Original Article

pISSN, eISSN 0125-5614
M Dent J 2025; 45 (2) : 81-92

# *In vitro* cytotoxicity evaluation of an experimental orthodontic adhesive containing gold nanoparticles

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**Objectives:** This study aimed to evaluate and compare the in vitro cytotoxicity of an experimental orthodontic adhesive containing 0.5% wt gold nanoparticles (AuNPs) with a conventional light-cured adhesive (Transbond XT) on human gingival fibroblast (HGF) cells using the MTT assay.

**Materials and Methods:** Both adhesives, comprising the AuNPs group and the Transbond group, were prepared and incubated in Dulbecco's Modified Eagle Medium (DMEM) under the same conditions with 20% concentration for 1, 7, 14, and 30 days. After each incubation, the whole medium was extracted for analysis, and the fresh medium was replenished at the same amount. Extracts were tested at concentrations of 20%, 2%, 0.2%, and 0.02%. HGF cells were seeded in 96-well plates 24 hours before cell exposure at each incubation and performing MTT assays. Cell viability was measured spectrophotometrically and analyzed using independent sample t-tests ($p<0.05$).

**Results:** At a 20% concentration, the AuNPs group exhibited significantly lower cell viability than the Transbond group across all time points, with severe cytotoxicity observed on Days 1 and 7, moderate on Day 14, and mild on Day 30. In contrast, the Transbond group consistently showed mild cytotoxicity. Both groups showed no cytotoxicity at lower concentrations (2%, 0.2%, 0.02%). Interestingly, at 2% and 0.2% concentrations, the AuNPs group had significantly higher cell viability than the Transbond group in most periods. A general trend of decreasing cytotoxicity over time was observed for both adhesives.

**Conclusions:** AuNPs adhesive showed higher cytotoxicity than the conventional adhesive at undiluted extract (20%) and both materials were non-cytotoxic at lower concentrations. These findings highlight the importance of concentration and aging in cytotoxicity outcomes and suggest that AuNPs adhesive may be biocompatible under clinical conditions. Further, in vivo studies are necessary to confirm the safety of AuNPs adhesive.

**Keywords:** 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), cell viability, cytotoxicity, gold nanoparticles (AuNPs), human gingival fibroblasts (HGF), orthodontic adhesive

**How to cite:** Lelapityamit T, Anuwongnukroh N, Dechkunakorn S, Wichai W, **Saengfai N**. *In vitro* cytotoxicity evaluation of an experimental orthodontic adhesive containing gold nanoparticles. M Dent J 2025;45(2): 81-92.

## Introduction

White spot lesions (WSLs), caused by enamel demineralization around orthodontic brackets, are among the most common side effects during fixed orthodontic treatment. This issue has led to a growing interest in modifying orthodontic adhesives with antibacterial agents to minimize the occurrence of WSLs [1]. Recently, the incorporation of nanoparticles into orthodontic adhesives offers a promising approach to enhance not only antimicrobial efficacy but also material properties [2]. However, some commonly used nanoparticles, such as silver (AgNPs), zinc oxide (ZnO), and copper oxide (CuO), have raised concerns about

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**Received:** 20 January 2025 **Revised:** 21 March 2025 **Accepted:** 28 March 2025

biocompatibility and demonstrated severe toxic effects in animal studies conducted in vitro [3-6].

Among the alternatives, gold nanoparticles (AuNPs) have emerged as a favorable candidate due to their superior biocompatibility, chemical stability, and antibacterial properties. AuNPs have been widely studied in medical applications and have shown relatively lower toxicity profiles than other metallic nanoparticles [7]. A previous study by Akarajarasrod *et al.* [8] indicated the antibacterial effect of experimental orthodontic adhesive containing AuNPs against *Streptococcus mutans* and *Streptococcus sobrinus*, the cariogenic bacteria that accumulate on metallic brackets. Despite these advantages, low clearance rates of AuNPs from circulatory systems and tissues may pose potential health risks [9]. Building upon that, the current study addresses the in-vitro biocompatibility of AuNPs-enhanced adhesives, particularly their cytotoxic effects, since these materials can release water-soluble components into saliva and the oral cavity, as well as direct interactions with nearby tissues such as the gingiva and periodontal ligaments [10].

