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# Cytotoxic Effects of Hydrogen Peroxide on Human Gingival Fibroblasts In Vitro

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### Clinical Relevance

This study aimed to elucidate the cytotoxic effects of an in-office bleaching agent on cultured human gingival fibroblasts.

## SUMMARY

**In-office bleaching is a popular treatment in modern esthetic dentistry. However, bleaching agents sometimes accidentally adhere to the gingiva and peripheral tissues, even when applied by well-trained dentists. This can lead to transient pain and whitish changes in the gingiva. Although these symptoms disappear within several hours, the effects of bleaching agents on gingiva have not been well described in the literature. The present study aimed to elucidate the cytotoxic effects of a bleaching agent on cultured human gingival fibroblasts (HGFs).**

**We performed a comprehensive analysis of the toxic effects of in-office bleaching agents on gingiva using cultured HGFs and DNA microarray. Survival rates of HGFs decreased with increases in the concentration of hydrogen peroxide, which became significant at concentrations of $1.5 \times 10^{-3}$% or higher at every time point. Concentrations lower than $1.5 \times 10^{-3}$% did not affect survival rates of HGFs. Cytotoxicity of hydrogen peroxide was significantly weakened by the addition of vitamin E. Stimulation by in-office bleaching agents triggered the proinflammatory cytokine tumor necrosis factor (TNF)–α cascade in gingival fibroblasts. As the TNF-α cascade can be inhibited by vitamin E additives, treatment with vitamin E may protect gingival fibroblasts against the toxic effects of an in-office bleaching agent.**

**The present results suggest that local administration of vitamin E to gingiva before in-**

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Figure 1. *Toxic effects of in-office bleaching agent on gingiva. Despite the application of petroleum jelly to protect the gingiva when performing in-office bleaching, the in-office bleaching agent sometimes comes into contact with the gingiva, as shown in the photo. When this occurs, the gingiva whitens and pain results, but the gingiva returns to its original state within 30 minutes and pain subsides.*

**office bleaching may be useful for preventing gingival irritation due to accidental adhesion of a bleaching agent.**

## INTRODUCTION

As patients' needs for dental treatment shift from traditional treatment including caries and dentures to esthetic treatment, an increasing number of people are visiting the dental office with hopes for whiter teeth. Acceptable vital tooth-whitening techniques include the "at-home"[1,2] and "in-office"[3,4] techniques. Over-the-counter (OTC) bleaching products are sold as cosmetics and are freely available through stores, pharmacies, and the Internet.[5]

Tooth sensitivity and gingival irritation are two of the most common side effects during/after at-home, in-office, and OTC bleaching.[6-11] Although in-office bleaching is a particularly popular method for bleaching, the bleaching agent may sometimes come into contact with the patient's gingiva or oral mucosa during the in-office bleaching procedure, even if the gingiva is protected with a light-cured resin or rubber dam and bleaching is performed by an experienced dentist (Figure 1). This may result in temporary whitening and pain in the gingiva or oral

mucosa, but the pain subsides within a few hours and the whitened spot eventually regains its original color.[11,12] This has also been reported with at-home bleaching.[13-16] However, this phenomenon has yet to be studied from a molecular biology perspective, and there are no studies reporting the prevention or treatment of gingival irritation. Therefore, when patients experience gingival irritation during at-home bleaching, dentists usually recommend to stop bleaching, change the concentration of the bleaching agent, or change bleaching materials.[17] We thus set out to determine the effects of in-office bleaching agents on the gingiva *in vitro*. We examined the following four points using cultured human gingival fibroblasts (HGFs) to investigate the toxicity of in-office bleaching agents on gingiva. 1) HGFs were stimulated with hydrogen peroxide ($H_2O_2$), and cytotoxicity was examined by Alamar blue staining. In clinical practice, it is commonly believed that vitamin E cream can be applied to gingiva that has come into contact with an in-office bleaching agent to relieve the signs, but there is no evidence to support this notion. To test this belief, 2) under these conditions, the protective effects of vitamin E on cytotoxicity were examined. 3) Phalloidin staining was then performed in order to examine the changes in cell morphology under each condition. 4) To comprehensively analyze the cell response triggered in HGFs by in-office bleaching agent stimulation, DNA microarray profiling was performed to assess changes in gene expression induced by $H_2O_2$ and vitamin E addition.

