A comparison of the *in vitro* cytotoxicity of conventional and resin modified glass ionomer cements

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Abstract

To evaluate cytotoxicity of experimental conventional and resin modified glass-ionomer cements on UMR-106 osteoblast cell cultures and cell cultures of NIH3T3 mouse fibroblasts specimens were prepared for every experimental material and divided into: group 1.Conventional glass-ionomer cements: GC Fuji IX GP Fast, GC Fuji Triage and Ketac Silver; group 2. Resin modified glass-ionomer cements: GC Fuji II LC, GC Fuji Plus and Vitrebond; group 3. Positive control was presented by specimens of composite Vit-l-ecence® and negative control-group 4. was presented by α-minimum essential medium for UMR-106 – osteoblast-like cells and Dulbecco's Modified Eagle's Medium for NIH3T3 mouse fibroblast cells. Both cell cultures were exposed to 10% of eluate of each single specimen of each experimental material. Experimental dishes were incubated for 24 h. Cell metabolism was evaluated using methyltetrazolium assay. Kruskal-Wallis test and Tukey-Kramer post hoc test for the materials evaluated on NIH3T3 mouse fibroblast cells, as well as UMR-106 osteoblast-like cells showed significantly more cytotoxicity of RMGICs, predominantly Vitrebond to both GICs and composite- Vit-l-ecence® .The lowest influence on cell's metabolism on UMR-106 osteoblas-like cells was shown by Ketac Silver and the lowest influence on cell's metabolism on NIH3T3 mouse fibroblast cells was shown by Fuji IX GP Fast. Statistical evaluation of sensitivity of cell lines UMR-106 osteoblast-like cells and NIH3T3 mouse fibroblast cells, using Mann-Whitney test, showed that NIH3T3 mouse fibroblast cells were more sensitive for the evaluation of cytotoxicity of dental materials.

KEY WORDS: conventional and resin-modified glass-ionomer cements, cytotoxicity tests, colorimetric assay

INTRODUCTION

Despite the fact that restorative dental materials and dental cements have improved their physico-chemical properties, level of intrinsic toxicity, or at least that part which appears in the evaluation *in vitro* remains rather high. Therefore, it is necessary to reevaluate physical, chemical and biological properties of these dental materials, since it is obvious that they do not meet the requirements either from the standpoint of biosafety, or even from the standpoint of longevity of restorations. When they emerged in the early seventies until today, glass-ionomer cements (GICs) are presented as biocompatible dental materials, with the possibility of chemical adhesion to tooth structure [1], the ability to release fluoride [2], ability to release-recharged-release fluoride [2], end enhance remineralization of caries lesion [3]. Subsequent development of glass-ionomer cements led to the emergence of hybrid versions of these materials known as resin modified glass-ionomer cements (RMGICs). It is believed that resin modified glass-ionomer cements combine the main advantages of glass-ionomer cements such as adhesion to tooth structure, fluoride release and biocompatibility, with easy handling of light polymerized composites [4]. They also show some adverse properties when used as restorative materials, and the level of biocompatibility is not always satisfactory [5]. Biocompatibility is an important characteristic of each material used in dentistry. Williams defined biocompatibility as "a material's ability to act in a particular application, with an acceptable response of the host". This definition encompasses several processes that occur during the interaction between tissues and artificial materials [6]. The biocompatibility of GICs depends of components eluted during the setting process. Having in mind that both components of GICs, glass powder and polyacidic liquid (acrylic acid – itaconic acid or copolymer maleic and acrylic acid), were composed of spectrum of chemical formulas, risk that these materials

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Submitted 30 Jun 2012/ Accepted 29 September 2012

can cause toxic effect *in vivo*, can be partially evaluated by tests performed *in vitro*. Important differences between the components of new hybrid materials and the conventional glass-ionomer cements, as well as different chemical processes during the polymerization, is an argument for continuation of the evaluation of their cytotoxicity. The aims of this double blind *in vitro* study were:

1. To evaluate cytotoxicity of experimental conventional and resin modified glass-ionomer cements on UMR-106 osteoblast cell cultures and cell cultures of NIH 3T3 mouse fibroblasts.
2. To evaluate statistical significance of differences in sensitive reaction of cell line UMR-106 osteoblast-like cells and cell line NIH3T3 mouse fibroblasts on cytotoxic influence of elutes of tested glass-ionomer cements.