Previous research on nanofilled dental adhesives has documented varying degrees of cytotoxicity. Even traditional composites without nanoparticles have shown some cytotoxic effects [11, 12]. Additionally, factors associated with AuNPs, such as particle size, shape, concentration, surface modifications, cellular uptake mechanisms, and toxicity response, may contribute to cytotoxicity [13]. This raises the question of whether nanofilled adhesives present a higher or lower biological risk.

This study selected human gingival fibroblasts (HGF), the first-line cells exposed to adhesives near the gingival sulcus, as the cell model to simulate real clinical exposure conditions. There are currently no studies in the literature addressing the biocompatibility of AuNPs adhesive. This study evaluates the cytotoxicity of experimental orthodontic adhesives containing AuNPs, compared with the conventional light-cured orthodontic adhesive Transbond XT on HGF using the MTT assay. Cytotoxicity will be assessed separately at varying concentrations, and trends over time will be observed descriptively. The results will provide a deeper understanding of the safety profile of AuNPs adhesive, which is critical for clinical acceptance and future development. The hypothesis was that there was no difference in the cytotoxicity level of AuNPs adhesive and conventional adhesive when tested on HGF.

## Materials and Methods

### Adhesive preparation

According to previous studies [8, 14], the composition of experimental light-cured orthodontic adhesives containing gold nanoparticles is listed as shown in Table 1. The centrifuge (Kubota) and food blender (Electrolux) were used to mix all compositions completely without any exposure to the light. Then, the experimental adhesive was degassed and stored in the nontransparent tube at room temperature before use. TransbondXT Light Cure Orthodontic Adhesive (3M, Unitek; Monrovia, CA, USA) will be a control adhesive for this study (Table 2). Both experimental and control uncured adhesive was pressed in the mold (thickness 1 mm and diameter 10 mm) between a mylar strip with a pressure of 500N. Then, light polymerization was done by VALO light curing device (Ultradent, Utah, USA, 1,400 mW/cm$^2$) for 12s (Figure 1).

Table 1 Experimental AuNPs adhesive composition

| List | Composition | Type of composition | Amount (wt%) |
|---|---|---|---|
| 1 | Bisphenol A glycerolate dimethacrylate (Bis-GMA) | Monomer | 20.65 |
| 2 | Triethylene glycol dimethacrylate (TEGDMA) | Monomer | 8.85 |
| 3 | Silanized barium borosilicate glass with particle size 0.7 micron | Filler | 69.5 |
| 4 | Gold nanoparticles (AuNPs) with spherical shape and particle size of 20 – 40 nm, stabilized by Cetyltrimethylammonium bromide (CTAB) Concentration = 3 mg/ml | Filler | 0.5 |
| 5 | Diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide (TPO) | Initiator | 0.5 |

(1-3 from Essington, PA, USA; 4 from the Department of Biochemistry, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand; 5 from St. Louis, MO, USA)

Table 2 Transbond™ XT adhesive composition

| List | Composition | Type of composition | Amount (wt%) |
|---|---|---|---|
| 1 | Bisphenol A Diglycidyl Ether Dimethacrylate (BIS-GMA) | Monomer | 14 |
| 2 | Bisphenol A Bis (2-Hydroxyethyl Ether) Dimethacrylate (BIS-EMA) | Monomer | 9 |
| 3 | Silane-treated quartz, Silane-treated silica | Filler | 77 |
| 4 | Camphorquinone (<1wt%), Other (<1wt%) | Initiator | <1 |