The following null hypotheses were tested: 1) HGFs were subjected to inflammation by $H_2O_2$ exposure, (2) damaged HGFs were stimulated with a proinflammatory cytokine, and (3) damaged HGFs were restored by vitamin E supplementation.

## METHODS AND MATERIALS

### Examination of Cytotoxicity

HGFs (ScienCell, Carlsbad, CA, USA) were grown in a gingival fibroblast medium (ScienCell) supplemented with 5% inactivated fetal bovine serum (FCS; ScienCell) until the cells formed a single layer at 37°C in the presence of 5% carbon dioxide on a 10-cm dish coated with poly-L-lysine (poly-L-lysine–coated 100-mm dish; Iwaki, Tokyo, Japan). In the present study, fibroblasts after four to six population doublings (4-6 PD fibroblasts) were used in experiments. To measure cytotoxicity (Figure 2), HGFs were seeded onto 96-well multiplates (poly-L-lysine–coated 96-well micro plate; Iwaki) with $1 \times 10^5$ cells/well and were incubated for 24 hours, after which cells were stimulated by $H_2O_2$ (Wako) at the concentrations shown in Figure 3, and were allowed to react for 90 seconds, 5 minutes, 10 minutes, 30 minutes, or 60 minutes. Next, Alamar Blue (Invitrogen, Carlsbad, CA, USA) was added, and cultures were incubated for a further 24 hours (Figure 4) and then measured at 540 nm with an absorbance microarray scanner (VersaMax; Molecular Devices, Tokyo, Japan).

### Effects of Vitamin E Under $H_2O_2$ Stimulation

During 15% $H_2O_2$ stimulation, 50, 100, 125, 250, and 375 μM of (±)-α-tocopherol (Wako; "vitamin E") was added to cultures and was allowed to react for 90 seconds. Alamar blue was then added and cytotoxicity was examined as described above.

### Examination of Changes in Cell Morphology

Cultured HGFs were seeded onto six-well dishes, and a control group, a 15% $H_2O_2$ group, and a 15% $H_2O_2$ + 250 μM vitamin E group were each allowed to react for 90 seconds. After incubation, cultures were fixed in 10% formalin and cells were stained with TRITC-labeled phalloidin.

### Examination of Changes in Expression of Various Genes by Microarray

Using samples under the same stimulation conditions as described in the previous section, microarray (Takara Bio Dragon Genomics Center, Mie, Japan) was used to examine which genes in the $H_2O_2$ stimulation-induced gene cluster had expression suppressed by addition of vitamin E.

### Statistical Analysis

Data were expressed as means ± standard deviation for the multiple experiments. Student *t*-tests were used for concentration of $H_2O_2$ and vitamin E with cell viability ($p<0.05$).

## RESULTS

### Cytotoxicity

Almost no cytotoxicity from $H_2O_2$ was seen in the low-concentration stimulation group ($1.5 \times 10^{-3}$% or less) at any duration other than 60 minutes. However, 60-minute stimulation resulted in cytotoxicity at all concentrations. Stimulation at 15% $H_2O_2$, which is similar to the concentration used in clinical practice, showed marked cytotoxicity, lowering cell survival by half at 90 seconds ($p<0.05$), which is the shortest time period examined (Figure 3).

