Dent Mater 10:298-303, September, 1994

Correlation of cytotoxicity with elemental release from mercury- and gallium-based dental alloys *in vitro*

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ABSTRACT

Objectives. An *in vitro* screening test was used to compare the cytotoxicity and elemental release from mercury- and gallium-based dental restorative materials.
Methods. The test employed three sequential extractions of the samples into cell-culture medium which were then used to evaluate the cytotoxicity of the samples and the release of elements from the samples. Cytotoxicity was measured by placing the extract in contact with Balb/c mouse fibroblasts for 24 h and measuring the succinic dehydrogenase activity of the cells. The release of elements was measured by means of atomic absorption spectrophotometry.
Results. Samples of Tytin (Kerr) showed no cytotoxicity compared to Teflon controls. Dispersalloy (Johnson and Johnson) was severely cytotoxic initially when Zn release was greatest, but was less toxic between 48 and 72 h as Zn release decreased. Gallium Alloy GF (Tokuriki Honten) was moderately cytotoxic after 8 h, and increased in cytotoxicity thereafter, which correlated with a substantial and persistent release of Ga from this material.
Significance. The results of the current study concurred with *in vivo* assessments of these materials, and the use of sequential extractions was useful in determining trends in the cytotoxicity and elemental release from these materials.

INTRODUCTION

Gallium-based alloys were introduced to dentistry in 1956 with the discovery that combinations of gallium and nickel produced a setting mass which was packable and had promising physical properties for oral restorations (Smith and Caul, 1956). Subsequently, alloys of gallium, copper, and tin (Smith *et al.*, 1956), gallium and palladium (Waterstrat and Longton, 1964), and gallium, palladium, and tin (Waterstrat, 1969) were reported. Modern formulations of these materials combine liquid alloys of gallium, indium and tin with a solid alloy of silver, palladium, tin, copper, and zinc (Horibe *et al.*, 1986; Okamoto and Horibe, 1991).

The biological response to gallium-based dental alloys has been of concern since their introduction (Waterstrat and Longton, 1964; Waterstrat, 1969). Although soluble gallium ions are relatively non-toxic compared to other elements such as cadmium (Kawahara *et al.*, 1968; Ferm and Carpenter, 1970; Domingo *et al.*, 1987), gallium ions significantly disrupt cellular metabolism (Aoki *et al.*, 1990; Wataha *et al.*, 1991). These adverse effects have been used to advantage in the development of Ga-based anti-cancer drugs, which have significant systemic toxicity, especially in the kidneys (Foster *et al.*, 1986). The biological safety of gallium-based dental alloys has been further questioned in light of reports (Lyon *et al.*, 1966; Langeland *et al.*, 1967; Kaga *et al.*, 1992a) which have demonstrated that these alloys elicit moderate to severe inflammatory responses when implanted subcutaneously and disintegrate over 6 mon. Clinically, dental restorations of gallium-based alloys have shown discoloration, tarnish, and roughness over periods of one year (Kim *et al.*, 1988; Yamashita *et al.*, 1989; Den *et al.*, 1991; Navarro *et al.*, 1993; Sakai *et al.*, 1993), which indicates that the *in vivo* deterioration of these alloys is significant.

New gallium-based alloys are being developed to improve the corrosion and biological properties of the earlier versions of these alloys, but there is a significant need for an *in vitro* screening test to measure the biological response to new formulations since animal and human experiments are expensive, time consuming, and controversial. There have been several previous reports of attempts to measure the *in vitro* cytotoxicity of gallium- and mercury-based alloys. Psarras *et al.* (1992) first exposed the alloys to saline for 1 or 10 wk, then tested alloy cytotoxicity indirectly through a filter barrier. They then exposed the samples to cell-culture medium for 24 h or 7 d and tested the cytotoxicity of the medium extracts. Although all of the samples caused mild to severe toxicity through the filters, none of the cell-culture extracts was severely cytotoxic; extracts from the specimens corroded for 10 wk were, however, slightly more toxic. The test described is too long to be useful as a screening test, and these authors did not measure the released elements which might be causing the cytotoxic responses.

