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Polymicrobial infection alter inflammatory microRNA in rat salivary glands during periodontal disease

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Abstract

Periodontal disease initiated by subgingival pathogens is linked with diminished secretion of saliva, and implies pathogenic bacteria dissemination to or affects secondary sites such as the salivary glands. MicroRNAs activated in response to bacteria may modulate immune responses against pathogens. Therefore, Sprague-Dawley rats were infected by oral lavage consisting of polymicrobial inocula, namely *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola*, or sham-infected for 12 weeks (n=6). We quantified inflammatory miRNA expression levels of miRNA-132, miR-146a, and miR-155 at secondary sites to the primary infection of the gingiva, including submandibular salivary glands, lacrimal glands, and pancreas. The presence of bacteria was detected *in situ* at secondary sites. Infected rat gingiva showed increased relative expression of miR-155. In contrast, miRNA-155 expression was decreased in submandibular salivary glands, along with positive identification of *P. gingivalis* in 2/6 and *T. denticola* in 1/6 rat salivary glands. Furthermore, miRNA-132 and miRNA-146a were significantly decreased in the pancreas of infected rats. This study is the first to show primary periodontal infections can alter miRNA profiles in secondary sites such as the salivary gland and pancreas. Whether these alterations contribute to pathologies of salivary glands in Sjögren's syndrome or of pancreas in diabetes warrants further investigation.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

Keywords

periodontal pathogens; polymicrobial infection; microRNA; submandibular glands; experimental periodontitis

1. INTRODUCTION

Periodontitis is a chronic polymicrobial dysbiotic inflammatory disease of the periodontal tissues and results in decreased tooth support [1, 2]. Periodontal disease results from a polymicrobial synergy among Gram-negative anaerobic microorganisms in subgingival biofilm including *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* which shifts relative abundance or influence of microbial species within the community known as “dysbiosis” thereby enabling the manifestation of periodontal disease [3]. Periodontal disease has also been linked to several systemic diseases, such as atherosclerosis [4], diabetes [5], rheumatoid arthritis [6], Alzheimer’s disease [7], adverse pregnancy outcomes [8], and respiratory diseases [9]. However, exact mechanisms contributing to development of these systemic diseases are unknown. Presumably, periodontal pathogens mediate disease progression by direct and indirect mechanisms through recurrent bacteremia resulting in inflammation at distal sites [10, 11]. Oral bacteria *P. gingivalis*, *T. denticola*, *T. forsythia*, and *Fusobacterium nucleatum* are capable of dissemination from the primary site of infection in the gingiva into vascular tissues and peripheral organs, as observed through the identification of bacterial 16S ribosomal RNA (rRNA) and bacterial genomic DNA in infected mice and rats at secondary sites [12–17]. Recent metagenomic analysis of ancient human ilial bone tissues from Otzi the Iceman, a 5,300-year-old Copper Age natural ice mummy, detected *T. denticola* corroborating evidence for haematogenous dissemination of this periodontal pathogen [18].

During the course of periodontal disease, the presence of pathogenic bacteria or their effector molecules activates local innate immune responses that typically result in inflammation of the host’s tissues that, if unchecked, ultimately results in progressive bacterial attachment and alveolar bone resorption characterized by gingival pocket formation and recession. Understanding the molecular mechanisms that govern the magnitude of inflammatory responses is vital and the study of microRNA becomes an indispensable tool at this juncture. MicroRNAs (miRNAs) are small (~20–22 nucleotides) non-coding, endogenously expressed, RNA sequences that can post-transcriptionally inhibit protein synthesis of their targeted messenger RNAs (mRNAs) [19]. MiRNAs are involved in regulation of various biological processes within cells, tissues, and in various pathological processes in autoimmune disorders and infection [20, 21]. The monocyte/macrophage inflammatory response to infection involves the differential expression of several miRNAs including miR-155, -146, and -132, which are critical for resolution of inflammation in a timely manner to avoid damage to the host.

