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The effect of expired orthodontic bonding material on primary human gingival fibroblasts: *In vitro* study

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Abstract

AIM: Composite resins and bonding agents are indispensable in orthodontic practice, necessitating a thorough understanding of their cytotoxic effects, particularly when expired. This study aimed to evaluate the influence of expired composite and bonding materials on human gingival fibroblasts (hGFs).

MATERIALS AND METHODS: Both expired and nonexpired composite resins and bonding agents were tested on hGFs using direct exposure methods. Viability assays, morphological evaluations, and wound healing assays were conducted at 24 and 72 hours post exposure.

RESULTS: Exposure to both expired and nonexpired materials led to significant reductions in hGF viability and alterations in morphology. Wound healing assays demonstrated impaired migratory abilities of hGFs following exposure to these materials.

CONCLUSION: The study highlights the importance of prudent material selection, handling, and monitoring in orthodontic practice to mitigate adverse effects on gingival tissues. Proper management of expired materials is crucial for cost-effectiveness, waste reduction, and patient safety. Further research into the long-term impacts of expired materials on gingival and periodontal health is imperative to ensure clinical treatment safety and efficacy. This investigation provides valuable insights into the biocompatibility of orthodontic bonding materials and emphasizes the necessity for continued vigilance in their usage to uphold patient welfare and treatment quality.

Keywords:

Bonding agent, composite, expired material, orthodontics

Introduction

Composite resins are among the popular and principal materials in dental practice.[1] They gained importance due to their physiochemical properties.[2] Furthermore, a flowable composite is frequently utilized by orthodontists during bonding of clear aligner attachments and other auxiliaries.[3] During these procedures, resin and other components of the dental material may contact the oral tissues, including the gingiva. Therefore, its toxicity to the surrounding oral tissues can be a concerning factor. Studies regarding the cytotoxicity of viable dental materials have been established.[4] However, experimental designs for research that investigate the extent of cytotoxicity of expired dental material are not common.

There are various *in vitro* methods to assess the cytotoxic effects of dental composites on the surrounding soft tissues such as cytotoxicity assay using (3-(4,5-dimethylthiazol-2-yl)-2,5-) diphenyltetrazolium bromide assay (MTT), LDH (lactate dehydrogenase) release assay, cell counting, and wound healing assay. Those involve direct or indirect exposure of the cultured cells to the material being studied.[5,6] The indirect exposure may involve the use of dental materials

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which can be immersed in the cell culture medium or artificial saliva for a specific period to allow leaching of components from the material into the solution. The resulting extract can then be used to treat cultured cells, and cytotoxicity can be evaluated using various assays.[7] The treated cells are then evaluated for any changes in morphology, viability, proliferation, or the release of inflammatory cytokines or other indicators of cellular changes.[8] Understanding the cytotoxic effects of daily used materials is crucial to ensure the biocompatibility and safe application of the material.[9]

Additionally, dental practices and clinics face the impasse of unused dental supplies and expired or nearly expired material; therefore, exploring their biocompatibility and viability to be safely used or not could be of clinical importance.[10]

Managing unused and expired dental supplies poses challenges for practices, affecting finances and patient safety. Therefore, assessing these materials is crucial for cost-effectiveness, waste reduction, and most importantly patient welfare.[10] Moreover, salvaging supplies instead of discarding them enhances resource utilization and reduces financial losses, thus contributing to sustainability efforts by minimizing waste and environmental impact.[9] Importantly, patient safety is paramount as expired materials can compromise dental restorations and pose risks.[11] In orthodontic clinics, composite and resin bonding agents are extensively used for bonding orthodontic attachments.[12] In this paper, we investigated the cytotoxicity of the expired bonding material specifically, flowable dental composite, and bonding agent on isolated human gingival fibroblasts (hGFs).

