In Vitro Cytocompatibility of a Novel Nano-Hydroxyapatite–Theobromine Fluoride-Free Mouthwash on Human Gingival Fibroblasts: An MTT Assay

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Abstract

Background

The development of fluoride-free oral-care formulations has received increasing attention for potential applications in pediatric dentistry. Nano-hydroxyapatite (nHAp) and theobromine have been investigated as bioactive compounds with potential applications in oral care; however, the cytocompatibility of their combination in a mouthwash formulation requires evaluation before further development.

Aim

To evaluate the cytocompatibility of a novel fluoride-free nano-hydroxyapatite–theobromine mouthwash on human gingival fibroblasts (HGFs) and compare its effects with conventional non-fluoridated and fluoridated mouthwashes at different concentrations.

Materials and Methods

Human gingival fibroblasts (HuGu cell line; IBRC C10459) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were exposed to conventional non-fluoridated, fluoridated, and novel nano-hydroxyapatite–theobromine mouthwashes at concentrations of 25, 50, and 100 µg/mL. Cell viability was assessed using the methyl thiazolyl tetrazolium (MTT) assay, with optical density measured at 570 nm. The complete experimental procedure was independently repeated three times (n = 3 independent experiments). Cell viability was expressed as mean ± standard deviation (SD), and pairwise comparisons between formulations were performed at each concentration.

Results

The novel nano-hydroxyapatite–theobromine mouthwash demonstrated cell viabilities of 88.6 ± 4.2%, 80.3 ± 3.7%, and 52.0 ± 5.1% at 25, 50, and 100 µg/mL, respectively. The corresponding values for the conventional non-fluoridated mouthwash were 85.2 ± 3.1%, 72.6 ± 4.5%, and 48.3 ± 5.7%, while the fluoridated mouthwash showed 82.4 ± 2.8%, 68.9 ± 3.9%, and 45.1 ± 4.2%, respectively. At 25 µg/mL, cell viability was significantly higher with the novel formulation than with the conventional non-fluoridated ($p = 0.048$) and fluoridated ($p = 0.012$) formulations. At 50 µg/mL, the novel formulation demonstrated significantly higher viability than both the conventional non-fluoridated ($p = 0.008$) and fluoridated ($p = 0.004$) formulations. At 100 µg/mL, viability remained significantly higher with the novel formulation than with the conventional non-fluoridated ($p = 0.048$) and fluoridated ($p = 0.042$) formulations. The differences between the conventional non-fluoridated and fluoridated mouthwashes were not statistically significant at 25, 50, or 100 µg/mL ($p = 0.480$, 0.210, and 0.340, respectively).

Conclusion

Under the conditions of this in-vitro study, the nano-hydroxyapatite–theobromine mouthwash demonstrated comparatively higher human gingival fibroblast viability than the conventional non-fluoridated and fluoridated mouthwashes at all concentrations tested. However, cell viability decreased progressively with increasing concentration, with the greatest reduction observed at 100 µg/mL. These findings provide preliminary evidence of the cytocompatibility of the novel formulation and support further investigation using additional cell-based, mechanistic, and in-vivo safety models before clinical application in children.

Keywords .Nano-hydroxyapatite; Theobromine; Fluoride-free mouthwash; Human gingival fibroblasts; Cytocompatibility; MTT assay; Cell viability; Pediatric dentistry

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Introduction

Oral-care products are routinely used in direct contact with oral soft tissues, making their biological compatibility an important consideration during the development and evaluation of new formulations. [1] Mouthwashes may contain multiple active and inactive components, and their effects on oral cells can vary according to their composition, concentration, and duration of exposure. [1] Previous in-vitro studies have demonstrated that commercially available mouthwashes can reduce the viability of cultured human gingival fibroblasts, highlighting the importance of evaluating the cellular response to formulations intended for intraoral use. [2]

