
# Ultrasonic Modulation of Gingival Stem Cell Mobilization

A Thesis

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by

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# ABSTRACT

**Background:** Ultrasonic scalers are routinely used in dental hygiene and periodontal therapy for removing plaque and calculus from the tooth and root surfaces. In medicine, ultrasonic tools are also used to accelerate wound healing and tissue regeneration. Studies showed that ultrasonic stimulation activates both bone and soft tissue regeneration in ligaments, joints, fractured bone, healing of wounds, skin rejuvenation, nerve stimulation and improving the strength and elasticity of scar tissue. Stem cells residing in mature tissues, also known as mesenchymal stem cells (MSCs), may play a key role in tissue regeneration and wound healing. In the oral cavity, MSCs have been isolated from bone marrow, dental pulp, dental follicles, periodontal ligaments, mucosa, and gingiva. As the connective tissue underlying the human oral mucosa is derived from the neural crest, identification of neural markers such as nestin may be used to monitor gingival MSC populations.

**Aim & Hypothesis:** We hypothesized that ultrasonic stimulation delivered to gingival tissues during piezoelectric scaling and root planing induces MSC mobilization. The aim of this study was to compare the expression of the MSCs marker nestin in gingival tissue after scaling and root planing (SRP) with or without the use of an ultrasonic scaler. A secondary aim of the study was to compare histological changes in the gingiva following SRP and ultrasonic scaling.

**Material & Methods:** This study was a randomized controlled clinical pilot trial designed to investigate the stem cell mobilization potential of ultrasonic SRP. Twelve subjects diagnosed

with severe periodontitis were enrolled, and 10 have completed the study. In each subject two non-adjacent teeth in the same quadrant with severe periodontitis, defined as inflammation with more than 4 mm interproximal attachment loss, were selected as study teeth. One study tooth in each subject was randomly assigned to SRP with hand instruments only, while the other study tooth received piezoelectric scaling in addition to SRP with hand instruments. Two gingival punch biopsies were taken from the interproximal papilla of each study tooth before and 1 week after the SRP. One set of biopsies was used for RNA extraction and nestin gene expression measurements with RT-PCR, while the other set of biopsies was sectioned and stained with hematoxylin and eosin for histological analysis.

**Results:** One biopsy from a Piezo treated tooth did not provide sufficient RNA and this subject was excluded from the nestin analysis, resulting in 9 study subjects with 36 biopsies. Nestin gene expression was detected in all 36 biopsies. The mean nestin signal, expressed as nestin/GAPDH ratio, was increased both after SRP and after SRP plus Piezo compared to before treatment levels. Combination therapy with SRP and Piezo yielded the strongest nestin signal. No statistical inference was performed as this was a pilot study. Descriptive histological analysis revealed heavy mononuclear cell infiltrates that was dominated by plasma cells in the before treatment samples. After SRP a decrease in mononuclear cell infiltrates and in an increase in vascularization, neutrophils, fibroblasts and endothelial cells were seen after SRP. All clinical parameters, including PD, CAL and BOP, markedly improved from the initial visit to the final examination.

**Conclusion:** The MSC marker nestin is readily detectable in gingival biopsies. Our data suggest that SRP with or without Piezo may induce stem cell mobilization. Histological analysis indicated a shift from chronic inflammation to acute inflammation and initiation of wound healing after SRP regardless of the use of Piezo. Thus, it may be concluded that SRP promotes gingival health not only by removing plaque and calculus but also by promoting stem cell mobilization, resolving chronic inflammation and accelerating wound healing.

# DEDICATION

*I'd like to dedicate this work to my grandmother, my parents, and the rest of my family for all of their support and encouragement.*

# ACKNOWLEDGMENTS

Special thanks to Dillon Hawley for his help with data collection and analysis. We are also thankful to Dr. Joseph Alroy for his help with the interpretation of the histological staining of the biopsies.

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# LIST OF ABBREVIATIONS

**Piezo:** Piezoelectric scaler

**PIEZO:** Treatment group receiving hand and Piezo instrumentations

**HAND:** Treatment group receiving hand instrumentations only

**MSC**: Mesenchymal stem cell

**SRP:** Scaling and root planing

**RT-PCR:** Reverse transcription polymerase chain reaction

**LIPUS:** Low intensity pulsed ultrasound

**ALP:** Alkaline phosphatase

**CFU-F:** Colony-forming unit-fibroblastic cells

**ICF:** Informed consent form

**OFD:** Open flap debridement

**PD:** Probing depth

**CAL:** Clinical attachment level

**BOP:** Bleeding on probing

**SD:** Standard deviation

**GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase

**TAE:** TRIS/EDTA/acetic acid

**H&E stain:** Hematoxylin and Eosin Stain

# LIST OF SYMBOLS

®: Registered Trademark

$^{TM}$: Trademark

# Ultrasonic Modulation of Gingival Stem Cell Mobilization

# Introduction

All periodontal treatment begins with removal of plaque and calculus. This treatment phase is also known as 'initial therapy'. The main goal of initial therapy of periodontitis is to eliminate both living bacteria in the oral biofilm and calcified biofilm microorganisms from the tooth and the root surface as well as from the periodontal pocket. Scaling and root planing (SRP) are the most frequently used procedures during initial therapy. SRP is effective in removing plaque and calculus up to 4-5 mm probing depth.[1] In deeper periodontal pockets most (57-68%) of the calculus remains on the root surface after SRP.[2] Thus for severe periodontitis the standard of care is SRP followed by open flap surgery.

In initial therapy two main groups of instruments may be used: hand instruments and ultrasonic instruments. Hand instruments include periodontal scalers and curettes, while ultrasonic devices may be magnetostrictive or piezoelectric. The working tip of an ultrasonic scaler vibrating at 25-30 kHz is inserted into the gingival sulcus and is used to remove plaque and calculus from the tooth and root surfaces. The vibrating tip breaks up calculus, removes plaque and also creates micro-bubbles that are thought to aid in disinfecting the gingival sulcus. Hand scalers and curettes are advanced into the gingival sulcus as well and their sharp working edges are used to remove plaque and calculus deposits from tooth and root surfaces. In terms of clinical utility, several recent systematic reviews [3, 4] and randomized clinical trials[5-7] have concluded that ultrasonic and hand instruments are equally effective in plaque and calculus removal, and both are considered standard of care in periodontal therapy. At

TUSDM, most patients receive both ultrasonic and hand instrumentations as standard initial therapy. However, hand scaling alone is considered equally effective and in selected populations where ultrasonic instrumentation is contraindicated, such as patients with root sensitivity or with excessive gag reflex or patients with certain pacemakers, hand scaling alone is routinely performed.

In medicine, ultrasonic tools are used to accelerate wound healing.[8] Ultrasound may have therapeutic applications on soft tissue and bone regeneration. Several studies have shown that ultrasonic stimulation activates both bone and soft tissue regenerations in ligaments, joints, fractured bones, healing of wounds, skin rejuvenation, nerve stimulation and improving the strength and elasticity of scar tissue.[8] Ultrasound stimulates soft tissue healing through its thermal and non-thermal mechanisms. The elevated temperature helps the soft tissue healing as a result of increasing blood flow. The non-thermal mechanism (cavitation and acoustic streaming) has been hypothesized to change diffusion rate and membrane permeability to ions at the cellular level, thus stimulating cells.[9] Ultrasound may help in wound contraction and scar tissue remodeling. These beneficial effects of ultrasound are used in treating skin conditions such as ulcers, skin lesions, and skin grafts. Stimulatory effect of ultrasound on osteogenesis are established in 1950s[10], and low intensity pulsed ultrasound (LIPUS) was approved as a bio-physical therapy for the treatment of bone fractures. LIPUS transmits an acoustic pressure wave at 30–100 $mW/cm^2$ through living tissues, resulting in biochemical events at the cellular level.[11, 12]

In periodontal tissues, LIPUS was shown to increase ALP activity *in vitro* and to accelerate

periodontal wound healing and bone repair animal studies[13, 14], suggesting that LIPUS may accelerate bone remodeling.[11] LIPUS and ultrasonic dental scalers are both ultrasonic, but the two procedures use different frequencies: LIPUS signals are composed of a 200-μs burst sine wave of 1.5 MHz repeated at a frequency of 1.0 kHz[13] while ultrasonic scalers operate continuously at 25 to 30 kHz. Even though ultrasonic scalers have been examined their effects on calculus removal and clinical healing, their actions on human periodontal wound healing and stem cell mobilization have not been investigated.

Oral tissues show remarkable regenerative potential. Wound healing is accelerated in gingiva compared to the skin[15] and wound healing in the mouth is considered 'scarless'. [15, 16] Fibroblasts cultured from the oral cavity of adults are comparable to fetal-derived fibroblasts in the number of potential cell divisions.[17] In oral connective tissues, similar to other tissues, mesenchymal stem cell (MSC) populations have been identified that represent a distinct cell type from fibroblasts and other connective tissue cells. MSCs are defined as fibroblast-like cells that are clonogenic and have the capacity for self-renewal, that is, making copies of themselves that have the same potential as the parent cell. In the oral cavity, MSCs have been isolated from human bone marrow, tooth dental pulp, exfoliated deciduous tooth pulp, papilla of immature teeth, dental follicles of impacted third molars, periodontal ligaments, mucosa, and gingiva.[18] The key properties of MSCs for promoting tissue regeneration are diverse differentiation potential, immunomodulation, stimulation of vasculogenesis and epithelialization.[18]

There are two main strategies for stem cell detection in oral tissues. In the first approach,

cells are grown from a tissue biopsy for establishing cultures of plastic-adherent fibroblasts. To this end, cells are either allowed to grow out of a connective tissue biopsy placed onto a plastic cell culture dish (explant culture method) or they are first enzymatically released from the connective tissue before seeding onto culture plates (enzymatic digestion method). Colony-forming unit-fibroblastic cells (CFU-F) technique is the technique that after seeding cells in very low density allows colonies to form a single cell. The largest colonies that develop are considered candidate MSCs. In human gingiva, the CFU-F technique has been used for MSCs isolation and gingival stem cells were identified by the presence of antigens CD73, CD90 and CD105, and the absence of CD34, CD25 and HLA-DR.[18]

Alternatively, the ability to differentiate into divergent cell types can be taken as evidence of 'stemness'. Of particular interest is a study by El-Bialy and coworkers who utilized neuronal differentiation markers to show that human gingival progenitor cells can be differentiated into different cell types by ultrasonic stimulation.[19] Abundant (40-80%) expression of MSC markers and neural crest stem cell markers was demonstrated in human oral mucosal biopsies.[20] Similarly, expression of the neural marker nestin was demonstrated in 30% of undifferentiated periodontal ligament stem cells in vitro.[21] In addition, Widera and co-workers demonstrated high levels of nestin expression in neurospheres generated from tissue removed during periodontal surgery.[22] The potential for neuronal differentiation of gingival and periodontal stem cells is not entirely surprising since the connective tissue underlying the human oral mucosa is derived from the neural crest.[23] Indeed, when human oral mucosal stem cells are transplanted into severe combined immunodeficiency mice, the resulting tumors consist of tissues derived from the neural crest.[20] Previous studies at TUSDM have

measured the expression of nestin by RT-PCR, immunofluorescence as well as western blotting on lacrimal glands. The results showed that nestin is expressed in non-injured tissue and that its expression increased following experimentally induced inflammation.[24, 25] Thus, we used nestin as an initial marker for stemness in gingival biopsies.