MiRNA (miR)-155 is involved in regulating toll-like receptor (TLR) 2/4-mediated NF-κB activation, thus limits the production of inflammatory cytokines [22, 23]. MiR-146a is another miRNA that is commonly implicated with the TLR-mediated pathways and

inflammatory cytokine inhibition, and shows significant increases after stimulation with bacterial lipopolysaccharide (LPS) in monocytes [23, 24]. MiR-146a acts as a negative feedback regulator of the innate immune response by targeting two adapter proteins, TRAF6 (TNF receptor–associated factor 6) and IRAK1 (IL-1 receptor-associated kinase 1), that are crucial for proinflammatory signaling [25]. MiR-132 has been shown to regulate neuronal morphogenesis and dendritic plasticity of cultured neurons [26]. MiR-132 may also be responsible for limiting inflammation in the mouse brain by targeting acetylcholinesterase [26]. MiR-132 can also modulate inflammation induced by early-stage Kaposi's sarcoma–associated herpesvirus (KSHV) infection. MiR-132 also shows significant increases after LPS stimulation and can also modulate antiviral immunity [23, 27].

Our previous study showed miR-146a was significantly increased in response to periodontal infections in the ApoE$^{-/-}$ mouse model of atherosclerosis in the periodontium and systemically at the spleen, suggesting that miRNAs associated with inflammation can be altered as a result of periodontal pathogens [28]. The mucus coat serves as a defense barrier protecting soft gingival tissue as well as oral mucosa from bacterial insults [28, 29]. And the development of bacterial infection can result from diminished saliva secretion [30]. Because periodontal disease development and inhibition of salivary gland function tend to be associated [29, 31–33], our current study continued the work of Rivera et al. on rat polymicrobial periodontal disease model [14] and evaluated the expression levels of miR-155, miR-146a, and miR-132 at sites peripheral or distant to periodontal infection - the submandibular salivary glands, lacrimal glands, and pancreas.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Inoculum Preparation

P. gingivalis FDC 381, *T. denticola* ATCC 35404, and *T. forsythi*a ATCC 43037 were grown in an anaerobic chamber at 37°C as described previously [14]. The mixed bacterial inoculum was prepared as described in Rivera et al. [14]. In brief, equal cell concentrations of *P. gingivalis*, *T. denticola*, and *T. forsythia* were sequentially suspended and mixed at 5 minute intervals in reduced transport fluid. The suspension was then mixed with an equal volume of 8% sterile carboxy methylcellulose (CMC) in phosphate-buffered saline (PBS).

2.2 Oral Infection and Sampling

This study was carried out in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Florida Institutional Animal Care and Use Committee (Protocol 201004367) [14]. Female Sprague-Dawley rats were obtained from Harlan laboratories (Harlan, Indianapolis, IN, USA) and fed powdered normal chow and water *ad libitum*. All rats were administered sulfamethoxazole (0.87 mg per mL) and trimethoprim (0.17 mg per ml) daily for 10 days in the drinking water to reduce endogenous oral flora and rat mouths were swabbed with 0.12% chlorhexidine gluconate mouth rinse to enhance subsequent colonization of periodontal bacteria [14]. Rats were randomly sorted into two groups (n = 6 per group): polymicrobial infected and sham-infected groups. The infected group was given polymicrobial inocula, as described above, of 1×10^9 cells via oral lavage

every other week for 12 weeks to establish a stable infection as described elsewhere [14]. The control was sham-infected with sterile 8% CMC following the same schedule [14]. Following 12 weeks of infection, rats were euthanized gingival tissues; submandibular salivary glands, lacrimal glands, and pancreas were collected and stored at −80°C until further usage.

2.3 Quantitative reverse transciptase PCR (qRT-PCR)

Total RNA including miRNAs were isolated from each tissue and individually processed using a miRNA isolation kit (Ambion mirVana miRNA Isolation Kit, Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. The quality and concentration of the RNA were assessed using a spectrophotometer (NanoDrop ND-1000 spectrophotometer, NanoDrop Technology Inc., Wilmington, DE, USA). Step-wise dilutions were made to standardize concentrations between samples. Subsequently, quantitative stem-loop reverse transcription and quantitative real-time PCR for miRNA-132, -155, and -146a (Life Technologies, Carlsbad, CA, USA) were used following the manufacturer's protocol to quantify our miRNAs of interest. The relative expression levels of miRNAs were determined following the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen [34].