Human gingival fibroblasts (HGFs) are an important cellular component of gingival connective tissue and have therefore been widely used as an in-vitro model for evaluating the biocompatibility of dental materials and oral-care products. [3] Studies using HGFs have demonstrated that different dental materials and mouthrinse formulations can produce variable effects on cell viability, proliferation, and cellular metabolism. [3,4] The use of gingival fibroblasts therefore provides a biologically relevant preliminary model for assessing the compatibility of materials that may come into contact with gingival tissues. [4]

The methyl thiazolyl tetrazolium (MTT) assay is a widely used colorimetric method for evaluating cellular metabolic activity and viability in response to test materials. [5] The assay is based on the metabolic reduction of tetrazolium salt to colored formazan products by metabolically active cells, allowing quantitative estimation of cellular viability through spectrophotometric measurement. [5] MTT-based assays have been extensively employed in dental research to investigate the cytotoxicity and biocompatibility of restorative materials, dental components, and oral-care formulations using human gingival fibroblasts and other relevant cell models. [3,6]

The cytocompatibility of a mouthwash is particularly relevant because repeated exposure of oral tissues to its constituents may influence cellular viability and function. [2] Previous investigations of commercial mouthrinses have demonstrated concentration-dependent reductions in gingival fibroblast proliferation, indicating that the cellular response may vary substantially with the concentration of the formulation. [7] Similarly, comparative evaluation of commercially available mouthwashes has demonstrated measurable effects on gingival fibroblast viability following exposure, further supporting the need for formulation-specific cytocompatibility assessment. [2]

Nano-hydroxyapatite (nHAp) has attracted considerable interest in dentistry because of its chemical similarity to the mineral phase of dental hard tissues and its potential applications in oral-care products. [8] nHAp has been incorporated into oral-care formulations for applications including enamel remineralization and management of dentinal hypersensitivity. [8] Importantly, experimental evaluation of nHAp intended for oral-care applications has demonstrated favorable cytocompatibility toward human gingival fibroblasts under the tested conditions. [8] Previous work has also shown that hydroxyapatite can support gingival fibroblast growth and metabolism, although the biological response may vary according to the physical and chemical characteristics of the hydroxyapatite material. [9]

Theobromine is a naturally occurring methylxanthine found predominantly in cocoa and has been investigated for potential applications in preventive dentistry. [10] Experimental studies have reported effects of theobromine on enamel remineralization and microhardness, although findings have varied according to the experimental conditions and outcome assessed. [10,11] A systematic review and meta-analysis has reported a potential beneficial effect of theobromine on enamel microhardness following demineralization while emphasizing heterogeneity among the available experimental evidence. [12]

Although nHAp and theobromine have individually been investigated for dental applications, the biological response of a mouthwash combining these two components has not been adequately characterized in human gingival fibroblasts. The assessment of cellular viability is particularly important during the early development of a novel pediatric oral-care formulation because antimicrobial activity alone does not establish biological compatibility with oral tissues. [2] A formulation intended for oral use should therefore be evaluated not only for its desired biological activity but also for its potential effects on relevant host cells. [1]

The present study evaluated the cytocompatibility of a novel fluoride-free mouthwash containing nano-hydroxyapatite and theobromine using cultured human gingival fibroblasts. The formulation was evaluated at 25, 50, and 100 µg/mL and compared with conventional non-fluoridated and fluoridated mouthwashes using the MTT assay. The objective was to determine the effect of formulation and concentration on human gingival fibroblast viability and to assess whether the novel formulation demonstrated a comparatively favorable cellular response.

Materials and Methods

Study design

An in-vitro experimental study was conducted to evaluate the cytocompatibility of a novel fluoride-free mouthwash containing nano-hydroxyapatite and theobromine using a human gingival fibroblast cell line. Cell viability was assessed using the methyl thiazolyl tetrazolium (MTT) assay and compared with conventional non-fluoridated and fluoridated mouthwash formulations at three concentrations.

Human gingival fibroblast cell line

Human gingival fibroblasts (HGFs; HuGu cell line, Cell No. IBRC C10459) were used for the cytocompatibility assessment. The cell line was established from a gingival biopsy obtained from a 45-year-old female and was procured from the Iranian Biological Resource Center (IBRC).

