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The Effect of Growth Hormone and Calcium Silicate-Based Sealer combination on the Viability, Migration, and Osteogenic Potential of Human Periodontal Ligament Stem Cells: In Vitro Study

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The Effect of Growth Hormone and Calcium Silicate-Based Sealer combination on the Viability, Migration, and Osteogenic Potential of Human Periodontal Ligament Stem Cells: *In Vitro* Study

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ABSTRACT

Background: Numerous strategies have been proposed for the management of apical periodontitis. Nonsurgical root canal therapy represents the primary conservative option before resorting to invasive surgical interventions. This study sought to evaluate the effects of growth hormone (GH) and Calcium Silicate-Based Sealer (EndoSeal MTA, ES) on the viability, migration, and osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs)

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In Vitro.

Methods: This experimental study included a sample size of eight per group. hPDLSCs were isolated from healthy premolars and characterized using flow cytometry. The cells were exposed to GH, ES eluates, or both. Viability was assessed via MTT assay. Cell migration was evaluated using scratch wound assay. Osteogenic potential was determined via Alizarin red staining and measurement of ALP activity and RUNX2, osteocalcin, and osteopontin expression.

Results: The ES/GH group had significantly enhanced cell viability ($p < 0.01$), wound closure (99.96% by day 3), highest calcium deposition (0.672 ± 0.045 nm), ALP activity (0.0542 ± 0.0026 nm), and upregulated osteogenic gene expression.

Conclusions: Under the limitations of this study, the combination of GH and EndoSeal MTA enhances hPDLSC bioactivity, proliferation, and osteogenic potential.

Keywords cell viability, growth hormone, Calcium Silicate-Based Sealer, osteogenic potential, stem cells.

INTRODUCTION

The successful healing of periapical tissues is desirable and essential for favorable outcomes in root canal therapy (1). In nonsurgical therapy, the thorough cleaning and shaping of root canals are a critical step to eliminate organic debris and bacterial load (2). Achieving a hermetic seal through proper obturation serves as a complementary measure to ensure long-term success (3). Calcium Silicate-Based Sealers have demonstrated desirable physicochemical characteristics, excellent biocompatibility, good bond strength, enhanced fracture resistance, minimal discoloration, favorable sealer distribution, and notable osteogenic potential (4). Although these properties accelerate periapical healing after endodontic treatment, the healing of periapical lesions with Calcium Silicate-Based Sealers remains somewhat unpredictable,

highlighting the need for adjunctive biological enhancement (5).

Among these adjuncts, growth hormone (GH), a single-chain peptide secreted by somatotroph cells in the anterior pituitary gland, has shown promising effects on tissue regeneration (6). It upregulates bone morphogenetic protein expression in human dental stem cells (HDSCs) (7, 8). However, research on the combined effects of GH and Calcium Silicate-Based Sealers are rare. The present study hypothesized that the combined use of GH with EndoSeal MTA and the use of each material alone will show no significant difference in influencing the viability, migration, and osteogenic potential of human periodontal ligament stem cells (hPDLSCs).

MATERIALS AND METHODS

Study Design, ethical approval and sample size calculation:

This in vitro study was approved by the Scientific Research Ethics Committee, Faculty of Dental Medicine, Al Azhar University (Cairo, Egypt) for Research on Human Subjects with the code (EC Ref No. 898/262). The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (9). Figure 1.

Sample size was calculated based on a previous study by Collado-González et al (10). using the G*Power software (University of Düsseldorf, Düsseldorf, Germany) (11), with α-error probability 0.05 (two-tailed) and power of study 95%. The minimal sample size was calculated to be eight samples per group for each test.

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Rationale/ Justification

To address the lack of evidence on the effect of growth hormone and calcium silicate-based sealer combination on osteogenic potential of hPDLSCs. As as one of periapical lesion treatment modalities.

AIM/HYPOTHESIS

To evaluate the effect of growth hormone and calcium silicate-based sealer combination on the viability, migration, and osteogenic potential of human periodontal ligament stem cells: *in vitro* study

ETHICAL APPROVAL

Approval no. (EC Ref No. 898/262).Informed consent obtained from all donors

SAMPLES

Human PDLSCe isolated from healthy adolscent extracted healthy premolars

EXPERIMENTAL AND CONTROL GROUPS, INCLUDE INDEPENDENT VARIABLES

Endoseal MTA sealer Group

Growth Hormone group

Endoseal MTA sealer Group and Growth Hormone group(Comination)

