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Assessment of Different Gingival Tissue Preparation Protocols for TNF-α Detection Using ELISA

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Abstract

Background: Tumor necrosis factor-alpha (TNF-α) is a critical pro-inflammatory cytokine involved in the pathogenesis of periodontitis. Accurate quantification of TNF-α from gingival tissues is essential for biomarker discovery and mechanistic studies. However, variability in tissue preparation protocols can significantly affect the yield and reliability of ELISA-based cytokine estimation.

Objective: To compare three distinct gingival tissue preparation protocols—mechanical homogenization in RIPA buffer, sonication in PBS with protease inhibitors, and enzymatic digestion followed by homogenization—for their efficiency in recovering TNF-α, as measured by ELISA.

Methods: Gingival tissue samples (n=30) from healthy individuals were divided and processed using the three protocols. Protein concentrations were normalized to 100 µg/mL, and TNF-α levels were quantified using a commercial ELISA kit. Data were statistically analyzed using one-way ANOVA and Tukey's post hoc test.

Results: Protocol B (PBS + sonication) yielded the highest mean TNF-α concentration (35.04 ± 3.75 pg/mL), significantly surpassing Protocol A (27.99 ± 4.97 pg/mL) and Protocol C (27.72 ± 4.76 pg/mL) ($p < 0.001$). No significant difference was observed between Protocols A and C.

Conclusion: The PBS-sonication protocol demonstrated superior efficiency in TNF-α recovery from gingival tissues, likely due to effective cell lysis with minimal protein degradation or interference. This method is recommended as a standardized approach for ELISA-based cytokine quantification in periodontal research, with potential applicability to other inflammatory biomarkers.

Keywords: Periodontal diseases, ELISA kit, Cytokines, Tobacco

Introduction

Periodontal diseases, particularly chronic periodontitis, are among the most prevalent inflammatory conditions affecting the oral cavity. They are characterized by

progressive destruction of the gingiva, periodontal ligament, and alveolar bone, ultimately leading to tooth loss if left untreated[1]. The pathogenesis of periodontitis is primarily driven by the host immune-inflammatory response to microbial biofilms, with a complex interplay of pro-inflammatory and anti-inflammatory cytokines mediating tissue breakdown and repair[2]. Among these, tumor necrosis factor-alpha (TNF-α) has emerged as a key pro-inflammatory mediator involved in the initiation and progression of periodontal inflammation[3,4].

TNF-α is primarily produced by activated macrophages and other immune cells in response to bacterial antigens, and it contributes to periodontal tissue destruction by promoting the expression of matrix metalloproteinases (MMPs), enhancing osteoclastogenesis, and inducing apoptosis in resident cells of the periodontium (5,6). Its levels have been found to be significantly elevated in gingival tissues, gingival crevicular fluid, and serum of patients with periodontitis compared to healthy individuals[7]. Consequently, accurate and reproducible quantification of TNF-α from gingival tissue samples is essential for understanding its role in disease pathogenesis and for evaluating potential therapeutic interventions[8].

The enzyme-linked immunosorbent assay (ELISA) remains one of the most widely used techniques for cytokine quantification due to its high sensitivity, specificity, and quantitative precision[9]. However, the accuracy of ELISA results is highly dependent on the quality and consistency of the sample processing protocol, particularly in solid tissues like gingiva, where fibrous and vascular components can affect protein extraction efficiency[10]. Various factors including the type of lysis buffer, inclusion of protease inhibitors, tissue homogenization method (mechanical, sonication, or enzymatic), and duration of extraction influence the yield and detectability of cytokines such as TNF-α[11,12]. Inadequate or inconsistent tissue preparation can lead to significant variability and loss of target proteins, thereby compromising data integrity and reproducibility across studies[13].

Despite the central role of TNF-α in periodontal inflammation and the widespread use of ELISA for its detection, there is a notable lack of standardized protocols for gingival tissue processing. Most studies employ different buffers and homogenization strategies without validating or comparing their efficiencies. This methodological gap necessitates a systematic evaluation of commonly used tissue preparation protocols to identify the most effective approach for extracting biologically active TNF-α from gingival tissues.

