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In vitro evaluation of experimental light activated gels for tooth bleaching – the response of L-929 cells, 3T3 cells and gingival fibroblasts†

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Dental bleaching is an important part of aesthetic dentistry. Various strategies have been created to enhance the bleaching efficacy. As one such strategy, light-activated nanoparticles that enable localized generation of reactive oxygen species have been developed. Here, we evaluated the cellular response to experimental hydrogels containing these materials in *in vitro* models. L-929 cells, 3T3 cells, and gingival fibroblasts were exposed to the hydrogels at 50%, 10%, 2%, 0.4%, 0.08%, 0.016%, and 0.0032%. The hydrogels contained TiO_2 nanoparticles, TiO_2/Ag nanoparticles, hydrogen peroxide (6% hydrogen peroxide), or no added component and were tested with and without exposure to blue LED light. Cells were exposed to gels for 24 h or for 30 min. The latter case mimics the clinical situation of a short bleaching gel exposure. Metabolic activity and cell viability were evaluated with MTT and neutral red assays, respectively. We found a dose-dependent reduction of formazan formation and neutral red staining with hydrogels containing TiO_2 nanoparticles or TiO_2/Ag nanoparticles. The strongest reduction, which was not dose-dependent in the evaluated concentrations, was found for the hydrogel containing hydrogen peroxide. Hydrogels with TiO_2 nanoparticles or TiO_2/Ag nanoparticles showed a similar response to hydrogel without particles. When the gels were removed by rinsing after 30 min of exposure without light illumination, gels based on TiO_2/Ag nanoparticles showed a stronger reduction of formazan formation and neutral red staining than gels based on TiO_2 particles. Similar effects were found with illuminated hydrogels. Exposure of cells for 30 min under illumination and consequent rinsing off the gel also showed that Ag-containing particles can have a higher impact on the metabolic activity and viability than particles from TiO_2 alone. Overall our results show that experimental bleaching gels containing TiO_2 or TiO_2/Ag nanoparticles show less cytotoxicity than hydrogen peroxide-containing gels. When gels were removed, gels containing TiO_2/Ag particles exhibited a stronger reduction of metabolic activity and viability than the gels containing TiO_2.

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Introduction

Aesthetics are highly valued in our society and are related to a high and increased demand from patients for teeth whitening or dental bleaching.[1–4] This increasing demand stimulates the development of dental bleaching products and systems. These systems include in-office approaches and home-bleaching kits.[5–11] The majority of the dental bleaching products are based on hydrogen peroxide or other reactive oxygen species such as carbamide peroxide which oxidizes discolorations, or chromophores, inside the teeth.[4,12–14] Carbamide peroxide is an agent composed by hydrogen peroxide and urea.

Currently, the use of hydrogen peroxide prevails in dental practice. Application of dental bleaching products without pro-

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fessional supervision of the dentist may result in tooth sensitivity,[13,15–19] gingival irritation,[13] alterations in dental structures like microhardness[20–22] and carcinogenic effects.[19,23] Hydrogen peroxide, even in low concentrations, is also considered to be toxic for oral soft tissues.[24] In this respect, the European Commission has issued the Council Directive 2011/84/EU regarding tooth whitening products in which these concerns about safety and adverse effects of high concentrations of hydrogen peroxide and hydrogen peroxide releasing compounds are reflected.[25] Therefore, it is stated that the application of hydrogen peroxide just up to a maximum of 6% may be safe under well-defined conditions when applied following the relevant treatment guidelines.[25] To improve bleaching efficiency while avoiding the use of hydrogen peroxide above 6%, research aims to develop novel bleaching technologies.

One of these approaches is the use of chairside bleaching lamps and laser activation to boost the bleaching efficacy.[9,26] The activation of photosensitizers or photocatalysts with the right wavelength of light, for example, can lead to heating of the bleaching gel or the production of reactive oxygen species. Light, especially that from lasers such as the KTP and Argon laser, can furthermore directly oxidize and bleach discolorations in teeth. Typically, however, the presence of hydrogen peroxide is necessary to achieve adequate whitening efficacies.[9,26]

Nanoparticles of a variety of materials are used in a wide range of applications in medicine and dentistry. The use of nanomaterials for light-based bleaching,[27] and for bactericidal effects[28–33] are two very promising examples. For bleaching, photocatalytic nanoparticles are used. These are typically semiconducting nanoparticles such as titanium dioxide (TiO_2) that have a bandgap in the UV or visible light.[34] When activated with the correct wavelength, an electron–hole pair will form and will initiate reduction and oxidation reactions at the surface of the particles. Adequate redox potential then allows the *in situ* generation of reactive oxygen species such as hydrogen peroxide and hydroxyl radicals.[35–37] Based on preliminary data from Coto *et al.* the here proposed experimental gels are feasible for bleaching purposes.[34] TiO_2 nanoparticles can be efficiently activated with UV light, whereas TiO_2/Ag nanoparticles can be activated efficiently with visible light including blue light.[34] Thus gels based on TiO_2/Ag nanoparticles could be a feasible approach for dental bleaching. Studies, however, which focus on the safety of nanoparticle-based bleaching gels, even at the *in vitro* level, are not available.

The aim of the present study, therefore, is to examine the effects of experimental bleaching gels containing either TiO_2 or TiO_2/Ag nanoparticles on the metabolic activity and viability of L-929 cells and 3T3 cells which represent the standard test murine cell lines for cytotoxicity and phototoxicity. Furthermore, as differences between these cell lines and primary human cells have been reported, the impact on human gingival fibroblasts (GF) was also evaluated.[38]

Hydrogen peroxide serves as a "positive" treatment group to assess toxicity, and the empty gel functions as a "negative" treatment group so that the effects of the TiO_2 or TiO_2/Ag nanoparticles can be distinguished from the effects of the carrier Gel. Cytotoxic effects of both the nanomaterials with ("illuminated") and without ("W/O") light were assessed.[34] The experiments were performed in a short-term exposure setting, which reflects the situation of bleaching (30 min group) and a long-term exposure setting which reflects the situation of insufficient cleaning (24 h group) and follows the standard timeline of cytotoxic testing (Fig. 1).

Experimental

Preparation of the experimental bleaching gels

The bleaching gels were provided by the Department of Material Sciences and Metallurgy, Cambridge University, Cambridge, United Kingdom.

The basic hydrogel (Gel) consist of 5 mg ml^{-1} Carbopol (Lubrizol, Wickliffe, OH, USA), 5% (v/v) propylene glycol (Sigma-Aldrich, St Louis, MO, USA), 5% (v/v) polyethylene glycol (Sigma-Aldrich), 1% (v/v) tween-20 (Sigma-Aldrich), and sodium dodecyl sulphate in water. Finally the pH was adjusted to 9 with sodium hydroxide.

The gel containing H_2O_2 (Gel-H_2O_2) was generated by supplementing the basic hydrogel with 6% hydrogen peroxide (Sigma-Aldrich) added just before use.