## Sample preparation

Both adhesives were then prepared for the test based on the International Organization for Standardization 10993-12: 2021 [15] and 10993-5: 2009(E) [16] by weighing Sample A (AuNPs group) at 1.043 g and Sample B (Transbond group) at 1.025 g. Both samples were sterilized using an autoclave at 121°C for 15 minutes. Subsequently, the samples were immersed in 5.215 mL DMEM, allocated for each sample to ensure a sample concentration of 20%. Then incubated in a 5% $CO_2$ at 37°C with 95% relative humidity for periods of 1, 7, 14, and 30 days. At the end of each incubation period, the total volume of the cell culture medium was extracted and stored for further analysis, while the fresh medium was replenished for continued incubation. The extract samples with 20% concentration were then diluted to achieve concentrations of 2%, 0.2%, and 0.02% for subsequent testing. Both sample groups underwent testing under these conditions (Figure 1).

## Cell preparation

A monolayer culture of human gingival fibroblast (HGF) cells (Lot No. 7009862) was established by suspending the cells at a concentration of $1 \times 10^5$ cells/mL in complete Dulbecco's Modified Eagle Medium (DMEM). A volume of 200 µL of the cell suspension, equivalent to $2 \times 10^4$ cells per well, was seeded into each well of a 96-well culture plate. The plates were then incubated in a $CO_2$ incubator at 37°C, with 95% relative humidity and 5% $CO_2$, for 24 hours to allow cell attachment and growth (Figure 1).

