

Evaluation of the cytotoxicity of elastomeric ligatures after sterilisation with 0.25% peracetic acid

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Introduction: Sterilisation using peracetic acid (PAA) has been advocated for orthodontic elastic bands. However, cane-loaded elastomeric ligatures can also become contaminated during processing, packaging, and manipulation before placement in the oral cavity and are therefore susceptible, and possible causes, of cross-contamination.

Aim: To test the hypothesis that 0.25% peracetic acid (PAA), following the sterilisation of elastomers, influences the cytotoxicity of elastomeric ligatures on L929 cell lines.

Materials and methods: Four hundred and eighty silver elastomeric ligatures were divided into 4 groups of 120 ligatures to produce, Group TP (latex natural, bulk pack, TP Orthodontics), Group M1 (Polyurethane, bulk pack, Morelli), Group M2 (Polyurethane, cane-loaded, Morelli) and Group U (Polyurethane, cane-loaded, Uniden). Of the 120 ligatures in each group, 100 were sterilised in 0.25% PAA at time intervals (N = 20) of 1 hour, 2 hours, 3 hours, 4 hours and 5 hours. The 20 remaining elastomeric ligatures in each group were not sterilised and served as controls. Cytotoxicity was assessed using L929 cell lines and a dye-uptake method. Analysis of variance (ANOVA), followed by the Tukey post hoc test ($p < 0.05$) determined statistical relevance.

Results: There was a significant difference between TP, Morelli and Uniden elastomers ($p < 0.05$), but no difference between the two types of Morelli elastomers at the 1 hour time interval. In addition, there was a significant difference between Group CC and the other groups assessed, except between Groups CC and TP at the 1 hour time interval. The non-sterilised elastomeric ligatures showed similar cell viability to that observed after 1 hour of standard sterilisation.

Conclusion: PAA did not significantly influence the cytotoxicity of elastomeric ligatures after a sterilisation time of 1 hour and is therefore recommended for clinical use.

(Aust Orthod J 2013; 29: 139-144)

Received for publication: January 2012

Accepted: May 2013

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Introduction

The rise in blood-borne diseases caused by viruses such as hepatitis C and HIV has generated an extensive review of infection control in dentistry.¹ There has been significant advancement and increasing use of sterilisation and disinfection techniques since the early 1990s. As a result, problems regarding the deterioration of instruments and an alteration of the

physical, biological and chemical properties of dental materials have appeared.¹⁻² Furthermore, studies have shown that sterilised orthodontic materials may be cytotoxic³ and cause harm.⁴

Cane-loaded elastomeric ligatures are orthodontic materials which are highly susceptible to cross-contamination¹ during processing, packaging, and manipulation by the dental assistant or orthodontist

Table 1. Experimental groups used for the cytotoxicity test.

Group	Commercial brand	Presentation	Lot	Reference number
TP	TP Orthodontics	bulk pack	0429010	361083
M1	Morelli	bulk pack	1132651	6003417
M2	Morelli	cane-loaded	1435294	6003317
U	Uniden	cane-loaded	071009-E1-4	000-1222

before being placed in the oral cavity. Elastomeric ligature placement usually occurs without prior disinfection or sterilisation. With the advent of tougher biohazard measures, this clinical conduct has been questioned.⁵⁻⁶

In an assessment of different elastomeric sterilisation methods, Pithon et al.⁷ determined that the use of ethylene oxide, ultraviolet radiation and gamma rays did not affect elastomeric cytotoxicity but 70% alcohol, 2% glutaraldehyde, autoclaving or microwaving increased cytotoxicity.

A clinical need has been established for elastomerics to be inert in the oral environment and capable of being sterilised whilst maintaining their elastic characteristics and pigmentation. Sterilisation with peracetic acid or peroxyacetic acid (PAA) has been suggested for orthodontic elastics and other dental materials due to minimal product deterioration and shorter sterilisation time compared with the use of glutaraldehyde.⁷ It has been reported that PAA does not produce toxic or mutagenic by-products after reaction with materials,⁸ as the PAA decomposition products are acetic acid, hydrogen peroxide, oxygen and water.⁹

The aim of the present study was to test the hypothesis that PAA does not influence the cytotoxicity of orthodontic elastomeric ligatures, which therefore remain safe for clinical use.