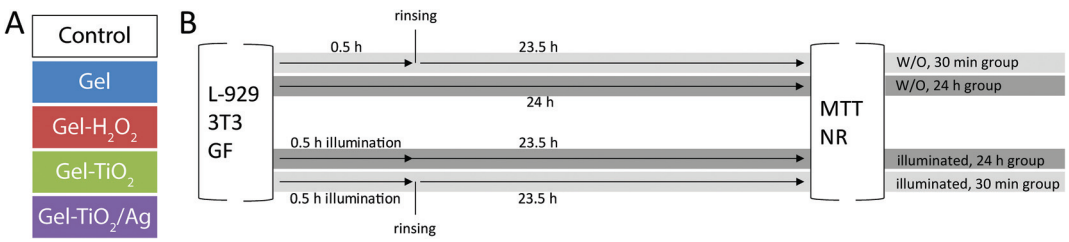

Fig. 1 Schematic drawing of the experimental protocol. L-929 cells, 3T3 cells, and gingival fibroblasts (GF) were treated with experimental bleaching gels with and without TiO_2 or TiO_2/Ag and gel with 6% hydrogen peroxide. Cells without gel treatment served as control. A representation of the experimental groups with the gels is given in (A). The two grey shades indicates the cells were either treated for 24 h with the gels (24 h group) or for 30 min followed by a rinsing step and further 23 h and 30 min incubation (30 min group), both without illumination ("W/O"). In a separate set of experiments, illumination was performed for the first 30 min of exposure to the gels ("illuminated"). All four gels were used in concentrations of 50%, 10%, 2%, 0.4%, 0.08%, 0.016%, and 0.0032%. A representation of the experimental protocol is given in (B).

For the experimental bleaching gels (Gel-TiO_2, Gel-TiO_2/Ag) 10 mg ml^{-1} TiO_2 or TiO_2/Ag nanoparticles were added to the basic hydrogel. The nanoparticles were manufactured, as described by Coto *et al.* 2017.[34] In brief, TiO_2 anatase powder (Sigma-Aldrich) with an average particle size of 25 nm was exposed to silver nitrate in deionised water (Sigma-Aldrich) at 3 mol%. Then a reducing agent was added and stirred at room temperature for 10 min followed by incubation at 85 °C until the solvent was evaporated. The powder was ground and incubated for 30 min at 300 °C, followed by rapid cooling.

Preparation of GF

After informed consent was given by the donors, GF were isolated from extracted third molars (1065/2013, Ethics Committee of the Medical University of Vienna, Vienna, Austria) following an established protocol.[38]

Cell culture

L-929 cells (American Type Culture Collection, Manassas, VA, USA), 3T3 cells (kindly provided by Pluchino *et al.* from the Department of Clinical Neurosciences, Cambridge University, Cambridge, United Kingdom) and human gingival fibroblasts (GF) were cultivated in cell culture medium (DMEM (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS; LifeTech, Vienna, Austria) and antibiotics), and were seeded in 96 well plates at 30 000 cells per cm^2. All experiments were performed at a temperature of 37 °C, 95% humidified atmosphere and 5% CO_2 concentration following previously published protocols with minor modifications.[38,39] Cells were stimulated with the gels (Gel, gel with 6% hydrogen peroxide, gel with TiO_2 nanoparticles, and gel with TiO_2/Ag nanoparticles) diluted in cell culture medium at a concentration of 50%, 10%, 2%, 0.4%, 0.08%, 0.016%, and 0.0032%. Respectively, gel with hydrogen peroxide resulted in a final hydrogen peroxide concentration of 3%, 0.6%, 0.12%, 0.024%, 0.0048%, 0.00096%, and 0.000192%. Pilot experiments showed that direct application of 100% gel onto cells was not technically feasible. Cells cultured without gels served as control. In one set of experiments, cells were incubated with the gels for one day. In a separate set of experiments, the gels were rinsed off with phosphate buffered saline (PBS) after 30 min of treatment and culture medium was refreshed. Cells were then cultured for one day. At the end of the culture period, cells were subjected to MTT and neutral red assays (see Fig. 1).

Light source

All experiments were performed with (Marked as "illuminated") and without illumination (marked as "W/O") for first 30 min of gel incubation with a blue LED light using the device MiniSun High Powered 50 W IP65 Blue LED Floodlight (100 V–240 V rating input, 50 W, blue colour; Mos Lighting Technology Co., Ltd, Shen Zhen, China). Illuminated specimens were positioned in an illumination chamber with a distance of 115 mm to the light source. For a schematic overview, see Fig. 1.

MTT assay

To evaluate the impact of the gels on the metabolic activity of the cells, MTT assays were used as previously published.[38] Cells were incubated with MTT at 1 mg ml^{-1} (Sigma-Aldrich) for the last 2 h. Formazan crystals were then solubilized with dimethyl sulfoxide. Absorbance at 550 nm was measured using the Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Neutral red assay

To measure the impact on cell viability, cells were incubated with neutral red (Sigma-Aldrich) as previously described.[40] Neutral red was used at 50 μg ml^{-1} in DMEM with antibiotics. After 2 h of incubation cells were rinsed, and neutral red was solubilized with a freshly prepared solvent containing 49 parts H_2O, 50 parts ethanol, and 1 part acetic acid. Absorbance at 540 nm was measured using the Synergy™ HTX Multi-Mode Microplate Reader (BioTek).

Statistical analysis

IBM SPSS Statistics Version 24 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis. ANOVA *post hoc* Dunnett-*T* test were performed. $p < 0.05$ was assigned as significant.

Results

The impact of experimental bleaching gels on L-929 cells without illumination

Hydrogen peroxide gels almost completely suppressed metabolic activity below 20% of control (Fig. 2A and B) and reduced cell viability (Fig. 2C and D) below 60%, for both 24 h and 30 min treatment protocols. The only exception was found in the 30 min group where viability was reduced upon treatment with hydrogen peroxide gels at a gel concentration of 0.0032% (Fig. 2D).

In the 24 h group all other gel formulations, including empty, TiO_2, and TiO_2/Ag, showed a dose–response for metabolic activity reduction (Fig. 2A) with levels of 11%, 21%, and 23%, respectively, compared to the control at the maximal dosage (MD) assessed which was 50% (v/v) of the gels. Also cell viability (Fig. 2C) of L-929 cells was reduced dose-dependently with levels of 32%, 28%, and 52% compared to the control at MD by gel alone and gels containing TiO_2 and TiO_2/Ag, respectively.

For the 30 min treatment (30 min group) cell metabolic activity was dose-dependently reduced by gel alone and gels containing TiO_2 and TiO_2/Ag with levels of 26%, 57%, and 15% of control at MD, respectively (Fig. 2B). Cell viability (Fig. 2D) of L-929 was reduced dose-dependently with levels of 55%, 86%, 39% compared to the control at MD by gel alone and gels containing TiO_2 and TiO_2/Ag, respectively.

To summarize the results with regard to the cytotoxicity capacity we found the following sequence: Gel-H_2O_2 > Gel-TiO_2/Ag > Gel and Gel-TiO_2.