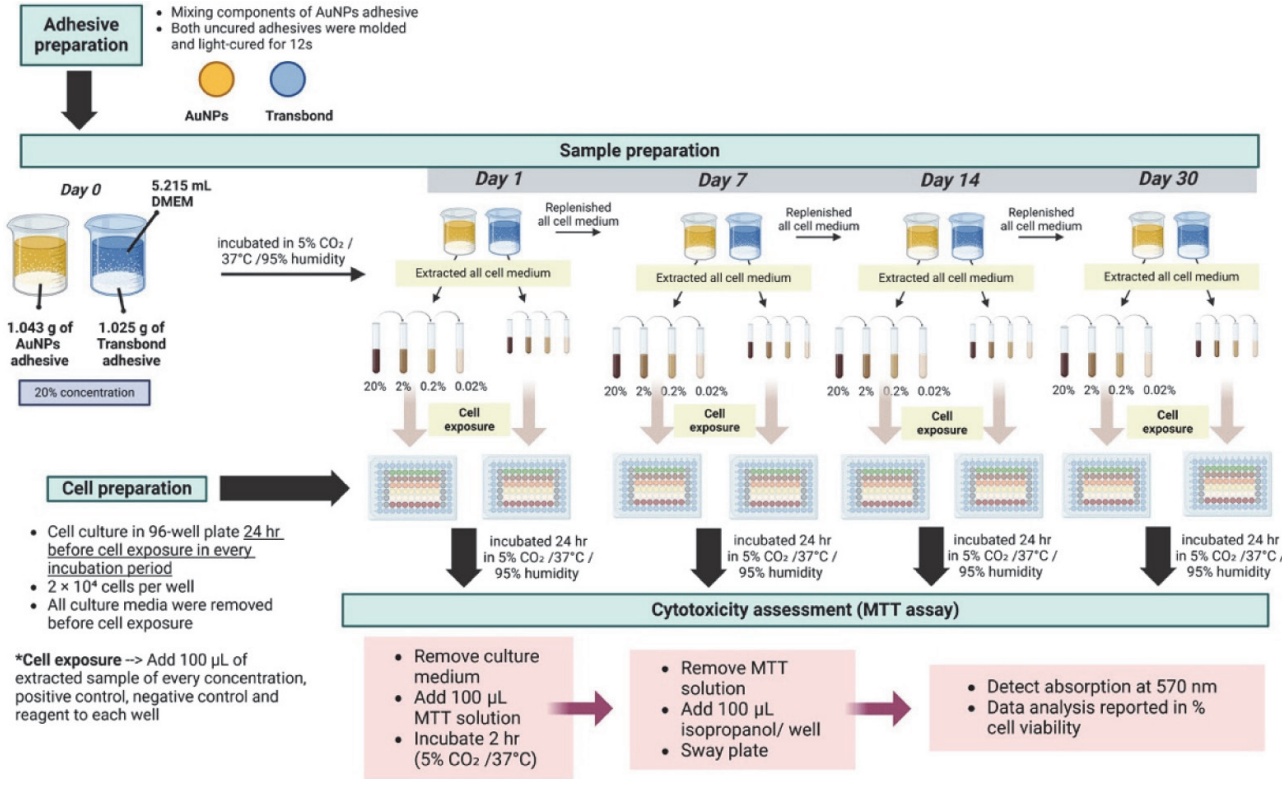


Figure 1 shows a schematic diagram of the research method from adhesive preparation to proliferation assay (created with BioRender)

## Cytotoxicity Assessment

This research was performed in a controlled in-vitro environment and approved by the Institutional Review Board, Faculty of Dentistry/ Faculty of Pharmacy, Mahidol University (COE.No.MU-DT/PY-IRB 2022/055.0212). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to evaluate the adhesive toxicity on HGF cell lines. A 96-well plate was divided into the AuNPs group and the Transbond group. Each plate (Figure 2) contained the samples and diluted samples at four concentrations (N=8/ concentration), the reagent control, which consisted of a cell culture medium (N=12), a negative control, Thermanox Plastic Coverslips (Nunc™ Naperville, IL, USA, Lot No. 600562) (N=8), with an extraction ratio of 6 $cm^2$/mL, and soaked in the extraction medium, and positive control, 0.1% ZDEC Polyurethane Film (RM-A, Lot No. A-223K) (N=8), with an extraction ratio of 6 $cm^2$/mL. Each well received 100 µL, which was exposed to cells and incubated in a 5% $CO_2$ at 37°C with 95% relative humidity for 24 hours before the MTT assay.

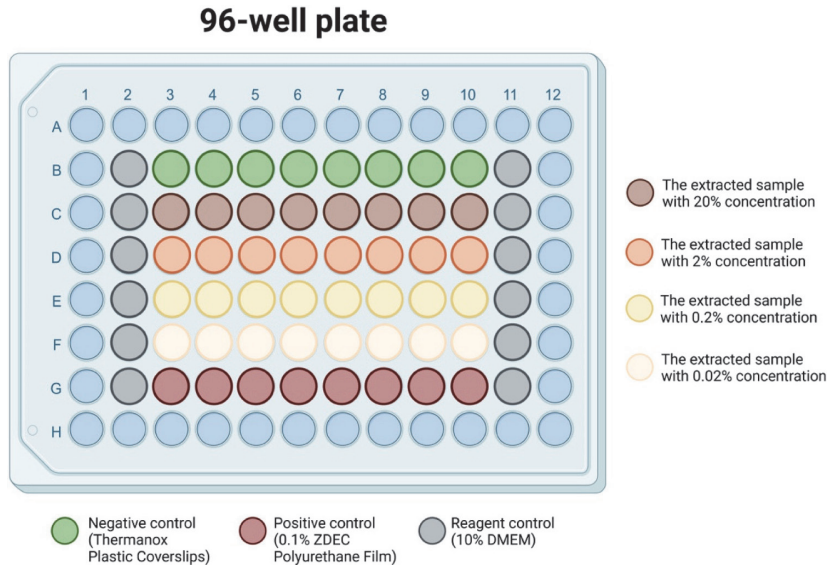


Figure 2 shows the preparation of a 96-well plate for cell exposure 24 hours prior to MTT assay at each incubation period (created with BioRender)

