Artificial saliva can stimulate chemokine expression in oral fibroblasts

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Running title: Artificial saliva and chemokines

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Abstract

Objectives: Artificial saliva is widely used to overcome reduced natural salivary flow. Natural saliva provokes the expression of chemokines in oral fibroblasts in vitro. However, if artificial saliva changes the expression of chemokines remains unknown.

Material and Methods: Here, we investigated the ability of four commercial preparations of artificial saliva to change the expression of chemokines in human oral fibroblasts and the human oral epithelial cell line HSC-2 by means of reverse transcription polymerase chain reaction and immunoassays. Mucins isolated from bovine submaxillary glands and recombinant human mucin 1 were included in the bioassay. Formazan formation and LIVE/DEAD® staining determined the impact of artificial saliva on cell viability. The involvement of signaling pathways was determined by pharmacologic inhibitors and western blotting.

Results: We report that Saliva Orthana® containing mucins, but not Aldiamed®, Glandosane® and Saliva natura® provoked a significantly increased expression of CXC ligand 8 (CXCL8, or interleukin 8), CXCL1, and CXCL2 in gingival fibroblasts, but not in HSC-2 cells. Immunoassays for CXCL8 and CXCL1 confirmed the translation at the protein level. The respective dilution of artificial saliva had no impact on formazan formation and LIVE/DEAD® staining. Supporting their potential role as the active component of artificial saliva, mucins isolated from bovine submaxillary glands and recombinant human mucin 1 also increased CXCL8, CXCL1, and CXCL2 expression in gingival fibroblasts. BAY 11-7082, a NF-κB inhibitor, blocked chemokine expression of Saliva Orthana® and bovine mucins.

Conclusions: Saliva Orthana® stimulated chemokine expression in gingival fibroblasts. A similar response was observed with mammalian mucins, suggesting that these glycosylated proteins contribute to the changes in gene expression provoked by the respective artificial saliva.

Clinical significance: Artificial saliva can incite a cellular response, if however the increased expression of chemokines by isolated fibroblasts in vitro translates into a clinical condition is not clear.

## Introduction

Saliva is a complex cocktail of compounds being produced by the submandibular and the parotid glands. Among these compounds are proteoglycans, enzymes, antibacterial compounds, and growth factors [1]. Saliva supports food digestion, oral hygiene, and prevents dental erosion [2, 3]. Healthy people produce around 0.75 to 1.5 liters per day [4]. However, Sjögren syndrome, radiotherapy, medication and aging are associated with xerostomia, also termed dry mouth disease [5]. The production of saliva can drop to almost zero, provoking multiple and severe symptoms such as mucositis, dysesthesia, and deep caries lesions [6, 7]. Xerostomia necessitates the use of extra lubricants, commonly termed artificial saliva.

Artificial saliva should closely mimic the composition and the biophysical properties of natural saliva [8]. Electrolytes provide the buffering capacity and support remineralization of the enamel surface, sugar alcohols serve as thickeners and sweeteners, and aroma compounds are odorants and increase the appeal of the product [9]. Enzymes such as amylase and lipases help the digestion of starch and lipids, respectively [10]. Glycoproteins change the viscoelastic properties allowing the formation of a stable film in the oral cavity [11]. Mucins are a group of high molecular-weight glycoproteins in saliva [12, 13]. Even though artificial saliva mimics the physiological counterpart, the composition of natural saliva is by far more complex than the substitutes [14]. The question arises what are the key components that make artificial saliva behave like natural saliva.

Natural saliva is commonly characterized by its chemical composition and viscoelastic properties [15]. Another way of characterizing natural saliva are bioassays, revealing the cellular responses. For example, natural saliva can provoke a robust increase in the expression of chemokines in oral fibroblasts [16]. Chemokines are a large family of small signaling proteins with their main function to recruit cells of the immune system to an inflammatory site [17]. CXC ligand 8 (CXCL8, or interleukin 8), CXCL1, and CXCL2 are among the chemokines that are expressed by gingival fibroblasts that were exposed to natural saliva [16]. The underlying intercellular signaling pathways that control the expression of the chemokines in response to whole saliva have recently been reported [16].

