Bone

An In Vitro Study of Alkaline Phosphatase Induction as a Quantitative Measure of the Osteoinductive Potential of FDA-Approved Nano-Bioactive Glass Combined with Demineralized Bone Matrix (DBM) vs Bioactive Glass Alone and DBM Alone

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Abstract:	Background The osteoinductive potential of bone graft substitutes is essential for bone regeneration. Bioactive glass (BAG) and demineralized bone matrix (DBM) are widely used biomaterials, the relative osteoinductive potential of these materials, alone or in combination, remains unclear. This study evaluates and compares the osteoinductive potential of nano-BAG+DBM (NanoFuse™ DBM), BAG alone and DBM alone using the C2C12 alkaline phosphatase (AP) induction assay, where AP activity serves as a quantitative marker of osteoinduction in vitro. Methods C2C12 murine myoblast cells were cultured in triplicate with four test materials: nano-BAG+DBM/Gel, BAG/Gel, DBM/Gel, and Wet/Frozen DBM. Wet/Frozen DBM served as a reference for native osteoinductive potential, while gel-based formulations were assessed for their ability to induce AP activity. AP activity was quantified spectrophotometrically at 410 nm after three days, with positive and negative controls included to validate assay performance. Results Wet/Frozen DBM exhibited the highest AP activity. Among gel-based formulations, nano-BAG+DBM/Gel induced the highest AP activity, suggesting a synergistic effect between BAG and DBM. DBM/Gel also demonstrated substantial AP activity but was less potent. BAG/Gel alone had the lowest AP activity, indicating limited osteoinductive potential.

	Conclusion NanoFuse™ DBM (nano-BAG+DBM) was superior to DBM or BAG alone in osteoinductive potential, supporting its use in bone formation. The greater AP activity observed suggests a synergistic effect between BAG and DBM, where bioactive ion release from BAG may enhance the osteoinductive signaling of DBM. Further in vivo studies are needed to confirm its long-term regenerative potential in bone healing.
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An *In Vitro* Study of Alkaline Phosphatase Induction as a Quantitative Measure of the Osteoinductive Potential of FDA-Approved Nano-Bioactive Glass Combined with Demineralized Bone Matrix (DBM) vs Bioactive Glass Alone and DBM Alone

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Structured Abstract

Background: The osteoinductive potential of bone graft substitutes is essential for bone regeneration. Bioactive glass (BAG) and demineralized bone matrix (DBM) are widely used biomaterials, the relative osteoinductive potential of these materials, alone or in combination, remains unclear. This study evaluates and compares the osteoinductive potential of nano-BAG+DBM (NanoFuse™ DBM), BAG alone and DBM alone using the C2C12 alkaline phosphatase (AP) induction assay, where AP activity serves as a quantitative marker of osteoinduction *in vitro*.

Methods: C2C12 murine myoblast cells were cultured in triplicate with four test materials: nano-BAG+DBM/Gel, BAG/Gel, DBM/Gel, and Wet/Frozen DBM. Wet/Frozen DBM served as a reference for native osteoinductive potential, while gel-based formulations were assessed for their ability to induce AP activity. AP activity was quantified spectrophotometrically at 410 nm after three days, with positive and negative controls included to validate assay performance.

Results: Wet/Frozen DBM exhibited the highest AP activity. Among gel-based formulations, nano-BAG+DBM/Gel induced the highest AP activity, suggesting a synergistic effect between BAG and DBM. DBM/Gel also demonstrated substantial AP activity but was less potent. BAG/Gel alone had the lowest AP activity, indicating limited osteoinductive potential.

Conclusion: NanoFuse™ DBM (nano-BAG+DBM) was superior to DBM or BAG alone in osteoinductive potential, supporting its use in bone formation. The greater AP activity observed suggests a synergistic effect between BAG and DBM, where bioactive ion release from BAG

may enhance the osteoinductive signaling of DBM. Further *in vivo* studies are needed to confirm its long-term regenerative potential in bone healing.

Keywords: NanoFuse; Osteoinduction, Bone Graft, Bioactive Glass (BAG), Demineralized bone matrix (DBM), Synergistic effect, *In Vitro* model.