After incubation, the extract from all groups was removed, and the cells were washed once with PBS (1x). 100 µL of 0.1% MTT solution, dissolved in DMEM without supplements or phenol red, was added to each well. The plates were then incubated in the $CO_2$ incubator under the same conditions for 2 hours. Then, the MTT solution was removed, and the cells were washed once with PBS (1x). Subsequently, 100 µL of isopropanol was added to each well and shaken for 30 minutes. The absorbance at 570 nm was measured using a microplate reader. A reduction in the number of viable cells leads to a corresponding decrease in the metabolic activity of the sample. This reduction is directly proportional to the amount of blue-violet formazan produced after being dissolved in alcohol, which is quantified by measuring the mean value of the optical density (OD) at 570 nm. The following equation was employed to determine the percentage reduction in cell viability relative to the blank control (reagent control) (Figure 1).

$$\text{Cell viability (\%)} = \left(\frac{OD \text{ of the test sample}}{OD \text{ of the blanks}}\right) \times 100\%$$

Cell viability was assessed based on the Dahl index [17, 18] as follows:

- Over 90% cell viability: no cytotoxicity
- Between 60%-90% cell viability: mild cytotoxicity
- Between 30%-59% cell viability: moderate cytotoxicity
- Less than 30% cell viability: severe cytotoxicity

## Statistical Analysis

The data, initially organized in Microsoft Excel, was imported into SPSS software, Version 29 (SPSS Inc., Chicago, IL, USA). Descriptive statistics, including mean and standard deviation, were used, and normality was evaluated through the Shapiro-Wilk test, confirming a normal distribution. An Independent sample t-test was employed to compare cytotoxic activity outcomes and to determine significant differences in cell viability between groups of each pre-incubation period assessed separately at varying concentrations. A significant level of $p<0.05$ was applied for all comparisons.

## Results

Table 3 shows the MTT assay results for both groups of orthodontic adhesives. At a 20% concentration, the AuNPs group demonstrated significantly lower cell viability percentages than the Transbond group across all time periods. The AuNPs group exhibited severe cytotoxicity on Day 1 and Day 7, moderate cytotoxicity on Day 14, and mild cytotoxicity on Day 30. In contrast, the Transbond group consistently showed mild cytotoxicity levels throughout all time periods.

Cell viability was significantly increased when the sample was diluted into 2%, 0.2%, and 0.02% concentrations. No cytotoxicity was represented in every incubation period in both groups. The AuNPs group exhibited significantly higher cell viability percentages than the Transbond group at a 2% concentration at all periods, at 0.2% concentration at all periods except day 30, and at 0.02% concentration at day 7 and day 14. For both adhesives, cytotoxicity progressively diminished over time (Figure 3).

**Table 3** Descriptive statistics and independent sample T-test of % cells viability in each sample concentration and incubation period

| % Cells Viability | | | | |
|---|---|---|---|---|
| Sample concentration | Incubation period | AuNPs adhesive (mean ± SD) % | Transbond adhesive (mean ± SD) % | Significance p-value |
| 20% | Day 1 | 10.27 ± 0.54 | 63.13 ± 0.93 | 0.000* |
| | Day 7 | 12.68 ± 0.76 | 64.58 ± 3.54 | 0.000* |
| | Day 14 | 32.88 ± 1.81 | 65.83 ± 1.60 | 0.000* |
| | Day 30 | 60.11 ± 3.54 | 71.47 ± 1.73 | 0.000* |
| 2% | Day 1 | 99.95 ± 4.09 | 90.18 ± 5.20 | 0.001* |
| | Day 7 | 100.19 ± 2.21 | 90.54 ± 1.47 | 0.000* |
| | Day 14 | 103.80 ± 2.21 | 91.87 ± 0.64 | 0.000* |
| | Day 30 | 104.94 ± 0.97 | 92.15 ± 0.40 | 0.000* |
| 0.2% | Day 1 | 100.19 ± 2.07 | 95.53 ± 2.07 | 0.001* |
| | Day 7 | 100.67 ± 2.41 | 96.05 ± 4.29 | 0.019* |
| | Day 14 | 107.14 ± 2.83 | 100.17 ± 5.14 | 0.005* |
| | Day 30 | 107.32 ± 3.02 | 106.29 ± 2.55 | 0.472 |
| 0.02% | Day 1 | 101.24 ± 6.20 | 98.26 ± 4.63 | 0.295 |
| | Day 7 | 102.10 ± 3.00 | 99.13 ± 0.48 | 0.027* |
| | Day 14 | 107.84 ± 2.60 | 100.42 ± 3.74 | 0.000* |
| | Day 30 | 108.51 ± 4.29 | 106.80 ± 1.74 | 0.322 |

