ANTIMICROBIAL EFFECTS ON *Streptococcus mutans* AND *Candida albicans* AND CYTOTOXICITY STUDY ON HUMAN PERIODONTAL LIGAMENT FIBROBLAST CELL LINE OF OXY-IONIC SOLUTION AT VARIOUS pH LEVELS - AN *IN VITRO* STUDY

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by

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LIST OF SYMBOLS

mV	Millivolt
°C	Degree Celsius
%	Percent
CFU/mL	Colony-Forming Units per Milliliter
µL	Microliter
mm	Millimeter
g	Relative Centrifugal Force
cm^2	Square Centimeters
μm^2	Square Micrometer

LIST OF ABBREVIATIONS

MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
SEM	Scanning Electron Microscopy
ECC	Early Childhood Caries
CHX	Chlorhexidine
hPDLFs	Human Periodontal Ligament Fibroblasts
EW	Electrolyzed Water
GBD	Global Burden of Disease
ECM	Extracellular Matrix
MMPs	Matrix Metalloproteinases
VSCs	Volatile Sulfur Compounds
CPC	Cetylpyridinium Chloride
ORP	Oxidation-Reduction Potential
NaCl	Sodium Chloride
HOCl	Hypochlorous Acid
NaOH	Sodium Hydroxide
ATP	Adenosine Triphosphate Assay
αMEM	Alpha Minimum Essential Medium
FBS	Fetal Bovine Serum
PenStrep	Penicillin-Streptomycin
PBS	Phosphate-Buffered Saline
ANOVA	Normality and homogeneity of variances
ROS	Reactive Oxygen Species

LIST OF APPENDICES

KESAN ANTIMIKROBIAL TERHADAP *Streptococcus mutans* DAN *Candida albicans* SERTA KAJIAN SITOTOKSISITI PADA SEL FIBROBLAS LIGAMEN PERIODONTAL MANUSIA DARI PELBAGAI TAHAP pH LARUTAN OXY-IONIC - KAJIAN *IN VITRO*

ABSTRAK

Larutan oxy-ionic yang mengandungi asid hipoklorus, natrium hidroksida, dan klorin aktif, telah mendapat pengiktirafan dalam penjagaan pergigian kerana sifat antimikrobial yang kuat, terutamanya terhadap patogen seperti *Streptococcus mutans* dan *Candida albicans*. Larutan ini dihasilkan melalui elektrolisis air garam. Walau bagaimanapun, pengaruh larutan ini di pelbagai tahap pH terhadap keberkesanan antimikrobial dan sitotoksisiti masih tidak pasti. Kajian ini menyiasat keberkesanan antimikrobial larutan oxy-ionic pada tahap pH yang berbeza (3, 5, dan 7) terhadap *Streptococcus mutans* dan *Candida albicans* serta sitotoksisiti mereka terhadap fibroblas ligamen periodontal manusia. Kajian ini menilai keberkesanan antimikrobial dan sitotoksisiti larutan oxy-ionic melalui tiga pendekatan. Kaedah resapan cakera dan pencairan mikro dalam kaldu mengukur kesan antimikrobial. Mikroskop elektron imbasan (SEM) memerhati perubahan morfologi mikrobial, manakala ujian ATP menilai sitotoksisiti terhadap fibroblas ligamen periodontal manusia. Larutan diuji pada pH 3, 5, dan 7 dengan kepekatan 100%, 75%, dan 25%, kecuali pH 7 yang hanya dinilai pada kepekatan 100%. Hasil "resapan cakera" menunjukkan bahawa larutan oxy-ionic pH 5 menghasilkan zon perencatan pertumbuhan microbial terbesar, dengan diameter 13.79 mm untuk *Streptococcus mutans* dan 19.67 mm untuk *Candida albicans*. Ujian "pencairan mikro dalam kaldu" juga mengesahkan larutan oxy-ionic pH 5 menunjukkan kepekatan minimum perencatan 50% untuk *Streptococcus mutans* dan 25% untuk *Candida albicans* untuk menghasilkan perencatan pertumbuhan mikrobial yang maksimum. Analisis mikroskop elektron imbasan menunjukkan kerosakan dinding sel mikrobial yang ketara dan terdapat perubahan morfologi apabila dirawat dengan larutan oxy-ionic pH 5. Hasil ujian ATP pada fibroblas ligamen periodontal manusia menunjukkan sitotoksisiti signifikan semua kepekatan (100%, 75%, 25%) pada pH 3, 5, dan 7 selepas 2 minit pendedahan. Walaupun daya hidup sel bertambah baik dengan masa pendedahan yang lebih lama sehingga 24 jam, ia masih berada di bawah ambang yang selamat. Penemuan ini mencadangkan bahawa larutan oxy-ionic pH 5 mencapai keberkesanan antimikrobial yang tinggi. Walau bagaimanapun, kajian lanjut diperlukan untuk mengoptimumkan kepekatan bagi memastikan keselamatan dan keberkesanan klinikal.