2.4 Fluorescence *in situ* hybridization (FISH) for Bacterial Localization

Modified FISH protocol [13] was used to identify periodontal bacteria that were metabolically active within tissues using probes specific for bacterial 16S rRNA [13, 35]. Submandibular salivary glands and lacrimal glands were fixed in 4% paraformaldehyde, paraffin embedded, cut sequentially, and mounted with two sections per slide. One section was used as a control and the other for bacterial detection. Sections were de-paraffinized by a series of xylene and ethanol washes of increasing dilution. Slides were blocked for 30 minutes at 37°C with Denhardt's reagent (Fisher Scientific, Waltham, MA, USA). Two hybridization solutions (900 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.01% SDS, 20% formamide) were made, one control and one mixed with 5 μg/mL of Alexafluor-568 3'-labeled oligonucleotide probe specific for 16S rRNA (Invitrogen, Carlsbad, CA, USA) of either *P. gingivalis*, *T. denticola*, or *T. forsythia*. Sections were covered with the respective hybridization buffers and placed in a dark, moist environment at 48°C for 3 hours. Slides were rinsed with pre-warmed washing buffer (20 mM Tris-HCl, pH7.5, 5 mM EDTA, 0.01% SDS, 0.225 M NaCl) and then incubated with washing buffer for 25 min in the dark. All solutions contained protectRNA (Sigma-Aldrich, St. Louis, MO, USA) to prevent RNA degradation. Slides were mounted and counterstained for fluorescence screening.

2.5 Image Acquisition

Hybridized slides were viewed with the Zeiss Axiovert 200M microscope fitted with a Zeiss AxioCam MRm camera (Carl Zeiss Microscopy, Thornwood, NY, USA). The Alexafluor-568 was visualized in the Cy3 channel. Unlabeled sections were used as background controls for Alexafluor-568. Background Cy3 noise was estimated by the control tissue unstained by Alexaflour-568 [13, 15].

2.6 Statistical Analyses

Two-tailed Student's t-test was used to compare miRNA expression between sham and infected groups for each miRNA in each tissue examined. In addition, a two-tailed nonparametric Mann-Whitney test was used to compare miRNA expression between sham and infected groups for miR-155 in the salivary gland. For all statistical analyses, $P < 0.05$ was considered significant.

3. RESULTS

3.1 MiRNAs are differentially expressed among tissues

Relative expressions of miRNAs were evaluated in gingival tissues, submandibular glands, lacrimal glands, and pancreas to determine the local and systemic alterations in miRNA expression following oral polymicrobial infection. Gingival and lacrimal gland tissue showed a general trend for elevated miR-132 and miR-146a in the infected group, which were not statistically significant (Figure 1ai, 1aii and Figure 2ai, 2aii). Of note, miR-155 was significantly elevated in the gingiva (Figure 1aiii). In contrast, submandibular glands exhibited a general decrease in miR-132, miR-146a, and miR-155 in the infected group (Figure 1b). Initial analysis of miR-155 in the submandibular salivary glands using a Student's t-test showed *P*-value of 0.0577. However, because variances were significantly different between the groups (F test, $P = 0.0006$), a two-tailed Mann-Whitney t-test was used, which resulted in *P*-value of 0.026 (Figure 1biii). Furthermore, pancreas tissues from the infected group exhibited a significant reduction in both miR-132 and miR-146a relative expression (Figure 2bi and 2bii).

3.2 Presence of *P. gingivalis* and *T. denticola* within the submandibular salivary glands

To further understand miRNA alterations of periodontal infection on the submandibular glands, bacterial presence was tested using species-specific probes that utilized complementary nucleotide sequences for the detection of metabolically active bacterial 16S rRNA. *P. gingivalis* was identified within the submandibular salivary glands of 2 out of 6 infected rats (Figure 3C–3F). The positive *P. gingivalis* labeling was localized in the perinuclear region of salivary gland cells as demonstrated by significant co-localization with the DAPI nuclear staining. In addition, *T. denticola* was also identified in submandibular salivary glands of one rat also positive for *P. gingivalis* (Figure 3H and 3I). The sham-infected rats showed no positive labeling for bacteria (Figure 3B and 3G). Furthermore, evaluation of the lacrimal glands of infected and sham-infected rats also showed no positive labeling for bacteria (data not shown).