* Significant = $p < 0.05$

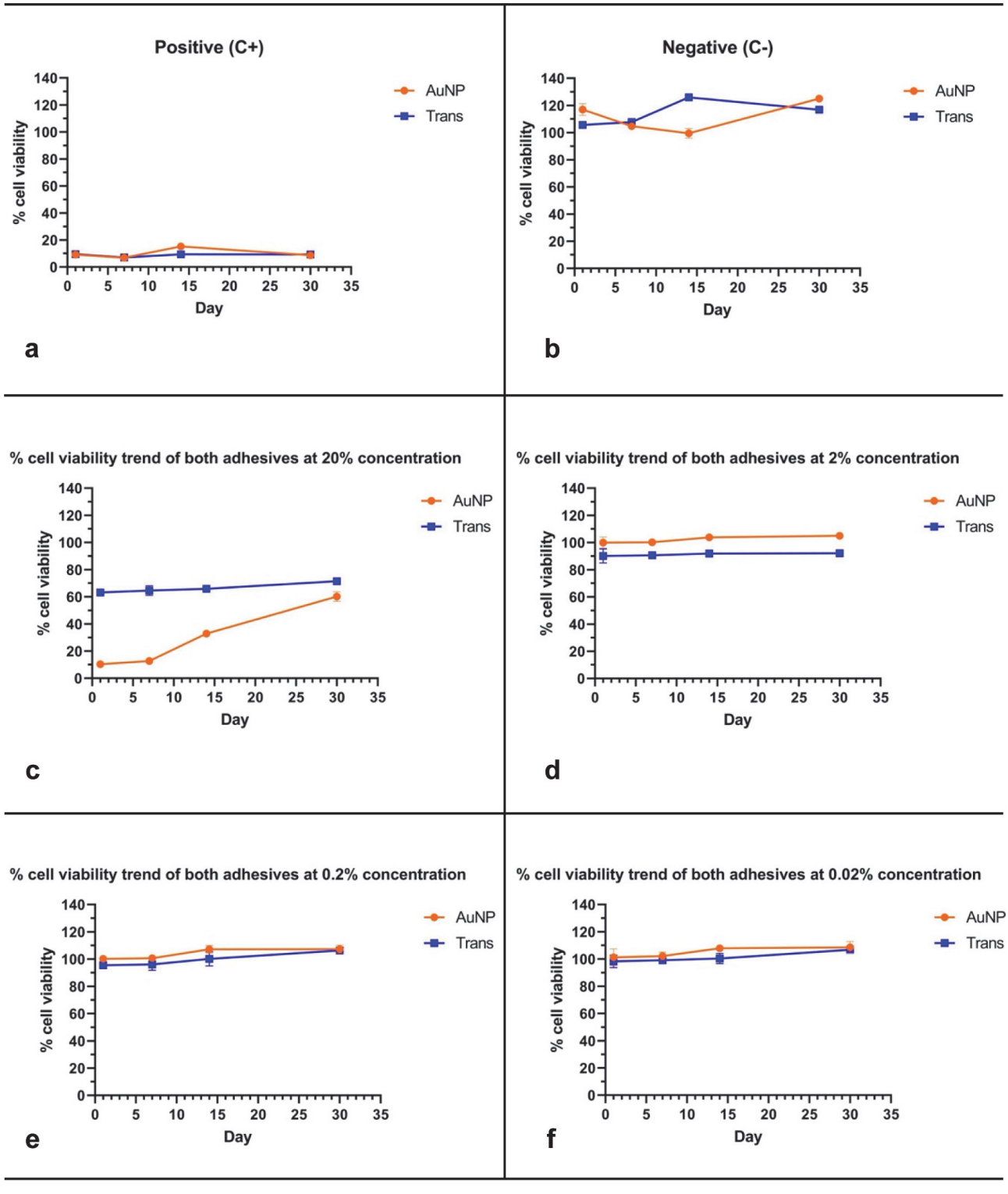


Figure 3 shows percent cell viability trend of human gingival fibroblast (HGF) cells (mean values) over time following exposure to: (a) positive control, (b) negative control, and extract dilutions of AuNP-containing adhesive and Transbond adhesive at (c) 20%, (d) 2%, (e) 0.2%, and (f) 0.02% concentrations.

## Discussion

In this study, 0.5 wt% AuNPs was selected for the investigation of cytotoxicity based on outcomes from a previous study by Akarajarasrod *et al.* [8] , which demonstrated that this concentration exhibited effective antimicrobial activity, a soft color, and rapid setting time compared to adhesive-containing 1.0 wt% AuNPs. This study evaluated the in vitro biocompatibility of Orthodontic adhesive containing gold nanoparticles compared to Transbond using the MTT assay method. The AuNPs adhesives showed significantly higher cytotoxicity on day 1, day 7, and day 14. However, the final cytotoxic level of the AuNPs group was shown at the same level as the Transbond group on day 30, and no cytotoxicity was reported in both groups at lower concentrations, which accepted the null hypothesis.

The variations in the biocompatibility of the adhesives observed in this study may be attributed to differences in their composition. The relatively high cytotoxicity of AuNPs adhesive can be attributed to a mixture of various monomers such as bisphenol A diglycidyl dimethacrylate (Bis-GMA) and triethylene glycol dimethacrylate (TEGDMA) [19]. For Transbond adhesive, the cytotoxicity is linked to the presence of bisphenol A-ethoxylate dimethacrylate (Bis-EMA) in its composition according to Malkoc et al [12]. Research has shown that cured bonding resins retain notable amounts of unpolymerized monomers and short-chain polymers within their structure [20, 21]. The release