Materials and Methods

Human gingival fibroblast isolation

Human gingival fibroblasts were obtained from a healthy adult patient following a crown lengthening operation after obtaining signed informed consent from the subject and approval of the Ethical Committee (approval no: 131-19, date: October 23, 2019) from an official governmental institution. The human gingival fibroblasts were isolated as described previously.[13] The gingival tissues were washed three times with phosphate-buffered saline (PBS) and incubated in 1 mg/ml dispase (Sigma, USA) overnight at 4°C. After removal of the epithelial layer, the connective tissues were cut into small pieces, cultured in a 25 ml flask in a complete cell growth medium, and incubated at 37°C in a humidified atmosphere of 5% CO2. The growth medium contains Dulbecco's modified Eagle medium (DMEM, Gibco, Thermo Scientific, USA) supplemented with 10% fatal bovine serum (HyClone, Thermo Scientific, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, USA), and 2.5 µg/ml amphotericin B (Gibco, Thermo Scientific, USA).

Composite and bonding agent preparation

The experimental design started at day (0) on mid of October 2023 and included the resin-bonded composite (Bis-GMA-free Nano-Flow Composite, CHL Medical Solutions SRL, Italy) and the adhesive agent (Bis-GMA-free and HEMA- free, Self-Etch Nano Dental Adhesive, CHL Medical Solutions SRL, Italy) expired in March and January 2023, respectively, and nonexpired (viable until September 2024 and April 2025, respectively), which were prepared into standardized desks using orthodontic rubber bands (Heavy 6 ½ oz., 4.8 mm, American Orthodontics, USA) as a confining frame to standardize the samples. LED light curing of both materials with the light cure tip at the level of the experimental 24-well plate edges (Bluephase G2 LED light cure, 1200 mW/cm2 intensity, ivoclar vivadent) for 20 seconds/sample was done.

Gingival fibroblasts cell viability by MTT assay

Gingival fibroblasts at the third passage were seeded at 5×10^4 cells/well in a 24-well plate and cultured in the growth medium and incubated for 24 hours in 5% CO2 at 37°C in a humidified environment. When the cells had reached 80% confluence after 24 hours of incubation, the resin-bonded composite and bonding agent (expired and unexpired) were placed directly on the fibroblast monolayer. Untreated fibroblasts were used as control. The cell viability was assessed at 24 and 72 hours by MTT (Thermo Fisher Scientific, USA) assay. The amount of formazan granules formed when MTT enters viable eukaryotic cells is closely correlated with the number of metabolically active cells.[14]

MTT assay was performed in 24-well plates to assess the cell viability of the fibroblasts. Briefly, the culture medium was removed from 24-well plates and replaced with 500 µl per well of the culture medium containing 0.5 mg/ml of MTT and incubated for 3 hours at 37°C. At the end of the incubation period, the medium was removed and DMSO: isopropanol (1:1) solvent solution was added at 400 µl per well to dissolve formazan crystals. Triplicates of 100 µl per well of the resulting purple solution were transported to a 96-well plate, and the optical density was read at 570 nm by a spectrophotometric Microplate Reader (SpectroStar Nano, BMG Lab). The optical density values were used to calculate cell viability relative to untreated control, which is set at 100%.

Wound healing assay

The composite and bonding agent (expired and unexpired) were prepared and left to set in 24-well

plates. The extract of the composite and bonding agent was produced by adding the growth culture medium to each material and incubated at 37°C for 4 days. The extract of each material was applied to assess the effect of the resin-bonded composite and bonding agent on fibroblast migration.

A two-well Ibidi silicone culture insert (Ibidi, Germany, 80206) was used to assess the fibroblast migration. The fibroblast cell suspension was adjusted to a cell concentration of 3×10^5 cells/ml, and 70 µl of the cell suspension was added to each well of the Culture-Insert 2 Well in 24-well plates to obtain a confluent cell layer after 24 hours. After reaching confluency, inserts were gently removed by sterilized tweezer and attached cells and the gap formed were checked under the microscope. Cells were washed with PBS to remove debris. The 4-day extract of the composite and bonding agent (expired and unexpired) were added to the cells. Images were taken at 0, 24, 48, and 72 hours under an inverted microscope (Nikon Eclips TS 100, Japan). Cell migration speed was analyzed using Fiji/ImageJ software.

