Original research

Cytotoxic effects of hydrogen peroxide on periodontal cells

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ABSTRACT

Objectives: To evaluate the in vitro cytotoxic effects of hydrogen peroxide (H_2O_2) on periodontal cells, based on the cellular viability and morphology of immortalized osteoblast and gingival fibroblast cultures, with different exposure times and concentrations.

Methods: Immortalized human gingival fibroblast and human fetal osteoblast cell lines were cultured, separately, in 96-well plates. After reaching confluency, they were exposed to H_2O_2 solutions at 16 different concentrations ranging between 0.05 µg/ml and 10 µg/ml for 1 h, 24 h or 72 h in triplicate assays (n=24), using culture media alone as the control. Cell viability was measured by previously established fluorometric methods, using a resazurin-based assay, and cell morphology by using an inverted microscope with integrated phase-contrast optics. Data were statistically analyzed using a one-way ANOVA with Tukey's and Dunnet's post hoc tests and Pearson correlation coefficient (r), as appropriate (α=0.05).

Results: H_2O_2 induced a decrease in cell viability to below 50% in fibroblasts and around 50% in osteoblasts, in all tested concentrations after 1h exposure, and a decrease in cell viability to above 70% after 24 h and 72 h (P<0.05). A significant negative correlation was detected between H_2O_2 and cell viability at 1 h and 72 h for osteoblast (r=-0.471) and HGF (r=-0.12) cells, respectively. The cell morphology analysis showed cell detachment and lower cell density, in agreement with these findings.

Conclusions: H_2O_2 induced cell alterations with moderate to severe cytotoxic effects in osteoblast and gingival fibroblasts. (Rev Port Estomatol Med Dent Cir Maxilofac. 2019;60(3):111-117)

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Efeitos citotóxicos do peróxido de hidrogénio em células periodontais

RESUMO

Palavras-chave:
Citotoxicidade
Fibroblasto
Periodonto
Peróxido de hidrogénio
Osteoblasto

Objectivos: Avaliar *in vitro* a citotoxicidade do Peróxido de Hidrogénio (H_2O_2) em células periodontais através do efeito de soluções H_2O_2 na viabilidade e morfologia de culturas de fibroblastos gengivais e osteoblastos humanos imortalizados, em diferentes concentrações e tempos de exposição.

Métodos: Foram usadas linhagens imortalizadas de fibroblastos gengivais e osteoblastos fetais humanos, as quais foram cultivadas, separadamente, em placas de 96 poços, que ao atingir a confluência, foram expostas a concentrações de H_2O_2 de 0,05 µg/ml a 10 µg/ml (16 concentrações diferentes), durante 1 h, 24 h ou 72 h, em ensaios triplicados (n=24), sendo utilizado exclusivamente meio de cultura como controlo. A viabilidade celular foi avaliada por métodos fluorométricos previamente descritos através da conversão da resazurina e a morfologia celular por microscopia ótica invertida com contraste de fase. Os dados foram analisados estatisticamente através do teste ANOVA one-way com post-hoc de Tukey e Dunnet's e do coeficiente de correlação de Pearson (r) conforme apropriado (α=0,05).

Resultados: O H_2O_2 induziu uma redução da viabilidade, superior a 50% tanto nos fibroblastos como nos osteoblastos, visível em todas as concentrações testadas após 1h de exposição e superior a 70% a 24h e 72h (p<0,05). Foi detetada uma correlação significativa e negativa aos tempos 1h e 72h para osteoblastos (r=-0,471) e fibroblastos (r=-0,12), respetivamente. A análise das micrografias obtidas apresentou destacamento celular e menor densidade celular em concordância com estes resultados.

Conclusão: A exposição ao H_2O_2 resultou em alterações celulares e efeitos citotóxicos de moderados a severos em fibroblastos gengivais e osteoblastos. (Rev Port Estomatol Med Dent Cir Maxilofac. 2019;60(3):111-117)

Introduction

Hydrogen peroxide (H_2O_2) is a well-described reactive oxygen species (ROS) commonly used in Dentistry procedures.[1] Namely, it is used in periodontal and peri-implantitis treatments including implant surface decontamination, as well as in tooth whitening products, mouth rinses and toothpastes.[2-6] H_2O_2 concentrations depend on the type of treatment: 30 µg/ml for peri-implantitis treatments and 30 µg/ml to 380 µg/ml for tooth bleaching. However, it has been well-described that H_2O_2 has a broad spectrum of biological effects that raise several concerns regarding its safety, even at low concentrations. Among those biological effects is the overproduction of ROS, which results in DNA, lipid and protein oxidation that may eventually lead to cell necrosis or apoptosis mechanisms in oral cells.[7,8]