1. Introduction

Bone healing is a complex biological process involving the coordination of cellular and molecular mechanisms to restore skeletal integrity following injury or surgical intervention [1-3]. A critical aspect of this process is osteoinduction, in which osteoprogenitor cells are stimulated to differentiate into osteoblasts, the bone-forming cells responsible for osteogenesis or the formation of new bone tissue. Osteogenesis is a tightly regulated process involving a sequence of cellular events driven by osteoinductive signaling molecules, such as bone morphogenetic proteins (BMPs). The foundation of osteoinduction was established by Marshall Urist, who identified bone morphogenetic proteins (BMPs) as potent regulators of osteogenesis [4, 5]. This breakthrough led to the development of demineralized bone matrix (DBM), an allograft-derived material that retains BMPs and other osteoinductive growth factors, making it one of the most widely used bone graft substitutes [6-8].

While natural bone regeneration is a tightly regulated process, severe bone defects and clinical conditions often necessitate the use of bone grafting materials to restore skeletal integrity [9-11]. Building on Urist's discovery, advances in biomaterials and tissue engineering have expanded the scope of bone grafting strategies, particularly in treating bone defects, non-unions, and spinal fusions, where natural bone regeneration alone is inadequate [12, 13]. Autograft bone harvested from a patient remains the gold standard due to its inherent osteogenic, osteoinductive, and osteoconductive properties. However, its use is limited by donor site morbidity and restricted availability, necessitating alternative grafting solutions [14, 15]. Consequently, researchers and clinicians have turned to allografts, xenografts, and synthetic substitutes, each offering distinct biological and mechanical properties for bone regeneration.

Among these alternatives, DBM has emerged as a key osteoinductive material due to its preserved BMP content, which promotes mesenchymal stem cell (MSC) differentiation into osteoblasts [16, 17]. Bioactive glass (BAG), a synthetic biomaterial developed by Hench et al. in the late 1960s, has gained attention for its ability to stimulate bone formation through ion release and surface bioactivity [18]. BAG, composed primarily of silica, calcium, and phosphate, which mimics the mineral composition of natural bone and fosters osteogenesis by releasing its biologically active ions, including calcium and silicate [19]. These calcium, phosphate and silica ions stimulate cellular responses, promote hydroxyapatite (HA) formation, and enhance integration with host bone. More recently, nano-BAG has been developed as a variant with higher surface area and enhanced bioactivity, potentially increasing its osteoinductive effects [20]. However, the osteogenic potential of DBM and BAG is highly dependent on their composition, formulation, and processing methods, necessitating further investigation into their comparative efficacy.

While DBM and BAG each exhibit distinct osteoinductive and osteoconductive properties, their combined use may create synergistic effects, enhancing osteoinduction beyond what either material can achieve alone. DBM serves as a natural reservoir of BMPs, while BAG promotes cellular activity and mineral deposition, suggesting that their co-administration could optimize bone. NanoFUSE® DBM (NanoFuse Biologics LLC, Burlington, MA), an FDA-approved bone graft substitute, was designed to harness these complementary mechanisms by integrating human-derived DBM with synthetic 45S5 bioactive glass [21]. Its formulation consists of 33% DBM cortical bone, 33% 45S5 BAG, and 33% porcine gelatin, providing a structural and biochemical environment conducive to osteogenesis. However, comparative studies assessing the

osteoinductive potential of BAG, DBM, and their combination remain limited, particularly in controlled *in vitro* settings.

Given the importance of osteoinduction in bone graft performance, *in vitro* assays provide a robust model for evaluating this property. A widely accepted approach involves measuring alkaline phosphatase (AP) activity, an early marker of osteogenic differentiation, to assess the osteoinductive capacity of bone graft materials [22, 23]. The C2C12 cell differentiation assay, which utilizes a murine myoblast line capable of differentiating into osteoblast-like cells in response to BMP signaling, serves as a reliable model for osteoinduction assessment.

Despite the promising osteoinductive properties of DBM and BAG, their comparative efficacy particularly in combination remains largely unexplored. This study provides the first quantitative assessment of nano-BAG+DBM, BAG alone and DBM alone using the C2C12 AP induction assay, offering critical insights into their potential synergistic effects. By evaluating AP activity as an early osteogenic marker, this information can aid in the development of optimized bone graft formulations, ultimately advancing regenerative strategies in orthopedic and spinal applications where robust bone formation is essential.