Statistical analysis

GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel were used for data collection, statistical analysis, and graphs. The results were expressed as mean ± standard error of the mean (SEM) and analyzed by unpaired *t*-test. The differences were significant if a *P* value is <0.05.

Results

In general, cell viability assay revealed significant effects of the composite and bonding agent on gingival fibroblast viability. Figure 1 illustrates hGF cell viability following 24 hours of treatment with the nonexpired composite (composite), expired composite (Exp. Composite), bonding agent (bonding), and expired bonding agent (Exp. Bonding). Expectedly, the control untreated hGF cells showed higher viability, which is considered a normal expected finding, while hGF cells treated with the composite (unexpired and expired) demonstrated less viability that is reduced almost to more than half viability of control hGF cells (0.3 OD). A statistical significance was found between hGF cells treated with the composite and expired composite ($P < 0.0371$). However, bonding (unexpired) demonstrated severe reduced survivability when compared to control hGF cells and composites (expired and unexpired) (0.1OD). Notably, hGFs treated with expired bonding showed decreased viability when compared to the unexpired composite but still severely reduced when compared to control hGF cells (0.2 OD). A statistical significance was found between hGF cells treated with bonding and expired bonding ($P < 0.0001$).

Figure 2 shows cell viability after 72 hours; the viability of hGFs was dramatically reduced. As healthy control hGF cells continued to show normal higher viability, hGF cells treated with the composite continued to show severe reduction in cellular viability at 72 h, while the expired composite still has some increased viability at 72 hours. A similar observation was seen in the case of treatment of hGFs with bonding (expired and unexpired) at 72 hours. Treatment was harmful and resulted in a considerable decrease in hGF cell viability. Statistically, a significant difference was found by unpaired *t*-test between hGF cells treated with the composite and expired composite for 72 h ($P < 0.0001$), similar in the case of treatment with bonding and expired bonding, which showed a statistically significant difference ($P < 0.0001$).

The effect of both composites and bonding (expired and nonexpired) on morphology of hGFs was also investigated. As shown in Figure 3, healthy control hGF cells showed normal cellular morphology (spindle-shaped cell) with no presence of apoptotic bodies. Interestingly, treatment of

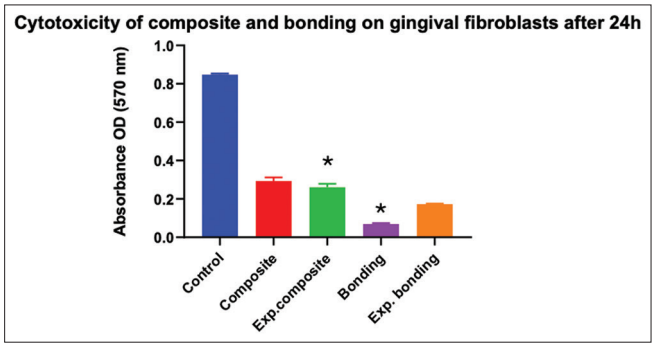

Figure 1: hGFs treated with composites, bonding, the expired composite (exp. composite), and expired bonding (exp. bonding) for 24 hours. Viability assay shows varying absorbance readings at 570 nm compared to control cells. Unpaired *t*-test shows statistical significance between the composite and expired composite ($P < 0.0371$). Similar statistics were found between bonding and expired bonding ($P < 0.0001$)

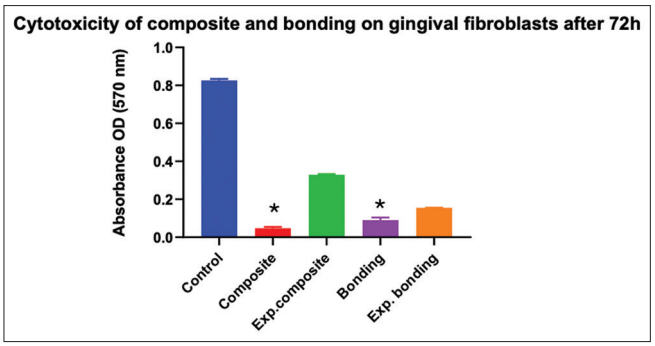

Figure 2: hGFs treated with composites, bonding, the expired composite (exp. composite), and expired bonding (exp. bonding) for 72 hours. Viability assay shows varying absorbance readings at 570 nm compared to control cells. Unpaired *t*-test shows statistical significance between the composite and expired composite ($P < 0.0001$). Similar statistics were found between bonding and expired bonding ($P < 0.0001$)