An *in vitro* test to measure the cytotoxicity of gallium-

Material	Batch Number	Hg	Ga	Ag	Sn	Cu	Pd	In	Zn	Powder/Liquid Ratio (g/g)
Powder:										
Gallium Alloy GF	Not Avail.			50.0	25.7	15.0	9.0		0.3	1.00/0.65
Dispersalloy*	180990A 0J519			69.6	17.7	11.8			0.67	1.00/1.00
Tytin	060188 1127			60.0	27.0	13.0				1.00/0.82
Gallium Metal	Not Avail.		100							
Liquid:										
Gallium Alloy GF			65.00	0.05	16.00			18.95		
Dispersalloy		100								
Tytin		100								

TABLE 1: COMPOSITION OF ALLOYS (WT. %)

* after deFreitas (1976)

based alloys has also been reported by Kaga *et al.* (1992b). These investigators also used a filter test to measure the cytotoxicity of a gallium-based alloy to human gingival fibroblasts after 24 h of exposure. They reported that the gallium-based alloy was less cytotoxic than Dispersalloy, but they did not carry the exposure beyond 24 h. Furthermore, although they measured the cytotoxicity of the individual elements which comprise the gallium-based alloy, they did not measure release of these elements from the samples themselves and were, therefore, only able to speculate about which element might be causing the cytotoxicity.

The purpose of the current study was to use an *in vitro* screening test to compare the cytotoxicity of dental amalgams to a gallium-based alloy which is used for dental restorations. This test offers several advantages over previous tests. First, by measuring the cytotoxicity of sequential extracts of the samples, trends in the cytotoxic response were identified. These trends were useful in predicting possible longer-term behavior from a short-term test. Second, by measuring the elements which were released into the cell-culture medium at different extraction intervals, trends in the corrosion of the samples were identified. And finally, by correlating the concentrations of released elements with the previous cytotoxicity data on the individual elements (Wataha *et al.*, 1991), the elements which were the most likely causes of the cytotoxicities of these samples were identified.

MATERIALS AND METHODS

Four materials were tested (Table 1). Gallium Alloy GF (Tokuriki Honten, Tokyo, Japan) was chosen because it has been previously tested *in vitro* (Kaga *et al.*, 1992b; Psarras *et al.*, 1992). Dispersalloy (Johnson and Johnson, New Brunswick, NJ, USA) and Tytin (Kerr, Romulus, MI, USA) were chosen because they are representative of mercury-based dental amalgams currently used as dental restorations. Pure gallium metal (99.99%, Johnson and Mathey, Ward Hill, MA, USA) was chosen to facilitate cytotoxicity comparisons with Gallium Alloy GF. Finally, Teflon (Small Parts, Inc., Miami Lakes, FL, USA) was chosen as a negative control. Six replicates of each material were mixed according to the manufacturers' directions. Gallium Alloy GF was triturated using a Vari-Mix III amalgamator (L.D. Caulk, Milford, DE, USA) on the L setting for 7 s. Dispersalloy was triturated on the M setting for 15 s, and Tytin on the M setting for 10 s. Cylindrical specimens (4 mm in diameter and 3 mm high) were prepared in a stainless steel mold with a pressure of 14 MPa according to the procedures outlined in ANSI/ADA specification #1 (ADA, 1980). The samples were then aged for 7 d at 37°C. Pure gallium specimens were melted at approximately 60°C and cast into a vinyl polysiloxane mold. For determination of Zn, separate samples 4 mm in diameter and 8 mm high were fabricated to provide more extract volume.

The samples were aged for 1 wk at 37°C and were then polished using clinically relevant procedures. Dispersalloy and Tytin samples were polished on 600 grit silicon carbide paper with water, then final polished with a pumice slurry and tin oxide slurry. Gallium Alloy GF was polished as specified by the manufacturer using medium and fine polishing points with intermittent soaks in water. After polishing, the samples were cleaned and disinfected for cytotoxicity testing by scrubbing each sample with a soft toothbrush and Alconox soap solution, followed by copious rinsing with double-distilled water, submersion in 95% ethanol, and four rinses with sterile double-distilled water. The samples were then stored at 25°C for 24 h in a sterile container. The total time between fabrication of the samples and immersion into cell-culture medium was approximately 2 wk.