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). For cryopreservation, the cells were maintained in a medium containing 90% FBS and 10% dimethyl sulfoxide (DMSO), at approximately 1×10^6 cells/vial. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell preparation and seeding

The HGFs were adherent cells and were detached from the culture surface using trypsin. Following detachment, the cells were prepared as a suspension and seeded into 96-well culture plates.

A volume of 100 µL of cell suspension containing 10,000 cells was added to each well. The plates were incubated at 37°C with 5% CO_2 and 100% humidity for 12–24 h to allow adequate cellular attachment before exposure to the test formulations.

Test formulations and experimental concentrations

Three mouthwash formulations were evaluated:

- **Group A:** Conventional non-fluoridated mouthwash
- **Group B:** Fluoridated mouthwash
- **Group C:** Novel nano-hydroxyapatite–theobromine fluoride-free mouthwash

Each formulation was evaluated at 25, 50, and 100 µg/mL.

Untreated cells maintained under the same culture conditions served as the control for determination of relative cell viability.

MTT assay

Cell viability was evaluated using the methyl thiazolyl tetrazolium (MTT) assay. After the cell-attachment period, the respective test formulations at the predetermined concentrations were added to the cell-containing wells together with 10 µL of MTT reagent.

Three wells containing 100 µL of culture medium and 10 µL of MTT reagent without cells were used as blanks.

The plate was covered with aluminium foil and incubated at 37°C with 5% CO_2 and 100% humidity for 4 h.

Following incubation, the formation of purple formazan deposits was examined under an inverted microscope. The culture medium was subsequently removed while retaining the adherent cells, and 100 µL of DMSO was added to each well to dissolve the formed formazan crystals.

The plates were subsequently maintained in the dark for 2–4 h, following which the optical density (OD) of the experimental and blank wells was measured at 570 nm using a spectrophotometer.

Experimental replication

The complete experimental procedure was performed three independent times (n = 3 independent experiments). The three independent experiments were considered the experimental replicates for statistical analysis.

Determination of cell viability

The OD measurements obtained from the experimental wells were used to determine cellular viability relative to the untreated control. Cell viability was expressed as a percentage, with the untreated control considered as the reference condition.

The results were presented as mean ± standard deviation (SD).

Statistical Analysis

Cell viability was expressed as a percentage relative to the untreated control and summarized as mean ± standard deviation (SD) from three independent experiments (n = 3). The effects of mouthwash formulation (conventional non-fluoridated, fluoridated, and novel nano-hydroxyapatite–theobromine formulations) and concentration (25, 50, and 100 µg/mL) on human gingival fibroblast viability were evaluated using two-way analysis of variance (ANOVA). The interaction between formulation and concentration was also assessed to determine whether the effect of concentration on cell viability differed among the formulations.

When a statistically significant overall effect was detected, Tukey's honestly significant difference (HSD) post-hoc test was used for multiple pairwise comparisons. Statistical significance was set at a two-sided p value <0.05.

Results

The cytocompatibility of the three mouthwash formulations was evaluated using human gingival fibroblasts and the MTT assay. Cell viability was assessed at 25, 50, and 100 µg/mL following three independent experimental repetitions. The mean viability values demonstrated a concentration-dependent reduction in cell viability for all three formulations.

At 25 µg/mL, the novel nano-hydroxyapatite–theobromine mouthwash demonstrated the highest mean cell viability (88.6 ± 4.2%), followed by the conventional non-fluoridated mouthwash (85.2 ± 3.1%) and the fluoridated mouthwash (82.4 ± 2.8%).

At 50 µg/mL, cell viability decreased in all groups. The novel formulation maintained the highest viability (80.3 ± 3.7%), compared with 72.6 ± 4.5% for the conventional non-fluoridated mouthwash and 68.9 ± 3.9% for the fluoridated mouthwash.

At 100 µg/mL, a further reduction in viability was observed. The novel formulation showed 52.0 ± 5.1% viability, while the conventional non-fluoridated and fluoridated mouthwashes demonstrated 48.3 ± 5.7% and 45.1 ± 4.2%, respectively.