Material and methods

Four hundred and eighty silver elastomeric ligatures from various manufacturers were obtained and divided into 4 groups of 120 ligatures. Groups were identified as Group TP (latex natural, bulk pack, TP Orthodontics, La Porte, IN, USA), Group M1 (Polyurethane, bulk pack, Morelli, Sorocaba, SP, Brazil), Group M2 (Polyurethane, cane-loaded, Morelli, Sorocaba, SP, Brazil) and Group U (Polyurethane, cane-loaded, Uniden, Sorocaba, SP, Brazil) (Table 1). The elastic

ligatures from TP Orthodontics and Morelli (Group M1) were converted into a cane-loaded system by using sterile straight Halsted mosquito forceps to standardise the groups (Quinelato, Rio Claro, São Paulo, Brazil).

Of the 120 ligatures in each group, 100 were further subdivided and sterilised in 100 ml of 0.25% peracetic acid (Proxitane®, Alfa, Curitiba, PR, Brazil) over five different time intervals (N = 20) of 1 hour (T1), 2 hours (T2), 3 hours (T3), 4 hours (T4) and 5 hours (T5). Subsequently, all ligatures were washed for 15 minutes in de-ionized water using the Milli-Q® purification system (Millipore, Bedford, MA, USA) to remove excess peracetic acid. The 20 remaining elastic ligatures in each group remained unsterilised and served as controls. The cytotoxicity of materials was determined in accordance with the evaluation and standardisation norms of ISO 10993-59.

Cell line, L929, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) (rat fibroblasts). The cells were cultivated in Eagle's minimal essential medium (MEM) (Cultilab, Campinas, São Paulo, Brazil) and supplemented with 2 mM of L-glutamine (Sigma, St. Louis, MO, USA), 50 µg/ml of gentamycin (Schering Plough, Kenilworth, NJ, USA), 2.5 µg/ml of fungizone (Bristol-Myers-Squibb, NY, USA), 0.25 ml of sodium bicarbonate solution (Merck, Darmstadt, Germany), 10 mM of HEPES (Sigma, St. Louis, MO, USA) and 10% of fetal calf serum (FCS) (Cultilab, Campinas, São Paulo, Brazil), and kept at 37°C in a 5% CO₂ environment.

Three additional cell groups were assessed in order to characterise and differentiate the cellular response. These comprised Group CC (cell control) in which the cells were not exposed to any material, Group C+ (positive control) in Tween 80® (polyoxyethylene-20-sorbitan) and C- (negative control) in PBS (phosphate-buffered saline) solution.