During oral care procedures, periodontal cells are potentially exposed to H_2O_2 from endogenous and exogenous sources. In periodontitis and peri-implantitis treatments, H_2O_2 is conventionally used as a decontaminating agent. A validated protocol for peri-implantitis is based on a 30-µg/ml solution of H_2O_2 applied on the implant surface.[3,9,10] During these treatments, connective tissue and bone in the peri-implant space are in contact with H_2O_2, thus raising questions on its toxicity and impact on tissue healing.

H_2O_2 is also the main active product in bleaching treatments. It has been demonstrated in vitro that, during internal whitening procedures, H_2O_2 is able to diffuse from the pulp chamber to the root surface.[11,12] This observation is supported by the documented risk of external root resorption associated with non-vital whitening procedures, in which the diffusion of H_2O_2 is considered to be the most probable etiological factor.[13,14] While the potential toxicity of H_2O_2 in external bleaching techniques has been previously assessed in pulp cells, little is known on the risks presented to periodontal cells by external and internal bleaching procedures, which may release up to 0.34 to 4.4 µg/ml of H_2O_2 into the periodontal space after 24 h of exposure.[1,15,16]

These considerations suggest a potential risk to periodontal cells, especially fibroblasts and osteoblasts. Human gingival fibroblasts (HGF) play an important role in the homeostasis of periodontal tissues since fibroblasts have an intrinsic ability to differentiate into other cells.[17] Osteoblasts, on the other hand, are the primary cells responsible for the formation of bone, by synthesizing the components of the extracellular matrix and regulating its mineralization.[18] While the current literature has focused mostly on the effects of H_2O_2 on the dental pulp after bleaching procedures, the effects of H_2O_2 on HGFs and osteoblasts may also be assessed using odontoblasts and dental pulp cells as models.

Previous studies using HGFs have shown that a high concentration of H_2O_2 (15% v/v) can delay cell division and induce alterations in cell morphology.[19] On the other hand, in studies

using osteoblasts, H_2O_2 has been described as inhibiting osteoblastic differentiation and inducing bone loss.[20] Previous reports have focused on high H_2O_2 concentrations, but the response of periodontal tissue cells to low-dose H_2O_2 (lower than 10 µg/ml), as observed in clinical conditions (peri-implantitis treatment, diffusion during whitening procedures), has not been described.

The aim of this study was to evaluate the cytotoxicity of H_2O_2 solutions with different concentrations on periodontal cells, using fibroblast and osteoblast cell lines. The secondary aims were to assess the effect of the H_2O_2 concentration on cell viability and cell morphology in different exposure times. The null hypotheses to be tested were: 1) Exposure to H_2O_2 does not decrease osteoblast viability; 2) Exposure to H_2O_2 does not decrease gingival fibroblast viability; 3) H_2O_2 concentration is not correlated with osteoblast viability; 4) H_2O_2 concentration is not correlated with gingival fibroblast viability.

Material and methods

HGF from an hTERT-immortalized cell line (ABM® Canada) were cultured at 37°C in a 100% humidified atmosphere containing 5% CO_2. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza®, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Biowest®, Nuaillé, France) and 1% penicillin/streptomycin (Biowest®, Nuaillé, France). At 80% confluence, the cells were trypsinated, centrifuged at 800 rpm and resuspended in culture media. Passage 7 was used for all the tests as displaying typical cell behavior for this cell line. All experiments were conducted at 37°C.

Human fetal osteoblasts hFOB 1.19 and a SV40-immortalized cell line (ATCC; American Culture Collection, Manassas, VA, USA) were used. Cells were cultured at 35°C in an atmosphere of 5% CO_2 and 100% humidity, in a culture medium composed of a 1:1 mixture of Ham's F12 Medium (Sigma-Aldrich 51651C, Hampshire, UK.) and DMEM (Biowhittaker, Lonza, Walksville, USA) supplemented with 0.3 mg/ml geneticin – G418 (Roche, Indiana, USA) and 10% FBS (Biowest, Nuaillé, France) until reaching 80% confluence. Cells were then trypsinated, centrifuged at 800 rpm and resuspended in culture media at an adequate density for the assays. Passage 8 was used for all tests, as displaying typical cell behavior for this cell line. All the experiments were conducted at 37ºC, which is the restrictive temperature for hFOB 1.19, since primary cell behavior is exhibited at this temperature, with little cell division and increased differentiation, thus providing a more representative model.

