ISSN 0973-2063 (online) 0973-8894 (print)

Bioinformation 22(7): 4521-4527 (2026)

BIOINFORMATION
Discovery at the interface of physical and biological sciences

BIOMEDICAL INFORMATICS

www.bioinformation.net

Volume 22(7)

Research Article

Received July 1, 2026; Revised July 31, 2026; Accepted July 31, 2026, Published July 31, 2026

DOI: 10.6026/973206300224521

SJIF 2026 (Scientific Journal Impact Factor for 2026) = 8.478
2022 Impact Factor (2023 Clarivate Inc. release) is 1.9

Edited by P Kangueane

Citation: Chaudhari *et al.* Bioinformation 22(7): 4521-4527 (2026)

Effect of EDTA demineralization duration on cryogenically nanogrinded dentin matrix: A pilot study

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ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 22(7): 4521-4527 (2026)

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Abstract:
The lack of standardized demineralization protocols limits the bioactivity of dentin-derived biomaterials. Therefore, it is of interest to evaluate the effect of different durations of 12% EDTA demineralization on cryogenically nanogrinded dentin matrix (nDDM). Nanogrinded dentin samples were treated with 12% EDTA for 0, 2, 5, 10, 30 and 60 minutes and analyzed using Fourier Transform Infrared (FTIR) spectroscopy, while biological performance was assessed by ELISA, MTT assay and qRT-PCR in human dental pulp stem cells (hDPSCs). FTIR showed preservation of non-collagen associated amide bands and the 5-minute group exhibited the highest cytocompatibility, increased BMP-2 and TGF-β1 release and enhanced expression of DMP-1 and DSPP. Prolonged demineralization beyond 5 minutes resulted in reduced biological performance and alterations in matrix-associated spectral characteristics, indicating excessive demineralization. Thus, data shows that 12% EDTA demineralization for 5 minutes provides an optimal balance between mineral removal and non-collagenous matrix preservation, supporting the regenerative potential for vital pulp therapy.

Keywords: Demineralized dentin matrix, Ethylenediaminetetraacetic acid (EDTA) demineralization, cryogenic nanogrinding, regenerative endodontics, fourier transform infrared spectroscopy (FTIR) spectroscopy, human dental pulp stem cells.

Background:
Vital pulp therapy requires biomaterials that not only seal the pulp tissue but also promote regeneration of the dentin–pulp complex **[1].** Conventional materials such as calcium hydroxide and mineral trioxide aggregate mainly function as passive barriers and do not fully reproduce the biological properties of natural dentin **[2].** Dentin is a mineralized connective tissue composed of inorganic minerals, organic matrix and water. The organic matrix contains type I collagen and several growth factors that regulate odontoblast differentiation and reparative dentinogenesis **[3].** Demineralized dentin matrix (DDM) has emerged as a biologically active scaffold because of its collagen-rich structure and retained bioactive molecules **[4].** Demineralization exposes the organic matrix and facilitates the release of growth factors, thereby enhancing regenerative potential **[5].** The biological performance of dentin matrix depends on particle size, preparation method and degree of demineralization **[6].** Nanogrinded dentin matrix provides increased surface area and improved bioactive molecule availability, which enhances cellular attachment and differentiation **[7].** The demineralization method significantly influences the structural integrity and bioactivity of dentin matrix **[8].** Ethylenediaminetetraacetic acid (EDTA) enables controlled mineral removal while preserving collagen architecture and growth factors, making it suitable for regenerative applications **[8].** The effectiveness of EDTA-mediated demineralization is influenced by exposure duration. Insufficient demineralization may limit the release of bioactive molecules, whereas prolonged exposure may adversely affect collagen and non-collagenous protein integrity and biological activity **[9].** Recent studies have highlighted the potential of dentin-derived biomaterials in regenerative endodontics; however, standardized protocols for preparation of nanostructured dentin matrices remain unavailable. Fourier Transform Infrared Spectroscopy (FTIR) is a reliable technique for evaluating mineral and organic matrix alterations following demineralization **[10, 11].** To the best of our knowledge, no previous study has investigated the influence of different durations of 12% EDTA demineralization on cryogenically nanogrinded dentin matrix (nDDM) **[10, 12].** Therefore, it is of interest to evaluate the physicochemical and biological effects of different EDTA exposure durations and identify an optimal demineralization protocol for regenerative endodontic applications.

