JURNAL ILMIAH MEDICAMENTO

Vol.11 No.2 (September 2025)
page 163-171
https://doi.org/10.36733/medicamento.v11i2.11435
e-ISSN: 2356-4814

Original Article

Proliferation of Nano Chitosan and Platelet Rich Plasma from Pre-osteoblast Cell with Ki67 as a Surrogate Marker in Vitro

Proliferasi Nano Kitosan dan *Platelet Rich Plasma* dari Sel *Pre-Osteoblast* dengan Ki67 sebagai Pengganti *Marker In Vitro*

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Submitted : 08-04-2025
Reviewed : 08-07-2025
Accepted : 20-09-2025

Keywords: hydroxyapatite, nano-chitosan, pre-osteoblast, proliferation, PRP.

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Abstract

This study investigated the effect of nano-chitosan mix with platelet rich plasma (PRP) to proliferation rate of pre-osteoblast cell with incubation time used in vitro culture system. The culture media of pre-osteoblast cell MC3T3-E1 used alpha-MEM, 2mm L-glutamine, 1mm sodium pyruvate, 10% FBS and 10% pen strep in 25cm^2 flask bottle and incubated in an incubator with 5% CO_2 at a temperature of 37^{o}C until the cell was confluent 70-80% and planting in well-24 to give treatments. Treatment was divided into two groups, nano-chitosan+PRP and hydroxyapatite+PRP. The proliferation of pre-osteoblast cells saw with immunocytochemical staining and proliferation of cells were counted and investigated with confocal laser scanning microscope (CLSM). The normality of sample data was analyzed with Shapiro-Wilk. Comparison test used independent sample t-test and one-way ANOVA (F-test). All data were analyzed with SPSS software. The experiment results showed that nano-chitosan+PRP can accelerate proliferation than hydroxyapatite+PRP of 0% and 10% concentrations. The independent sample t-test showed there were a significant difference ($p=0.010<\propto$) from proliferation rate mean (0%) between treatment group nano-chitosan+PRP (1076.3±176.4au) and treatment group hydroxyapatite+PRP (659.5±272.7au) on five days incubation time, and proliferation mean (10%) between treatment group of nano-chitosan+PRP (710.3±109.7au) and hydroxyapatite+PRP (581.8±76.4au) on seven days incubation time. Based on proliferation mean (0%) and (10%), the treatment group of nano-chitosan+PRP with five- and seven-days incubation have higher mean than 0% and 10% on treatment group nano-chitosan and PRP and can accelerate bone healing with incubation time of five and seven days compared to treatment group of hydroxyapatite+PRP.

Kata Kunci: hydroksiapatit, *nano-chitosan*, proliferasi, PRP, sel preosteoblas.

Abstrak

Penelitian ini bertujuan untuk menyelidiki efek campuran nano-chitosan dengan plasma kaya platelet (PRP) terhadap laju proliferasi sel pre-osteoblast dalam sistem kultur in vitro dengan waktu inkubasi yang digunakan. Medium kultur sel pre-osteoblast MC3T3-E1 menggunakan alpha-MEM, 2 mm L-glutamin, 1 mm natrium piruvat, 10% FBS, dan 10% penisilin-streptomisin dalam botol flask 25 cm^2 dan diinkubasi dalam inkubator dengan 5% CO_2 pada suhu 37°C hingga sel mencapai kepadatan 70-80% dan ditanam dalam plate 24 sumur untuk pemberian perlakuan. Perlakuan dibagi menjadi dua kelompok, nano-chitosan+PRP dan hydroxyapatite+PRP. Proliferasi sel pre-osteoblast diamati dengan pewarnaan imunositokimia, dan jumlah sel yang berkembang biak dihitung dan dianalisis menggunakan mikroskop laser pemindaian konfokal (CLSM). Normalitas data sampel dianalisis dengan uji Shapiro-Wilk. Uji perbandingan menggunakan uji t sampel independen dan ANOVA satu arah (uji F). Semua data dianalisis menggunakan perangkat lunak SPSS. Hasil eksperimen menunjukkan bahwa nano-chitosan+PRP dapat mempercepat proliferasi dibandingkan dengan hidroksiapatit+PRP pada konsentrasi 0% dan 10%. Uji t sampel independen menunjukkan adanya perbedaan yang signifikan ($p=0.010<\alpha$) antara rata-rata laju proliferasi (0%) pada kelompok perlakuan nano-chitosan+PRP (1076.3±176.4au) dan kelompok perlakuan hidroksiapatit+PRP (659,5±272,7au) pada waktu inkubasi lima hari, serta rata-rata proliferasi (10%) antara kelompok perlakuan nano-chitosan+PRP (710,3±109,7au) dan hidroksiapatit+PRP (581,8±76,4au) pada waktu inkubasi tujuh hari. Berdasarkan rata-rata proliferasi (0%) dan (10%), kelompok perlakuan nano-chitosan+PRP dengan waktu inkubasi lima dan tujuh hari memiliki rata-rata yang lebih tinggi daripada 0% dan 10% pada kelompok perlakuan nano-chitosan dan PRP, dan dapat mempercepat penyembuhan tulang dengan waktu inkubasi lima dan tujuh hari dibandingkan dengan kelompok perlakuan hidroksiapatit+PRP.

