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In Vitro Comparison of the Viability and Proliferation of Human Gingival Fibroblasts and Osteoblast-like MG-63 Cells on Three Different Temporary Cements Used in Dental Implants

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A B S T R A C T

Objectives: Retained cement is an idiopathic factor contributing to early implant loss, making the selection of cement with minimal toxicity to peri-implant hard and soft tissues crucial. This study aimed to evaluate the cytotoxicity of three types of temporary cements and titanium specimens cemented with each, following direct exposure to human gingival fibroblasts (HGF) and MG-63 osteoblast-like cells.

Materials and Methods: In this in vitro study, zinc oxide-eugenol (ZOE), eugenol-free zinc oxide (ZONE), and resin (R) cements were prepared in cylindrical forms of similar dimensions. Each cement was applied to titanium disks to create cemented titanium samples. Cytotoxicity was evaluated using the MTT assay at 24 hours, 72 hours, and 7 days. Cytotoxicity was assessed on HGF and MG-63 osteoblast-like cells using the MTT assay at 24, 72 hours, and 7 days. Data analysis involved two-way and one-way ANOVA, with Tukey's post-hoc tests, and statistical significance was defined as $P<0.05$.

Results: All cements significantly reduced cell viability in both cell lines. None of the cements demonstrated cellular viability percentages above the minimum threshold (70%) required for biocompatibility. The cytotoxicity of the cemented titanium disks was not significantly different from that of the cement-only samples ($P>0.05$). Additionally, there were no significant differences in the sensitivity of MG-63 osteoblast-like cells and HGF cells to the evaluated cements.

Conclusion: The composition of the cement played a significant role in the host cell response. This study demonstrated that dental cements could induce tissue toxicity in the gingiva and bones, ultimately affecting implant survival.

Keywords: Materials Testing; Dental Cements; Cytotoxicity Tests, Immunologic; Titanium; Zinc Oxide-Eugenol Cement

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INTRODUCTION

One of the most critical decisions in implant-supported prostheses is to select the most appropriate method to achieve retention for the restoration using temporary or permanent cements or screws. One of the advantages of cemented restorations is the possibility of compensating the improper position of the implant and improving the esthetic appearance due to the absence of the screw access hole [1]. Inherent mechanical problems, such as screw loosening or fracture, are the most common disadvantages of screw-retained restorations [2]. The absence of the screw access hole is one of the essential advantages of cemented restorations, which in addition to esthetic,

provides a proper occlusal morphology due to an intact ceramic surface in the occlusal area [2]. The feasibility of removing the crown in cemented implant-supported prostheses may be an important factor in selecting the type of cement used. As a result, temporary cements have been considered for these restorations [3]. Many clinicians justify the use of weak cements due to the precise fit between the implant and prosthetic components [4]. Temporary cement can cause fewer biological problems (such as peri-implantitis) since they are soluble, and the extra cement is washed away. However, this solubility in the oral environment might lead to mechanical problems [5]. Both temporary and permanent cements have resulted in a high survival rate in cemented restorations [6].

Peri-implant disease refers to pathologic inflammatory changes in the tissues around an implant under load [7]. Similar to the progression from gingivitis to periodontitis, the factors that determine the progression of the disease from mucositis to peri-implantitis remain unknown. However, it seems that the duration of exposure to microbial biofilm on loaded implants is a crucial factor [8, 9]. In the early stages of peri-implant diseases, local risk factors, such as residual cement, may play a significant role [8]. Sometimes, bacterial infections around implants might occur after non-microbial incidents, resulting in pathogenic microbiota, an example of which is a peri-implant infection due to subgingival residual cement [10]. According to a recent systematic review, approximately 33-100% of implants with peri-implantitis had residual cement [11]. One possible reason for the presence of residual cement in tissues is the subgingival placement of the implant restoration margin for esthetic reasons [12]. In contrast to teeth, peri-implant tissues exhibit lower resistance to pressure due to the absence of attachment to the implant surface and the positioning of the connective tissue fibers parallel to the fixture surface [13]. Therefore, the cement may be pushed deeper into the gingival sulcus, remaining undetected [14].

