Histological, immunohistochemical and biomolecular analysis of the effect of chlorhexidine digluconate on early wound healing in human gingival tissues.

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Abstract:

Aim: to evaluate the *in vivo* effect of the post-surgical use of chlorhexidine digluconate (CHX) mouthrinse on the gingival tissue.

Materials and Methods: Gingival biopsies were obtained in three patients 24 hours after surgery with indication of post-surgical 0.12% CHX mouthrinse and were compared with those obtained from the same patients without antiseptic use indication. Changes in collagen production, cell proliferation and apoptosis were examined by histological and Ki-67/P53 immunohistochemical analysis. To understand the mechanism through which CHX influence the cellular behavior, fibrotic markers (COL1A1, α-SMA), proapoptotic protein (BAX) and wound healing-related genes (RAC1, SERPINE1, TIMP1) expression were analyzed by Quantitative real-time PCR.

Results: 24 hours after surgery, CHX was able to (1) increase collagen deposition, (2) reduce proliferation cell ability and increase the expression of proapoptotic molecules, (3) increase fibrotic markers expression and myofibroblasts differentiation, (4) reduce expression of RAC1 and trigger expression of SERPINE1 and TIMP1, showing a pattern associated with "scar wound healing response".

Conclusions: The results of this study demonstrate a CHX-induced fibrotic transformation, leading to scar periodontal tissue repair. Therefore, the results highlight the need to further investigate in order to define a post-surgical clinical protocol that provides a strategic and personalized use of CHX in the first hours after surgery.

Abstract:

Obiettivo: valutare l'effetto *in vivo* dell'utilizzo post-chirurgico della clorexidina (CHX) nel tessuto gengivale.

Materiali e Metodi: a 24 ore dall'intervento chirurgico, sono state eseguite biopsie a livello della gengiva aderente in tre pazienti che hanno effettuato sciacqui con CHX 0.12% e sono state confrontate con quelle prelevate dagli stessi pazienti per un ulteriore intervento senza la prescrizione dell'antisettico. Sono stati esaminati la produzione di collagene, la proliferazione cellulare e l'apoptosi mediante analisi istologica e immunoistochimica (Ki-67/P53). Per comprendere il meccanismo attraverso il quale la CHX influenza il comportamento cellulare, sono stati analizzati marcatori fibrotici (COL1A1, α-SMA), proteine pro-apoptotiche (BAX) e geni correlati alla guarigione delle ferite (RAC1, SERPINE1, TIMP1) mediante Quantitative real-time PCR.

Risultati: a 24 ore dall'intervento chirurgico, la CHX ha determinato (1) l'aumento della deposizione di collagene, (2) la riduzione della capacità proliferativa delle cellule e l'aumento dell'espressione di molecole proapoptotiche, (3) l'aumento dell'espressione dei marcatori fibrotici e della differenziazione dei miofibroblasti, (4) la riduzione dell'espressione di RAC1 e l'induzione dell'espressione di SERPINE1 e TIMP1, mostrando un pattern associato ad una "risposta di riparazione tissutale con cicatrice".

Conclusioni: i risultati di questo studio dimostrano una trasformazione fibrotica indotta da CHX, che potrebbe portare alla riparazione del tessuto parodontale con presenza di cicatrice. Pertanto, i risultati evidenziano la necessità di investigare ulteriormente al fine di definire un protocollo clinico post-chirurgico che preveda un uso strategico e personalizzato della CHX nelle prime ore post-intervento.

Introduction

Wound healing is an extremely complex physiological process that depends on multiple factors.[1] The presence of oral biofilm, the main etiological factor of periodontal and peri-implant diseases, may jeopardize the repair process.[2] Thus, especially after surgical procedures in which mechanical plaque control cannot be performed, is extremely important the reduction of plaque accumulation by means of antimicrobial agents.[3]

Chlorhexidine digluconate (CHX), a bisbiguanide broad-spectrum antiseptic with antibacterial action, is widely used as therapeutic agent in periodontology. Numerous studies have demonstrated its ability in reducing oral biofilm deposition.[4-6] Moreover, penetrating biofilms, CHX has a bactericidal action,[7] reaching a substantivity of 12 hours.[8]

Although different effects have been reported based on different available concentrations, it has been concluded that twice daily rinses with 15 ml of 0.12% CHX are enough for effective plaque control in the oral cavity.[9]

However, side effects such as desquamation of the oral mucosa, soreness, increased calculus formation and tooth pigmentation have been reported in the literature, suggesting it use only for short periods.[10]

A recent systematic review,[11] concluded that CHX helps in reducing biofilm formation and gingival inflammation after periodontal and implant surgery and that a 0.12% concentration should be indicated in order to reduce the adverse effects.

Due to the above mentioned bactericidal and bacteriostatic activities[4-6] and to the absence of toxic systemic effects reported,[12] CHX has been considered the gold standard for antiseptic treatment of the oral cavity.[9] Nevertheless, a recent *in vitro* study evaluating its impact in controlling oral biofilms showed an initial drop in biofilm bacterial cell concentration followed by a quick recovery after its use. The authors concluded that CHX can be ineffective in maintaining oral health since presents a temporal effect and, as a broad-spectrum antiseptic, it can also affect the endogenous oral microbiota, increasing the risk of microbial dysbiosis, leading

in turn to the development of oral diseases.[13]