Positive Control Group

Negative Control group

***Sample number specified in each methodolgy section**

OUTCOME(S) ASSESSED, INCLUDE DEPENDENT VARIABLES AND TYPE

Cells viability (MTT assay), (Wound Closure testing) (Scratch assay),Alizarin red assay ,ALP activity assay, PCR Osteogenic Gene Marker Expression (RUX2, Osteocalcin, Osteopontin (SPP)) In vitro

DEPENDENT VARIABLES TYPE:

Wound Closure testing :Qualitative

MTT assay, (Scratch assay),Alizarin red assay ,ALP activity assay, PCR Osteogenic Gene Marker Expression (RUX2, Osteocalcin, Osteopontin (SPP) :Quantitative

:

METHOD USED TO ASSESS THE OUTCOME (S) AND WHO ASSESSED THE OUTCOME(S)

Flow cytomerty,CD markers

Cells viability (MTT assay), (Wound Closure testing) (Scratch assay),Alizarin red assay ,ALP activity assay, PCR Osteogenic Gene Marker Expression (RUX2, Osteocalcin, Osteopontin (SPP))

RESULTS

All ES showed 100% cell viability, GH 0.1 and 0.05 µg/mL reaching 100%

ES/GH group had the highest wound closure

ES and GH increased mineralization

The ES/GH group had the highest expression levels of RUNX2, osteocalcin, and osteopontin genes

CONCLUSION(S)

The combination of GH and EndoSeal MTA enhances hPDLSC bioactivity, proliferation, and osteogenic potential.

FUNDING DETAILS

No funding

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Figure (1): PRILE 2021 flowchart outlining the methodology and principal findings involved in this study.

Material Preparation: (Eluate Preparation)

Eluates from tested materials were collected aseptically in line with ISO 10993-12. Cultured cells were exposed to the extracts and eluates as per ISO 10993-5 to simulate clinical conditions. The eluates were prepared in complete medium (Dulbecco's modified Eagle medium) DMEM with 10% (FBS, L-glutamine, and penicillin–streptomycin) and used undiluted or at serial dilutions.

Endoseal MTA (EndoSeal, Maruchi, Seoul, Korea) was mixed as per manufacturer's instructions. Sealer disks (5 mm × 2 mm) were made aseptically, sterilized with UV for 15 minutes, and cured at 37 °C for 48 hours.

Recombinant human GH (SEDICO, Egypt) was prepared as per supplier guidelines and suspended in complete medium (DMEM with 10% FBS, L-glutamine, and penicillin–streptomycin) for cell assays.

Periodontal Ligament Stem Cell Isolation:

Periodontal ligament (PDL) stem cells were isolated from three healthy premolars extracted for orthodontic reasons after obtaining informed consent, following the protocol by Dahak et al. (12). PDL fragments were digested with collagenase and dispase in DMEM/F12 with FBS and antibiotics. The cultures were maintained at 37 °C with 5% CO2, and fourth-passage cells were used for experiments with at least three replicates per group.

Characterization by MSC Surface Marker Expression:

Following the method of Feng et al. (13) and Seo et al. (14), the hPDLSCs were characterized by immunophenotyping using antibodies against CD45, CD34, CD73, CD90, CD105, and HLA-DR, Figure 2 (A).

Multilineage Differentiation Potential:

The multilineage differentiation capacity of hPDLSCs was measured in a 24-well plate using a differentiation identification kit (R&D Systems, Minneapolis, MN, USA). The cultures were then photographed on an inverted microscope (TCM 400). Images were captured using a digital camera (Canon, Woodhatch, UK), Figure 2 (B).