## Aim and Hypothesis

The aim of this study was to compare the gene expression of the stem cell marker nestin in periodontal tissue after scaling and root planing (SRP) with or without the use of an ultrasonic device.

We hypothesized that ultrasonic stimulation delivered to gingival tissues during routine piezoelectric scaling and root planning induces MSCs mobilization.

# Research Design

**Patient population and clinical parameters**

The study population consisted of patients recruited from the Department of Periodontology at TUSDM who were diagnosed with severe periodontitis. The protocol was approved by the Tufts Health Sciences Institutional Review Board. (IRB #12113) All participants were informed about the details of the study, treatment and follow up schedules, and signed the informed consent form. Comprehensive oral and periodontal examination was performed at the screening visit.

Inclusion criteria were as follow:

- Adults (18 years or older)
- Patients having at least two non-adjacent teeth in the same quadrant with severe periodontitis, defined as presence of gingival inflammation and more than 4 mm clinical attachment loss, and judged to be in need of surgical periodontal treatment.[26]

Exclusion criteria were as follow:

- Subjects with recent (within the past 12 months) treatment for periodontitis
- Subjects whose teeth were beyond repair and would not benefit from periodontal treatment (hopeless teeth)
- Subjects with contraindications to periodontal SRP or gingival surgery according to standard clinical practice, including but not limited to patients with hematologic disorders, bleeding disorders, oral cancer, uncontrolled diabetes (HbA1c>7), stage II

hypertension (systolic blood pressure >160 mm Hg or diastolic blood pressure >100 mm Hg), uncontrolled thyroid disease and pregnancy (self-reported).

- Subjects with contraindications to ultrasonic instrumentation, including but not limited to patients with pacemaker or with excessive gag reflex.
- Subjects that were unable or unwilling to sign the informed consent form (ICF).
- Subjects who did not understand English.

**Study design**

This was a prospective randomized controlled clinical trial designed to investigate the stem-cell inducing potential of piezoelectric scaling and root planing (SRP). Patients with severe periodontitis and in need of open flap debridement were considered as study subjects. The study followed a split-mouth design. Of the two non-adjacent teeth in each patient that were selected for the study, one tooth was randomly assigned to receive SRP with hand instruments and Piezo (PIEZO), while the other tooth received SRP with hand instruments only (HAND). Following SRP, all subjects underwent open flap debridement (OFD) on both study teeth. Tissue samples were taken before the SRP and during the open flap debridement. Tissue samples were coded and thus the evaluator of tissue samples was blinded as to the treatment assignments. The primary outcome of the study was the relative level of gene expression for the stem cell marker nestin in gingival biopsy samples. Secondary outcomes included inflammatory cell counts determined histologically, as well as clinical changes in probing pocket depth, recession, attachment level, and bleeding on probing. Biopsies were analyzed as outlined in the study procedure section.

## Sample Size and Statistical Analysis

Comprehensive literature search was performed on multiple databases (PubMed, Web of Science and Scopus) with the help of a TUSDM research librarian. No data was found in the literature with regards to changes in gingival/periodontal stem cell markers or stem cell numbers after SRP with either hand instruments or ultrasonic instruments. Therefore, a pilot study with a convenience sample of 13 subjects, allowing for ~20% dropout with a final projected sample size of 10 was proposed to obtain initial numbers and to estimate mean difference and variability in stem cell marker levels and stem cell counts after SRP. For continuous outcomes (stem cell marker level, probing pocket depth, and clinical attachment level), means and standard deviations were computed; for bleeding on probing, the percentage of bleeding sites was computed in each group before and after treatment and then the mean and standard deviation of the percentage of bleeding sites were calculated. Once these pilot data were obtained, an adequately powered clinical study could be designed.

## Randomization

Study teeth were selected based on periodontal diagnosis and only teeth with severe periodontitis (having more than 4 mm attachment loss and periodontal inflammation) were included. The two selected teeth were not adjacent to each other. If more than 2 teeth fulfilled these criteria, teeth with the most favorable prognosis, i.e. teeth with attachment loss closest to 5 mm were selected. A randomization list was generated using the "sample" function of

the statistical software package R (Version 3.1.2). The randomization list was used at Visit 2 to determine which tooth was to be treated with hand instruments alone and which tooth was treated with Piezo in addition to hand instruments.

# Materials and Methods

## Study Procedures

All surgical procedures and clinical measurements were performed by the same investigator (C.S.).

Visit 1: Screening

- All the participants were explained the details of the study purpose and follow up schedule and signed appropriate consent form.
- Medical history and demographic information were collected.
- Periodontal examination was performed, including measuring probing depth (PD), recession, clinical attachment level (CAL), and bleeding on probing (BOP).
- Inclusion and exclusion criteria were considered and eligibility for the study was determined.
- Intraoral photographs were taken and oral hygiene instructions were given.

Visit 2: Gingival biopsy, randomization, SRP (within 2 months after Visit 1)

- Subjects were anesthetized with appropriate local anesthetic (2% Lidocaine with 1:100,000 epinephrine) injection to the quadrants to be treated. Two gingival biopsies from each tooth included in the study ('study tooth') were taken from the periodontal pocket to be treated with a 2 mm diameter tissue punch (Miltex® Biopsy Dermal

Punch). The tissues removed by the biopsies were part of the tissue area that would be removed during OFD at Visit 3, thus no healthy tissue was removed.

- Two teeth that selected for this study were randomized using statistical software package R (Version 3.1.2). Each subject was assigned the number 0 or 1 according to the randomization list. 0 meant that the lower tooth number received a treatment with hand instruments plus Piezo and 1 meant the higher tooth number was treated with hand instruments alone.
- Both study teeth were treated with SRP using hand instruments. In addition, all adjacent teeth in the quadrant needing SRP were treated with hand instruments. The tooth randomized to SRP with Piezo scaling was then be treated with a Piezo scaler. Hemostasis at all SRP and biopsy sites were achieved by compression using wet gauze. Ibuprofen 800 mg was prescribed as needed for post-operative discomfort.

Visit 3: Open flap debridement (7-10 days after Visit 2)

- OFD was performed on both study teeth and any adjacent tooth in the quadrant in need of this treatment.[27] Open flap debridement involved administration of local anesthesia, placement of incisions around the tooth and in the gingiva, separation of gingival tissue from underlying structures, removal of inflamed gingival tissue surrounding the teeth and suturing the elevated gingival flap in place. After this surgery, the two 2 mm tissues that were removed during OFD were saved and later analyzed for stem cell content. (**Figure 1)**
- Post-operative pain medication and oral hygiene instructions for wound care were given.

- The gingival biopsy samples collected at Visits 2 and 3 were placed into labeled containers and evaluated for stem cell marker levels in the laboratory.

Visit 4: Periodontal examination (6-8 weeks after Visit 2)

- A comprehensive periodontal examination including measurements of PD, recession, CAL, and BOP were recorded.

**Laboratory analysis**

Laboratory analysis was performed on biopsies obtained at Visits 2 and 3. With two biopsies taken from around each study tooth at the SRP visit and two biopsies collected from the excised tissue removed at the OFD visit, we collected a total of 4 biopsies per study tooth and 8 biopsies per study subject. One of each pair of biopsies was used for RNA extraction and nestin gene expression analysis with RT-PCR, providing data on nestin expression before and after SRP and with or without Piezo. The second half of the biopsies were used for histology.

**RNA analysis**

Collected biopsies were stored in RNAlater® Stabilization Solution. For RNA extraction, biological samples were first lysed with adding 700 $\mu L$ of QlA201 Lysis Reagent and homogenized (IKA® homogenizer) in the presence of a highly denaturing guanidine containing buffer, which immediately inactivates RNases® to ensure samples protection and

purification of intact RNA. After adding 140 $\mu L$ chloroform and vortexing for 15 seconds, samples were centrifuged for 15 minutes at 12,000 x g at 4 ℃. Ethanol was added to provide appropriate binding conditions for precipitation of the RNA. Samples were then applied to RNeasy® Mini spin columns, where the total RNA binds to a membrane and contaminants are efficiently washed away. Finally, high-quality RNA was then eluted in 40 $\mu L$ water. The extracted RNA was quantified and the sample purity ratio (260/280nm) was determined with NanoDrop™ Spectrophotometer. The ratio of absorbance at these wavelengths was used as a measure of purity in both nucleic acid and protein extractions. A ratio of ~2.0 is generally accepted as "pure" for RNA.