Furthermore, since 1970s, several studies have reported noxious effects on many cells as macrophages,[14] leucocytes[15] and skin epithelial cells.[16] Bassetti and Kallenberger in 1980[17] through an animal experimental model have demonstrated that intensive post-surgical rinsing with high concentrations of CHX could delay and impair the wound repair process. In addition, many recent studies showed cytotoxic effects in human periodontal tissues cells, such as gingival epithelial cells,[18] gingival fibroblasts,[19-21] bone[22] and periodontal ligament cells.[23]

Faria et al.[20] observed that CHX induced apoptosis of cultured fibroblasts at lower concentrations and necrosis at higher concentrations. Mariotti e Rumpf [19] postulated that CHX can reduce both collagen and non-collagen proteins production and proliferation of human gingival fibroblasts (HGFs), even in a very low concentration. This was confirmed in a very recent *in vitro* study in which cells were exposed to a concentration diluted 100-fold when compared to their current uses in clinical practice.[24] Another recent *in vitro* study using HGFs showed that a concentration ≥0.04% inhibits cell proliferation, affects cells morphology and induces apoptosis. These effects are concentration and time-dependent. The authors concluded that post-surgical applications of CHX should be limited.[25]

All the above-mentioned *in vitro* studies allow to understand that CHX is not harmless to oral tissues, mainly in the wound healing process. However, it is important to highlight that *in vitro* assays cannot represent the oral environment as a whole and this could be a limitation.[26]

Chen et al.,[27] have demonstrated that the main transcriptional changes in the wound healing occur in the first 12-24 hours. In fact, we have observed significant changes in myofibroblast differentiation, fibrotic markers and wound healing genes expression of oral soft tissues derived-fibroblasts 24 hours after surgery when compared to baseline.[28,29] In addition, it has been demonstrated that until the first 24 hours the biofilm is primarily populated by gram-positive cocci, and gram-negative anaerobic bacteria rapidly increase and predominate after 48 hours.[30,31]

Considering all the aforementioned, immediate post-surgical use of CHX might not be necessary. This could be of beneficial effect on the healing process, since the most important changes in tissue repair occurs in the very early stages.

To date, no *in vivo* study has been conducted evaluating the CHX effects on gingival tissue behavior in the early wound healing process.

Therefore, the aim of the present study was to evaluate the *in vivo* effect of post-surgical CHX mouthrinse on the gingival tissue 24 hours after injury. Our hypothesis was that CHX impairs the wound healing potential by: 1) reducing the proliferation ability, 2) increasing cell apoptosis, fibrotic markers expression and myofibroblasts differentiation and 3) modifying early wound healing-related genes expression, as determined by histological, immunohistochemical and biomolecular analyses of human gingival biopsies.

Materials and Methods

- **Ethics statements**

The study protocol (ClinicalTrial.gov-NCT04276129) was approved by Sapienza University of Rome Ethics Committee (Ref.5315-Prot.1066/19). Each participant signed an informed consent in accordance with the Declaration of Helsinki (1975, revised in 2013).

- **Study design and patient selection**

The present pilot study involved three systemically healthy adult patients (mean age 39.3 ± 5.44) who undergone at least two periodontal surgery procedures and who agreed to be "volunteer" for biopsy collection procedures by signing an informed consent. Patients who underwent antibiotic or anti-inflammatory drug consumption during the previous six months, patients in pregnancy or lactation period and smokers were excluded from the study.

The subjects were enrolled at the clinical center in the Section of Periodontology, Sapienza University of Rome, Department of Oral and Maxillo-Facial Sciences. Each patient underwent two surgical procedures and was treated in split mouth design to either post-surgical CHX mouthrinses indication (treatment group - CHX) or non post-surgical mouthrinses indication (no treatment group - NT).

Biopsies from buccal attached gingiva (G) were harvested 24 hours after surgical procedures.

- **Surgical procedures and collection of human gingival tissues samples**

All surgical procedures and biopsies were performed by the same operator (MR). At the end of the surgical procedure, primary closure was obtained at the level of the vertical releasing incisions (VRIs). In the treatment group, 0.12% CHX mouthrinses (15ml/30s) were indicated two times/day. Therefore, at the time of the biopsy collection, the patients had already performed two mouthrinses. In the NT group, patients did not perform any mouthrinse after surgery. Twenty-four hours after the surgical procedure, G biopsies were harvested at the level of the VRIs with a biopsy punch of 2.0 mm diameter.

The biopsy areas healed by second intention and sutures were removed at 1 week.

- **Histological analysis**

Gingival biopsies were fixed in 10% neutral buffered formalin and processed for paraffin embedding. Blocks of paraffin were cut at 3 μm thickness using a Leica microtome. Sections were deparaffinized in xylene, rehydrated through graded alcohol series and stained with Hematoxylin–Eosin (HE) and trichrome Masson according to standard protocols.

- **Immunohistochemistry**

For immunohistochemical (ICH) evaluation, the sections were treated by boiling in citrate buffer (0.01 mol/l, pH 6) in microwave (750 W) for antigen retrieval. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Slides were then incubated overnight at 4 °C with the following primary antibodies (dilution 1:100): vimentin (clone V9, Dako, Glostrup, Denmark), Col1a1 (clone 3G3, Santa Cruz Biotechnology, Santa Cruz, USA), αSMA (clone 1A4, Sigma-Aldrich, Milano, Italy), Ki67 (clone MIB1, Dako), p53 (clone DO-7, Dako). The Universal Dako Labelled Streptavidin-Biotin 2 System, Horseradish Peroxidase (Dako LSAB2 + System-HRP; Dako) was used to label the primary antibody. The reaction product was visualized with diaminobenzidine (DAB) substrate–chromogen (Dako) and counterstained with Mayer haematoxylin (Sigma). Negative control slides were obtained by omitting the primary antibody.

