Annals of Clinical and Analytical Medicine
Original Research

In vitro evaluation of the cytotoxic effects of alkasite restorative material on human dental pulp stem cells

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Abstract

Aim: The clinical suitability of restorative dental materials is determined by evaluating their biocompatibility, and physical and chemical properties. The present study examined the cytotoxic effects of three different dental restorative materials on human dental pulp stem cells (DPSCs).

Material and Methods: In this study, composite, high-viscosity glass ionomer cement (HVGIC), and an alkasite were used. In total, 12 samples of each material were prepared for cytotoxicity assays. Cytotoxic effects were determined by considering biomaterial releases. Cell viability and proliferation were observed and analyzed at intervals of 24 and 72 hours using both the methyl-thiazole-diphenyl-tetrazolium (MTT) and xCELLigence cytotoxicity assays. Data were calculated using the RTCA-DP integrated software of the xCELLigence system and the GraphPad Prism 9.1.1 program. Data from the proliferation experiments were statistically evaluated using the Two-way ANOVA test.

Results: Alkasite exhibited the highest cytotoxicity, whereas HVGIC and composite did not exhibit any significant difference compared with the control. There was no difference between the two time points in the cytotoxicity of composite and alkasite in the MTT assay. However, the cytotoxicity of HVGIC was higher at 72-hours than at 24-hours. Similar results were obtained with both assays. Although alkasite exhibited higher cytotoxicity than composite and HVGIC, all materials exerted slightly cytotoxic effects (60%–90% cell viability) on DPSCs.

Discussion: Considering its aesthetic, and mechanical properties, alkasite can be clinically preferred instead of other materials in cavities that are not close to the dental pulp.

Keywords

Alkasite, Composite, Glass Ionomer Cement, Cytotoxicity, Dental Pulp

DOI: 10.4328/ACAM.21846 Received: 2023-08-05 Accepted: 2023-09-06 Published Online: 2023-09-12 Printed: 2023-11-01 Ann Clin Anal Med 2023;14(11):1044-1048
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This study was approved by the Ethics Committee of Biruni University (Date: 2019-11-07, No: 2019/34-12)

Introduction

Restorative materials should exhibit good mechanical properties, biocompatibility, and fluoride-releasing ability [1]. Amalgam, composites, compomers, and glass ionomer cements represent some common dental materials used for the restoration of primary and permanent dentition [2]. Amalgam has been used most commonly for many years as it exhibits good, long-lasting biomechanical properties; however, the presence of mercury in its composition and its non-aesthetic properties have made it less popular among dental patients lately [3]. In contrast, first developed in 1962, composite resins meet the increasing aesthetic and masticatory needs of patients while also exhibiting good retention [4].

The popularity of fluoride-releasing dental materials has increased recently because of their role in caries prevention. In this regard, glass ionomer cements are considered particularly advantageous because they are biocompatible, release fluoride, and chemically bond to enamel and dentin [5]. However, they also have certain disadvantages, such as sensitivity to moisture and poor mechanical strength. Although resin- modified and HVGICs can minimize these disadvantages, further improvement is necessary [6].

Cention N (Ivoclar Vivadent, Liechtenstein) is a newly developed dental material and is classified as an "alkasite," which has been defined as a subgroup of composite materials. Cention N is an aesthetic and highly resistant material, which is particularly suitable for the posterior region of oral cavity. The presence of alkaline fillers in its composition enables the release of fluoride, calcium, and hydroxyl ions that can prevent demineralization and enhance remineralization. Therefore, the manufacturer of Cention N states that it combines the best properties of amalgam and glass ionomer cement. Cention N is a urethane dimethacrylate (UDMA) based self-curing restorative material with optional additional light-curing [7].

In addition to the physical and chemical properties of restorative materials, their clinical suitability is determined by their biocompatibility the ability of a material to create an appropriate biological response around application [8]. It is crucial that restorative materials used in clinical practice do not cause systemic or local cytotoxicity in the oral mucosa, gingiva, and pulpal tissues adjacent to the material [9].

This study aimed to evaluate the cytotoxicity of a newly developed alkasite restorative material on DPSC using methyl-thiazole-diphenyl-tetrazolium (MTT) and xCELLigence assays and compare this with a composite and a high-viscosity glass ionomer cement (HVGIC) routinely used in the clinic. To the best of our knowledge, this is the first study to examine the cytotoxic effect of alkasite material on dental pulp stem cells. Our two hypotheses were that the cytotoxicity of alkasite is higher than that of HVGIC and like that of composite.