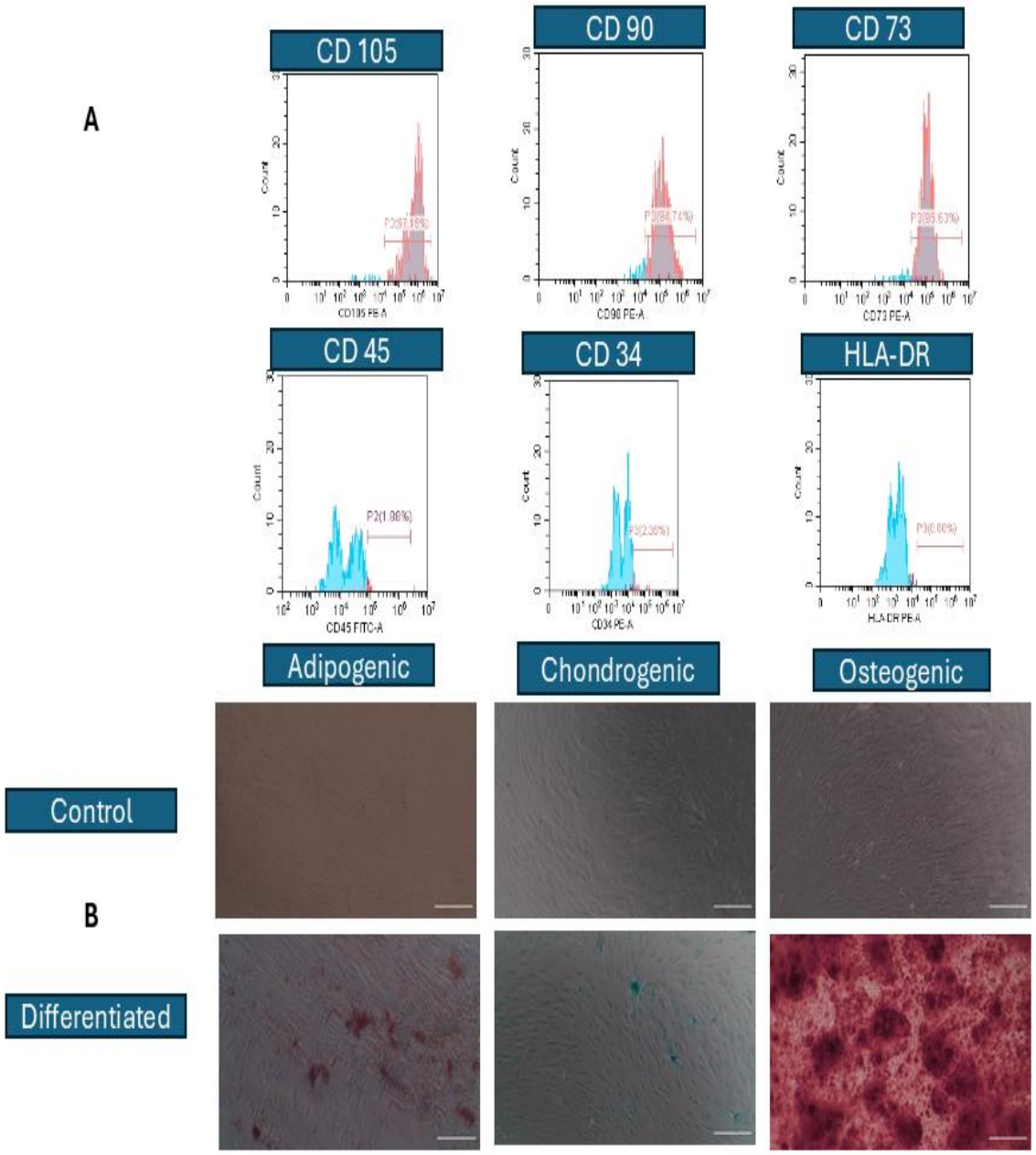


Figure (2) (A). Flow cytometry analysis revealed that 97.15% of the PDLSCs were CD105 positive, 94.74% were CD90 positive, and 95.63% were CD73 positive; only 1.88% of the hPDLSCs were CD45 positive, 2.35% were CD34 positive, and 0.00% were HLA-DR positive. (B). Oil red O was used to stain lipid droplets after adipogenic differentiation, Alcian blue was used to stain enriched proteoglycans after chondrogenic differentiation, and Alizarin red S (ARS) was used to stain the calcium-rich extracellular matrix after osteogenic differentiation.

Cell Viability Assessment (MTT Assay):

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze dilutions of the experimental sealer eluate and GH to establish their ideal concentrations. The test cells were maintained in DMEM (Sigma, St. Louis, MO, USA) at a density of 5 × 10 5 cells/well, equilibrating at 37 °C (n = 4 per sealer condition), confirming previous findings. On the following day, the cells were diluted with sealer eluate (undiluted, 1:1, 1:2, and 1:4) and GH concentrations (6.4–0.05 μg/ml) in half-log serial dilutions. After 1, 3, and 7 days, the cultures stopped. Violet formazan crystals were solubilized in DMSO and quantified at 570 (620) nm using a microplate reader (12).

Cell Proliferation and Migration Assessment (Scratch Wound Assay):

The hPDLSCs were kept on sterile six-well plates until 95%–100% confluence. Each well was wounded linearly with a sterile P200 pipette tip. The cells were then divided into four experimental conditions: one well was left untreated as a negative control (medium alone); the second was exposed to undiluted extracellular stem cell (ES) eluate, called the ES group; the third received GH, called the GH group; and the fourth was treated with both. With a phase-contrast inverted microscope, scratch digital micrographs were taken at 0, 24, 48, and 72 hours. Three-time imaging with ImageJ (National Institutes of Health, Bethesda, MD, USA)

quantified the wound areas.

