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Comparative Osteoinductive Potential of BMP-2, Bioactive Glass, and Demineralized Bone Matrix: An In Vitro Alkaline Phosphatase Assay Study

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ABSTRACT

Background: The rising number of spinal fusion procedures has increased the demand for effective bone graft substitutes. Although recombinant human bone morphogenetic protein-2 is clinically used for its osteoinductive properties, dose-dependent complications limit its broader application. Demineralized bone matrix (DBM) and bioactive glass (BAG) are alternative materials, but their comparative and combined osteogenic potential remains unclear. This study evaluated the in vitro osteoinductive activity of BMP-2, DBM, BAG, and a composite nano-BAG + DBM formulation.

Methods: An in vitro C2C12 alkaline phosphatase (ALP) assay was used to assess osteogenic differentiation following exposure to BMP-2 (50 ng/mL) and test materials at 20 and 50 mg/mL. Gel-based formulations were standardized to 1 g total weight and included the following: nano-BAG + DBM (33:33:33 of cortical DBM, 45S5 BAG, and porcine gelatin; marketed as NanoFuse DBM), BAG + Gel (50:50 BAG and gelatin), and DBM + Gel (50:50 DBM and gelatin). Wet/frozen DBM (100% DBM) served as the native reference. ALP activity was measured at 410 nm and normalized to total protein content.

Results: Wet/frozen DBM exhibited the highest ALP activity (>94.420 units/mg protein), followed by nano-BAG + DBM at 50 mg/mL, which exceeded the assay's upper detection limit (>92.473 units/mg). DBM + Gel showed moderate activity, while BAG + Gel and the negative control showed minimal induction. BMP-2 at 50 ng/mL demonstrated lower activity (31.700 units/mg) than nano-BAG + DBM.

Clinical Relevance: NanoFuse DBM demonstrated dose-dependent osteoinductive activity and may offer a safer, more efficient alternative to BMP-2 and traditional grafts in spinal fusion, trauma, and joint reconstruction.

Conclusions: NanoFuse DBM demonstrated dose-dependent osteoinductive activity and outperformed DBM, BAG, and BMP-2 at the tested dose. These findings support its potential as a bone graft substitute in spinal fusion and other orthopedic applications where improved biological performance and safety are critical. Further research is needed to optimize BMP-2 dosing and evaluate NanoFuse DBM's in vivo efficacy.

Level of Evidence: 5.

Biologics

Keywords: NanoFuse, osteoinduction, bone graft, bioactive glass (BAG), demineralized bone matrix (DBM), synergistic effect, in vitro model, infuse, trauma, joint replacement

INTRODUCTION

Spinal fusion is one of the most frequently performed procedures in orthopedic and neurosurgical practice. As spinal fusion surgeries continue to rise globally, so does the demand for biological materials that can reliably promote bone regeneration. Achieving solid arthrodesis requires osteoinductive activity to stimulate new bone formation across fusion sites, particularly in patients with comorbidities or compromised healing potential. While autografts remain the gold standard due to their inherent osteogenic, osteoinductive, and osteoconductive properties, clinical limitations such as donor site morbidity, variability in graft quality, and limited availability have led to increased interest in alternative bone graft substitutes. Recombinant human bone morphogenetic protein-2, demineralized bone matrix (DBM), and bioactive glass (BAG) are widely used in spinal fusion and other orthopedic applications for this purpose.

Successful bone regeneration depends on a series of coordinated cellular and molecular processes that drive

osteogenesis—the formation of mineralized bone tissue. A key factor in this process is osteoinduction, where mesenchymal progenitor cells are recruited and induced to differentiate into osteoblasts. Marshall Urist's discovery of BMPs was pivotal in advancing this understanding and led to the development of DBM as a bone graft material.[1–5] DBM contains endogenous growth factors such as BMP-2, BMP-4, and BMP-7; however, its biological activity can vary with donor characteristics and processing methods, leading to inconsistent clinical results. This variability has prompted the development of alternative and combinatory materials to improve the consistency and efficacy of osteoinductive performance in spinal fusion.[6–8]