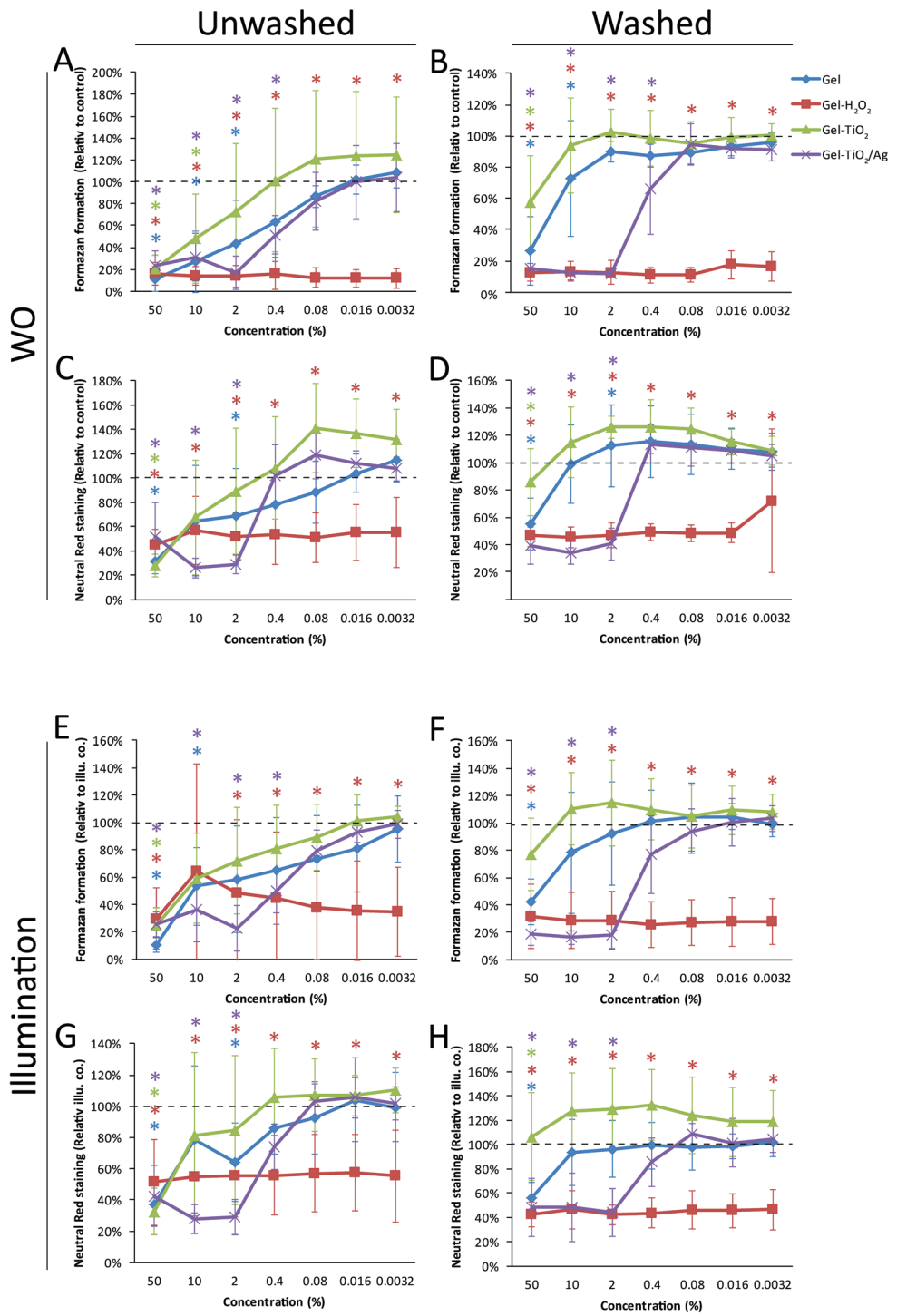

Fig. 2 The impact of experimental bleaching gels on L-929 cells without and with illumination. Without illumination, treatment of L-929 cells with the experimental bleaching gels (*i.e.* with TiO_2 or TiO_2/Ag) reduced metabolic activity (A, B) and cell viability (C, D) dose-dependently. Gel with 6% hydrogen peroxide and gel without supplementation served as controls. Cells were either exposed to the gels for 24 h (24 h group: A, C) or were rinsed after 30 min of exposure and cultured for further 23 h and 30 min (30 min group: B, D). Data are presented as mean ± standard deviation relative to untreated cells (control) (N = 9). With illumination, treatment of L-929 cells with the experimental bleaching gels (*i.e.* with TiO_2 or TiO_2/Ag) reduced metabolic activity (E, F) and cell viability (G, H) dose-dependently. Gel with 6% hydrogen peroxide and gel without supplementation served as illuminated controls. Cells were either exposed to the gels for 24 h (24 h group: E, G) or were rinsed after 30 min of exposure and cultured for further 23 h and 30 min (30 min group: F, H). Data are presented as mean ± standard deviation relative to cells which had also been exposed to illumination (illuminated control, illu. co.) (N = 9) *p < 0.05. The colour of the star labels statistical significance of the respective test group *vs.* the control (with or without illumination). The colour of the error bars indicates the standard deviation of the respective group.

The impact of experimental bleaching gels on L-929 cells after illumination

Metabolic activity and viability levels of L-929 exposed to illumination were 83% and 95% of controls for the 24 h group and 77% and 91% for the 30 min group (Table 1). The gel containing hydrogen peroxide showed the strongest reduction in metabolic activity and cell viability leading to levels below 60% of illuminated control with no dose–response for both, the 24 h and 30 min treatment protocol (Fig. 2E–H). Overall the toxicity profiles for hydrogen peroxide after illumination are similar to those of L-929 without illumination.

In the group treated for 24 h (24 h group) with the empty gel, TiO_2-containing gel, and TiO_2/Ag-containing gel a dose–response for metabolic activity (Fig. 2E) with levels of 11%, 24%, and 25% compared to the illuminated control at MD, respectively, was observed. Also cell viability of L-929 cells was reduced dose-dependently (Fig. 2G) with levels of 37%, 33%, and 42% compared to the illuminated control at MD, respectively.

In the 30 min group cell metabolic activity was decreased dose-dependently by gel alone and gels containing TiO_2 and TiO_2/Ag with levels of 42%, 77%, and 19% of illuminated control at MD, respectively (Fig. 2F). Viability was reduced dose-dependently with levels of 56%, 107%, and 48% compared to the illuminated control at MD, respectively (Fig. 2H).

In summary we found the following sequence with regard to the cytotoxicity capacity: Gel-H_2O_2 > Gel-TiO_2/Ag > Gel and Gel-TiO_2. The negative impact on cells by hydrogen peroxide-containing gels was not dominantly modulated by the reduction of exposure time (Fig. 2A–D *versus* E, F).

The impact of experimental bleaching gels on 3T3 cells without illumination

In the 24 h group exposure of 3T3 cells to the experimental bleaching gels dose-dependently decreased metabolic activity (Fig. 3A–D). On 3T3 cells, different cytotoxic profiles were observed with regard to cell viability. Gel containing H_2O_2 did not show a dose–response and metabolic activity (Fig. 3A) as well as cell viability (Fig. 3C) were decreased. In the group treated for 24 h (24 h group) when compared to each other Gel, Gel-TiO_2, and Gel-TiO_2/Ag showed a similar dose–response on 3T3 cells. Metabolic activity reached levels of 23%, 18%, and 37% upon exposure to Gel, Gel-TiO_2, and Gel-TiO_2/Ag at MD (Fig. 3A). Cell viability was reduced to 27%, 28%, and 25% (Fig. 3C). The 30 min protocol (30 min group) did not reduce the toxicity for gels with or without nanoparticles (Fig. 3B and D). Overall, 3T3 showed a strong variation in the response to the gels.