A small number of studies have considered the corrosion of titanium implants as one of the factors contributing to the incidence of peri-implantitis and implant failure [15]. The high resistance of titanium and its alloys against corrosion is due to the formation of a titanium oxide layer that serves as a protective layer to prevent further oxidation of the metal. The stability of this layer depends on the physicochemical conditions of the surrounding environment (such as an acidic pH resulting from the inflammatory process, oral bacteria, or the use of solutions that can adversely affect the implant surface, implant micromovements, abnormal loading, and the subsequent abrasion) [16]. Titanium's corrosion is a cause of concern because large amounts of metallic ions and debris are produced during this process, the accumulation of which can lead to adverse peri-implant tissue reactions [15]. Several studies have shown that acidic cements and fluoride ion-releasing cements increase titanium's susceptibility to corrosion [16-20]. A study by Kinani et al demonstrated that eugenol reduced titanium's bacterial corrosion [21]. Although numerous studies have investigated the cytotoxic effects of various cements, only one study has examined the biocompatibility of the cemented titanium interface with the cells of hard and soft tissues [22].

Since the best solution for peri-implant disease is prevention, it is necessary to use cements with the least toxicity in the peri-implant hard and soft tissues to improve implant longevity. Since the effects of different cement components on the cells of hard and soft tissues are not yet fully understood, the present study aimed to evaluate the effects of three different temporary cements, i.e., zinc oxide eugenol (ZOE), eugenol-free zinc oxide (ZONE), and resin (R) cement, on HGF cell line as a representative of peri-implant soft tissue and MG-63 osteoblast-like cells as a representative of peri-implant hard tissues. Cemented titanium disks were used in addition to cement alone to evaluate the effect of the cements on titanium's corrosion and its subsequent cytotoxicity. Besides, the tests were carried out at different time intervals after the cement setting to determine when the cement exhibited the highest cytotoxicity (i.e., the lowest cell viability percentage).

MATERIALS AND METHODS

The present in vitro study was approved by the Ethics Committee under the code IR.TUMS.REC.1397.142.

Sampling and sample size determination

According to a study by Marvin et al. [22], sample size calculation was performed using one-way ANOVA with PASS 11 software at α= 0.05, β=0.2, standard deviation=9%, and effect size=1.36. The minimum required sample size for each group was determined to be N=3. To accommodate the MTT assay at three different time intervals (24 hours, 3 days, and 7 days), nine samples were included in each group. The sample size requirement based on two-way ANOVA was smaller than this.

Preparation of cement samples

Three different types of temporary cement were used in the present study (Table 1). All the cements were sourced from a single company (Kerr) to eliminate potential variations in composition between cements manufactured by different companies.

All cement samples were prepared under aseptic conditions according to the manufacturer's instructions. After mixing the base and the catalyst, each cement was placed in a syringe with an internal diameter of 5mm. The syringe piston was adjusted so that the cement block height would measure approximately 3mm after it had fully set (Figure 1A). Therefore, cylindrical cement molds were prepared, measuring 5mm in diameter and 3mm in height. These mold dimensions provided a contact surface of 15.7 or approximately 16mm^2 with the cells.

Preparation of cement-titanium disk samples

Grade 4 titanium disks measuring 5mm in diameter and 3mm in height were prepared. After mixing, each cement sample was applied to the disk surface to a thickness of 0.5–1mm using a corrosion-resistant spatula (Figure 1B). All the samples were sterilized with gamma rays after preparation.

Preparation of the cells

Two cell lines were used in the present study:

1. Human gingival fibroblast cell line (HGF1-PI 1, Pasteur, Tehran, Iran) (NCBI code: C165) as a representative of peri-implant soft tissue.
2. Human osteoblast-like cell line (MG-63, Pasteur, Tehran, Iran) (NCBI code: C555) as a representative of peri-implant hard tissue.

Each cell line was cultured in a 75mL cell culture flask in DMEM (Dulbecco's Modified Eagles Medium) nutrient culture medium containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) and incubated at 5% CO2, 95% moisture, and 37ºC.