of these residual substances is believed to be the primary factor contributing to the short-term cytotoxic effects of composite resins [22, 23]. The biodegradation of composite resins elutes leachable substances, which can contribute to toxic effects similar to those caused by the original monomers [24]. Bis-GMA was identified as releasing at the highest level among several time points in a literature review by Gorgen et al. [25] , and it was compared to TEGDMA, Bis-EMA, and UDMA [26]. The higher cytotoxicity level in the AuNPs group was associated with a higher amount of Bis-GMA in the adhesive (Table 1 & 2). Moreover, lower molecular weight monomers like TEGDMA can be easily released [20] and persist for 32 days [27], which revealed a mild cytotoxic effect on Day 30 in the AuNPs group. In contrast, Bis-EMA, which is included in Transbond XT, has a higher molecular weight than Bis-GMA and TEGDMA, which reduced mobility, potentially contributing to the lower cytotoxicity observed in that group.

The cytotoxicity of gold nanoparticles (AuNPs) can be influenced by various factors, including their size, shape, surface properties, and concentration. These characteristics can compromise cell membrane integrity, a common issue with metallic nanoparticles [13]. Due to their high surface area-to-volume ratio, nanoparticles tend to exhibit more pronounced toxic effects compared to larger particles [11]. This study used spherical AuNPs ranging from 20 to 40 nm in diameter, with cetyltrimethylammonium bromide (CTAB) as a stabilizer to prevent particle aggregation. Among different morphologies, gold nanospheres and nanorods have been reported to display higher toxicity [13]. CTAB, a frequently used surfactant in AuNP synthesis, has also linked to cell membrane disruption, leading to structural rearrangements and resulting in cell death [28].

