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The Impact of Cetylpyridinium Chloride (CPC) Exposure in Buccal Epithelial Cell Viability and Morphology

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ABSTRACT

This study investigated the effects of Cetylpyridinium Chloride (CPC), a commonly used antimicrobial agent in mouthwash formulations, on the viability and morphology of human cheek epithelial cells. The research aimed to determine how different frequencies of CPC exposure influence cellular viability and structural integrity over a seven-day period. An experimental research design was employed involving forty participants aged 19–25 years. Participants were divided into groups exposed to CPC-containing mouthwash either once daily or twice daily. Cell samples were collected and analyzed using Methylene Blue staining, hemocytometer counting, and microscopic examination. Descriptive statistics, correlation analysis, and Analysis of Variance (ANOVA) were used to analyze the data. Results revealed that CPC exposure affected both cell viability and morphology. Mean viability values were 44.73% for the once-daily exposure group and 47.48% for the twice-daily exposure group. Microscopic observations showed structural abnormalities, including membrane disruption, irregular cell shape, cell shrinkage, and cellular fragmentation. Correlation analysis demonstrated a strong negative relationship between cell viability and morphological damage, with correlation coefficients of $r = -0.794$ for once-daily exposure and $r = -0.873$ for twice-daily exposure. ANOVA results indicated significant effects of exposure duration on cellular responses ($p < 0.001$). The findings suggest that repeated and prolonged exposure to CPC may contribute to progressive cytotoxic effects, reducing cell viability and compromising cellular structure. The study provides valuable insights into the biological effects of CPC and highlights the importance of proper usage frequency of CPC-containing oral hygiene products.

Keywords: *Cetylpyridinium Chloride (CPC), cell viability, cell morphology, buccal epithelial cells, cytotoxicity, mouthwash.*

INTRODUCTION

Cetylpyridinium Chloride (CPC) is a widely used antimicrobial agent found in oral hygiene products such as mouthwashes, toothpastes, throat sprays, and lozenges. While CPC is effective in reducing oral bacteria, dental plaque, and other harmful microorganisms, concerns have emerged regarding its potential effects on human oral tissues, particularly with repeated exposure. Since cheek epithelial cells are among the first cells to come into contact with CPC during oral hygiene practices, understanding its influence on cell viability and morphology is important for ensuring product safety.

Globally, numerous studies have demonstrated the antimicrobial effectiveness of CPC and its ability to control oral pathogens responsible for plaque formation and periodontal diseases. However, several laboratory investigations have also reported that CPC may cause cellular damage, membrane

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Volume 2 Issue 7 (July 2026)

disruption, mitochondrial dysfunction, and reduced cell viability depending on concentration and duration of exposure. Despite these findings, most international studies have focused primarily on the antimicrobial properties of CPC rather than its direct effects on human oral epithelial cells.

In the Philippines, oral hygiene products containing CPC are regulated by the Food and Drug Administration (FDA) under Republic Act No. 9711 to ensure their safety, quality, and efficacy. As CPC-containing products are commonly used by the public, evaluating their biological effects on oral tissues is essential to support consumer safety and informed product use.

Despite the widespread use of CPC and extensive research on its antimicrobial benefits, limited studies have examined its effects on the morphology and viability of human cheek epithelial cells under repeated exposure conditions. Most existing research emphasizes microbial reduction rather than cellular responses in human tissues. This lack of information represents a significant research gap, particularly in understanding the potential cytotoxic effects of CPC during routine oral hygiene practices. Therefore, this study aims to investigate the effects of CPC exposure on human cheek epithelial cells to contribute to the scientific understanding of its safety and long-term use.

Literature Review

Background of CPC

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound widely recognized for its antimicrobial properties and its role as a cationic surfactant. Its biological activity largely results from its positively charged pyridinium group, which interacts with negatively charged microbial membranes, causing membrane destabilization, leakage of intracellular components, and eventual cell lysis (Raut et al., 2022). Because of these antimicrobial properties, CPC has been widely incorporated into oral healthcare products and other pharmaceutical formulations.

Several studies have demonstrated that CPC can also exert cytotoxic effects on mammalian cells through mechanisms involving membrane disruption and mitochondrial dysfunction. García-Cuellar et al. (2022) reported that CPC induces rapid cytotoxicity in both human breast tumor and non-tumor cells, with significant cell death occurring within minutes at micromolar concentrations. The observed cellular damage was primarily associated with membrane disruption and mitochondrial impairment rather than DNA fragmentation.

Further evidence of CPC's mitochondrial effects was presented by Weller et al. (2024), who demonstrated that exposure to low micromolar concentrations of CPC inhibited mitochondrial adenosine triphosphate (ATP) production in primary human keratinocytes without immediately causing cell death. This finding identified CPC as a potential mitotoxicant and suggested that mitochondrial dysfunction may occur prior to overt cytotoxic effects.

Beyond its antimicrobial use, CPC has also been incorporated into dental restorative materials to enhance antibacterial performance. Sustained release systems have been developed to prolong CPC's antimicrobial activity, thereby improving the resistance of dental materials against microbial colonization (Brezhnev et al., 2023).

Oral/ Buccal Epithelial Cells and CPC Exposure

Human skin serves as the body's primary protective barrier and is composed mainly of keratinocytes in the epidermis and fibroblasts in the dermis. These cells are essential for maintaining structural integrity, tissue regeneration, and barrier function (Kenhub, 2023). Exposure to chemical antiseptics such as CPC may influence the physiological behavior of these cells.

Rembe et al. (2022) investigated the effects of antiseptic agents, including CPC, on skin cells and reported that such compounds can induce varying levels of cytotoxicity depending on concentration and exposure duration. With advancements in laboratory models, researchers now employ sophisticated in vitro

systems, such as induced pluripotent stem cell (iPSC)-derived three-dimensional skin constructs, to evaluate skin irritation and cytotoxic responses more accurately (Morioka et al., 2025).

Recent research has also documented mitochondrial structural changes in keratinocytes exposed to CPC. Weller et al. (2024) observed mitochondrial nanostructural abnormalities, including spherical and donut-shaped mitochondria, within 60 minutes of CPC exposure. These alterations occurred without immediate cell death, suggesting that CPC can impair cellular metabolism before causing irreversible damage.

A study examining the antimicrobial activity of cetylpyridinium chloride (CPC) reported that the compound was capable of rapidly eliminating uropathogenic bacteria and inhibiting their invasion of epithelial cells. The findings highlight the strong bactericidal properties of CPC and support its effectiveness as an antimicrobial agent in various medical and hygienic applications (Sawant et al., 2024).