MATERIALS AND METHODS

Materials and manufacture of specimens

For the evaluation of cytotoxicity of glass-ionomer cements on UMR-106 osteoblast-like cells and NIH3T3 mouse fibroblast cells, six glass-ionomer cements divided in two groups were used. First group was presented by conventional glass-ionomer cements GC Fuji IX GP Fast, GC Fuji Triage (GC Corporation) and Ketac Silver (3MESPE), and second group, resin modified glass-ionomer cements, was presented by GC Fuji II LC, GC Fuji Plus (GC Corporation) and Vitrebond (3MESPE). Group 7. (positive control) was presented by specimens of composite Vit-l-ecence® (Ultradent Products, Inc. USA), and negative control group was presented by α-minimum essential medium (α-MEM, GIBCO® CO.USA) for UMR-106 osteoblast-like cells and Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co. St. Louis, MO) for NIH3T3 mouse fibroblast cells. Six groups of disc shaped specimens, 4 mm in diameter and 2 mm of thickness, were prepared for each experimental material, following the manufacturer's directions. In order to avoid external contamination of cell cultures, all specimens were sterilsed using UV light in Labconco Purifier Class II (Labconco CO.) for 24 hours.

Elution samples

After the sterilization, the GICs and RMGICs samples were placed in two 96 well tissue culture plates (Falcon MICROTEST™ 96 Tissue Culture Plate Becton Dickinson Labware) one for UMR-106 osteoblast like cells and the other for NIH3T3 mouse fibroblast cells. Each chamber was filled with 100μl of culture medium (α-MEM for UMR-106 osteoblast like cells and DMEM for NIH3T3 mouse fibroblast cells). The medium, with the immersed specimens, were maintained for 72 hours in a humidified incubator at 37 °C with 95% air and 5% CO_2. The medium was retained for toxicity testing.

Cell Culture

In this *in vitro* study UMR -106 osteoblast like cells and NIH3T3 mouse fibroblast cells were used. UMR-106 osteoblast like cells were cultivated in experimental culture flask T-25 (culture flasks Becton Dickinson Falcon, Franklin Lakes, NJ) on α-MEM, (GIBCO® CO.USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and 1% AA-liquid (GIBCO® CO.USA.) containing 10 000 units/ml penicillinum in G-sodium, 10,000 μg/ml streptomycin sulphate, 25 μg/ml amphotericin B as antimycotic diluted in 0.85 % saline. Cultures were incubated at 37 °C in humidified atmosphere with 95% air and 5% CO_2 until confluent. Cellular growth and medium pH was monitored daily using phase contrast microscopy Nikon TMS (Nikon USA, Melville, NY) and pH meter (SympHony SB-301 pH/ISE meter VWR Scientific-Product, USA). Cells were grown to density $1x\ 10^4$ cells / cm^2. NIH3T3 mouse fibroblast cells (ATCC CCL 163, clone A31; American Type culture collection, Rockville, MD) were cultivated in experimental culture flask T-25 on DMEM (Sigma Chemical Co. St. Louis, MO), supplemented with 10% (v/v) fetal calf serum (FCS, Collaborative research, Bedford, MA) and 1% AA-liquid, (GIBCO® CO.USA) containing 10,000 units/ml penicillinum in G-sodium, 10,000 μg/ml streptomycin sulphate, 25 μg/ml amphotericin B as antimycotic diluted in 0.85% saline. Cultures were incubated at 37°C in humidified atmosphere with 95% air and 5% CO_2 until confluent. Cellular growth and medium pH were monitored daily using phase contrast microscopy and pH meter. Cells were grown to density $1x\ 10^4$ cells / cm^2. 24 hours before experiment, both types of cell cultures were plated at $3x\ 10^4$ cells/ cm^2 in two 96-well tissue culture plates (Falcon MICROTEST™ 96 Tissue Culture Plate Becton Dickinson Labware), one experimental plate for every cell culture, containing 100 μl DMEM for NIH3T3 mouse fibroblast cells, and 100 μl α-MEM for UMR-106 osteoblast like cells.