Bone grafting continues to be a cornerstone in spinal fusion surgery, particularly in the treatment of degenerative disc disease, deformity, trauma, and other structural pathologies.[9–11] Although autografts possess favorable biological properties, their limitations have driven ongoing innovation in synthetic and allograft-based options.[12,13] Current research focuses on materials that maintain or enhance biologic efficacy while minimizing complication risk.[14,15]

Among synthetic alternatives, BAG has emerged as an osteoinductive material that facilitates bone formation through the release of biologically active ions. Developed by Larry Hench, 45S5 BAG is composed of silica, calcium, and phosphate, which promote hydroxyapatite formation and support bone integration.[16–18] These components enable BAG to serve as a scaffold that supports osteoblast adhesion, proliferation, and differentiation while promoting mineralization.[19] More recently, nano-scale BAG has been developed to increase surface area and ion release, thereby enhancing its bioactivity and osteogenic potential.[20]

Both DBM and BAG independently exhibit osteogenic properties, but their combination may yield synergistic effects. NanoFuse DBM (NanoFuse Biologics LLC, Burlington, MA) is an US Food and Drug Administration–approved synthetic bone graft that combines 33% cortical DBM with 33% 45S5 BAG and 33% porcine gelatin. This unique formulation is designed to optimize osteoinductive performance by leveraging DBM's endogenous growth factors and BAG's bioactive ion release.[21] Despite the clinical relevance of these materials, no prior in vitro study has directly compared the osteoinductive potential of BMP-2, DBM, BAG, and nano-BAG + DBM in a controlled model.

Although BMP-2 remains a clinical benchmark for osteoinduction, its use is limited by dose-dependent complications such as heterotopic ossification, inflammation, and osteolysis, particularly at the INFUSE (Medtronic, MN, USA) dose of 1.5 mg/mL. Additionally, the optimal and safest dosing of BMP-2 remains undefined, and its efficacy relative to newer materials like nano-BAG + DBM is not well established.

To address this gap, the present study offers the first in vitro comparison of BMP-2, nano-BAG + DBM, BAG alone, and DBM alone using the C2C12 alkaline phosphatase (ALP) induction assay—a validated marker of early osteogenic differentiation. By quantifying ALP activity, this study aims to clarify the relative and potential synergistic effects of these materials and inform future strategies for selecting biologics in spinal fusion surgery.

METHODS

Study Design

To assess the osteoinductive potential of BMP-2, nano-BAG + DBM (NanoFuse DBM), BAG alone, and DBM alone, an in vitro C2C12 cell differentiation assay was performed. ALP activity was used as a quantitative marker of early osteogenic differentiation. This study was designed to evaluate the relative and potential synergistic effects of BAG and DBM in comparison to BMP-2, a clinically established osteoinductive factor.

Cell Culture and Experimental Design

C2C12 murine myoblast cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified incubator with 5% CO_2. For differentiation studies, cells were seeded at a density of 2×10^4 cells per well in 24-well plates and incubated overnight to allow for adhesion.

Once adherent, cells were exposed to 1 of 5 experimental conditions:

- BMP-2 (50 ng/mL): Positive control for osteogenic induction. A BMP-2 concentration of 50 ng/mL was selected based on prior studies demonstrating effective induction of ALP activity in C2C12 cells at this dose.[22,23]
- Nano-BAG + DBM + Gel (20 mg/mL and 50 mg/mL): A composite of 33% cortical DBM, 33% nano-sized 45S5 BAG, and 33% porcine gelatin by weight (marketed as NanoFuse DBM).
- BAG + Gel (20 mg/mL and 50 mg/mL): 50% BAG and 50% porcine gelatin.
- DBM + Gel (20 mg/mL and 50 mg/mL): 50% DBM and 50% porcine gelatin.

- Wet/frozen DBM (20 mg/mL and 50 mg/mL): 100% DBM, serving as a reference for native osteoinductive potential.

Each DBM-containing formulation was derived from a distinct production lot. A negative control group (untreated cells in growth medium) was included to establish baseline ALP activity. Cells were incubated for 3 days, with daily monitoring for attachment, proliferation, and any cytotoxic effects of the test materials.