After sample quantification and qualification, reverse transcription PCR (QIAGEN® OneStep Ahead RT-PCR Kit) was used to detect nestin RNA expression. All RNA samples were optimized for 1$\mu g$/2 $\mu L$ of total RNA based on each sample's concentrations. PCR reaction solutions were prepared that contained dNTPs, primers, template RNA, DNA polymerase and a buffer solution. For the reactions, OneStep Adhead RT-PCR Master Mix, 2.5x 10 $\mu L$, OneStep Ahead RT-Mix, 25x 1 $\mu L$, RNase-Free Water 9.5 $\mu L$, Forward primer 1.25 $\mu L$, Reverse primer 1.25 $\mu L$ and Template RNA 2 $\mu L$ were mixed in the individual PCR tubes. The total reaction volumes were 25 $\mu L$. Each mix was added to a PCR tube for each reaction and then the temperate RNA was added. Each RNA sample was prepared into 2 different primer mix, one with nestin and one with human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The DNA sequence of the nestin primers followed those published by Kim et al.[28] The DNA sequence of nestin primers were, forward primer 5'-CCA

GAA ACT CAA GCA CCA C-3' and reverse primer 5'-TTT TCC ACT CCA GCC ATC C-3'.

All PCR reactions were placed in the thermal cycler and then amplified with the following thermal cycler program: one cycle of reverse transcription 10 minutes at 50 ℃, initial PCR activation 5 minutes at 95 ℃, denaturation 15 seconds at 95 ℃, annealing 15 seconds at 55 ℃, extension 1 minute at 72 ℃ and final extension 2 minutes at 72 ℃ for a total of 40 cycles. (**Figure 2**)

After the cycling, RT-PCR products were analyzed with gel electrophoresis. 1.0 g agarose was measured and mixed with 100 *mL* TRIS/EDTA/acetic acid (TAE) buffer in a microwavable flask. The agarose suspension was microwaved for 1 minute until the agarose was completely dissolved; then, 10 $\mu L$ of ethidium bromide was added to the agarose solution. The prepared solution was poured into a gel tray with the well comb in place. The poured gel was allowed to set at room temperature for 30 minutes until it had completely solidified.

Each 20 $\mu L$ DNA sample was mixed with 6 $\mu L$ DNA Loading Dye. DNA ladder (1 $\mu L$) was added together with 4 $\mu L$ DNA Loading Dye and 15 $\mu L$ RNase-Free Water. Then 20 $\mu L$ of each DNA mixture was loaded onto a 1.0 % agarose gel mixed with ethidium bromide. For visualizing the fragments, agarose gel electrophoresis was used.

An electric current of 100 volts for 40 minutes was used to separate the DNA molecules across the agarose gel.

**Histology**

Embedding, sectioning and staining of histological samples were performed in the Histology Laboratory at Tufts Medical Center. Hematoxylin and Eosin stain (H&E stain) was performed for 5 of the 9 subjects' histology samples in order to obtain the morphological information. Slides were de-paraffinized in xylene and rehydrated in the descending concentrations of ethanol solutions (100, 96, 80% ethanol) and tap water. All samples were stained in Hematoxylin solution for 30 minutes and washed in tap water for 5 minutes until sections were turning blue. Then all samples were differentiated in 70% ethanol for 5 seconds in order to remove excess dye and washed in water for 5 minutes. Samples were stained in Eosin solution for 10 minutes and washed for 5 minutes in water. Slides were dehydrated in 96% ethanol and xylene mounted with coverslips.

# Results

Twelve subjects were screened and all twelve qualified for this randomized controlled clinical pilot study. One patient dropped out of the study after screening and another after Visit 2, both due to scheduling conflicts. These 2 subjects were not included in date analysis. Ten subjects completed all clinical procedures. All subjects were diagnosed with severe periodontitis according to a comprehensive oral and periodontal examination performed at the screening visit. All subjects were treated at TUSDM Department of Periodontology (Boston, MA, USA) from September 2016 to June 2017. Due to insufficient RNA concentration, sample #37, subject #11 had to be excluded from data analysis. Thus, a total of 9 subjects (6 males and 3 females, aged 36-60; mean 47.7 ± 9.4 years) were evaluated. **Table 1** shows study subject characteristics. Clinical measurements of PD, CAL and BOP (% of positive sites per tooth) were taken at Visit 1 (screening) and Visit 4 (final follow-up). PD, CAL and BOP were all decreased in both treatment groups at Visit 4 (**Table 2)**.

The first set of biopsies was removed at Visit 2, before SRP or ultrasonic scaling ('before' samples, **Figure 3**). At Visit 3, 7-8 days after the SRP performed, open flap debridement (OFD) was performed according to standard clinical practice. In this patient population, there was no re-evaluation of the periodontal status after SRP as the study teeth selected were all diagnosed with severe periodontitis in need of surgical treatment at the initial screening. During surgery, tissues were removed and two 2 mm tissue biopsies on each tooth were saved ('after' samples, **Figure 4**).

**RNA analysis**

All 10 subjects' RNA samples (R01-32, R35-42) have been initially analyzed for nestin expression, but due to the failure of sample #37 only 9 subjects were included in the final analysis. RNA samples were quantified with NanoDrop™ Spectrophotometer at 260 and 280 nm wavelengths. The ratio of absorbance at these wavelengths was used as a measure of purity in both nucleic acid and protein extractions. All samples' ratio of absorbance at these wavelengths were close to 2, indicating sufficient RNA purity (**Figure 5**). All samples' 260/280 ratio and RNA sample concentration (ng/μL) are shown in **Table 3**.

All samples were analyzed with RT-PCR using nestin-specific primers, followed by gel electrophoresis. Each sample's RT-PCR gel with nestin gene expression, GAPDH gene expression, and DNA ladder was captured by MultiDoc-It™ Imaging System (**Figure 6**). Nestin and GAPDH signal intensity was determined densitometrically using computer image analysis software (ImageJ, NIH, Bethesda, MD). Gene expression data are expressed as mean integrated density. All samples' mean integrated density was calculated then the mean background integrated density in each area were calculated and subtracted from each sample's mean integrated density. The background subtracted mean integrated density of both nestin and GAPDH in each sample was calculated. The ratio of nestin and GAPDH mean integrated density, a dimensionless number, is reported as nestin gene expression level. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is a housekeeping gene commonly used to normalize gene expression data. Nestin/GAPDH gene expression ratio in individual subjects before SRP with hand instruments only and with Piezo and after SRP with hand instruments only and with Piezo of 9 patients were calculated and shown in **Table 4** and

**Figure 7**. The mean ± SD relative gene expression ratio of before SRP with hand instruments only was 0.24 ± 0.13 and before with Piezo was 0.42 ± 0.34. The mean ± SD relative expression ratio of after 1 week of SRP with hand instruments only was 0.33 ± 0.19 and after 1 week with Piezo was 0.65 ± 0.45. There was a trend for increasing relative gene expression in both groups after SRP. Combination treatment with SRP + Piezo resulted in the strongest increase in the nestin signal. No statistical hypothesis testing was performed as this was a pilot study.

## Histology

Histology slides of before and after SRP with hand instruments only and with Piezo are shown in **Figure 8-10**.

**Figure 8** shows a typical tissue sample from a severe chronic periodontitis patient before treatment. Low magnification image (**Figure 8-a**) shows the entire tissue plug removed by tissue punch. On the top shows stratified squamous cell epithelial layer (arrow). The submucosa shows isolated islands of eosinophilic areas with heavy cellular infiltrate. Medium magnification (**Figure 8-b**) shows inflammatory cell infiltrate (arrow) and collagenous tissue with embedded fibroblasts (arrow head). Higher magnification (**Figure 8-c**) shows that the majority of cellular infiltrate consists of mononuclear cells with basophilic cytoplasm and eccentric nucleus, consistent with plasma cell morphology (arrowhead). Other cell types visible are fibroblasts (star) and endothelial cells (arrow).

**Figure 9** shows a histology slide of SRP with using Piezo after one week. Low magnification image (**Figure 9-a**) shows the entire biopsy removed during OFD at Visit 3. The submucosa shows isolated islands of eosinophilic areas with less cellular infiltrate in the connective tissue. Medium magnification (**Figure 9-b**) shows inflammatory cell infiltrate and presence of multiple capillaries in the connective tissue (arrow). Bone fragment is seen in connective tissue (star). Higher magnification (**Figure 9-c**) shows that the majority of cellular infiltrate consists of neutrophils (arrow head) and fibroblasts (arrow) with fewer plasma cells in the connective tissue.

**Figure 10** shows a histology slide of SRP with hand instruments only after one week. Low magnification image (**Figure 10-a**) shows the entire biopsy removed during OFD at Visit 3. Similar to **Figure 9**, the submucosa shows isolated islands of eosinophilic areas with less cellular infiltrate in the connective tissue. Medium magnification (**Figure 10-b**) shows inflammatory cell infiltrates and presence of multiple capillaries and endothelial cells (arrow). Higher magnification (**Figure 10-c**) shows that the majority of cellular infiltrate consists of neutrophils (arrow head) and some plasma cells (arrow) and fibroblasts (star).

In summary, mononuclear cells and plasma cells characteristic of chronic inflammation dominate the histology image before SRP, while neutrophils, fibroblasts, endothelial cells and capillaries consistent with acute inflammation and wound healing become more evident in the histology slides after 1 week of SRP both with or without Piezo application.

## Discussion

This study demonstrates that the stem cell marker nestin is detectable in human gingival tissues and its expression increases after non-surgical periodontal therapy. On the histological level, we have shown that SRP with or without Piezo scaling results in decreased cellular evidence of chronic inflammation and increased cellular signature of acute inflammation, revascularization, and tissue repair.

SRP and ultrasonic instruments are used interchangeably in current clinical practice and both were shown to be equally effective in removing plaque and calculus.[3-7] Our findings show that SRP and Piezo are also both capable of inducing nestin expression, indicating that stem cells may be mobilized after non-surgical periodontal therapy. This confirms and extends earlier findings that a multitude of oral tissues such as connective tissue, bone marrow, oral mucosa, and periodontal tissues contain MSCs.[18] Our findings are novel in that they indicate that SRP performed with hand instruments as well as with ultrasonic instrument can induce and further mobilize MSCs in the periodontium. It is widely accepted that MSCs play a key role in promoting tissue regeneration including multipotent differentiation, stimulation of vasculogenesis and epithelialization. Thus, it may be concluded that SRP promotes gingival health not only by removing plaque and calculus but also by promoting stem cell function, resolving chronic inflammation and accelerating wound healing.