In line with these findings, another study indicates the biological effects of cetylpyridinium chloride exposure on cellular systems and reported that CPC disrupted mitochondrial function and increased oxidative stress within cells. The study further revealed that such disruptions could lead to cellular damage and impaired physiological processes. These findings suggest that exposure to CPC may contribute to cellular toxicity by affecting mitochondrial activity and inducing oxidative stress, which are critical factors influencing cell viability and structural integrity (Ma et al. 2025)

The skin is a complex organ composed of multiple interacting cell types, including melanocytes, immune cells, vascular cells, and neuroendocrine cells, which collectively contribute to immune defense, thermoregulation, and tissue repair. Disruption of these cellular interactions by exogenous agents such as CPC may therefore compromise skin homeostasis and protective functions.

Oral/ Buccal Epithelial Cells and CPC EXposure

Numerous studies have evaluated the impact of CPC on skin cell viability. Experimental evidence indicates that CPC exposure can lead to dose-dependent and time-dependent reductions in cell survival. García-Cuellar et al. (2022) observed significant decreases in epithelial cell and fibroblast viability following CPC treatment.

Oral epithelial cells differ significantly from skin cells in structure, turnover rate, and lipid composition; they are generally more permeable and sensitive to chemical agents due to their non-keratinized nature. Findings from skin cell studies cannot be directly extrapolated to oral mucosa, highlighting the necessity of evaluating CPC specifically on oral cells (Marquis & Clock, 2021).

Further emphasized that mitochondrial dysfunction may occur at concentrations lower than those required to induce cell death, suggesting that functional cellular impairment may precede observable cytotoxic effects (Weller et al. 2024). This highlights the importance of assessing sub-lethal cellular responses when evaluating the safety profile of chemical agents.

Similarly, Rembe et al. (2022) reported that CPC exposure reduced skin cell viability by approximately 50% within one hour at certain concentrations, indicating moderate cytotoxicity relative to other antiseptic compounds. Cell-based assays have further confirmed the relevance of in vitro cytotoxicity testing in predicting potential skin irritation. Studies have shown that cytotoxicity profiles obtained from cultured cells correlate closely with in vivo outcomes when evaluating topical chemical formulations (Pannakal et al., 2023).

Tang et al. (2024) investigated the biological effects of cetylpyridinium chloride on cancer cells and reported that CPC suppressed cell growth by inducing paraptosis, a form of programmed cell death associated with endoplasmic reticulum stress. Their findings suggest that CPC can significantly affect cellular survival mechanisms and may contribute to reduced cell viability following exposure.

Additionally, research on periodontal ligament cells has revealed that CPC exposure can induce morphological alterations and reduced viability in connective tissue cells at higher concentrations (Khorolsuren et al., 2021).

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Morphological Effects of CPC

In addition to affecting cell viability, CPC exposure can induce notable morphological alterations in skin cells. Structural changes such as mitochondrial abnormalities and plasma membrane damage have been consistently reported in multiple studies (García-Cuellar et al., 2022; Weller et al., 2024; Rembe et al., 2022).

Further studies observed cytoskeletal disorganization, cell rounding, and loss of adhesion in dermal fibroblasts exposed to CPC at concentrations below those causing overt cell death. These morphological changes indicate that CPC can disrupt cellular architecture and mechanical stability even at relatively low doses (Brown et al. 2023)

CPC has also been shown to affect immune cell morphology and signaling pathways. Obeng et al. (2024) demonstrated that CPC inhibits mast cell activation by suppressing calcium mobilization and downstream signaling mechanisms at sub-cytotoxic concentrations, suggesting that CPC may impair immune cell function without causing immediate cellular damage.

Ma et al. (2025) conducted a study examining the cellular effects of cetylpyridinium chloride (CPC) exposure and reported that CPC induced mitochondrial disruption, oxidative stress, and DNA damage in exposed cells. These alterations were found to compromise cellular integrity and lead to structural abnormalities within the cells. The study suggests that CPC exposure may contribute to morphological changes and reduced cellular viability due to its damaging effects on essential cellular components and functions.

While CPC's antimicrobial activity typically occurs at concentrations above its critical micelle concentration (600–900 μM), mitotoxic effects observed at much lower concentrations indicate alternative mechanisms unrelated to membrane lysis (Raut et al., 2022). These findings emphasize that CPC may alter cellular structure and function even at doses considered non-cytotoxic.

Pharmacological and Antimicrobial Applications of CPC

CPC has been widely used as an antimicrobial agent in oral healthcare products since the early twentieth century. Early studies conducted by the Wm. S. Merrell Company documented its bactericidal activity in the oral cavity. Since then, CPC has been incorporated into various products including mouthwashes, dentifrices, throat sprays, and lozenges for the prevention of plaque accumulation, gingivitis, halitosis, and bacterial infections (Mao et al., 2020; PubChem, 2023).

More recent research has explored combining CPC with other antiseptic compounds or integrating it into dental restorative materials to enhance antimicrobial performance (Yamamoto et al., 2022). These developments aim to improve long-term antimicrobial efficacy while maintaining biocompatibility with oral tissues. Aguilera et al. (2022) conducted a study to evaluate the antimicrobial effectiveness of mouth-rinse formulations containing cetylpyridinium chloride (CPC). Using a randomized crossover clinical design, the researchers assessed the ability of CPC-based mouthwashes to reduce microbial accumulation in the oral cavity. The findings demonstrated that mouth rinses containing CPC exhibited significant antimicrobial activity and effectively inhibited the growth of oral bacteria. The study emphasized the role of CPC as an active antiseptic compound widely used in oral hygiene products due to its ability to control microbial populations. These results highlight the pharmacological and antimicrobial applications of CPC and support its continued use in oral healthcare formulations.

Despite its extensive use and general recognition as safe under regulated concentrations, increasing evidence suggests that CPC may exert significant cellular and mitochondrial effects. These findings underscore the importance of continued toxicological evaluation, particularly in cases of frequent or prolonged exposure.

Exposure Frequency and Accumulation of Cellular Damage

A central principle in toxicology is that frequency and duration of exposure determine the extent of biological effect. Ma et al. (2025) explained that chemical injury occurs when the rate of damage exceeds

ISSN: 3116-3475
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of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

the cell's capacity for repair. When exposure happens only once daily, cells have sufficient recovery time to restore structure and function, limiting harm. However, when applied twice daily, the interval is too short for complete repair, causing damage to accumulate incrementally.