Kata kunci: Larutan oxy-ionic, keberkesanan antimikrobial, *Streptococcus mutans*, *Candida albicans*, sitotoksisiti, fibroblas ligamen periodontal manusia

ANTIMICROBIAL EFFECTS ON *Streptococcus mutans* AND *Candida albicans* AND CYTOTOXICITY STUDY ON HUMAN PERIODONTAL LIGAMENT FIBROBLAST CELL LINE OF OXY-IONIC SOLUTION AT VARIOUS pH LEVELS - AN *IN VITRO* STUDY

ABSTRACT

Oxy-ionic solutions, comprising hypochlorous acid, sodium hydroxide, and active chlorine, have gained recognition in dental care for their strong antimicrobial properties, especially against pathogens like *Streptococcus mutans* and *Candida albicans*. These solutions are produced through the electrolysis of saline water. However, the influence of pH on their antimicrobial effectiveness and cytotoxicity remains uncertain. This study investigates the antimicrobial efficacy of oxy-ionic solutions at varying pH levels (3, 5, and 7) against *Streptococcus mutans* and *Candida albicans* and their cytotoxicity on human periodontal ligament fibroblasts. This study evaluated the antimicrobial efficacy and cytotoxicity of oxy-ionic solutions using three complementary approaches. First, disk diffusion and broth microdilution assays quantified antimicrobial effects. Second, scanning electron microscopy (SEM) revealed microbial morphological changes. Third, ATP assays determined cytotoxicity against human periodontal ligament fibroblasts. The oxy-ionic solutions were tested at pH 3, 5, and 7 with concentrations of 100%, 75%, and 25%, except for pH 7 which was assessed only at 100% concentration. Disk diffusion results revealed that the pH 5 solution exhibited the largest zones of growth inhibition, with diameters of 13.79 mm for *Streptococcus mutans* and 19.67 mm for *Candida albicans*, indicating the strongest antimicrobial efficacy among the tested pH levels. Broth microdilution assays further confirmed this, with the pH 5 solution demonstrating the minimum inhibitory concentration of 50% for *Streptococcus mutans* and 25% for *Candida albicans*. Scanning electron microscopy analysis showed significant microbial cell wall damage and morphological changes when treated with the pH 5 solution. The ATP assay results on human periodontal ligament fibroblasts showed that all concentrations of oxy-ionic solutions at pH 3, pH 5, and pH 7 exhibited significant cytotoxicity after 2 minutes of exposure. Although cell viability improved with extended exposure time to 24 hours, it still remained below safe thresholds. These findings suggest that there is a therapeutic potential in the dental care of oxy-ionic solution pH 5 based on high antimicrobial effects, however further study on the dilution of the solution is necessary to ensure clinical safety and effectiveness.

Key words: Oxy-ionic solution, antimicrobial efficacy, *Streptococcus mutans*, *Candida albicans*, cytotoxicity, human periodontal ligament fibroblasts

CHAPTER 1

INTRODUCTION

1.1 Background of The Study

Dental caries, a prevalent chronic infectious condition of the oral cavity, involves the destruction of tooth hard tissue due to bacterial activity and is particularly common among children. It occurs at any age but is more prevalent in children and is closely related to oral microbiota (Lu et al., 2019). Consuming excessive carbohydrates can lead to tooth decay by promoting acid production and disrupting the saliva's buffering capacity. The microbial shift in oral biofilms from good to bad promotes enamel loss and demineralization of mineral hydroxyapatite crystals (Sharma et al., 2018).

A primary factor in caries development is the formation of biofilms that are rich in acid-producing and acid-tolerant bacteria like *Streptococcus mutans* (Martins et al., 2019). Their ability to produce high levels of acid, withstand acidic environments, and form robust biofilms in the presence of sucrose marks them as key culprits in dental caries development (Abranches et al., 2018). *Candida albicans*, a fungal pathogen, is often co-isolated with *Streptococcus mutans* at carious sites in patients with early childhood caries (ECC), dentinal, and root caries (Katrak et al., 2023). Moreover, the co-infection of *Streptococcus mutans* and *Candida albicans* is linked to persistent oral infections. Such infections tend to exacerbate caries prevalence in children and increase the likelihood of recurrence post-clinical intervention (Li et al., 2023).

Chlorhexidine (CHX) is a commonly used disinfectant in oral healthcare. Despite its efficacy, CHX has limitations and potential adverse effects that must be considered. These disadvantages including taste alteration, tooth discoloration, and xerostomia must be carefully considered (Moein et al., 2020). Moreover, CHX may have cytotoxic effects on human periodontal ligament fibroblasts (hPDLFs), which are critical for maintaining periodontal health (Mirhadi et al., 2014). These cells are responsible for the structural integrity and normal function of periodontal ligaments. Any damage or alteration to hPDLFs can lead to chronic periodontal disease (Naruishi, 2022).

Recent trends in oral healthcare have explored the potential of alternative disinfectants like oxy-ionic solution, also referred to as Electrolyzed Water (EW), which are strong oxidizers with a broad pH range of production, typically centered around neutrality for most applications. These solutions, notable for their high oxidation-reduction potential, are effective against a spectrum of pathogens, including bacteria, fungi, and viruses, and have demonstrated efficacy as surface disinfectants without leading to microbial resistance (Chen et al., 2022).