Sections were analyzed using a Leica microscope coupled to a digital camera. Two independent pathologists, blinded to the treatment, observed the immunostaining and, subsequently, images were captured. The staining intensity for αSMA, vimentin and Col1a1 was determined using a semi-quantitative score (0, no staining; 1, low staining; 2, moderate staining; 3, strong staining).[32,33] This evaluation was performed by two independent investigators blinded to the treatment, who observed five microscopic fields for each of the three sections randomly selected for each case using the objective ×20.

Immunohistochemical staining for the nuclear proliferation-associated antigen Ki-67 and for p53 was estimated as the percentage of stained nuclei among all nuclei visible in the field. The analysis was performed by two blinded examiners. The number of cells with Ki67/p53-positive nuclei was evaluated in 10 random microscopic fields in each cell preparation and expressed as percentage of Ki67/p53-positive nuclei per optical field.

- **Quantitative real- time PCR (qRT-PCR)**

Total RNA from CHX and NT gingival biopsies of the three enrolled patients were extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions, and was reverse transcribed using High Capacity RNA to cDNA Kit (Applied Biosystems by Life Technologies, Carlsbad, USA). cDNAs were then used for amplification of BAX, Col1a1, αSMA, RAC1, SERPINE1 and TIMP1, using the appropriate TaqMan gene expression assay kits (Applied Biosystems). A total of 2 μl/well of template was added to the sample wells along with TaqMan Universal PCR master mix at a concentration of 1x and water to a volume of 25 μl/well. Assays were conducted in triplicate on an ABI 7500 Real Time instrument (Applied Biosystems) using the following conditions: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min, repeated 40 times. Relative quantification was performed using GAPDH mRNA as an endogenous control.

- **Statistical analysis**

Data were analysed on Prism 8.0 (GraphPad Software, La Jolla, USA) and are shown as mean ± SD from three independent experiments conducted in triplicate. Two-tailed unpaired Student's t test was used for statistical analysis. P values < 0.05 was considered statistically significant.

Results

- **CHX post-surgical mouthrinse increases fibrotic markers expression and myofibroblasts differentiation**

HE staining revealed in both NT and CHX group a thick gingival mucosa, with deep and branching epithelial ridges sometimes joined by epithelial bridges. Subjacent chorion was full of collagen bundles, appearing as a dense and homogeneous structure (Figure 1A). Collagen deposition was further revealed with Masson's trichrome staining (Figure 1B). As for CHX group, HE staining showed the presence of enlarged, polymorphic and polymetric nuclei, indicative of activated cells, in the epithelial layer (Figure 1C, upper panel), and a more extensive fibrosis in the chorion (Figure 1C, lower panel).

Afterwards, the expression levels of fibrosis markers were analyzed with IHC staining. We incubated serial sections of each biopsy belonging to the two groups (NT and CHX) with the following antibodies: anti-αSMA, anti-Col1a1 and anti-vimentin. For αSMA, normal vessels' smooth muscle immunoreactivity was used as an internal positive control, while αSMA-positive stromal cells, showing cytoplasmic immunostaining, were considered to be myofibroblasts. NT samples showed an extremely weak positivity in the mesenchymal cells, while cells of blood vessels were labeled. In the CHX group, we noted a higher number of blood vessels in the chorionic papillae and the deep chorion compared to NT samples (Figure 2A), and we also observed the presence of cells with cytoplasmic positivity localized in the basal epithelial layer, particularly in the deep and prickle cell layers (Figure 2B).

As for the fibrotic marker Collagen 1a1 (Col1a1), its expression was localized in the subepithelial layer, and it was significantly higher in CHX biopsies with respect to NT group (Figure 2C).

Immunostaining for vimentin, specific for cells of mesenchymal origin, showed few positive cells concentrated mainly in the subepithelial layer. We observed no significant differences both in the amount of positive cells and in their location between samples from NT and CXH group.

The semiquantitative evaluation for αSMA, Col1a1 and vimentin staining intensity was reported in Table 1.

The expression of αSMA and Col1a1 was also assessed at mRNA level by qRT-PCR analysis in gingival biopsies of three patients subjected or not to CHX mouthrinses in the 24 hours between surgical intervention and biopsy collection. Our results confirmed a significant increase in αSMA expression in the CHX biopsies of all the three patients (3.6, 2.3 and 3.6-fold, respectively) (Fig. 3A). The same trend was observed for Col1a1, with a consistent increase in the CHX biopsies of all patients (2.9, 2.3 and 34.4-fold, respectively) (Figure 3B).

- **CHX influences the expression of key genes involved in early wound healing**

We then investigated the effect of CHX on the expression of some genes previously shown to play a role in the early wound healing process,[29] in two out of the three enrolled patients. We first evaluated RAC1, a member of the Rho family of small GTPases that promotes healing and that has been previously shown to increase in gingival tissue 24 hours after injury.[28,29] Interestingly, we observed a significant downmodulation of RAC1 expression at 24 hours in CHX biopsies of both patients (0.2 and 0.02-fold, respectively) (Figure 4A), thus suggesting that CHX might impair gingival wound healing. SERPINE1 and TIMP1 were also evaluated. Such genes, involved in collagen deposition and fibrosis, were previously shown to remain stable in gingival tissue at 24 hours after injury. In our study, we observed an increase of them in CHX biopsies of both patients (1.6 and 3.0-fold for SERPINE1; 3.4 and 11.8-fold for TIMP1, respectively; Figure 4B, C).