How to cite: (citation style AMA 11th Ed.)
Wedagama DM. Proliferation of Nano Chitosan and Platelet Rich Plasma from Pre-osteoblast Cell with Ki67 as a Surrogate Marker in Vitro. *J. Ilm. Medicam.*, 2025:11(2), 163-171, DOI: 10.36733/medicamento.v11i2.11435

INTRODUCTION

Lately, many medical advancements are being made to aid bond defects caused by tumor re-sectioning of the bone, apex re-sectioning of tooth or bone caused by trauma. Room forming on surgical method required bone healing (bone remodeling).[1] To accelerate the process of bone healing, a bone graft material is needed which is biocompatible and made from natural chitosan. It is capable of acting as a physical barrier and serves in the osteo-conduction, osteo-induction, and osteogenesis.[2–4] Nano chitosan is a biopolymer which is biocompatible, biodegradable and anti-microorganism that can accelerate bone formation and wound healing through the properties of smaller particles, namely nano particles, where nano particles are smart materials that easily adapt to tissue.[5,6] The platelet-rich plasma (PRP) on nano chitosan as a scaffold is expected to function as the delivery vehicle for cells.[7] It also regulates the process of bone healing by acting as an extracellular matrix and maintain the space and shape of the bone defect remodeling. Thus, Hydroxyapatite (HA) is the crystal-like compound that acts as a scaffold.[8–10] Hypothetically, it is found out that nano chitosan plus a platelet-rich plasma (PRP) can accelerate proliferation compared with hydroxyapatite plus a platelet-rich plasma (PRP). For that reason, the process of bone healing by nano chitosan plus platelet-rich plasma (PRP) treatment is preferred.[11,12]

Modeling and Bone Healing

Bone Modeling is a term used to describe changes in bone structure during skeleton formation, growth and maturation.[13] Modeling leads to the process of changing the size and shape of bones which happens until the end of puberty, but the increase of density still occurs until four decades.[8] Medium regeneration of bone healing is a process that occurs continuously by replacing the old bone with new ones.[3,14] The place where remodeling occurred are termed basic multicellular remodeling units (BMus). It occurs between two to eight weeks as the formation of bone takes longer than the bone resorption. The remodeling process occurs since bone growth until the end of life.[10] The remodeling process includes two activities namely: bone resorption followed by bone formation process, the first process known as osteoclast activity while the latter known as osteoblast activity.[4,9,15] The remodeling process involves two main cell, osteoblasts and osteoclasts, and both are derived from marrow cells bone (bone marrow).[16] Osteoblasts are derived from pluripotent mesenchymal stem cells which is the fibroblast colony-forming units (CFU-F).[17,18] On the other hand, osteoclasts are derived from hematopoietic stem cells that is granulocyte-macrophage of colony-forming units (CFU-GM).[9,14,19]