Inflammatory signal transduction is carried out by intracellular nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein (MAP) kinase including ERK, JNK, and p38 [18]. Pharmacologic inhibitors can reveal the involvement of the signaling pathways in gene expression. For example, chemokines such as CXCL8 play a crucial role in wound healing involving activation of NF-κB and MAP kinase pathways [19]. Myofibroblasts and neutrophils also require NF-κB and MAPK signaling for the secretion of CXCL1 [20] and CXCL2 [21], respectively. Whole saliva involves NF-κB, p38, and ERK to increase CXCL8 expression [16].

However, it still remains unclear how mucins contribute to the inflammatory response of natural saliva to oral fibroblasts. Here, we take advantage of this bioassay to evaluate the impact of various commercial preparations of artificial saliva on the chemokine expression.

## Materials and Methods

### Primary cell cultures of gingival fibroblast and oral epithelial cells

Human gingival fibroblasts and were harvested after informed consent was obtained (Ethics Committee of Bern, Switzerland). Gingival fibroblasts and oral epithelial cells (HSC-2 cell line) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Linz, Austria) and antibiotics (Life Technologies, Carlsbad, CA, USA) for 24 hours at 37°C, 5% CO2, and 95% humidity. In total, three strains of gingival fibroblasts and HSC-2 cells were established and less than 10 passages were used for the experiments. For all experiments, cells were seeded at a concentration of 30,000 cells/cm² into culture dishes one day prior to stimulation.

### Stimulation of gingival fibroblasts and oral epithelial cells

Gingival fibroblasts and HSC-2 cells were incubated with a 10-fold dilution of artificial saliva Orthana® (A.S. Pharma, Hampshire, UK), Aldiamed® (Certmedica International GmbH, Aschaffenburg, Germany), Glandosane® (Cell pharm, Bad Vilbel, Germany), and Saliva natura® (Medac GmbH, Wedel, Germany). Cells were also incubated with 50 µg/mL mucin from bovine submaxillary glands Type I-S (Sigma-Aldrich, St. Louis, MO) or 25 µg/mL recombinant human mucin 1 (Hölzel Diagnostika, Cologne, Germany). For positive controls, cells were incubated with a 10-fold dilution of human sterile saliva, as described recently [16]. If not otherwise indicated, cells were exposed to artificial and natural saliva and the mucins for 6 h for gene expression analysis, and 24 h for immunoassays and viability assay. The metabolic activity was determined by the conversion of MTT (Sigma, St. Louis, MO) into formazan crystals. LIVE/DEAD® staining was performed with a kit from Enzo Life Sciences AG (Lausen, Switzerland).

### Reverse transcription polymerase chain reaction (RT-PCR) and immunoassay

Prior RT-PCR analysis each sample was digested with 180 Units Deoxyribonuclease I (F. Hoffmann-La Roche, Basel, BS, Switzerland). Reverse transcription (RT) was performed with Transcriptor Universal cDNA Master (Roche). RT-PCR was done with the FastStart Universal SYBR Green Master (Roche). To quantify cDNA in the samples, the 7500 Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA) was used. Primer designing was done online via the Universal ProbeLibrary Assay Design Center (Roche), and sequences are provided in Table 1. Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method [22]. The immunoassay for human CXCL8 and CXCL1 was obtained from R&D Systems (Minneapolis, MN, USA).

### Inhibition of chemokine signal activation pathways

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein (MAP) kinase related inflammatory pathways were inhibited using BAY11-7082 (Enzo Life Sciences, Inc., Farmingdale, NY), SB203580, U0126 and SP600125 (all Santa Cruz Biotechnology, Santa Cruz, CA). Pharmacological inhibitors were used at 10 µM for blocking NF-κB, p38, ERK, and JNK, respectively. Cells were exposed to Saliva Orthana® (10 fold dilution) and mucins from bovine submaxillary glands (50 µg/ml) for 6 hours, after which their mRNA were harvested and analyzed using RT-PCR.