ALP Assay

At the end of the incubation period, cells were gently rinsed with cold phosphate-buffered saline to remove non-adherent material and residual media. Cell lysis was performed using 0.2% Triton X-100, followed by freeze-thaw cycles to ensure complete membrane disruption. ALP activity was then measured using a colorimetric assay in which 50 μL of cell lysate was incubated with 150 μL of 0.3 mM p-nitrophenyl phosphate in 2-amino-2-methyl-1-propanol buffer (pH 10.5) at 37°C for 30 minutes. The enzymatic reaction, which produces yellow-colored p-nitrophenol upon ALP-mediated cleavage of p-nitrophenyl phosphate, was terminated by adding 50 μL of 1.0 N NaOH. Absorbance was measured at 410 nm using a microplate reader, and results were recorded as optical density at 410 nm (OD_{410}). This wavelength corresponds to the peak absorbance of p-nitrophenol and is commonly used to quantify ALP activity in bone biology assays.[22] ALP activity was normalized to total protein content to ensure accurate comparison across experimental groups.

Validation Controls and Criteria

To confirm the validity of the assay, several quality control measures were applied. The BMP-2 positive control was required to generate ALP activity at least twice that of the negative control. An ALP standard (300 U/mL) served as a reference enzyme control and was expected to yield OD_{410} values at least twice that of the assay buffer blank. Negative controls, including untreated cells and assay buffer blanks, were required to produce OD_{410} values below 0.100 to confirm low background interference.

Data Analysis and Interpretation

All experimental conditions were performed in triplicate. Results were expressed as mean ± SD and percent relative SD. Given the pilot nature of the study and limited sample sizes, formal inferential statistical testing, such as analysis of variance, was not performed. Instead, descriptive statistics were used to compare trends in osteoinductive activity across groups. ALP activity was used to classify osteoinductive potential using the following thresholds: OD_{410} values at least twice that of the negative control were classified as osteogenic; values below this threshold were considered to exhibit minimal or no osteoinductive activity. Samples exceeding the upper assay limit (UAL) were classified as highly osteoinductive, while those below the limit of quantification were categorized as inactive.

RESULTS

The assay met all predefined validation criteria, confirming its reliability and reproducibility. The BMP-2 (50 ng/mL) and wet/frozen DBM groups, included as positive controls, exhibited robust osteogenic activity, as anticipated. All negative controls (untreated cells and assay blanks) yielded low absorbance values below the defined threshold, confirming minimal background interference. No protocol deviations occurred during the study.

Among all tested groups, wet/frozen DBM demonstrated the highest ALP activity, with both 20 mg/mL and 50 mg/mL concentrations exceeding the UAL. At 50 mg/mL, nano-BAG + DBM + Gel induced a strong osteoinductive response, exceeding the UAL and achieving greater ALP activity than BMP-2. DBM + Gel at 50 mg/mL also exceeded the UAL, exhibiting greater activity than BMP-2, but remained slightly lower than nano-BAG + DBM + Gel.

At 20 mg/mL, both nano-BAG + DBM + Gel and DBM + Gel produced moderate ALP activity. While their responses were above the negative control, they did not exceed the UAL, indicating a dose-dependent osteoinductive effect. In contrast, BAG + Gel showed the lowest ALP activity across both concentrations. Its values remained below the limit of quantification and were comparable to the negative control, indicating minimal to no osteoinductive potential.

Detailed quantitative comparisons of ALP activity across all groups are presented in Tables 1 and 2, with graphical representation in Figure.

DISCUSSION

Summary of Key Findings

This study provides a controlled in vitro comparison of the osteoinductive potential of BMP-2, DBM, BAG, and nano-BAG + DBM using the C2C12 alkaline phosphatase assay. The results demonstrate that nano-BAG + DBM exhibits strong, dose-dependent osteoinductive

Table 1. Summary of ALP activity across test groups and concentrations.

Test Groups	Sample Number	Concentration Tested	Specific Activity ALP Units/mg Protein
BMP-2	-	50 ng/mL	31.700
BAG/Gel	SN001	50 mg/mL	<LOQ (<8.132)
		20 mg/mL	<LOQ (<6.277)
Nano-BAG + DBM/Gel	SN002	50 mg/mL	>Upper assay limit (>92.473)
		20 mg/mL	19.974
DBM/Gel	SN003	50 mg/mL	>Upper assay limit (>72.569)
		20	13.815
Wet/Frozen DBM	SN004	50 mg/mL	>Upper assay limit (>94.420)
		20 mg/mL	>Upper assay limit (>64.885)