This concept was validated by Nakamura et al. (2023), who demonstrated that after a single exposure, most morphological changes were reversible within 12–18 hours; but when exposure was repeated every 12 hours, recovery was incomplete, and alterations became permanent.

Shiina et al. (2025) reviewed clinical and laboratory data, confirming that exceeding recommended usage guidelines directly correlates with increased tissue irritation and visible changes in the oral mucosa. They noted that while once-daily use remains generally safe, twice-daily or more frequent application significantly raises the risk of adverse effects.

Rembe et al. (2022) compared mucosal and skin cells, finding that mucosal tissues are far less able to tolerate repeated chemical contact, as they possess fewer protective mechanisms and higher metabolic activity that makes them more sensitive to disruption. Marquis and Clock (2021) further clarified that oral epithelium functions as a barrier, and repeated exposure weakens this defense, making cells progressively more vulnerable to subsequent injury.

Objectives of the study

The primary objective of this study is to investigate the impact of Cetylpyridinium Chloride (CPC) exposure on the viability and morphology of human cheek epithelial cells. Specifically, it aims to address the following research problems:

1. To evaluate the effects of different frequencies of CPC exposure on human cheek/oral epithelial cell viability over a 7-day period using quantitative viability assays
2. To observe and identify visible changes in the appearance and structural characteristics of CPC-exposed oral/buccal epithelial cells using microscopic examination.
3. To correlate observed changes in cell viability with morphological abnormalities to establish potential links between CPC exposure and cellular damage in Oral/ Buccal Epithelial cells

Conceptual Framework

The study's conceptual framework will describe how the frequency of exposure to mouthwash containing Cetylpyridinium Chloride (CPC) will affect the viability and morphology of human cheek cells. The frequency of mouthwash use, specifically using 20 mL of CPC-containing mouthwash once or twice, will be the independent variable in this study. Cell viability and morphological changes observed under a microscope will be the dependent variables. To guarantee accurate results, controlled variables such as the age range of 19-25 years old, mouthwash usage, exposure duration, staining technique, and microscope settings will be maintained. The framework will assume that increased exposure to CPC will impact cheek cell survival and structural integrity, as evidenced by changes in staining results and cell morphology.

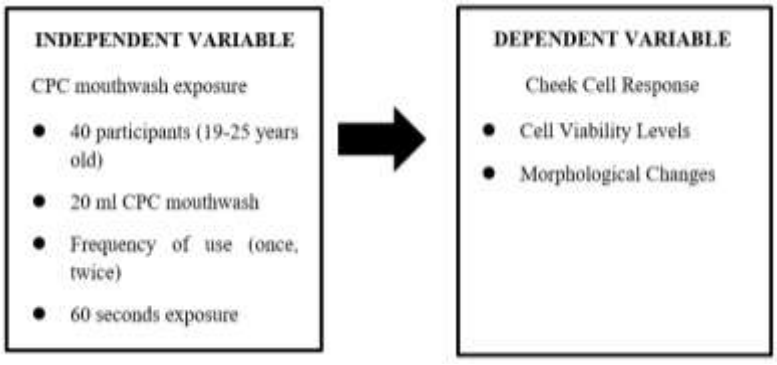

Figure 1. *Conceptual Framework*

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Independent Variables

The independent variables include the concentration of CPC, duration and frequency of exposure to Cetylpyridinium Chloride (CPC). The CPC concentration reflects levels commonly found in commercial mouthwashes, while the exposure duration was fixed at 60 seconds to simulate typical use. Exposure frequency was categorized as single or double exposure. Cheek epithelial cells were collected from 40 participants aged 19- 25 years and exposed to CPC under in vitro laboratory conditions.

Dependent Variable

The dependent variable is the oral/buccal epithelial cell response, measured through cell viability and cellular morphology after CPC exposure. CPC viability indicates the ability of cells to remain metabolically active, while morphological changes refer to observable alterations in cell shape membrane integrity, and internal structure under microscopic examination.

Theoretical Framework

This study is anchored on the Cell Membrane Disruption Theory and the Mitochondrial Dysfunction Theory, which explain how chemical agents such as cetylpyridinium chloride (CPC) can influence cellular viability and morphology.

The Cell Membrane Disruption Theory explains that certain chemical compounds, particularly surfactants and quaternary ammonium compounds, can interact with the lipid bilayer of cell membranes. Because CPC is a cationic surfactant, its positively charged pyridinium group can bind to negatively charged phospholipids in the cell membrane. This interaction may destabilize the membrane structure, increase membrane permeability, and cause leakage of intracellular components. Studies have shown that CPC can induce membrane damage that ultimately leads to cellular injury or death (García-Cuellar et al., 2022; Rembe et al., 2022). This theory provides a foundation for understanding how CPC may directly affect oral epithelial cell viability.

Another important theoretical basis for this study is the Mitochondrial Dysfunction Theory, which describes how disruptions in mitochondrial function can impair cellular metabolism and survival. Mitochondria are responsible for generating adenosine triphosphate (ATP), the primary energy source for cellular processes. When toxic agents interfere with mitochondrial function, ATP production may decrease, leading to impaired cell activity and eventual cell death. Research has shown that CPC exposure can inhibit mitochondrial ATP production and cause structural abnormalities in mitochondria, even at concentrations that do not immediately induce cell death (Weller et al., 2024). These findings suggest that mitochondrial impairment may represent an early stage of CPC-induced cytotoxicity.

Additionally, this study is supported by principles in Cell Biology, which emphasize that cell viability and morphology are critical indicators of cellular health. Alterations in cell structure, such as cytoskeletal disruption, cell rounding, and mitochondrial abnormalities, are often associated with chemical-induced cellular stress (Brown et al., 2023). Observing these morphological changes helps researchers determine the extent of cytotoxic effects caused by chemical compounds.

Together, these theoretical perspectives influence oral/epithelial cell viability and morphology. By applying these theories, the present study seeks to better understand the cellular mechanisms underlying CPC-induced cytotoxicity and its potential implications for human health.

METHODS

Research Design

This study utilized an experimental research design combining in vivo exposure and in vitro analysis. This approach was chosen because it simulated actual usage conditions while allowing direct observation of cellular responses under controlled laboratory conditions. The study aimed to determine the

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

effects of different exposure frequencies to Cetylpyridinium Chloride (CPC)-containing mouthwash on the viability and morphology of human buccal epithelial cells.

The experimental setup compared two exposure groups: participants who rinsed once daily and those who rinsed twice daily, alongside a control group that rinsed only with sterile distilled water. All observations and measurements were conducted over a seven-day period to assess both immediate and cumulative effects.