- **CHX increases the expression of apoptotic markers and reduces the proliferative ability of gingival cells.**

In order to understand the molecular events underlying the effect of CHX on early gingival wound healing, the expressions of proteins related to proliferation and apoptosis were examined by IHC analysis. As compared with NT group, the Ki67 proliferation marker was significantly downregulated in the CHX group

(Fig. 5A), as indicated by the percentage of stained nuclei reported in Figure 5B (26.8% vs 42.8% of NT, *p < 0.05).

So, we assessed if the reduced proliferation could be accompanied by an induction of apoptosis. To this aim, we evaluated the expression of the tumor suppressor gene p53, a key regulator of cell death under multiple physiological and pathological conditions. In our *in vivo* model, IHC analysis showed that p53 expression was slightly higher in the CHX group (Figure 6A), with a modest but not statistically significant increase of the percentage of stained nuclei in CHX samples (18,1% vs 14.2% of NT, Figure 6B).

Interestingly, when analyzing the expression of the proapoptotic BAX protein in gingival tissue by Real Time PCR, we found a significantly higher expression of BAX in the CHX biopsies of all the enrolled patients (1.5, 2.4 and 3.7-fold, respectively) (Figure 6C), thus indicating a potential p53-independent proapoptotic effect of CHX post-surgical treatment on gingival tissue.

Discussion

Chlorhexidine is considered as the gold standard in the antiseptic treatment of the oral cavity.[9] Nevertheless, time and dose-dependent cytotoxic effect of CHX in human fibroblasts has been demonstrated in previous *in vitro* studies,[19,26,34] delaying wound healing or increasing wound dehiscence rates. [35-37]

The present study was designed to investigate the *in vivo* effect of post-surgical 0.12% CHX mouthrinse in the early phase of gingival tissue repair to understand its role on cell behavior, including (1) proliferation, (2) apoptosis, (3) fibrotic markers expression, (4) myofibroblasts differentiation and (5) early wound healing-related genes expression through a histological, immunohistochemical and biomolecular analysis of human gingival biopsies. All these processes are involved in the soft tissue wound healing response after surgical procedure.

Our findings demonstrate that, 24 hours after injury, CHX is able to (a) reduce cell proliferation and increase the expression of proapoptotic molecules, (b) increase fibrotic markers expression and myofibroblasts differentiation, (c) reduce expression of RAC1 and trigger expression of SERPINE1 and TIMP1.

In our *in vivo* experimental setting, we observed that Ki67 proliferation marker was significantly downregulated in the CHX group compared with NT group, confirming the anti-proliferative effects of CHX in gingival tissue *in vivo*, in agreement with those obtained *in vitro* by other authors.[19,25,26,38-40] Many citotoxic agents modulates the balance between cell proliferation and cell death.[41]Cell death can occur through different pathways that can culminate in autophagy, necrosis or apoptosis.[25] These mechanisms may play an important role in the scarring response. In fact, it has been reported the ability of apoptotic cells to induce myofibroblasts differentiation and proliferation.[42,43]

Gianelli et al.[34] reported that after 1 min treatment, nearly 50% of fibroblastic and endothelial cells treated with 0.12% CHX exhibited apoptotic nuclei. In the present study, we did not observe a significant increase in the percentage of stained nuclei after CHX treatment through IHC analysis using p53 as a marker of apoptosis. However, we can infer a proapoptotic potential of CHX consistently with higher expression of the gene BAX. Thus, our results confirmed *in vivo* the detrimental effect of CHX in reducing cell viability, and led us to hypothesize that CHX mouthrinse could trigger a p53-independent apoptosis.

Fibroblasts become activated upon wounding, as evidenced by expression of α-SMA, proliferation and migration to the wound area, and ECM deposition.[44]

In our previous studies,[28,29] we demonstrated a downregulation of α-SMA and Col1a1 in gingival tissue 24 hours after injury, in line with clinical observation of reduced scar formation in this tissue. Instead of, the alveolar mucosal (M) tissue showed the opposite response, according to the clinical observation of scar tissue repair. We observed that CHX-treated G tissue present similar behavior to M tissue suggesting that it could induce a “fibrotic response”.

The effect of CHX on collagen production was reported by Mariotti e Rumpf.[19] The authors postulated that, at concentrations which have little effect on cellular proliferation, it can significantly reduce both HGFs collagen and non-collagen protein production. Consistent with these findings, a very recent study showed decreased COL1 expression after CHX treatment.[24] Here, we observed the opposite response, and this could be related with the differences between *in vitro/in vivo* analysis.[26] It is noteworthy that these features are similar to

those reported in adult skin fibroblasts, which show a reduction in genes associated with proliferation and an enrichment for GO terms ECM production and remodeling-related with increasing age.[45] Additionally, it is interesting to mention that CHX intraperitoneal injection has been reported as the most commonly used method to create a peritoneal fibrosis animal model[46-48] showing increased expression of transforming growth factor β1 (TGF- β1), α-SMA, type I collagen, and vascular endothelial growth factor (VEGF).[47]

Based on our group's previous results,[29] it was still interesting to further investigate the findings based on previously assessed genes related to scar wound healing. Through qRT-PCR analysis, we evaluated the expression of RAC1, TIMP1 and SERPINE1 genes. Noteworthy, we observed that gingival tissue after CHX treatment present the same pattern observed in alveolar mucosal-derived fibroblasts[29] showing RAC1 downmodulation and TIMP1 and SERPINE1 upregulation. These results are in line with the evidence of an increase in collagen deposition mediated by CHX mouthrinses.