The oxy-ionic solution, which has shown potential for promoting antimicrobial efficacy and tissue regeneration. However, there is a lack of comprehensive understanding regarding the antimicrobial efficacy of oxy-ionic solution against *Streptococcus mutans* and *Candida albicans*, as well as its safety profile for human periodontal fibroblasts (Deasy et al., 2018; Mohammed et al., 2011).

In this study, we aim to bridge this knowledge gap by investigating the cytotoxic effects of oxy-ionic solution at various pH levels on hPDLFs as well as antimicrobial efficacy against *Streptococcus mutans*, and *Candida albicans*.

1.2 Problem Statement

Despite significant advancements in oral healthcare, dental caries and periodontal diseases remain prevalent global health concerns. While conventional mouthwashes such as chlorhexidine and fluoride rinses are commonly used in clinical practice, they are associated with several well-documented drawbacks including tooth discoloration, the emergence of microbial resistance, and potential mucosal irritation. Streptococcus mutans and Candida albicans have been consistently identified as primary etiological agents in dental caries pathogenesis due to their remarkable capacity to form tenacious biofilms, produce and endure highly acidic environments particularly in sucrose-rich conditions, and their frequent co-occurrence at carious lesions (Katrak et al., 2023; Martins et al., 2019).

hPDLFs play a pivotal role in maintaining periodontal homeostasis and tissue integrity. The emerging oxy-ionic solution technology presents several potential advantages over traditional formulations, including its production through saline electrolysis which eliminates the need for chemical additives, the ability to precisely modulate pH for optimized performance, and demonstrated broad-spectrum antimicrobial activity without reported side effects of tooth staining. Nevertheless, there remains a critical knowledge gap regarding the comprehensive therapeutic potential of oxy-ionic solutions, particularly their effects on hPDLFs and their antimicrobial efficacy against these key oral pathogens at varying pH levels.

1.3 Research Questions

1. How does the antimicrobial efficacy of oxy-ionic solution against *Streptococcus mutans* and *Candida albicans* vary with various pH, and how does it compare to fluoride mouthwash and CHX.

2. What morphological changes do *Streptococcus mutans* and *Candida albicans* undergo when treated with various pH levels of oxy-ionic solution, fluoride mouthwash and CHX.

3. What are the cytotoxic effects of oxy-ionic solution at different pH levels on hPDLFs, and how do these effects compare to those of fluoride mouthwash?

1.4 Research Hypotheses

1. The antimicrobial efficacy of oxy-ionic solution against *Streptococcus mutans* and *Candida albicans* is dependent on pH and is comparable or superior to that of fluoride mouthwash and CHX at optimal pH levels.

2. Treatment with oxy-ionic solution, fluoride mouthwash and CHX induces significant morphological changes in *Streptococcus mutans* and *Candida albicans*, reflecting the underlying antimicrobial mechanisms.

3. Oxy-ionic solution at different pH levels exhibit varying degrees of cytotoxicity on hPDLFs, with certain pH levels being as safe as or safer than fluoride mouthwash for cellular health.

1.5 Research Objectives

1.5.1 General Objective

To investigate the therapeutic potential of oxy-ionic solution with various pH levels in dental care by assessing their antimicrobial efficacy against *Streptococcus mutans* and *Candida albicans*, and their cytotoxic effects on hPDLFs.

1.5.2 Specific Objectives

1. To compare the antimicrobial efficacy of oxy-ionic solution at different pH levels (pH 3, pH 5, and pH 7) with fluoride mouthwash and CHX on *Streptococcus mutans* and *Candida albicans* using disc diffusion assays and broth microdilution methods.

2. To compare the morphological changes in *Streptococcus mutans* and *Candida albicans* using SEM after exposure to various pH levels of oxy-ionic solution (pH 3, pH 5, and pH 7), fluoride mouthwash, and CHX.

3. To compare the cytotoxic effects of oxy-ionic solution at different pH levels (pH 3, pH 5, and pH 7) on hPDLFs and fluoride mouthwash using the ATP assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Oral Diseases

Oral diseases, despite being a global public health challenge that significantly impacts socioeconomic status, often remain underrecognized. According to GBD 2021 findings, oral diseases remain a growing global burden, with dental caries affecting 2.5 billion people and severe periodontal disease ranking as the 11th most prevalent condition worldwide (GBD 2021 Collaborators, 2024). Notably, dental caries is the most prevalent chronic disease among children, while periodontal disease is a leading cause of tooth loss in adults. Due to their high prevalence and chronic nature, oral health issues account for some of the highest healthcare costs in various countries (Jin et al., 2016).

Dental caries primarily results from prolonged exposure to low pH environments, leading to tooth decay, while periodontal disease involves complex inflammatory processes that can be exacerbated by periodontal tissue inflammation. Existing research indicates that these oral diseases are multifactorial, involving dietary habits, oral hygiene practices, and genetic predispositions, among other factors. The interaction of these factors highlights the complexity of oral disease progression, suggesting that treatment and prevention strategies must consider multiple aspects, including environmental pH, inflammatory responses, and other relevant factors (Shrestha et al., 2022).