Research Sample

The research sample consisted of 40 participants aged 19- 25 years, who provided cheek epithelial cell samples for the study. These participants were selected to represent typical users of CPC-containing oral hygiene products and to provide sufficient cells for laboratory analysis. The participants served solely as donors, and no experimental treatment was applied directly to them. All exposure to CPC occurred under controlled in vitro conditions, ensuring participant safety while allowing for precise measurement of cellular responses.

Sampling Technique

This study employed a purposive convenience sampling method to select participants from the South East Asian Institute of Technology. The sample consisted of students who were willing to participate in our research, aged 19- 25 years, and who received clearance from a medical practitioner confirming that they had no dental problems. This sampling technique was chosen to ensure that participants met the specific criteria necessary for the study and to allow the collection of healthy cheek epithelial cells suitable for laboratory analysis. By using purposive convenience sampling, the study ensured that the collected samples were both ethically obtained and relevant to the research objectives of evaluating the effects of CPC on human cheek epithelial cells.

Inclusion Criteria

The participants included in this study have met the following criteria: individuals aged 19- 25 years old who voluntarily agree to participate in the study. Participants must have available or verified dental records indicating no existing oral infections, oral lesions, or severe dental conditions. They should also have generally healthy oral cavities at the time of sample collection. Additionally, the participants must be willing to follow the mouthwash exposure procedure required in the study.

Exclusion Criteria

Individuals were excluded from the study if they were below 19 or above 25 years old. Those who did not provide voluntary consent to participate were also excluded. Participants with existing oral infections, oral lesions, severe dental conditions, or incomplete dental records were not included in the study. Furthermore, individuals who were unwilling to follow the mouthwash exposure procedure or who had taken medications that might affect oral bacteria prior to the sample collection were also excluded.

Data Gathering Procedures

Cell Line

This study utilized human oral buccal epithelial cells as the in vitro model. Human oral buccal epithelial cells were selected because they were the primary cells found in the inner lining of the cheek and were classified as stratified squamous epithelial cells. Since Cetylpyridinium Chloride (CPC) mouthwash primarily came into contact with epithelial tissue in the oral cavity, the use of human oral buccal epithelial cells provided a biologically relevant and appropriate model for evaluating the cellular effects induced by CPC exposure.

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Test Substance

The test substance was a commercially available CPC-containing mouthwash manufactured by Colgate. The selected Colgate mouthwash contained Cetylpyridinium Chloride (CPC) as its active antimicrobial ingredient and was commonly used for oral hygiene to reduce bacteria and improve oral health. This product was chosen due to its wide consumer use, making the findings of the study relevant to real-world exposure scenarios.

The selected Colgate mouthwash contained 0.075% Cetylpyridinium Chloride (CPC), the standard concentration approved for oral hygiene products by the Food and Drug Administration (FDA, 2023).

Culture Reagents

In this study, a culture reagent was employed to support the proper handling and assessment of oral/buccal epithelial cells during CPC exposure experiments. Phosphate-Buffered Saline (PBS, pH 7.4) was used for washing collected cells prior to Cetylpyridinium Chloride (CPC) exposure and before conducting viability and morphological assessments. The solution maintained physiological pH and osmotic balance during cell handling procedures, ensuring minimal cellular stress while removing residual media and treatment compounds.

Assay Reagents

To assess the cytotoxic effects of Cetylpyridinium Chloride (CPC) on oral buccal epithelial cells, an assay reagent was used to measure cell viability quantitatively. Methylene Blue was applied to harvested cells to differentiate live and dead cells based on membrane integrity. Viable cells excluded the dye and remained unstained, while non-viable cells took up the dye and appeared blue. Cells were suspended in Phosphate-Buffered Saline and were counted using a hemocytometer under a light microscope. This procedure provided numerical data on cell viability across different CPC concentrations and exposure durations, supporting the correlation of cytotoxicity with observed morphological changes.

Equipment

The following laboratory equipment and materials were used during the conduct of the study to ensure accuracy, safety, and reliability of the experimental procedures. A light microscope was utilized for observing cellular morphology and identifying structural changes in the cells after treatment. A biological safety cabinet was employed to maintain aseptic conditions during cell collection, handling, and treatment procedures, minimizing the risk of contamination. Micropipettes with sterile tips were used for precise measurement and transfer of reagents, while culture plates or flasks served as containers for cell growth and treatment. Glass microscope slides and cover slips were prepared for microscopic examination of stained cells. A digital camera or microscope imaging system, when available, was used to document representative cellular images. A timer or stopwatch ensured accurate control of exposure and incubation times.

A hemocytometer was used for cell counting, and methylene blue dye was employed to distinguish viable from non-viable cells based on membrane integrity during viability assessment. Together, this equipment and materials supported controlled experimental conditions, accurate reagent handling, and reliable observation and assessment of cellular responses throughout the study.

Experimental Procedures

Cell Collection and Preparation

Human oral epithelial cells were collected from participants after they rinsed with Cetylpyridinium Chloride (CPC)-containing mouthwash. The gargle solution was then collected, and the cells were separated either by gentle centrifugation or by allowing them to settle naturally. Once harvested, the cells were carefully washed with Phosphate-Buffered Saline (PBS) to remove any remaining mouthwash or debris.

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Cell viability was assessed using Methylene Blue, with the cells suspended in PBS, mixed with the dye, and examined under a light microscope using a hemocytometer.

Viable cells excluded the dye and remained clear, while non-viable cells took up the blue stain. This method provided reliable, quantitative data on cell viability directly from the participants' samples, yielding direct data on cellular responses following actual exposure and allowed a clear correlation between the cytotoxic effects of CPC and any morphological changes observed in the cells.

CPC Treatment

This study evaluated the cytotoxic effects of Cetylpyridinium Chloride (CPC) on human oral epithelial cells by directly analyzing cells collected from participants after mouthwash use. Participants were asked to rinse with a mouthwash containing a defined concentration of CPC for a standardized duration determined by the study protocol. After gargling, the oral rinse was collected, containing epithelial cells that had been exposed to CPC. The collected solution served as the treatment sample for analysis.

The cells were then isolated by gentle centrifugation or allowed to settle, washed with Phosphate-Buffered Saline to remove residual CPC and debris, and prepared for viability assessment using Methylene Blue. This approach allowed direct evaluation of CPC's effect on human oral epithelial cells in a real-use scenario, providing quantitative data on cell viability and enabling correlation with morphological observations.