Induction of Osteogenic Differentiation in hPDLSCs

The ells were cultured on a six-well plate to initiate osteogenic differentiation. On the subsequent day, the medium was altered to OsteoDiff (Miltenyi Biotec, San Diego, CA, USA). The composition comprised α-MEM, 10% fetal bovine serum, 2.5 mg/L ascorbic acid-2-phosphate (Sigma Aldrich, Steinheim, Germany), 0.1 μM dexamethasone (Sigma), and 10 mM beta-glycerophosphate (Merck, Darmstadt, Germany). The cells underwent treatment for 14 days in the media containing eluate from ES, GH, or a combination of both.

Cell Mineralization Assessment (Alizarin Red Assay):

Mineralization assay was performed 14 days after osteogenic induction as described by Dahake et al. (12). The cell cultures were fixed in 70% (v/v) ethanol and then incubated with 40 mM Alizarin red S (Sigma, St. Louis, MO, USA) for 30 minutes. Excess dye was removed, and the preparations were visualized using an inverted microscope. For the quantification of mineralization, the dye was solubilized in 10% glacial acetic acid, yielding a yellow supernatant at 405 nm (15).

Alkaline Phosphatase Enzyme Activity Assessment (ALP Assay):

For the measurement of ALP activity in hPDLSCs after 14 days of differentiation, the cells were collected and evaluated using the LabAssay™ Kit methodology (Wako Pure Chemicals, Tokyo, Japan). The wells were rinsed with PBS to remove nonadherent cells. For monolayer lysis, 0.1% Triton X-100 in 1 mL of PBS was used. The lysate was ultrasonically disrupted for 30 seconds to disaggregate. After 10 minutes of centrifugation at 12,000xg, the supernatant was collected and treated with 10 mM para-nitrophenylphosphate. After 90 minutes at 37 °C, p-NP was measured photometrically at 405 nm (16).

Osteogenic Gene Marker Expression Assessment (RUX2, Osteocalcin, and Osteopontin

(SPP)):

Runt-related transcription factor 2 (RUNX2), osteocalcin (OC), and Osteopontin (SPP) were assessed using RT-qPCR to describe cellular differentiation. Total cellular mRNA was extracted from the TRIzol-lysed samples (TRIzol, Zymo Research Corp., Irvine, CA, USA) in accordance with the manufacturer's instructions. Complementary DNA was generated utilizing the miScript Reverse Transcription Kit (QIAGEN). Transcript quantification was performed using the miScript PCR Kit. Target mRNA expression levels were standardized to β-actin.

Statistical Analysis

The Kolmogorov–Smirnov test was utilized to evaluate the normality of the data. One-way and two-way ANOVA were utilized for group comparisons, followed by Tukey's post hoc test for pairwise analysis. The significance level was set at $p < 0.05$. Data analysis was performed with SPSS 23.0 (SPSS, Inc., Chicago, IL, USA) for Windows.

RESULTS

Evaluation of Cell Viability (MTT Assay):

All ES eluates showed 100% cell viability, so undiluted eluate was used. GH concentrations above 0.05 µg/mL produced over 70% viability, with 0.1 and 0.05 µg/mL reaching 100% at all time points; therefore, 0.1 µg/mL was used with ES (Figure 3, A, B). MTT assays showed that ES/GH had the highest viability on days 1 and 3, followed by GH, and ES had the lowest; the differences were significant ($p < 0.001$). On day 7, no significant differences were found among the groups. Only the ES group showed significant changes in viability over time; the GH and ES/GH groups remained stable (Figure 4, A).