Abbreviations: ALP, alkaline phosphatase; BAG, bioactive glass; BMP-2, Bone morphogenetic protein-2; DBM, demineralized bone matrix; LOQ, limit of quantification; Nano-BAG+ DBM, nano-bioactive glass and demineralized bone matrix combination.

activity, exceeding BMP-2 (50 ng/mL) and its individual components (Table 1 and Figure). Wet/frozen DBM showed the highest ALP activity overall, likely reflecting preserved native BMP content and sustained growth factor release. Among gel-based formulations, nano-BAG + DBM demonstrated superior osteogenic activity related to DBM and BAG alone, supporting a synergistic interaction between BAG's bioactive ion release and DBM's endogenous growth factors. BAG alone demonstrated minimal activity, consistent with its primarily osteoconductive role. Notably, BMP-2 at 50 ng/mL induced lower ALP activity than both nano-BAG + DBM and DBM + Gel (Table 2 and Figure), suggesting that this dose may have been below the optimal osteogenic threshold. Further investigation of BMP-2 dosing is needed to determine whether higher concentrations can match the osteoinductive potential of nano-BAG + DBM while avoiding dose-related complications.

Comparison With Existing Research

These findings are consistent with prior work demonstrating DBM's osteoinductive capacity through gradual BMP release and matrix signaling.[24] In contrast, BMP-2 is known for rapid release kinetics, which can lead to transient stimulation and complications such as heterotopic ossification, osteolysis, and inflammation at higher doses.[25,26] While both BMP-2 and DBM are clinically used, DBM offers advantages in safety, availability, and sustained activity—although its efficacy is influenced by donor variability and processing.[27] Preclinical studies suggest that BAG can enhance osteogenesis when combined with DBM by releasing calcium and phosphate ions that stimulate osteoblast differentiation.[28–30] Our findings reinforce this interaction, with nano-BAG amplifying DBM-induced signaling, potentially improving BMP retention and osteoinductive outcomes.

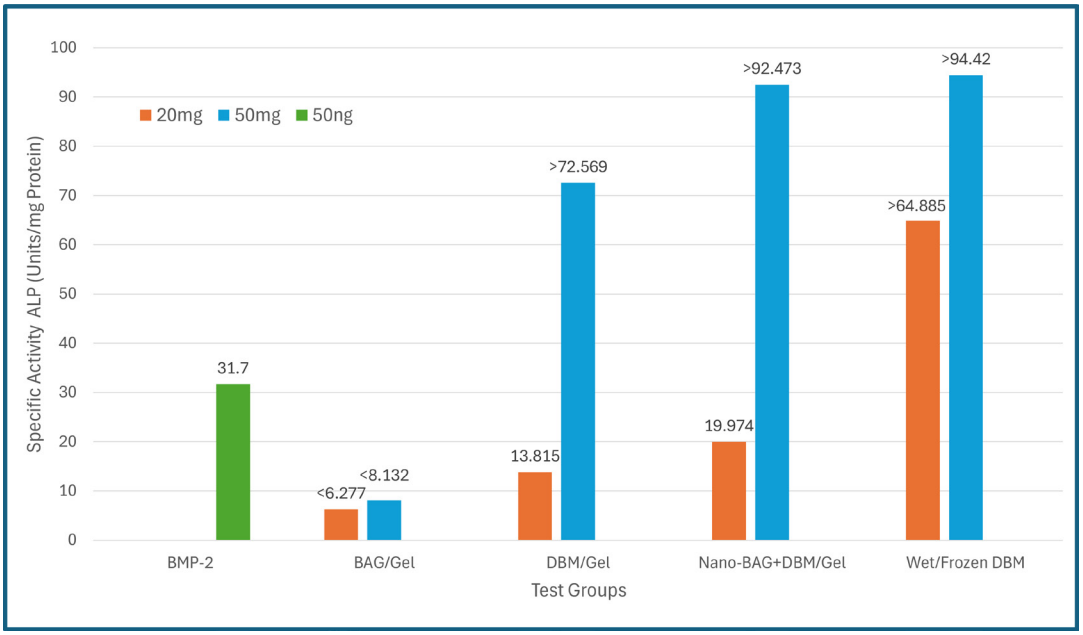

Figure. Alkaline phosphatase (ALP) activity comparison indicating osteoinductive potential across test groups and concentrations. BMP-2, bone morphogenetic protein-2; BAG + Gel, bioactive glass in gel paste; DBM + Gel, demineralized bone matrix in gel paste; Nano-BAG + DBM + Gel, Nano-Bioactive glass and demineralized bone matrix combination in gel paste; Wet/Frozen DBM, wet/frozen demineralized bone matrix.