hGFs with composites for 24 hours demonstrated severe effects on cellular morphology as cells showed shrinkage and underwent cell death when compared to healthy hGF controls. However, the expired composite showed a lesser effect on cell morphology and proliferation as some cells underwent shrinkage with the presence of apoptotic bodies. Similar findings were observed when hGFs were treated with bonding, but the effect was less marked when compared to composites. Also, expired bonding showed less effect as the cells continued to grow and proliferate normally when compared to control cells.

At 72 hours, healthy hGF control cells continued to demonstrate normal cellular morphology. However, composites had a severe effect on hGFs, leading them to disintegrate and fragment, all of which are indicators of cell death [Figure 4]. While the expired composite started to show effects on hGFs cells, cells started to display shrinkage and the presence of apoptotic bodies are evident at 72 hours. In the context of bonding, this resulted in cellular death and cell shrinkage, as seen with composites, whereas in the case of expired bonding, hGFs just started to show indicators of the cell death process (cellular shrinkage and the presence of apoptotic bodies).

The wound healing assay is a common technique that studies cell migration and wound closure *in vitro*; when using hGFs cells, we can study the ability of cells to migrate and close an artificial gap created in a cell monolayer using sterilized inserts.[15] The results of this assay provide valuable insights into the migratory and proliferative abilities of hGF cells, which are crucial processes in the wound healing and tissue

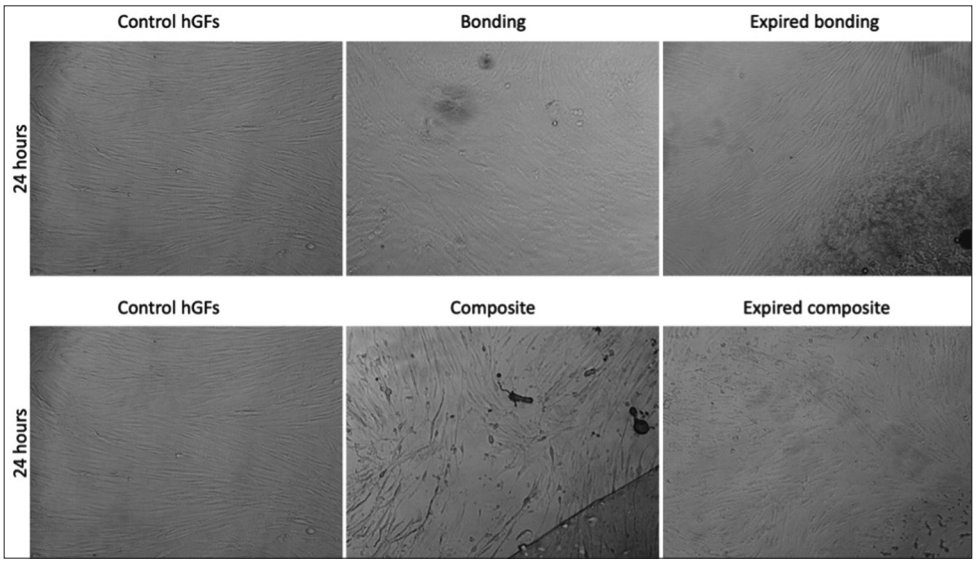

Figure 3: Treatment of hGFs with composites and bonding (expired and nonexpired) for 24 hours