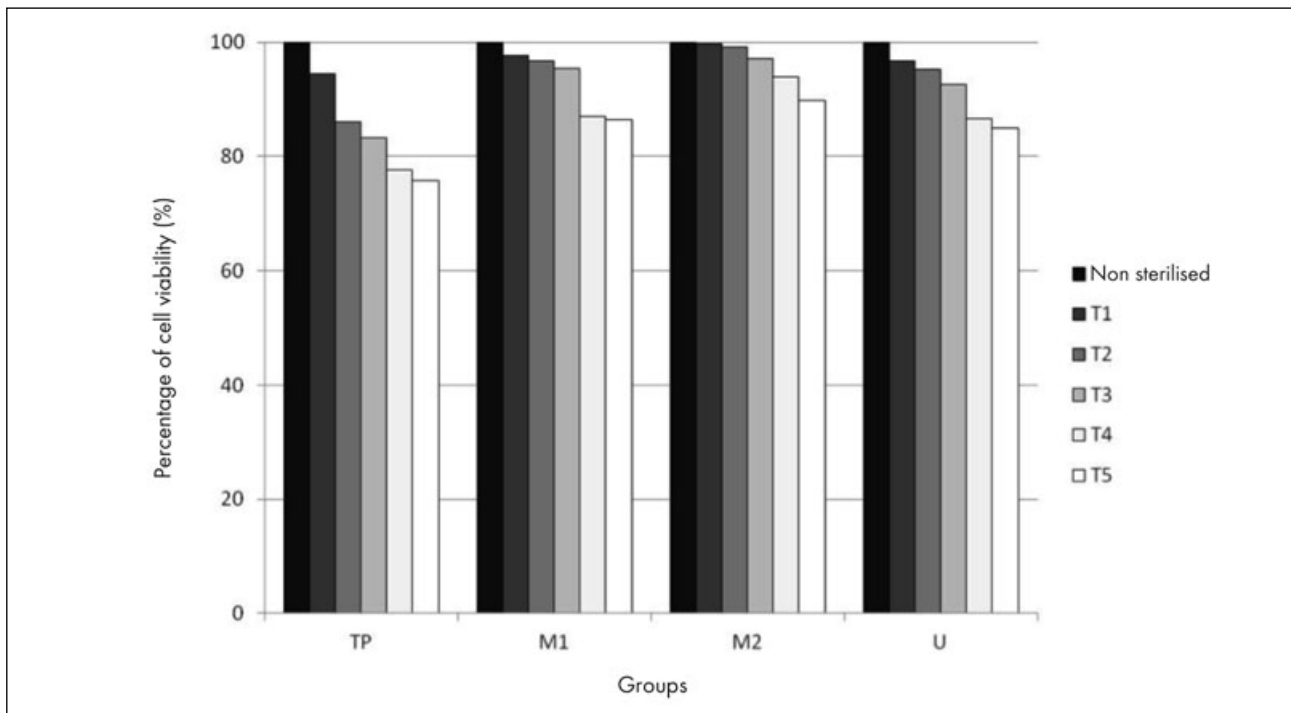


Figure 1. Percentage difference of the cell viability decrease of elastics in different time intervals (T1-T5).

Following sterilisation of the elastomeric ligatures, samples of ligatures were placed in 24-well plates containing a culture medium (MEM) (Cultilab, Campinas, São Paulo, Brazil). The culture medium was replaced every 24 hours, the supernatants collected and the effect on the L929 cells was assessed. The supernatants were placed, in triplicate, in a 96-well plate containing a confluent monolayer of L929 cells and incubated for 24 hours at 37°C in a 5% CO₂ environment.

After the incubation period, cell viability was determined by a dye uptake technique¹⁰ which involved the addition of 100 µl of 0.01% neutral red (Sigma, St. Louis, MO, USA) to the culture medium in each microplate well. A further 3 hour incubation at 37°C allowed the dye to penetrate into the live cells following which 100 µl of 4% formaldehyde solution (Reagen, Rio de Janeiro, Brazil) was added to the PBS (NaCl 130 mM; KCl 2 mM; Na₂HPO₄ 2H₂O 6 mM; K₂HPO₄ 1mM, pH 7.2) for five minutes to promote fixation of the cells to the plates.

Dye extraction was performed by the use of 100 µl of 1% acetic acid solution (Vetec, Rio de Janeiro, Brazil) in 50% methanol (Reagen, Rio de Janeiro, Brazil). Readings were performed in a spectrophotometer

(BioTek, Winooski, VT, USA) at a wavelength of 492 nm ($\lambda = 492$ nm) over 20 minutes.

A post hoc comparison was performed to determine cytotoxicity ranking.¹¹⁻¹² Statistical calculations were undertaken by a one-way analysis of variance (ANOVA) followed by a Tukey post hoc test (SPSS 13.0®, SPSS Inc., Chicago, IL, USA). *P* values less than .05 ($p < 0.05$) were accepted to indicate significant differences. Each culture well was considered as an individual sample.

Results

The results indicated that Group TP (TP Orthodontics) maintained greater cell viability at all time periods assessed. Groups M1 and M2 (Morelli, bulk pack and cane-loaded, respectively) showed similar cell viability throughout the experiment. Group U (Uniden) showed low cell viability at a time interval of 1 hour and beyond. There was a statistically significant difference between the TP, Morelli and Uniden brands ($p < 0.05$) but there was no statistically significant difference ($p < 0.05$) between the two types of Morelli brand at the 1 hour time interval (Table II).