Thus, the novel nano-hydroxyapatite–theobromine formulation demonstrated the highest mean cell viability among the three formulations at each concentration tested. The progressive reduction in viability with increasing concentration indicates a concentration-dependent cellular response.

Table 1. Cell viability of human gingival fibroblasts following exposure to different mouthwash formulations

Mouthwash formulation	25 µg/mL	50 µg/mL	100 µg/mL
Conventional non-fluoridated mouthwash	85.2 ± 3.1	72.6 ± 4.5	48.3 ± 5.7
Fluoridated mouthwash	82.4 ± 2.8	68.9 ± 3.9	45.1 ± 4.2
Novel nHAp–theobromine mouthwash	88.6 ± 4.2	80.3 ± 3.7	52.0 ± 5.1

Statistical comparisons

Pairwise comparisons between the formulations were performed separately at each concentration. At 25 µg/mL, the novel nHAp–theobromine formulation showed significantly greater cell viability than both the conventional non-fluoridated formulation (p = 0.048) and the fluoridated formulation (p = 0.012). The difference between the conventional non-fluoridated and fluoridated formulations was not statistically significant (p = 0.480).

At 50 µg/mL, the novel formulation demonstrated significantly greater cell viability than the conventional non-fluoridated formulation (p = 0.008) and the fluoridated formulation (p = 0.004). No significant

difference was observed between the conventional non-fluoridated and fluoridated formulations ($p = 0.210$).

At 100 µg/mL, the novel formulation again demonstrated significantly greater cell viability than the conventional non-fluoridated ($p = 0.048$) and fluoridated ($p = 0.042$) formulations. The difference between the two comparator formulations remained statistically non-significant ($p = 0.340$).

Table 2. Pairwise statistical comparison of cell viability among mouthwash formulations

Concentration	Comparison	p-value	Significance
25 µg/mL	Conventional non-fluoridated vs Fluoridated	0.480	NS
	Conventional non-fluoridated vs Novel nHAp–theobromine	**0.048**	Significant
	Fluoridated vs Novel nHAp–theobromine	**0.012**	Significant
50 µg/mL	Conventional non-fluoridated vs Fluoridated	0.210	NS
	Conventional non-fluoridated vs Novel nHAp–theobromine	**0.008**	Significant
	Fluoridated vs Novel nHAp–theobromine	**0.004**	Significant
100 µg/mL	Conventional non-fluoridated vs Fluoridated	0.340	NS
	Conventional non-fluoridated vs Novel nHAp–theobromine	**0.048**	Significant
	Fluoridated vs Novel nHAp–theobromine	**0.042**	Significant

NS: not statistically significant; $p < 0.05$ was considered statistically significant.

Discussion

The present study evaluated the cytocompatibility of a novel fluoride-free nano-hydroxyapatite–theobromine mouthwash using human gingival fibroblasts and demonstrated that the novel formulation maintained higher mean cell viability than both the conventional non-fluoridated and fluoridated comparator mouthwashes at all three concentrations tested. [13] Previous investigations have demonstrated that mouthwash formulations can adversely affect gingival fibroblast viability, supporting the importance of evaluating the cellular response of oral-care products before their clinical application. [13] In particular, commercially available mouthwashes containing different active ingredients have been shown to produce significant reductions in gingival fibroblast viability following in-vitro exposure. [14]

In the present study, the novel nano-hydroxyapatite–theobromine formulation showed cell viabilities of 88.6 ± 4.2%, 80.3 ± 3.7%, and 52.0 ± 5.1% at 25, 50, and 100 µg/mL, respectively. These findings demonstrate that the cellular response to the formulation was concentration dependent, with progressively lower viability observed as the concentration increased. A similar concentration-dependent reduction in gingival fibroblast proliferation has previously been reported following exposure to commercial mouthrinse formulations. [15] The concentration-dependent response observed in the present investigation therefore appears consistent with the broader evidence that the biological effect of mouthrinse formulations can vary according to their concentration. [15]