Both cells were cultured, separately, in 96-well plates with a cell density of 5.0 x 10^3 cells/well. After 24 h of incubation, the culture medium was replaced by an H_2O_2 solution for 1 h, 24 h or 72 h.

From the initial 30 µg/ml H_2O_2 solution (Sigma-Aldrich, St. Louis, MO, USA), 16 H_2O_2 solutions were prepared with concentrations ranging between 0.05 µg/ml and 10 µg/ml (0.05 µg/ml; 0.10 µg/ml; 0.15 µg/ml; 0.20 µg/ml; 0.25 µg/ml; 0.30 µg/ml; 0.35 µg/ml; 0.40 µg/ml; 0.50 µg/ml; 0.75 µg/ml; 1.0 µg/ml; 1.5 µg/ml; 3.0 µg/ml; 5.0 µg/ml; 10.0 µg/ml). These concentrations were determined according to the potential H_2O_2 range of exposure

during clinical treatment and, thus, IC50 assessment was not performed. The solutions were diluted with an appropriate medium according to the cell line. Cells were incubated with H_2O_2 solutions for 1 h, 24 h and 72 h, and, in order to simulate clinical conditions, solutions were not renovated, and culture media alone was used as control. Each group had a final sample size of 24 wells, based on triplicate assays of n=8 wells each.

Cytotoxicity was assessed based on cell viability and proliferation assays using a 20% resazurin solution (Sigma-Aldrich, St. Louis, MO, USA), according to the ability of living cells to irreversibly convert a redox dye (resazurin) into a fluorescent final product (resorufin). The conversion rate was measured as the fluorescence intensity after 1 h, 24 h and 72 h of culture. After incubation at 37°C for 3 h, a cell-viability assay was performed following the manufacturer's instructions, and the fluorescence intensity was recorded at excitation/emission wavelengths of 560/590 nm in a luminescence spectrometer (PerkinElmer LS 50B, Waltham MA, USA). The results were expressed in arbitrary units (A.U.). The mean of three consecutive measurements in each well was considered as the final result.

Cytotoxicity was evaluated based on the cell viability relative to the controls as a percentage, according to the following formulas:

$$\text{Viability (\%)} = 100 \times \frac{\textbf{Sample Fluorescente Intensity}}{\textbf{Control Fluorescente Intensity}}$$

$$\textbf{Cytotoxicity (\%) = Viability (\%) control – Viability (\%) test}$$

For cell morphology evaluation, the cells were observed under an inverted microscope with integrated phase-contrast optics (Olympus CK2, Tokyo, Japan). The micrographs were obtained at 250x magnification with a NIKON® D60 (Tokyo, Japan) camera mounted with an appropriate lens adapter, and were analyzed by two independent observers, considering cell density, adhesion and morphology.

Statistical analysis was performed using IBM® SPSS® Statistics 24, Inc., Chicago, IL, EUA. Normality was assessed using the Kolmogorov-Smirnov test. The differences of viability between the H_2O_2 concentrations at different time points were analyzed by one-way analysis of variance (ANOVA), using Tukey's and Dunnet's post hoc tests, as appropriate. Pearson correlation coefficients were used to correlate H_2O_2 concentrations, exposure time and cell viability, with P<0.05 considered as significant. The strength of the resulting correlations was described using established criteria.[21] The results were expressed as the mean ± 95% confidence interval. The significance was set at an alpha value of 0.05 and a beta value of 0.80.

Results

A decrease in cell viability was observed for all H_2O_2 concentrations in both cell lines. In HGF, it was more marked in the 0.05-0.75 µg/ml range at 1 h, 24 h and 72 h (P<0.05), while in osteoblasts it was less pronounced in the lowest H_2O_2 concentration (0.05 µg/ml) with a 23.5% (14.49%; 32.51%) decrease. The lowest viability values occurred at a concentration of 0.35 µg/ml and remained stable up to the highest concentration (10.0 µg/ml).