Both adhesives in this study show an increasing trend in % cell viability, which aligns with previous studies by Malkoc *et al.* [12] and Ahrari *et al.* [22], which both reported time-dependent reductions in cytotoxicity

of orthodontic adhesives. Malkoc *et al.* demonstrated that freshly cured orthodontic composites exhibited higher cytotoxicity on fibroblasts, while Ahrari *et al.* similarly observed an initial cytotoxic response that diminished with time. This reduction is attributed to aging effects, in which longer pre-incubation periods lead to the complete polymerization of adhesives and decreased monomer release, thereby lowering the concentration of cytotoxic components in the extract, supported by many studies [27, 29-31]. Solanki *et al.* [27] also reported decreasing cytotoxicity levels on both HGF cells and L929 cells after 48 hours of exposure in a systematic review.

This study demonstrated that using a lower concentration of both adhesives resulted in reduced cytotoxicity effects with no observable cytotoxicity. However, it is crucial to emphasize that in vitro cytotoxicity tests have inherent limitations and do not fully mimic the complex oral environment or biological consequences. In cell culture, the cell surface directly binds and absorbs chemicals, leading to exaggerated toxic outcomes. Conversely, real-life exposure in the oral cavity involves dynamic factors such as saliva flow, enzymatic activity, tissue barriers, and mechanical dilution [32], which reduce the actual concentration of leachable substances like monomers or 0.5 wt% AuNPs [33]. The use of 20% extract in MTT assays represents a worst-case scenario, while lower tested concentrations (2%, 0.2%, 0.02%) may more closely reflect potential clinical exposure, considering the rapid dilution capacity of saliva, which ranges from 0.3 to 7 mL/min [34]. In vitro tests are thus best viewed as “accelerated models” to detect early cellular responses under controlled conditions [35]. While they are valuable for standardizing results and comparing materials, caution must be taken when referring to in vivo contexts. Further research, including animal studies, salivary simulation models, toxicokinetic analyses, and clinical trials, is needed to more accurately assess the safety of AuNP adhesives in clinical applications.

For the selection of cells and method in this study, HGF cell lines were utilized because they are the predominant cell type in gingival connective tissue [36] and are the first to be exposed to orthodontic adhesives placed near the gingival sulcus, especially when excess material extended subgingival [37]. Additionally, HGFs have high metabolic activity and sensitivity to leachable substances [38], making them a relevant model for cytotoxicity evaluation. Some studies particularly used L929 fibroblasts due to ease of cultivation and consistent biological response. However, a shorter lifespan and slower growth present certain challenges [14, 36]. The widely used MTT assay technique was also utilized in this study, as it is considered a reliable and efficient method for assessing cell viability, as present in several studies [38, 39].

Incorporating nanoparticles into orthodontic adhesives offers antibacterial properties that can help reduce plaque formation and caries risk. However, the use of nanomaterials also introduces potential biological hazards since orthodontic adhesives come into contact with gingival and oral tissues, ensuring their biocompatibility is essential for safe clinical application. The results of this *in vitro* study highlight that while AuNPs adhesive exhibit higher initial cytotoxicity compared to conventional orthodontic adhesives at higher concentrations, their biocompatibility improves significantly over time and with dilution. These findings support further exploration into developing orthodontic adhesives that balance antimicrobial performance with improved biocompatibility. Specifically, optimizing nanoparticle concentration, modifying their

surface, shape, and size, and refining adhesive chemistry could reduce early cytotoxic effects. In clinical settings, particular care should be taken to eliminate excess adhesives, especially in subgingival and interproximal areas. Further research is needed to explore additional properties of AuNPs adhesive, especially in vivo experiments and evaluations of mechanical properties to ensure their safety, efficacy, and functional durability in the complex oral environment.

## Conclusion

The cytotoxicity level of AuNPs adhesive is higher than conventional adhesive at 20% concentration. However, no cytotoxicity was observed at lower concentrations in both groups. The findings of this research will contribute to understanding the safety profile of these advanced materials and guide their development forfurther investigation and clinical use in orthodontics.

### Abbreviations

AuNPs: Gold nanoparticles; DMEM: Dulbecco's Modified Eagle Medium; HGF: Human Gingival Fibroblasts; MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide

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