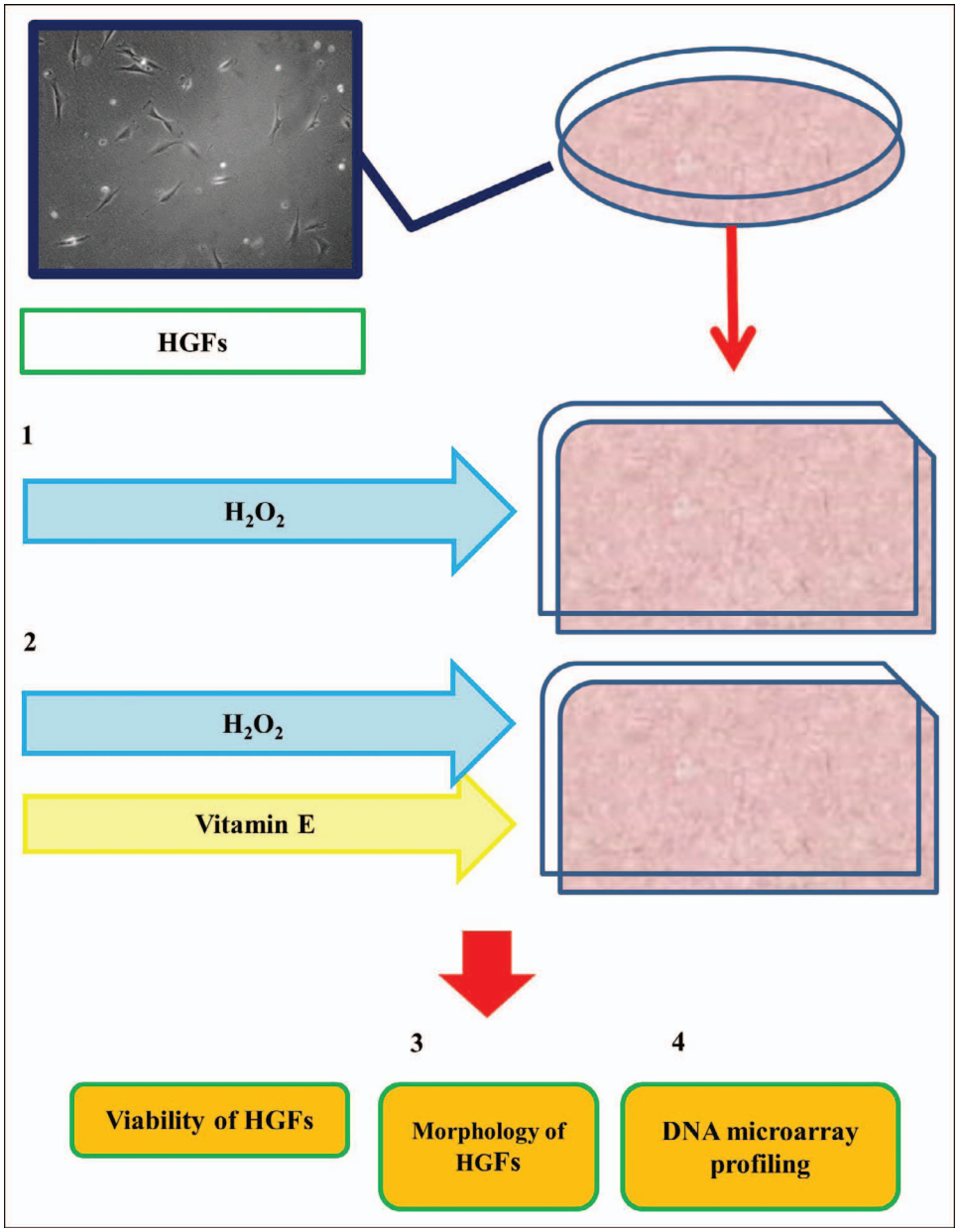


Figure 2. *Experimental methods. Human gingival fibroblasts were used to analyze cell survival rate, cellular morphological changes, and changes in gene expression* in vitro *after $H_2O_2$ stimulation and/or addition of vitamin E.*

### Examination of Cytotoxicity Suppression by Vitamin E

As HGF cytotoxicity from $H_2O_2$ stimulation was demonstrated in the results of the Cytotoxicity section above, we then examined whether addition of vitamin E as an antioxidant acts to suppress cytotoxicity from $H_2O_2$. As an experimental condition, 15% $H_2O_2$ was used, as it resulted in the most severe cytotoxicity effects in HGFs in the Cytotoxicity section, and it is the concentration closest to that used in clinical practice. A stimulation duration of 90 seconds was used, as this is when patients begin to feel pain when the in-office bleaching agent contacts the gingiva. Similarly to the Cytotoxicity section above, 50, 100, 125, 250, and 375 μM vitamin E was added to cultured HGFs. Cell survival, which was cut in half by $H_2O_2$ stimulation, recovered to control levels with the addition of 250 μM vitamin E (Figure 5).

### Observation of Changes in Cell Morphology

Cultured HGFs are normally spindle shaped (Figure 6a). The cell morphology of HGFs exposed to $H_2O_2$ stimulation changed dramatically (Figure 6b). Specifically, the spindle-shaped fibroblasts became circular after $H_2O_2$ stimulation (Figure 6b, arrow). Addition of vitamin E suppressed the rounding of