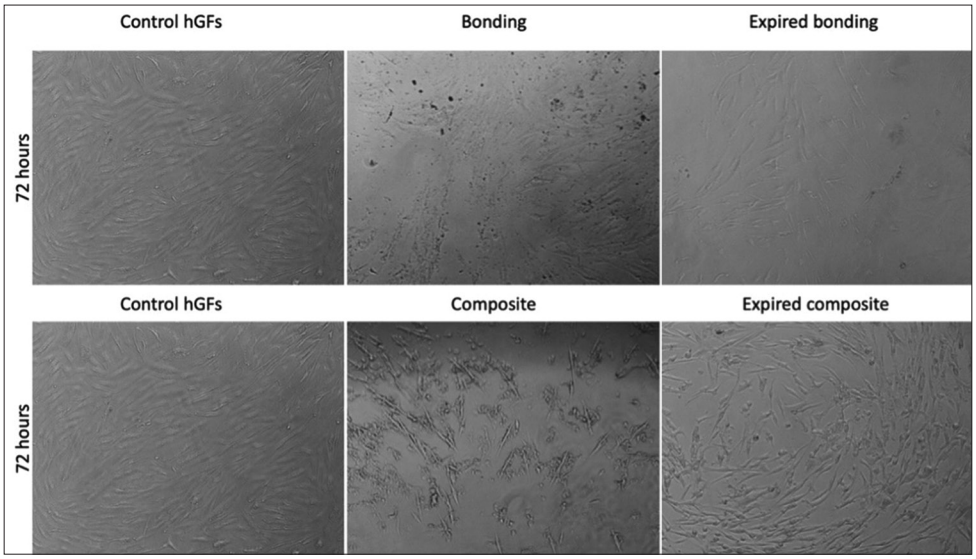

Figure 4: Treatment of hGFs with composites and bonding (expired and nonexpired) for 72 hours. It illustrates the significant impact of treating hGFs for 72 hours

repair mechanisms of the gingiva. Thus, we sought to investigate the effect of expired and nonexpired materials on the migration ability of hGF cells when a wound (scratch) is induced.

In Figure 5, the hGF migratory ability to close an artificial gap was tested after adding the materials of interest for 24 and 72 hours. Notably, at 0 hour, the wound (artificial gap) is similar in size for all conditions (control, composite, expired composite, bonding, and expired bonding). At 24 hours, control hGF cells were able to close the gap about more than half of original gap, which means the cells have higher ability to migrate. While in case of adding composites (nonexpired and expired) to cells, both affected the cell migratory ability by inducing apoptosis and shrinkage of cells; however, in expired composite conditions, hGFs were still able to migrate to close the gap more than in the case of campsite and slower than control hGF cells at 24 hours. The bonding agent in both conditions (unexpired and expired) was toxic on hGF cells and induced severe apoptosis, cell death, and fragmentation at 24 hours.

Finally, at 72 hours, control hGF cells were able to entirely close the gap, exhibiting a normal cellular shape and a greater capacity for migration. However, with composites, hGF cells showed the capacity for recovery, which was more visible in the expired composite, where hGF cells migrated to bridge the gap but with fewer cells than healthy control hGFs. The bonding agent continued to damage cells in both conditions (nonexpired and expired), causing severe cell death and fragmentation at 72 hours.

Discussion

Exploring the biocompatibility and viability of expired and nonexpired dental bonding materials is of clinical importance; hence, managing unused, expired, or nearly expiring dental supplies poses a significant challenge for dental practices. Our study's findings reveal significant impacts of both composites and bonding agents, regardless of expiration status, on the viability, morphology, and migratory abilities of hGF cells. After 24 hours, untreated hGFs exhibited expectedly higher viability, while those treated with composites, both expired and nonexpired, displayed a significant reduction in viability, nearly halving compared to controls. Bonding treatments, especially nonexpired, led to severe reductions in viability, surpassing even the impact of composite-treated cells. Notably, expired bonding showed increased viability compared to the nonexpired composite but still experienced a notable reduction of cell proliferation compared to control hGFs. A similar study has shown the cytotoxicity of direct interaction of composite material on hGF cells and pulpal cells at molecular levels.[16] Furthermore, the impact of different composite types such as BisGMA, TEGDMA, and UDMA on hGF cells has been reported to induce similar cytotoxicity to what we found in our results.[17]

In context of morphological changes, control hGFs maintained their typical spindle-shaped morphology without any apoptotic features. In contrast, treatment with composites for 24 hours induced severe cellular changes, including shrinkage and signs of cell death, with the expired composite displaying a milder effect.