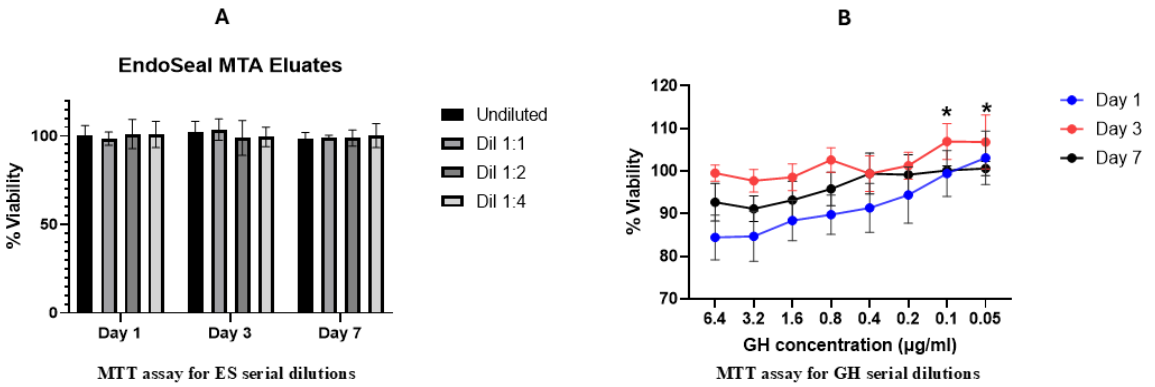


Figure (3)(A): MTT assay for EndoSeal Eluates. Serial dilutions were prepared from the undiluted eluate with 1:1, 1:2 and 1:4 ratios. No significant difference between the different dilutions and the undiluted eluate. All groups showed 100% viability on day 1,3, and 7. (B). MTT assay results using serial dilutions of GH. *Indicates the concentration at which GH possessed 100% viability or more at the three determined time points.

Evaluation of Cell Proliferation and Migration (Scratch Wound Assay):

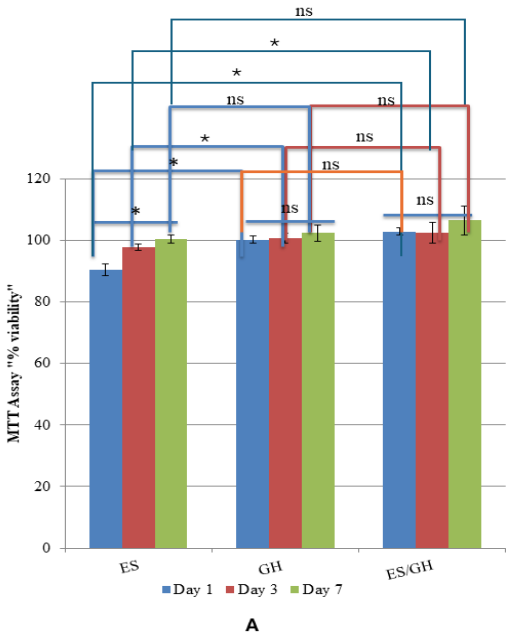
On days 1 and 2, the ES/GH group had the highest wound closure (81.48 ± 2, 98.41 ± 0.75), whereas the controls had the lowest. By day 3, wound closure increased for all groups with no significant differences (Figure 4B).

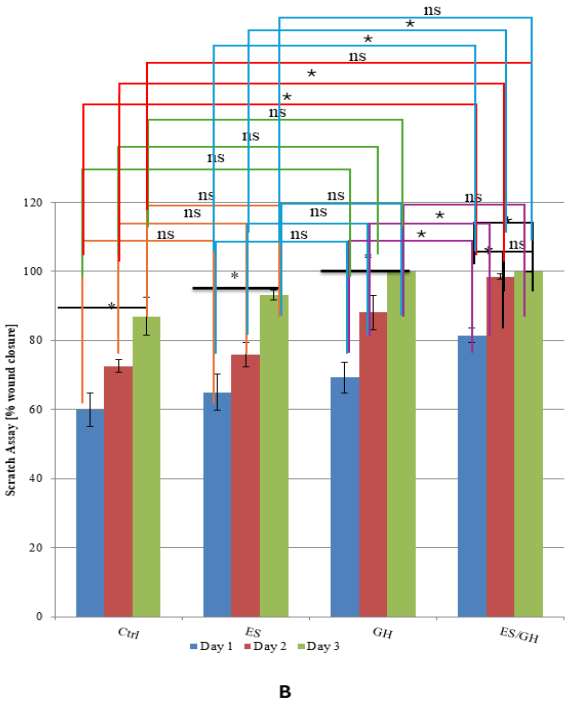
Evaluation of Cell Mineralization (Alizarin Red Assay):

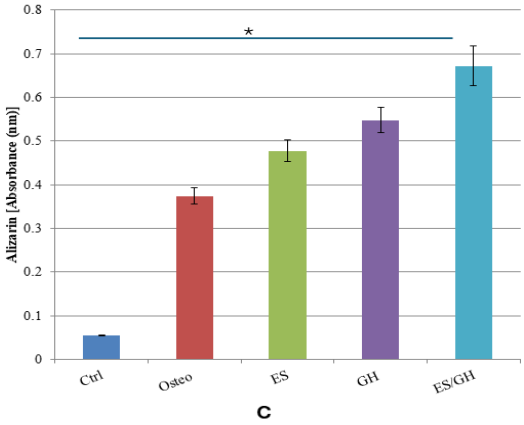
ES eluate and GH increased mineralization, but the ES/GH group had the highest calcium nodule deposition (Alizarin red absorbance 0.672, p = 0.001;(Figure 4C).