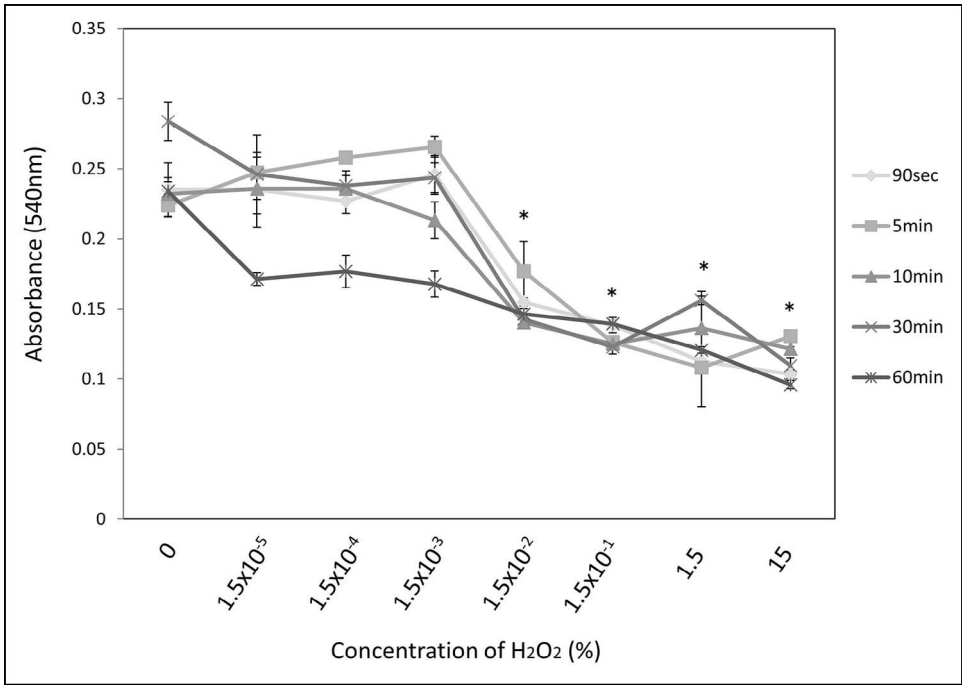


Figure 3. *Gingival fibroblast cytotoxicity after $H_2O_2$ stimulation. Cytotoxicity is low with $H_2O_2$ concentrations of 1.5 × $10^{-3}$% or higher. The significance of the differences was assessed by* t*-test (*$p<0.05$*).*

cell morphology caused by $H_2O_2$ stimulation (Figure 6c).

### Examination of Changes in Gene Expression by Microarray

Using the same stimulation samples as in the section above, microarray was used to analyze the gene clusters that were suppressed from the addition of vitamin E among the gene cluster induced by $H_2O_2$ stimulation. The results confirmed that $H_2O_2$ stimulation induced the expression of genes associated with the proinflammatory cytokine TNF-α cascade (Table 1). Expression of these genes was suppressed with vitamin E addition. These results demonstrate that $H_2O_2$ stimulation response includes triggering the proinflammatory cytokine TNF-α cascade and that addition of vitamin E suppresses the response of this cascade (Figure 7).

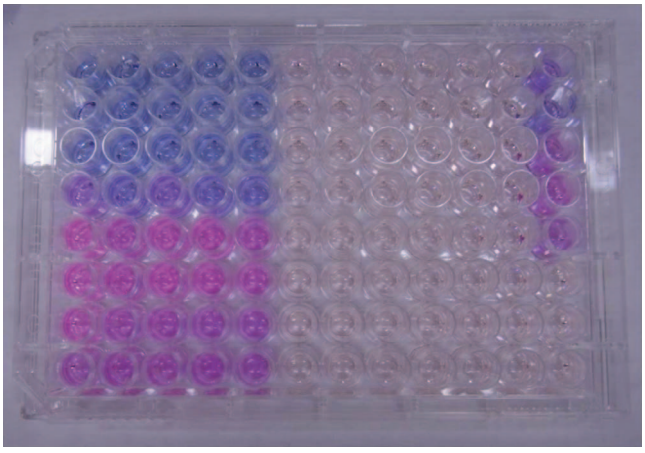

Figure 4. *Alamar blue staining. Red indicates high cytotoxicity.*

## DISCUSSION

We conducted *in vitro* examinations of signs such as whitening of the gingiva and pain that may result from tooth whitening. No studies to this effect in HGFs have been reported. These signs may arise from at-home bleaching agents[6,7,9] but mostly result from in-office bleaching agents[11,12] and OTC products.[10]