4. DISCUSSION

Chronic periodontal disease fosters bacteria that can hematogenously disseminate and trigger significant systemic disease processes in non-oral sites. Periodontal lesions have been shown to be reservoirs for chronic recurrent systemic bacteremia [12, 13, 15, 36]. The current study was designed to explore the effects of chronic periodontal disease with potential bacteremia and translocation of bacteria on the immune response, as quantified in terms of miRNA expression profiles, at secondary sites of infection. Our previous study,

Rivera et al. showed that periodontal disease as well as the systemic immune responses to periodontal pathogens were evidenced in the infected group of rats by significant alveolar bone resorption, inflammation of the epithelium, and increased specific antibodies against *P. gingivalis*, *T. forsythia*, and *T. denticola* in polymicrobial infected rats [14]. Our current findings indicate increased inflammatory miR-155 in the gingiva of infected rats. Interestingly, decreased miR-155 was evident in the submandibular salivary glands and miR-132 and miR-146a were both reduced in the pancreas of infected rats.

In vivo and *in vitro* studies together demonstrate the complexity of oral bacterial involvement in miRNA expression patterns. Gram-negative bacterial cell walls contain LPS endotoxins, which are well known to increase miR-146a and miR-155 in RAW264.7 macrophages and miR-146a, miR-132, and miR-155 in THP-1 monocytes *in vitro* [23, 37]. A previous study of human periodontal disease gingiva found miR-146a increased but miR-155 reduced relative to healthy tissues [38]. Interestingly, similar to our current infection model, another recent study showed miR-155 increased in human periodontal disease gingiva in comparison to healthy tissues [39]. Conversely, oral pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* monoinfections or polymicrobial infections in human THP-1 monocytes resulted in distinct differential miRNA expression patterns *in vitro*. Interestingly, live *T. denticola* alone inhibited miR-146a expression and live *P. gingivalis* or *T. denticola* alone inhibited miR-132 expression when compared with heat-killed bacteria. Of note, both live and heat-killed *P. gingivalis*, *T. denticola*, and *T. forsythia* failed to increase miR-155 expression *in vitro* [28]. Similarly, we showed miR-132 and miR-146a were reduced in pancreas and miR-155 reduced in submandibular salivary glands in the periodontitis rat model. Therefore, these results support a possible mechanism for oral Gram-negative bacteria to modulate miRNA responses during infection *in vivo*.

Altered miRNA expression may be due to dissemination of oral pathogens, although the exact mechanisms are still unknown. In our study, metabolically active *P. gingivalis* and *T. denticola* was identified in infected rat submandibular salivary glands. It is unlikely that these bacteria were present before oral inoculation in these rats, as they are not found in their natural oral flora. In addition, an antibiotic course was given prior to infection to these rat and sham-infected rats showed no presence of these bacteria. Therefore, bacterial presence in submandibular salivary glands provides strong evidence of haematogenous or possible retrograde dissemination of these bacteria from the site of the oral cavity. As such, it is possible to speculate that oral bacterial dissemination may act directly to inhibit inflammatory miR-155 expression in the salivary glands. miRNA expression level may be increased in infected animals at the beginning of infection course and then diminish during subsequent infections.

Surprisingly, the pancreas of infected rats showed significant reductions in miR-132 and miR-146a. Although the pancreas were not evaluated for presence of oral periodontal pathogens, it is possible that periodontal pathogens may also alter pancreatic inflammatory miRNA expression patterns. For instance, diabetes, a disease in which blood sugar levels are dysregulated is an important risk factor for more severe and progressive periodontitis, and periodontitis in turn can also exacerbate the progression of diabetes [40]. This bidirectional

association between periodontal disease and diabetes implicates the potential role of periodontal pathogens on modulation of inflammation through miRNAs.