MATERIALS AND METHODS

Ethical approval for the use of experimental animals was obtained from the Ethics Committee of Mahasaraswati University (Approval No. 03.0019/KEP-Unmas/VIII/2025). The primary material used in this study was nano-chitosan derived from Nephropidae shells collected from the Bali Sea, processed into nanoparticles using ball milling techniques. Platelet-rich plasma (PRP) was prepared from blood donated by the research team, with support from the Indonesian Red Cross, Malang Branch. PRP was isolated via centrifugation. The Ki67 proliferation marker was sourced from mouse origin, and pre-osteoblast MC3T3-E1 cells (ATCC murine cell line) were cultured in standard growth medium.

Pre-osteoblasts were seeded in multi-well plates and allocated into four experimental groups to assess proliferative and osteogenic responses under different treatments: (1) nano-chitosan + PRP, (2) micro-chitosan + PRP, (3) bone graft + PRP, and (4) ascorbic acid (positive control for osteogenic differentiation). Each group was prepared in multiple replicates to ensure statistical reliability. Cultures were incubated for two time points: 5 and 7 days. After each incubation period, assays were performed to quantify cellular proliferation and differentiation. Proliferation was evaluated using a standardized protocol, with measurements taken under two conditions (0% and 10%). Differentiation was assessed to determine the maturation of pre-osteoblasts into osteoblasts. All quantitative data for proliferation and differentiation were expressed in arbitrary units (AU).

Pre-osteoblast Cell Culture

MC3T3-E1 pre-osteoblast cells (ATCC, murine cell line) were cultured in α-MEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin in 25 cm^2 culture flasks. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching 70–80% confluence. Upon confluence, cells were harvested and seeded into 24-well plates for experimental treatments. Platelet-rich plasma (PRP) was prepared from blood samples collected from the investigators, following standard centrifugation protocols, with assistance from the Indonesian Red Cross (PMI) Malang Branch. Pre-osteoblast cultures were treated with nano-chitosan and PRP and incubated for 5 or 7 days prior to analysis (**Figure 1**).

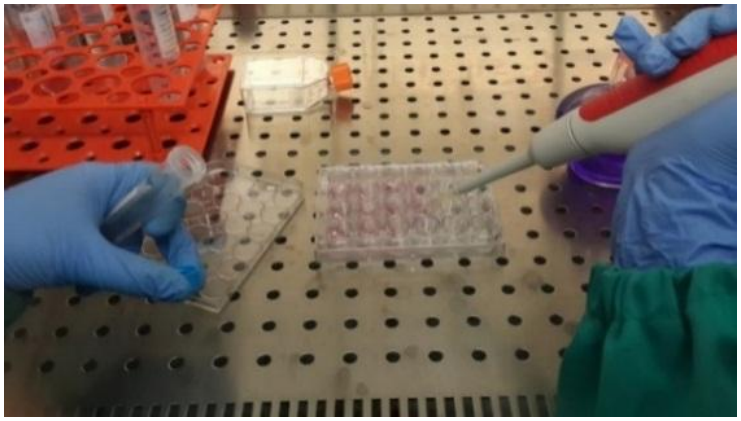

Figure 1. Platting Pre-Osteoblasts Cell Culture with Chitosan Nano Hydroxyapatite + + PRP and PRP

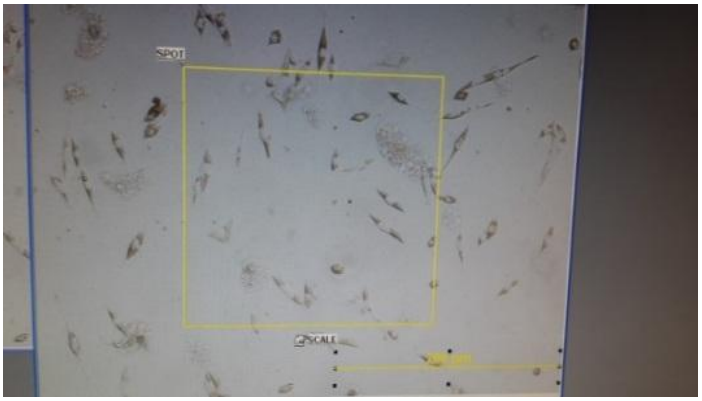

Figure 2. Growth Pre-Osteoblasts Cells after Incubation in an Incubator with an Electron Microscope (magnification 200µm)