Material and Methods

This study was approved by the Ethics Committee of Biruni University (Date: 07-11-2019, No: 2019/34-12) and was conducted in accordance with the World Medical Association Declaration of Helsinki.

Dental materials tested in this study

This study examined the cytotoxic effects of a composite (Gradia Direct, GC Europe, Belgium), a HVGIC; Equia Forte, GC Europe, Belgium), and an alkasite restorative material (Cention N, Ivoclar Vivadent, Liechtenstein) on DPSC.

Preparation of samples

In total, 12 specimens of each material were prepared under sterile conditions in a laminar flow chamber (Heal Force, China) and placed into cylindrical Teflon molds (5.0 mm diameter × 2.0 mm height). Thereafter, the lower and upper surfaces of the materials were covered with transparent matrix tape to prevent the formation of an oxygen inhibition layer; the polymerization phase was initiated by placing the Teflon molds between two glass coverslips to remove excess material and prevent air bubble formation. The materials were cured or set in accordance with the manufacturers' recommendations. An amalgamator device (GC Europe, Belgium) was used to mix materials in capsule form, and a light device (Elipar™ S10; 3M ESPE, St. Paul, MN, USA) was used to polymerize light-cured restorative materials. The biomaterials were sterilized using ultraviolet light for 30 minutes.

Cell culture and experimental design

Human DPSCs (CELPROGEN, 36086-01, USA) were supplied as a cell line and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO, USA), 100 U / mL penicillin / streptomycin (Sigma Aldrich, St. Louis, MO, USA), 100 U / mL L-glutamine (Sigma Aldrich, St. Louis, MO, USA), and 100 U / mL sodium pyruvate at 37°C under 5% CO2 humidified air. Third passage DPSCs were detached using a 0.05% trypsin–EDTA solution (Sigma Aldrich, St. Louis, MO, USA) and a monolayer was cultured at a concentration of 5 × 105 in 25 cm flask containing DMEM medium.

A total of 12 samples of each material were divided into three subgroups containing four samples each. Freshly prepared samples were placed in 10 ml DMEM and incubated for 24 and 72 hours to obtain eluates.

Determination of cell viability using MTT assay

Cytotoxic effects of the three tested materials on cell viability and proliferation were evaluated using the MTT assay (Sigma Aldrich Inc., St. Louis, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a stable tetrazolium salt. The production of NAD(P)H in the glycolytic pathways of living cells can decrease and cause formation of formazan crystals, the concentration of which is directly proportional to the number of living cells at the end of the experiment [10].

In this study, 3×10^4 cells were grown, placed on 96-well plates, and incubated at 37°C for 24 hours. The next day, 100 µl of different concentrations of the medium in which the biomaterials were stored for 24 and 72 hours were applied to the 96-well plates. To allow examination of the effects of these substances on cell viability over time, 10 µl of MTT was added 24 hours after the application of the medium and left to incubate for 4 hours at 37°C in the dark. Thereafter, 100 µl of the solubilization solution was added to each well and the plate was kept in the incubator overnight. The absorbance

(optical density) of the samples was measured using a spectrophotometer (ELISA reader) at 590 nm.

Proliferation and cytotoxicity assay of DPSCs using xCELLigence assay

The xCELLigence system (Roche Applied Science, and ACEA Biosciences) was used to assess the survival of DPSCs upon exposure to various dental materials over time. Physiologic changes in the cells were identified and measured by the electronic impedance of the sensor electrodes. This real-time monitoring system provides quantitative information on the biological status of cells, including cell number, viability, and morphology; the cell index system displays the relative changes in the electrical impedance.

By following the procedure, 200 µL of the cell suspensions were seeded into a 16-well E-plate (30,000 cells/well; well volume: 250 µL; base diameter of well: 5 mm) in a laminar flow cabinet, placed in the incubator at 37°C and 5% CO2, and monitored using the RTCA-DP system at 15-minute time intervals for up to 72 hours with or without dental materials. Control samples received only medium and, in accordance with the xCELLigence technical manual, at least three repetitions of each experimental condition were performed to facilitate statistical evaluation [10].

Statistical analysis

Data were calculated using the RTCA-DP integrated software of the xCELLigence system and the GraphPad Prism 9.1.1 (GraphPad Software, Inc) program. Data from the proliferation experiments were statistically evaluated using the two-way ANOVA test, and a p-value of <0.05 was considered statistically significant.

Ethical Approval

Ethics Committee approval for the study was obtained.