Many studies have been carried out on tooth hypersensitivity from in-office or at-home bleaching agents.[6-15] From *in vitro* studies, it has been concluded that whitening agents histologically penetrate the dentin[18] and do not damage the pulp.[19,20] In current dental practice, pain incurred during the procedure is generally treated with medication or fluoride or by stopping the procedure.[21-25] Several studies examining plaque control as an index have shown that whitening agents reduce plaque on the gingiva and reduce gingival inflammation.[26,27] Hydrogen peroxide and carbamide peroxide have been used for debridement during endodontic therapy,[28] in mouth rinses to reduce plaque in individuals with gingivitis,[29,30] and for treatment of periodontal diseases.[31]

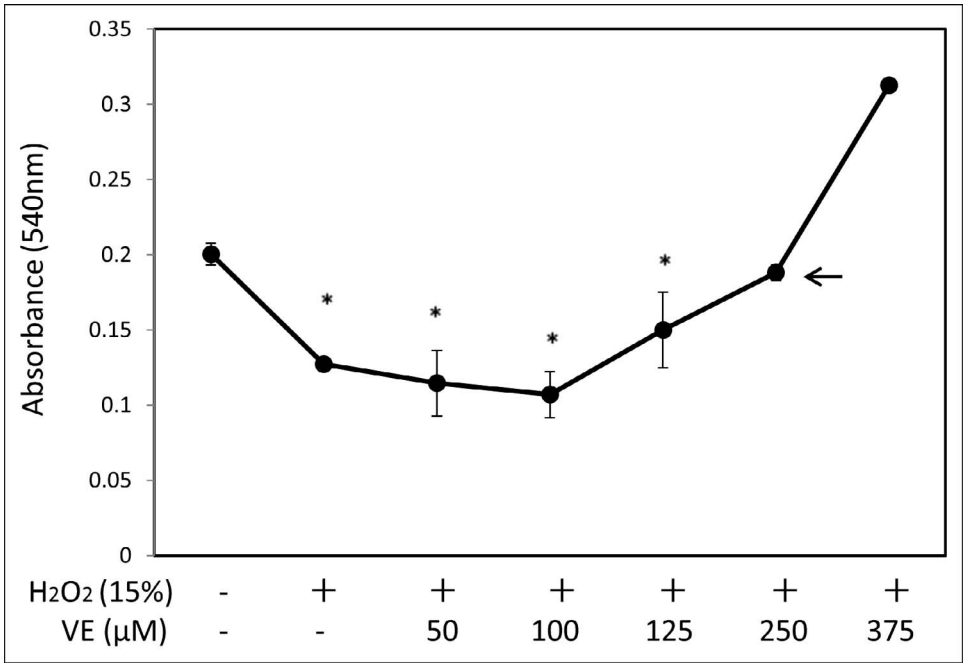


Figure 5. *Examination of cytotoxicity suppression by vitamin E. Various concentrations of vitamin E were added to human gingival fibroblasts treated with the $H_2O_2$ concentration obtained in cytotoxicity tests ($1.5 \times 10^{-3}$%). After a 90-second reaction time, Alamar blue was used to test cytotoxicity. Cell survival, which was cut in half by $H_2O_2$ stimulation, recovered to control levels with the addition of 250 μM of vitamin E. The significance of the differences was assessed by* t*-test (*$p<0.05$*).*

Gingival fibroblasts are known to be affected by $H_2O_2$.[32,33] However, studies have shown that fibroblasts in other parts of the body are more strongly affected.[34] In the present study, highly concentrated $H_2O_2$ caused inflammation in gingival fibroblasts and had toxic effects, with marked changes in cell morphology. One study found that higher concentrations of $H_2O_2$ caused senescence-like changes in cells.[33] It has long been known from *in vivo* animal experiments that $H_2O_2$ causes acute inflammation and even edema in skin.[35-40] Simon and others[41] have shown $H_2O_2$ to cause necrosis in human fibroblasts. Cells are protected by various types of enzymes and other substances and manage the immune system that responds to inflammation.[42,43] Inferring from the above studies, it is possible that $H_2O_2$ penetrates the cell membrane to cause damage, so that enzymes cannot do their job.

The $H_2O_2$ stimulation duration in the present study was 90 seconds. In our own experience in actual clinical practice, patients begin to complain of pain about 90 seconds after the in-office bleaching agent contacts the gingiva. At the same time, the gingiva whitens, and 60 minutes after touching the gingiva, both the pain and the whitening have completely disappeared (Figure 1).