2. Materials and Methods

2.1. Study Design

This study utilized an *in vitro* C2C12 cell differentiation assay to evaluate the osteoinductive potential of four test materials and two controls. Three test materials were gel-based formulations, each weighing 5 g, and stored at room temperature: BAG alone (BAG/Gel, SN001), nano-BAG+DBM (nano-BAG+DBM/Gel, SN002, NanoFuse™ DBM), and DBM alone (DBM/Gel, SN003). The fourth material, wet/frozen DBM (SN004), weighed 1 g and was

stored at -10 to -20°C to compare its osteoinductive effects with the gel-based formulations. The negative control consisted of C2C12 cells cultured in growth media alone, establishing a baseline for AP activity. The positive control included C2C12 cells treated with human bone morphogenic protein-2 (BMP-2) to confirm osteogenic induction. All test materials were prepared, stored under the specified conditions, and used in the assay accordingly.

2.2. Cell Culture and Differentiation Assay

C2C12 myoblast cells were maintained under standard culture conditions at 37°C in a humidified atmosphere with 5% CO_2, using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and L-glutamine. Cells were seeded into 24-well plates at a density of 1×10^4 cells per well and allowed to adhere overnight. After adherence, the culture medium was replaced with differentiation media containing 2% FBS to minimize proliferation and promote differentiation. Test materials from each of the four test groups were added at 20 mg and 50 mg concentrations, with each condition tested in triplicate wells. BMP-2 was added to positive control wells. Cells were incubated for three days at 37 ± 2°C in a $5 \pm 2\%$ CO_2 atmosphere and monitored daily for adherence, contamination, and any cytotoxic effects of the test materials.

2.3. Alkaline Phosphatase (AP) Assay

At the end of the incubation period, cells were washed with cold phosphate-buffered saline (PBS) to remove residual media. Cells were lysed using 0.5% Triton X-100 in PBS, followed by three freeze-thaw cycles to ensure complete membrane disruption. AP activity was quantified using a colorimetric assay, in which 50 µL of each clarified lysate was incubated with 150 µL of 0.3 mM p-nitrophenyl phosphate (p-NPP) in 2-amino-2-methyl-1-propanol buffer (pH 10.5) for

30 minutes at 37°C. The reaction was stopped by adding 50 µL of 1.0 N NaOH, and the amount of p-nitrophenol released was measured spectrophotometrically at 410 nm using a plate reader. Alkaline phosphatase catalyzes the conversion of p-nitrophenyl phosphate (pNPP) into p-nitrophenol, a reaction that produces a yellow color detectable at 410 nm (OD_{410}). The intensity of this color change correlates with AP activity, meaning that higher AP activity results in greater absorbance and indicates a stronger osteoinductive response.

To standardize AP activity across different samples, specific activity was calculated and expressed as AP units per milligram of total protein (AP units/mg protein). This measure normalizes enzyme activity to total protein content, allowing for direct comparison of osteoinductive potential across different test groups. Higher specific AP activity values indicate greater AP induction and, by extension, stronger osteoinductive potential. Samples exceeding the upper assay limit (UAL) were classified as having high osteoinductive potential, while those below the limit of quantification (LOQ) were considered to have minimal or no osteoinductive response.

2.4. Controls and Assay Validation Criteria

The negative control for the C2C12 cell differentiation assay established a baseline for AP activity. The negative control for the AP assay was the AMP buffer (pH 10.5) plus substrate (p-NPP) blank, ensuring no background enzyme activity from the assay reagents. The positive control for the differentiation assay was C2C12 cells treated with BMP-2, serving as a reference for osteogenic induction, while the positive control for the AP assay was 300 U/mL alkaline phosphatase, confirming the functionality of the AP assay itself. The Wet/frozen DBM test was included as an additional positive control for DBM-based test material to compare the

osteoinductive potential of different DBM formulations, specifically gel-based versus wet/frozen DBM.

To validate the assay, the following criteria were established:

- The OD_{410} value for BMP-2-treated cells must be at least twice that of the negative control (C2C12 cells + growth media).
- The OD_{410} value for the 300 U/mL alkaline phosphatase standard must be at least twice that of the AMP buffer plus substrate blank.
- The OD_{410} value for the negative controls (C2C12 cells plus growth media and AMP buffer plus substrate blank) must not exceed 0.100.