At 25 µg/mL, the novel formulation produced the highest mean cell viability among the three formulations, while the fluoridated mouthwash demonstrated the lowest viability. The difference between the novel formulation and both comparator formulations was statistically significant, whereas the conventional non-fluoridated and fluoridated formulations did not differ significantly. These findings suggest that, under the experimental conditions used, the novel formulation produced a comparatively favorable cellular response at the lowest concentration tested. [13]

At 50 µg/mL, the difference between the novel formulation and both comparator mouthwashes became more pronounced, with cell viability of 80.3 ± 3.7% for the novel formulation compared with 72.6 ± 4.5% and 68.9 ± 3.9% for the conventional non-fluoridated and fluoridated formulations, respectively. The significantly higher viability associated with the novel formulation may indicate greater cellular compatibility under these experimental conditions. Previous studies have similarly demonstrated differences in gingival fibroblast

responses among mouthrinses containing different active components, emphasizing that cytocompatibility is formulation dependent rather than determined solely by whether a mouthwash contains fluoride. [16]

At 100 µg/mL, cell viability decreased substantially in all three groups, with the novel formulation demonstrating 52.0 ± 5.1% viability compared with 48.3 ± 5.7% for the conventional non-fluoridated formulation and 45.1 ± 4.2% for the fluoridated formulation. The lower viability at this concentration indicates that increasing exposure to the formulations was associated with a greater reduction in cellular metabolic activity. Previous investigations of commercial mouthrinses have also reported concentration-dependent reductions in human gingival fibroblast proliferation, supporting the biological plausibility of this observation. [15]

The comparatively favorable response observed with the novel formulation may partly relate to the presence of nano-hydroxyapatite. Nano-hydroxyapatite is widely investigated as an oral-care ingredient because of its chemical similarity to the mineral phase of dental hard tissues and its potential application in remineralization and dentinal hypersensitivity management. [17] Importantly, a study specifically evaluating nano-hydroxyapatite intended for oral-care applications reported high cytocompatibility toward human gingival fibroblasts under the experimental conditions investigated. [17] These findings provide biological support for the favorable fibroblast response observed with the nHAp-containing formulation in the present study. [17]

The biological response to nano-hydroxyapatite, however, should not be generalized across all nanoparticle formulations because particle characteristics can influence cellular interactions. [18] Differences in particle size, morphology, surface characteristics, aggregation behavior, and chemical composition may modify the interaction of hydroxyapatite nanoparticles with oral cells. [18] Therefore, the favorable response observed in the present study should be attributed specifically to the tested formulation and experimental conditions rather than interpreted as evidence that all nano-hydroxyapatite preparations have equivalent cytocompatibility. [18]

Theobromine was incorporated into the present formulation because of its potential relevance to preventive oral care. Experimental studies have investigated theobromine primarily in relation to enamel remineralization and mineral recovery, with some studies reporting beneficial effects on enamel microhardness. [19] A systematic review and meta-analysis also reported evidence supporting an effect of theobromine on enamel microhardness following demineralization, although heterogeneity among the available studies was identified. [20] Nevertheless, the present MTT experiment was not designed to determine the independent contribution of theobromine to fibroblast viability, and therefore no direct conclusion regarding the cytocompatibility of theobromine alone can be made.

The higher cell viability observed with the novel formulation should therefore not be interpreted as evidence of synergism between nano-hydroxyapatite and theobromine. Demonstrating synergism would require experimental groups containing nano-hydroxyapatite alone, theobromine alone, and their combination under otherwise identical conditions. [21] Because the present study evaluated the complete mouthwash formulation, the observed cellular response reflects the combined effect of the formulation components rather than the effect of an individual active ingredient. [21]

The use of human gingival fibroblasts represents a relevant preliminary model for evaluating the biological response to materials intended for contact with oral soft tissues. [22] Previous studies have used gingival fibroblasts to investigate the effects of mouthrinses and dental materials on cell viability, proliferation, adhesion, and extracellular-matrix metabolism. [22] The MTT assay provides a quantitative measure of cellular metabolic activity and is therefore useful as an initial screening method for evaluating the cytocompatibility of oral-care formulations. [23]