Therefore, the present study aims to assess and compare different gingival tissue preparation protocols for TNF-α quantification using ELISA. By identifying a standardized and optimized method, this study seeks to enhance the accuracy and reproducibility of TNF-α estimation, thereby contributing to improved biomarker research in periodontal disease.

Materials and Methods

Study Design and Ethics

This was an in vitro comparative study conducted to evaluate three gingival tissue preparation protocols for tumor necrosis factor-alpha (TNF-α) quantification using ELISA. The study was approved by the Institutional Ethics Committee of All India Institute of Medical Sciences, Mangalagiri (Approval No: AIIMS/MG/ IEC/2022-23/213 and dated November 11, 2022), and informed consent was obtained from all participants. The study adhered to the principles of the Declaration of Helsinki.

Tissue Collection

Gingival tissue samples were collected from 30 periodontally healthy individuals (aged 25–55 years) undergoing flap surgery or therapeutic tooth extraction. Exclusion criteria included systemic inflammatory diseases, recent antibiotic or anti-inflammatory therapy (within 3 months), tobacco use, and pregnancy. Freshly excised tissues (~20–30 mg) were rinsed in cold phosphate-buffered saline (PBS), and stored at –80°C until processing.

Sample Processing Protocols

Each gingival tissue sample was divided into three equal portions and subjected to one of the following protocols:

- **Protocol A – Mechanical Homogenization with RIPA Buffer**

  Samples were homogenized on ice in 300 µL RIPA buffer (Thermo Fisher Scientific) with protease inhibitors (Sigma-Aldrich) using a motorized homogenizer. The lysates were incubated on ice for 30 minutes and centrifuged at 12,000 × g for 15 minutes at 4°C. Supernatants were collected and stored at –80°C.

- **Protocol B – Sonication with PBS + Protease Inhibitors**

  Tissues were suspended in 300 µL PBS with protease inhibitors and sonicated at 30% amplitude in three 10-second pulses on ice. Lysates were centrifuged as above.

- **Protocol C – Enzymatic Digestion + Mechanical Homogenization**

  Samples were digested with 1 mg/mL collagenase IV (Gibco) at 37°C for 60 minutes, then homogenized in PBS containing protease inhibitors. Lysates were centrifuged and supernatants stored.

Table 1: Comparison of Gingival Tissue Preparation Protocols for TNF-α Estimation by ELISA

Feature	Protocol A	Protocol B	Protocol C
Buffer Used	RIPA buffer + protease inhibitor cocktail	PBS + protease inhibitor cocktail	Collagenase IV (1 mg/mL) digestion + PBS + inhibitors
Homogenization Method	Mechanical homogenization (motorized)	Sonication (probe-based, 30% amplitude, 3 × 10 s)	Enzymatic digestion (37°C, 60 min) + mechanical homogenization
Total Time for Processing	~45 minutes	~40 minutes	~90 minutes
Centrifugation Parameters	12,000 × g for 15 min at 4°C	12,000 × g for 15 min at 4°C	12,000 × g for 15 min at 4°C
Protein Quantification	BCA assay, adjusted to 100 µg/mL before ELISA	Same	Same
ELISA Compatibility	High	Moderate to high	Variable (dependent on digestion efficacy)
Advantages	High yield, effective lysis of cellular proteins	Rapid, avoids use of detergents	Facilitates ECM breakdown, may enhance cytokine release

Limitations	Use of strong detergents may interfere with some assays	Risk of overheating during sonication	Longer processing time; possible cytokine degradation during digestion

Protein Estimation

Total protein was quantified using the biuret method as per manufacturer instructions. All samples were normalized to 100 µg/mL before ELISA.

Protein Normalization Protocol

Protein concentrations in all tissue lysates were determined using the biuret method on clinical chemistry analyser, following the manufacturer's protocol. A standard curve was constructed using known concentrations of bovine serum albumin (BSA) to calculate the protein concentration of each sample.

Following quantification, all samples were normalized to a final concentration of 100 µg/mL using cold phosphate-buffered saline (PBS) as the diluent. The dilution factor for each sample was calculated using the formula:

Volume of sample to use=$100 / C_{measured} \times V$

Where:

- $C_{measured}$ is the measured concentration in µg/mL
- V is the desired final volume of diluted sample (e.g., 500 µL or 1 mL)
- PBS was added to reach the final volume

If a sample had a protein concentration lower than 100 µg/mL, it was excluded or reprocessed to concentrate the lysate using centrifugal filters (Amicon Ultra-0.5 mL, 10K MWCO, Millipore Sigma).