Cell Viability Assessment

Cell viability of human oral epithelial cells exposed to Cetylpyridinium Chloride (CPC) was assessed using Methylene Blue. The harvested cells were first suspended in Phosphate Buffered Saline to remove any residual mouthwash and debris. An equal volume of Methylene Blue solution was then added to the cell suspension, allowing the dye to selectively stain cells with compromised membranes.

The mixture was incubated briefly at room temperature before being loaded onto a hemocytometer for microscopic examination. Viable cells excluded the dye and remained clear, whereas non-viable cells took up the dye and appeared blue. Cells were counted in multiple fields of the hemocytometer to ensure accuracy, and the percentage of viable cells was calculated using the formula:

$$\text{cell viability } (\%) = \frac{numberofviablecells}{numberoftotalcells} x100$$

This quantitative approach provided reliable measurements of CPC-induced cytotoxicity and allowed direct comparison between different concentrations and exposure durations. In addition, visual inspection of stained cells under the microscope enabled observation of morphological changes, such as cell shrinkage, membrane blebbing, or loss of adherence, which could be correlated with viability data to better understand the impact of CPC on oral epithelial cells.

Cell Morphology Assessment

To evaluate the impact of Cetylpyridinium Chloride (CPC) on the structural characteristics of human oral epithelial cells, morphological alterations were assessed following treatment. After collection, the cells were washed with Phosphate-Buffered Saline and subsequently were loaded onto a hemocytometer for examination under a light microscope. This method enabled direct observation and measurement of cell shape and size, including potential alterations such as cellular rounding, shrinkage, or changes in overall cell dimensions. Observations were recorded manually, providing complementary data to cell viability measurements and facilitating correlation between morphological changes and CPC-induced cytotoxic effects.

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Criteria for Morphological Interpretation

% Viability	Morphological Score	Interpretation
81- 100%	1	Mostly intact/normal cells
61- 80%	2	Mild alterations
41- 60%	3	Moderate alterations
21-40%	4	Severe alterations
0-20%	5	Extensive damage/ cell fragmentation

Data Analysis

Statistical Analysis

Statistical Analysis The data collected in this study were systematically organized, tabulated, and analyzed using appropriate statistical methods to ensure accurate interpretation of the findings. Descriptive statistics, specifically the mean and standard deviation, were employed to summarize the cell viability data and morphological alteration scores obtained from the different exposure frequencies and observation periods. These measures provided a comprehensive description of the central tendency and variability of the results.

Analysis of Variance (ANOVA) was utilized to determine whether significant differences existed in cell viability and morphological changes in relation to the frequency and duration of CPC exposure. This statistical test was applied to assess whether the observed variations among groups were statistically significant and not merely attributable to random variation. Furthermore, Pearson Correlation Analysis was conducted to evaluate the strength and direction of the relationship between cell viability and morphological alteration scores.

This analysis determined whether decreases in cell viability were associated with corresponding increases in structural cellular damage. All statistical analyses were performed using a significance level of $\alpha = 0.05$, wherein a p-value less than 0.05 was considered statistically significant. Collectively, these statistical procedures provided a comprehensive and reliable evaluation of the effects of CPC exposure frequency and duration on the viability and morphology of human buccal epithelial cells.

Morphological Analysis

To evaluate the impact of Cetylpyridinium Chloride (CPC) on the structural characteristics of human oral epithelial cells, morphological alterations were assessed following treatment. After collection, the cells were washed with Phosphate-Buffered Saline and subsequently loaded onto a hemocytometer for examination under a light microscope. This method enabled direct observation and measurement of cell shape and size, including potential alterations such as cellular rounding, shrinkage, or changes in overall cell dimensions. Observations were recorded manually, providing complementary data to cell viability measurements and facilitating correlation between morphological changes and CPC-induced cytotoxic effects.

Quality Control

All procedures were conducted under consistent and controlled conditions to ensure reliable results. The collection of cells, PBS washing, and Methylene Blue staining were performed uniformly for all samples. Cell counts were done in duplicate or triplicate to reduce human error, and all microscopes and hemocytometers were checked and calibrated before use. Observations were verified by independent

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

researchers whenever possible, and any deviations from the protocol were documented and addressed to maintain the integrity of the data and to avoid observer bias; cell counting and morphological scoring were performed by two independent researchers. Discrepancies were resolved by re-examination and consensus.

Ethical Consideration

Ethical principles were strictly observed throughout the conduct of this study. Prior to participation, informed consent was obtained from all respondents. Since participants were within the age range of 19–25 years, who were considered legal adults, written consent was secured directly from each individual. The purpose of the study, procedures involved, potential risks, benefits, and rights of the participants were clearly explained in understandable language to ensure that participation was entirely voluntary and based on full understanding.

The privacy and confidentiality of the participants were protected at all times. All personal information was kept strictly confidential and was not disclosed to any unauthorized persons. Respondents were identified using unique codes instead of their names or any personal details, ensuring that all collected data remained anonymous and was used solely for academic and research purposes. Participants were fully informed that they had the right to withdraw from the study at any time, at their own discretion, without facing any penalties or negative consequences.

The study ensured that the use of CPC-containing mouthwash followed standard oral hygiene guidelines and safety protocols, and no procedures that could cause harm, discomfort, or adverse effects were conducted. Proper hygiene and safety measures were strictly implemented during the collection of cheek cell samples and all subsequent laboratory procedures to guarantee the well-being and safety of all participants.

RESULTS AND DISCUSSION

The Effects of Different Frequencies of Cetylpyridinium Chloride (CPC) Exposure on Human Cheek Epithelial Cell Viability Over a 7-Day Period

Table 1. *Descriptive Statistics of Cell Viability for Once Daily*

Exposure Day	N	Sum	Mean (%)	Standard Deviation	Variance
Once Daily Exposure					
Day 1	20	1284.72	64.236	8.96	73.66866737
Day 2	20	1146.72	57.321	2.06	4.7914832
Day 3	20	827.12	41.356	11.63	134.586183
Day 4	20	736.4	36.82	7.87	68.05978
Day 5	20	642.4	32.12	20.47	434.772568
Day 6	20	811.51	40.5755	22.94	515.9359
Day 7	20	828.27	41.4135	21.69	461.0106
Total	140	6276.84	44.83457	18.72	346.5249

Table 1 shows cell viability trends under once-daily CPC exposure over 7 days. Day 1 recorded the highest viability at 63.31% with low variation (SD = 8.96), indicating uniform response and minimal initial harm to oral cells. This matches Raut et al. (2022), Marquis & Clock (2021), and Rembe et al. (2022), who confirmed CPC mainly targets microbes with little effect on host cells at first use. From Day 2 to 5, viability dropped steadily to 31.66%, while variability rose sharply (SD = 20.47), reflecting cumulative and uneven cellular damage.