It is important to highlight that, although it has been demonstrated a higher tolerance of human tissues for antiseptic solutions *in vivo* compared to *in vitro* tissue culture,[49] in the present study we demonstrated that even after only two mouthrinses with 0.12% CHX, gingival tissue behavior is modified, altering the normal wound healing repair response 24 hours after injury.

Undoubtedly, our study presents some limitations, since the evaluation was carried out in only three patients and in a single period-time. Moreover, the data obtained here should be paralleled with a clinical evaluation through an accurate assessment of the healing characteristics.[50,51] Although our results should be extended to solve the aforementioned issues, the *in vivo* data obtained in the present work confirms previous *in vitro* findings and provide additional *in vivo* evidence to understand the potential of CHX to negatively interfere in the early phase of human gingival tissue wound healing. However, because of a small sample size, the results should be cautiously interpreted.

One of the main strengths of this study is that the effect of CHX was evaluated *in vivo*, through a human biopsy wound model. Although through an *in vitro* assay a better quantitative analysis can be achieved, without the interference of other *in vivo* factors,[52] surgical wounds present particular conditions to consider, such as vascularization, local and systemic inflammatory responses after injury, mechanical forces affecting tissue repair process, multiple cell layers and presence of saliva and crevicular fluid. All these features are not present in a monolayer culture and this could produce relevant changes in the oral tissue response. In fact, we observed some differences between our results and the *in vitro* performed studies and many similarities with *in vivo* animal studies performed in other medical fields. Therefore, *in vivo* evaluations appear to be critical to elucidate the mechanisms impairing the wound healing process after the post-surgical use of CHX mouthrinses.

Conclusion

The present research was designed to evaluate the *in vivo* effect of post-surgical CHX mouthrinse in the gingival tissue 24 hours after injury. The results of this investigation showed significant changes in the expression of BAX, Col1a1, α-SMA, RAC1, SERPINE1 and TIMP1 in CHX-treated gingival biopsies when compared with NT group. These findings further support that features such as increased collagen deposition, myofibroblasts differentiation and cell apoptosis, as well as reduced cell proliferation, could be relevant for a CHX-induced fibrotic transformation, leading to scar tissue repair. Therefore, the results highlight the need to further investigate in order to define a post-surgical clinical protocol that provides a strategic and personalized use of CHX in the first hours after surgery.

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Tables

Table 1. Immunohistochemical scoring of staining intensity for α-SMA, Col1a1 and Vimentin

Patient	IHC score[a]					
	αSMA		Col1a1		Vimentin	
	NT	CHX	NT	CHX	NT	CHX
1	0	1	2	3	1	1
2	0	1	1	2	1	1
3	0	1	1	3	1	1

IHC, immunohistochemistry; NT, no treatment group; CHX, chlorhexidine mouthrinses group.
[a] Staining intensity scores were as follows: 0, no staining; 1, low staining; 2, moderate staining; 3, strong staining.[31,32]

Figures

Figure 1

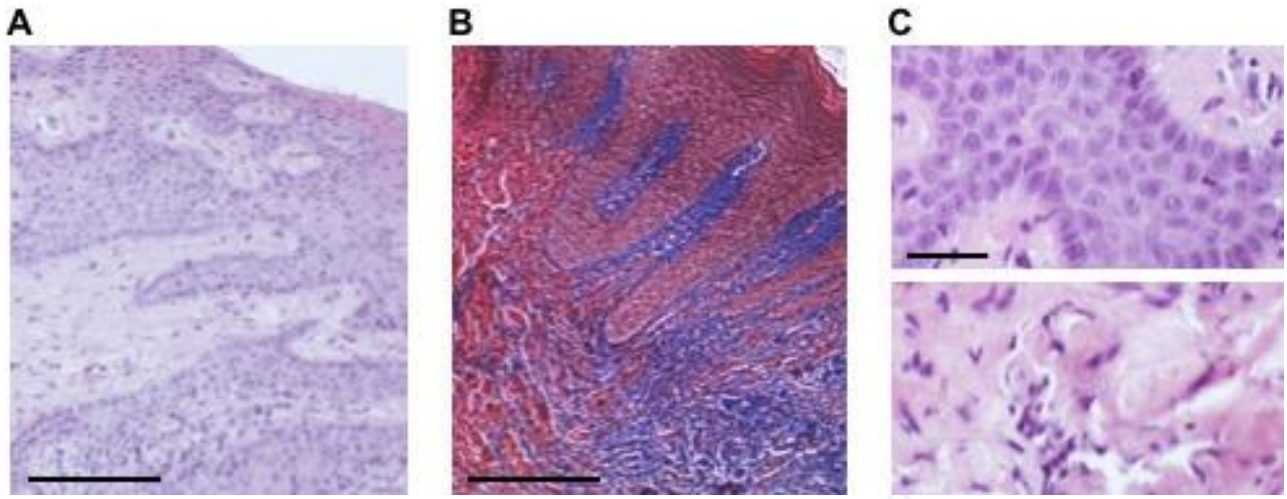

Figure 1. Histological characterization of gingival biopsies. A) Representative photomicrograph of sections of gingival biopsies showing elongated and branched epithelial ridges and subjacent chorion full of a dense and homogeneous structure of collagen bundles. HE staining, scale bar 100 μm. **B)** Representative photomicrograph of sections of gingival biopsies showing collagen bundles in the deep chorion (blue). Trichromic Masson staining, scale bar 100 μm. **C)** Representative photomicrographs of histological alterations observed in CHX biopsies, such as enlarged and polymorphic nuclei in the epithelial layer (upper panel) and enhanced fibrosis in the deep chorion (lower panel). HE staining, scale bar 25 μm.