The impact of experimental bleaching gels on 3T3 cells after illumination

Metabolic activity and viability levels of 3T3 exposed to illumination were 26% and 61% of controls for the 24 h group and 101% and 78% for the 30 min group (Table 1). Incubation of 3T3 cells with the experimental bleaching gels and light exposure showed high variations in metabolic activity and cell viability (Fig. 3EH). Also, hydrogen peroxide-containing gel showed high variations in this setting. No dose–response for hydrogen peroxide can be found. In the group treated for 24 h (24 h group) metabolic activity reached levels of 57%, 84%, and 195% upon exposure to Gel, Gel-TiO_2, and Gel-TiO_2/Ag at MD (Fig. 3E). Cell viability at MD was 66%, 88%, and 103% compared to the illuminated control when exposed to Gel, Gel-TiO_2, and Gel-TiO_2/Ag (Fig. 3G). Short time exposure for 30 min (30 min group) with illumination showed similar effects (Fig. 3F and H). The profiles were similar for all gels compared without illumination (Fig. 3B, D, F and H). Also when exposed to illumination 3T3 showed a strong variation in their response to the gels.

The impact of experimental bleaching gels on GF cells without illumination

Hydrogen peroxide-containing gel decreased the metabolic activity (Fig. 4A and B) and cell viability (Fig. 4C and D) of GF below 40%. No dose–response was observed for gel with hydrogen peroxide. Exceptions were found in the 30 min group where metabolic activity of GF were above 40% for the gel concentration of 0.0032% and for viability for the gel concentration of 0.016% and 0.0032% (Fig. 4B and D).

For GF in the 24 h group all other gels, including gel without particles, gel with TiO_2, and gel with TiO_2/Ag, showed a dose–response for metabolic activity (Fig. 4A) with levels of 13%, 17%, and 46% compared to the control at MD, respectively. Also viability (Fig. 4C) of GF was reduced dose-dependently with levels of 18%, 22%, and 43% compared to the control at MD, respectively.

GF in the 30 min treatment (30 min group) showed a dose-dependent reduction of metabolic activity by gel alone and

Table 1 Metabolic activity and viability of cells exposed to illumination relative to cells without illumination

	24 h group		30 min group	
	Metabolic activity	Viability	Metabolic activity	Viability
L-929	83% ± 39%*	95% ± 39%	77% ± 21%*	91% ± 22%
3T3	26% ± 14%*	61% ± 30%*	101% ± 64%	78% ± 22%*
GF	104% ± 17%	91% ± 14%*	111% ± 27%*	106% ± 24%

Here we show metabolic activity and viability of L-929 cells, 3T3 cells and fibroblasts from the gingiva (GF) exposed to illumination in comparison to the cells without illumination (100%). Data are presented as mean ± standard deviation relative to untreated cells (control) ($N = 9$)
*$p < 0.05$.

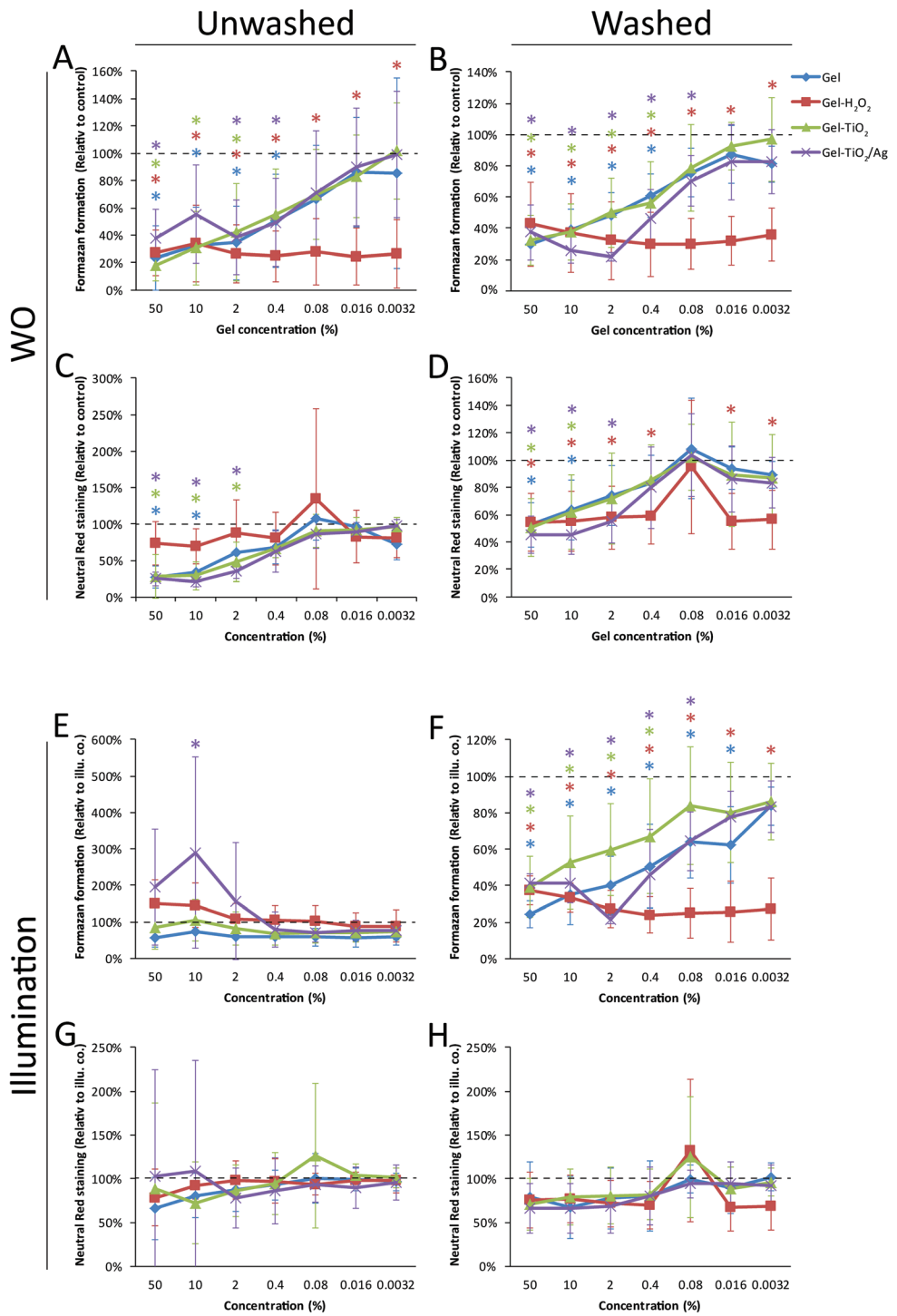

Fig. 3 The impact of experimental bleaching gels on 3T3 cells without and with illumination. Without illumination, treatment of 3T3 cells with the experimental bleaching gels (*i.e.* with TiO_2 or TiO_2/Ag) reduced metabolic activity (A, B) and cell viability (C, D) dose-dependently. Gel with 6% hydrogen peroxide and gel without supplementation served as controls. Cells were either exposed to the gels for 24 h (24 h group: A, C) or were rinsed after 30 min of exposure and cultured for further 23 h and 30 min (30 min group: B, D). Data are presented as mean ± standard deviation relative to untreated cells (control) (N = 9). With illumination, treatment of 3T3 cells with the experimental bleaching gels (*i.e.* with TiO_2 or TiO_2/Ag) reduced metabolic activity (E, F) and cell viability (G, H) dose-dependently. Gel with 6% hydrogen peroxide and gel without supplementation served as illuminated controls. Cells were either exposed to the gels for 24 h (24 h group: E, G) or were rinsed after 30 min of exposure and cultured for further 23 h and 30 min (30 min group: F, H). Data are presented as mean ± standard deviation relative to cells which had also been exposed to illumination (illuminated control, illu. Co.) (N = 9) *p < 0.05. The colour of the star labels statistical significance of the respective test group *vs.* the control (with or without illumination). The colour of the error bars indicates the standard deviation of the respective group.

