Original Article

pISSN, eISSN 0125-5614
M Dent J 2022; 42 (2) : 137-144

Cytotoxic effect of gutta percha solvents on human periodontal ligament fibroblasts

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Objective: The aims of this study were to evaluate cytotoxic effects of gutta percha solvents: industrial solvent (xylene), solvent containing d-limonene (GP-solvent), and solvent composed of essential oils and surfactants (GuttaClear) at various concentrations and two exposure times on cell viability of human periodontal ligament fibroblasts (HPDLFs).

Materials and Methods: The cytotoxicity of xylene, GP-solvent, and GuttaClear in different dilutions as 1:200, 1:400, 1:800,1:1600, 1:3200, 1:6400, 1:12800, and 1:25600 dilutions of each solvent, to human periodontal ligament fibroblasts were measured by MTT assay. As the tested solvent could not be totally miscible in DMEM, thus 10% dimethyl sulfoxide was used as a solubilizer to improve the solubility of the substances, and ultrasonic activation was used to facilitate the contact of solvents. The mean percentages of cell viability after 30 minutes and 24 hours exposed to various dilutions of solvents were evaluated and compared the difference of cell viability among experimental groups.

Results: The cytotoxicity of each solvent was relatively dose dependent. HPDLF cells showed the viability above 80% when exposed to 1:12800 (0.008%) and 1:25600 (0.004%) concentrations of all solvents in both 30 minutes and 24 hours exposure times. The solvents became toxic to HPDLFs at the different concentrations over 1:6400 (0.016%), 1:3200 (0.031%) and 1:800 (0.001%) for GuttaClear, GP-solvent, and xylene, respectively, regardless of the exposure times.

Conclusion: All tested gutta percha solvents showed cytotoxicity to HPDLFs in a dose-dependent manner, which arranged in that descending orders: GuttaClear, GP-solvent, and xylene. At high concentrations, all of the solvents showed similar severe toxic effects.

Keywords: cytotoxicity, GP-solvent, GuttaClear, gutta percha solvent, root canal retreatment, xylene

How to cite: Kespichayawattana N, Jantarat J, Ounjai P, Wongtrakoongate P. Cytotoxic effect of gutta percha solvents on human periodontal fibroblasts. M Dent J 2022; 42: 137-144.

Introduction

Many studies reveal that the success rate of root canal treatment varies from 82 – 98% [1, 2]. Although the incidence of post-treatment disease is low, appropriate management is still required. To avoid tooth extraction, non-surgical root canal retreatment should be considered as one of the treatment choices [3]. In root canal retreatment, adequate cleaning and disinfection of the root canal system must be achieved. Removal of existing root canal filling is required to gain access to the root canal system [4, 5]. Several techniques are suggested for the gutta percha removal, such as the use of heat, ultrasonic, hand, and rotary instruments [6-8]. Nevertheless, using only the

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Received: 13 November 2021 Revised: 25 March 2022 Accepted: 28 March 2022

thermo-mechanical techniques for removing gutta percha, several complications may occur, such as ledge, transportation, or broken instrument. The use of chemical solvents reduces excessive force during negotiation, so the occurrence of transportation decreases [8]. Thus, using a solvent in combination with other techniques is recommended to soften and remove gutta percha, providing an easier, faster, and safer procedure for gutta percha removal [9-11].