It is important to note that the lacrimal glands did not exhibit any significant alterations in the inflammatory miRNA profiles in this study; therefore we hypothesize a possible supporting role for a direct effect of bacteria on the miRNA expression profiles in the salivary glands rather than a general systemic response. Our study has a limitation in that we used only one time point of infection i.e. 12 weeks, and in the small number of rats used. Further studies with multiple time points of infection would be critical for determining the timing and specific cell populations involved in infection-associated miRNA expression. Hence, there is an urgent need to expand our current study to further understand the mechanisms by which periodontal pathogens may directly affect the differential expression of miRNAs in the local and distant sites *in vivo*.

5. CONCLUSIONS

It is postulated that heavy microbial growth, which may occur in the setting of poor oral hygiene as well as resulting from decreased salivary flow, play complimentary roles in the pathogenesis of acute bacterial infections of the salivary glands [41, 42]. However, the role of periodontal disease in Sjögren's syndrome, a chronic autoimmune disease of the exocrine glands, still remains controversial. Earlier studies suggest Sjögren's syndrome was not associated with increased periodontal disease [43–45]. However, more recent studies indicate Sjögren's syndrome is associated with higher gingival inflammation and increased periodontal disease [46–49]. Interestingly, periodontal disease development is also associated with inhibition of salivary gland function [29, 31–33]. The role of miRNA in modulating the development of diseases such as Sjögren's syndrome is a new avenue offering plausible mechanisms for observed associations with periodontal disease. The implications of periodontal disease pathogens affecting miRNA expression of the salivary glands have not been previously explored. Our study is the first to identify the alterations of miRNAs in salivary glands of rats with chronic periodontal disease, potentially as a result of translocation of pathogenic bacteria from the primary site of infection. Thus, our study highlights these intricate associations between periodontal disease, diabetes, and Sjögren's syndrome and emphasizes the need to initiate further investigations to better understand these conditions.

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HIGHLIGHTS

- We show that the relative expression of miRNA-155 was increased in periodontal bacteria-infected rat gingiva.
- We observed decreased miRNA-155 expression in submandibular salivary glands, along with positive identification of *P. gingivalis* and *T. denticola* in rat salivary glands.
- We found miRNA-132 and miRNA-146a expression to significantly decrease in the pancreas of infected rats.
- We report for the first time that primary periodontal infections can alter miRNA profiles in secondary sites such as the salivary gland and pancreas.