Identification of Pre-osteoblast Cell Proliferation

Pre-osteoblasts proliferating cell staining were washed with PBS 3x10 min, incubation with 0.05 % Triton - X in PBS for 15 minutes. The color red ties Ki 67 primary antibody (mouse host) by administering anti-mouse secondary antibodies labeled rhodamine C. Proliferation of cells with test immunocytochemical was observed through a confocal laser scanning microscope (CLSM) (**Figure 2**).

Immunocytochemistry Featured

Immunocytochemistry was done to study the distribution of specific enzymes in the cell structure intact to normal or full band detect the cell component that is bio-macromolecules such as proteins, carbohydrates, among others. In this examination, it was done to see the proliferation of cell staining by pre-osteoblasts. Cell proliferation by immunocytochemistry was observed through a confocal laser scanning microscope (CLSM) (**Figure 3**).

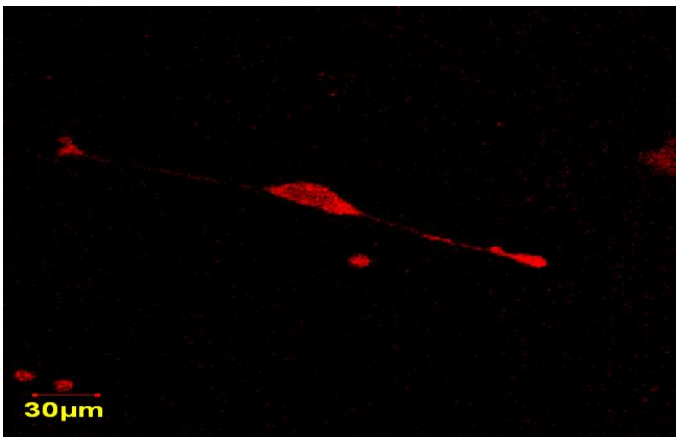

Figure 3. Overview of Pre-Osteoblasts Cell Proliferation in Immunocytochemistry*
*Note: the red Figure shows the absorption bonding primer Ki67 antibodies. It is with anti-mouse secondary antibodies labeled rhodamine C.

Data Analysis

Data analysis included six types of calculations. Normality of the sample data was assessed using the Shapiro–Wilk test. Group comparisons were performed using an independent samples t-test. All statistical analyses were conducted using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

Cellular proliferation of MC3T3-E1 pre-osteoblasts was quantified by Ki67 immunocytochemistry and expressed in arbitrary units (AU). Ki67 is a validated nuclear marker of cells in active phases of the cell cycle (G1, S, G2, M) and is absent in quiescent G0 cells, supporting its use for proliferation assessment in vitro.[20]

Table 1. Result of data normality test

Treatment Group	*p-value*	
	Proliferation (0%)	Proliferation (10%)
Nano Chitosan + PRP (5 days)	0.996	0.473
Nano Chitosan + PRP (7 days)	0.489*	0.867**
Hydroxyapatite + PRP (5 days)	0.122	0.620
Hydroxyapatite + PRP (7 days)	0.159	0.066

Interpretation: A p-value < 0.05 indicates that the data deviate significantly from a normal distribution, whereas a p-value > 0.05 suggests that the data are normally distributed.

The Shapiro–Wilk test indicated that the p-values for all observation groups were greater than the significance level (α = 0.05) (**Table 1**), confirming that the data met the assumptions of normality. Therefore, the dataset satisfied the prerequisites for parametric testing.

Table 2. Comparison results between incubation time of 0% proliferation

Treatment Group	Incubation Time		*p-value*
	5 days	7days	(t test)
Nano Chitosan + PRP	1076.3±176.4**	374.6±72.9	0.000 <∝
Hydroxyapatite + PRP	659.5±272.7*	368.7±95.8	0.033 <∝

Interpretation: A p-value < 0.05 indicates a statistically significant difference between groups, whereas a p-value > 0.05 indicates no statistically significant difference.

