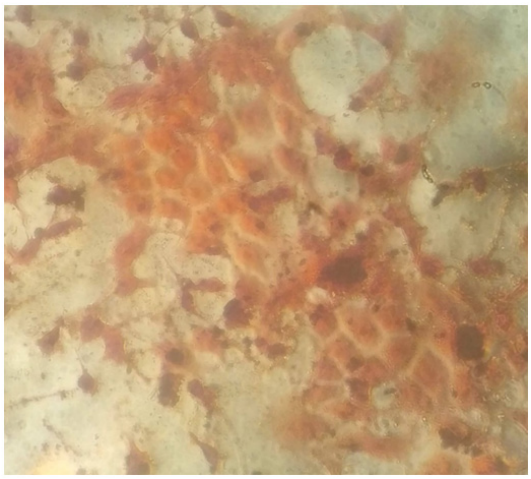

Fig. 9. Von Kossa staining of MG63cells treated with 5ppm of gold nanoparticles.

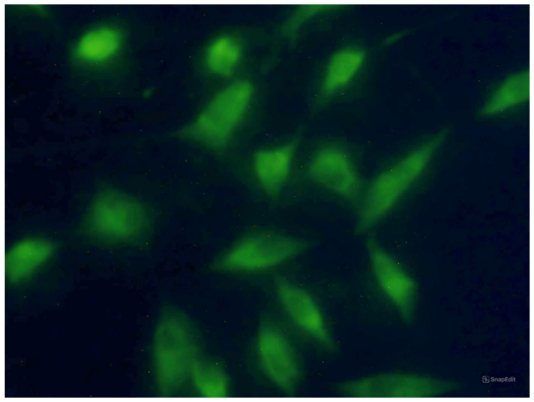

Fig. 10. Focal adhesion proteins vinculin expression (Control).

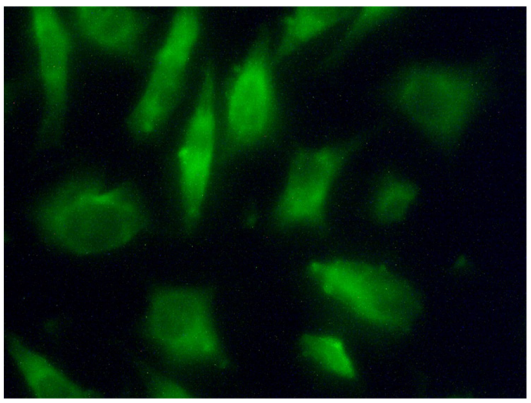

Fig. 11. Focal adhesion proteins vinculin expression in the presence of gold nanoparticles

Table 1. Percentages of proliferation rates in different time periods at different concentrations

Control	24 hrs	48 hrs	72 hrs
5PPM GNPs	46%	54%	57%
	104.4%	109.7%	107.4%

biological compatibility, bone formation potential, and antibacterial capabilities of GNP-coated titanium discs.

Study Constraints

The data could provide for further alternative hypotheses and interpretations of the research. Here are some potential areas to consider.

Table 2. Comparison of groups at different time points ANOVA

Time points	Groups	Mean	SD	Mean rank	H-value	P-value
24hrs	Control	41.00	7.00	2.00	7.2000	0.0270*
	5ppm GNPs	100.33	3.72	8.00		
48hrs	Control	53.03	6.50	2.00	7.2000	0.0270*
	5ppm GNPs	102.27	6.48	8.00		
72hrs	Control	49.03	12.11	2.00	7.2610	0.0270*
	5ppm GNPs	101.63	5.20	8.00		

Table 3. Comparison of groups at different time points ANOVA

Group (alkaline phosphatase)	Mean & standard division
1. Loaded 5ppm	0.23+/-0.005
2. Control	0.22+/-0.005

Particle size influence: The study was carried out with 20 nm GNPs, but other particle sizes may result in other observations. The effect of these small or large particles on cell behaviour and antimicrobial properties may be distinct.

Potential systemic effects: The study is limited to local effects, but as GNPs can enter the bloodstream, systemic effects must also be considered. It could be a potential interaction with the immune system or getting stuck in other organs.

Investigate alternative coating methods

A layer-by-layer specific method to coat titanium discs with GNPs was used in the current study for further research on how the coating might affect the distribution, stability, and efficacy of the GNP layer.

Address long-term stability and effectiveness

Consider hypothesizing about the long-term stability of the GNP coating and its potential impact on implant performance over extended periods.

Addressing these alternative hypotheses and adequate interpretations would constitute a more complete analysis as the implant tissue interface is complex and the implant success is multi-factorial. This approach would also be useful for the identification of possible areas for future research and to highlight several complexities associated with the development of improved implant surfaces.

This study's findings are encouraging, yet it has constraints. The antimicrobial tests were conducted with GNP in suspension, which may not entirely reflect the behaviour of GNP when adhered to a surface. Future research should include antimicrobial testing on both coated and uncoated titanium samples. Additionally, more in-depth studies are required to explore the in vivo osteogenic differentiation process and evaluate the long-term biocompatibility and effectiveness of GNP-coated implants.

Clinical Implications of the Study

Microbial contamination and infection pose significant challenges in implantology, often leading to implant failure. The localized antimicrobial properties of GNP, combined with their ability to enhance osteogenesis, make them a promising candidate for surface modification of titanium implants. By reducing infection risks and promoting better integration with bone tissue, GNP-coated implants could lead to improved

clinical outcomes and longevity. This study offers useful information that may be used to enhance the safety and more effective implant materials, with GNP playing a crucial role in advancing biomedical implant technology.

CONCLUSION

Through this study, we show that coating titanium implants with gold nanoparticle (GNP) enables significant improvement to both osteogenic activity and antimicrobial effect. We also generated 20 nm spherical nGNPs through a green process that resulted in strong surface properties that favor the MG63 cell attachment, proliferation and differentiation. It observed these effects along with preferred focal adhesion dynamics such as increased vinculin expression and organization. Moreover, the antibacterial activity of the GNP coated titanium discs was effective against S. aureus and E. coli and could be used as a strong MIC value (indicating strong inhibition of microbial growth).

These findings highlight the potential of GNP coatings as a dual-functionality surface treatment for titanium implants, addressing two critical challenges in implantology: Osteointegration enhancement and infection prevention. The capacity of GNPs to either induce localized antimicrobial effects and promote bone cell growth simultaneously suggests that future development of such composite GNPs due to a more stable and long lasting implant has benefits to the reduction of implant failure and thereby improved patient outcomes.

Inaive future research should focus on in vivo validation of these results, in particular soon to assess whether GNPs coated implants are biocompatible for long term use, osteogenic differentiating and antimicrobial properties. In general, the integration of GNPs into titanium surface yields a promising advance in biomedical implant technology, increasing the safety, efficacy and clinical longevity of titanium base implants.

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Conflict of Interest

The author(s) do not have any conflict of interest

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

Authors' Contribution

Dr. Santosh Nelogi made substantial contributions to conception and design of the study, drafting the manuscript and revising it critically and have given final approval of the version to be published; Dr.Kiran Kaur have been involved in substantial contributions data collection and data analysis; Dr.Prashant Kani have been involved in data collection and data analysis; Dr Amit Porwal have been involved in data collection and data analysis; Arvind Halgekar have been involved in data collection and data analysis; Varky Santosh involved in data interpretation

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