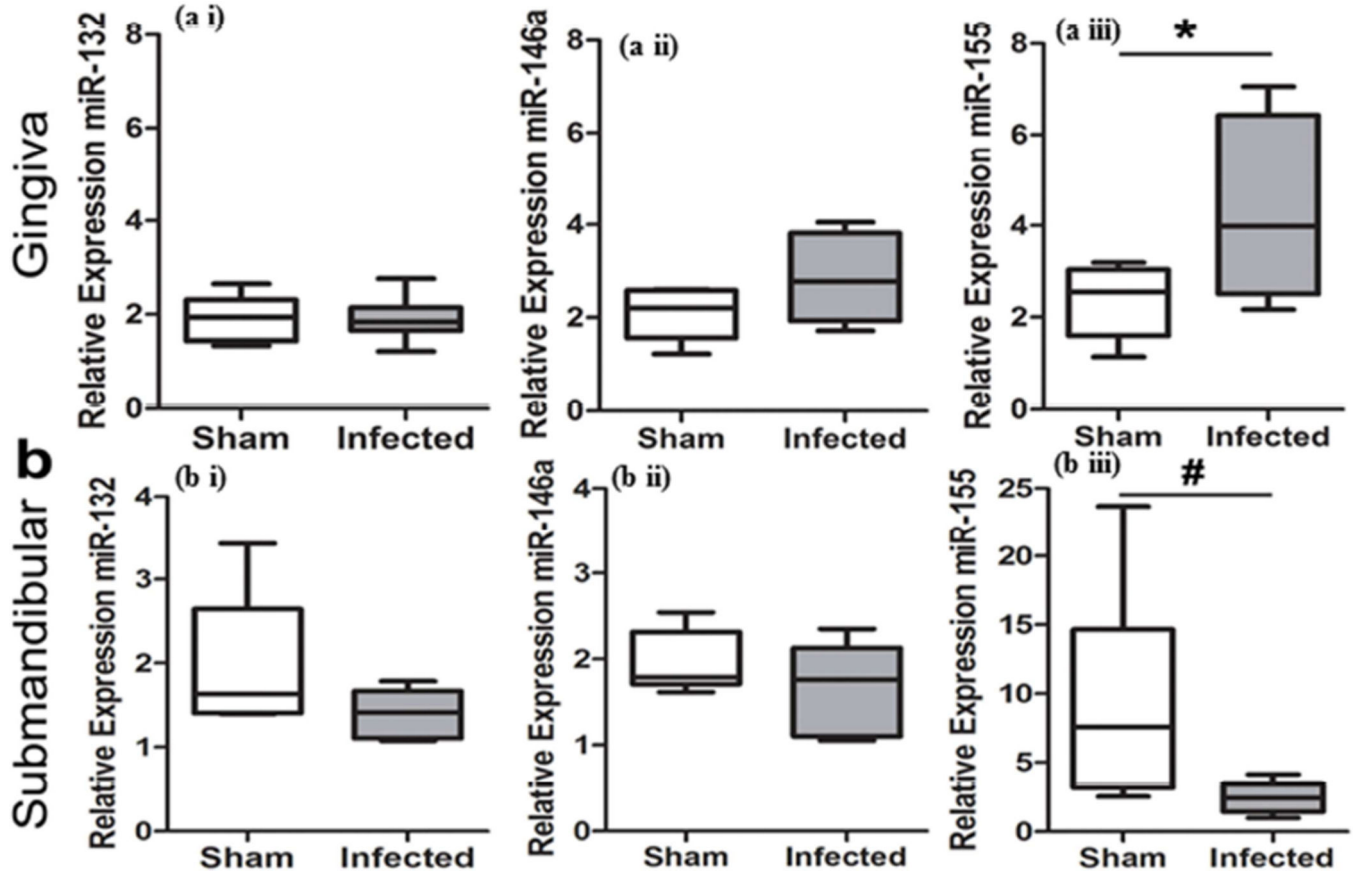

Figure 1.

Polymicrobial infection with periodontal pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* alters miRNA expression levels in the gingiva and submandibular salivary glands following 12 weeks of infection in rats. Total RNAs from **a)** gingiva, **b)** submandibular salivary glands, were evaluated by qRT-PCR to determine the relative miRNA expression levels of miR-132 (a i and b i), -146a (a ii and b ii), and -155(a iii and b iii) between infected and sham-infected rats (n = 6). Middle line indicates mean, box indicates the upper and lower quartiles, and whiskers indicate minimum and maximum values. **P* < 0.05 by Student's two-tailed t-test, #*P* < 0.05 Mann-Whitney two-tailed t-test.