After 1 h of exposure to H_2O_2, viability decreased to below 50% in HGF (Figure 1A) and around 50% in osteoblasts (Figure 2A). After 24 h of incubation (Figure 1B and 2B), an increase in the number of cells in the control wells was evident. However, in the first tested concentration (0.05 μg/ml), viability significantly decreased to approximately 25% in HGFs (P<0.05) and osteoblasts (P<0.05). After 72 h of H_2O_2 exposure (Figure 1C and 2C), the differences detected between the control and the concentrations studied reflected a decrease of over 80% viability in HGFs and osteoblasts (P<0.05) without statistically significant differences between the different concentrations tested.

A very small but significant negative correlation (r =-0.164, P<0.01) between H_2O_2 concentration and cell viability was reported for osteoblasts. When separate time points were considered, H_2O_2 concentration was significantly correlated with osteoblast viability at 1h of exposure (r=-0.471, P<0.01) and with HGF viability at 72h of exposure (r = -0.12, P<0.05). When cell viability was correlated to exposure time, a significant negative correlation (P<0.01) with moderate effect was detected for both cell lines, with r =-0.573 for osteoblasts and r =-0.403 for HGF, corresponding to lower viability with exposure time increase.

HGF micrographs showed that, as a result of the exposure to increasing H_2O_2 concentrations, cell morphology changes were observed from fusiform to rounded and flattened cells, with various stages of cell detachment in all the exposure times (1 h, 24 h and 72 h). Figures 3 and 4 show the micro-

graphs obtained using phase-contrast microscopy for the osteoblasts and HGF cultures, respectively. In the control wells, normal osteoblast and fibroblast morphologies were observed, showing adhered elongated cells spread with filamentous extensions, which indicate a correct cell attachment. However, with higher H_2O_2 concentrations, alterations in cell morphology were evident, with round and flattened cells, which are compatible with various stages of cell detachment. A perceived increase in the cell density in the control wells, but not in the H_2O_2-exposed wells, was observed from 1 h to 72 h.

Discussion

H_2O_2 has been widely used for a number of preventive and therapeutic applications in Dentistry.[1-6] While generally regarded as a safe agent for these applications, H_2O_2 has a strong oxidant potential. Its effects in teeth and pulpal tissues have been well studied;[22-27] however, an exhaustive screening of the potential toxicity of H_2O_2 within the clinically relevant concentration range in periodontal cells has not yet been performed. In the present work, we evaluated the in vitro effects of H_2O_2 exposure on the cell viability of osteoblasts and fibroblasts. Cytotoxicity was classified, in accordance with the ISO standard 10993-5:2009, as non-cytotoxic at > 80% cell viability; slightly cytotoxic at 80-50% cell viability;
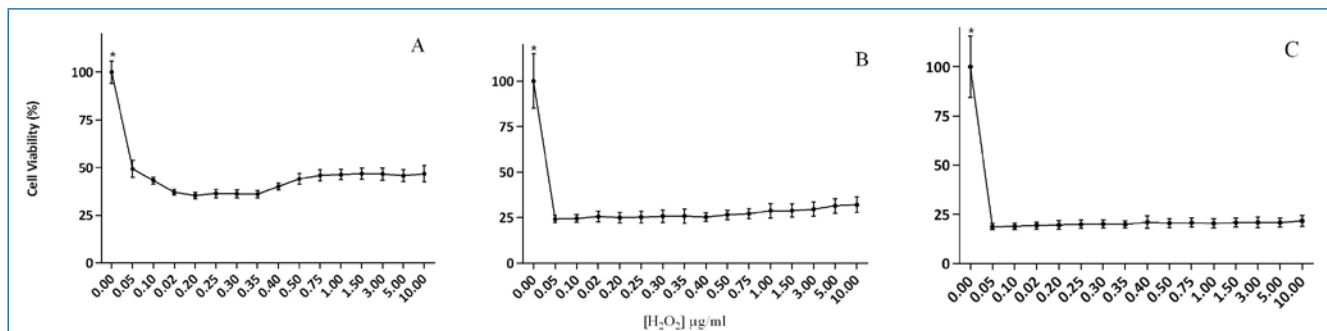