The cells were passaged several times and cultured in special cell culture plates for testing in their best growth phase (logarithmic phase) and used for the quantitative MTT test. To this end, on the first day, the cells in the logarithmic growth phase were cultured in a complete cell culture medium (DMEM containing FBS and antibiotics) in each well of a 24-well plate at 105 cells/1mL. The culture procedure was carried out under sterile conditions under a Cl II biologic hood. The plates were incubated for 24 hours at 95% moisture and 37ºC.

Table 1. Composition of the materials evaluated in this study. All cements were manufactured by Kerr.

Proprietary name	Cement type	Composition
Temp-Bond	Zinc oxide-eugenol self-cured temporary cement	Base: zinc oxide Catalyst: dehydrated zinc acetate, oligomer, rosin, and eugenol
Temp-Bond NE	Eugenol-free zinc oxide self-cured temporary cement	Base: zinc oxide Catalyst: dehydrated zinc acetate,) R)-p-mentha-3,8(9)-diene oligomer, rosin
Temp-Bond Clear	Translucent methacrylate resin dual-cured temporary cement	Base: non-polymerized urethane acrylate monomers Catalyst: non-polymerized urethane acrylate monomer, dibutyl phenolate

Fig 1. (A) Cement samples prepared using a piston syringe. (B) Cement-titanium disk samples.

Treatment of the cultured cells with the test materials

On the second day, the cells were microscopically evaluated to confirm their viability and absence of contamination. Each well was carefully aspirated and replaced with fresh complete culture medium containing serum and antibiotics. Test materials were then introduced into each well under sterile conditions. The direct contact method, in accordance with ISO 10993-5 and ISO 10993-12 standards, was employed to closely simulate in vitro conditions and assess the cytotoxicity of the cements. The cement molds (with an approximate contact area of 16mm^2) occupied >10% of the surface area of each well (approximately 51mm^2) in 24-well plates. Two control groups, were included in this study. The positive control group consisted of healthy cells (normal proliferation, without cytotoxicity) in a conventional cell culture medium (without cement or disk). The negative control group included cells in DMSO (dimethyl sulfoxide). Three wells were included for each test sample and each control group.

Evaluation of viability and proliferation of the cells with the MTT quantitative assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to evaluate the effect of the test materials on the viability and proliferation of cell lines. This assay, quantitatively determines mitochondrial activity, and has a direct relationship with cell proliferation. MTT, a yellow tetrazolium salt, is reduced by the dehydrogenase enzyme in the active mitochondria of viable cells and converted to the violet metabolite formazan, which is an insoluble crystal. The formed crystals are then dissolved in an appropriate solvent, and the optical density (OD) of the resulting solution is determined by a spectrophotometer at 500–600nm wavelength. A reduction in the number of viable cells in the test samples leads to a decrease in its total metabolic activity. This decrease is directly associated with a decrease in the formation of violet crystals and mitochondrial activity. MG-63 and HGF cell lines were evaluated at 24- and 72-hour and 1-week intervals after treatment with MTT to determine the effect of cements on their viability. At each interval, the wells were completely evacuated, 100µL of MTT was added and the plates were returned to the incubator and incubated for 3 hours at 37ºC. After formazan crystal formation the medium in each well was evaluated and replaced with 100 µL of isopropanol solvent to dissolve the crystals and show the violet color. The plate was placed in an ELISA reader, and the OD was determined at 570nm (specific for MMT) and 620nm (the reference wavelength).

Statistical analysis of MTT results

The means ± standard deviations (SD) of OD values were calculated for all 3 wells of each sample. The following formula was used to report the percentage of viability compared to the positive control group.

$$Cell\ viability(\%)=\frac{Abs\ treatment\ group-Abs\ blank}{Abs\ Control\ group-Abs\ blank}\times 100$$

According to ISO 10993-5 standards, a material is classified as cytotoxic if it reduces cell viability to less than 70% compared to the control. Materials with cell viability greater than 90% are considered non-toxic, while those with cell viability between 60% and 90% are categorized as having low toxicity. Materials with cell viability between 30%

and 59% are classified as moderately cytotoxic, and those with cell viability below 30% are deemed highly cytotoxic.