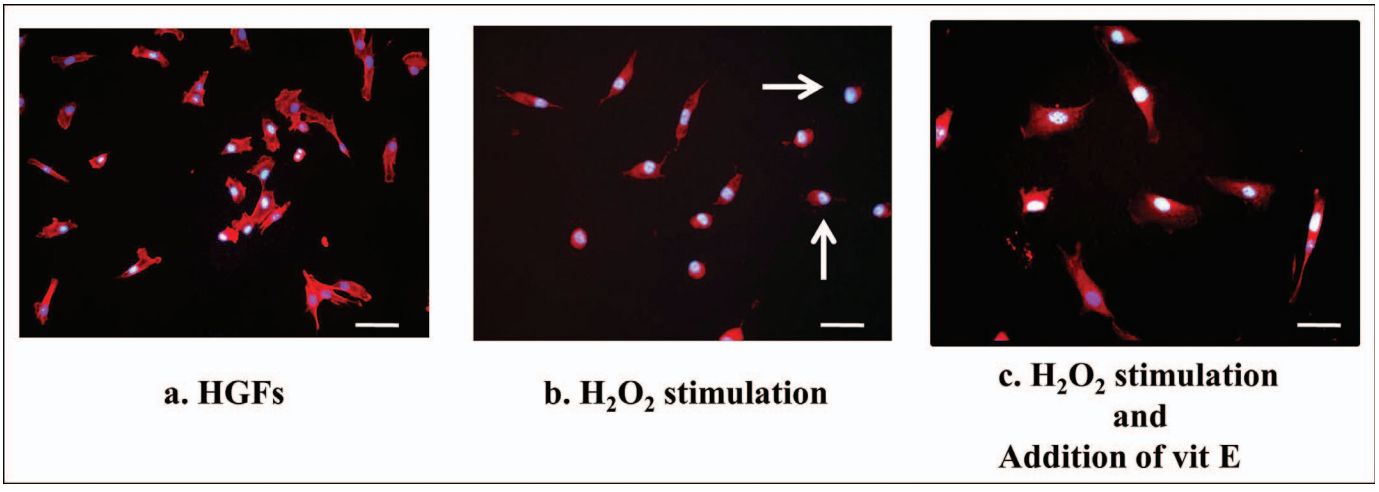


Figure 6. *Changes in cell morphology (phalloidin staining). Cultured human gingival fibroblasts were divided into (a): control group, (b): 15% $H_2O_2$ group, and (c): 15% $H_2O_2$ + 250 μM vitamin E group and were allowed to react for 90 seconds. Scale bars = 30 μm.*

Table 1: *Gene Clusters Induced by $H_2O_2$ Stimulation and Suppressed by Addition of Vitamin E. Average of* log2 expression ratio *(Test/control).*