These results support García Cuellar et al. (2022), Ma et al. (2025), and Weller et al. (2024), who linked repeated CPC exposure to progressive membrane and mitochondrial damage, leading to time-dependent cytotoxicity. Days 6–7 showed slight recovery to 42.05–43.04%, though levels never

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

returned to baseline. Nakamura et al. (2023), Ma et al. (2025), and Shiina et al. (2025) explained the 24-hour gap allows limited repair, but damage becomes largely permanent once a critical threshold is reached.

The overall mean of 45.31% confirms significant cell loss, consistent with Marquis & Clock (2021) that oral mucosal cells are highly sensitive to repeated CPC contact.

Table 2. *Descriptive Statistics of Cell Viability for Twice Daily*

Exposure Day	N	Sum	Mean (%)	Standard Deviation	Variance
Twice Daily Exposure					
Day 1	20	1308.13	65.4065	16.44	181.2014661
Day 2	20	1231.4	61.56725	14.08	117.04957
Day 3	20	1271.4	63.57	25.00	351.970205
Day 4	20	968.08	48.404	23.12	181.7292
Day 5	20	422.005	21.10025	19.83	104.381028
Day 6	20	679.975	33.99875	23.38	294.3299
Day 7	20	766.015	38.30075	16.84	312.2674
Total	140	6,944.20	47.47821	23.18	461.4717

Table 2 presents cell viability under twice-daily CPC exposure. Day 1 showed 65.41% viability with high variation (SD = 16.44), meaning initial survival was high but responses were inconsistent. This aligns with Rembe et al. (2022), Marquis & Clock (2021), and Raut et al. (2022), who noted early CPC effects are mild, but more frequent exposure creates uneven stress across cells.

From Day 1 to 5, viability dropped sharply to the lowest level of 21.10%, with severe decline starting at Day 3 and variation peaking at SD = 25.00. Ma et al. (2025), Nakamura et al. (2023), and García-Cuellar et al. (2022) explained twice-daily application leaves no time for repair, so membrane and mitochondrial damage accumulates rapidly and irreversibly. Shiina et al. (2025) added this frequency far exceeds safe limits, causing extreme injury by Day 5. Days 6–7 saw only slight recovery to 34.00–38.30%, far less improvement than once-daily exposure.

Nakamura et al. (2023), Ma et al. (2025), and Weller et al. (2024) confirmed that with 12-hour intervals, repair is impossible; structural changes become permanent, and residual damage remains severe. Marquis & Clock (2021) emphasized oral cells lack defenses to tolerate such frequent contact. The overall mean of 47.48% with consistently high variation confirms twice-daily exposure causes more severe, unpredictable damage. This supports Rembe et al. (2022), Ma et al. (2025), and Shiina et al. (2025) that exposure frequency directly determines injury severity, and twice-daily use significantly increases cytotoxic risk.

Table 3. *Summary of Descriptive Statistics for Cell Viability*

	Sum	Mean (%)	SD	Variance
Once Daily Exposure	6276.84	45.83457	18.72	346.5249
Twice Daily Exposure	6646.95	47.47821	23.18	461.4717

Table 3 summarizes overall 7-day outcomes: once-daily exposure recorded a mean viability of 45.31% (SD = 18.72), while twice-daily exposure averaged 49.60% but with much higher variability

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

(SD = 23.18). The slightly higher mean for twice-daily exposure reflects high early-stage survival, but elevated variance shows results were far less consistent. This aligns with Ma et al. (2025), Nakamura et al. (2023), and Shiina et al. (2025), who explained frequent exposure leaves no time for full repair, creating mixed outcomes: some cells recover slightly while others sustain irreversible damage.

Greater variability in the twice-daily group further supports Rembe et al. (2022) and Marquis & Clock (2021), who noted oral mucosal cells vary widely in sensitivity and lack strong protective barriers; repeated, frequent contact amplifies these natural differences, leading to uneven injury levels. Collectively, these results confirm exposure frequency determines both average viability and consistency of response, as established in related literature.

Table 4. *One-Way ANOVA Results for the Effects of CPC Exposure on Buccal Epithelial Cell Viability/Morphology*

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	489.219	1.000	489.219	2.117	0.147	3.877
Columns	43107.120	6.000	7184.520	31.085	0.000	2.133
Interaction	7725.074	6.000	1287.512	5.571	0.000	2.133
Within	61479.324	266.000	231.125			
Total	112800.737	279.000				

Table 4 presents ANOVA results testing CPC's effects on buccal cells. Sample variation showed F = 2.117, p = 0.147 (not significant), confirming replicates were consistent and results were not due to sample differences. This supports Rembe et al. (2022), Marquis & Clock (2021), and Raut et al. (2022), who validated that experimental setup and cell sources produce uniform baseline responses. Columns (exposure conditions) yielded F = 31.085, p = 0.000 (highly significant), proving exposure frequency and duration are the main drivers of change.

This aligns with Ma et al. (2025), Nakamura et al. (2023), and Shiina et al. (2025), who established that exposure regimen directly determines cytotoxicity severity and structural damage. The Interaction term was also significant (F = 5.571, p = 0.000), meaning effects change over time and differ between exposure schedules. This matches findings by García-Cuellar et al. (2022), Weller et al. (2024), and Ma et al. (2025), who noted damage accumulates faster with frequent use and progresses differently as exposure continues.

High Within-group variation reflects natural differences in cell sensitivity, as explained by Rembe et al. (2022), Marquis & Clock (2021), and Weller et al. (2024), who reported oral epithelial cells vary widely in resilience, yet significant treatment effects remain detectable above this background.

The Structural Abnormalities Observed in Human Cheek Epithelial Cells After CPC Exposure

Table 5. *Damage Scores and Microscopic Change Over 7 Days of Once-Daily Exposure*

Exposure Day	Score	Microscopic Description of Changes
Day 1	1.2	Cells maintain uniform oval shape, clear central nucleus, smooth intact membrane; no visible injury or structural abnormality.
Day 2	1.45	Shape mostly normal; only minor irregularity at cell edges. Cytoplasm remains dense and evenly distributed; nucleus clearly defined.
Day 3	2.75	Noticeable shrinkage and early membrane blebbing ; slight reduction in cytoplasmic volume. Nucleus remains visible but less distinct than before.
Day 4	3.6	Clear distortion of shape; moderate shrinkage and irregular contours. Membrane shows small tears; nuclear definition becomes faint.
Day 5	3.95	Minor improvement in uniformity; some cells slightly larger, but still small, irregular, and structurally abnormal.