In order to identify MSC expressions of various stem cell markers have been used.[18] The expression of the neural marker nestin in human oral mucosa had been shown in studies[22, 23]

and previous study showed the ability to measure the expression of nestin by RT-PCR.[24, 25] However, one marker may not be sufficient to confirm MSCs identity. Human oral mucosal fibroblasts have been reported to express MSC markers such as CD90, CD105 and CD146.[20] To support the further findings of MSC expression in the human gingival tissue of ultrasonic application, the detections and quantifications of other MSC markers expression will be necessary.

Histological sections showed extensive areas of inflammatory infiltrates that decreased in size after SRP with or without use of Piezo. Before the procedure there were mainly monocyte and plasma cell infiltration in the connective tissue that indicated chronic inflammation. After SRP with hand instruments only or hand instruments plus application of Piezo, there were more neutrophil infiltration, increased fibroblasts and fewer plasma cells. As evidence of re-vascularization, the presence of multiple capillaries increased in the connective tissue. That indicated healing phase of tissue following treatment. Histological signs of tissue healing were similar between both with or without Piezo application.

All clinical parameters, including PD, CAL and BOP, markedly improved from Visit 1 to Visit 4; however, these numbers reflect the combined effect of SRP with or without Piezo and the OFD performed at Visit 3. It is likely that the reduction in PD is mainly due to the OFD; thus no conclusions may be drawn with regards to the additional benefits of Piezo towards clinical parameters.

**Limitations and future perspectives**

In this study, the RT-PCR technique was used to detect nestin gene expression and quantified by mean integrated density of RT-PCR gel image. Although RT-PCR technique is widely accepted and commonly used in detection of RNA expression. The use of quantitative PCR (q-PCR) is needed. As in standard PCR, DNA is amplified by 3 repeating steps: denaturation, annealing and elongation. However, in qPCR, fluorescent labeling enables the collection of data as PCR progresses. Q-PCR technique may be considered for future experiments because of its higher sensitivity and quantification.

To further document nestin expression, immunohistochemistry may be performed on histological sections already collected from the tissue biopsies. Immunohistochemistry would not only allow quantification of nestin positive cells but would also provide information on tissue distribution of MSCs in the periodontium. Additional markers of MSCs such as CD90, CD105 and CD146 may be assessed with RT-PCR, qPCR and immunohistochemistry to confirm MSC mobilization as suggested by nestin expression.

To correlate final clinical outcomes with gene expression and histological findings, an additional biopsy may be taken at Visit 4 (6 weeks after SRP) and OFD would be performed at Visit 4 instead of Visit 3. This would allow for monitoring gene expression and histology with standard clinical outcome measurements 1 and 6 weeks after SRP.

A definitive clinical trial may be designed using the outcome measurements from this pilot study. A sample size calculation was conducted using nQuery Advisor (Version 7.0) to

determine the necessary sample size for an adequately powered future study with a split-mouth design. The calculation took the relative gene expression (nestin/GAPDH) after treatment as the outcome of interest. Based on the effect size obtained in the pilot study (a mean difference of 0.315 between the PIEZO group and the HAND group, with a standard deviation of 0.358), the calculation indicated that a sample size of n = 13 would be adequate to obtain a Type I error rate of $\alpha = 0.05$ and a power of 80%. From this study, we suggest the split mouth design randomized clinical trial to confirm our findings.

# Conclusion

The stem cell marker nestin is readily detectable in gingival biopsies and it appears to increase after SRP. Our data suggest that SRP with or without Piezo may induce stem cell mobilization. Histological analysis indicates a shift from chronic inflammation to acute inflammation and wound healing after SRP regardless of the use of the Piezo scaler. Thus, it may be concluded that SRP promotes gingival health not only by removing plaque and calculus but also by promoting stem cell function, resolving chronic inflammation and accelerating wound healing.

# References


1. Stambaugh RV, Dragoo M, Smith DM, Carasali L. The limits of subgingival scaling. *The International journal of periodontics & restorative dentistry* 1981;1:30-41.

2. Rabbani GM, Ash MM, Jr., Caffesse RG. The effectiveness of subgingival scaling and root planing in calculus removal. *Journal of periodontology* 1981;52:119-123.

3. Tunkel J, Heinecke A, Flemmig TF. A systematic review of efficacy of machine-driven and manual subgingival debridement in the treatment of chronic periodontitis. *Journal of clinical periodontology* 2002;29 Suppl 3:72-81; discussion 90-71.

4. Hallmon WW, Rees TD. Local anti-infective therapy: mechanical and physical approaches. A systematic review. *Annals of periodontology* 2003;8:99-114.

5. Ioannou I, Dimitriadis N, Papadimitriou K, Sakellari D, Vouros I, Konstantinidis A. Hand instrumentation versus ultrasonic debridement in the treatment of chronic periodontitis: a randomized clinical and microbiological trial. *Journal of clinical periodontology* 2009;36:132-141.

6. Tomasi C, Bertelle A, Dellasega E, Wennstrom JL. Full-mouth ultrasonic debridement and risk of disease recurrence: a 1-year follow-up. *Journal of clinical periodontology* 2006;33:626-631.

7. Wennstrom JL, Tomasi C, Bertelle A, Dellasega E. Full-mouth ultrasonic debridement versus quadrant scaling and root planing as an initial approach in the treatment of chronic periodontitis. *Journal of clinical periodontology* 2005;32:851-859.

8. Paliwal S, Mitragotri S. Therapeutic opportunities in biological responses of ultrasound. *Ultrasonics* 2008;48:271-278.

9. Dinno MA, Dyson M, Young SR, Mortimer AJ, Hart J, Crum LA. The significance of membrane changes in the safe and effective use of therapeutic and diagnostic ultrasound. *Physics in medicine and biology* 1989;34:1543-1552.

10. Maintz G. [Animal experiments in the study of the effect of ultrasonic waves on bone regeneration]. *Strahlentherapie* 1950;82:631-638.

11. Rego EB, Takata T, Tanne K, Tanaka E. Current status of low intensity pulsed ultrasound for dental purposes. *The open dentistry journal* 2012;6:220-225.

12. Ren L, Yang Z, Song J, Wang Z, Deng F, Li W. Involvement of p38 MAPK pathway in low intensity pulsed ultrasound induced osteogenic differentiation of human periodontal ligament cells. *Ultrasonics* 2013;53:686-690.

13. Ikai H, Tamura T, Watanabe T, et al. Low-intensity pulsed ultrasound accelerates periodontal wound healing after flap surgery. *Journal of periodontal research* 2008;43:212-216.

14. Inubushi T, Tanaka E, Rego EB, et al. Effects of ultrasound on the proliferation and differentiation of cementoblast lineage cells. *Journal of periodontology* 2008;79:1984-1990.

15. Szpaderska AM, Zuckerman JD, DiPietro LA. Differential injury responses in oral mucosal and cutaneous wounds. *Journal of dental research* 2003;82:621-626.

16. Hallock GG. In utero cleft lip repair in A/J mice. *Plastic and reconstructive surgery* 1985;75:785-790.

17. Schor SL, Ellis I, Irwin CR, et al. Subpopulations of fetal-like gingival fibroblasts: characterisation and potential significance for wound healing and the progression of periodontal disease. *Oral diseases* 1996;2:155-166.

18. Fournier BP, Larjava H, Hakkinen L. Gingiva as a source of stem cells with therapeutic potential. *Stem cells and development* 2013;22:3157-3177.

19. El-Bialy T, Alhadlaq A, Wong B, Kucharski C. Ultrasound effect on neural differentiation of gingival stem/progenitor cells. *Annals of biomedical engineering* 2014;42:1406-1412.

20. Marynka-Kalmani K, Treves S, Yafee M, et al. The lamina propria of adult human oral mucosa harbors a novel stem cell population. *Stem cells (Dayton, Ohio)* 2010;28:984-995.

21. Foudah D, Monfrini M, Donzelli E, et al. Expression of neural markers by undifferentiated mesenchymal-like stem cells from different sources. *Journal of immunology research* 2014;2014:987678.

22. Widera D, Grimm WD, Moebius JM, et al. Highly efficient neural differentiation of human somatic stem cells, isolated by minimally invasive periodontal surgery. *Stem cells and development* 2007;16:447-460.

23. Achilleos A, Trainor PA. Neural crest stem cells: discovery, properties and potential for therapy. *Cell research* 2012;22:288-304.

24. You S, Kublin CL, Avidan O, Miyasaki D, Zoukhri D. Isolation and propagation of mesenchymal stem cells from the lacrimal gland. *Investigative ophthalmology & visual science* 2011;52:2087-2094.

25. Zoukhri D, Fix A, Alroy J, Kublin CL. Mechanisms of murine lacrimal gland repair after experimentally induced inflammation. *Investigative ophthalmology & visual science* 2008;49:4399-4406.

26. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Annals of periodontology* 1999;4:1-6.

27. Ramfjord SP, Nissle RR. The modified widman flap. *Journal of periodontology* 1974;45:601-607.

28. Kim J, Kang HM, Kim H, et al. Ex vivo characteristics of human amniotic membrane-derived stem cells. *Cloning and stem cells* 2007;9:581-594.