Figure 1. Line charts showing the mean relative percentage of HGF viability comparing to control wells, for the three time points assessed (A – 1 h; B – 24 h; C – 72 h). Control values correspond to the 0.00 μg/ml H_2O_2 point. Error bars show 95% confidence interval limits. * P<0.001, ANOVA One-way, Dunnet's post hoc. n=24
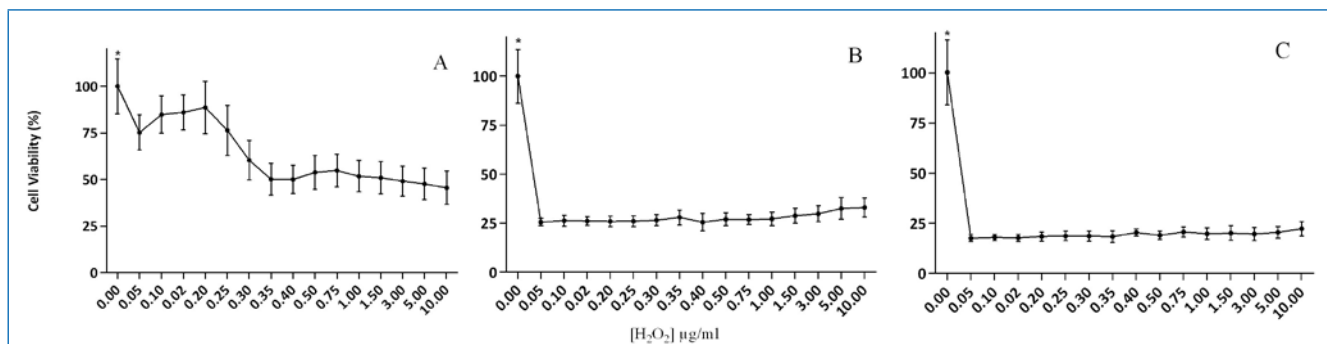

Figure 2. Line charts showing the mean relative percentage of hFOB viability comparing to control wells, for the three time-points assessed (A – 1 h; B – 24 h; C – 72 h). Control values correspond to the 0.00 μg/ml H_2O_2 point. Error bars show 95% confidence interval limits. * P<0.001, ANOVA One-way Dunnet's post hoc. n=24
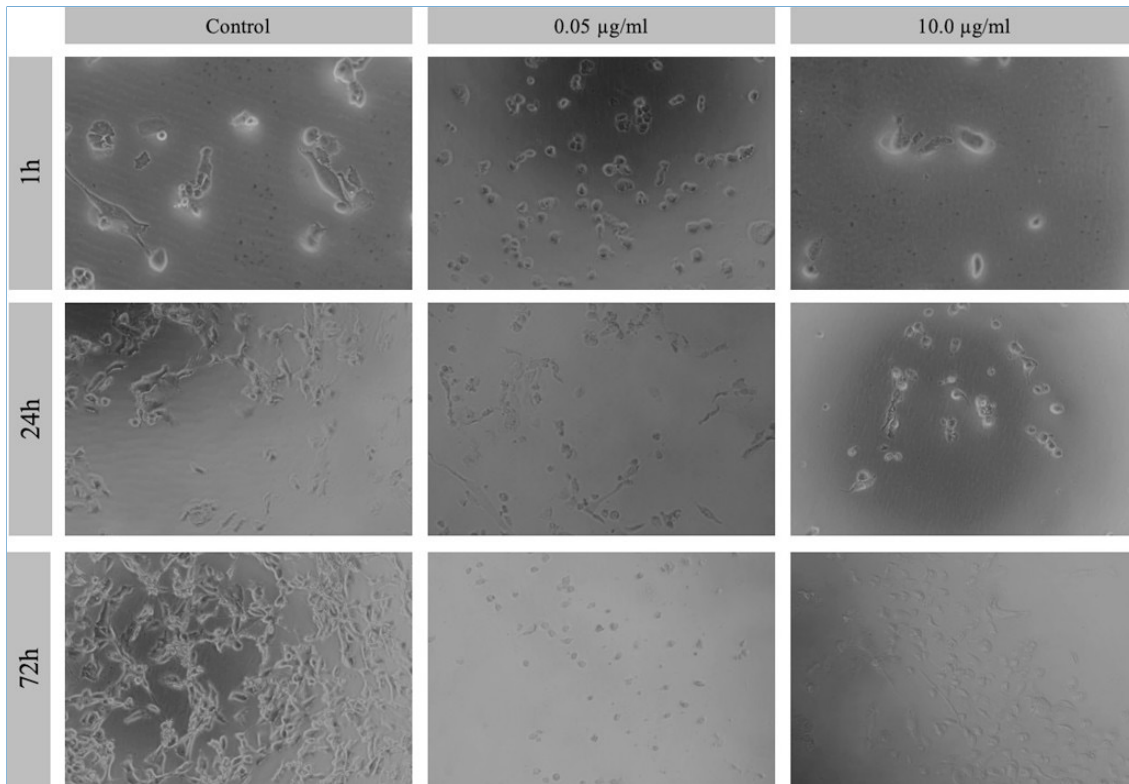
Figure 3. hFOB morphology after H$_2$O$_2$ exposure for 1 h, 24 h and 72 h using 0.00, 0.15 and 10 µg/ml H2O2 solutions. Phase-contrast micrographs were obtained at 25x magnification.