Test material and controls

After the incubation period of 24 hours, complete culture medium in 96 well tissue culture plates with NIH3T3 mouse fibroblast cells were replaced with 90 μl of fresh DMEM and 10 μl of extract DMEM which represents 10% eluate of specimens of conventional and resin modified glass-ionomer cements previously incubated 72 hours in DMEM. Procedure was repeated for 96 well tissue culture plates with UMR-106 osteoblaste like cells and complete culture medium was replaced with 90 μl of fresh α MEM and 10 μl of extract α MEM which represents 10% eluate of specimens of conventional and resin modified glass-ionomer cements previously incubated 72 hours in α-MEM. This way, both cell cultures were exposed to 10% of elu-

ate of each single specimen of each experimental material. Each experiment was performed using 12 representative areas, for each material as well as for positive and negative control group. Experimental dishes were incubated for 24 hours at 37 °C with 5% CO_2 and 95% air. In order to ensure reproducibility, the experiment was conducted in triplicate.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] cytotoxicity assay

Citotoxicity of conventional glass-ionomer cements (GC Fuji IX GP Fast, GC Fuji Triage (GC Corporation) and Ketac Silver (3MESPE), and resin modified glass-ionomer cements, presented by GC Fuji II LC, GC Fuji Plus (GC Corporation) and Vitrebond (3MESPE), was evaluated by cell metabolic activity measured by succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial respiration of the cells [7]. Following the procedure, previously described in detail by Mossman[7], citotoxicity of six experimental glass-ionomer cements was evaluated by methyltetrazolium (MTT) assay. After 24 hr. of incubation of cells with the GICs and RMGICs eluates, the basal MTT scores were obtained by spectrophotometer (Safire² Tecan Group Ltd.) using a test wavelength of 570 nm.

Statistical analysis

Statistical evaluation of cytotoxicity of conventional GICs: GC Fuji IX GP Fast, GC Fuji Triage (GC Corporation) and Ketac Silver (3MESPE), and RMGICs: GC Fuji II LC, GC Fuji Plus (GC Corporation) and Vitrebond (3MESPE) were performed by statistical software SPSS for Windows 15.0 (SPSS Inc. USA).

RESULTS

Kruskal-Wallis test (Table 1.) and Tukey-Kramer post hoc test (Table 2.) for the materials evaluated on NIH_3T_3 mouse fibroblast cells, shows significantly more cytotoxicity of RMGICs, predominantly Vitrebond ($Z= -5.5835$, $p< 0.0001$). The Vitrebond (3MESPE) showed significantly higher cytotoxicity than all other materials as well as positive and negative control. Fuji IX GP Fast (GC Corporation) showed the lowest influence on cell's metabolism as well as the lowest cytotoxicity on NIH_3T_3 mouse fibroblast cells ($Z =1.9719$, $p<0.0001$). Kruskal-Wallis test (Table 3.) and Tukey-Kramer post hoc test (Table 4.) for the materials evaluated on UMR-106 osteoblast-like cells, showed severe cytotoxicity of RMGIC Vitrebond ($Z= -5.5835$, $p<0.0001$). Comparing with all tested materials, Vitrebond (3MESPE) significantly diminished UMR-106 osteoblas-like cell's metabolism. The lowest influence on cell's metabolism as well as the lowest cytotoxicity on UMR-106 osteoblast-like cells was shown by Ketac Silver (3MESPE), ($Z= 2.7142$, $p<0.0001$). Statistical evaluation of sensitivity of cell lines UMR-106 osteoblast-like cells and NIH 3T3 mouse fibroblast cells, using Mann-Whitney test, showed that NIH_3T_3 mouse

TABLE 1. Results of Kruskal-Wallis test of cytotoxicity of experimental glass-ionomer cements evaluated on NIH3T3 mouse fibroblast cells

Material	Number of measurements	Sum of Ranks	Mean Rank	Z-value	Median
Negative control	12	1045.00	87.08	5.1293	0.78365
Fuji Triage	12	724.00	60.33	1.5731	0.68695
Fuji IX GP fast	12	760.00	63.33	1.9719	0.695
Ketac Silver	12	632.00	52.67	0.5539	0.6623
Vitrebond	12	78.00	6.50	-5.5835	0.10145
Fuji plus	12	503.50	41.96	-0.8696	0.62655
Fuji II LC	12	418.50	34.88	-1.8113	0.6262
Positive control Vit-l-escence®	12	495.00	41.25	-0.9638	0.6336

Vitrebond was the most cytotoxic experimental material ($Z= -5.5835$; $p< 0,0001$).