29. Basic Principles of RT-qPCR: https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molec…nter/molecular-biology-resource-library/basic-principles-rt-qpcr.html

# APPENDICES

Appendix A: Tables

Appendix B: Figures

# Appendix A: Tables

**Table 1:** Patient characteristics (gender and mean ± SD age)

| | |
|---|---|
| Total subjects | 9 |
| Gender (%) | |
| Female | 3 (33.3%) |
| Male | 6 (66.7%) |
| Age | 47.7 ± 9.4 |

**Table 2:** Mean ± SD of clinical parameters in patients: PD: Pocket depth, CAL: Clinical attachment level, BOP: Bleeding on probing, OFD: Open flap debridement

| | Before Piezo | Before Hand | After Piezo + OFD | After Hand + OFD |
|---|---|---|---|---|
| PD (mm) | 4.6 ± 0.7 | 4.3 ± 0.7 | 2.8 ± 0.2 | 2.9 ± 0.2 |
| CAL (mm) | 5.3 ± 1.3 | 4.8 ± 0.7 | 4.4 ± 1.0 | 4.1 ± 1.1 |
| BOP (% positive site/tooth) | 81.5 ± 24.2 | 75.9 ± 29.0 | 14.8 ± 19.4 | 20.4 ± 23.2 |

**Table 3:** Ratio of absorbance (260/280) and sample concentration (ng/μL) were measured and listed.

| Sample # | 260/280 | ng/uL |
|---|---|---|
| R01 | 2.03 | 199.4 |
| R02 | 2.01 | 389.2 |
| R03 | 1.95 | 120 |
| R04 | 2.04 | 532.1 |
| R05 | 2.03 | 255.7 |
| R06 | 2.07 | 626.6 |
| R07 | 2.07 | 1082.6 |
| R08 | 2.05 | 507.4 |
| R09 | 2.06 | 702.4 |
| R10 | 2.08 | 783.4 |
| R11 | 1.99 | 358.4 |
| R12 | 1.99 | 350.8 |
| R13 | 2.05 | 686.7 |
| R14 | 1.92 | 164.5 |
| R15 | 2.07 | 852.1 |
| R16 | 2.09 | 507.7 |
| R17 | 2.01 | 303.1 |
| R18 | 2.02 | 236.9 |
| R19 | 1.98 | 357.1 |
| R20 | 2.04 | 555.5 |
| R21 | 2.06 | 150.6 |
| R22 | 2.02 | 641.9 |
| R23 | 2.04 | 348.7 |
| R24 | 2.02 | 519 |
| R25 | 2.04 | 319.9 |
| R26 | 2.03 | 209.8 |
| R27 | 2.03 | 739.5 |
| R28 | 2.03 | 613.4 |
| R29 | 2 | 210 |
| R30 | 2.03 | 164 |
| R31 | 2.01 | 456.2 |
| R32 | 2.06 | 336.9 |
| R35 | 2.05 | 202.8 |
| R36 | 2 | 379.4 |
| R37 | 1.88 | 28 |
| R38 | 1.73 | 62.6 |
| R39 | 2.18 | 12.9 |
| R40 | 2.08 | 96.8 |
| R41 | 1.8 | 61.3 |
| R42 | 2.04 | 193.6 |

**Table 4:** Mean integrated density was calculated for each sample using ImageJ image analysis software. Nestin/GAPDH gene expression ratio (Ratio) was determined with RT-PCR in gingival biopsies from subjects undergoing SRP. Subject #11 (highlighted in red) was excluded from the analysis as the Piezo After sample failed to yield any GAPDH signal.

| Patient | Time | Treatment | Nestin | GAPDH | Ratio (Nestin / GAPDH) |
|---|---|---|---|---|---|
| 1 | Before | Hand | 991.1 | 6666.6 | 0.15 |
| 1 | Before | Piezo | 1445.2 | 7163.8 | 0.20 |
| 1 | After | Hand | 588.1 | 8069.0 | 0.07 |
| 1 | After | Piezo | 1421.6 | 6925.0 | 0.21 |
| 2 | Before | Hand | 742.3 | 7992.6 | 0.09 |
| 2 | Before | Piezo | 9858.6 | 7781.7 | 1.27 |
| 2 | After | Hand | 813.5 | 7753.4 | 0.10 |
| 2 | After | Piezo | 6252.5 | 8730.5 | 0.72 |
| 3 | Before | Hand | 1940.9 | 8450.4 | 0.23 |
| 3 | Before | Piezo | 894.5 | 3421.9 | 0.26 |
| 3 | After | Hand | 3435.3 | 9839.7 | 0.35 |
| 3 | After | Piezo | 3090.2 | 9476.1 | 0.33 |
| 4 | Before | Hand | 1613.5 | 8661.4 | 0.19 |
| 4 | Before | Piezo | 1309.4 | 8571.3 | 0.15 |
| 4 | After | Hand | 2148.7 | 9403.0 | 0.23 |
| 4 | After | Piezo | 841.6 | 6960.6 | 0.12 |
| 5 | Before | Hand | 4001.8 | 32041.4 | 0.12 |
| 5 | Before | Piezo | 9584.9 | 25981.0 | 0.37 |
| 5 | After | Hand | 22175.6 | 33168.5 | 0.67 |
| 5 | After | Piezo | 33909.8 | 22376.0 | 1.52 |
| 6 | Before | Hand | 2383.0 | 12576.5 | 0.19 |
| 6 | Before | Piezo | 4200.7 | 11036.6 | 0.38 |
| 6 | After | Hand | 4784.7 | 16243.7 | 0.29 |
| 6 | After | Piezo | 5495.3 | 11917.7 | 0.46 |
| 7 | Before | Hand | 4645.6 | 16072.5 | 0.29 |
| 7 | Before | Piezo | 3794.7 | 17355.0 | 0.22 |
| 7 | After | Hand | 8484.5 | 17490.7 | 0.49 |
| 7 | After | Piezo | 8763.1 | 18090.0 | 0.48 |
| 8 | Before | Hand | 7544.0 | 15376.5 | 0.49 |
| 8 | Before | Piezo | 7673.4 | 15310.1 | 0.50 |
| 8 | After | Hand | 8062.0 | 15734.8 | 0.51 |
| 8 | After | Piezo | 16675.3 | 16940.5 | 0.98 |
| 11 | Before | Hand | 1498.1 | 16034.5 | 0.09 |
| 11 | Before | Piezo | 1275.9 | 13494.9 | 0.09 |
| 11 | After | Hand | 7967.2 | 9027.6 | 0.88 |
| 11 | After | Piezo | — | — | — |
| 12 | Before | Hand | 6079.7 | 14907.4 | 0.41 |
| 12 | Before | Piezo | 5119.4 | 11509.4 | 0.44 |
| 12 | After | Hand | 4920.0 | 17577.4 | 0.28 |
| 12 | After | Piezo | 19115.7 | 18752.5 | 1.02 |