Western blot analysis

Human gingival fibroblasts were cultivated with serum free medium overnight. Afterwards, cells were stimulated with a 10-fold dilution of Saliva Orthana® and bovine mucins (50 µg/ml) for 30 min. Whole human saliva, IL-1 (10 ng/ml) and tumor necrosis factor alpha (TNF-α, 10 ng/ml) served as positive, and serum-free medium as negative control. Sodium dodecyl sulfate (SDS) containing cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Whatman, GE Healthcare, General Electric Company, Fairfield, CT). Primary antibody binding was accomplished with specific Phospho-NF-kB and β-actin antibodies (Cell Signaling Technology, Danvers, MA). Quantitative secondary antibody bindings were detected by near-infrared absorbing dyes with the appropriate imaging system (LI-COR Biosciences; Lincoln, NE).

Statistical Analysis

Data were compared using Kruskal-Wallis and post-hoc Mann-Whitney U tests, or Friedman tests. Significance was assigned at the $p < 0.05$ level. Statistical analysis was performed using Graph PadPrism 6.0 (GraphPad Software Inc., San Diego, USA).

Results

Viability of gingival fibroblasts exposed to artificial saliva

To rule out a possible toxicity in the bioassay, gingival fibroblasts were exposed to various concentrations of artificial saliva as indicated in Figure 1. At a 10-fold dilution all four preparations of artificial saliva reached the control levels of formazan formation. In support of these observations, the cell morphology and the LIVE/DEAD® staining indicated that a 10-fold dilution of artificial saliva had no visible adverse effects (Figure 2). Thus, further experiments were performed with a 10-fold dilution of artificial saliva.

Chemokine expression of gingival fibroblasts and oral epithelial cells exposed to artificial saliva

Next, we determined if artificial saliva could cause a change in the expression of a panel of chemokines. Among the four preparations of artificial saliva, only Saliva Orthana®, but not Aldiamed®, Glandosane® and Saliva natura® provoked a strong increase of CXCL8, CXCL1 and CXCL2 expression (Figure 3) in gingival fibroblasts. In contrast to natural saliva, which also increased CXCL3, CXCL6, and CCL20, no considerable changes of these chemokines were observed in gingival fibroblasts with either of the artificial saliva preparations. HSC-2 cells exposed to any artificial saliva preparation do not elevate expression levels of chemokines (Table 2). The CXCL8 and CXCL1 production in response to Saliva Orthana® was also evident at the protein level (Figure 4). To support the selected conditions for the bioassay we performed a dose- and time-response experiment. Saliva Orthana® increased CXCL8 expression at a maximum at the conditions selected for the bioassay (Figure 5).

Mammalian mucins increased CXCL8 expression of gingival fibroblasts

Saliva Orthana® contains mucins, in contrast to the other three preparations of artificial saliva. In line with the possible role of the mucins to cause the expression of chemokines in gingival fibroblasts, mucins purified from bovine submandibular glands and recombinant human mucin 1 stimulated the expression of CXCL8 in oral fibroblasts (Figure 6).

Inhibiting NF-κB pathway reduces gingival chemokine expression

Gingival fibroblasts were co-cultivated with NF-κB and MAP kinase inhibitors and stimulated with Saliva Orthana® or bovine mucins. CXCL8, CXCL1 and CXCL2 expression was significantly reduced blocking NF-κB signal pathway with BAY11-7082. The p38 inhibitor reduced CXCL8 and CXCL1 chemokines when stimulated with Saliva Orthana®. Blocking ERK and JNK pathway via U0126 and SP600125 showed that they are not involved in signal transduction of CXCL8, CXCL1 and CXCL2 (Figure 7a). Gingival fibroblasts were exposed to a 10-fold dilution of Saliva Orthana®, whole saliva, and mucins from bovine submaxillary glands. All preparations enhanced phospho-NF-κB signaling in gingival fibroblasts indicating that mucin-containing artificial saliva can activate the NF-κB pathway in gingival fibroblasts (Figure 7b).

Discussion

Despite the global use of artificial saliva in xerostomia patients, a potential cellular response to artificial saliva has not been investigated so far. In the present report we show that Saliva Orthana®, a product containing mucins, provoked a significant increase in CXCL8, CXCL1, and CXCL2 expression in gingival fibroblasts but not in oral epithelial cells. The effects were also observed with mucins purified from bovine salivary glands, or recombinant mucin 1. These data suggest a possible cellular response to the mucins in Saliva Orthana®. The impact of Saliva Orthana® and the mucins to the expression of chemokines is by far less pronounced than the broad spectrum response to whole natural saliva [16]. These data suggest that Saliva Orthana® and isolated mucins only partially mimic the excessive inflammatory response of oral fibroblasts exposed to natural saliva.