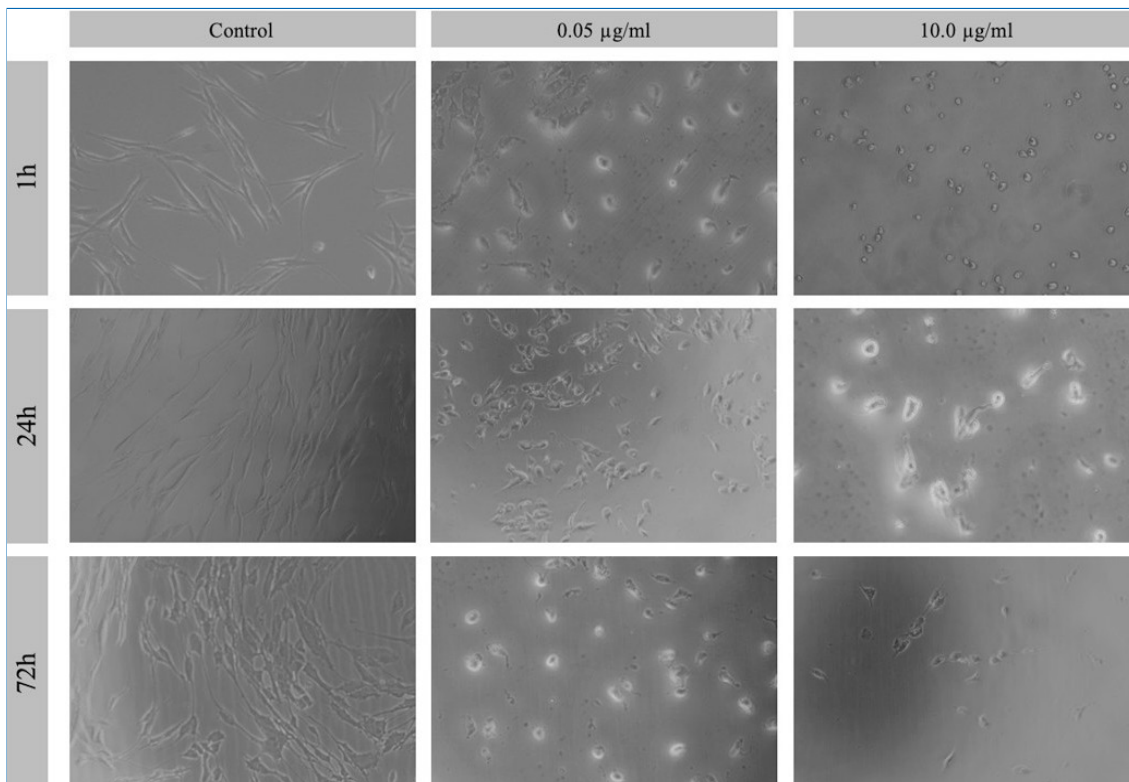**Figure 4.** HGF morphology after H$_2$O$_2$ exposure for 1 h, 24 h and 72 h using 0.00, 0.15 and 10 µg/ml H$_2$O$_2$ solutions. Phase-contrast micrographs were obtained at 25x magnification.

moderately cytotoxic at 50-30% cell viability; and severely cytotoxic at <30% cell viability (ISO 10993-5:2009).