Evaluation of Alkaline Phosphatase Enzyme Activity (ALP Assay):

The ALP activity was higher in the ES and GH groups than in the controls, with the ES/GH group showing the greatest increase (0.0542, p = 0.001). No significant difference was found between the ES and GH groups (Figure 4D).







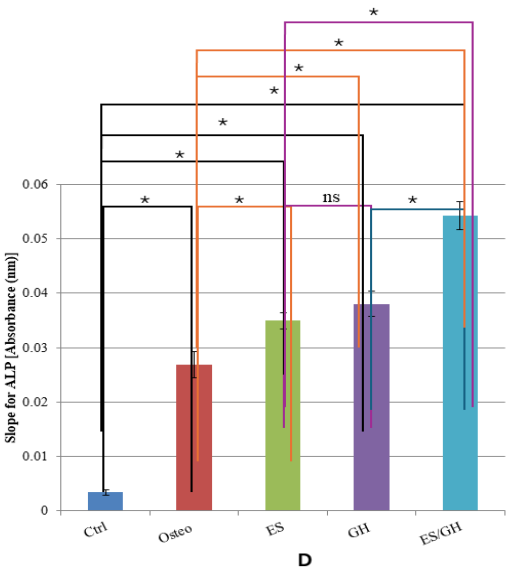


Figure (4): (A). Comparison of the percentage viability of the different groups at different times via the MTT assay. (B). Comparison between different times regarding the scratch assay (% wound closure). (C). Comparison between different groups regarding alizarin (absorbance (nm)) (D). Comparison between different groups regarding slope for ALP (absorbance (nm)).

Evaluation of Osteogenic Gene Marker Expression (RUX2, Osteocalcin, and Osteopontin (SPP)):

The ES/GH group had the highest expression levels of RUNX2, osteocalcin, and osteopontin genes ($p < 0.05$), which were significantly higher than those in the control and Osteo groups. No significant differences were found between the ES/GH and GH groups or between the ES and Osteo groups (Figure 5).