The samples (geometrical surface area of 63 mm^2) were extracted individually into cell-culture medium as follows. Each sample was submerged in 1.0 mL of cell-culture medium consisting of Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY, USA), 3% NuSerum serum supplement (Collaborative Research, Bedford, MA, USA), 125 units/mL of penicillin, 125 µg/mL of streptomycin, 2 mmol/L glutamine, 10 µg/mL of gentamycin, and 28 mmol/L of HEPES, pH = 7.2 (Gibco BRL). The samples were then incubated 8 h at 37°C in sealed, sterile polyethylene centrifuge tubes. After 8 h, the medium was removed and transferred to a separate vial. Another mL of medium was added, and the samples were incubated for 40 h. The medium was removed, then replaced, and the samples were incubated for a final 24 h. The cytotoxicity of the extracts (0-8 h, 8-48 h, and 48-72 h) and elements released into them were measured. For determinations of the release of zinc, the 4 x 8 mm samples were extracted into 2.0 mL of medium to maintain the same surface area to volume ratio. Pilot experiments showed that the release of Zn from either 4 x 3 or 4 x 8 mm samples was equivalent.

The cytotoxicity of the extracts was tested on Balb/c 3T3 cells (ATCC CCL 163, clone A31, Rockville, MD, USA) because these cells are recommended by the International Standards Organization for cytotoxicity screening (ISO, 1993) and because the investigators had previous data on the cytotoxicity of the individual elements on these cells (Wataha *et al.*, 1991). The cells were cultured and plated into 96-well dishes (0.33 cm^2/well) at 25,000 cells/cm^2 in 200 µL of medium. After incubation at 37°C and 5% CO_2 for 24 h, the

TABLE 2: CONDITIONS FOR ATOMIC ABSORPTION DETECTION				
Element	Method	Wavelength (nm)	Slit Width (nm)	Detection Limit (ppb)
Ag	GFA*	328.1	0.5	6.8
Cu	GFA	327.4	0.5	16.3
Ga	GFA	294.4	0.5	1600
In	GFA	325.6	0.5	24
Hg	CV**	253.7	0.5	54
Zn	AA***	213.9	1.0	82

* Graphite Furnace Atomization
** Cold Vapor Method
*** Air-Acetylene Flame Atomization

medium was removed, and 100 μL of the alloy extracts were added to each well. After an additional 24 h, the mitochondrial activity of the cells was assessed by measuring the succinic dehydrogenase (SDH) activity. SDH activity was measured using the MTT technique as previously described (Pearse, 1972; Wataha *et al.,* 1991).

The concentration of the elements in the extracts was measured by means of atomic absorption spectrophotometry (Varian Model AA20, Varian, Mulgrave, Australia). Conditions for the analysis and detection limits are listed in Table 2. For the graphite furnace method, samples were diluted to the appropriate concentrations with 10% v/v HNO_3, except for silver which was diluted with 40% v/v HNO_3. For mercury, the cold vapor method (Dominski, 1985) was employed using a cold vapor generator (VGA-76, Varian), and samples were diluted with 5% by volume HNO_3 and 5% by volume HCl. For the air-acetylene flame method, 30% HNO_3 was used as a diluent.

The cytotoxicity of the extracts was calculated by expressing the SDH activity of the cells as a percentage of the Teflon negative controls. Three standard errors of the mean were used to represent 95% confidence intervals of these values. Differences between the cytotoxicities were assessed using ANOVA and Tukey multiple comparison intervals ($p = 0.05$). The concentrations of elements released into the cell-culture medium were averaged, and three standard errors of the means were computed.

RESULTS

The Tytin extracts were not significantly different in cytotoxicity from Teflon in any of the extraction intervals (Fig. 1). Dispersalloy extracts were severely toxic for the 0-8 and 8-48 h intervals, but were only moderately toxic for the 48-72 h interval. Gallium Alloy GF extracts were moderately toxic for all intervals, but the cytotoxicity increased from a 25% depression at 0-8 h to more than 45% at the 8-48 and 48-72 h intervals. The gallium metal was moderately toxic at all three intervals.