The independent samples t-test revealed a statistically significant difference ($p < 0.001$) in mean proliferation (0%) for the nano-chitosan + PRP treatment group between the two incubation periods: 5 days (1076.3 ± 176.4 AU) and 7 days (374.6 ± 72.9 AU). In contrast, the hydroxyapatite + PRP group showed no

statistically significant difference (p = 0.033 > α) between 5 days (659.5 ± 272.7 AU) and 7 days (368.7 ± 95.8 AU) (**Table 2**).

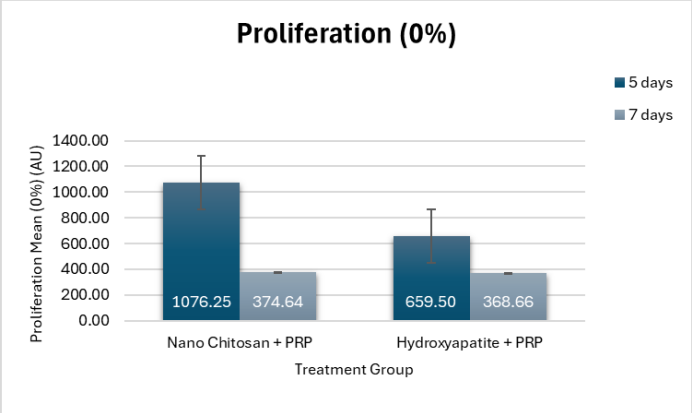

Figure 4. Histogram of Mean Proliferation (0 %) (incubation five days and seven days)*

Interpretation: For the treatment groups (nano-chitosan + PRP and hydroxyapatite + PRP), means sharing the same letter indicates no statistically significant difference, whereas means with different letters indicate a statistically significant difference.

Figure 4 presents a histogram of mean proliferation (0%) after 5 and 7 days of incubation in two treatment groups. The highest proliferation was observed in the nano-chitosan + PRP group at 5 days (1076.3 ± 176.4 AU), which was significantly greater than all other groups.

Table 3. Results of the comparison between the time incubation on proliferation (10 %)

Treatment Group	Incubation Time		*p-value*
	5 days	7days	
Nano Chitosan + PRP	241.9±96.7	710.3±109.7**	0.000 <∝
Hydroxyapatite + PRP	396.4±74.1	581.8±76.4*	0.002 <∝

Interpretation: For the t-test, a p-value < 0.05 indicates a statistically significant difference between groups, whereas a p-value > 0.05 indicates no statistically significant difference.

The independent samples t-test revealed a statistically significant difference (p < 0.001) in mean proliferation (10%) for the nano-chitosan + PRP treatment group between the two incubation periods: 5 days (241.9 ± 96.7 AU) and 7 days (710.3 ± 109.7 AU) (**Table 3**). Based on these values, proliferation after 7 days was substantially higher compared to 5 days, indicating that the combination of nano-chitosan and platelet-rich plasma (PRP) markedly enhances cellular proliferation over time. This suggests that nano-chitosan + PRP may accelerate bone healing within a 7-day incubation period.

In contrast, the hydroxyapatite + PRP group showed a smaller difference between incubation periods, with mean proliferation values of 396.4 ± 74.0 AU at 5 days and 581.8 ± 76.4 AU at 7 days. Although the p-value was 0.033 (which is below α = 0.05), the magnitude of change was less pronounced compared to the nano-chitosan + PRP group. This indicates that while hydroxyapatite + PRP also promotes proliferation over time, its effect is more moderate.

When comparing the two treatments, nano-chitosan + PRP demonstrated a significantly greater and faster proliferative response than hydroxyapatite + PRP after 7 days. These findings suggest that nano-chitosan + PRP is a more effective option for accelerating bone healing, whereas hydroxyapatite + PRP may be more suitable for applications requiring gradual, sustained regeneration, particularly when scaffold stability is a priority.