Materials and Methods:
Study design:
This *in vitro* pilot study evaluated the effect of different durations of 12% ethylenediaminetetraacetic acid (EDTA)-mediated demineralization on nanogrinded dentin matrix (nDDM). Physicochemical properties were assessed using Fourier Transform Infrared (FTIR) spectroscopy and biological performance was evaluated using cytocompatibility (MTT assay), growth factor release (ELISA) and odontogenic gene

expression (qRT-PCR). The objective was to identify the optimal demineralization duration for preserving collagen matrix and bioactive proteins.

Ethical considerations:
The study was approved by the Institutional Ethics Committee of Jaipur Dental College, Jaipur (Approval No: MUVU/PHD/2023/1158). Informed consent was obtained from patients for the use of extracted teeth.

Sample size and grouping:
As a pilot study, formal sample size calculation was not performed. Samples were randomly allocated into six groups: T0 (0 min, control), T2 (2 min), T5 (5 min), T10 (10 min), T30 (30 min) and T60 (60 min).

Collection and preparation of dentin matrix:
Extracted human permanent teeth indicated for periodontal or orthodontic reasons were collected. Teeth with caries, restorations, fractures, or developmental defects were excluded. Teeth were cleaned using ultrasonic scalers and disinfected in 0.5% chloramine-T solution for 24 hours, followed by rinsing with sterile saline. Teeth were sectioned using a diamond disc under water irrigation. Enamel, cementum and pulp tissue were removed to isolate dentin, which was ground using a sterile metallic pestle. Dentin samples were sterilized by gamma irradiation (15.79 kGy) at a certified facility.

Preparation of nanogrinded dentin matrix:
Dentin samples were dried and subjected to cryogenic nanogrinding using a vibratory cryomill (Fritsch Pulversette Cryomill 2013, Germany) under liquid nitrogen at temperatures below −150°C to obtain nanoscale particles **[13]**. The overall preparation protocol of nanogrinded dentin matrix is illustrated in **Figure 1**.

EDTA treatment and FTIR analysis:
Nanogrinded dentin samples were demineralized using 12% EDTA for respective durations (T0–T60) **[6].** Samples were rinsed with distilled water and saline to remove residual EDTA. FTIR analysis was performed using a Perkin Elmer UATR Spectrum Two spectrometer in the range of 4000–400 cm^{-1}. Peaks corresponding to amide I, II and III (organic matrix) and phosphate and carbonate groups (mineral phase) were analyzed.

Preparation of nDDM and MTA extracts:
NDDM from each group was incubated in complete culture medium for 24 hours to obtain conditioned extracts, followed by filtration using a 0.22 µm membrane filter. Mineral trioxide aggregate (ProRoot MTA, Dentsply Tulsa Dental, USA) was prepared according to manufacturer's instructions, allowed to set, incubated in culture medium for 24 hours and filtered before use **[14, 15].**

Cell culture:
Human dental pulp stem cells (hDPSCs) were obtained from the institutional biobank of Saveetha Dental College, Chennai. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose; Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin and maintained at 37°C in 5% CO_2.

Cytocompatibility assessment (MTT Assay):
Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 hours. Cells were treated with extracts from the T5 group and MTA group. After 1, 7, 14 and 21 days, MTT reagent was added and incubated for 4 hours. Formazan crystals were dissolved in dimethyl sulfoxide and absorbance was measured at 570 nm **[16].**

Growth factor quantification (ELISA):
Supernatants were analyzed for BMP-2 and TGF-β1 using ELISA kits (Krishgen Biosystems, India) according to manufacturer's instructions. Optical density was measured at 450 nm **[17].**

Gene expression analysis (qRT-PCR):
Total RNA was extracted (Takara Bio, Japan) and cDNA was synthesized using PrimeScript™ RT Reagent Kit. qRT-PCR was performed using TB Green® Premix Ex Taq™ II (Takara Bio). Target genes included DMP-1 and DSPP, with GAPDH as the housekeeping gene. Relative expression was calculated using the 2^-ΔΔCt method **[18].**

Statistical analysis:
As a pilot study, formal sample size calculation and inferential statistical analysis were not performed. Data were analyzed descriptively. FTIR results were evaluated qualitatively and semi-quantitatively. Biological assay values (MTT, ELISA, qRT-PCR) were expressed as mean of duplicate experiments.