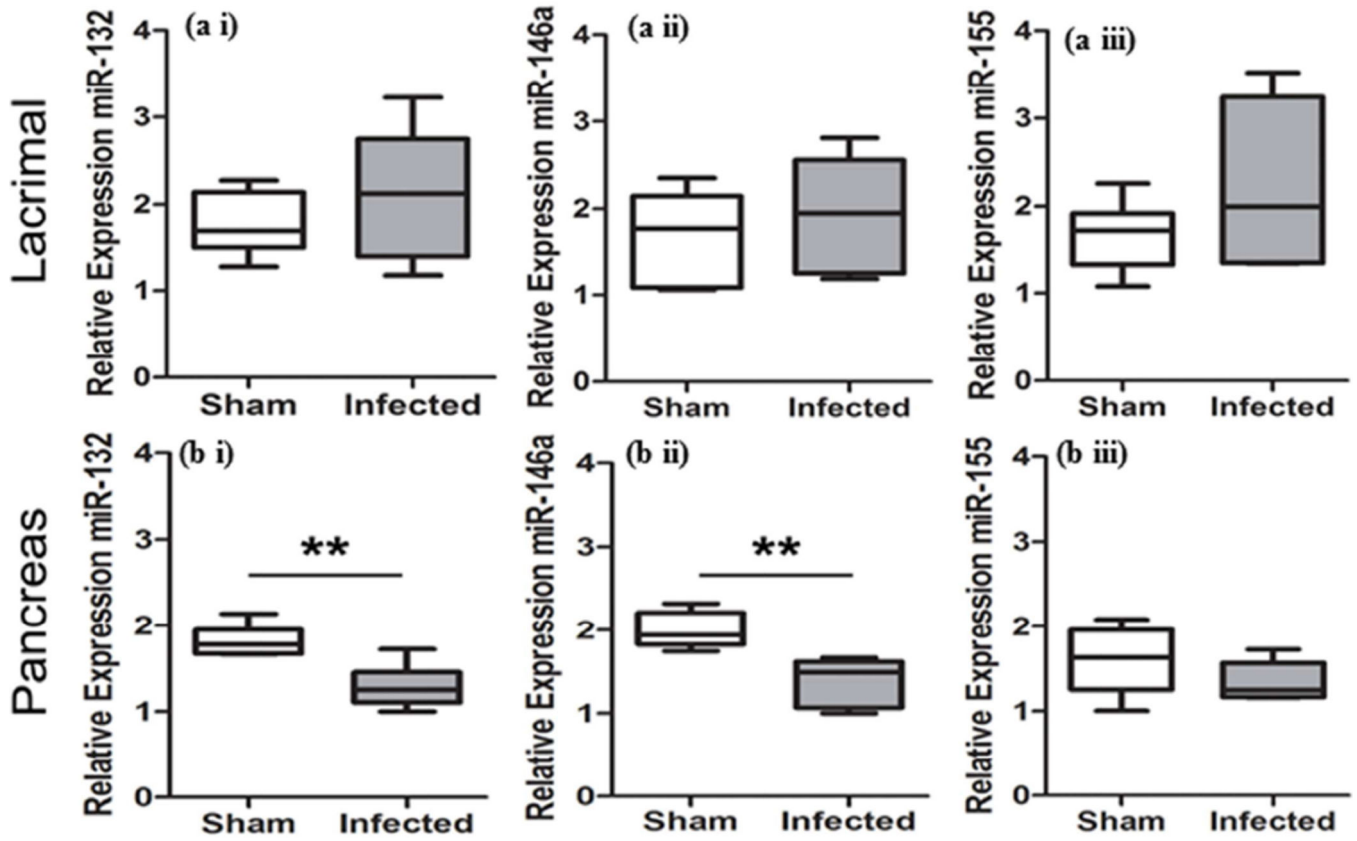

Figure 2.

Polymicrobial infection with periodontal pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* alters miRNA expression levels in lacrimal glands and pancreas following 12 weeks of infection in rats **a)** lacrimal glands, and **b)** pancreas were evaluated by qRT-PCR to determine the relative miRNA expression levels of miR-132 (ai and bi), -146a (aii and bii), and -155 (aiii and biii) between infected and sham-infected rats (n = 6). Middle line indicates mean, box indicates the upper and lower quartiles, and whiskers indicate minimum and maximum values. $^{**}P < 0.01$ by Student's two-tailed t-test.