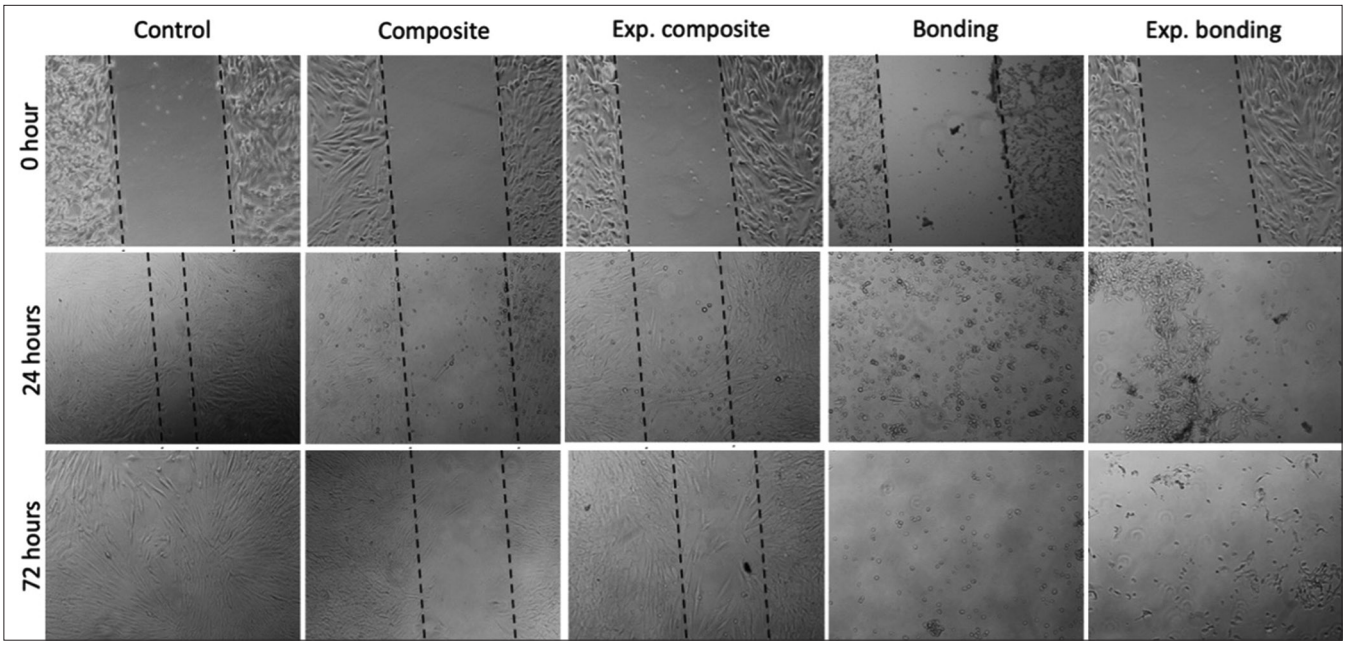

Figure 5: Wound (scratch) assay using hGF cells and treated with composites, bonding, the expired composite, and expired bonding for 24 and 72 hours

Similar trends were observed with bonding treatments, although the impact was less pronounced. Expired bonding showed the least effect on morphology, with cells displaying normal growth patterns compared to controls.

By the 72-hour mark, untreated hGFs continued to exhibit normal morphology and viability. However, composite treatments continued to induce severe reductions in viability and significant morphological changes, with the expired composite showing signs of effect. Similarly, bonding treatments resulted in cellular death and shrinkage, with expired bonding exhibiting early signs of cellular stress compared to controls. A study by Yang Y *et al*.[18] reported DNA double-strand breaks in hGF cells treated indirectly with composite eluates. Our study has shown severe cytotoxicity of direct contact of composites and hGF cells, which further support our findings.