Two-way ANOVA was used to evaluate the effect of sample type and time parameters. Then, one-way ANOVA was used due to the interaction between the samples, followed by post hoc Tukey tests. Statistical significance was set at $P<0.05$. A paired t-test was used to compare the viability of the HGF and MG-63 cell line in terms of the test materials.

RESULTS

There was no significant difference in cell viability among the study groups in either HGF or MG-63 cell lines. Additionally, no significant differences in cell viability were observed between the negative control and any of the study groups ($P>0.05$) (Figures 2 and 3).

After direct exposure to the study materials, cell viability did not differ significantly between the cell lines. A comparison of cytotoxicity at different time points is reported in Table 2.

Considering the viability percentage of MG-63 cells in 24 hours, R and ZOE cements exhibited high toxicity (viability percentage <30%), and ZONE exhibited moderate cytotoxicity (viability percentage of 30–59%). Considering the viability percentage of HGF cells in 24 hours, all three cements exhibited moderate cytotoxicity (viability percentage of 30–59%).

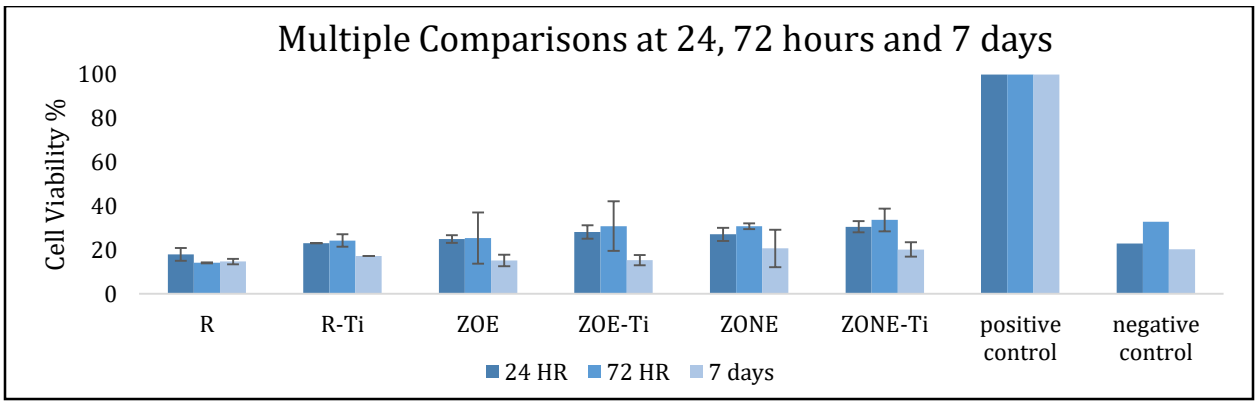

Fig 2. Comparison of mean MG-63 cell viability at 24- and 72-hour and 7-day intervals between the study groups after direct exposure (error bar: 95% confidence interval). Cell viability was not significantly different between the test groups. R: resin cement, ZOE: zinc oxide eugenol cement, ZONE: zinc oxide non eugenol cement, R-Ti: resin cemented titanium, ZOE-Ti: zinc oxide eugenol cemented titanium, ZONE-Ti: zinc oxide non eugenol cemented titanium