Table 2. Protein concentration and final result.

Sample	Sample Description	OD410-1	OD410-2	OD410-3	OD410 Mean	Protein Normalized Mean	Protein SD	Protein %RSD	Protein Result, mg/ML	U/mL ALP	Specific activity ALP, U/mg Protein
Lysis Buffer Blank	-	-0.008	-0.003	0.007	0.000	-	0.005	-	-	-	-
BMP-2	-	0.214	0.240	0.263	0.239	0.239	0.025	10.46	20.158	0.630	31.700
Cell Lysate	-	0.205	0.223	0.230	0.219	0.219	0.013	5.94	18.675	<0.100	<LOQ (<5.355)
BAG/Gel	SN001@ 50 mg/well	0.123	0.141	0.137	0.133	0.133	0.008	6.02	12.207	<0.100	<LOQ (<8.132)
BAG/Gel	SN001@ 20 mg/well	0.176	0.186	0.185	0.182	0.182	0.006	3.30	15.981	<0.100	<LOQ (<6.277)
Nano-BAG + DBM/Gel	SN002@ 50 mg/well	0.105	0.113	0.122	0.113	0.113	0.009	7.98	10.814	>1.000	>UAL (>92.473)
Nano-BAG + DBM/Gel	SN002@ 20 mg/well	0.196	0.206	0.175	0.192	0.192	0.016	8.33	16.672	0.333	19.974
DBM/Gel	SN003@ 50 mg/well	0.166	0.167	0.127	0.153	0.153	0.023	15.03	13.780	>1.000	>UAL (>72.569)
DBM/Gel	SN003@ 20 mg/well	0.199	0.176	0.162	0.179	0.170	0.019	10.61	15.708	0.217	13.815
Wet/Frozen DBM	SN004@ 50 mg/well	0.134	0.184	0.001	0.110	0.110	0.022	20.00	10.591	>1.000	>UAL(>94.420)
Wet/Frozen DBM	SN004@ 20 mg/well	0.192	0.165	0.167	0.175	0.175	0.015	8.57	15.412	>1.000	>UAL (>64.885)

Abbreviations: ALP, alkaline phosphatase; BAG, bioactive glass; BMP-2, bone morphogenetic protein-2; DBM, demineralized bone matrix; LOQ, limit of quantification; Nano-BAG+ DBM, nano-bioactive glass and demineralized bone matrix combination; OD_{410}, optical density measured at 410 nm; %RSD, percent relative SD; UAL, upper assay limit.
Note: ALP standard curve. Slope 0.01348425, Intercept -0.03281584, 10U.

Although this study did not include mechanistic assays to directly confirm synergy between nano-BAG and DBM, prior studies have reported that combining DBM with BAG can enhance bone formation compared with each material alone. Pajamaki et al demonstrated that DBM combined with BAG improved bone regeneration in rat models compared with DBM alone.[28] The bioactive ions released by BAG, including calcium and phosphate, are known to stimulate osteoblast differentiation and matrix mineralization,[31] potentially amplifying the osteoinductive signals provided by DBM's endogenous growth factors. This interaction may underlie the enhanced ALP activity observed in our composite formulation, though further mechanistic studies are warranted to confirm this effect. Huber et al previously showed that DBM can both retain and gradually release BMP-2,[32] while Maddox et al emphasized the role of processing in DBM efficacy.[33] Our study builds on these findings by demonstrating that nano-BAG enhances DBM-mediated signaling, potentially improving BMP retention and osteoinductive outcomes.

Study Limitations and Future Research

This study has several limitations that inform the interpretation of results. The C2C12 ALP assay models early osteogenic differentiation but does not capture later stages of bone formation or the complexities of the in vivo environment, such as immune modulation, vascularization, and mechanical loading.