Results

Alkasite treated DPSCs had low cell proliferation rates related to the MTT assay after 24 h

The MTT cytotoxicity assay was used to observe and analyze cell survival at 24 and 72 hours intervals. All restorative materials exhibited varying levels of cytotoxicity against DPSCs, and alkasite samples showed significantly lower cell proliferation after 24 hours than the HVGIC sample (p = 0.002). Although the alkasite and composite samples exhibited no significant changes in DPSC proliferation over time (24–72 hours), the HVGIC samples demonstrated a significant decrease (79.7%) in proliferation rates after 72 hours (p = 0.01) (Figure 1).

No statistically significant difference was observed between the materials and the control after 72 hours; however, all materials in this study exhibited numerical decreases in cell viability. Therefore, they were found to be slightly cytotoxic (Table 1).

XCELLigence assay results

The effects of the HVGIC, alkasite, and composite samples on DPSC cells were monitored for 72 hours using a real-time cytotoxicity analysis system. Cell index values increased from 0.23 before the application of HVGIC and composite samples to the DPSC cells to 0.51 24 hours after the application. In contrast, the cell index value after alkasite application was 0.35. The cell index value increased in the first hour after applying of the release medium of alkasite (24th hour); however, the cells reached a plateau after that (25th hour), and the cell viability decreased compared with the control.

Monitoring the viability of cells released from the HVGIC and composite samples and the applied medium for 72 hours using the xCELLigence system showed that the cell index values were similar to that of the control, with continuous cell growth and proliferation cessation being observed after a while. A significant decrease in DPSC proliferation was observed in the

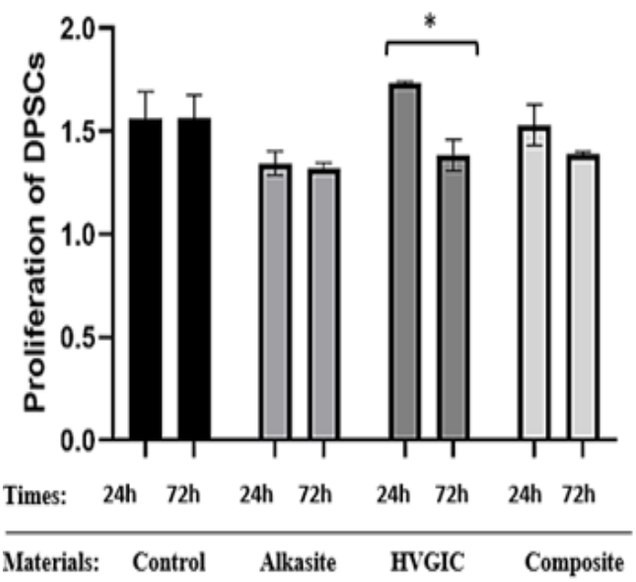

Figure 1. Change in DPSC proliferation by time, observed with MTT.

Table 1. Change in DPSC proliferation by time, observed with MTT.

Time	CTRL		Alkasite			HVGIC			Composite		
	Total amount of cells/µm²	Viability (%)	Total amount of cells/µm²	Viability (%)	p value	Total amount of cells/µm²	Viability (%)	p value	Total amount of cells/µm²	Viability (%)	p value
24 h	1.467	100	1.305	88.9	0.136	1.73	117.9	0.277	1.46	99.5	0.9888
72 h	1.484	100	1.339	90.2	0.079	1.33	89.6	0.003*	1.396	94.1	0.2731

Table 2. Change in DPSC proliferation by time, observed using xCELLigence system.

Time	CTRL		Alkasite		p value	HVGIC		p value	Composite		p value
24-hour impedance values	0.5139	0.6915	0.3094	0.3406	0.0064*	10.345	0.9573	0.0007*	0.7203	0.847	0.059
72-hour impedance values	0.5961	0.6027	0.154	0.1497	0.0003*	0.4867	0.4662	0.2329	0.4592	0.4662	0.1699

alkasite group after 24 and 72 hours compared with the control ($p < 0.01$), whereas a significant increase was detected in the HVGIC and composite groups after 24 hours compared with the alkasite group. Although no statistically significant difference was noted between the HVGIC and composite groups after 24 hours, the increase in proliferation observed in the HVGIC group compared with the control was statistically significant. Alkasite demonstrated the highest cytotoxicity on cell viability after 72 hours (Table 2).

No differences were observed in the cytotoxicity effects of alkasite restorative material on DPSCs, when comparing the two time points, whereas both HVGIC and composite exhibited a significant increase in cytotoxicity at 72 hours compared with 24-hour treatment of the materials.