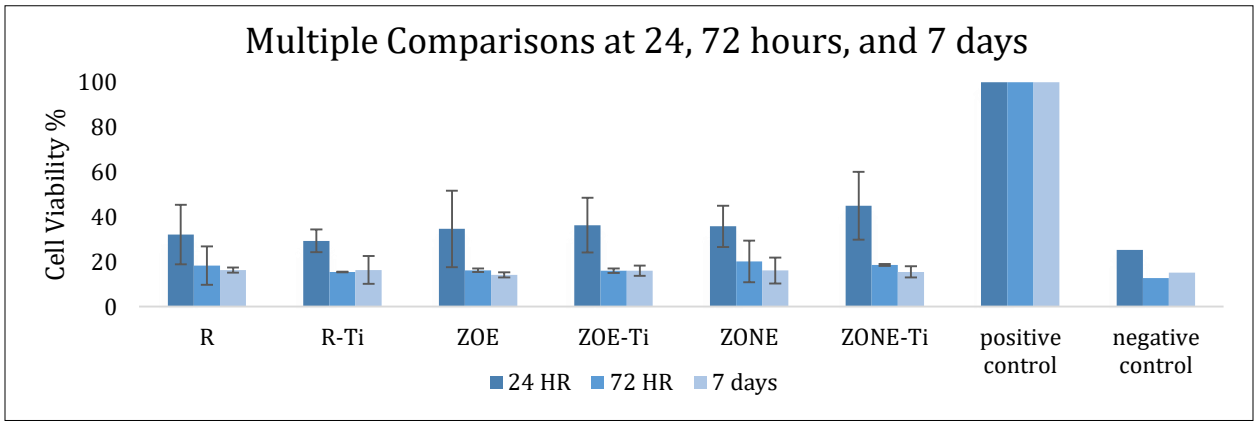

Fig 3. Comparison of mean human gingival fibroblasts (HGF) cell viability at 24- and 72-hour and 7-day intervals between the study groups after direct exposure (error bar: 95% confidence interval). Cell viability was not significantly different between the test groups. R: resin cement, ZOE: zinc oxide eugenol cement, ZONE: zinc oxide non eugenol cement, R-Ti: resin cemented titanium, ZOE-Ti: zinc oxide eugenol cemented titanium, ZONE-Ti: zinc oxide non eugenol cemented titanium

Table 2. Comparison of cytotoxicity at different times

Samples	Cell lines	Time	P
Resin cement	MG-63	24h vs 72h	0.638
		24h vs 7 days	<0.001*
		72h vs 7 days	<0.001*
	HGF	24h vs 72h	0.81
		24h vs 7 days	0.985
		72h vs 7 days	0.72
Resin cemented titanium	MG-63	24h vs 72h	0.048*
		24h vs 7 days	<0.001*
		72h vs 7 days	<0.001*
	HGF	24h vs 72h	0.584
		24h vs 7 days	0.785
		72h vs 7 days	0.276
zinc oxide eugenol cement	MG-63	24h vs 72h	0.832
		24h vs 7 days	0.267
		72h vs 7 days	0.52
	HGF	24h vs 72h	0.615
		24h vs 7 days	0.191
		72h vs 7 days	0.588
zinc oxide eugenol cemented titanium	MG-63	24h vs 72h	0.581
		24h vs 7 days	0.422
		72h vs 7 days	0.949
	HGF	24h vs 72h	0.174
		24h vs 7 days	0.855
		72h vs 7 days	0.339
zinc oxide non eugenol cement	MG-63	24h vs 72h	0.711
		24h vs 7 days	0.121
		72h vs 7 days	0.333
	HGF	24h vs 72h	0.791
		24h vs 7 days	0.96
		72h vs 7 days	0.919
zinc oxide non eugenol cemented titanium	MG-63	24h vs 72h	0.316
		24h vs 7 days	0.021*
		72h vs 7 days	0.148
	HGF	24h vs 72h	0.103
		24h vs 7 days	0.241
		72h vs 7 days	0.790
Positive control	MG-63	24h vs 72h	0.563
		24h vs 7 days	<0.001*
		72h vs 7 days	<0.001*
	HGF	24h vs 72h	0.01*
		24h vs 7 days	<0.001*
		72h vs 7 days	0.003*
Negative control	MG-63	24h vs 72h	0.54
		24h vs 7 days	0.181
		72h vs 7 days	0.64
	HGF	24h vs 72h	0.367
		24h vs 7 days	0.444
		72h vs 7 days	0.072

* P< 0.05, significant differences between two different times

DISCUSSION

The present study aimed to evaluate the biocompatibility of three temporary cements_ ZOE, ZONE, and R cements (both alone and placed on a titanium disk) in contact with fibroblasts and osteoblast-like cells, representatives for soft and hard tissues, respectively. None of the cements evaluated exhibited cell viability ≥70%, the minimum threshold required for a material to be considered biocompatible.