## Appendix B: Figures

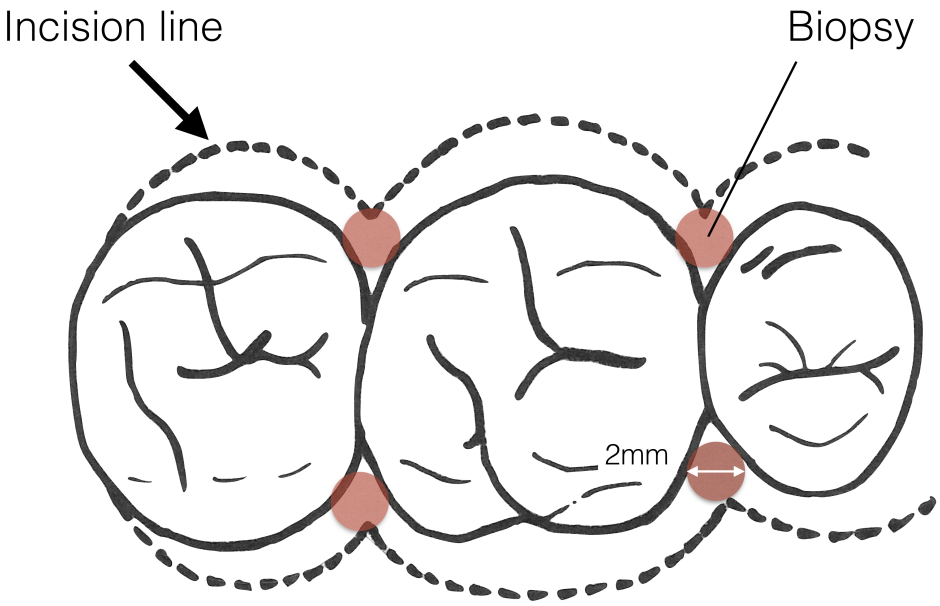


**Figure 1:** Biopsy sites (orange circles) and OFD incision lines

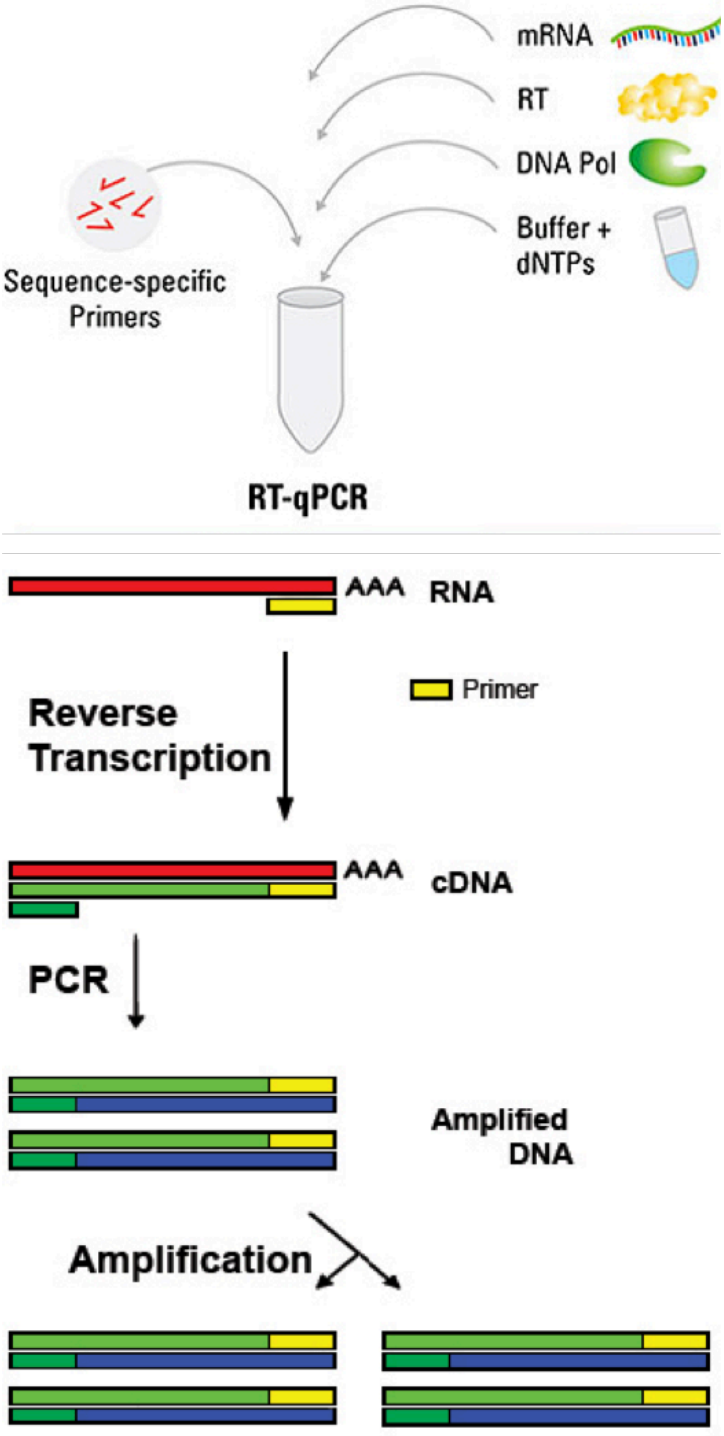


**Figure 2:** In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR.[29]

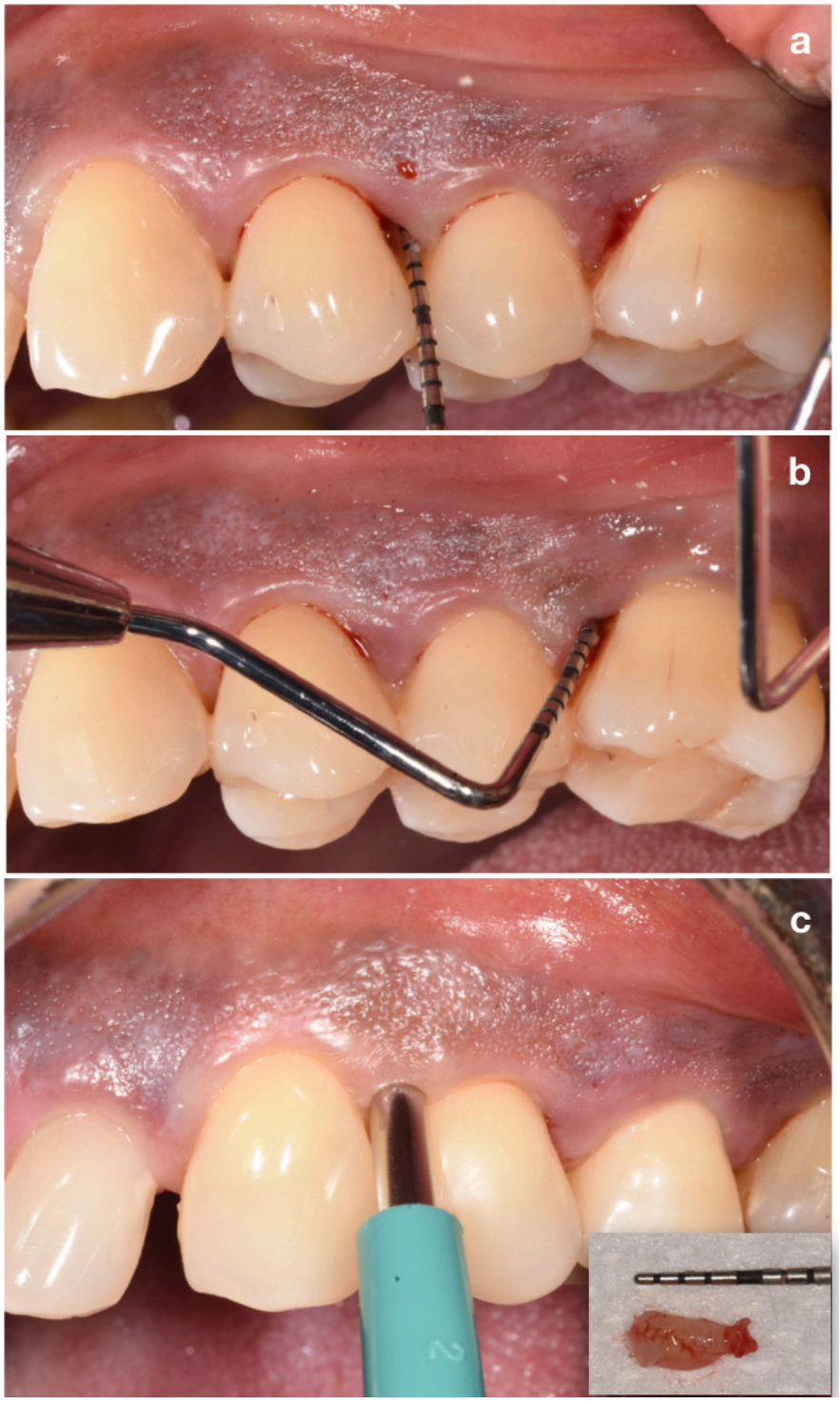


**Figure 3:** Tissue biopsies were performed total 4 sites prior to SRP: (a)(b) Tooth #12 and #14 were selected in this study subject. (c) 2mm sterile single-use biopsy punch was used. Biopsies were approximately 2mm in diameter and 5 mm in length (insert). Mesial buccal of each selected tooth biopsy was used for RNA analysis and mesial palatal of each selected tooth biopsy was used for histology.

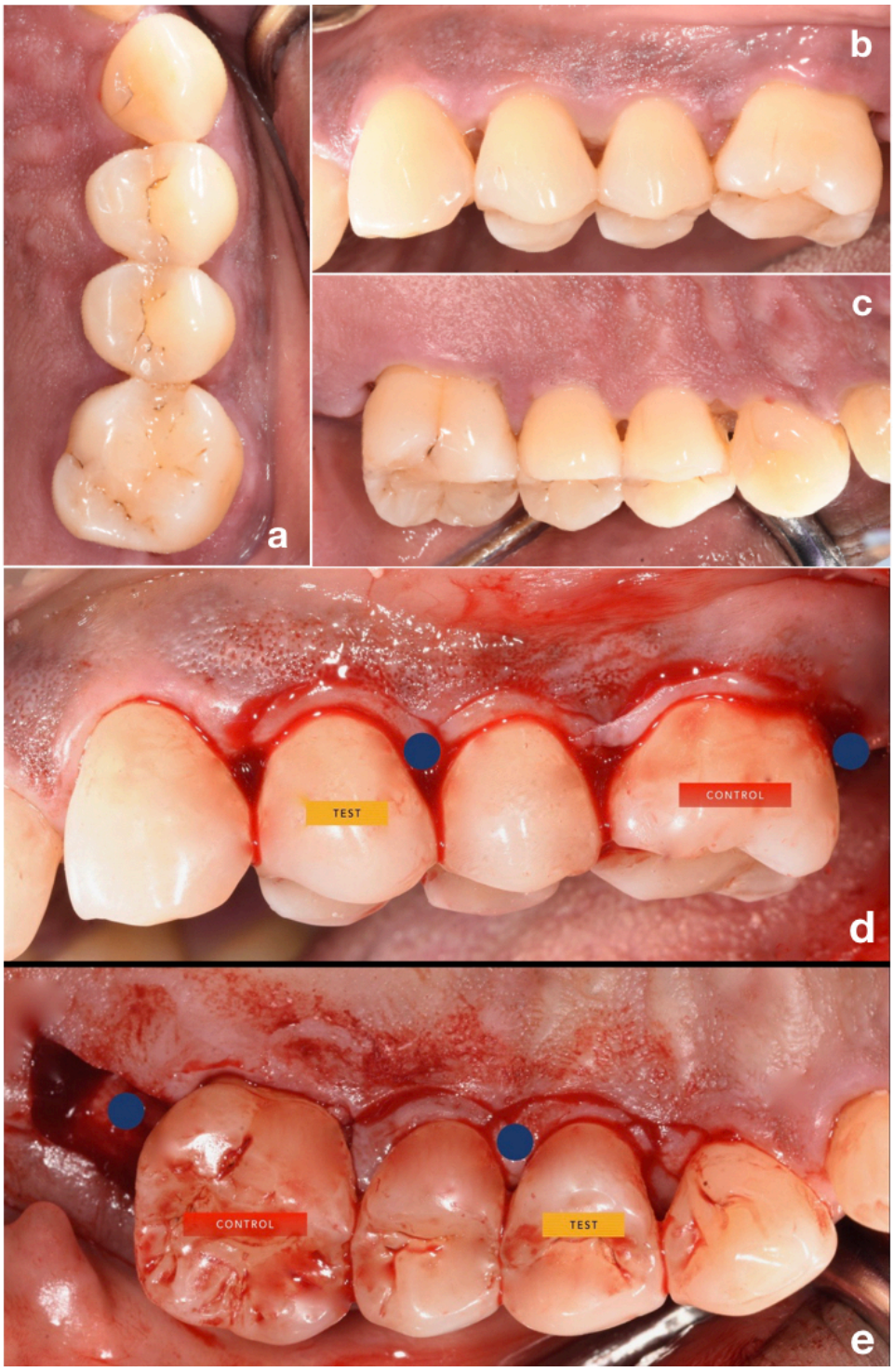


**Figure 4:** Tissue were taken from total 4 sites during surgery: (a-c) Clinical pictures pre-operation after 7 days after Visit 2. (d)(e) The submarginal incision was made on both buccal and palatal site of tooth #12-#14 and distal wedge was made distal of #14 according to bone sounding. Then tissue was removed. Distal buccal of each selected tooth was used for RNA analysis and distal palatal of each selected tooth biopsy was used for histology. (blue dot)