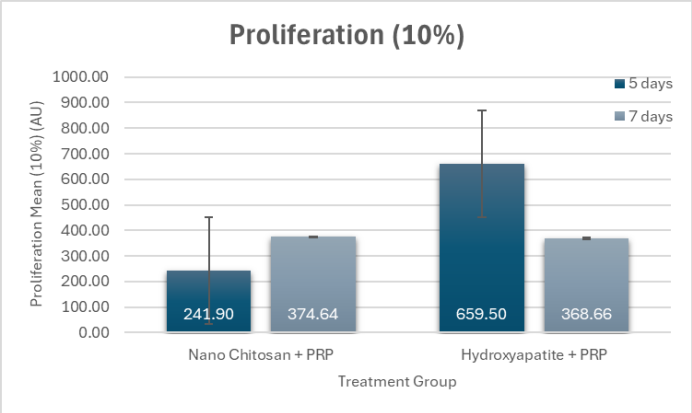

Figure 5. Histogram of Proliferation Mean (10 %) (incubation five days and seven days)

Interpretation: For the treatment groups (nano-chitosan + PRP and hydroxyapatite + PRP), means that share the same letter indicate no statistically significant difference, whereas means with different letters indicate a statistically significant difference.

Figure 5 illustrates a histogram showing the mean proliferation rate (10%) after 5-day and 7-day incubation periods for two treatment groups. The nano-chitosan + PRP group exhibited a markedly higher proliferation compared to the hydroxyapatite + PRP group, and this difference was statistically significant.

Table 4. The comparison results in proving the hypothesis

Treatment Group	Treatment group		*p-value*
	Nano Chitosan Mean ± SD	Hydroxyapatite Mean ± SD	
Proliferation (0%) (au)	1076.3±176.4**	659.5±272.7*	0.010 <∝
Proliferation (10%) (au)	710.4±109.7	581.8±76.4	0.040 <∝

Interpretation: For the t-test, a p-value < 0.05 indicates a statistically significant difference between groups, whereas a p-value > 0.05 indicates no statistically significant difference.

Table 4 presents the comparative results of mean proliferation rates (0% and 10%) for both treatment groups—nano-chitosan + PRP and hydroxyapatite + PRP—across 5-day and 7-day incubation periods. Based on the average proliferation values at both 0% and 10%, the nano-chitosan + PRP group consistently exhibited substantially higher proliferation compared to the hydroxyapatite + PRP group at both time points. This finding suggests that the combination of nano-chitosan and PRP significantly enhances cellular proliferation and may accelerate bone healing within 5 to 7 days of incubation, outperforming the hydroxyapatite + PRP treatment.

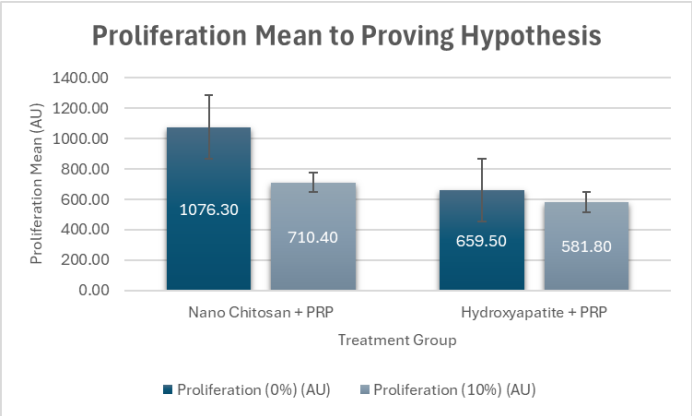

Figure 6. Histogram of Proliferation Mean to Proving Hypothesis*

* Description: The comparison involves two treatment groups: nano-chitosan + PRP and hydroxyapatite + PRP.