Results:
FTIR spectroscopy showed distinct spectral variations among dentin samples subjected to different EDTA durations (T0–T60) (**Figure 2**). The control group (T0) showed prominent phosphate (PO_4^{3-}) peaks at ~1005–1010 cm^{-1} and clear amide I (~1643 cm^{-1}) and amide II (~1411 cm^{-1}) bands. With increasing EDTA exposure, a progressive reduction in phosphate peak intensity was observed. Amide bands showed slight shifts toward lower wavenumbers and carboxylate (COO^-) bands became more prominent in treated groups. At T2, a reduction in phosphate intensity with shifts in amide I (~1636–1638 cm^{-1}) and amide II (~1406–1414 cm^{-1}) was observed. At T5, increased intensity of organic matrix-related peaks and more distinct COO^- bands were noted. At longer durations (T10–T60), further reduction in mineral-associated peaks was observed, along with variation in organic matrix-associated bands. Absorbance values of selected FTIR bands are presented in **Table 1**. The phosphate band at 1021 cm^{-1} decreased from T0 (0.0510) to lower values at subsequent time points. Carboxylate-associated bands at 1408 cm^{-1} and 1577 cm^{-1} showed an increasing trend up to intermediate durations, followed by variation at later time

ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 22(7): 4521-4527 (2026)

points. The carbonate band at 872 cm^{-1} showed no consistent trend. Carboxylate Signal Changes The asymmetric COO^- band (~1577 cm^{-1}) increased and reached higher intensity at T5 compared to T0. The symmetric COO^- band (~1405–1408 cm^{-1}) also showed increased intensity following EDTA treatment. At later time points (T10–T60), a relative decrease in these bands was observed. FTIR analysis demonstrated a time-dependent reduction in phosphate peaks with increasing EDTA exposure. Organic matrix-related peaks showed increased prominence at intermediate durations, with variation at extended exposure times. Cytocompatibility assessment (MTT assay) demonstrated that Nanogrinded DDM (T5) showed higher cell viability compared to control and MTA groups at all-time points (Day 1, 7, 14 and 21) (**Figure 3**). Cell viability increased from Day 1 to Day 14 and remained stable at Day 21. The MTA group showed moderate viability, while the control group showed baseline levels. Growth factor analysis (ELISA) revealed that BMP-2: BMP-2 levels were higher in the nanogrinded DDM group compared to control and MTA at both Day 7 and Day 14 (**Figure 4**), with increased levels at Day 14. TGF-β1 levels were higher in the nanogrinded DDM group compared to control and MTA groups at both time points, with maximum levels at Day 14 (**Figure 4**). Gene Expression (qRT-PCR) showed DMP-1: DMP-1 expression was higher in the nanogrinded DDM group compared to control and MTA groups at Day 7, 14 and 21 (**Figure 5**), with increased expression at later time points. DSPP expression was higher in the nanogrinded DDM group compared to control and MTA groups, with peak expression at Day 14 and sustained levels at Day 21 (**Figure 5**).