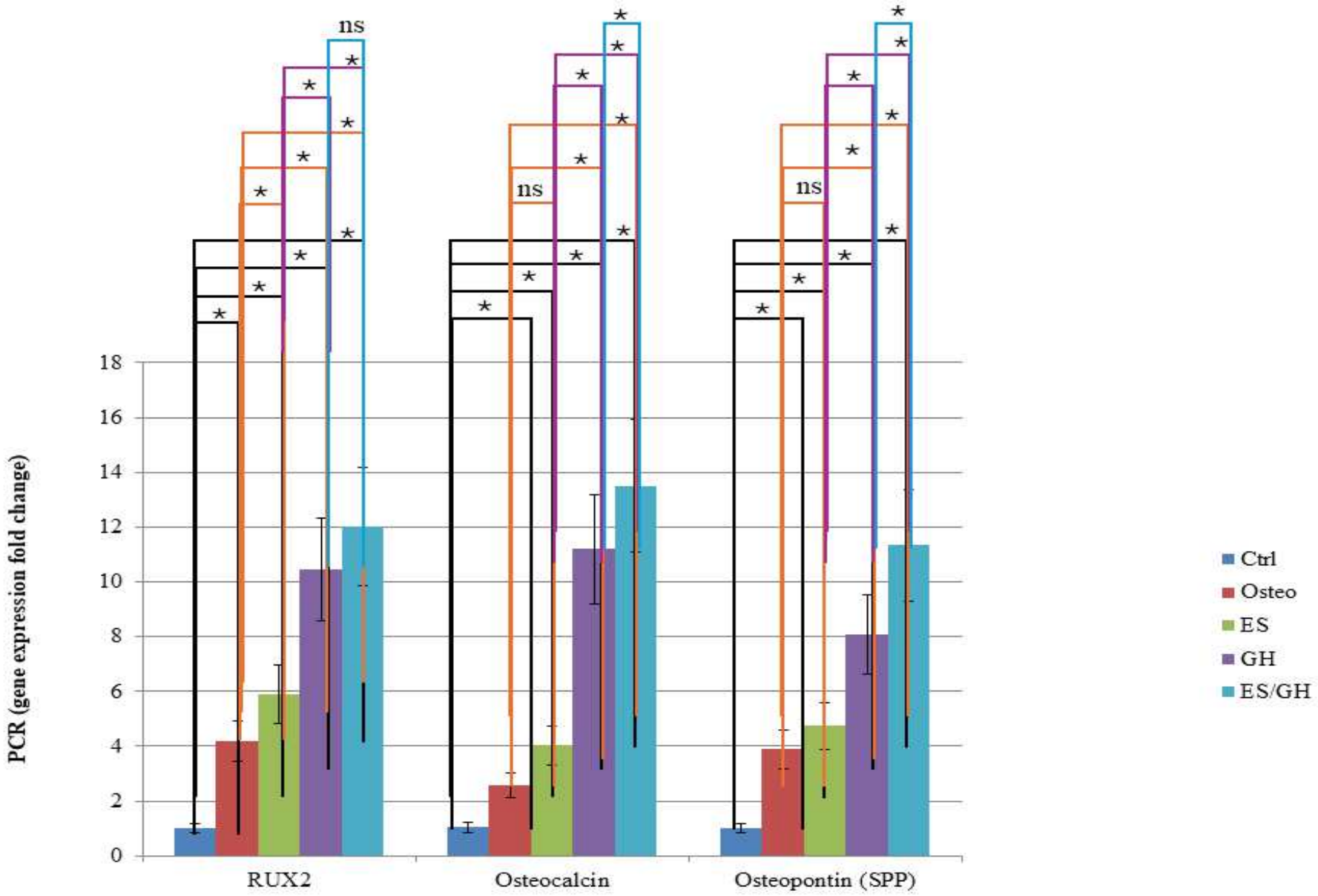


Figure (5): Comparison between different groups regarding PCR.

DISCUSSION

Following appropriate nonsurgical root canal therapy, periapical wound healing transpires (17). Biocompatible and bioactive root canal sealers may expedite the healing of apical periodontitis. This study aimed to assess the synergistic effects of GH and EndoSeal MTA (ES) on the viability and osteogenic differentiation capability of hPDLSC *In Vitro* (9).

In previous studies, GH and insulin-like growth factor I (IGF-I) upregulated BMP-2 and BMP-4 mRNA expression in human dental pulp fibroblasts, with peak induction at 4–8 hours and physiological concentrations (6, 18). MTA also improved the healing of large periapical lesions over 6 years and modulated periapical healing in rat molars, supporting its role in periapical tissue regeneration (19, 20).

The cytocompatibility of the sealer eluates and GH with hPDLSCs was evaluated by two tests. MTT test quantified cell viability, and cell migration assay assessed cell proliferation and migration (21). According to ISO 10993-5 criteria, sealers are cytotoxic if cell viability falls below 70% (20). MTT assay showed that all sealer eluates and GH (0.1, 0.05 μg/mL) achieved 100% cell viability. The hPDLSCs treated with ES/GH or GH alone showed the highest viability, consistent with prior reports stating that EndoSeal MTA enhances cell viability and migration over other sealers (7, 22-24). EndoSeal MTA has minimal cytotoxicity at clinical doses (25). Cell migration assay showed that the ES/GH group had the highest wound closure, indicating enhanced cell adhesion, growth, and differentiation. This result aligns with previous findings stating that MTA and GH together improve cell adhesion (7). MTA releases ions such as calcium and silicon, activating ERK and p38 MAPK pathways to promote chemotaxis. GH enhances cell migration by increasing chemokine receptors and actin remodeling. Together, they may accelerate hPDLSC migration for tissue regeneration.

Osteogenic potential was assessed by Alizarin red S staining for mineralization, ALP

activity as an early marker, and osteogenic gene expression measurement. Runx2, osteocalcin, and osteopontin were used to confirm osteoblast differentiation and commitment (25-29). Alizarin red and ALP assays showed that the ES/GH and GH groups had more calcified nodules than the ES alone, Osteo, or control group (7, 12, 22). GH synergizes with these pathways by increasing IGF-1 production, which enhances RUNX2 expression and osteoblastic activity (30).

With regard to osteogenic gene markers (RUNX2, osteocalcin, and SPP), the ES/GH group showed the highest percentage of gene expression. This observation is in accordance with previous results showing that EndoSeal MTA and GH achieved high levels of osteogenic gene expression (31,32). Regarding RUNX2, MTA releases calcium (Ca^{2+}) and silicon (Si^{4+}) ions, which stimulate BMP/Smad and Wnt/β-catenin pathways. These pathways directly upregulate RUNX2, a master transcription factor for osteoblast differentiation. While GH stimulates IGF-1 production in target cells, IGF-1 activates PI3K/Akt and MAPK/ERK pathways, which enhance RUNX2 expression and activity (30).