2.1.1 Dental Caries

Dental caries, commonly known as tooth decay, represents one of the most prevalent global oral health challenges affecting individuals of all ages. According to the World Health Organization, it is the most frequent childhood condition and a prevalent chronic disease among adults (WHO, 2022). The etiology of dental caries is multifactorial, involving the dynamic interplay among cariogenic bacteria such as *Streptococcus mutans*, fermentable carbohydrate consumption, and the oral environmental factors, including saliva, which modulates the biofilm's pH and facilitates remineralization (Bowen, 2016; Marsh, 2003; Moynihan, 2002; Ten Cate, 1999).

Caries initiate with the formation of a dental biofilm, where bacterial fermentation processes produce acids, leading to demineralization of the tooth enamel. Early stages manifest as white spot lesions on enamel surfaces, potentially progressing to cavitation if the decay process is not intercepted (Figure 2.1). Although fluoride mouthwash and regular oral hygiene can significantly reduce caries incidence, this condition remains a significant public health issue globally, particularly affecting vulnerable populations in low and middle-income countries (Petersen et al., 2005).

In response to the widespread impact of dental caries, ongoing research seeks to innovate in antimicrobial agents, bioactive materials, and enhanced dental restorative materials, aiming to refine prevention and treatment strategies (Featherstone, 2000). This endeavor is critical as dental caries pose a significant global health burden, necessitating comprehensive strategies encompassing public health interventions, community awareness, and scientific advancements.

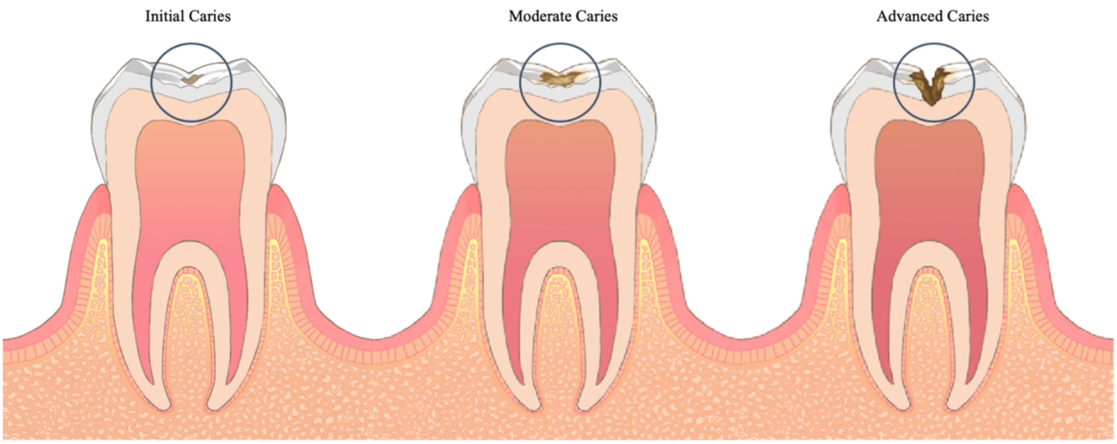

Figure 2.1 An illustration depicting the progression of dental caries from initial lesions to extensive cavitation (Source: www.shika-sozai.com)

2.1.2 Periodontitis

Periodontitis, commonly called gum disease, is among the most prevalent oral health challenges worldwide, affecting individuals of all ages. According to the World Health Organization, it is a widespread chronic oral disease in adults (Salari et al., 2022). The etiology of periodontitis is multifactorial, involving the interaction of periodontal pathogens, such as *Porphyromonas gingivalis*, alongside other pathogens, including *Streptococcus mutans* and *Candida albicans*. These organisms contribute to the condition by forming biofilms and secreting toxins that induce gingival inflammation and subsequent tissue degradation (Hajishengallis & Lamont, 2012; Johansson et al., 2016).

The progression of periodontitis begins with the formation of dental plaque, a biofilm that harbors bacteria-producing toxins leading to gum inflammation. Initial symptoms often include gum redness and bleeding, potentially advancing to periodontal pocket formation and tooth mobility if not adequately addressed (Figure 2.2). hPDLFs play a crucial role in maintaining periodontal health and the structural integrity of the periodontium. During the progression of periodontitis, hPDLFs are directly affected by the inflammatory response and pathogen-induced tissue degradation, leading to impaired function and contributing to the disease's advancement (Naruishi, 2022).

Despite significant reductions in incidence rates through improved oral hygiene and regular dental cleanings, periodontitis remains an important public health issue, especially in low and middle-income countries(Tonetti et al., 2018).In response to its extensive impact, ongoing research is focused on developing new antimicrobial agents, bioactive materials, and advanced dental restorative materials to enhance prevention and treatment strategies (Slots, 2017). Understanding the response of hPDLFs to these treatments is essential, as they are integral to periodontal regeneration and repair. This effort is crucial as periodontitis poses a significant global health burden, necessitating comprehensive approaches that include public health interventions, community awareness, and scientific progress.