TABLE 2. Tukey-Kramer test of multiple comparison for the experimental glass ionomer cements evaluated on NIH3T3 mouse fibroblast cells

Material	Code	Number of measurements	Mean value	Differences between the groups	Median
Vitrebond	E	12	0.1032417	G, H, F, D, B, C, A	0.78365
Fuji II LC	G	12	0.61465	E, B, C, A	0.68695
Positive control Vit-l-escence®	H	12	0.6246417	E, C, A	0.695
Fuji plus	F	12	0.635025	E, A	0.6623
Ketac Silver	D	12	0.6686	E, A	0.10145
Fuji Triage	B	12	0.6931916	E, G, A	0.62655
Fuji IX GP fast	C	12	0.6961833	E, G, H, A	0.6262
Negative control	A	12	0.796475	E, G, H, F, D, B, C	0.6336

The RMGIC Vitrebond showed significantly higher cytotoxicity than all other materials as well as positive and negative control.

TABLE 3. Results of Kruskal-Wallis test for the experimental glass ionomer cements evaluated on UMR-106 osteoblast-like cells

Material	N° of measurements	Sum of Ranks	Mean Rank	Z-value	Median
Negative control	12	917.00	76.42	3.7112	0.8692
Fuji Triage	12	597.00	49.75	0.1662	0.73535
Fuji IX GP fast	12	717.00	59.75	1.4956	0.7672
Ketac Silver	12	827.00	68.92	2.7142	0.80005
Vitrebond	12	78.00	6.50	-5.5835	0.2753
Fuji plus	12	364.00	30.33	-2.4151	0.6578
Fuji II LC	12	495.00	41.25	-0.9638	0.6892
Positive control Vit-l-escence®	12	661.00	55.08	0.8752	0.73895

Evaluation on UMR-106 osteoblast-like cells showed severe cytotoxicity of Vitrebond ($Z= -5.5835$; $p< 0.0001$).

TABLE 4. Tukey-Kramer multiple comparison tests for the experimental glass ionomer cements evaluated on UMR-106 osteoblast-like cells

Material	Code	No.of measurements	Mean value	Differences between the groups	Median
Vitrebond	E	12	0.2571583	F, G, B, H, C, D, A	0.8692
Fuji plus	F	12	0.6554	E, C, D, A	0.73535
Fuji II LC	G	12	0.6968583	E, A	0.7672
Fuji Triage	B	12	0.733175	E, A	0.80005
Positive control Vit-l-escence®	H	12	0.7555417	E	0.2753
Fuji IX GP fast	C	12	0.7826083	E, F	0.6578
Ketac Silver	D	12	0.8031917	E, F	0.6892
Negative control	A	12	0.862125	E, F, G, B	0.73895

The lowest cytotoxicity on UMR-106 osteoblast-like cells was shown by Ketac Silver.

fibroblast cells were more sensitive for the evaluation of cytotoxicity of dental materials (Z= -4.666, p=0.0001).