2.5. Evaluation Criteria

Once the assay was validated, the osteogenic potential of the four test groups was evaluated by comparing their OD_{410} values to the negative and positive controls. The following criteria were applied:

- If the mean OD_{410} value of a test material was at least twice that of the negative control, it was classified as positive for AP induction, indicating osteoinductive potential.
- If the mean OD_{410} value was less than twice that of the negative control, it was classified as negative for AP induction, suggesting little or no osteoinductive activity.

This evaluation provided a quantitative comparison of the four test materials, determining their ability to induce AP activity in C2C12 cells.

2.6. Statistical Analysis

Data was collected in triplicate for each experimental condition. Statistical analyses included calculations of mean values, standard deviation (SD), and percent relative standard deviation (%RSD).

3. Results

The assay's validity was confirmed, as all positive and negative controls met predefined acceptance criteria, thereby ensuring reliability and reproducibility (Table 1). BMP-2 and Wet/Frozen DBM served as positive controls, demonstrating strong osteoinductive activity, as expected. No protocol deviations were observed, further reinforcing the accuracy and consistency of the findings. AP activity varied across the test groups, reflecting differences in osteoinductive potential based on composition and concentration. The detailed results are presented in Table 2 and Figure 1.

The highest AP activity was observed in the Wet/Frozen DBM group, where both concentrations surpassed the UAL. The 50 mg/well sample reached >94.420 AP units/mg protein, while the 20 mg/well sample measured >64.885 AP units/mg protein. Among the gel-based formulations, nano-BAG+DBM/Gel demonstrated the strongest osteoinductive response, ranking second overall to Wet/Frozen DBM. The 50 mg/well sample exceeded the UAL (>92.473 AP units/mg protein), while the 20 mg/well sample induced moderate AP activity (0.333 U/mL, 19.974 AP units/mg protein). Although DBM/Gel alone also induced substantial AP activity, its response was lower than nano-BAG+DBM/Gel at both concentrations. The 50 mg/well DBM/Gel sample exceeded the UAL (>72.569 AP units/mg protein), while the 20 mg/well sample exhibited moderate AP activity (0.217 U/mL, 13.815 AP units/mg protein). In contrast, BAG/Gel alone displayed the lowest osteoinductive potential, with AP activity below the LOQ, comparable to the negative control.

4. Discussion

4.1. Brief Summary

This study evaluates and compares the osteoinductive potential of an FDA-approved nano-bioactive glass (BAG) and DBM combination (nano-BAG+DBM), DBM alone and BAG alone using the C2C12 alkaline phosphatase (AP) induction assay in an established *in vitro* model. While both BAG and DBM are widely used in bone grafting applications, their individual limitations necessitate exploring complementary formulations that enhance osteoinductive activity. By using AP activity as a quantitative marker of osteoinduction, this study provides new insights into how these materials compare in stimulating osteogenic differentiation and their potential clinical applications.

4.2. Key Findings

The highest AP activity was observed in the Wet/Frozen DBM control, confirming its strong intrinsic osteoinductive properties due to the presence of native BMPs and other osteogenic factors. Among the gel test groups, the nano-BAG+DBM/Gel formulation exhibited significantly greater AP activity than either BAG or DBM alone, indicating a synergistic effect between bioactive ion release from BAG and the osteoinductive factors within DBM. While DBM/Gel alone exhibited moderate osteoinductive potential, its effect was less pronounced than the nano-BAG+DBM combination, suggesting that the addition of nano-BAG enhances DBM's osteoinductive properties, possibly through BMP retention and osteogenic signaling. In contrast, BAG alone failed to induce significant AP activity, confirming its function as an osteoconductive material. These findings suggest that nano-BAG+DBM/Gel provides an optimal balance of osteoconductive and osteoinductive properties, making it a more effective bone graft alternative than either component alone [24, 25]. These results support the use of BAG as a scaffold that

requires additional biologic components, such as DBM, to achieve meaningful osteoinductive effects.