In the wound healing assay, control cells demonstrated superior migratory capacity, with composite treatments hindering migration significantly, though the expired composite allowed for slower yet observable migration as a result of lost efficacy of active ingredients which are harmful for gingival health. Bonding agents, however, induced severe cellular damage, including apoptosis and fragmentation, regardless of expiration status. Overall, while expired agents displayed less damaging effects, they still adversely affected hGF behavior, suggesting careful consideration of their usage in clinical settings. To our knowledge, our study is the first to utilize wound healing assay in testing the effect of composites on the migratory ability of hGF cells. However, a study by Frese C *et al*.[19] showed a lower adhesion ability of hGF cells to different composite types and suggested limiting the use of composites in subgingival restorations, which support our findings of the effect of composites on reducing cellular adhesion and migration.

A limitation of our experimental study is the exclusive focus on assessing the biocompatibility of dental materials on a single cell type, namely, hGFs. However, the oral cavity mucosa, gingival tissue, and periodontal structures comprise a diverse array of cell types, including epithelial cells and various immune cells. Consequently, the interactions between dental materials and these complex cellular environments may differ significantly from our observations with hGFs alone. Therefore, future investigations should consider employing more comprehensive models, such as three-dimensional (3D) cell culture systems, to better mimic the multicellular and multidimensional nature of oral tissues.

Composites and bonding materials undergo a process of polymerization and are linked to the degree of conversion. As a result, the expiry of these materials is expected to have an impact on both polymerization efficiency and rate of conversion, resulting in the release of unreacted monomers, weakly cross-linked polymers, and the formation of reactive oxygen species.[20,21] These factors can have a major impact on cellular health, leading to cellular damage and toxicity.[22] As these were not investigated in our work, further *in vitro* investigations of these aspects are strongly advised.

Orthodontic treatment often involves the use of composite materials for bonding brackets or other attachments to teeth. Understanding the effects of bonding materials on gingival fibroblasts is crucial for assessing their impact on soft tissue health during orthodontic therapy. Our findings suggest that both expired and nonexpired composites and bonding agents can adversely affect gingival fibroblast viability and morphology. Using expired composites and bonding substances in orthodontic bracket bonding can lead to reduced bonding strength, frequent debonding, and poor treatment mechanics.[23] These challenges can lengthen treatment time, increase patient discomfort, and potentially cause long-term enamel damage.[24] Therefore, clinical training in orthodontics should emphasize the skills of applying these materials on dental structure solely, minimizing their proximity to the gingiva, and avoiding frequent debonds and rebonds of orthodontic brackets and other attachments, as possible. Nevertheless, the multieffects of using the expired composite and bonding materials in various restorative dental procedures have been reported to result in compromised mechanical strength, adhesion, and longevity of restorations.[25] These issues result in higher risks of clinical failure, secondary caries, pulpal inflammation, and overall poor patient outcomes.[26] For optimal results, all bonding materials must be utilized within their expiration date and preserve their intended chemical qualities.

Dental materials represent a substantial portion of the operating expenses in dental practice, and improper handling or storage can lead to financial losses due to expiration.[27] We highlight the importance of salvaging and utilizing the expiring bonding supplies in preclinical settings for dental students to use on artificial teeth during preclinical training, instead of discarding them. This proactive approach may optimize resource utilization, enhance cost-effectiveness, reduce the environmental burden of the excess dental material waste, and optimize sustainability.[28]

Conclusion

In conclusion, the study highlights the considerable impact of composites and bonding agents on the viability and morphology of hGF cells, regardless of their

expiration status. Treatment with these agents resulted in significant reductions in cell viability and induced morphological changes indicative of cellular stress and apoptosis. Interestingly, while expired variants showed some alleviation of these effects compared to their nonexpired counterparts, they still notably affected cell behavior. These findings underscore the importance of carefully selecting and monitoring the application of dental materials to minimize adverse effects on gingival tissues during orthodontic and dental procedures. Further research into the long-term effects of these materials on periodontal health and wound healing processes is warranted to optimize patient outcomes and ensure the safety and efficacy of clinical treatments.

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Conflicts of interest

There are no conflicts of interest.

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