Figure 2.2 An illustration depicting the progression of periodontal disease from initial gingivitis to advanced periodontitis (Source: www.shika-sozai.com)

2.2 Etiology

Dental diseases like caries and periodontitis predominantly stem from biofilm formation, a complex aggregation of microorganisms, including *Streptococcus mutans* and *Candida albicans*, adhering to oral surfaces. These organisms secrete a matrix that facilitates further bacterial adhesion and colonization, protecting the community from external insults and immune responses, thus perpetuating oral diseases (Salehi et al., 2020).

The disruption of the oral microbial balance, known as dysbiosis, plays a crucial role in the pathogenesis of these conditions. Overgrowth of pathogenic bacteria, particularly when *Streptococcus mutans* is accompanied by *Candida albicans*, leads to diseases by altering the ecological equilibrium within the oral cavity. This imbalance can provoke caries through acidogenic activity and initiate periodontal inflammation due to enhanced virulence (Hajishengallis & Lamont, 2012).

hPDLFs are essential in maintaining the structural integrity and function of the periodontal ligament (Naruishi, 2022). During periodontal disease, the inflammatory environment and increased virulence of pathogens can damage hPDLFs, impairing their ability to repair and regenerate periodontal tissues. The interaction between biofilms and hPDLFs is crucial in understanding the progression of periodontitis, as these cells are directly involved in responding to bacterial insults and orchestrating tissue repair processes (Martínez-García & Hernández-Lemus, 2021).

Furthermore, the systemic implications of oral health extend to conditions such as cardiovascular disease and diabetes, evidencing a significant link between oral bacterial profiles and broader health challenges. Pathogens from oral infections can enter the systemic circulation, influencing or exacerbating systemic inflammatory responses (Lockhart et al., 2012).

2.3 *Streptococcus mutans*

Streptococcus mutans is a Gram-positive, facultative anaerobic bacterium commonly found in the human oral cavity. It is highly cariogenic due to a range of virulence factors, including its ability to produce extracellular polysaccharides that enhance adherence, generate acids through carbohydrate fermentation (acidogenicity), and survive in low pH environments (aciduricity). In addition, it forms robust biofilms and secretes bacteriocins to outcompete other microorganisms, further contributing to its persistence and pathogenicity (Y. Lin et al., 2021).

Streptococcus mutans is a key contributor to dental caries, primarily due to its capacity to form acidic biofilms from the fermentation of dietary sugars in the oral environment. This process facilitates the adherence and colonization of these bacteria on dental surfaces and accelerates enamel breakdown by acid production. Such biofilms are not merely protective shelters for the bacteria but are also integral to their mechanism of causing tooth decay (Bowen & Koo, 2011).

One of the significant challenges in treating infections caused by *Streptococcus mutans* is the bacterium's increasing resistance to conventional antibiotics. This resistance arises from both the protective nature of the biofilm, which impedes antibiotic penetration, and the habitual use of antibiotics, which promotes the selection of resistant strains. The clinical implications include more complex management of oral infections and higher recurrence rates (Al-Shamahy et al., 2019).

In response, research has pivoted towards discovering and developing new therapeutic approaches that target the specific properties of *Streptococcus mutans*, such as biofilm-forming capabilities and metabolic pathways for acid production. Innovations in this area include agents that disrupt biofilm integrity or inhibit acid synthesis, aiming to reduce the bacterium's pathogenicity without fostering resistance (Lemos et al., 2019).

2.3.1 Genetic Variability and Adaptation

Research highlights that the genetic diversity within *Streptococcus mutans* significantly impacts its ability to adapt to the oral environment. Genetic variations affect its acidogenic and biofilm-forming capabilities, which are essential for its role in caries development. Understanding these genetic factors is crucial for developing targeted therapies to mitigate its virulence (Bedoya-Correa et al., 2019).

2.3.2 Host Immune Interaction

Streptococcus mutans interacts with the host immune system in ways that facilitate its evasion of immune responses. This includes the expression of surface proteins that inhibit immune cell activation and the secretion of proteases that contribute to tissue destruction, aiding deeper tissue invasion. Insights into these interactions suggest potential avenues for immunomodulatory therapies (Lemos et al., 2019).

2.3.3 Antibiotic Resistance and Therapeutic Strategies

The increasing antibiotic resistance of *Streptococcus mutans* underscores the need for novel therapeutic strategies. Research focuses on agents that can disrupt biofilm integrity and inhibit metabolic pathways critical to *Streptococcus mutans*' pathogenicity, offering alternatives to traditional antibiotic treatments (Shrestha et al., 2022).

2.3.4 Microbial Interactions in Biofilms

The interaction of *Streptococcus mutans* with other oral microbes, particularly *Candida albicans*, within biofilms, can enhance their collective resistance and pathogenicity. Understanding these interactions is vital for developing effective treatment strategies that target microbial communities within biofilms (Jin et al., 2024).