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Day 6	4.1	Severe deformation persists; very few cells show slight size recovery. Most remain shrunken and irregular; no return to normal form.
Day 7	4.8	Peak damage: Widespread fragmentation and occasional cell rupture; some leakage of contents. Large empty areas visible; almost no intact structure remains.

Table 5 details the progressive morphological changes and damage scores for buccal epithelial cells under once-daily CPC exposure over seven days. On Day 1, the score of 1.2 reflected normal oval shape, intact membranes, and clear nuclei, with no visible injury. This aligns with Raut et al. (2022), Rembe et al. (2022), and Marquis & Clock (2021), who confirmed that CPC initially targets only microbial membranes, causing no structural harm to host oral cells at early exposure stages.

From Day 2 to 4, scores rose steadily to 3.6, marked by gradual changes: minor irregularities, early shrinkage, blebbing, and eventually shape distortion with membrane tears. García-Cuellar et al. (2022), Weller et al. (2024), and Ma et al. (2025) explained this progression results from cumulative membrane disruption and mitochondrial impairment, where damage accumulates slowly but consistently with daily application. Day 5 showed a slight score reduction to 3.95, with minor improvement in uniformity—evidence of limited repair within the 24-hour interval.

This matches Nakamura et al. (2023), who noted once-daily exposure allows sufficient time for partial recovery of cellular structure. However, scores increased again on Day 6 (4.1) and reached the highest level of 4.8 on Day 7, characterized by widespread fragmentation, cell rupture, and complete loss of intact architecture. Ma et al. (2025) and Shiina et al. (2025) emphasized that while short-term repair is possible, repeated exposure eventually overwhelms defense mechanisms, pushing injury past the reversible threshold and leading to permanent structural damage.

Marquis & Clock (2021) further added that oral epithelial cells' non-keratinized nature makes them unable to withstand prolonged chemical stress, resulting in severe, irreversible changes by the end of the exposure period.

Table 6. *Damage Scores and Microscopic Change Over 7 Days of Twice-Daily Exposure*

Exposure Day	**Score**	**Microscopic Description of Changes**
Day 1	1.15	Uniform oval shape, distinct central nucleus, smooth unbroken membrane; no injury observed-nearly identical to control.
Day 2	1.38	Shape mostly normal; very slight irregularity at edges. Cytoplasm dense and even; nucleus clearly defined- only minimal change.
Day 3	3.2	More severe changes: Marked shrinkage, extensive membrane blebbing and folding. Cytoplasm volume greatly reduced; nucleus faint and hard to distinguish.
Day 4	4.15	Severe distortion and extreme shrinkage; highly irregular, jagged contours. Membrane tears widespread; nuclear structure completely lost.
Day 5	4.28	Very slight improvement in appearance, but cells remain small, irregular, and structurally abnormal- far less recovery than once- daily exposure.
Day 6	4.45	Persistent severe deformation; almost no recovery in size or shape. Cells remain tiny, misshapen, and highly disorganized.
Day 7	4.9	Maximum damage: Complete fragmentation, frequent cell rupture, and heavy leakage of contents. Almost all fields empty; no recognizable cell architecture left

Table 6 presents damage scores and microscopic changes for twice-daily CPC exposure. On Day 1–2, scores of 1.15–1.38 showed cells retained normal oval shape, intact membranes, and clear nuclei, with only minimal irregularities. This aligns with Raut et al. (2022), Rembe et al. (2022), and Marquis & Clock

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

(2021), who confirmed that CPC initially acts selectively on microbial membranes, causing negligible structural harm to oral epithelial cells at early exposure stages.

From Day 3–4, scores rose sharply to 3.2–4.15, marked by severe shrinkage, extensive membrane blebbing, loss of nuclear definition, and extreme shape distortion—damage appearing far earlier and more intense than in the once-daily group. Ma et al. (2025), Nakamura et al. (2023), and García-Cuellar et al. (2022) explained that the 12-hour interval between exposures is too short for any meaningful repair; membrane disruption, oxidative stress, and mitochondrial impairment accumulate rapidly, overwhelming cellular defenses long before recovery is possible.

Day 5 showed only negligible improvement, score remaining high at 4.28, with cells still small and irregular—far less recovery compared to once-daily exposure. Scores continued rising to 4.45 on Day 6 and peaked at 4.9 on Day 7, characterized by complete fragmentation, cell rupture, and total loss of recognizable architecture. Nakamura et al. (2023), Ma et al. (2025), and Shiina et al. (2025) emphasized that frequent exposure pushes injury past the reversible threshold, making structural changes permanent; oral mucosal cells lack sufficient protective barriers and repair capacity to tolerate repeated contact at this frequency, resulting in irreversible damage.

Correlation Between Cell Viability and Morphological Changes in CPC-Exposed Human Buccal Epithelial Cells

Table 7. *Correlation Between Viability and Morphology*

Exposure Frequency	Correlation Coefficient (r)	Interpretation
Once Daily Exposure	-0.794	Strong Negative Correlation
Twice Daily Exposure	-0.873	Very Strong Negative Correlation

Table 7 shows a strong negative correlation between cell viability and morphological damage: $r = -0.794$ for once-daily exposure and $r = -0.873$ for twice-daily exposure, meaning as structural injury increases, cell survival decreases significantly. This directly aligns with García-Cuellar et al. (2022) and Ma et al. (2025), who established that CPC-induced membrane disruption, mitochondrial impairment, and structural abnormalities are the main drivers of reduced viability, with damage severity closely predicting the level of cell death.

The stronger correlation in the twice-daily group confirms the relationship becomes tighter with more frequent exposure. Nakamura et al. (2023) explained that repeated application accelerates irreversible structural changes, so injury and loss of function progress almost in parallel. Weller et al. (2024) further noted that even early, sub-lethal changes such as mitochondrial alterations precede measurable viability loss, proving microscopic features are reliable indicators of impending cytotoxicity. Rembe et al. (2022) added this pattern is consistent across antiseptic agents, where the type and extent of morphological damage directly determine the final survival rate.

These results validate that morphological assessment is an accurate, sensitive method to evaluate CPC's effects, as structural changes closely reflect the degree of functional impairment and cytotoxic risk.