4.3. Comparison with Similar Research

The results align with previous findings that DBM serves as a more effective long-term osteoinductive agent than single-factor BMP-2 due to its sustained release of multiple growth factors [26]. The enhanced performance of nano-BAG+DBM in this study is further supported by prior animal studies, which have demonstrated that bioactive glass enhances bone induction and formation when combined with DBM [27-29]. Studies have shown that bioactive ions released from BAG enhance osteogenic differentiation, thereby contributing to cell signaling for osteogenesis [30-32]. Incorporating DBM into a scaffold may influence its degradation rate and bioresportion, potentially affecting the controlled release of osteoinductive factors [31]. These factors may further support the observed synergistic effects in this study

4.4. Limitations and Future Research

Despite these promising results, this study has limitations. The C2C12 AP assay measures early-stage osteogenic differentiation, but it does not assess later stages of bone formation, such as mineralization and matrix deposition. Additionally, as an *in vitro* model, it does not fully replicate the complex physiological environment of bone healing. Variability in DBM composition across donors may also affect reproducibility.

4.5. Clinical Relevance

The FDA-approved nano-BAG+DBM formulation demonstrated superior osteoinductive potential, making it a viable candidate for bone grafting applications. Unlike autografts and allografts, which pose risks such as donor site morbidity and immune rejection, synthetic-biological hybrid materials like nano-BAG+DBM offer a potentially safer and more effective

alternative. Given its enhanced osteoinductive response compared to DBM alone, nano-BAG+DBM may be particularly useful in challenging clinical scenarios, such as non-unions, large bone defects, and spinal fusion procedures.

4.6. Implications for Future Research

To further validate these findings, future studies should focus on *in vivo* models to assess the long-term regenerative potential of nano-BAG+DBM in bone healing. Given the variability in DBM composition across donors, additional research is needed to evaluate batch-to-batch consistency and reproducibility in DBM-based composites. Optimizing the nano-BAG:DBM ratio may further enhance osteoinductive potential, ensuring a balanced formulation that maximizes both bioactivity and structural integrity. Investigating the underlying molecular mechanisms, particularly key signaling pathways such as BMP/Smad, Wnt/β-catenin, and Mitogen-Activated Protein Kinase (MAPK), will provide deeper insights into the synergistic effects observed in this study. Biomechanical testing should be conducted to determine the load-bearing capacity and mechanical stability of BAG+DBM composites in clinical applications.

5. Conclusion

This study provides quantitative evidence that the FDA-approved nano-BAG+DBM formulation exhibits significantly greater osteoinductive potential than either BAG or DBM alone, as indicated by elevated AP activity in C2C12 cells. The combination of bioactive glass and DBM leverages both osteoconductive and osteoinductive mechanisms, creating a highly effective bone graft substitute. Given these findings, nano-BAG+DBM represents a promising alternative to traditional bone grafting materials. However, further *in vivo* research is needed to confirm its

efficacy in clinical applications and optimize its formulation for enhanced regenerative outcomes.

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Data availability

Data will be made available on request.

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Table 1: Validation of Controls and Assay Criteria for Alkaline Phosphatase Activity.

Controls and Validity Criteria	**Result**
BMP>=2X the negative control (cell Lysate)	PASS
AP Standard Curve R2 is > 0.98	PASS
Protein Standard Curve R2 is >0.98	PASS
The Cell Lysate negative control <= 0.100	PASS
The Lysis Buffer Blank negative control <=0.100	PASS