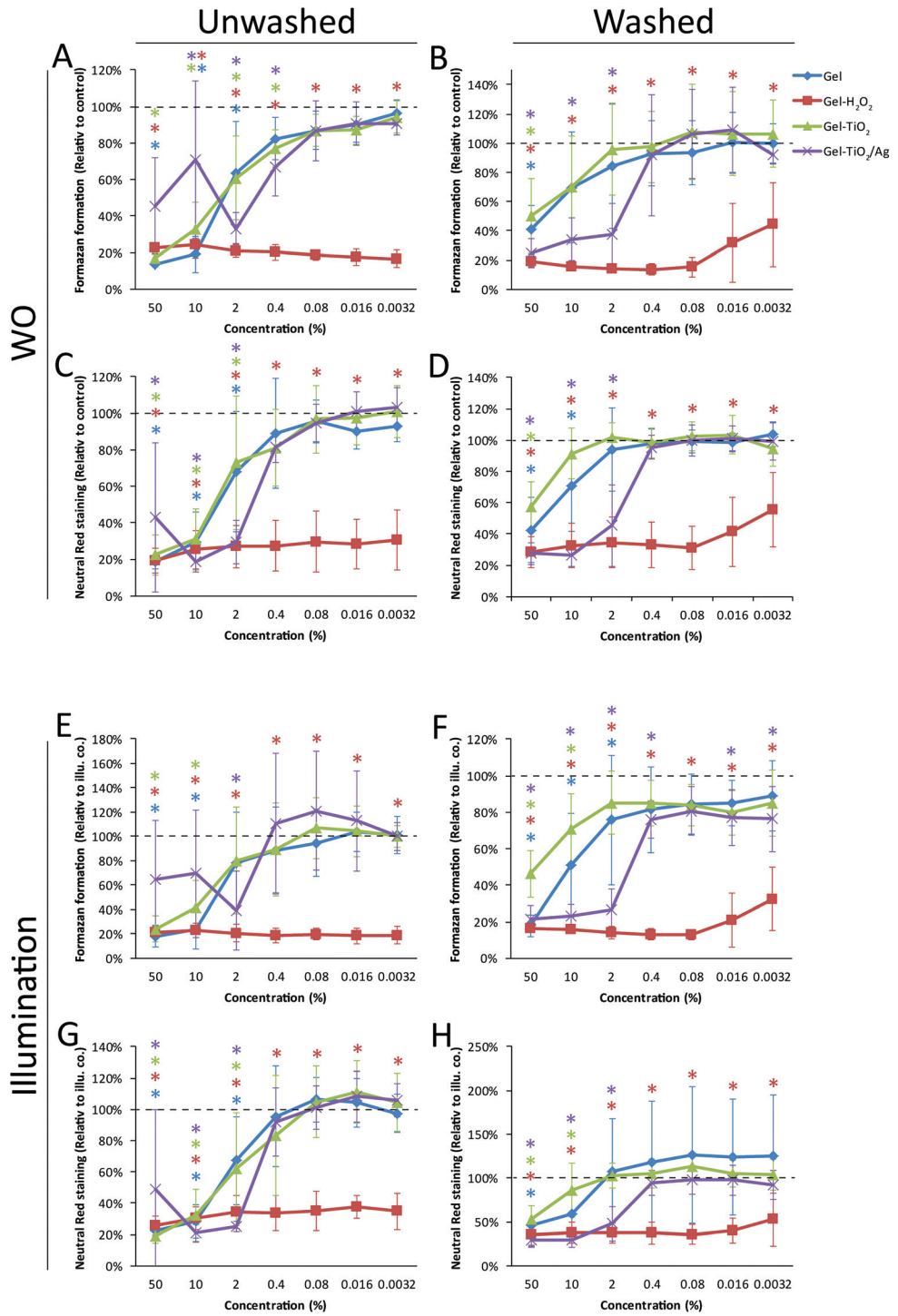

Fig. 4 The impact of experimental bleaching gels on gingival fibroblasts without and with illumination. Without illumination, treatment of gingival fibroblasts with the experimental bleaching gels (*i.e.* with TiO_2 or TiO_2/Ag) reduced metabolic activity (A, B) and cell viability (C, D) dose-dependently. Gel with 6% hydrogen peroxide and gel without supplementation served as controls. Cells were either exposed to the gels for 24 h (24 h group: A, C) or were rinsed after 30 min of exposure and cultured for further 23 h and 30 min (30 min group: B, D). Data are presented as mean ± standard deviation relative to untreated cells (control) (N = 9). With illumination, treatment of gingival fibroblasts with the experimental bleaching gels (*i.e.* with TiO_2 or TiO_2/Ag) reduced metabolic activity (E, F) and cell viability (G, H) dose-dependently. Gel with 6% hydrogen peroxide and gel without supplementation served as illuminated controls. Cells were either exposed to the gels for 24 h (24 h group: E, G) or were rinsed after 30 min of exposure and cultured for further 23 h and 30 min (30 min group: F, H). Data are presented as mean ± standard deviation relative to cells which had also been exposed to illumination (illuminated control, illu. co.) (N = 9) *p < 0.05. The colour of the star labels statistical significance of the respective test group *vs.* the control (with or without illumination). The colour of the error bars indicates the standard deviation of the respective group.

gels containing TiO_2 and TiO_2/Ag with levels of 41%, 50%, and 25% of control at MD, respectively (Fig. 4B). GF viability was decreased dose-dependently with levels of 42%, 57%, and 27% for gel alone and gels containing TiO_2 and TiO_2/Ag compared to the control at MD, respectively (Fig. 4D).

TiO_2 nanoparticle-containing gel showed a similar dose-dependent reduction as TiO_2/Ag nanoparticle-containing gels. Gel without supplementations had the lowest impact on metabolic activity and viability. Short time exposure revealed a stronger reduction of cell viability by TiO_2/Ag nanoparticle-containing gel than gel with TiO_2 nanoparticles or gel without supplementation. For these gels, short-term exposure reduced the impact on the cells. The negative impact on cells by hydrogen peroxide-containing gels was not dominantly modulated by the reduction of exposure time.

To summarize the results with regard to the cytotoxic capacity we found the following sequence: Gel-H_2O_2 > Gel-TiO_2/Ag > Gel and Gel-TiO_2.