| Name of Gene | Function | $H_2O_2$ | Vitamin E |
|---|---|---|---|
| Homo sapiens cation channel, sperm associated 2 (CATSPER2) | Protein binding | 2.844 | −2.838 |
| | Voltage-gated calcium channel activity | | |
| Homo sapiens calsyntenin 3 (CLSTN3) | Calcium ion binding | 2.04 | −2.681 |
| Homo sapiens deleted in liver cancer 1 (DLC1) | Protein binding | 1.68 | −1.55 |
| Homo sapiens ephrin-B3 (EFNB3) | Axon guidance | 1.68 | −1.81 |
| Homo sapiens Fc receptor-like 5 (FCRL5) | Receptor activity association to diseases | 3.84 | −3.3 |
| Homo sapiens growth hormone 1 (GH1) | Cytokine-cytokine receptor interaction | 1.90 | −2.053 |
| Homo sapiens guanine nucleotide binding protein (G protein) | GTPase activity | 1.745 | −1.669 |
| Homo sapiens kinesin family member 26A (KIF26A) | ATP binding | 1.99 | −4.39 |
| Homo sapiens Kruppel-like factor 6 (KLF6) | DNA binding | 1.776 | −1.55 |
| Homo sapiens nuclear receptor subfamily 1, group H, member 4 (NR1H4) | Protein binding | 3.054 | −2.50 |
| Homo sapiens olfactory receptor, family 2, subfamily H, member 1 (OR2H1) | G-protein coupled receptor activity | 2.629 | −2.23 |
| | olfactory receptor activity | | |
| Homo sapiens olfactory receptor, family 3, subfamily A, member 4 pseudogene (OR3A4P) | Odorant receptor | 2.132 | −1.76 |
| Homo sapiens outer dense fiber of sperm tails 4 (ODF4) | Unknown | 2.4745 | −2.015 |
| Homo sapiens phosphate regulating endopeptidase homolog, X-linked (PHEX) | Zinc ion activity | 1.562 | −1.53 |
| | Aminopeptodase activity | | |
| | Metalloendopeptise activity | | |
| Homo sapiens phospholipase C, zeta 1 (PLCZ1) | Calcium ion binding | 3.205 | −2.879 |
| Homo sapiens POU class 4 homeobox 1 (POU4F1) | DNA binding transcription factor activity | 2.968 | −2.618 |
| Homo sapiens RUN domain containing 3B (RUNDC3B), transcript variant 1 | Unknown | 2.647 | −1.962 |
| Homo sapiens scavenger receptor cysteine rich domain containing (5 domains) (SSC5D) | Scavenger receptor activity | 1.518 | −1.862 |
| Homo sapiens small nucleolar RNA, C/D box 88C (SNORD88C) | Unknown | 2.088 | −2.51 |
| Homo sapiens small nucleolar RNA, H/ACA box 30 (SNORA30) | Unknown | 1.82 | −1.92 |
| Homo sapiens transcription factor 20 (AR1) (TCF20) | DNA binding | 1.58 | −1.70 |
| Homo sapiens transmembrane protein 236 (TMEM236) | Unknown | 2.72 | −2.24 |
| Homo sapiens tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10) | Cytokine-cytokine receptor interaction | 1.920 | −2.07 |
| Homo sapiens tumor necrosis factor receptor superfamily, member 19 (TNFRSF19) | Cytokine-cytokine receptor interaction | 2.268 | −1.62 |
| Homo sapiens tumor necrosis factor receptor superfamily, member 4 (TNFRSF4) | Cytokine-cytokine receptor interaction | 2.667 | −2.454 |
| Homo sapiens V-set and transmembrane domain containing 2 like (VSTM2L) | Protein binding | 1.518 | −1.790 |
| Homo sapiens zinc finger and BTB domain containing 22 (ZBTB22) | DNA binding, zinc ion binding | 2.018 | −1.981 |
| Netrin G1 | Protein binding | 2.057 | −1.864 |

Recent studies have reported gingival irritation after at-home bleaching.[13,14] Kirsten and others[16] reported that patients experienced gingival irritation from at-home bleaching both immediately after the procedure and up to 45 days following treatment. On the other hand, no participants reported gingival sensitivity at 6 months posttreatment with 10% carbamide peroxide.[44] They performed observations at 3, 6, and 47 months posttreatment, but we believe that gingival irritation is acute and spontaneous. During at-home bleaching, patients were not monitored by a dentist; thus, gingival irritation could not be established with certainty.

Ghalili and others[15] found that gingival irritation disappeared in 10 minutes using OTC products. Al