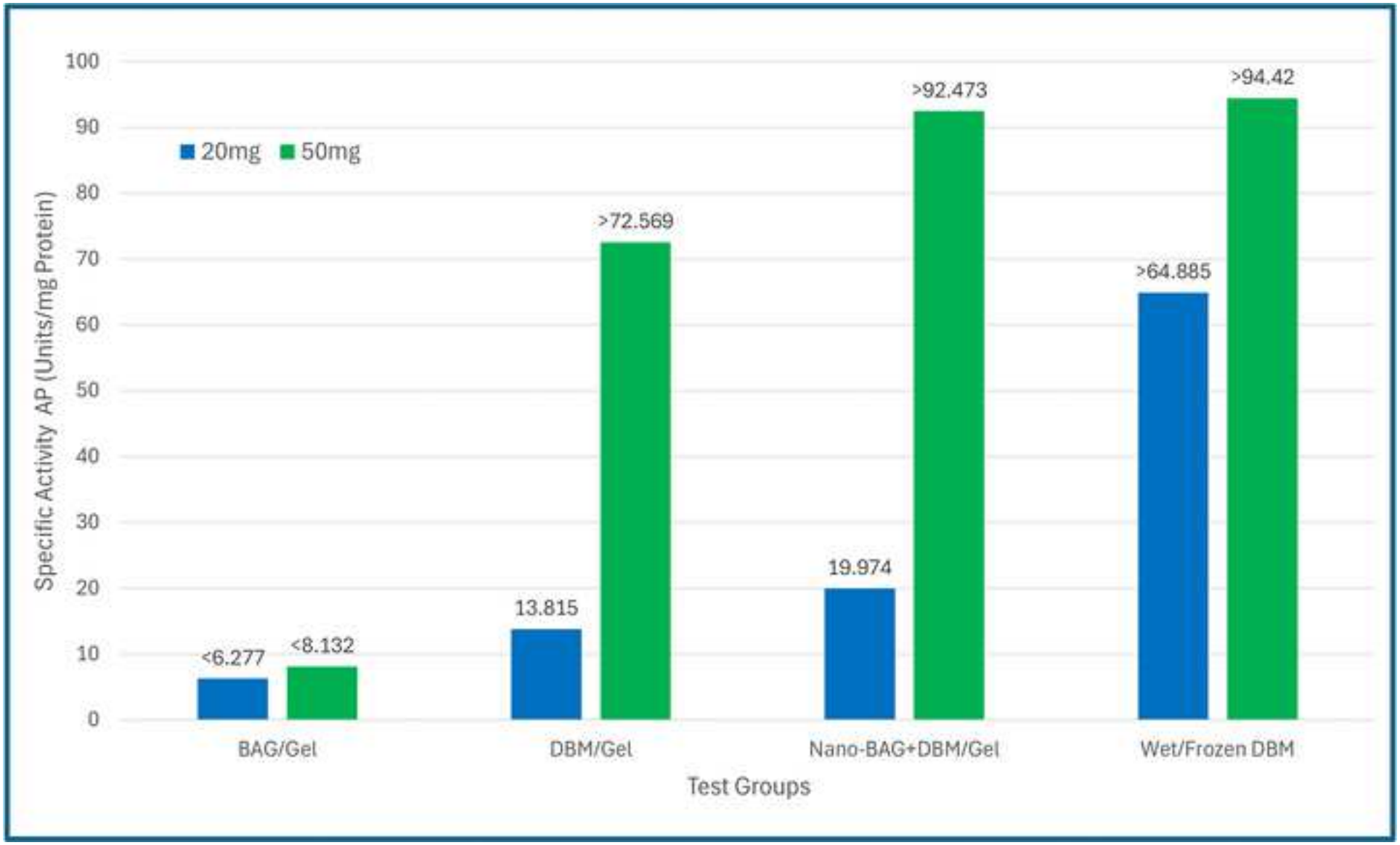
BMP, bone morphogenetic proteins; AP, Alkaline Phosphatase

Table 2: Summary of Alkaline Phosphatase Activity Results Across Test Groups and Concentrations.

Test Groups	Sample Number	Concentration Tested (mg/well)	Specific Activity AP Units/mg Protein
BAG/Gel	SN001	50	< LOQ (< 8.132)
		20	< LOQ (< 6.277)
Nano-BAG+DBM/Gel	SN002	50	> Upper Assay Limit (> 92.473)
		20	19.974
DBM/Gel	SN003	50	> Upper Assay Limit (> 72.569)
		20	13.815
Wet/Frozen DBM	SN004	50	> Upper Assay Limit (> 94.420)
		20	> Upper Assay Limit (> 64.885)

BAG, Bioactive glass; DBM, Demineralized Bone Matrix; Nano-BAG+DBM, Nano-Bioactive glass and Demineralized Bone Matrix combination; AP, Alkaline Phosphatase; LOQ, Limit of Quantification.

Figure(s)


100
90
80
70
60
50
40
30
20
10
0
Specific Activity AP (Units/mg Protein)
20mg
50mg
<6.277
<8.132
13.815
>72.569
19.974
>92.473
>64.885
>94.42
BAG/Gel
DBM/Gel
Nano-BAG+DBM/Gel
Wet/Frozen DBM
Test Groups

Highlights

1. Nano-BAG+DBM exhibited greater osteoinductive potential than DBM or BAG alone.
2. Nano-BAG+DBM induced the highest AP activity among gel-based formulations.
3. DBM alone demonstrated substantial AP activity but was surpassed by Nano-BAG+DBM.
4. BAG alone showed the lowest osteoinductive potential.
5. DBM in gel formulation showed lower AP activity than Wet/Frozen DBM.