The present findings suggest that artificial saliva can be distinguished by the containing of mucins, which are linear, heavily O-glycosylated proteins with multiple roles [23, 24]. It is therefore possible that the biological response of an oral connective tissue is differentially affected by mucins. In support of this idea, CXCL8 staining was increased in the epithelium of patients with cystic fibrosis and colocalized with mucins [25]. Mucins are correlated with the severity of periodontal disease [26]. However, mucin 2 can even be inversely correlated with CXCL8 expression, at least in pediatric patients with Crohn's disease [27]. Also, different splice variants differ in their ability to modulate CXCL8 expression [28]. Thus, there is an accumulating reasoning that mucins may provoke the expression of chemokines, even though the molecular mechanisms are unclear.

The molecular mechanisms at least require NF-κB and partially MAP kinase signaling to mediate the chemokine expression in response to Saliva Orthana® and mucins. However, the gene expression pattern by p38 blocking

is not identical between Saliva Orthana® and purified mucins, at least at the level of statistics. Another hint towards the complexity of the situation comes from experiments with recombinant mucin 1 versus purified mucins. Recombinant mucin 1 increased CXCL8, but not CXCL1 and CXCL2 expression, indicating that the cellular response cannot be explained by one single molecular mechanism alone. On communication level, cell signal with mucins is based on the binding to receptors including epidermal growth factor receptor and related subfamilies [29], however, blocking of the respective receptor had no impact on the inflammatory response to whole saliva [16]. Thus, the data presented here are basic observations, with an yet unknown molecular mechanism.

The clinical relevance of the present observation remains a matter of speculations. The overall questions are whether or not artificial saliva contributes to oral wound healing and what the underlying cellular molecular mechanisms are. Theoretically, the increased CXCL8, CXCL1, and CXCL2 expression by gingival fibroblasts upon exposure to artificial saliva with mucins attract cells of the innate immune system that in turn advance the healing process. In support of this assumption, chronic gastric ulcer healing was significantly impaired in male mucin 2-deficient mice [30]. Moreover, administration of mucin 3-epidermal growth factor-like domains reduced mucosal ulceration in experimental acute colitis [31]. Extensive preclinical research by Bonder et al. supports a role of saliva in wound healing [32, 33]. Today, however, no preclinical studies are available answering if artificial saliva modulates oral wound healing and if mucins play a role in this context.

Future studies should provide an explanation on how Saliva Orthana® increases chemokine expression, if the response of gingival fibroblasts can be explained by the mucins, and why the response is far less compared to whole saliva. Further research is also possibly based on our observations with Saliva Natura® that contains plant mucins from Eriodictyon californicum but failed to provoke a change in chemokine expression. It will also be important to understand the fibroblasts´ response to mucins at the molecular level, for example the ligand-receptor interaction. Thus, while it cannot point out how Saliva Orthana® and mucins activate inflammatory NF-κB signaling and chemokine expression, the study clearly provides the scientific basis to better understand possible cellular activity of artificial saliva in its various compositions.