TNF-α Quantification: TNF-α concentrations were measured using a commercially available ELISA kit (R&D Systems) following the manufacturer's instructions. All standards and samples were assayed in duplicate. Optical density was read at 450 nm using a microplate reader (BioTek ELx808), and cytokine levels were extrapolated from a four-parameter logistic standard curve.

Statistical Analysis

Data analysis was performed using SPSS Version 26.0 (IBM Corp.). Normality was assessed with the Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare TNF-α concentrations across protocols. A p value <.05 was considered statistically significant.

Results

A total of 30 gingival tissue samples were processed using three distinct protocols (n = 30 per group) to assess TNF-α concentration via ELISA. Descriptive statistics revealed that Protocol B (PBS with protease inhibitors followed by sonication) yielded the highest mean TNF-α concentration (35.04 ± 3.75 pg/mL), followed by Protocol A (RIPA buffer + mechanical homogenization) (27.99 ± 4.97 pg/mL) and Protocol C (collagenase digestion + homogenization) (27.72 ± 4.76 pg/mL).

Normality of data distribution was confirmed using the Shapiro-Wilk test for all groups ($p > 0.05$). A one-way ANOVA demonstrated a statistically significant difference in TNF-α concentrations among the three protocols ($F(2,87) = 25.25$, $p < 0.001$).

Subsequent Tukey's post hoc analysis indicated that Protocol B yielded significantly higher TNF-α levels compared to both Protocol A ($p < 0.001$) and Protocol C ($p < 0.001$). No significant difference was observed between Protocol A and Protocol C ($p > 0.05$).

These findings suggest that the sonication-based PBS method (Protocol B) offers superior recovery of TNF-α

from gingival tissue samples compared to detergent- or enzyme-based protocols.

Table 2: TNF-α concentrations (pg/mL) across different gingival tissue preparation protocols.

Protocol B (PBS + sonication) showed significantly higher levels compared to Protocols A and C.

Protocol	Mean (pg/mL)	SD	Min	Max
Protocol A	27.99	4.97	20.65	38.91
Protocol B	35.04	3.75	29.54	42.90
Protocol C	27.72	4.76	20.41	38.42

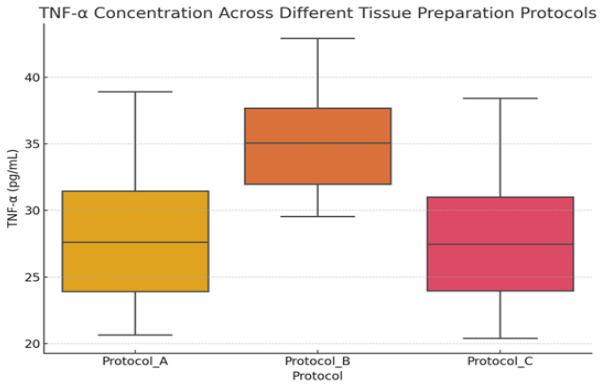

Figure1: TNF Alpha Concentrations in different protocols

Discussion

The accurate quantification of TNF-α in gingival tissues is critical for understanding its role in the pathogenesis of periodontitis, a disease hallmarked by chronic inflammation and tissue destruction. Despite the widespread use of ELISA for cytokine detection, variability in sample preparation protocols continues to be a limiting factor for inter-study comparability and biomarker reliability. This study evaluated three distinct protocols for gingival tissue processing and found that the PBS-based sonication method (Protocol B) significantly outperformed mechanical (Protocol A) and enzymatic homogenization (Protocol C) in TNF-α recovery.

Our findings are consistent with previous research indicating that sample homogenization and buffer composition substantially affect cytokine detection efficiency. Specifically, we observed that Protocol B yielded the highest mean TNF-α concentration (35.04 ± 3.75 pg/mL), significantly greater than both Protocol A and Protocol C ($p < 0.001$). The use of PBS supplemented with protease inhibitors and gentle sonication likely contributes to improved cell lysis while preserving protein integrity. This aligns with the study by de Jager and Rijkers, who emphasized that low-detergent, isotonic buffers minimize protein denaturation and interference in solid-phase immunoassays like ELISA [14].