At time intervals of 2, 3, 4 and 5 hours, there was a statistically significant difference between Group

Table II. Number of cells (Mean), standard deviation and statistical analysis of groups of elastic bands tested.

	Non sterilised			T1		T2		T3		T4		T5	
	Mean (SD)	Stat.		Mean (SD)	Stat.	Mean (SD)	Stat.	Mean (SD)	Stat.	Mean (SD)	Stat.	Mean (SD)	Stat.
TP	689.4 (22.2)	A		651.1 (42.2)	A	592.2 (13.4)	A	574.1 (35.5)	A	535.5 (28.9)	A	522.2 (56.2)	A
M1	589.4 (17.3)	B		575.6 (76.2)	B	570.1 (28.2)	A	562.2 (87.1)	A	512.7 (45.8)	A	509.2 (24.5)	A
M2	573.1 (28.2)	B		571.2 (27.8)	B	568.2 (45.2)	A	556.2 (59.1)	A	533.6 (76.2)	A	514.5 (55.2)	A
U	104.1 (14.5)	C		100.6 (34.5)	C	99.1 (45.3)	B	96.3 (11.3)	B	90.2 (18.2)	B	88.3 (45.6)	B
CC	714.6 (55.2)	A		714.6 (55.2)	A	714.6 (55.2)	C	714.6 (55.2)	C	714.6 (55.2)	C	714.6 (55.2)	C
C+	102.1 (12.3)	C		102.1 (12.3)	C	102.1 (12.3)	B	102.1 (12.3)	B	102.1 (12.3)	B	102.1 (12.3)	D
C-	704.6 (34.1)	A		704.6 (34.1)	A	704.6 (34.1)	C	704.6 (34.1)	C	704.6 (34.1)	C	704.6 (34.1)	C

Stat. = statistical analysis: Equal letters correspond to the absence of statistical differences ($p > 0.05$)
SD = standard deviation

Uniden and the other groups ($p < 0.05$). However, there was no statistically significant difference between Groups TP and Morelli (bulk pack and cane-loaded). Statistically significant differences were observed between Group CC and all other groups assessed, except between Groups CC and TP at the 1 hour time interval.

For the non-sterilised elastomerics, cell viability was evident and considered as 100%. The non-sterilised groups of elastomerics showed similar cell viability to that observed after a standard sterilisation protocol of 1 hour. As the sterilisation time increased, a gradual decrease in cell viability occurred (Figure 1).

Group TP showed the highest percentage difference of viable cells between the time intervals. The Groups M1 and M2 showed similar percentage differences, while Group U maintained this variable more subtly (Figure 1).

Discussion

So that patients and professionals can be protected, infection control authorities have recommended that disinfection must be performed if the sterilisation of materials which come into contact with the oral environment is not possible.¹³ Because of the large number of bacterial species in the oral cavity,¹ diseases such as influenza, tuberculosis, herpes and hepatitis B, may be transmitted during dental operative procedures. However, effective sterilisation or disinfection usually prevents the dissemination of pathogens.¹⁴

A concentration of 0.25% peracetic acid (PAA) has been identified as a chemical alternative to sterilise critical and semi-critical devices to prevent cross-

contamination.⁵ Its odour is less pungent than glutaraldehyde and it is not affected by glass and by most plastics.⁶ The aim of this study was to determine whether PAA could be successfully used to sterilise elastomers without producing adverse effects.

Cellular alterations may be observed by different methods,¹⁵⁻¹⁷ but in the present study, the cellular incorporation of neutral red was measured.^{3,13,18-20} This method has previously been widely used for orthodontic materials.^{3,19-21} The cytotoxicity of materials is determined by spectrophotometry, which records the amount of dye incorporated into the cells and which directly relates to the number of viable cells. The cells used were derived from the L929 line of mouse fibroblasts which are comparable to human gingival fibroblasts²²⁻²³ and therefore provide an appropriate model for cytotoxicity tests of materials and cells related to the oral cavity.