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Figures

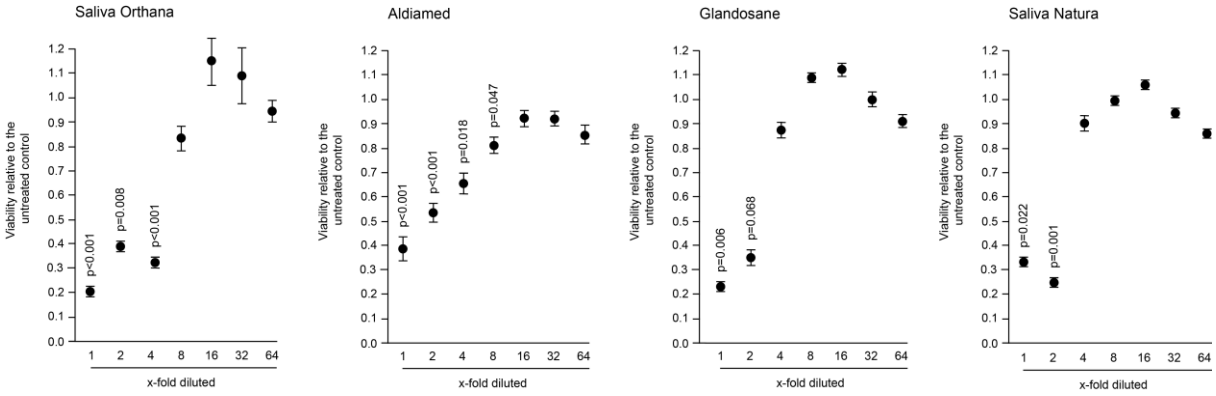


Figure 1: Viability of gingival fibroblasts exposed to artificial saliva

Gingival fibroblasts were exposed for 24 hours to artificial saliva at the indicated dilutions with serum-free medium. Formazan formation was performed to measure cell viability. Data were normalized to the levels of control cultures with serum-free medium alone. Data points represent the mean ± standard deviation of three independent experiments with two cell donors each. Not indicated are p-values > 0.1.

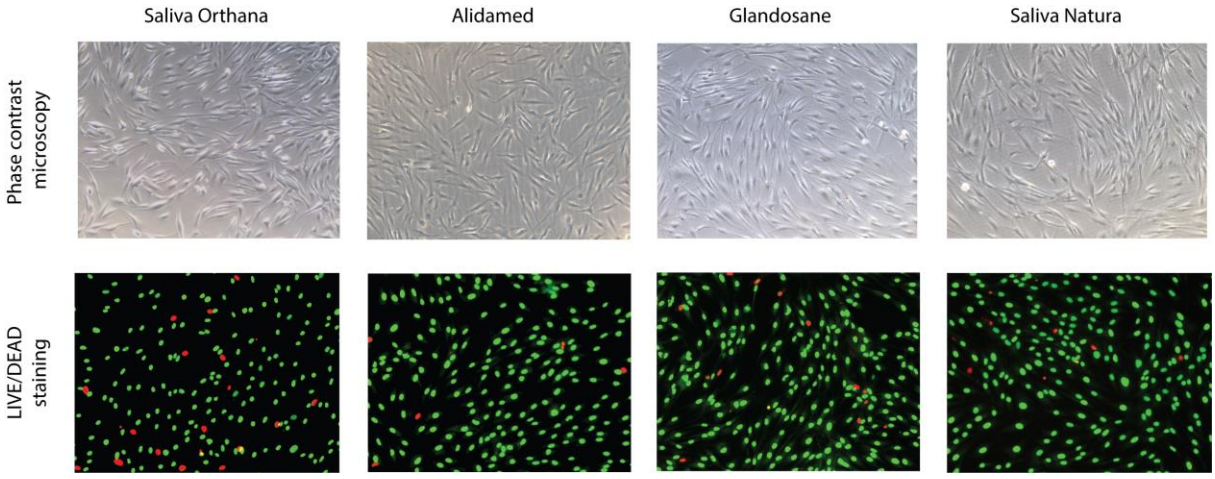


Figure 2: Phase contrast and LIVE/DEAD® staining microscopy

Gingival fibroblasts were exposed for 24 hours to 10-fold diluted artificial saliva. Phase contrast microscopy revealed a regular fibroblastic morphology, independent of the artificial saliva. LIVE/DEAD® staining showed that most of the cells are green, indicating that the cells were alive (10-fold magnification). Cells were washed prior to taking pictures because Aldiamed covers the cells with a dense precipitation layer.