2.4 *Candida albicans*

Candida albicans is a polymorphic, opportunistic fungal pathogen commonly found as a commensal organism in the human oral cavity, gastrointestinal tract, and genitourinary tract. Its ability to switch between yeast, pseudohyphal, and hyphal forms under different environmental conditions is a key virulence factor that facilitates tissue invasion and immune evasion. Additionally, *C. albicans* exhibits strong adhesion properties, secretes hydrolytic enzymes (such as proteases and phospholipases), and forms highly structured biofilms that contribute to its persistence and resistance in host environments (Talapko et al., 2021).

Candida albicans engage in a complex symbiotic relationship with *Streptococcus mutans* within the oral microbiome, significantly impacting dental health. This interaction fosters robust biofilm formation that not only enhances their survival and pathogenicity but also increases their resistance to antimicrobial treatments. Such synergistic relationships within biofilms are crucial contributors to the progression of oral diseases like dental caries and periodontitis, underscoring the challenges in managing these infections effectively (Li et al., 2023).

The treatment of *Candida albicans* infections is notably complicated by its developed resistance to common antifungal agents. This resistance poses a significant problem, especially in immunocompromised individuals, where infections can recur and become challenging to manage, often requiring more complex and prolonged treatment strategies (Fisher et al., 2022; Vitiello et al., 2023).

In response to these challenges, current research is intensely focused on developing innovative therapeutic approaches that can effectively counteract *Candida albicans'* biofilm-forming abilities and their resistance mechanisms. New treatment strategies include the development of drugs that disrupt the biofilm architecture and specifically target key fungal metabolic pathways, offering hope for more effective management of these persistent infections (Černáková et al., 2019).

2.4.1 Genetic and Environmental Interactions

The interaction between *Candida albicans* and *Streptococcus mutans* involves complex genetic and environmental factors that enhance their capacity to colonize and damage host tissues. Understanding the genetic determinants that facilitate these interactions can lead to targeted interventions that disrupt the co-adhesion and co-aggregation in biofilms (Li et al., 2022).

2.4.2 Emerging Treatment Modalities

Research into new treatment modalities focuses on overcoming the limitations of current antifungal therapies. Innovations include the development of small molecule inhibitors that prevent biofilm formation and novel antifungal peptides that target specific components of the fungal cell wall (Fisher et al., 2022).

2.5 Human Periodontal Ligament Fibroblasts

hPDLFs are the primary cell type in the periodontal ligament, directly involved in the connection and support between teeth and the alveolar bone. hPDLFs play a crucial role in maintaining the function and integrity of the periodontal ligament. When studying periodontal diseases and related treatments, selecting hPDLFs can provide results that are more reflective of actual conditions, as these cells can simulate the environment of periodontal tissues in vitro. This is supported by their ability to differentiate into various cell types and their involvement in periodontal wound-healing processes (Chang et al., 2023).

Furthermore, hPDLFs not only secrete a rich extracellular matrix (ECM) composed of collagen, elastic fibers, and glycosaminoglycans, which provide structural support to the periodontal ligament but also regulate the activity of matrix metalloproteinases (MMPs), which are involved in the degradation and reconstruction of the ECM. This is essential for the repair and regeneration of periodontal tissues. Moreover, hPDLFs can exhibit biomechanical responses when subjected to mechanical stress, regulating the synthesis and degradation of the ECM to adapt to the constantly changing mechanical environment (Kasaj et al., 2012; Radzki et al., 2024).

Additionally, hPDLFs can regulate bone remodeling by secreting growth factors and cytokines such as bone morphogenetic proteins and insulin-like growth factors. These interactions with osteoblasts and other periodontal cells promote bone formation and the reconstruction of periodontal tissues. Consequently, these functions make hPDLFs an ideal cell model for studying the pathophysiology and treatment of periodontal diseases (Nomura et al., 2012).

Although gingival fibroblasts (GFs) are commonly used in oral cytotoxicity studies due to their ease of access and stable proliferation in vitro, they differ significantly from hPDLFs in both function and origin. GFs are derived from the gingival connective tissue and primarily reflect soft tissue responses, whereas hPDLFs originate from the periodontal ligament, which is directly involved in tooth anchorage and periodontal regeneration. Compared to GFs, hPDLFs exhibit stronger osteogenic potential, higher responsiveness to mechanical stress, and more accurate simulation of periodontal healing processes. Therefore, for this study focused on evaluating potential clinical applications of oxy-ionic solutions in periodontal environments, hPDLFs are considered a more representative and appropriate in vitro model.

2.5.1 Role in Periodontal Health

Periodontitis is a chronic inflammatory disease characterized by complex interactions between pathogenic microorganisms and host immune responses. Several key cell types are involved in the initiation and progression of periodontitis, including neutrophils, macrophages, T lymphocytes, B lymphocytes, and resident cells such as gingival fibroblasts and periodontal ligament fibroblasts. Neutrophils act as the first line of defense, migrating rapidly to infection sites to phagocytose pathogens. Macrophages and dendritic cells process and present antigens, activating adaptive immune responses. T and B cells orchestrate chronic inflammation through cytokine production and antibody-mediated mechanisms. Among resident cells, hPDLFs play a dual role in maintaining tissue homeostasis and modulating inflammatory responses by producing various mediators (Lin et al., 2022).