BMP-2 was tested at a single concentration (50 ng/mL), while nano-BAG + DBM was evaluated at 2 doses. This limits direct comparison and raises the question of whether higher BMP-2 doses could elicit comparable responses. Establishing comprehensive dose-response curves for BMP-2 and the tested materials will be important for benchmarking osteoinductive efficacy.

Additionally, only ALP activity was measured; future studies should incorporate markers of mineralization and late-stage differentiation, such as Alizarin Red staining and osteocalcin expression.

DBM used across formulations originated from different production batches, potentially introducing donor-dependent variability. Standardizing DBM sourcing would help minimize this factor. Moreover, we did not evaluate a 50:50 DBM to BAG formulation, focusing instead on the 33:33:33 ratio in the NanoFuse DBM product. Testing alternative ratios may further clarify the contributions of DBM and BAG.

Some conditions exceeded the ALP UAL, and as such, the actual magnitude of osteoinductive activity remains unknown. Serial dilutions were not performed to bring these samples within the assay's linear detection range, as the study was designed to compare relative trends rather than precise quantification at high activity levels. Addressing this in future studies will enable more accurate comparisons. Similarly, although triplicates and SDs were reported, formal statistical analyses such as analysis of variance or post hoc testing were not conducted due to the pilot nature and small sample size.

Larger studies with appropriate statistical methods, alongside in vivo investigations, are needed to validate

these findings and assess the long-term performance, scalability, and cost-effectiveness of nano-BAG + DBM in clinical applications.

Clinical Relevance

Nano-BAG + DBM combination demonstrated enhanced osteogenic activity and may offer a scalable, off-the-shelf alternative to autografts and allografts. Unlike donor-derived grafts, which are limited by availability and risk of morbidity, synthetic-biological composites like nano-BAG + DBM provide consistent composition and performance. The observed activity of nano-BAG + DBM relative to BMP-2 at the tested dose suggests that it may offer an alternative that could reduce reliance on high-dose BMP-2 formulations, which have been associated with dose-related adverse effects. Additionally, the synergy between BAG and DBM may allow for reduced DBM content per graft, improving material efficiency while maintaining biological effectiveness. This is particularly valuable in spinal fusion, non-unions, joint reconstruction, and large bone defects where reliable osteoinduction is essential. Future work should also explore the use of nano-BAG as a BMP carrier to prolong BMP-2 activity and minimize toxicity.

Furthermore, the scalable manufacturing process and synthetic components of nano-BAG + DBM could offer advantages in cost-effectiveness compared with recombinant BMP-2 products, which are often expensive and constrained by dosing-related complications. Nevertheless, clinical translation will require rigorous in vivo studies, long-term outcome assessments, and economic evaluations to validate the utility of nano-BAG+ DBM across diverse orthopedic applications.

All experimental assays were conducted by an independent contract research organization (AppTec, Inc.) following standardized, validated protocols. Data collection, assay controls, and analysis were performed according to predefined validity criteria. The study authors were not involved in the direct execution of laboratory testing, helping to further mitigate bias in data generation and interpretation.

CONCLUSION

This in vitro study of early osteogenic differentiation demonstrates that nano-BAG + DBM, a formulation combining nano-sized BAG with DBM, enhances osteoinductive potential based on increased ALP activity compared with DBM or BAG alone or BMP-2 at 50 ng/mL. This effect may reflect a synergistic effect between the release of bioactive ions and DBM growth factors. However, these findings are limited to early-stage markers, and further in vivo studies are necessary to confirm the clinical relevance, efficacy, and safety. Given its dual osteoinductive and osteoconductive properties, nano-BAG + DBM represents a promising alternative to conventional bone graft materials, particularly in clinical settings requiring reliable bone regeneration, such as spinal fusion, joint reconstruction, trauma, non-unions, and large bone defects. The ability of nano-BAG to potentiate DBM's osteoinductive activity may also improve DBM utilization efficiency, allowing for reduced graft volume without compromising biologic performance. While BMP-2 served as a benchmark in this study, the single dose tested (50 ng/mL) may have been subtherapeutic, underscoring the need for further investigation. Future studies should explore a full BMP-2 dose-response curve to determine the threshold required to match or exceed the osteoinductive potential of nano-BAG + DBM while carefully evaluating safety, cost-effectiveness, and clinical feasibility.

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