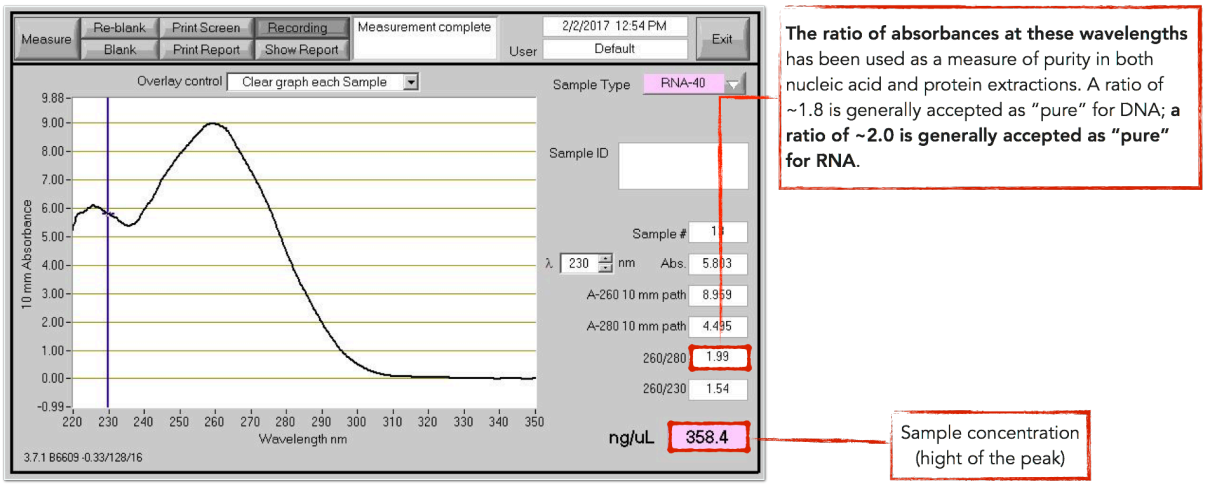


**Figure 5:** All RNA samples were quantified and qualified with NanoDrop™ Spectrophotometer. Absorbance at 260 and 280 nm were determined. The ratio of (260/280) was calculated. Sample concentration was calculated from the 260 values.

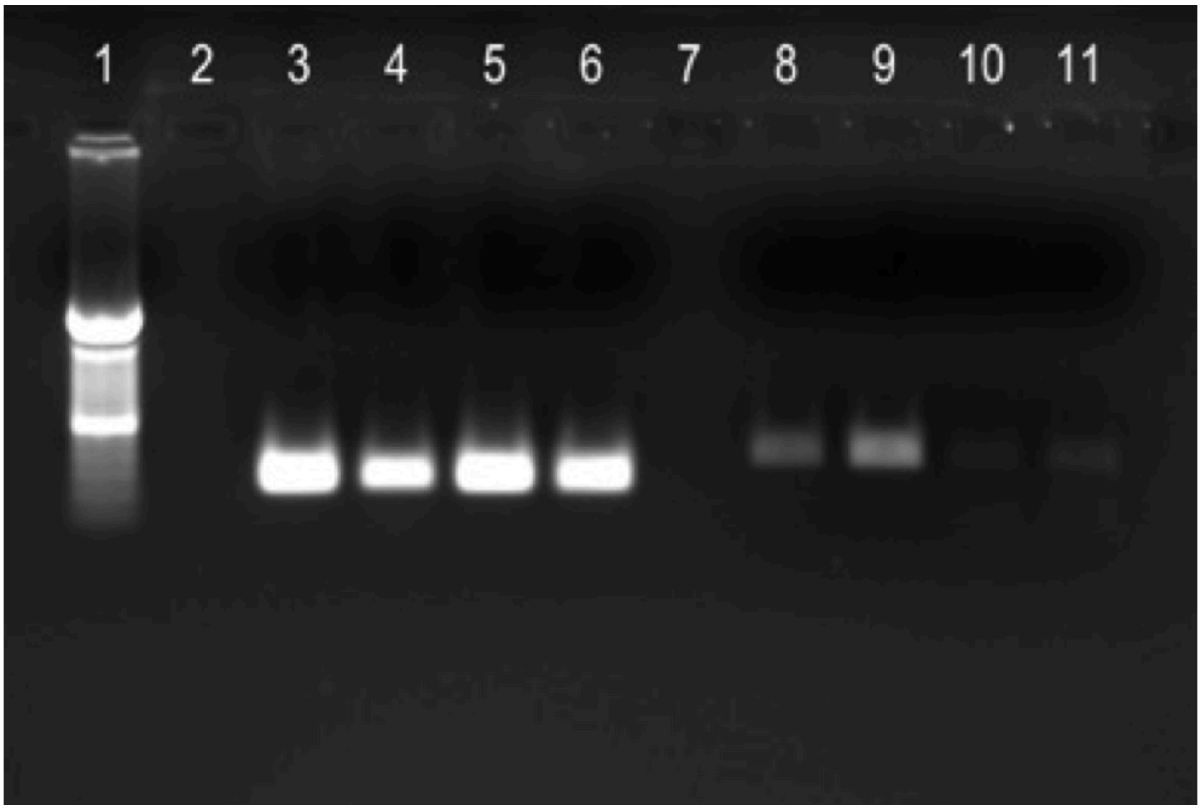


**Figure 6:** RT-PCR gel showing GAPDH (lanes 3-6) and nestin (lane 8-11) gene expression levels before (lane 5, 6, 10, 11) and after (lane 3,4,8,9) SRP. Lanes 3, 5, 8 and 10 show and SRP only; lanes 4, 6, 9 and 11 show hand and ultrasonic SRP. Lane 1: DNA size standards; lanes 2 and 7 are blank.

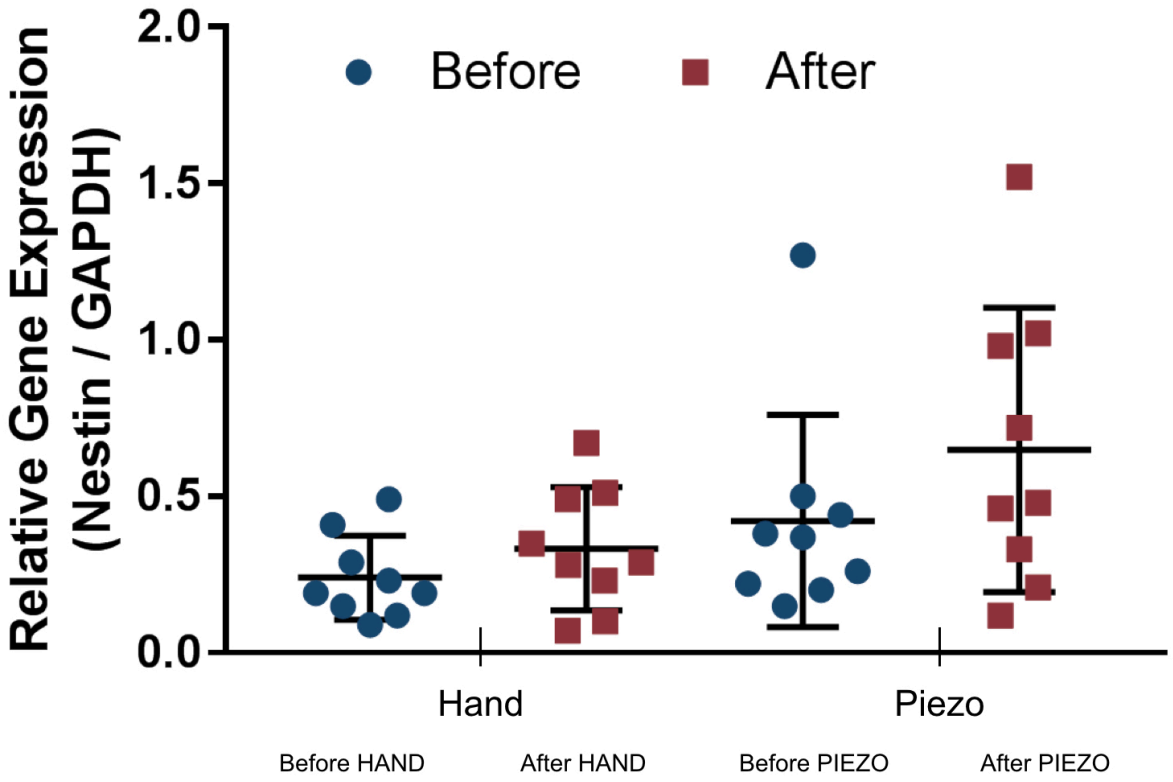


**Figure 7:** Nestin/GAPDH gene expression ratio as determined with RT-PCR in individual subjects. X-axis describes the treatments and Y-axis describes nestin/GAPDH gene expression ratio. Before HAND: baseline before HAND. After HAND: 1 week post-HAND. Before PIEZO: baseline before PIEZO. After PIEZO: 1 week post-PIEZO.