Figure 6 illustrates a histogram comparing the mean proliferation rate (0%) for the two treatment groups during the 5-day incubation period. The nano-chitosan + PRP group (1076.3 ± 176.4 AU) exhibited a higher proliferation rate than the hydroxyapatite + PRP group (659.5 ± 272.7 AU). This difference was

statistically significant ($p = 0.010 < \alpha$), indicating that nano-chitosan + PRP promotes greater cellular proliferation under these conditions.

Similarly, the mean proliferation (10%) in the nano-chitosan + PRP group after 7 days of incubation (710.3 ± 109.7 AU) was higher than that observed in the hydroxyapatite + PRP group (581.8 ± 76.4 AU) during the same period. This difference was statistically significant ($p = 0.040 < \alpha$), supporting the hypothesis that pre-osteoblast proliferation (Ki67) in vitro is greater with nano-chitosan + PRP compared to hydroxyapatite + PRP. These findings reinforce the role of PRP in accelerating bone regeneration during the early phases of healing, although previous studies have reported mixed results regarding its statistical significance across different models.[21]

Overall, there was a significant difference ($p < 0.05$) in mean proliferation (0% and 10%) between the two treatment groups across both incubation periods (5 and 7 days). The nano-chitosan + PRP group consistently demonstrated higher proliferation values than the hydroxyapatite + PRP group, indicating that nano-chitosan + PRP can accelerate bone healing more effectively. Notably, cell proliferation showed optimal results at 5% concentration during the 7-day incubation period. Previous research suggests that chitosan can minimize oxidative stress (as indicated by peroxide values), influence color stability, and reduce microbial load in samples.[22,23] Furthermore, nano-chitosan, with a deacetylation degree greater than 90%, has been shown to enhance pre-osteoblast proliferation and induce new bone formation more effectively than conventional chitosan, including in femoral defect models in goats.

The combination of chitosan and PRP has potential applications in complex or chronic wound healing and in soft and hard tissue regeneration, such as post-extraction sites requiring sustained release of growth factors. PRP provides concentrated bioactive mediators (e.g., PDGF, TGF-β, VEGF) that can stimulate cell proliferation and early osteogenic signaling, which is well-documented in oral and maxillofacial contexts.[24,25] In parallel, chitosan's cationic nature, biocompatibility, and modifiable nanoscale surface topography can improve protein adsorption, cell adhesion, and local factor presentation, thereby potentiating osteogenic responses. The synergy of nano-scale chitosan (greater surface area and adsorption capacity) with PRP likely underpins the stronger, time-dependent gains seen here.[20,26]

Within the limits of an in vitro model, the present findings suggest nano-chitosan + PRP may offer a more efficacious early-phase proliferative stimulus than hydroxyapatite + PRP—a desirable attribute for applications targeting rapid early cellular recruitment (e.g., alveolar ridge preservation or peri-implant defects). Nevertheless, translation to clinical outcomes depends on additional properties (matrix mineralization, vascularization, mechanical stability), domains where HAp's scaffolding and long-term dimensional integrity may remain advantageous.[27,28] Rigorous in vivo studies and controlled clinical trials are warranted to determine whether the proliferative advantage observed here translates into accelerated and durable bone formation in dental indications.[24,25,27]

CONCLUSIONS

The findings of this study indicate that the combination of nano-chitosan and platelet-rich plasma (PRP) resulted in higher mean proliferation values under both 0% and 10% conditions compared to hydroxyapatite + PRP, across both 5-day and 7-day incubation periods. These results suggest that nano-chitosan + PRP can accelerate pre-osteoblast proliferation more effectively than hydroxyapatite + PRP, thereby potentially enhancing the bone healing process within a shorter timeframe. The superior performance of nano-chitosan + PRP highlights its promise as a bioactive material for promoting early bone regeneration in dental applications.

RECOMMENDATIONS

Future research should further investigate the effect of nano-chitosan combined with platelet-rich plasma (PRP) on pre-osteoblast proliferation under varying concentrations (e.g., 0% and 10%) and extended

incubation periods in an in vitro culture system. Additional studies could also explore different PRP concentrations, nano-chitosan particle sizes, and their synergistic impact on osteogenic differentiation and mineralization to better understand their potential in accelerating bone regeneration for dental applications.

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