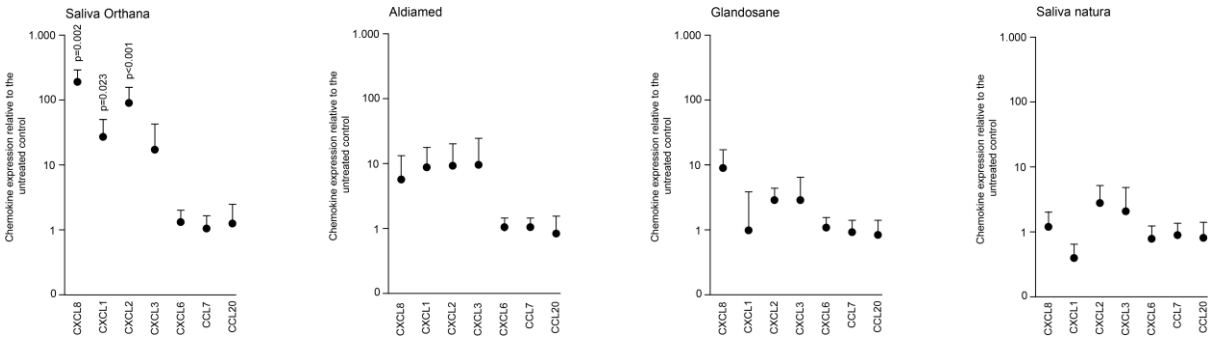


Figure 3: Chemokine response of oral fibroblasts exposed to artificial saliva

Gingival fibroblasts were exposed to a 10-fold diluted concentration of artificial saliva for 6 hours. A panel of chemokines was included in the RT-PCR assay. Data were normalized to expression levels of control cultures with serum-free medium alone. Circles represent the mean ± standard deviation of six experiments with two cell donors. Not indicated are p-values > 0.1.

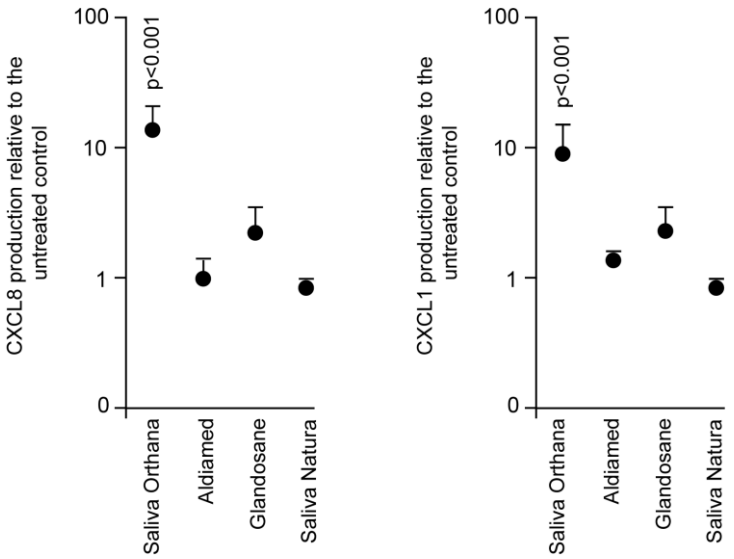


Figure 4: CXCL8 and CXCL1 protein production in response to artificial saliva

Gingival fibroblasts were exposed to a 10-fold diluted concentration of artificial saliva for 24 hours. CXCL8 and CXCL1 protein production were measured with immunoassays. Data were normalized to expression levels of control cultures with serum-free medium alone. Circles represent the mean ± standard deviation of six independent experiments. Not indicated are p-values > 0.1.