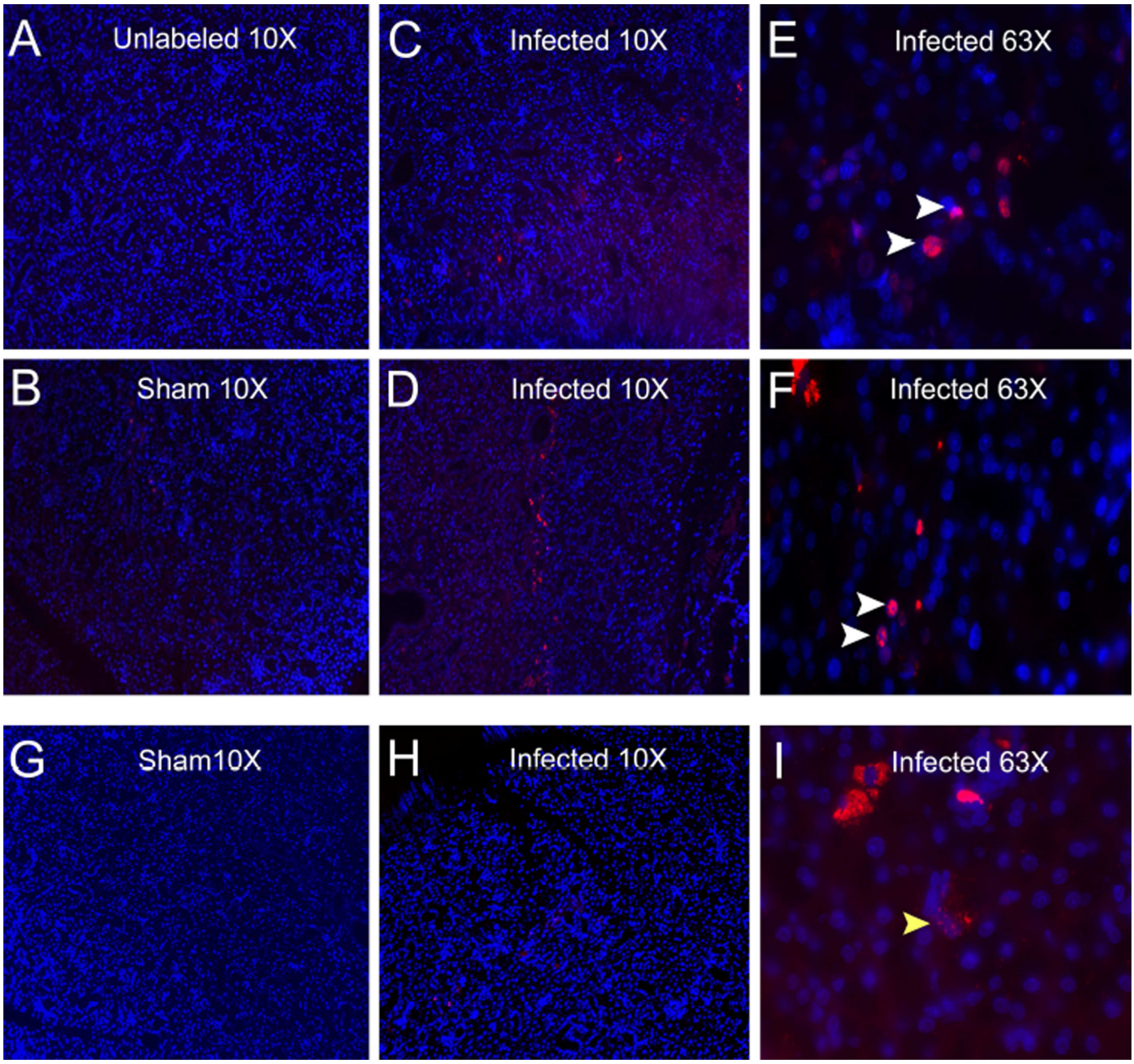

Figure 3.

Polymicrobial infection with periodontal pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* results in hematogenous dissemination from the oral cavity into the salivary glands of rats. Fluorescence *in situ* hybridization of rat submandibular salivary glands identifies presence of 16S rRNA for oral bacteria (red) counter-stained with DAPI (blue). **A)** Control unlabeled salivary gland, **B)** representative sham-infected rat salivary gland, **C)** Representative salivary gland tissue sections of polymicrobial infected rat #2 showing *P. gingivalis* at lower magnification 10× and E) at higher magnification 63×. **D)** Representative salivary gland tissue sections of polymicrobial infected rat #3 showing presence of *P. gingivalis* (10× magnification). and F) at higher magnification 63×. White arrow heads

indicate perinuclear localization of *P. gingivalis*. **G)** Representative sham-infected salivary gland and **H)** Representative salivary gland tissue sections of polymicrobial infected rat #3 showing the presence of *T. denticola*. (10× lower magnification). **I)** Under higher magnification (63×). Yellow arrow head indicate localization of *T. denticola*.