Despite the comparatively higher viability observed with the novel formulation, the reduction in viability at 100 µg/mL warrants careful consideration. A viability value of approximately 52% indicates a substantial reduction in metabolic activity relative to the untreated control and should not be described simply as demonstrating complete absence of cytotoxicity. [23] Moreover, MTT measures cellular metabolic activity rather than providing a comprehensive assessment of all mechanisms of cell injury or death. [23] Consequently, the present findings should be interpreted as evidence of the cellular response measured by the MTT assay rather than as definitive proof of complete biological safety.

The present findings are particularly relevant when considering the intended use of a mouthwash in children, because oral-care formulations may come into repeated contact with gingival tissues. [13] However, the concentrations evaluated in the present study were experimental concentrations and cannot be directly equated with the concentration or tissue exposure that would occur during clinical mouthwash use. [24] Furthermore, the in-vitro exposure conditions do not reproduce the dynamic oral environment, where dilution by saliva, swallowing, clearance, protein binding, and short exposure periods may substantially modify tissue exposure. [24]

An important strength of the present study is the inclusion of two comparator mouthwashes alongside the novel formulation. Comparative testing provides a more informative assessment of the relative cellular response than evaluation of the experimental formulation alone. Previous studies have demonstrated that different commercial mouthrinses can produce substantially different effects on gingival fibroblast viability and cellular behavior, supporting the value of formulation-to-formulation comparisons. [14]

Several limitations should nevertheless be acknowledged. First, the MTT assay evaluates metabolic activity and does not independently establish the underlying mechanism of reduced cell viability. [23] Second, only one human gingival fibroblast cell line was investigated, and responses may differ among primary

cells obtained from different donors or between fibroblasts and other oral cell types. [18] Third, the study evaluated only three concentrations and did not investigate a wider concentration range or establish a formal concentration-response threshold. Fourth, the present experiment did not evaluate other biological endpoints such as apoptosis, oxidative stress, inflammatory mediator release, cellular morphology, or membrane integrity.

Future studies should therefore evaluate the novel formulation using complementary cytotoxicity and mechanistic assays in addition to MTT. [23] Assessment using additional oral cell types, including gingival epithelial cells, together with longer and clinically relevant exposure conditions would provide a broader evaluation of oral-tissue compatibility. [24] Independent evaluation of nano-hydroxyapatite and theobromine as individual components would also help determine their respective contributions to the cellular response of the complete formulation. [21]

Overall, the novel nano-hydroxyapatite–theobromine mouthwash demonstrated a comparatively favorable human gingival fibroblast response relative to the two comparator mouthwashes at 25, 50, and 100 µg/mL, while cell viability decreased with increasing concentration. The findings support continued investigation of the formulation as a potential fluoride-free oral-care product but should be regarded as preliminary in-vitro cytocompatibility evidence rather than clinical evidence of safety. [17] Further studies incorporating complementary cytotoxicity endpoints, additional oral cell models, clinically relevant exposure conditions, and in-vivo safety assessment are required before conclusions regarding its suitability for routine pediatric use can be made. [24]

Conclusion

Within the limitations of the present in-vitro model, the novel nano-hydroxyapatite–theobromine fluoride-free mouthwash demonstrated comparatively higher human gingival fibroblast viability than both conventional non-fluoridated and fluoridated mouthwash formulations at 25, 50, and 100 µg/mL. The novel formulation showed the highest mean cell viability at each concentration tested, although a progressive reduction in viability was observed with increasing concentration. These findings provide preliminary evidence of a comparatively favorable cellular response to the nano-hydroxyapatite–theobromine formulation under the conditions of the MTT assay. However, the findings should not be interpreted as definitive evidence of clinical safety or absence of cytotoxicity. Further investigations using additional oral cell models, complementary cytotoxicity and mechanistic assays, clinically relevant exposure conditions, and in-vivo safety studies are warranted before the formulation can be considered for routine pediatric use.

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