The results of our study showed, for the first time, that, in both the osteoblast and gingival fibroblast cell lines tested, exposure to a 0.05-µg/ml solution of H_2O_2 (10-fold lower than the reportedly safe concentration of 0.68 µg/ml) elicited a cell-viability decrease of up to 50% (P<0.05), thus rejecting the first two null hypotheses. This effect was more pronounced with longer incubation times. According to some authors, exposure to H_2O_2 concentrations lower than 0.68 µg/ml is considered safe regardless of the cell type, resulting in limited effects in many animal cells.[28] A significant impact on the cell viability would only be expected in exposures to concentrations over 1.7 µg/ml.[7] As stated before, our results contradict this assumption, suggesting that H_2O_2 toxicity for periodontal cells might be underestimated. This divergence could be explained by the use of distinct and more-resistant cell lines in these previous studies, or by other differences in experimental design, including broader concentration ranges or shorter exposure times.

Different toxicity effects were observed in the two cell types used in this assay. After the exposure of HGF to the lowest-concentration H_2O_2 solution (0.05 µg/ml), mean viability decreases of 50% – slightly cytotoxic – (1 h) and 80% – severely cytotoxic (24 and 72 h) – were observed. In previous studies with human fibroblast cell lines, concentrations of H_2O_2 ranging between 1.7 and 42.5 µg/ml resulted in a proliferation decrease, morphology alterations and cell death.[(29–34)] However, these studies were based on shorter exposure times (90 seconds), while, in our study, the shortest exposure was 1 h, and most were performed in types of fibroblasts other that gingival fibroblasts. Our results presented a similar decrease in cell viability at a 100-fold lower concentration of H_2O_2 for the HGF cell line (0.05 µg/ml), thus suggesting that the tolerance threshold for gingival fibroblasts may be lower than previously reported, considering exposure times comprised between 1 h and 72 h.[1,19] However, this effect might be specifically related to the cell line used in this study, which may be more sensitive to H_2O_2 exposure than other cell lines and primary cells.

Higher resistance to H_2O_2 after 1 h of exposure, with non-cytotoxic to slightly cytotoxic effects (25-50% viability decrease) but similar severely cytotoxic effects in longer exposures, was observed in hFOBs. A previous study with hFOB cells reported a safe threshold of a 24-h exposure to 3.4 µg/ml H_2O_2.[35] Our results suggest that this limit is potentially below a concentration of 0.05 µg/ml of H_2O_2 in osteoblasts, particularly after 24 h of exposure. This difference may result from the use of a different method for viability assessment, as the authors of the previous study used formazan reduction. While these methods are generally considered equivalent, some reports state different performances of resazurin and formazan methods, with resazurin generally causing higher sensitivity.[36,37]

A small negative overall correlation between H_2O_2 concentration and cell viability was detected in osteoblasts, specifically at 1 h of exposure (moderate effect). It can be therefore speculated that osteoblasts may have a dose-dependent response to short-term H_2O_2 exposures. The results also suggest that, within this range of concentrations, there is a dose-dependent response in the viability of HGFs at 72 h of exposure (small effect), thus rejecting the third and fourth null hypotheses.

An important limitation of our study is that only one cell line was used for each cell type. Thus, further studies using different cell lines and primary osteoblasts or fibroblasts should be undertaken to confirm these findings. IC50 was not calculated because the scope of this study was to evaluate the effects of clinically relevant concentrations on cell viability. However, further studies should characterize the toxicological profile of H_2O_2 exposure in these cell types in order to fully elucidate on the safe threshold concentration of this agent, as well as its impacts on cell function and differentiation. Finally, this was an *in vitro* study, with the inherent limitations that turn direct extrapolation to in vivo impossible, namely the absence of antioxidant mechanisms, or the rate of H_2O_2 degradation.

Within the limits of this study, the results suggest that H_2O_2 exposure in the range of concentrations used in peri-implantitis debridement and in internal bleaching procedures, potentially reaching the periodontal space, may severely decrease periodontal cell viability. These findings raise new questions regarding H_2O_2 safety for those applications. Whether this cytotoxicity happens *in vivo* and to what extent H_2O_2 exposure impacts cell function is yet to determine. These observations need to be integrated into more complex models to clarify the role of endogenous and exogenous antioxidant systems in reverting these alterations.

Conclusions

The results of this study suggest that direct exposure to H_2O_2 within clinically relevant concentrations resulted in moderate to severe cytotoxic effects in periodontal cells – osteoblasts and gingival fibroblasts in vitro. A small negative correlation between H_2O_2 concentration and cell viability was observed in osteoblasts but not in fibroblasts.

Further studies are necessary to determine the exact safety threshold of the direct application of H_2O_2 on these cells, as well as its impact on cell differentiation and function.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors have no conflicts of interest to declare.

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