Table 1: Absorbance values of selected FTIR bands at different time intervals

	T0	T2	T5	T10	T30	T60
Band 872 (cm^{-1})/Band A	0.0139	0.0119	0.0149	0.0048	0.0028	0.0086
Band 1021(cm^{-1})/ Band B	0.051	0.0362	0.0532	0.0193	0.017	0.0379
Band 1408 (cm^{-1})/ Band C1	0.0163	0.0202	0.0207	0.026	0.0265	0.0259
Band 1577 (cm^{-1})/Band C2	0.011	0.0196	0.0248	0.0271	0.033	0.028

Figure 1: Preparation of nanogrinded dentin matrix

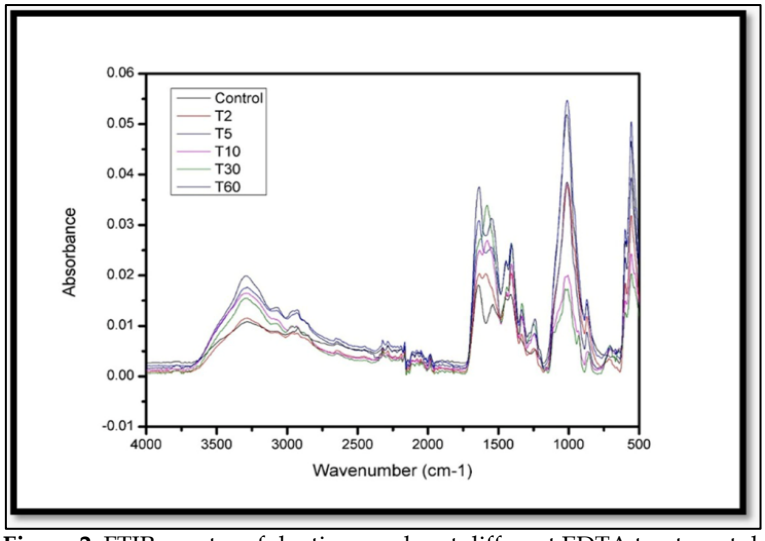

Figure 2: FTIR spectra of dentin samples at different EDTA treatment durations

ISSN 0973-2063 (online) 0973-8894 (print)
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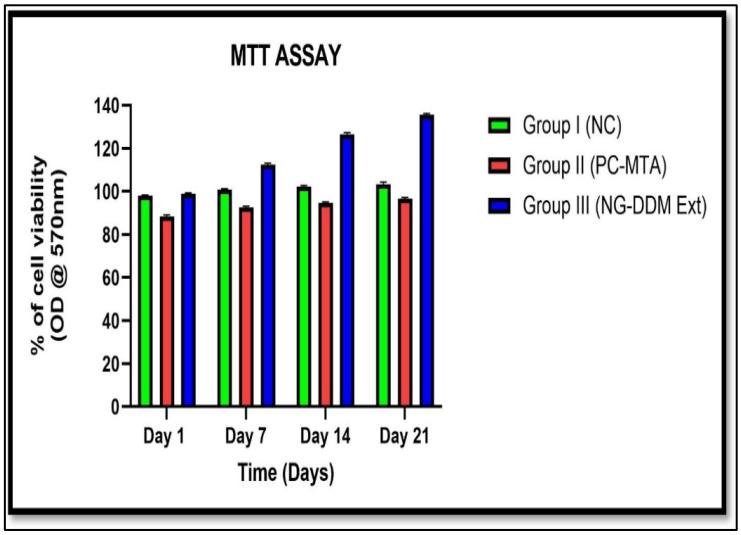