The role of hPDLFs in periodontal health is indispensable (Naruishi, 2022). They play a crucial part in defending against periodontal disease by recognizing and responding to bacterial infections. They produce inflammatory mediators such as cytokines and chemokines to regulate the inflammatory response, thus protecting periodontal tissues from further damage. These inflammatory mediators, including interleukins and tumor necrosis factors, recruit immune cells to the infection site, clearing pathogens and limiting the spread of infection (Goel et al., 2020).

Moreover, hPDLFs possess multipotent stem cell characteristics, enabling them to differentiate into various cell types under appropriate conditions, including osteoblasts, fibroblasts, and chondrocytes. This capability is essential for the repair and regeneration of periodontal tissues. In periodontal tissue injury, hPDLFs can secrete growth factors and cytokines, promoting angiogenesis, osteogenesis, and fibroblast activity, thereby accelerating the repair of damaged tissues (Shimono et al., 2003). This multipotency positions hPDLFs as highly valuable in periodontal regenerative medicine.

Due to these properties and functions, hPDLFs not only play a pivotal role in the prevention and treatment of periodontal diseases but also provide a crucial cellular foundation for periodontal regeneration. Therefore, in-depth research into the biological characteristics and mechanisms of action of hPDLFs is vital for developing new periodontal disease treatment strategies and ensuring biosafety (Li et al., 2019).

2.5.2 Justification for Using hPDLFs Instead of Gingival Fibroblasts

Although mouthwashes primarily act on gingival tissues and tooth surfaces, their active ingredients can penetrate deeper into periodontal pockets or sulcular areas, especially in patients with gingival recession or active inflammation. In such cases, even surface-applied solutions may interact with deeper cells, including those in the periodontal ligament (Maita-Véliz et al., 2022).

Moreover, hPDLFs are more relevant than gingival fibroblasts (GFs) when evaluating not only superficial cytotoxicity but also the long-term biological impact on periodontal regeneration and tissue remodeling. hPDLFs exhibit higher alkaline phosphatase activity, stronger collagen matrix production, and greater osteogenic potential compared to GFs, making them a more suitable in vitro model for periodontal studies (Wen et al., 2025).

In orthodontics, hPDLFs are mechanosensitive cells that play a critical role in mediating alveolar bone remodeling during tooth movement. They respond to mechanical forces by regulating cytokine secretion, extracellular matrixremodeling, and osteogenic signaling, which are all essential to periodontal adaptation under orthodontic loading (Rojasawasthien et al., 2025).

Although GFs are advantageous due to their accessibility and faster proliferation, the use of hPDLFs in this study provides a more clinically meaningful assessment of cytotoxic effects, especially in scenarios involving periodontal stress, inflammation, or orthodontic treatment.

2.6 Impact of pH on Oral Microbial Dynamics and Cellular Health

In the oral microbiome, the symbiotic interactions between *Candida albicans* and bacteria like *Streptococcus mutans* significantly influence the formation and resilience of biofilms. These interactions are particularly enhanced in varied pH environments, with acidic conditions fostering the virulence of both organisms, thereby complicating treatment due to increased resistance to antimicrobial agents. Research shows that biofilms become more robust under such conditions, protecting the microbial community from external threats (Li et al., 2001).

The challenge of treating infections caused by *Candida albicans* is compounded by its ability to develop resistance to antifungal treatments. This resistance is especially problematic in immunocompromised patients and is influenced by pH fluctuations, which can alter the efficacy of therapeutic agents, making the management of these infections more complex (Ellepola & Samaranayake, 2017).

Furthermore, various pH levels can significantly impact the health of oral cells, especially on the hPDLFs. Acidic environments can induce cytotoxic effects and inflammatory responses in hPDLFs, compromising their viability, proliferation, and function. This not only affects the structural integrity and repair capabilities of periodontal tissues but also contributes to the overall pathogenesis of periodontal diseases (Jin et al., 2022; Zhang et al., 2024).

Advancements in therapeutic strategies are crucial in addressing these challenges. Innovative treatments are being developed to disrupt biofilm architecture and target metabolic pathways influenced by pH changes, aiming to enhance the efficacy of treatments against these persistent oral pathogens (Marsh et al., 2011). Additionally, understanding the impact of pH on oral cellular health is essential for developing therapeutic approaches that protect cellular integrity while effectively managing microbial infections (Zhang et al., 2024).

2.6.1 Microbial Interactions and pH Influence

The intricate relationship between *Candida albicans* and *Streptococcus mutans* within the oral biofilms is significantly affected by pH levels (Martorano-Fernandes et al., 2023). These conditions not only influence their survival but also their ability to cause disease (Haque et al., 2023). Research has demonstrated that acidic environments boost acid production by *Streptococcus mutans*, thereby increasing the pathogenicity of both organisms within the biofilm. (Hosida et al., 2022).

2.6.2 Challenges in Antifungal Resistance

Resistance to antifungal agents in *Candida albicans* has become a pressing issue, especially under various pH conditions that affect the fungus's biofilm structure and resistance mechanisms. Research has indicated that environmental stresses, including pH changes, can trigger genetic adaptations in *Candida* that lead to enhanced resistance (Chaillot et al., 2017; Sobel & Akins, 2022).