Figure 2

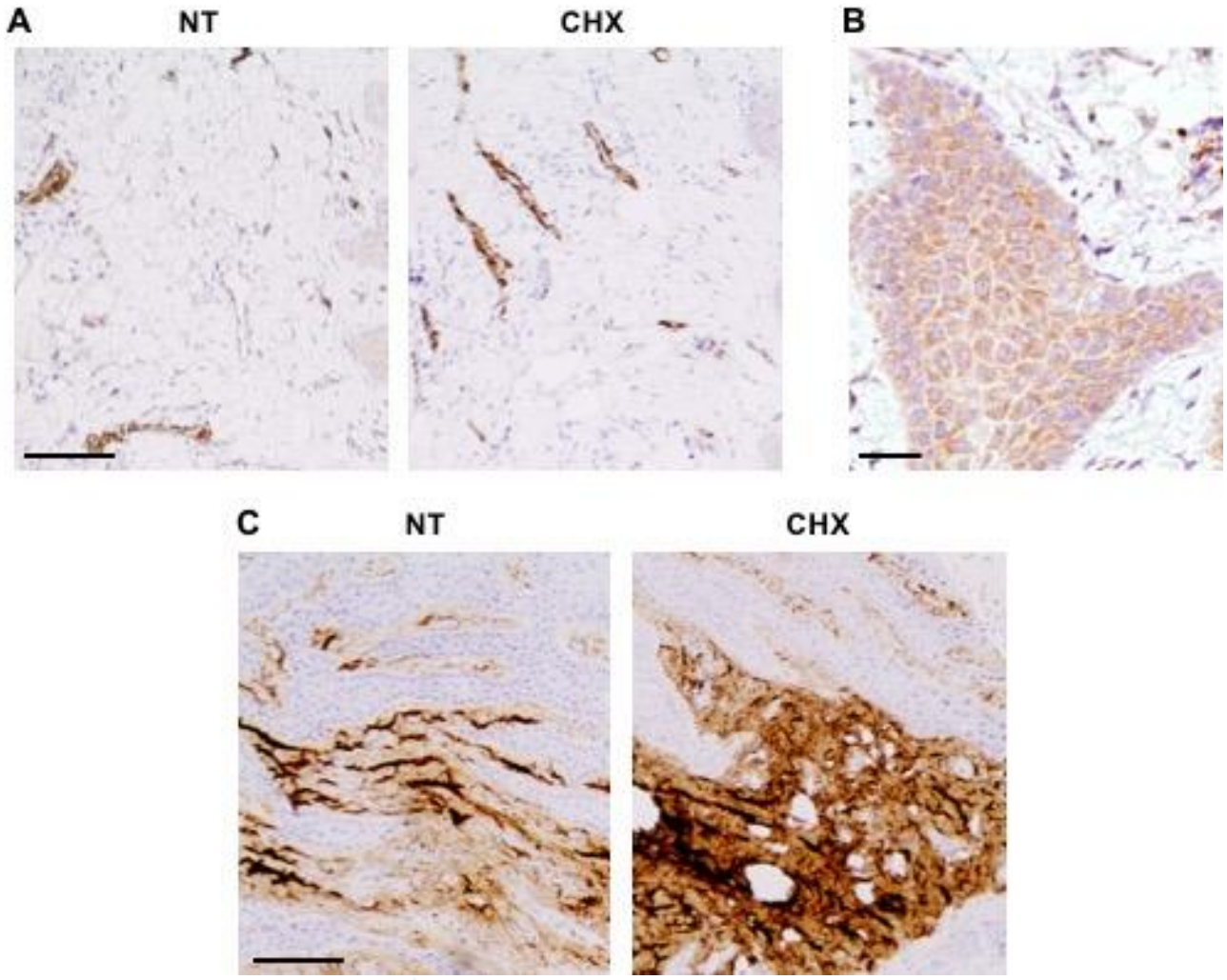

Figure 2. CHX increases protein expression of fibrotic markers αSMA and Col1A1 in gingival tissues. **A)** Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-αSMA. Scale bar 100 μm. **B)** Representative photomicrograph of cytoplasmic staining for αSMA in the epithelial layer observed in CHX biopsies. Scale bar 25 μm. **C)** Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-Col1a1 antibodies. Scale bar 100 μm.

Figure 3

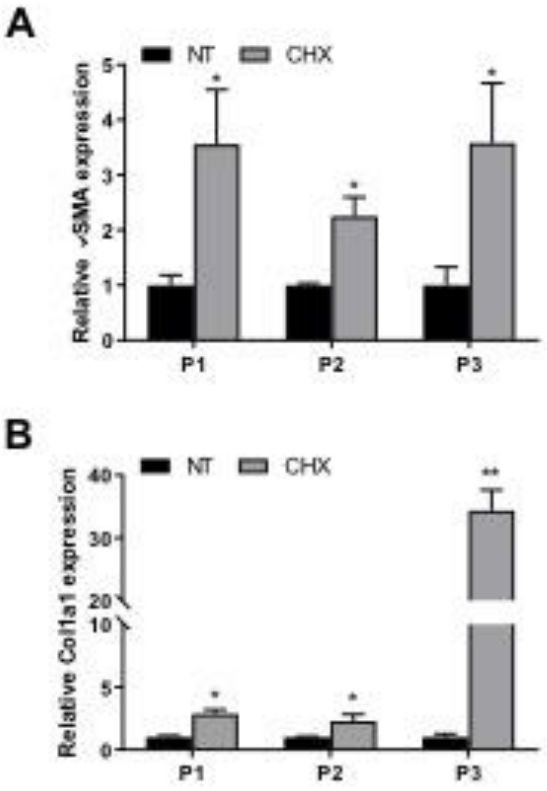

Figure 3. CHX increases mRNA expression of fibrotic markers αSMA and Col1A1 in gingival tissues. Quantitative real-time PCR analysis of αSMA **(A)** and Col1a1 **(B)** mRNA expression in NT and CHX biopsies of three patients. Relative mRNA levels are shown as fold value of the NT levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *p < 0.05 and **p < 0.005 vs NT.