The Tytin samples released levels of mercury which were generally below the detection limits, but released low levels of silver and significant levels of Cu, especially in the 0-8 h interval (Table 3). Little or no Zn release was detected. Dispersalloy samples released low levels of mercury, and levels of Cu and Ag which were lower than for the Tytin samples. However, Dispersalloy released significant levels of Zn in the initial intervals (0-8 and 8-48 h). Gallium Alloy GF samples

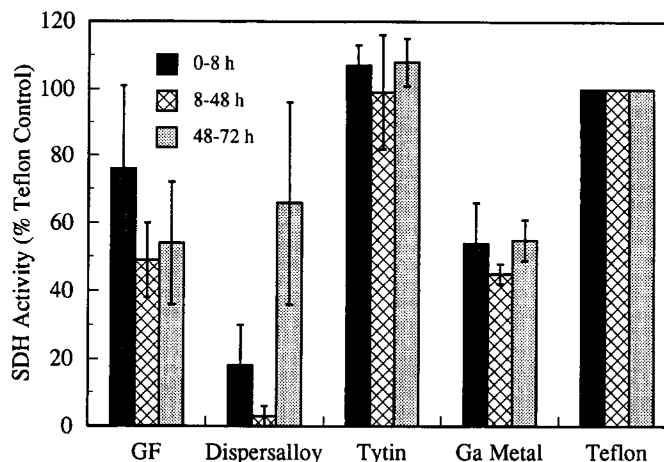

Fig. 1. **Cytotoxicity of extracts of gallium- and mercury-based dental alloys. Extraction intervals were 0-8, 8-48, 48-72 h. Balb/c 3T3 mouse fibroblasts were exposed to the extracts for 24 h. The succinic dehydrogenase (SDH) activity was measured and expressed as a percentage of the Teflon negative control. Error bars are three standard errors of the mean. ANOVA (with Tukey multiple comparison intervals at p = 0.05) showed that Teflon and Tytin were not different in any time interval, but that Gallium Alloy GF (GF) and the Ga metal were significantly more cytotoxic than Teflon in all intervals. Dispersalloy extracts were significantly more cytotoxic than GF only for the 0-8 and 8-48 h intervals.**

released > 7000 parts per billion (ppb) of gallium in all intervals, but lower levels of Ag, Cu, and In. The levels of Ag and Cu were slightly higher in the 0-8 h interval. Palladium and zinc were not measured in the Gallium Alloy GF extracts because the release of these elements was below detectable levels in preliminary experiments. The gallium metal released > 17,000 ppb of gallium in all intervals. Zinc at concentrations of 130 ppb was detected in the Teflon extracts as expected, but concentrations of the other elements in the native medium were below detection limits (Table 2). Because of severe matrix interferences, the concentrations of tin were not measured in any of the extracts.

For the Dispersalloy and Tytin samples, the release rates were the highest from 0-8 h for Ag and Cu, but increased slightly for Zn (Table 4). The rate of release of Zn was ten times less for Tytin than for Dispersalloy, while the release of Cu was ten times greater for Tytin than for Dispersalloy. For the Gallium Alloy GF samples, the rates of release showed no clear pattern, but did not decrease over time. The release of Ga from gallium metal was faster in the 0-8 h interval than for the Gallium Alloy GF samples, but was roughly equivalent thereafter. Rates of Hg release were not calculated because the release of Hg was at or below detection limits.