2.6.3 Emerging Therapeutic Approaches

Novel therapeutic approaches focus on disrupting the biofilm and targeting metabolic activities specific to the acidic and alkaline environments of the oral cavity. A promising approach involves developing pH-sensitive drug delivery systems that activate in response to biofilm acidity with the goal of improving the penetration and effectiveness of antimicrobial agents. (He et al., 2023; Li et al., 2023a).

2.6.4 Impact of pH on hPDLFs

The effects of pH on hPDLFs are multifaceted, impacting cell viability, proliferation, morphology, function, and signal transduction. In acidic environments (pH below 7), the viability and proliferation of hPDLFs significantly decrease due to cell membrane damage and metabolic interference, leading to apoptosis or cell death. Acidic conditions also cause hPDLFs to undergo significant morphological changes, such as contraction and reduced cell volume. Conversely, in alkaline environments (pH above 7), cell growth and proliferation are similarly inhibited, and cells may exhibit swelling, irregular morphology, or membrane rupture (Jin et al., 2022; Narayanan & Bartold, 1996).

Changes in pH also affect cell function. Acidic environments can inhibit collagen synthesis, whereas moderately alkaline conditions may promote collagen production (Narayanan & Bartold, 1996). Additionally, pH levels regulate the activity of MMPs, which influence the degradation and repair of periodontal tissues (Jin et al., 2022). Finally, variations in pH can disrupt the balance of intracellular and extracellular ion concentrations, affecting signal transduction pathways and resulting in altered hPDLFs proliferation, differentiation, and stress responses (Kim, 2021).

Understanding the multifaceted impact of pH on hPDLFs is crucial for research on periodontal health and disease, providing insights into cellular responses and potential therapeutic approaches.

2.7 Mouthwash

Initially, mouthwashes were simple herbal concoctions used in ancient civilizations for oral hygiene. Over time, advances in science transformed these into sophisticated solution, incorporating a range of chemical and natural agents to address specific oral health issues comprehensively (Gurudath et al., 2012).

2.7.1 Types and Composition

2.7.1(a) Cosmetic Mouthwashes

Cosmetic mouthwashes are primarily designed to temporarily freshen breath without offering therapeutic benefits. Studies have shown that these mouthwashes often contain ingredients that neutralize volatile sulfur compounds (VSCs), which are a significant cause of bad breath. For instance, a clinical trial highlighted that even a single rinse can significantly reduce the levels of VSCs in morning breath, thereby improving breath freshness (Luzi et al., 2023). For example, a study found that a mouthwash containing chlorhexidine and essential oils significantly reduced VSC levels for up to 12 hours after use, compared to a placebo mouthwash (Rodriguez-Archilla & Muñoz-Martin, 2019).

Another research pointed out that cosmetic mouthwashes assist in physically removing food debris and bacteria, indirectly aiding in breath freshness by mechanical cleansing rather than chemical or biological means (Pawar et al., 2022). For instance, mouthwashes with mild surfactants helped dislodge particles and bacteria from the mouth surfaces, which contributed to fresher breath, like the effects of brushing teeth (Mangulkar, 2020).

2.7.1(b) Therapeutic Mouthwashes

Therapeutic mouthwashes are classified into those with chemical components and those primarily based on natural substances, like herbal mouthwashes.

2.7.1(b)(i) Chemical Component Mouthwashes

These mouthwashes include active ingredients such as CHX, cetylpyridinium chloride (CPC), and fluoride mouthwash, which are aimed at combating dental conditions like gingivitis and dental caries. Chlorhexidine is notably effective at reducing dental plaque and managing gingivitis. Fluoride mouthwash aids in tooth remineralization. On the other hand, CPC targets oral bacteria, improving overall oral hygiene (Oo et al., 2023a).

CHX has long been recognized for its broad-spectrum antimicrobial properties, particularly against oral pathogens involved in gingivitis and periodontal diseases (Guerra et al., 2019). Its mechanism primarily involves altering bacterial cell walls and precipitating cell contents, which effectively reduces plaque formation and prevents gingival inflammation (Brunello et al., 2022). Studies highlight CHX's superior efficacy in maintaining oral hygiene post-dental procedures and in daily dental care routines to manage gingivitis. CHX maintains its antimicrobial efficacy across a wide pH range but is most effective at a neutral pH (de Souza-Filho et al., 2008). It is generally stable and has a long shelf life when stored at room temperature. However, it is sensitive to light and should be stored in opaque containers (Mcdonnell & Russell, 1999). The typical concentration of CHX in mouthwash formulations is 0.12% to 0.2% (Jones, 1997). However, its use is often limited to short-term due to side effects such as tooth staining and altered taste sensation, which may affect patient compliance (Liu et al., 2023). This highlights the need for balanced usage, optimizing its benefits while minimizing adverse effects.

Fluoride mouthwashes are pivotal in preventive dental care, primarily due to their ability to enhance tooth remineralization (Marinho et al., 2016). Fluoride mouthwash ions act by integrating into tooth enamel, thereby enhancing resistance to