Figure 4

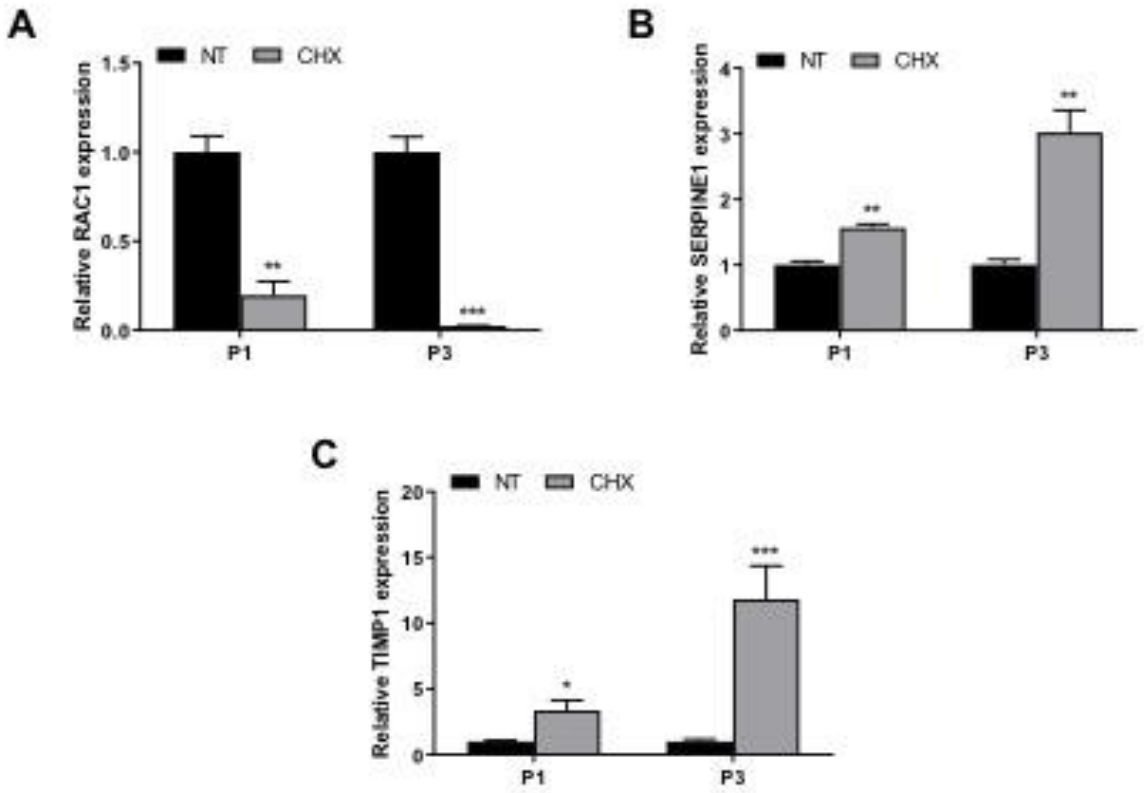

Figure 4. CHX modulates mRNA expression of genes involved in early wound healing. Quantitative real-time PCR analysis of RAC1 **(A)**, SERPINE1 **(B)** and TIMP1 **(C)** mRNA expression in NT and CHX biopsies of two patients. Relative mRNA levels are shown as fold value of the NT levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$ vs NT.