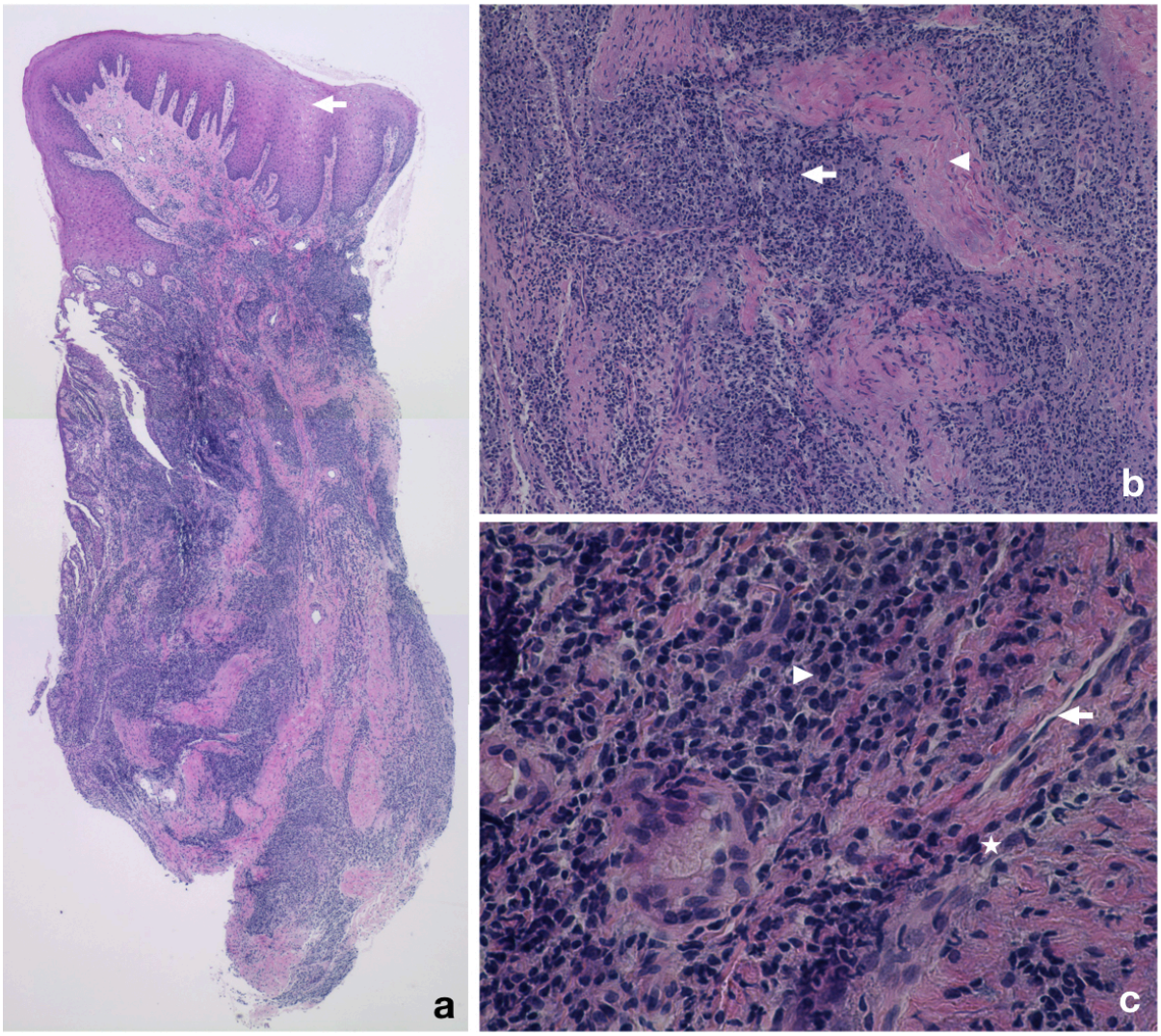


**Figure 8:** Histology samples in gingival biopsies from subject before SRP: (a) Typical tissue sample from severe chronic periodontitis patient before treatment. Low magnification image (original magnification x4) shows the entire tissue plug removed by tissue punch. On the top shows stratified squamous cell epithelial layer (arrow). The submucosa shows isolated island of eosinophilic areas with heavy cellular infiltrate. (b) Medium magnification (original magnification x10) shows inflammatory cell infiltrate (arrow) and collagenous tissue with embedded fibroblasts (arrowhead). (c) High magnification (Original magnification x40) shows that the majority of cellular infiltrate consists of mononuclear cells with basophilic cytoplasm consistent with plasma cells morphology (arrow head). Other cell types visible are fibroblasts (star) and endothelial cells (arrow).

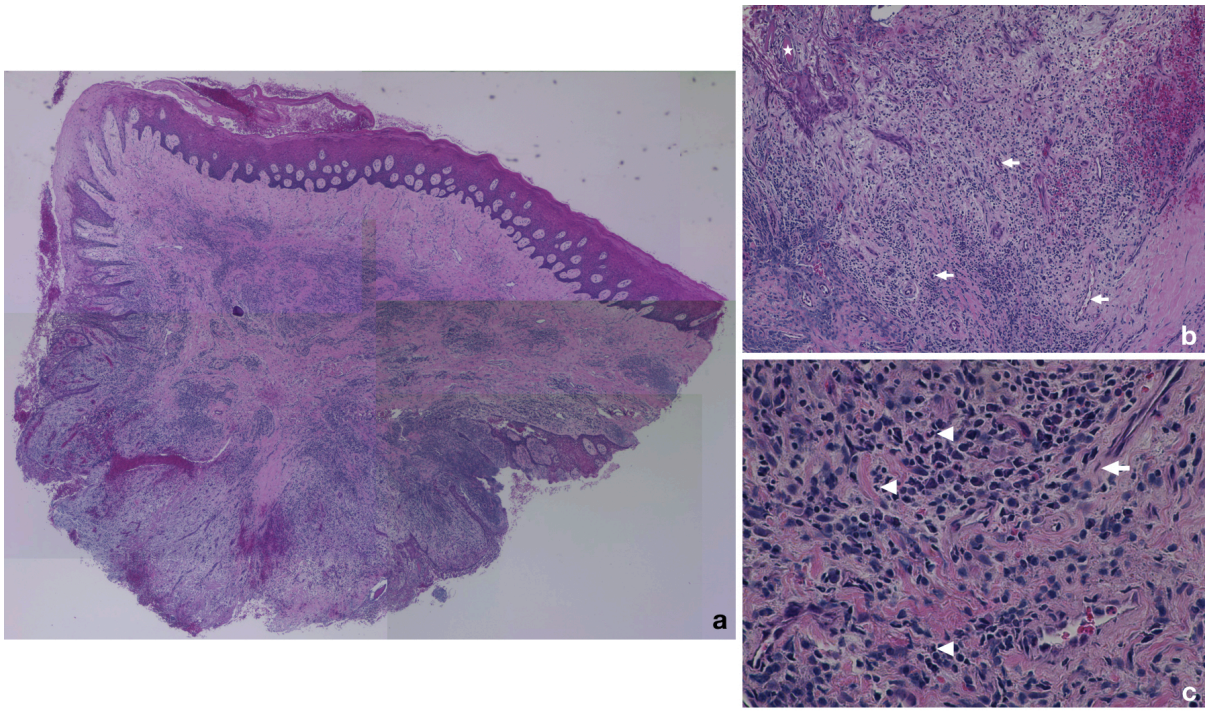


**Figure 9:** Histology samples in gingival biopsies from subjects after 1 week of PIEZO: (a) Low magnification image (original magnification x4) shows the entire tissue that surgically removed. The submucosa shows isolated island of eosinophilic areas with less cellular infiltrate in connective tissue. (b) Medium magnification (original magnification x10) shows inflammatory cell infiltrate and presence of multiple capillaries in the connective tissue (arrow). Bone segment is seen in connective tissue (star). (c) High magnification (original magnification x40) shows that the majority of cellular infiltrate consists of neutrophils (arrow head) and fibroblasts (arrow) and lesser plasma cells are seen in the connective tissue.

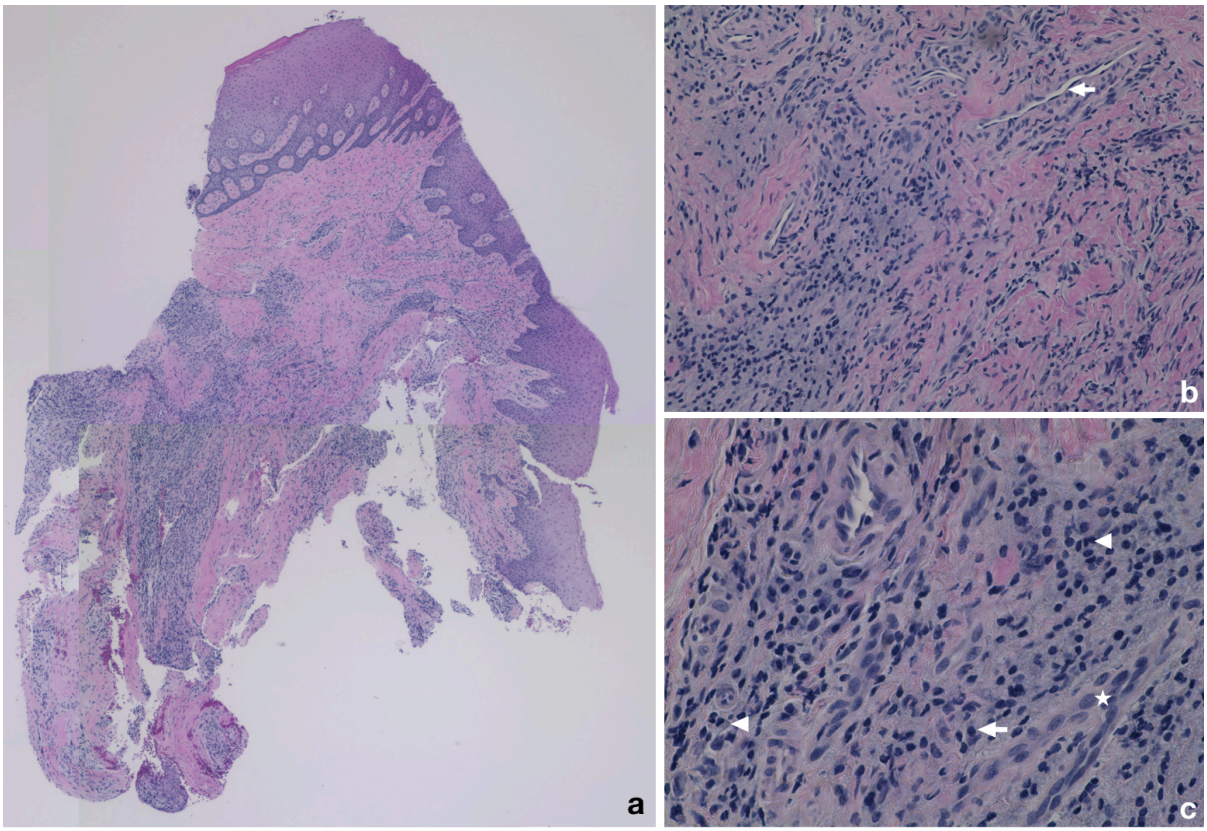


**Figure 10:** Histology samples in gingival biopsies from subjects after 1 week of HAND: (a) Low magnification image (original magnification x4) shows the entire tissue that surgically removed. The submucosa shows isolated island of eosinophilic areas with less cellular infiltrate in connective tissue. (b) Medium magnification (original magnification x10) shows inflammatory cell infiltrate and presence of multiple capillaries and endothelial cells (arrow). (c) High magnification (original magnification x40) shows that the majority of cellular infiltrate consists of neutrophils (arrow head) and some plasma cells (arrow) and fibroblasts (star).