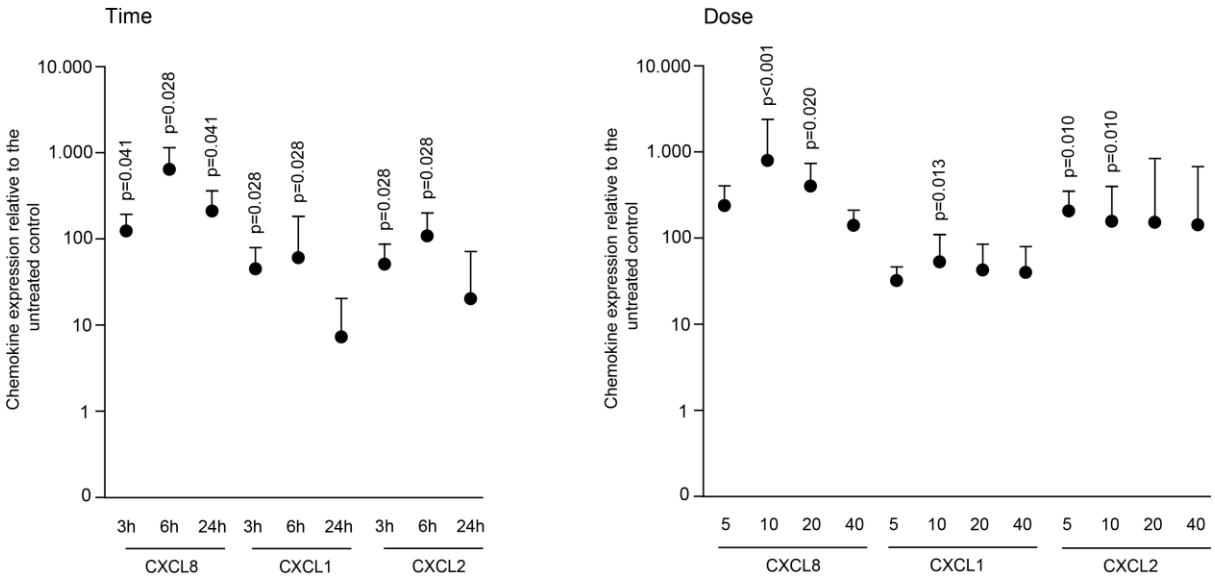


Figure 5: Time- and dose-dependent mRNA expression of CXCL8, CXCL1 and CXCL2 to Saliva Orthana®

Gingival fibroblasts were exposed to Saliva Orthana® at indicated time points and with the indicated dilutions. CXCL8 expression was measured with RT-PCR. Data were normalized to expression levels of control cultures with serum-free medium alone. Circles represent the mean ± standard deviation of three experiments with two cell donors. Not indicated are p-values > 0.1.

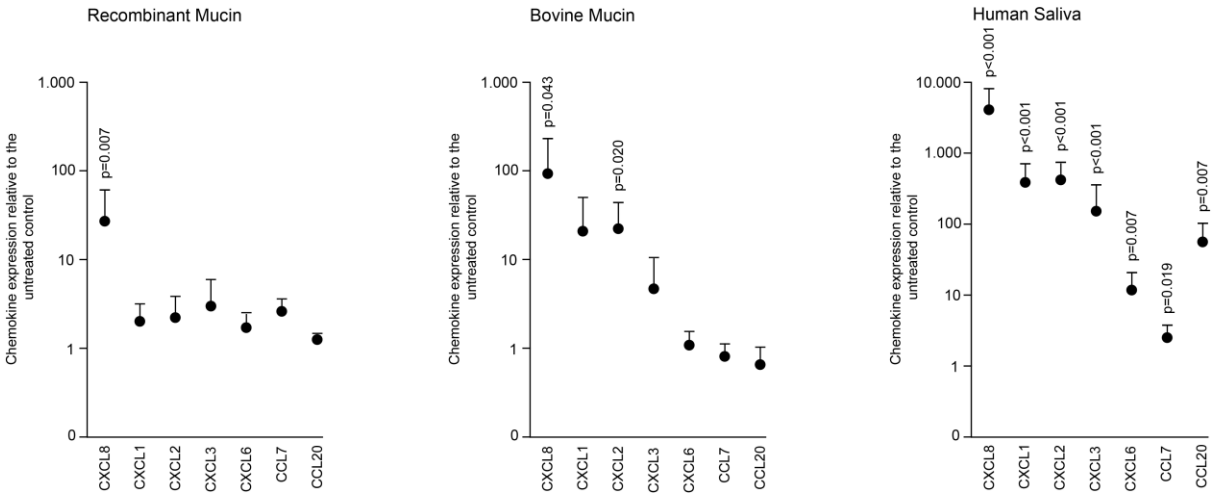


Figure 6: Chemokine expression of gingival fibroblasts to different mucin origins

Mucins from different origins were utilized to stimulate oral fibroblasts. Cells were stimulated with recombinant human mucin 1 (25 µg/ml), mucins from bovine submaxillary glands (50 µg/ml), and sterile-filtered saliva. After 6 hours of exposure mRNA was collected and subjected to RT-PCR. Data were normalized to expression levels of control cultures with serum-free medium alone. Circles represent the mean ± standard deviation of three experiments with two cell donors. Not indicated are p-values > 0.1.