DISCUSSION

To determine which element(s) were contributing to the cytotoxicity of these materials, the concentrations of elements released (Table 3) were compared with the concentrations of the elements required to cause a 50% reduction in SDH activity (Table 5), which were published previously (Wataha *et al.,* 1991). Tytin showed little or no cytotoxicity in spite of significant Cu release. However, the levels of Cu required (>15,000 ppb) to cause inhibition of SDH activity were not present in the extracts (Wataha *et al.,* 1991). Similarly, levels of Ag and Zn did not approach toxic levels in any interval. For Dispersalloy, the levels of Ag and Cu which were released were not sufficient to cause cytotoxicity, but the levels of Zn

TABLE 3: CONCENTRATIONS OF ELEMENTS RELEASED INTO CELL-CULTURE MEDIUM (ppb)

Extraction Period (h)	Hg	Ga	Ag	Cu	In	Zn
Gallium Alloy GF						
0 - 8		7000 ± 4000	42 ± 27	260 ± 140	100 ± 70	NM
8 - 48		22000 ± 8000	24 ± 14	50 ± 28	1680 ± 900	NM
48 - 72		24000 ± 8000	20 ± 15	55 ± 50	600 ± 250	NM
Dispersalloy						
0 - 8	16 ± 15		22 ± 5	185 ± 76		730 ± 240
8 - 48	90 ± 30		40 ± 19	200 ± 220		1300 ± 400
48 - 72	5 ± 5		21 ± 16	100 ± 50		470 ± 460
Tytin						
0 - 8	20 ± 10		35 ± 27	1740 ± 500		131 ± 17
8 - 48	46 ± 42		26 ± 17	1600 ± 600		160 ± 60
48 - 72	9 ± 6		32 ± 38	270 ± 80		190 ± 15
Gallium Metal						
0 - 8		17000 ± 600				
8 - 48		28000 ± 700				
48 - 72		21000 ± 300				

Note: Sn was not measured (NM). Pd and Zn were not measured for Gallium Alloy GF. The values given are means ± standard deviations of n = 6. The concentration of Zn in normal cell-culture medium was 130 ± 40 ppb. Other elements were not detected. Surface area of the samples to volume of medium was 63 mm²/1.0 mL. Vertical bars connect cells with values which are not statistically different (p = 0.05, Tukey multiple comparison intervals).

TABLE 4: RATES OF RELEASE OF ELEMENTS INTO CELL-CULTURE MEDIUM (ppb/h)

Extraction Period (h)	Hg	Ga	Ag	Cu	In	Zn
Gallium Alloy GF						
0 - 8		880	5.2	3.3	13	NA
8 - 48		550	0.6	1.3	42	NA
48 - 72		1000	0.8	2.3	25	NA
Dispersalloy						
0 - 8	NA		2.7	23		75
8 - 48	NA		1.0	5		29
48 - 72	NA		0.9	4		14
Tytin						
0 - 8	NA		4.3	210		0
8 - 48	NA		0.7	40		0.8
48 - 72	NA		1.3	11		2.5
Gallium Metal						
0 - 8		2100				
8 - 48		700				
48 - 72		875				

Note: Values marked NA are not available either because the release was lower than the detection limit (as with Hg) or the element was not measured in the medium (Zn). 95% confidence intervals are approximately 30% at p = 0.05. Vertical bars connect cells with values which are not statistically different (p = 0.05, Tukey multiple comparison intervals).

TABLE 5: CONCENTRATIONS OF ELEMENTS REQUIRED TO CAUSE 50% TOXICITY

Element	Conc. Required to Cause 50% Toxicity (ppb)[a]
Ag^{+1}	600
Cu^{+2}	15,000
Ga^{+3}	14,000
In^{+3}	>50,000
Pd^{+2}	38,000
Zn^{+2}	1,800

[a] Succinic dehydrogenase activity on Balb/c 3T3 cells, 16,000 cells/cm², 24 h exposure. Adapted from Wataha *et al.* (1991).

(730 and 1300 ppb at 0-8 and 8-48 h, respectively) approached cytotoxic levels (Table 5) and were most likely responsible for the cytotoxicity. The cytotoxicity of the samples decreased somewhat in the 48-72 h interval when the concentration of released Zn decreased. For Gallium Alloy GF, the amount of Ga released was more than sufficient to cause 100% toxicity (Table 5), but levels of Ag, Cu, and In were insignificant. The toxicity of the gallium-alloy extracts was somewhat less than might be expected since the released Ga levels were greater than those required to cause 50% toxicity. The reduced cytotoxicity of gallium-based alloy in the current study may have been caused by the higher cell densities (25,000 cells/cm²) which were used compared to previous investigations (16,000 cells/cm²) (Wataha *et al.,* 1991). It has been shown that for Ga, higher cell densities significantly reduce the toxic response of Balb/c 3T3 cells (Wataha *et al.,* 1993). The gallium metal released levels of gallium which were also cytotoxic. The toxicity of the 0-8 h extracts was greater for the gallium metal than for the gallium-based alloy, which correlated well with the higher release of Ga from the metal *vs.* the alloy in this interval. Furthermore, for the 8-48 and 48-72 h intervals, the amount of gallium released correlated with the cytotoxicities of the gallium metal or alloy. In these intervals, the cytotoxicities were not statistically different.