Discussion

Dental materials, which have better mechanical and chemical properties, are gradually developed for more aesthetic and long-lasting restorations of decayed teeth. In addition to having good mechanical, chemical, and aesthetic properties, dental materials should also exhibit suitable biocompatibility as they can directly or indirectly affect the surrounding structures via the substances they release during and after setting. These leachable substances can migrate through the dentinal tubules and damage the dental pulp, highlighting the importance of understanding the cytotoxic effects of restorative dental materials in deep cavities [11-13]. The present study examined and compared the cytotoxicity of a newly developed alkasite material, HVGIC, and composite on DPSCs and found that all three materials exhibited slightly cytotoxic effects, although this was statistically significant only in the alkasite group.

The number of ions and residual monomers released from the material, its composition, and the concentration of filler particles play a role in its cytotoxicity [14]. The present study found no statistically significant differences in cytotoxicity among the control, HVGIC, and composite, although the alkasite material demonstrated significant cytotoxic effects. This may be attributed to the chemical composition of the material: in contrast to composites that released only monomers and HVGICs that released only ions, alkasites were capable of releasing both (particularly, UDMA and fluoride), which potentially increased their cytotoxicity. In addition, previous studies have found that the amount of residual monomer increases with higher filler content in resin-based materials, thus decreasing the cell proliferation [15-22]. The filler content of the alkasite restorative material (78.4%) was higher than that of the composite (73%) examined in this study, which might have resulted in greater cytotoxic effects. To the best of our knowledge, only a single previous study by Awad et al. [16] has investigated the effects of alkasites on human gingival fibroblast cells and found greater cytotoxicity than that of composites. The results of the present study were in accordance with this.

Da Silva et al. [23] have suggested that a material is considered nontoxic or slightly toxic if the cell viability exceeds 90% or ranges between 60%–90%, respectively. In accordance with this, all materials evaluated in the present study were considered slightly cytotoxic after 72 hours, and these findings agreed with those of previous studies [16-22].

An increase in cell proliferation was also observed in the HVGIC group after 24 hours, and this was in accordance with the finding of Ersahan et al. [13] who observed no cytotoxicity with HVGIC in their study. On the contrary, they reported an increase in cell proliferation, suggesting that the material used was biocompatible. This might be attributed to the small-particle glass-filler technology used, low-setting exothermic reaction, and rapid neutralization [19].

No significant differences between the two time points were observed in the alkasite group, which is a fluoride-releasing dual-cure material. This means that the setting reaction of alkasite begins when the powder and liquid are mixed and can be accelerated further with additional light-curing. The cytotoxic effects of fluoride-releasing materials can also be affected by the amount of fluoride released [16,20,21]. Egil [22] reported that the amount of fluoride released by alkasite was lower than that released by HVGIC. Therefore, both the shorter polymerization time and the less amount of ions released might be effective in maintaining the cytotoxicity of alkasite.

The xCELLigence assay performed in this study revealed that the cytotoxicity of the composite increased after 72 hours, which is in agreement with the findings of previous studies [23, 24]; this could be attributed to an increase in monomer release with degradation over time.

The present study used the MTT and xCELLigence assays to investigate the cytotoxic effects of restorative dental materials on human DPSCs. The MTT assay is considered one of the most reliable biocompatibility assays because of its rapid results and sensitivity, although the use of end-point qualitative measures of cell fitness is a major limitation [23,24]. To the best of our knowledge, very few studies to date have evaluated the cytotoxicity of dental materials using both assays and, although they yielded similar results in the present study, the findings of the xCELLigence assay were more accurate and detailed [25].

The cytotoxicity of alkasite material was higher than that of any of the other materials assessed in this study. Therefore, our first hypothesis was confirmed, and our second hypothesis was refuted. The formation of a partial barrier to protect the pulp in deep cavities with increased dentin permeability can reduce the cytotoxic potential of dental materials. In addition to the mechanical properties of dental materials, an understanding of its cytotoxic effects is essential to allow appropriate material selection and increase treatment success.

Conclusions

Alkasite demonstrated acceptable cytotoxicity on DPSCs after a 72-hour but was more cytotoxic than HVGIC and composite, which demonstrated similar cytotoxicity. The MTT and xCELLigence assays yielded similar results.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with

the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Funding: *This work was supported by Research Fund of the Biruni University, Project Number: Biruni-BAP-2019-02-09. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

Conflict of interest
The authors declare no conflict of interest.

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How to cite this article:
Tugba Elgun, Belen Sırınoglu Capan, Canan Duman, Nazli Ece Gungor Ordueri. In vitro evaluation of the cytotoxic effects of alkasite restorative material on human dental pulp stem cells. Ann Clin Anal Med 2023;14(11):1044-1048

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