Figure 5

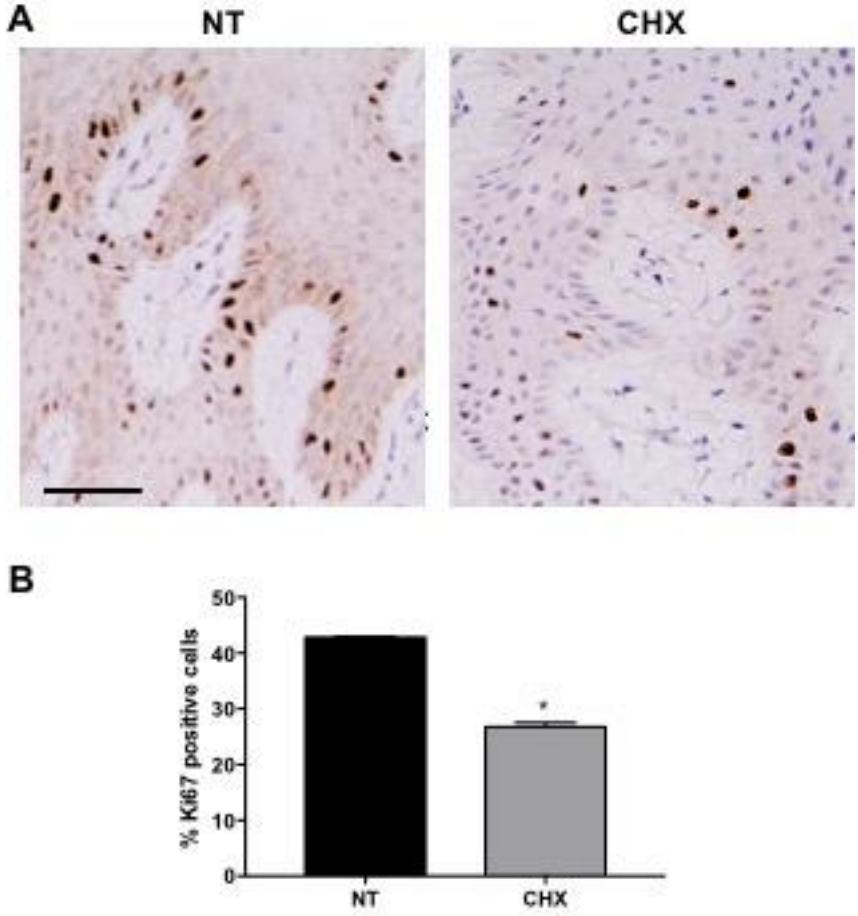

Figure 5. CHX shows an antiproliferative effect in gingival tissues. A) Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-Ki67 antibodies. Scale bar 50 μm. **B)** Mean percentage of Ki67 immunopositive cells. *p < 0.05 vs NT.

Figure 6

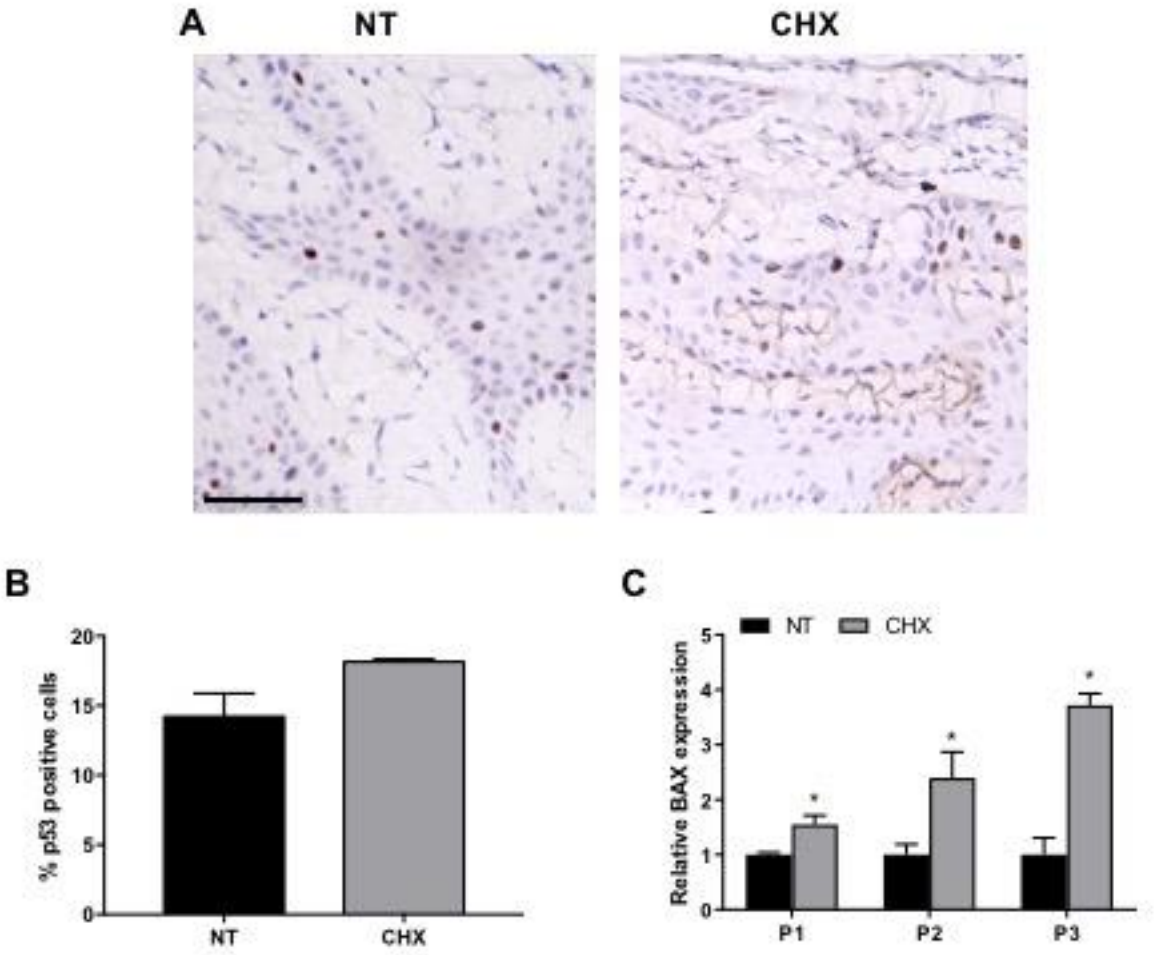

Figure 6. CHX shows a proapoptotic effect in gingival tissues. A) Representative photomicrographs of sections of NT and CHX gingival biopsies stained with anti-p53 antibodies. Scale bar 50 μm. **B)** Mean percentage of p53 immunopositive cells. **C)** Quantitative real-time PCR analysis of BAX mRNA expression in NT and CHX biopsies of three patients. Relative mRNA levels are shown as fold value of the NT levels. mRNA levels were normalized to GAPDH mRNA expression. Each experiment was performed in triplicate. Error bars represent standard deviations. *$p < 0.05$ vs NT.