The current study demonstrated that the use of multiple extraction intervals combined with knowledge about which elements were released was valuable in projecting longer-term cytotoxicity behavior from short-term tests. In the 0-8 and 8-48 h extraction intervals, the cytotoxicity of Gallium Alloy GF was greater than that of Tytin, but less than Dispersalloy. However, in the 48-72 h extraction interval, the cytotoxicity of

Dispersalloy was reduced because the Zn release was decreasing. Thus, the longer-term trend in cytotoxicity favored Dispersalloy over Gallium Alloy GF because the release of elements from Dispersalloy was decreasing, whereas the release of Ga from the gallium-based alloy and its cytotoxicity showed no signs of decreasing. Longer-term tests are needed to verify these observations.

This work agrees with reports that the cytotoxicity of Gallium Alloy GF was less than that of Dispersalloy after 24 h (Kaga *et al.,* 1992b). However, because of the long-term trends in the release of elements, a 24 h test by itself is probably not predictive of longer-term behavior. In contrast, the results of the current study did not agree with other work which has shown that gallium-based alloy samples were no more cytotoxic than those from Dispersalloy even after 7 d of extraction (Psarras *et al.,* 1992). It is possible that the precorrosion of samples in saline used by these investigators or differences in cell-culture variables were responsible for these discrepancies. The results of the current study also agree with several groups who have reported that gallium-based alloys show severe corrosion and disintegration of samples when implanted subcutaneously in animals for up to 6 mon (Lyon *et al.,* 1966; Langeland *et al.,* 1967; Kaga *et al.,* 1992a). The current study shows that Gallium Alloy GF released substantial amounts of gallium which did not abate after 72 h of exposure to cell-culture medium. This finding correlates well with the behavior of these alloys *in vivo* for periods up to 6 mon. The significant corrosion of these alloys observed in the current study also correlated well with reports of significant tarnish, roughness, and discoloration in Ga-based alloys which have been placed intraorally (Kim *et al.,* 1988; Yamashita *et al.,* 1989; Den *et al.,* 1991).

In conclusion, the current study demonstrated that a 72 h test for measuring *in vitro* cytotoxicity was successful in discriminating between dental amalgam and gallium-based alloys. The use of sequential extracts over the 72 h period permitted observation of trends in both the elemental release from the materials and their cytotoxicities, and these trends correlated well with *in vivo* tests which have been previously published (Langeland *et al.,* 1967; Kaga *et al.,* 1992b; Kim *et al.,* 1988; Yamashita *et al.,* 1989; Den *et al.,* 1991; Navarro *et al.,* 1993). The cytotoxicity of Tytin was low throughout all extraction intervals, which correlated well with the low concentrations of elements released from this material. The cytotoxicity of Dispersalloy was severe initially, but improved between 48 and 72 h as the Zn release decreased. The cytotoxicity of Gallium Alloy GF was moderate initially but increased after 8 h, which correlated with a substantial and persistent Ga release from this material. This design of the cytotoxicity test reported here should be useful in evaluating new formulations of gallium-based and other dental restorative materials which are developed.

ACKNOWLEDGMENTS

The investigators gratefully acknowledge the support of the National Institutes of Health through Grants DE-10178 and DE-09296. The authors also thank Tokuriki Honten (Tokyo, Japan) for supplying the gallium-based alloy, and Ms. Lois Rockwell for her expert assistance with the atomic absorption spectrophotometry.

Received May 20, 1994 / Accepted August 17, 1994

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