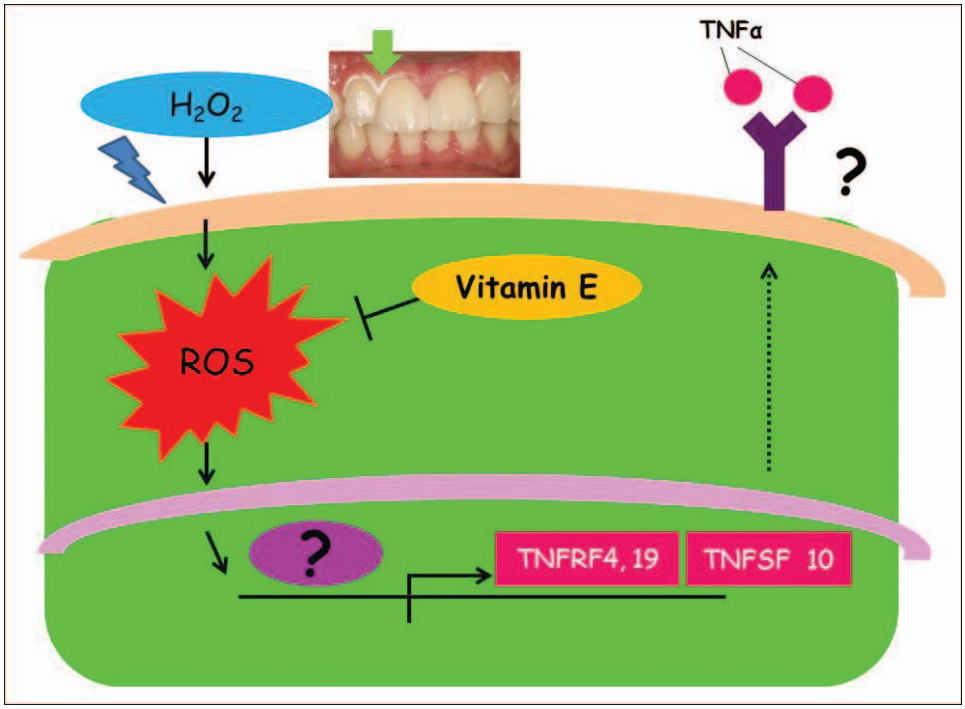


Figure 7. *Inflammatory response pathway from $H_2O_2$ stimulation via TNF-α.*

Shethri and others[12] reported that hypersensitivity and gingival irritation disappeared within two days after in-office bleaching. Previous investigations have shown that 15% of patients reported gingival irritation after in-office bleaching, but it was possible to safely control contact of the bleaching gel with the gingival margin by using light-cured gingival dams.[45] Therefore, it is difficult to compare previously reported gingival irritation results with our observations. The cytotoxic effects produced *in vitro* cannot be immediately applied *in vivo*.

$H_2O_2$ is commonly used in dentistry for tooth bleaching and cleaning, among other purposes. $H_2O_2$ is a form of active oxygen that can cause oxidative damage to fatty acids, biological membranes, and DNA. Therefore, humans are endowed with defenses against $H_2O_2$, and vitamin E is particularly effective at preventing damage by $H_2O_2$.[46,47]

As demonstrated by microarray, the genes induced by $H_2O_2$ stimulation and suppressed by the addition of vitamin E are shown in Table 1. Among the genes listed in Table 1, TNFSF10 belongs to the tumor necrosis factor (TNF)–α ligand superfamily. TNFRSF4 and TNFRSF19 belong to the TNF-α receptor superfamily (TNFRSF). Other authors have examined the gingiva and proinflammatory cytokines. Firat and others[27] examined two types of in-office bleaching agent and one type of at-home bleaching agent to examine periodontal tissue inflammation indices such as gingival index, plaque index, bleeding on probing, and gingival crevicular fluid and found that interleukin (IL)–1"β expression increased with in-office bleaching but that there was no change in the expression of IL-10. However, there have been no reports to date on TNFRF4, TNFRF19, or TNFSF10.

In inflamed tissue, macrophages and other cells of the innate immune system synthesize TNF-α,[48-50] a proinflammatory cytokine, to fight off infection and treat tissue damage. This TNF-α then binds to cell surface receptors and induces the production of other cytokines, triggering and maintaining inflammation. In this study, it is possible that the in-office bleaching agent that came into contact with the gingiva triggered a cellular response through the inflammatory cascade via TNF-α (Figure 7). This cellular response may be suppressed by addition of vitamin E.

When using vitamin E in clinical practice, most dental offices in Japan currently use commercially available vitamin E cream. A recent study has shown that vitamin E added to cells that have been stimulated by $H_2O_2$ restores the cell membrane.[47] Such reports suggest that application of vitamin E to gingiva that has come into contact with an in-office bleaching agent could restore the cell membrane in areas that have been damaged. The results of the present study demonstrate that applying vitamin E

to the gingiva for protection prior to in-office bleaching is useful for suppressing cytotoxicity in HGFs caused by the in-office bleaching agent. The results also support a method for responding to unpleasant signs arising from tooth whitening. Moreover, while host defense mechanisms may be able to protect the tissues and cells from the toxic effects of $H_2O_2$ in in-office bleaching agents, the effects of longer clinical contact between gingival tissues and $H_2O_2$ remain uncertain.

The three null hypotheses were accepted. HGFs exhibited inflammation after $H_2O_2$ exposure, damaged HGFs expressed proinflammatory cytokines, and damaged HGFs were restored by vitamin E supplementation.

## CONCLUSIONS

1. $H_2O_2$ affected HGFs and induced proinflammatory cytokines.
2. Supplemental vitamin E is able to repair HGFs.
3. Pretreatment with vitamin E supplementation before in-office bleaching can help to reduce gingival irritation.
4. Further studies are necessary to develop a vitamin E cream to protect the gingiva.

### Human Subjects Statement

This study was conducted at the Department of Clinical Cariology and Aesthetic Dentistry, Showa University, School of Dentistry in Tokyo, Japan.

### Conflict of Interest

The authors of this article certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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