DISCUSSION

The comparison of the cytotoxic effects of tested conventional and resin modified glass –ionomer cements clearly indicates that there are significant differences between the various materials. Results of MTT citotoxicity assay of GICs and RMGICs, on both cell lines used in this study, showed intense cytotoxic effect of RMGIC Vitrebond (3MESPE). The other RMGICs used in this study, GC Fuji II LC and GC Fuji plus (GC Corporation), showed significant decrease of cell metabolism, especially UMR-106 osteoblast-like cells metabolism comparing even with composit material Vit-l-escence® (Ultradent Products, Inc. USA), which was used as a positive control. This finding was unexpected, and proves superior biological performance of at least one composite material over RMGICs. There is some discrepancy in literature about the reasons for pulp irritation and inflammatory reaction after the placement of different GICs and RMGICs [8]. The fact that glass-ionomer cements were formed by reaction of a fluoraluminosilicate glass powder with an aqueous solution of acidic polymers such as polyacrilic acid or acrylic acid /itaconic acid copolymers and that RMGICs contains hydrophilic monomers and polymerization initiators, shows that large number of components can be responsible for cytotoxic effect [1]. The powder of GICs contains SiO_2, Al_2O_3, CaF_2 and Na_3AlF_6. During the mixing process, free metal ions: Al^{3+}, Ca^{2+}, Na^{+} are dissolved from the powder to the liquid. These metal ions, Al^{3+}, Ca^{2+}, Na^{+} are considerably non-toxic or non-irritant for living cells or tissues. SiO_2, as a base substance of glass powder, does no damage as well [1]. Therefore, the main factor for the mild cytotoxicity may be in the liquid of polyacrilic acid. Most of the authors considered that the unbound free monomers, released during the polymerization of dental composites and RMGICs, are responsible for the cytotoxic effect of these materials and there is an evidence in the literature that cytotoxicity is related to some additional mechanisms such as short-term release of free monomers which occurs during the monomer - polymer conversion [8, 9]. This release of free monomers is due to irregular photopolymerization, chemical, thermal or mechanical factors. Due to the industrial process of improving of RMGICs, the amount of unbound monomers has been decreased, but still there is no complete conversion during the polymerization process. Although the quantity of residual monomers is less than1,5-5%, this is enough to contribute to cytotoxic effects of those materials which is proved in tests *in vitro* [10]. Results of this *in vitro* study coincide with the results of Geurtzen W. et al. who confirmed that Vitrebond is extremely cytotoxic to cells culture, while Fuji II LC shows a moderate inhibition of cell growth [11]. The authors considered that this effect caused by Vitrebond may be mainly produced by decomposition products of the initiator diphenyliodoniumchloride, especially chlorine benzene, iodine benzene and bromide benzene which were not found in other RMGICs [11]. Moderate inhibition of growth of NIH3T3 mouse fibroblast cells and mild cytotoxic effect on UMR-106 osteoblast-like cells, which RMGIC Fuji II LC showed in this study, can be considered as a result of released 2-hydroxy-ethyl-methacrylate (HEMA) and champhorquinone, which are components of liquid part of this RMGIC. Besides the fact that cytotoxic effect of HEMA was confirmed by a number of studies [5, 8, 11, 12, 13], photo-initiator camphorquinone, show some cytotoxic effects in cell cultures as well [14]. Demage of primary human fibroblast DNA as a result of influence of camphorquinone was confirmed by Schweikl et al. [12]. Present study showed no significant difference between the level of citotoxicity of RMGIC Fuji II LC and resin composite Vit-l-ecence®, on both cell lines. This concurs with the results of Schedle et al. who showed that the cytotoxicity of RMGICs and resin composite is conducted by the same model within the similar boundaries [15]. A moderate cytotoxicity of Fuji II LC was confirmed by Stanislawski et al., [4] who pointed out that expressed cytotoxicity was dependent of dose of cytotoxic components and in the case of Fuji II LC decreases with the time. A moderate cytotoxicity of RMGIC GC Fuji plus, which was seen in present study, might be explain with the presence of urethanedimethacrylate (UDMA) in the liquid component of this RMGIC. Probable mechanism of influence of this monomer is alteration of lipid bilayer of

cell membrane, which changes membrane's permeability and leads to the solubilisation of this structure [13]. The results found in this study indicate that the cytotoxic alterations of NIH3T3 mouse fibroblast cells and UMR-106 osteoblas-like cells induced by conventional glass-ionomer cements were significantly lower. Conventional GIC Ketac Silver (3MESPE) showed mild cytotoxic effect on NIH3T3 mouse fibroblast cells, significantly different only from Vitrebond and negative control. Citotoxic responses of UMR-106 osteoblast-like cells, produced by Ketac Silver was the lowest response recorded in this study on this cell line. This observation indicated that Ketac Silver is the less toxic material tested on UMR-106 osteoblast-like cells in this study. Blackman et al. showed that Ketac Silver induced mild inflammatory response which decreases over time. In that *in vivo* study, Blackman et al. [16] observed that Ketac Silver is relatively biocompatible, that concurs with the results of our study. The present *in vitro* study demonstrated that GIC GC Fuji IX GP fast (GC Corporation) induced the lowest cytotoxic effect on NIH3T3 mouse fibroblast cells which concurs with the results of Costa CAS et al. [5] who showed that GC Fuji IX GP fast was the least cytotoxic experimental material evaluated in their study on an odontoblast cell line (MDPC-23). On the other hand, when tested on UMR-106 osteoblast-like cells, GC Fuji IX GP fast showed mild cytotoxic effects with no significant difference, comparing with Ketac Silver.