The impact of experimental bleaching gels on GF cells after illumination

Metabolic activity and viability levels of GF exposed to illumination were 104% and 91% of controls for the 24 h group and 111% and 106% for the 30 min group (Table 1). Hydrogen peroxide-containing gel strongly decreased metabolic activity and cell viability of GF below 50% of illuminated control, but no dose–response was found (Fig. 4E–H). The only exception was found in the 30 min group where viability was reduced upon treatment with hydrogen peroxide gels at a concentration of 0.0032% (Fig. 4H). Hydrogen peroxide toxicity profiles were similar to GF profiles without illumination.

In the 24 h group empty, TiO_2, and TiO_2/Ag-containing gels induced a dose-depending reduction of both metabolic activity in GF (Fig. 4E) with levels of 18%, 24%, and 64% compared to the illuminated control at MD, respectively. Also viability of GF (Fig. 4G) was reduced dose-dependently with levels of 23%, 19%, and 49% compared to the illuminated control at MD, respectively.

GF treated for 30 min (30 min group) with gel alone and gels containing TiO_2 and TiO_2/Ag revealed reduced metabolic activity with levels of 18%, 46%, and 22% of illuminated control at MD, respectively (Fig. 4F). Viability of GF was reduced dose-dependently with levels of 45%, 53%, and 29% compared to the illuminated control at MD, respectively (Fig. 4H).

The negative impact on cells by hydrogen peroxide-containing gels was not dominantly modulated by the reduction of exposure time.

To summarize the results with regard to the cytotoxicity capacity we found the following sequence: Gel-H_2O_2 > Gel-TiO_2/Ag > Gel and Gel-TiO_2.

Discussion

In dental tooth bleaching, we face the need to reduce the concentration of hydrogen peroxide for safety purposes, while still trying to facilitate adequate bleaching efficiencies. Reduction of the hydrogen peroxide concentration in combination with decreased bleaching time can reduce the risk of potential adverse effects such as cytotoxic effects of the bleaching agents, due to hydrogen peroxide, but also diminishes the aesthetic outcome.[41] While there is evidence that enzymes reduce the toxicity of hydrogen peroxide *in vivo*, some adverse effects remain.[42,43] Therefore, more effective approaches for bleaching are in development. A well-adapted system of a light source and an adequate photocatalytic nanoparticle bleaching gel could allow localized production of reactive oxygen species, including hydrogen peroxide and hydroxyl radicals, and boost the bleaching effect while reducing potential adverse side effects. Clinical results based on different concentrations of hydrogen peroxide, photocatalysts, blue LED and laser light support this hypothesis.[44] Based on preliminary data from Coto *et al.* the proposed experimental TiO_2/Ag Gel are feasible for bleaching purposes when applied with blue LED lamps.[34] Experimental bleaching gels using such approaches still needed to be assessed for their biocompatibility through *in vitro* testing for toxic effects.

Here we evaluated experimental nanoparticle-based bleaching gels for their impact on the metabolic activity and viability of the L-929 cells, 3T3 cells, and GF. According to *in vitro* experiments at Cambridge University, blue LED light activation of gels containing visible-light activated TiO_2/Ag nanoparticles resulted in adequate bleaching, even without the addition of hydrogen peroxide.[34] In our study, we thus evaluated both the experimental bleaching gels without and with light activation under blue LED light. Scanning electron microscope evaluation of both the experimental bleaching gels can be found in the ESI Fig. 1.† Overall, 3T3 cells even without the presence of gels showed to be more sensitive to the illumination protocol with blue light compared to L-929 and GF with regard to metabolic activity and viability in the 24 h group while this effect was not found in the 30 min group. Based on these findings we presented the results relative to the respective control group either with or without illumination (Table 1). We found that gel containing 6% hydrogen peroxide strongly reduced metabolic activity and cell viability of L-929, 3T3, and GF cells, even at dilutions of 0.0032%.

In general, gels containing nanoparticles showed much lower toxicity then those containing hydrogen peroxide. Although differences were observed between the three cell lines that were tested, TiO_2/Ag-containing gel was more toxic then empty gels or gels containing TiO_2. Understandably, the shorter treatments of 30 min, mimicking typical dental bleaching procedures, showed lower cytotoxicity (*i.e.* metabolic activity and cell viability) compared to the 24 h treatment protocols. For clinically relevant 30 min treatments, significant reduction of metabolic activity and cell viability was typically observed at lower concentrations for gels with TiO_2/Ag compared to empty gels or those with TiO_2. Nevertheless, with cytotoxic effects of 2% dilutions, TiO_2/Ag gels can be deemed much safer than the here applied hydrogen peroxide gel, which show toxicity at dilutions of 0.0032%. Similar effects were observed when the treatment was performed with illumination using the blue LED light.

Our data suggest that there is some cytotoxicity associated with the hydrogel. This can be either contributed to components inside the gel or the reduced content of cell culture medium. Supplementation of TiO_2 with Ag can modulate their impact on oral soft tissue cells. TiO_2-containing gels are not more cytotoxic then the empty gel. This is an interesting finding given earlier research suggesting that TiO_2 can induce IL-6 production in GF (Oberoi *et al.* submitted, unpublished results). Hence proper isolation during the bleaching gel application and cleaning after the bleaching process are important issues to consider when applying this strategy[45] and Oberoi *et al.* submitted (unpublished observation). Based on our study this cleaning step is of relevance in particular when modified TiO_2 particles are used that contain Ag.

Nanocomposites of TiO_2 and Ag show increased toxicity compared to TiO_2 alone, albeit a relatively small effect. This effect should be attributed to the presence of Ag. Our finding is interesting given that Ag nanoparticles and ions are deemed bactericidal but save for eukaryotic cells.[46] However, toxic effects of Ag-containing biomaterials have been described.[47] As TiO_2/Ag will be active effectively under blue light and TiO_2 effectively with UV light, it is possible that in addition to the direct effects of silver,[47] also photocatalytically activity is involved in the impact of the nanoparticles on the cells.[34]

It has to be mentioned that all gels used for these initial experiments are pure experimental bleaching gels without CE certificate. Our *in vitro* results, therefore, provide data on their cytotoxicity on L-929, 3T3, and gingival fibroblasts and do not provide conclusive data on their safety or biocompatibility in the clinical situations.

A limitation of the study is that we did not test the pure experimental bleaching gels which would be applied clinically but dilutions of the gels. In our experiments, we aimed to test the gels in an *in vitro* cell culture model which mimics the *in vivo* situation. However, the application of pure bleaching gel was not feasible in cell culture. This can be understood because of the need of cell culture medium in these reductionistic settings. Furthermore, the high viscosity of the pure gel makes its handling in these settings challenging. Hence the gels were applied at 50% in cell culture medium.

When the gels are diluted in culture medium and applied to the cell culture, the nanoparticles sediment onto the cells. Thus, it is possible that the local concentration to which the cells were exposed is higher than the concentration of nanoparticles that can be found in the *in vivo* situation. However, in a clinical setting, it is possible that due to ineffective cleaning after a whitening procedure, nanoparticles can accumulate *e.g.* in periodontal pockets leading to higher local concentrations.