As the end-product of the reaction of hydrogen peroxide with acetic acid, PAA is considered an efficient sterilisation method against a wide range of microorganisms when used at low active concentrations.^{22,24-26} However, it is imperative to know if the method of sterilisation alters the properties of dental materials, in this case orthodontic elastomers.

PAA is commonly used for a sterilisation period of 1 hour at a concentration of 0.25%.^{22,24-26} The present study found that the elastic ligatures manufactured by TP Orthodontics and Morelli showed cell viability higher than 80% after a 1 hour sterilisation period. These values were found to be similar to the non-sterilised control elastic ligatures. It was concluded that a sterilisation process of 1 hour was sufficient

and did not result in noticeable adverse effects on cell viability.

However, a sterilisation period longer than 1 hour produced a higher cell cytotoxic effect. This suggested that the use of PAA for a prolonged sterilisation period or the re-sterilisation of elastic ligatures which had previously been exposed to PAA, may lead to a higher release of bioactive components which affect the viability of the fibroblast.

The elastic ligatures manufactured by Uniden produced low cell viability over all experimental time periods. It is possible that a different manufacturing process and/or the presence of stabilising substances in the composition of these ligatures may be responsible for the performance differences, since all tested ligatures were made of the same raw material.

Studies^{21,27} which have analysed the possible cytotoxic effects of dyes used in the fabrication of intra-oral elastic ligatures revealed that the colourless and coloured elastomerics caused cytotoxic effects in in vitro experimental conditions. The conclusion drawn was that pigmentation was unlikely to influence the cytotoxicity of elastics.

Santos et al.³ compared the cytotoxicity of latex-free elastomeric ligatures (American Orthodontics and Unitek) with those of polyurethane (Morelli, GAC, Tecnident) and those that contained latex (TP Orthodontics). Latex-free elastomeric ligatures were found to cause less cell lysis than latex or polyurethane ligatures, the latter being more cytotoxic. The elastic ligatures made by TP Orthodontics maintained greater cell viability while the Uniden brand produced the least cell viability among the groups assessed. However, cell viability was still higher than 50%,³ in support of the findings of the present study. In an examination of the degradation in strength of elastomeric ligatures, Simões²⁸ assessed different brands and types (cane-loaded and modular) after sterilisation in 0.25% peracetic acid and found that all assessed brands suffered minimal degradation without exhibiting any statistically significant differences.

According to Ceretta,²⁹ a cytotoxic chemical product must be considered when cell population reduction is higher than 10%. In the present study, cell reduction was 5.5% after 1 hour, which was much less than other reported methods of sterilisation.⁵ In addition, PAA was considered a viable alternative method of sterilisation, compared with ethylene oxide, UV radiation and gamma rays, because it required shorter

sterilisation time,⁵ and was an acceptable method for clinical or public health application. This does not apply for UV radiation and gamma rays, which are mainly for industrial use.

The comparison of 0.25% peracetic acid on the cytotoxicity of test samples against an unsterilised control sample, revealed that the TP Orthodontics ligatures were affected by PAA, even though greater cell viability existed over all assessed time intervals. The Uniden brand was least affected by PAA, but produced the lowest cell viability of the groups. A possible reason why PAA had a greater influence on elastic ligatures made by TP Orthodontics is perhaps because they are made from natural latex compared with Morelli and Uniden ligatures, which are made of polyurethane. PAA has been shown to react with types of vinyl, synthetic and natural rubbers, depending on contact time.^{8,28}

As elastic ligatures are in close contact with the gingiva and oral mucosa, care regarding their potential cytotoxic effects is recommended. However, the results found in in vitro tests must not be over-estimated and further clinical studies are needed to confirm the present findings.

Conclusion

It is concluded that PAA did not significantly influence the cytotoxicity of elastomeric ligatures at the recommended sterilisation time of 1 hour. The TP Orthodontics brand showed greater cell viability, but was more susceptible to the influence of PAA compared with the other brands. However, while clinical testing is required, PAA is recommended for routine use for the disinfection of elastomeric ligatures.

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