In implant-supported restorations, dental cements enable passive fitting of the framework and provide long-term retention of the crown [23]. Various studies have reported inflammatory reactions due to cement remaining in subgingival tissues, leading to the initial stages of peri-implantitis [24, 25]. However, cemented restorations remain popular among clinicians for several reasons, including the ability to achieve a more favorable occlusal relationship, easier management, better distribution of axial stresses on prosthetic components and implant-bone interface, improved esthetic appearance, simpler fabrication, and lower cost [2,14,25,26].

No standard criteria have been established for selecting dental cements. During cementation of the crown, the cement comes into contact with the gingival soft tissue. If the cement remains subgingivally, it may affect the alveolar bone. Since the cellular response is a critical factor in selecting the type of cement, it is important to understand the potential effects of the cement's composition on the biocompatibility of peri-implant hard and soft tissues, as well as the corrosion susceptibility of implant component materials. Proper cement selection can reduce the risk of complications that may lead to implant failure.

In the present in vitro study, a standardized protocol was used to evaluate and compare the cytotoxic potential of temporary cements used in implant dentistry. Cytotoxicity tests in cell cultures are among the most critical methods to evaluate the biocompatibility of dental materials. These in vitro tests are reproducible, cost-effective, applicable, and appropriate for assessing the biological properties of dental materials, allowing for the evaluation of a large number of materials at specific time intervals [27,28].

Various laboratory tests and cell lines are used to assess the cytotoxicity of dental materials. The MTT assay is an effective technique for cell viability analysis. In this assay, MTT is converted to violet formazan in cell mitochondria. The dissolution of violet formazan produces a colored solution, and this solution's optical density (OD) is measured by a spectrophotometer, yielding numeric values. The cytotoxic response is classified as severe (<30%), moderate (30–60%), mild (60–90%), and nontoxic (>90%). Previous studies have shown that the cytotoxicity test results are different in human and animal cells, and these cells mount different responses to toxic materials. Human diploid cells have different mitotic rates, mitochondrial function, and growth rates compared to aneuploid cells [29], and they are generally more resistant to toxic materials [30]. Undoubtedly, cytotoxicity evaluation of human cells directly extracted from relevant tissues is more clinically relevant [31]. Therefore, human cell lines were used in the present study.

In the present study, the cytotoxicity of all the three cement types evaluated, i.e., Temp-Bond (ZOE), Temp-Bond NE (ZONE), and Temp-Bond Clear (resin methacrylate cement) were not significantly different from the negative control group. All three cement types exhibited moderate to severe (viability percentage of <70%) cytotoxicity and significantly decreased the viability of HGF and MG-63 cells. In a study by Rodriguez et al [32], similar to the present study, R (Premier Implant Cement) and ZOE (TempoCem) cements significantly decreased the viability of HGF cells compared to the positive control group (the cell culture without cement components) after 24 hours; however, the decrease was not significant in MC3T3E1 preosteoblasts. Besides, in contrast to the present study, ZONE cement (Temp-Bond NE) did not significantly decrease cell viability in contact with HGF and MC3T3E1 preosteo-

blasts. In a study by Marvin et al [22], similar to the present study, both the R (RelyxTM Unicem 2 Clicker) and ZOE (Intermediate Restorative Material) cements were severely cytotoxic to MC3T3E1 cells after half an hour and 24 hours. Both cements were cytotoxic to the HGF cell line (cell viability <70%) after half an hour; however, cell viability was >70% after 24 hours, in contrast to the present study. In a study by Gallegos et al [33], similar to the present study, R (RelyX Unicem 2 Clicker) cement was highly toxic to the MC3T3E1 and HGF cell lines after 3 and 7 days.

Consistent with the present study, the study by Kwon et al [34] also found no significant difference in cytotoxicity between ZOE cement (Relyx Temp E) and ZONE cement (RelyxTemp NE and ESTemp NE). They reported that most of the cytotoxicity was due to the presence of zinc ions in both cements.