Conclusion

Overall, we showed that experimental bleaching gels containing TiO_2 or TiO_2/Ag show less cytotoxicity than hydrogen peroxide-containing gels. Although the empty gel and the gel containing TiO_2 had a similar toxicity dose–response, the gel containing TiO_2/Ag was shown to be more cytotoxic *in vitro*.

Although more research needs to be conducted on its *in vivo* safety and bleaching efficacy, this study conclusively shows that photocatalytic nanoparticles are less cytotoxic than hydrogen peroxide, and could thus serve as a compelling alternative for aesthetic dental whitening.

Abbreviations

Ag	Silver
CE	European conformity
h	Hours
H_2O_2	Hydrogen peroxide
LED	Light emitting diode
mbar	Millibar
MD	Maximal dose, defined as 50% (v/v)
min	Minutes
NPs	Nanoparticles
SEM	Scanning electron microscope
TiO_2	Titanium dioxide
UV	Ultraviolet
W/O	Without (illumination)

Conflicts of interest

HA, MC, GD, CK, RVK, AM, HAS-T, RJGDM, JV, PV have no conflict of interest.

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References

1 C. D. Presoto, J. F. Bortolatto, P. P. F. de Carvalho, T. C. Trevisan, M. C. Floros and O. B. de O. Junior, New Parameter for In-Office Dental Bleaching, *Case Rep. Dent.*, 2016, **2016**, 6034757. Q5 Q6

2 A. Joiner and W. Luo, Tooth colour and whiteness: A review, *J. Dent.*, 2017, **67S**, S3–S10.

3 J. Montero, C. Gómez-Polo, J. A. Santos, M. Portillo, M. C. Lorenzo and A. Albaladejo, Contributions of dental colour to the physical attractiveness stereotype, *J. Oral Rehabil.*, 2014, **41**, 768–782.

4 A. Joiner, The bleaching of teeth: a review of the literature, *J. Dent.*, 2006, **34**, 412–419.

5 S. Gurgan, F. Y. Cakir and E. Yazici, Different light-activated in-office bleaching systems: a clinical evaluation, *Lasers Med. Sci.*, 2010, **25**, 817–822.

6 L. C. do A. G. de Almeida, H. Riehl, P. H. dos Santos, M. L. M. M. Sundfeld and A. L. F. Briso, Clinical evaluation of the effectiveness of different bleaching therapies in vital teeth, *Int. J. Periodontics Restorative Dent.*, 2012, **32**, 303–309.

7 P. F. L. Dawson, M. O. Sharif, A. B. Smith and P. A. Brunton, A clinical study comparing the efficacy and sensitivity of home vs combined whitening, *Oper. Dent.*, 2011, **36**, 460–466.

8 C. R. Serraglio, L. Zanella, K. B. Dalla-Vecchia and S. A. Rodrigues Junior, Efficacy and safety of over-the-counter whitening strips as compared to home-whitening with 10% carbamide peroxide gel–systematic review of RCTs and metanalysis, *Clin. Oral Investig.*, 2016, **20**, 1–14.

9 R. J. G. De Moor, J. Verheyen, A. Diachuk, P. Verheyen, M. A. Meire, P. J. De Coster, F. Keulemans, M. De Bruyne and L. J. Walsh, Insight in the chemistry of laser-activated dental bleaching, *Sci. World J.*, 2015, **2015**, 650492.

10 J. Martín, P. Vildósola, C. Bersezio, A. Herrera, J. Bortolatto, J. R. C. Saad, O. B. Oliveira and E. Fernández, Effectiveness of 6% hydrogen peroxide concentration for tooth bleaching —A double-blind, randomized clinical trial, *J. Dent.*, 2015, **43**, 965–972.

11 In-office dental bleaching with light vs. without light: A systematic review and meta-analysis, *Br. Dent. J.*, 2018, **224**, 622.

12 E. Klaric Sever, Z. Budimir, M. Cerovac, M. Stambuk, M. Par, D. Negovetic Vranic and Z. Tarle, Clinical and patient reported outcomes of bleaching effectiveness, *Acta Odontol. Scand.*, 2018, **76**, 30–38.

13 I. Luque-Martinez, A. Reis, M. Schroeder, M. A. Muñoz, A. D. Loguercio, D. Masterson and L. C. Maia, Comparison of efficacy of tray-delivered carbamide and hydrogen peroxide for at-home bleaching: a systematic review and meta-analysis, *Clin. Oral Investig.*, 2016, **20**, 1419–1433.

14 A. Majeed, I. Farooq, S. R. Grobler and M. H. Moola, In vitro evaluation of variances between real and declared concentration of hydrogen peroxide in various tooth-whitening products, *Acta Odontol. Scand.*, 2015, **73**, 387–390.

15 K. Chemin, M. Rezende, A. D. Loguercio, A. Reis and S. Kossatz, Effectiveness of and Dental Sensitivity to At-home Bleaching With 4 and 10 Hydrogen Peroxide: A Randomized, Triple-blind Clinical Trial, *Oper. Dent.*, 2018, **43**, 232–240.

16 M. Rezende, K. Chemin, S. C. Vaez, A. C. Peixoto, J. de F. Rabelo, S. S. L. Braga, A. L. Faria-E-Silva, G. R. da Silva, C. J. Soares, A. D. Loguercio and A. Reis, Effect of topical application of dipyrone on dental sensitivity reduction after in-office dental bleaching: A randomized, triple-blind multicenter clinical trial, *J. Am. Dent. Assoc.*, 2018, **149**, 363–371.

17 B. Rossi, P. M. Freitas, T. K. Tedesco, F. Gonçalves and L. S. Ferreira, Tooth color changes and sensitivity in patients undergoing dental bleaching with 10% hydrogen peroxide using customized trays or strips: a randomized clinical trial, *Minerva Stomatol.*, 2018, **67**, 55–61.

18 A. M. Kielbassa, M. Maier, A.-K. Gieren and E. Eliav, Tooth sensitivity during and after vital tooth bleaching: A systematic review on an unsolved problem, *Quintessence Int.*, 2015, **46**, 881–897.

19 C. J. Tredwin, S. Naik, N. J. Lewis and C. Scully, Hydrogen peroxide tooth-whitening (bleaching) products: review of adverse effects and safety issues, *Br. Dent. J.*, 2006, **200**, 371–376.

20 A. L. B. Jurema, M. Y. de Souza, C. R. G. Torres, A. B. Borges and T. M. F. Caneppele, Effect of pH on whitening efficacy of 35% hydrogen peroxide and enamel microhardness, *J. Esthet. Restor. Dent.*, 2018, **30**, E39–E44.

21 G. C. Lopes, L. Bonissoni, L. N. Baratieri, L. C. C. Vieira and S. Monteiro, Effect of bleaching agents on the hardness and morphology of enamel, *J. Esthet. Restor. Dent.*, 2002, **14**, 24–30.

22 R. F. Lia Mondelli, T. R. C. Garrido Gabriel, F. A. Piola Rizzante, A. C. Magalhães, J. F. Soares Bombonatti and S. K. Ishikiriama, Do different bleaching protocols affect the enamel microhardness?, *Eur. J. Dent.*, 2015, **9**, 25–30.