Figure 3: MTT assay – cytocompatibility of hDPSCs

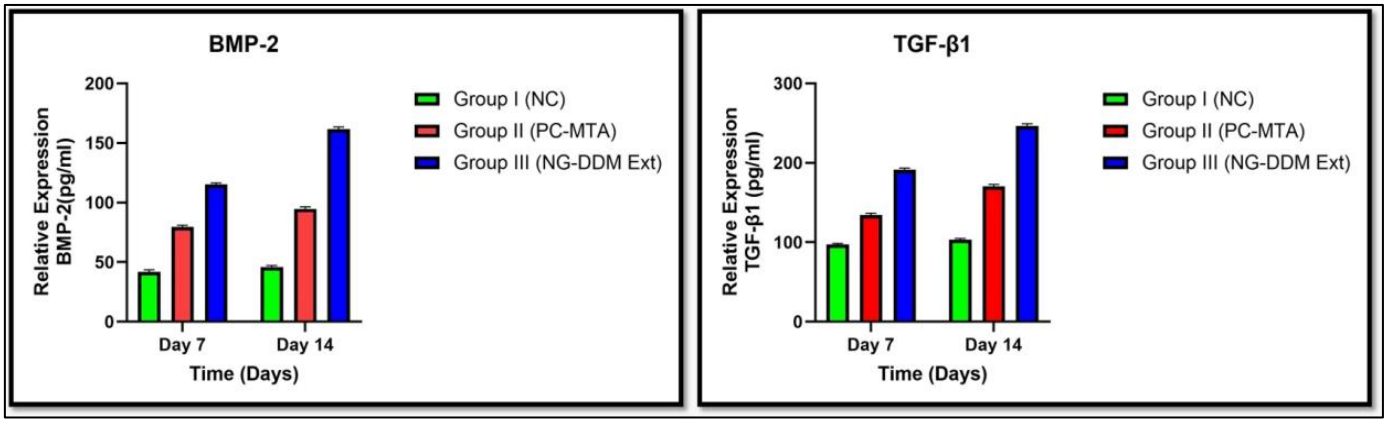

Figure 4: ELISA – BMP-2 & TGF-β1 secretion by hDPSCs

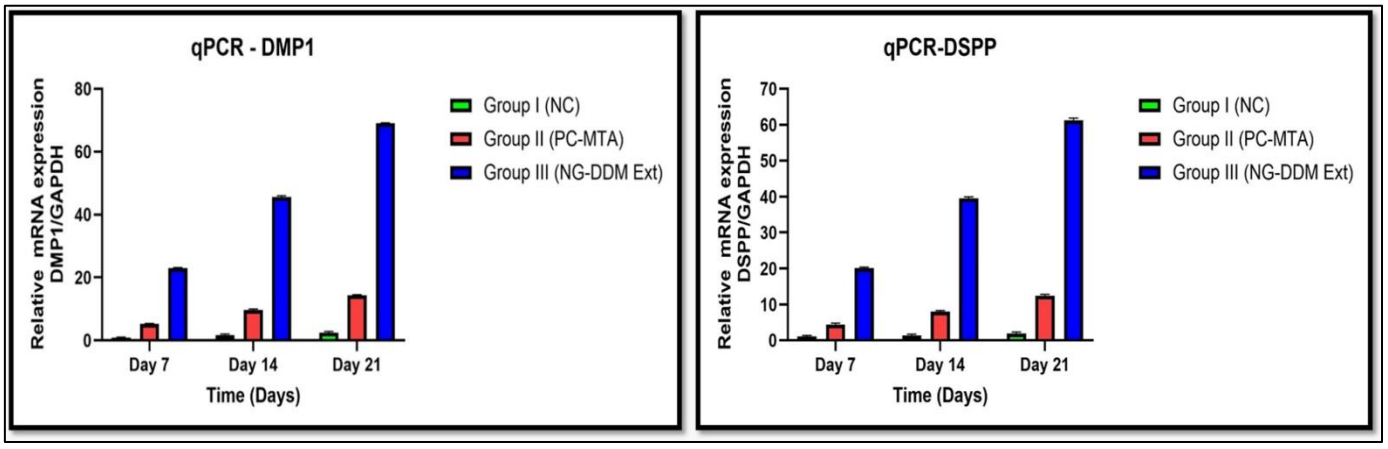

Figure 5: qRT-PCR – DMP-1 & DSPP gene expression

Discussion:

The present pilot study evaluated the effect of different durations of EDTA-mediated demineralization on nanogrinded dentin matrix (nDDM) with respect to physicochemical characteristics and biological performance. Dentin matrix acts as a reservoir of bioactive molecules, including bone