Generally, the cytotoxicity of a cement depends on its chemical composition. The cytotoxicity of ZOE and ZONE cements is attributed to their chemical composition, including the release of eugenol from ZOE cement [35] and zinc ions from both cements [35,36]. A high concentration of eugenol might be cytotoxic to fibroblasts and osteoblasts and can even lead to necrosis or a disruption in the healing process in a dose-dependent manner. At low concentrations, contact dermatitis and delayed hyper-sensitivity reactions are possible [37]. It has been reported that eugenol can inhibit the growth and proliferation of osteoblasts in a dose-dependent manner [38]. R cement contains acrylate-based monomers that can cause contact dermatitis and pupal injuries and should not be in contact with skin, eyes, and soft tissues for a long time.

The cement remaining in the oral environment can react with the titanium oxide layer on the implant components' surface. A study by Saba et al [39] showed that the amount of corrosion in their uncemented titanium and the titanium cemented with R cement was significantly less than that of titanium cemented with ZOE. However, Kinani et al [21] reported that eugenol inhibits titanium corrosion in artificial saliva. Eugenol protects titanium against bacterial invasion and inhibits the dissolution of titanium ions. Cement corrosion can adversely affect the soft and hard tissue cells by releasing metallic ions. Therefore, it was hypothesized that the biocompatibility of the cement–titanium interface, through cement corrosion, might differ from that of the cement alone. However, in the present study, in both cell lines, there were no significant differences in the cemented titanium disks' cytotoxicity compared to each other and compared to the cement samples alone. Since the cements used in the present study did not have an acidic nature and did not release fluoride ions, it is logical that they did not affect the correction of titanium and the subsequent cytotoxicity. Besides, according to studies by Turpin et al [20], and Demirel et al [17], zinc eugenate did not affect titanium corrosion due to its neutral nature. In a study by Marvin et al [22], in the HGF cell line, the cytotoxicity of titanium cemented with ZOE was significantly lower than the cement alone after half an hour; however, after 24 hours, no significant differences were observed. In the MC3T3E1 preosteoblast cell line, the cytotoxicity of titanium cemented with ZOE was not significantly different from the cement alone after half an hour; however, it was significantly lower after 24 hours. The researchers concluded that placing ZOE cement on titanium decreased its cytotoxic effects. Concerning the R cement, in both cell lines, cemented titanium's cytotoxicity was not significantly different from the cement alone after half an hour and 24 hours.

According to the study by Marvin et al [22], in the HGF cell line, the cytotoxicity of ZOE and R cements were significantly lower than the MC3T3E1 preosteoblasts after half an hour and 24 hours. They concluded that this cement's cytotoxicity to the soft tissue cells decreased over time, and preosteoblasts were more sensitive to cement cytotoxicity than gingival fibroblasts. In contrast, in a study by Rodriguez et al [32], R and ZOE cements significantly decreased HGF viability compared to MC3T3E1 preosteoblasts. They concluded that the gingival fibroblasts were more sensitive to the cement cytotoxicity than

preosteoblasts. In the present study, the three cement types did not exhibit any significant differences in HGF and MG-63 cells' viability. The cell type affected the cytotoxicity results. The osteoblasts used in the present study (MG-63) were derived from human osteosarcoma; however, the MC3T3E1 osteoblasts were derived from the mouse calvarium. The proliferation rate of MG-63 cells is higher than that of MC3T3E1 cells [40], which may explain the lack of significant difference in the sensitivity of MG-63 and HFG cells in the present study, compared to the study by Marvin et al [22].

CONCLUSION

Under the limitations of the present study, the following conclusions can be made:

- There was no significant difference in cytotoxicity between the evaluated cement samples.
- In all the cements evaluated, cell viability was <40%, which is below the minimum threshold required for biocompatibility. Therefore, none of the cements was superior to the other.
- There were no significant differences in cytotoxicity between the cement samples alone and cemented titanium samples.
- No significant differences were observed in the sensitivity of HGF cells and MG-63 osteoblasts to the tested cements.

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CONFLICT OF INTEREST STATEMENT

None declared.

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