Fuji Triage (GC Corporation), third material in this group of conventional glass-ionomer cements, showed mild cytotoxic effects on NIH3T3 mouse fibroblast cells significantly lower when compared to the experimental RMGIC Vitrebond and Fuji II LC. A moderate cytotoxic effect which Fuji Triage showed on UMR-106 osteoblas-like cells was higher than cytotoxic effect showed on this cell line by other two experimental conventional glass-ionomer cements and even positive control although this difference wasn't significant. Several *in vitro* studies that assessed the cytotoxicity of conventional and resin-modified GICs have supported the concept that leachable components of these materials are responsible for their adverse effect on experimental cell culture [11, 14, 17]. In this present study after the preparation, which followed the manufacturer's instructions, all the samples were sterilsed for 24 hours in order to avoid external contamination of cell cultures. This means that all leachable components were released from GICs and RMGICs not as a result of insufficient polymerization but as a result of melting of material. However, in the present investigation the leachable components of the experimental GICs and RMGICs in the culture medium were not assessed. Numerous studies confirmed that the rank of relative toxicity of tested materials significantly varied depending on the type of cell lines used for testing [18, 19]. Geurtsen et al. [17] reported that various primary cell types derived from human oral tissues, like human pulp fibroblasts and human periodontal ligament fibroblasts did not consistently respond as less sensitive to toxic effects of dental resin components compared to the continuous 3T3 cell line. Moharamzadeh et al. [19], confirmed that the variability of cell responses were higher in primary human periodontal ligament cells and pulp fibroblasts compared to 3T3 fibroblasts. Present study showed the differences in the responses of NIH3T3 mouse fibroblast cells as well as UMR-106 osteoblast-like cells during the testing of cytotoxic properties of conventional GICs and RMGICs. Based on experimental results of MTT assay in this study, Mann-Whitney test showed that NIH3T3 mouse fibroblast cells were significantly more sensitive than UMR-106 osteoblast-like cells where Z= -4,666 and p=0.0001. Aalthough results of cytotoxicity testing of dental materials depended on cell type as well as the assay system used in the study our data support the concept which suggested that RMGICs are more cytotoxic comparing to conventional GICs. It should be emphasized that the present results should not be interpreted as an indicator that any of the GICs examined have significant potential for pulpal toxicity when placed onto intact dentin.

CONCLUSION

Based on applied methodology, the results of this study have shown:

- The evaluation of citotoxicity of glass-ionomer cements on cell lines NIH3T3 mouse fibroblast cells and UMR-106 osteoblast-like cells, RMGICs showed more citotoxicity than conventional glass-ionomer cements. Vitrebond (3MESPE) showed significantly higher cytotoxicity than all other materials while Fuji IX GP Fast (GC Corporation) and Ketac Silver (3MESPE) were the least cytotoxic tested materials.
- Cell line of NIH3T3 mouse fibroblast cells was significantly more sensitive to cytotoxic influence of elutes of tested glass-ionomer cements than cell line UMR-106 osteoblast-like cells.
- With the aim to achieve a comparable results and further development of screening tests, international standardize protocol for the preparation of the specimens, including determination of the surfaces of the specimens as well as cell lines, and volume of the cell lines, is needed. This protocol will improve accurate estimation of the risk of dental materials usage.

ACKNOWLEDGEMENTS

This study was supported by Western University, Canada and The Dental Faculty, University of Sarajevo with the international agreement that is held between both Universities. The authors gratefully acknowledge colleagues at The Division of Oral Biology at Schulich School of Medicine & Dentistry, Western University, Canada for their skilful technical assistance.

DECLARATION OF INTEREST

The authors have no conflict of interest to declare.

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