In contrast, Protocol A employed RIPA buffer, a commonly used detergent-rich solution. While RIPA effectively lyses a wide range of cell types, its inclusion of ionic detergents such as SDS and sodium deoxycholate can compromise antigen-antibody interactions and reduce ELISA sensitivity[15]. Our results reflect this limitation, as TNF-α levels were significantly lower despite comparable tissue input. Álvarez et al. also reported diminished cytokine recovery when using detergent-based buffers for gingival tissues, further supporting our findings [16].

Protocol C, which involved enzymatic digestion with collagenase prior to homogenization, also produced lower TNF-α levels. While enzymatic disaggregation is often used to isolate single cells or solubilize dense connective tissues, prolonged exposure to enzymes like collagenase and trypsin can result in cytokine degradation or altered epitope conformation[17]. This likely explains the poor yield observed in our samples. Similar concerns were raised by Beikler et al., who reported loss of pro-

inflammatory cytokines due to over-digestion or insufficient inhibition of residual enzymatic activity[18].

Peer comparison with studies assessing gingival cytokine profiles further validates our findings. In a study by Gomes et al., gingival biopsies processed using mechanical homogenization in detergent-free buffer yielded TNF-α levels comparable to our Protocol B, suggesting the robustness of sonication-based methods [19]. Moreover, Kinney et al. emphasized the need for standardization in biomarker discovery studies and showed significant variation in cytokine detection based solely on processing methods[20].

An important implication of our findings is the necessity for methodological standardization in periodontal biomarker studies. The choice of buffer, mechanical disruption method, and preservation strategy should be tailored to the target protein's stability profile and the intended detection platform. Protocol B, being both technically straightforward and reproducible, represents a compelling candidate for standard protocol development in tissue-based cytokine studies.

Limitations

While our study provides clear evidence supporting the superiority of PBS-sonication-based processing for TNF-α detection, it is limited in scope to a single cytokine. Other inflammatory markers, particularly those bound within cellular compartments or extracellular matrices, may respond differently to lysis conditions. Additionally, our study did not stratify samples based on disease severity or clinical status, which may influence cytokine expression levels and tissue characteristics. Future studies should include broader cytokine panels and validate findings across different disease stages and tissue sources.

Conclusion

The quantification of TNF-α in gingival tissue serves as a valuable proxy for assessing inflammatory activity and disease progression in periodontal pathology. However, the accuracy and reproducibility of TNF-α measurements are critically dependent on the efficiency of tissue homogenization and cytokine preservation during sample processing.

This study systematically compared three widely applicable tissue processing protocols—mechanical homogenization using RIPA buffer, sonication in PBS with protease inhibitors, and enzymatic digestion followed by homogenization—to identify the most suitable approach for recovering TNF-α from gingival biopsies. Our findings demonstrate that the PBS-sonication method (Protocol B) yielded significantly higher TNF-α levels than both detergent-based (Protocol A) and enzyme-based (Protocol C) preparations. This protocol not only preserved cytokine integrity but also provided a cleaner matrix compatible with ELISA, ensuring more accurate quantification.

The superiority of Protocol B is likely attributed to its balanced combination of effective cell lysis, protease inhibition, and minimal chemical interference. These characteristics make it particularly suitable for sensitive immunoassays and highlight the importance of non-denaturing, low-detergent conditions for cytokine extraction. In contrast, Protocols A and C may compromise protein recovery through detergent interference or enzymatic degradation, respectively.

Our findings support the standardization of PBS-based sonication protocols in future periodontal biomarker studies to improve the reproducibility and comparability of results across laboratories. Furthermore, adopting such optimized tissue processing workflows will enhance the

utility of TNF-α and potentially other cytokines as diagnostic and prognostic biomarkers in periodontal research.

While the study focused exclusively on TNF-α, the methodological insights derived from this work may extend to the broader class of inflammatory mediators implicated in periodontal disease. Future investigations should validate this protocol for multiplex cytokine profiling and assess its applicability in clinical samples across varying disease severities.

In conclusion, sonication in PBS with protease inhibitors offers a superior and reproducible method for TNF-α extraction from gingival tissues, establishing a methodological benchmark for cytokine studies in periodontology and related fields.

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