morphogenetic proteins and transforming growth factor-β, which are essential for dentin regeneration and pulp healing **[19, 20]**. Demineralized dentin matrix (DDM) has been investigated as a regenerative biomaterial due to its structural and biochemical similarity to bone **[21].** Controlled demineralization exposes the collagen matrix and facilitates the release of non-collagenous proteins, thereby enhancing biological activity **[22].** Sterilization of dentin-derived biomaterials is essential for clinical and *in vitro* use **[23].** Gamma irradiation is an effective method due to its penetration capacity and ability to eliminate microbial contamination **[24].** In the present study, irradiation at 15.79 kGy preserved the structural integrity of dentin, as supported by the maintenance of FTIR spectral characteristics. Cryogenic nanogrinding was used to obtain nanoscale dentin particles. This method minimizes thermal degradation and preserves collagen and associated bioactive components **[9].** Reduction in particle size increases surface area, which may enhance the availability of bioactive molecules. Previous studies have reported improved biological performance with reduced particle size **[25].** EDTA, as a chelating agent, enables controlled removal of the mineral phase while preserving the organic matrix **[26].** FTIR findings in the present study demonstrated a reduction in phosphate peaks with preservation of amide bands, indicating effective demineralization with retention of organic components which is consistent with previous reports on extended demineralization **[8].** Among the tested durations, 12% EDTA for 5 minutes showed a balanced profile between mineral removal and matrix preservation. Prolonged exposure resulted in variation in organic-related peaks, suggesting potential alteration of matrix components. Biological findings were consistent with the physicochemical observations. NDDM treated with 12% EDTA for 5 minutes demonstrated higher cell viability compared to control and MTA. This may be attributed to preservation of the dentin extracellular matrix, which supports cellular attachment and proliferation **[27, 5].** EDTA-mediated demineralization also preserves bioactive proteins by minimizing denaturation of the organic matrix **[27].** Increased BMP-2 and TGF-β1 levels observed in the present study indicate enhanced biological activity of nDDM. These growth factors are involved in cell proliferation, differentiation and matrix formation, contributing to dentin regeneration **[28].** The observed increase at later time points suggests sustained release following demineralization. Upregulation of odontogenic markers DMP-1 and DSPP further indicates differentiation of human dental pulp stem cells into odontoblast-like cells **[28].** These findings are consistent with previous reports on dentin-derived biomaterials **[29].** Under the conditions of the present study, Mineral trioxide aggregate (MTA) showed comparatively lower biological activity. Although MTA is biocompatible and promotes healing, its effect is primarily related to calcium ion release and alkaline pH rather than intrinsic biological signaling **[29].** In contrast, nDDM provides both a structural scaffold and endogenous bioactive molecules that may enhance cellular responses and tissue reneration **[21].** Other calcium silicate-based materials, such as Biodentine, show improved bioactivity but lack the biological complexity of dentin-derived matrices **[30].** Previous studies have reported EDTA demineralization durations of 10–15 minutes **[8].** However, limited data are available on nanogrinded dentin matrix, indicating the novelty of the present study. These findings may contribute toward developing a standardized protocol for nDDM preparation in regenerative applications. This study has limitations, including its *in vitro* design, small sample size and absence of inferential statistical analysis. Further studies with larger sample sizes, ultrastructural characterizations, advanced protein analysis and *in vivo* validation are required.

Conclusion:

Cryogenically nanogrinded DDM prepared using 12% EDTA for 5 minutes showed favorable biological performance with enhanced cytocompatibility, increased growth factor release and improved odontogenic differentiation compared to MTA. Cryogenic nanogrinding preserved collagen structure and non-collagenous bioactive components, while gamma irradiation provided effective sterilization without compromising matrix integrity. These findings suggest that this preparation protocol may serve as a promising approach for developing a biologically active dentin matrix for regenerative endodontic applications.

Advancement to knowledge:

This study identifies an optimal 5-minute 12% EDTA demineralization protocol for cryogenically nanogrinded dentin matrix. The findings show improved preservation of non-collagenous protein and collagen structure, enhanced growth factor release and increased odontogenic differentiation of hDPSCs. The study contributes toward standardization of biologically active dentin-derived scaffolds for regenerative endodontic applications.

Acknowledgment:

This *in vitro* pilot study forms a part of the PhD thesis work of the first author submitted to Jaipur dental college, Maharaj Vinayak Global University, Jaipur. The authors acknowledge Indian Institute of Technology, Kanpur for providing facilities for cryogenic nanogrinding. The authors also thank AV processors for gamma irradiation of the samples. The authors acknowledge Saveetha Dental College for providing hDPSC samples getting through their biobank facility.

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