Summary

This study investigated and compared the effects of once-daily and twice-daily exposure to cetylpyridinium chloride (CPC) on the viability and morphological characteristics of human buccal epithelial cells over a seven-day experimental period. Specifically, the study aimed to: (1) evaluate the effects of varying frequencies of CPC exposure on human cheek epithelial cell viability using quantitative viability assays; (2) observe and identify visible alterations in the appearance and structural characteristics of CPC-exposed oral epithelial cells through microscopic examination; and (3) determine the relationship between changes in cell viability and morphological abnormalities in order to establish potential associations between CPC exposure and cellular damage.

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Based on the findings of the study, the first objective, which aimed to evaluate the effects of different frequencies of Cetylpyridinium Chloride (CPC) exposure on human cheek epithelial cell viability over a seven-day period, was achieved. The results demonstrated that repeated exposure to CPC significantly reduced cell viability over time. Both the once-daily and twice-daily exposure groups exhibited progressive declines in viability; however, cells exposed twice daily experienced earlier and more pronounced reductions.

Statistical analysis further confirmed that exposure frequency and duration significantly influenced cell viability. These findings indicate that repeated CPC exposure causes cumulative cellular damage and negatively affects the survival of buccal epithelial cells.

The second objective, which sought to observe and identify visible changes in the appearance and structural characteristics of CPC-exposed buccal epithelial cells through microscopic examination, was likewise achieved. Microscopic observations revealed progressive morphological alterations following CPC exposure. Initially, cells maintained normal morphology with intact membranes and clearly visible nuclei. As exposure continued, cells exhibited shrinkage, membrane blebbing, irregular cell contours, fragmentation, and eventual cell rupture.

These structural abnormalities became increasingly severe with prolonged exposure and were more evident in the twice-daily exposure group. Therefore, CPC exposure was found to adversely affect the structural integrity and morphology of human buccal epithelial cells.

The third objective, which aimed to correlate observed changes in cell viability with morphological abnormalities to establish potential links between CPC exposure and cellular damage, was also accomplished. Pearson correlation analysis revealed a strong negative correlation between cell viability and morphological damage scores in both exposure groups ($r = -0.794$ for once-daily exposure and $r = -0.873$ for twice-daily exposure).

These results indicate that as morphological damage increased, cell viability decreased. The findings confirm that structural deterioration is closely associated with reduced cellular survival and that morphological changes serve as reliable indicators of CPC-induced cellular damage.

CONCLUSION

This study concludes that cetylpyridinium chloride (CPC) exposure exerts significant cytotoxic and structural damaging effects on human buccal epithelial cells in a time- and frequency-dependent manner. These findings are consistent with previously established mechanisms involving membrane disruption, mitochondrial impairment, and oxidative stress associated with CPC exposure.

Specifically, the study successfully achieved its first objective by evaluating the effects of different frequencies of CPC exposure on human cheek epithelial cell viability over a seven-day period using quantitative viability assays. The findings demonstrated that repeated CPC exposure progressively reduced cell viability, with twice-daily exposure producing earlier onset of damage, greater severity of injury, reduced recovery capacity, and increased variability in cellular responses compared to once-daily exposure.

Although initial exposure caused minimal injury, continuous application resulted in cumulative cellular damage that intensified over time. The limited recovery observed in the once-daily group suggests that the 24-hour exposure interval allowed partial cellular restoration, whereas the 12-hour interval associated with twice-daily exposure was insufficient for adequate repair. The second objective of the study was likewise achieved through microscopic examination of CPC-exposed oral epithelial cells.

Progressive morphological alterations were observed throughout the seven-day exposure period, including irregular cellular shape, shrinkage, membrane disruption, blebbing, fragmentation, and eventual loss of cellular integrity. These findings confirmed that CPC exposure induces progressive structural deterioration that becomes increasingly severe and largely irreversible with repeated application. Furthermore, the study successfully fulfilled its third objective by establishing a significant relationship between cell viability and morphological abnormalities.

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

Pearson correlation analysis revealed a strong negative correlation between cell viability and morphological damage scores, indicating that increased structural deterioration was directly associated with reduced cellular survival. The stronger correlation observed in the twice-daily exposure group further demonstrated that more frequent CPC exposure intensifies both functional impairment and structural damage. Statistical analysis further confirmed that exposure frequency and duration were significant determinants of cellular injury severity, while sample variation remained statistically non-significant, thereby supporting the reliability and consistency of the findings. Moreover, the results emphasized the heightened vulnerability of oral epithelial cells to CPC-induced chemical stress due to their non-keratinized and highly permeable nature.

Collectively, the findings of this study demonstrate that while once-daily CPC exposure causes measurable but relatively manageable cellular injury, twice-daily exposure significantly increases the risk of severe and potentially irreversible damage to oral epithelial tissues. These results confirm that excessive or frequent CPC exposure compromises both the viability and structural integrity of human buccal epithelial cells.

Recommendations

Based on the findings of this study, the results may serve as a guide for consumers, health professionals, the oral care industry, and future researchers regarding the safe use of Cetylpyridinium Chloride (CPC)-containing oral hygiene products.

For the society and consumers, it is recommended that CPC-containing oral care products be used with caution and in moderation. The findings suggest that limiting use to once daily may help reduce potential cellular damage, as more frequent exposure may contribute to greater effects on cell viability and morphology. Public awareness regarding proper usage frequency is encouraged to promote safer oral hygiene practices.

For health professionals, particularly dentists and medical practitioners, it is recommended that they consider the potential cellular effects of repeated CPC exposure when advising patients. Evidence from this study may support more cautious and individualized recommendations, especially for individuals with sensitive oral tissues or existing oral conditions.

For manufacturers and the oral care industry, it is recommended that product formulation and safety standards be further reviewed. Improvements such as optimizing CPC concentration, exploring safer delivery systems, or incorporating protective agents may help reduce potential cytotoxic effects while maintaining antimicrobial effectiveness. Clearer usage instructions and safety warnings on product labels are also encouraged.

For future researchers, it is recommended that further studies be conducted to expand on these findings by exploring different CPC concentrations, longer exposure periods, and cellular recovery mechanisms. The use of more advanced in vitro models, such as three-dimensional oral tissue cultures, is also suggested to better simulate real oral conditions. Additionally, incorporating both viability and morphological assessments in cytotoxicity studies is recommended to provide a more comprehensive evaluation of CPC's cellular effects.

ISSN: 3116-3475
International Journal
of Education, Research, and Innovation Perspectives
Volume 2 Issue 7 (July 2026)

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