For osteocalcin, Calcium Silicate-Based Sealers continuously release Ca^{2+} and Si^{4+} ions. These ions stimulate osteoblastic differentiation by activating BMP/Smad and Wnt/β-catenin pathways, both of which drive osteocalcin transcription via RUNX2. GH stimulates local IGF-1 release, which activates PI3K/Akt and MAPK/ERK pathways (30, 32). When GH and MTA are applied together, MTA supplies bioactive ionic cues and alkaline pH and then activates BMP/Smad and Wnt/β-catenin signaling, while GH supplies systemic and local anabolic cues via IGF-1, ERK, and PI3K/Akt pathways. Their combination sustains the signal over time, ensuring higher and prolonged OCN gene expression compared with that when either agent alone was applied.

Finally, for osteopontin, MTA stimulates SPP expression through integrin-mediated

signaling and activation of p38 MAPK and ERK1/2 pathways (31). GH enhances osteopontin expression indirectly by promoting osteoblast maturation and matrix protein synthesis through IGF-1-dependent transcriptional regulation (34-37).

CONCLUSIONS

Combining GH with EndoSeal MTA has led to noticeably better outcomes than the use of either material alone. This combination increased the viability, migration, and bone-forming potential of hPDLSCs. The high ALP activity, great calcium deposition, and strong expression of key osteogenic markers all indicate a clear regenerative effect. These results suggest that this approach could offer a promising nonsurgical option for supporting bone healing in periapical lesions. Further research is needed to explore its potential clinical applications.

Declarations

Ethics approval and consent to participate

This in vitro study was approved by the Scientific Research Ethics Committee, Faculty of Dental Medicine, Al Azhar University (Cairo, Egypt) for Research on Human Subjects with the code (EC Ref No. 898/262). The study was conducted in accordance with the Declaration of Helsinki and relevant institutional and national guidelines and regulations. Extracted human teeth used in this in vitro study were obtained after written informed consent had been obtained from the patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

All authors contributed equally. All authors read and approved the final manuscript.

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List of abbreviation

Abbreviation	Full Term
GH	Growth Hormone
ES	EndoSeal MTA
MTA	Mineral Trioxide Aggregate
hPDLSCs	Human Periodontal Ligament Stem Cells
PDL	Periodontal Ligament
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
MSC	Mesenchymal Stem Cell
HLA-DR	Human Leukocyte Antigen–DR
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Abbreviation	Full Term
DMSO	Dimethyl Sulfoxide
ALP	Alkaline Phosphatase
ARS	Alizarin Red S
RUNX2	Runt-related Transcription Factor 2
OC	Osteocalcin
SPP	Osteopontin
PBS	Phosphate-Buffered Saline
p-NP	para-Nitrophenol
PRILE	Preferred Reporting Items for Laboratory Studies in Endodontology
ISO	International Organization for Standardization
IGF-I	Insulin-like Growth Factor I
BMP	Bone Morphogenetic Protein
MAPK	Mitogen-Activated Protein Kinase
ERK	Extracellular Signal-Regulated Kinase
PI3K/Akt	Phosphoinositide 3-Kinase/Protein Kinase B Pathway

383 Figure legends

Figure (1)	PRILE 2021 flowchart outlining the methodology and principal findings involved in this study.
Figure (2)	(A). Flow cytometry analysis revealed that 97.15% of the PDLSCs were CD105 positive, 94.74% were CD90 positive, and 95.63% were CD73

	positive; only 1.88% of the hPDLSCs were CD45 positive, 2.35% were CD34 positive, and 0.00% were HLA-DR positive. (B). Oil red O was used to stain lipid droplets after adipogenic differentiation, Alcian blue was used to stain enriched proteoglycans after chondrogenic differentiation, and Alizarin red S (ARS) was used to stain the calcium-rich extracellular matrix after osteogenic differentiation.
Figure (3)	(A): MTT assay for EndoSeal Eluates. Serial dilutions were prepared from the undiluted eluate with 1:1, 1:2 and 1:4 ratios. No significant difference between the different dilutions and the undiluted eluate. All groups showed 100% viability on day 1,3, and 7. (B). MTT assay results using serial dilutions of GH. *Indicates the concentration at which GH possessed 100% viability or more at the three determined time points.
Figure (4)	(A). Comparison of the percentage viability of the different groups at different times via the MTT assay. (B). Comparison between different times regarding the scratch assay (% wound closure). (C). Comparison between different groups regarding alizarin (absorbance (nm)) (D). Comparison between different groups regarding slope for ALP (absorbance (nm)).
Figure (5)	Comparison between different groups regarding PCR

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