23 S. Naik, C. J. Tredwin and C. Scully, Hydrogen peroxide tooth-whitening (bleaching): review of safety in relation to possible carcinogenesis, *Oral Oncol.*, 2006, **42**, 668–674.

24 L. J. Walsh, Safety issues relating to the use of hydrogen peroxide in dentistry, *Aust. Dent. J.*, 2000, **45**, 257–269; quiz 289.

25 COUNCIL DIRECTIVE 2011/84/EU of 20 September 2011, amending Directive 76/768/EEC, concerning cosmetic products, for the purpose of adapting Annex III thereto to technical progress, *Official Journal of the European Union*, L 283/36–38.

26 R. J. G. De Moor, J. Verheyen, P. Verheyen, A. Diachuk, M. A. Meire, P. J. De Coster, M. De Bruyne and F. Keulemans, Laser teeth bleaching: evaluation of eventual side effects on enamel and the pulp and the efficiency in vitro and in vivo, *Sci. World J.*, 2015, **2015**, 835405.

27 J. F. Bortolatto, T. C. Trevisan, P. S. I. Bernardi, E. Fernandez, L. N. Dovigo, A. D. Loguercio, O. Batista de Oliveira Junior and H. Pretel, A novel approach for in-office tooth bleaching with 6% H2O2/TiO_N and LED/laser system-a controlled, triple-blinded, randomized clinical trial, *Lasers Med. Sci.*, 2016, **31**, 437–444.

28 M. A. Pérez-Díaz, L. Boegli, G. James, C. Velasquillo, R. Sánchez-Sánchez, R.-E. Martínez-Martínez, G. A. Martínez-Castañón and F. Martinez-Gutierrez, Silver nanoparticles with antimicrobial activities against Streptococcus mutans and their cytotoxic effect, *Mater. Sci. Eng., C*, 2015, **55**, 360–366.

29 F. Martinez-Gutierrez, L. Boegli, A. Agostinho, E. M. Sánchez, H. Bach, F. Ruiz and G. James, Anti-biofilm activity of silver nanoparticles against different microorganisms, *Biofouling*, 2013, **29**, 651–660.

30 K. Markowska, A. M. Grudniak and K. I. Wolska, Silver nanoparticles as an alternative strategy against bacterial biofilms, *Acta Biochim. Pol.*, 2013, **60**, 523–530.

31 G. Quiram, F. Montagner, K. L. Palmer, M. C. Stefan, K. E. Washington and D. C. Rodrigues, Novel Chlorhexidine-Loaded Polymeric Nanoparticles for Root Canal Treatment, *J. Funct. Biomater.*, DOI: 10.3390/jfb9020029.

32 C. Covarrubias, D. Trepiana and C. Corral, Synthesis of hybrid copper-chitosan nanoparticles with antibacterial activity against cariogenic Streptococcus mutans, *Dent. Mater. J.*, DOI: 10.4012/dmj.2017-195.

33 J. M. Martinez-Andrade, M. Avalos-Borja, A. R. Vilchis-Nestor, L. O. Sanchez-Vargas and E. Castro-Longoria, Dual function of EDTA with silver nanoparticles for root canal treatment-A novel modification, *PLoS One*, 2018, **13**, e0190866.

34 M. Coto, G. Divitini, A. Dey, S. Krishnamurthy, N. Ullah, C. Ducati and R. V. Kumar, Tuning the properties of a black TiO 2 -Ag visible light photocatalyst produced by a rapid one-pot chemical reduction, *Mater. Today Chem.*, 2017, **4**, 142–149.

35 A. Kishi, M. Otsuki, A. Sadr, M. Ikeda and J. Tagami, Effect of light units on tooth bleaching with visible-light activating titanium dioxide photocatalyst, *Dent. Mater. J.*, 2011, **30**, 723–729.

36 Y. Suyama, M. Otsuki, S. Ogisu, R. Kishikawa, J. Tagami, M. Ikeda, H. Kurata and T. Cho, Effects of light sources and visible light-activated titanium dioxide photocatalyst on bleaching, *Dent. Mater. J.*, 2009, **28**, 693–699.

37 K. Sakai, J. Kato, H. Kurata, T. Nakazawa, G. Akashi, A. Kameyama and Y. Hirai, The amounts of hydroxyl radicals generated by titanium dioxide and 3.5% hydrogen peroxide under 405-nm diode laser irradiation, *Laser Phys.*, 2007, **17**, 1062–1066.

38 C. Kurzmann, K. Janjić, H. Shokoohi-Tabrizi, M. Edelmayer, M. Pensch, A. Moritz and H. Agis, Evaluation of Resins for Stereolithographic 3D-Printed Surgical Guides: The Response of L929 Cells and Human Gingival Fibroblasts, *BioMed Res. Int.*, 2017, **2017**, 4057612.

39 K. Janjić, C. Kurzmann, A. Moritz and H. Agis, Expression of circadian core clock genes in fibroblasts of human gingiva and periodontal ligament is modulated by L-Mimosine and hypoxia in monolayer and spheroid cultures, *Arch. Oral Biol.*, 2017, **79**, 95–99.

40 J. CONSIDERATION, OECD GUIDELINE FOR TESTING OF CHEMICALS.

41 D. G. Soares, A. P. D. Ribeiro, F. da Silveira Vargas, J. Hebling and C. A. de Souza Costa, Efficacy and cytotoxicity of a bleaching gel after short application times on dental enamel, *Clin. Oral Investig.*, 2013, **17**, 1901–1909.

42 D. A. Tipton, S. D. Braxton and M. K. Dabbous, Role of saliva and salivary components as modulators of bleaching agent toxicity to human gingival fibroblasts in vitro, *J. Periodontol.*, 1995, **66**, 766–774.

43 D. A. Tipton, S. D. Braxton and M. K. Dabbous, Effects of a bleaching agent on human gingival fibroblasts, *J. Periodontol.*, 1995, **66**, 7–13.

44 P. Vildósola, J. Bottner, F. Avalos, I. Godoy, J. Martín and E. Fernández, Teeth bleaching with low concentrations of hydrogen peroxide (6%) and catalyzed by LED blue (450±10 nm) and laser infrared (808±10 nm) light for in-office treatment: Randomized clinical trial 1-year follow-up, *J. Esthet. Restor. Dent.*, 2017, **29**, 339–345.

45 X. He, E. Hartlieb, L. Rothmund, J. Waschke, X. Wu, K. L. Van Landuyt, S. Milz, B. Michalke, R. Hickel, F.-X. Reichl and C. Högg, Intracellular uptake and toxicity of three different Titanium particles, *Dent. Mater.*, 2015, **31**, 734–744.

46 M. Składanowski, P. Golinska, K. Rudnicka, H. Dahm and M. Rai, Evaluation of cytotoxicity, immune compatibility and antibacterial activity of biogenic silver nanoparticles, *Med. Microbiol. Immunol.*, 2016, **205**, 603–613.

47 B. Cvikl, S. C. Hess, R. J. Miron, H. Agis, D. Bosshardt, T. Attin, P. R. Schmidlin and A. Lussi, Response of human dental pulp cells to a silver-containing PLGA/TCP-nanofabric as a potential antibacterial regenerative pulp-capping material, *BMC Oral Health*, 2017, **17**, 57.