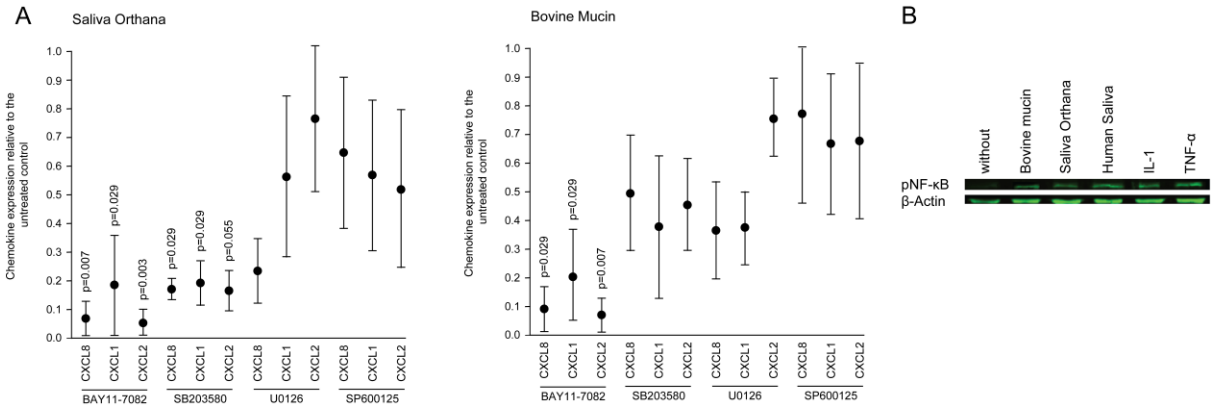


Figure 7: NF-κB and mitogen-activated protein kinase inhibition and activation of gingival fibroblast chemokine expression

Gingival fibroblasts were exposed to Saliva Orthana® and mucins from bovine submaxillary glands (50 µg/ml). (A) Signal pathways for NF-κB and mitogen-activated protein (MAP) kinase were blocked with BAY11-7082, SB203580, U0126 and SP600125 for NF-κB, p38, ERK and JNK respectively. After 6 hours of exposure mRNA was collected and subjected to RT-PCR. (B) Saliva Orthana® and bovine mucins increased Phospho-NF-κB signaling in gingival fibroblasts. Human Saliva, 10-fold dilution, Interleukin (IL)-1 and Tumor necrosis factor (TNF)-α, both 10 ng/ml, served as positive, and serum free medium as negative control. Data were normalized to expression levels of control cultures with serum-free medium alone. Circles represent the mean ± standard deviation of four experiments with two cell donors. Not indicated are p-values > 0.1.

Tables

Table 1: Primer sequences of the investigated chemokines

| Gene | Forward Primer | Reverse Primer | Reference |
|---|---|---|---|
| CXCL8 | aacttctccacaaccctctg | ttggcagccttcctgatttc | [34] |
| CXCL1 | tcctgcatcccccatagtta | cttcaggaacagccaccagt | [16] |
| CXCL2 | cccatggttaagaaaatcatcg | cttcaggaacagccaccaat | [16] |
| CXCL3 | aaatcatcgaaaagatactgaacaag | ggtaagggcagggaccac | [16] |
| CXCL6 | gtccttcgggctccttgt | cagcacagcagagacaggac | [16] |
| CCL7 | gaaagcctctgcagcacttc | aatctgtagcagcaggtagttgaa | [16] |
| CCL20 | gctgctttgatgtcagtgct | gcagtcaaagttgcttgctg | [16] |

Table 2: Chemokines expression of oral epithelial cells exposed to artificial saliva

| | Saliva Orthana | SD | Aldiamed | SD | Glandosane | SD | Saliva Natura | SD |
|---|---|---|---|---|---|---|---|---|
| CXCL8 | 3,24 | 1,29 | 0,36 | 0,26 | 6,50 | 5,06 | 1,75 | 0,70 |