In the past, chloroform was widely used as the most effective solvent but had a serious concern. According to the International Agency for Research on Cancer (IARC), chloroform is classified into a group 2B carcinogen and is a potential risk to a dental practitioner [12]. Consequently, alternative choices of solvent have been proposed [13]. Industrial solvents (such as xylene, xylol, and halothane), natural essential oil (such as eucalyptol oil, turpentine oil, pomelo oil, and orange oil), and the solvent contained d-limonene (GP-solvent) were investigated. However, the effectiveness of alternative solvents is still less than chloroform. Nowadays, xylene and GP solvent are commercially available and clinically used as non-carcinogenic solvents. Xylene has been widely used for a long time. The major problem of using xylene is the toxic effect of inhaling xylene vapor. Xylene affects the central nervous system causing symptoms such as headache, dizziness, nausea, and vomiting. GP solvent is considered as the effective solvent, but it is expensive and must be imported. There was an attempt to develop a new gutta percha solvent, which effectively dissolves gutta percha and is also safe for dentists, patients, and working environments. New formulation of gutta percha solvent composed of essential oils and surfactants (GuttaClear) demonstrated the effectiveness on softening and dissolving gutta percha, which was comparable to xylene and GP-solvent [14, 15]. However, one of the major concerns in using the solvent is extrusion beyond the apex of root canal during the gutta percha removal [16]. Extruding of solvents may irritate and affect the healing of periapical tissue. Many studies revealed that gutta percha solvents presented various cytotoxicity on mouse fibroblast cells line (L929), macrophages, and gingival fibroblast cells [17-21]. However, no previous studies tested the effect of gutta percha solvent on human periodontal fibroblasts (HPDLFs), which are responsible for the regeneration of the periodontium and healing of periapical tissue. Therefore, the present study aimed to evaluate the cytotoxic effect of xylene, d-limonene containing solvent, and new natural gutta percha solvent at various dilutions and two exposure times (30 minutes and 24 hours) on human periodontal ligament fibroblasts.

Materials and Methods

Human periodontal ligament fibroblast cells preparation

Human periodontal ligament fibroblasts (HPDLF, 2630, ScienCell Research Laboratories, CA, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, NY, USA) supplemented with 10% fetal bovine serum, 100 u/mL penicillin, and 100 µg/mL streptomycin at 37°C in 95% humidified, and 5% CO_2 which media was changed every 2 days. After HPDLFs reached 80% confluence, trypsinization with trypsin-EDTA was performed. The cells were sub-cultured and incubated at 37°C and 5% CO_2. The cell culture was examined daily under an inverted microscope (Nikon Model TMS, Kanagawa, Japan).

Test materials preparation

Three experiment groups were tested according to the solvent used as follows: Group 1: Xylene (Chulalongkorn University, Bangkok,

Thailand), Group 2: d-limonene containing solvent (GP-Solvent, Nippon Shika Yakuhin Ltd. Shimonoseki, Japan), and Group 3: new natural gutta percha solvent (GuttaClear, MDent, Bangkok, Thailand). Tested solvents were serially diluted with DMEM. As the substances tested could not be totally miscible in DMEM: thus, 10% dimethyl sulfoxide (DMSO; Sigma, MO, USA) was used as a solubilizer to improve the solubility of the substances, and ultrasonic activation was used to facilitate the contact of solvents.

All solvents were diluted to 1:10 dilution by mixing 100 µL of the solvent containing 10% DMSO with 900 µL of DMEM. Sonication of 1:10 diluted solvent was performed using an ultrasonic device (VS-100-III; AS ONE Inc, Japan) under 28 kHz and repeated 3 cycles for 5 minutes each. Then serial dilution was performed by mixing the solvents with DMEM. Tested solvents were divided into 8 experimental groups as 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, and 1:25600 dilutions of each solvent. A culture medium with 0.1% DMSO was used as the control group to confirm that there was no effect of DMSO to the cells.

Cytotoxicity testing procedure

1. Exposure period

A subconfluent monolayer culture of HPDLFs (passages 4-12) was removed from a culture flask by trypsinization using 0.25% trypsin solution, and the cells were collected in the culture medium. The cell suspense was counted and prepared at a cell density of 1×10^5 cells/mL. The HPDLFs were added 2×10^4 cells per well into 96-well flat-bottom plates and incubated in a chamber with 95% humidified atmosphere and 5% CO_2 at 37 ˚C for 24 hours, to ensure that the cells were in the exponential phase of growth. After a 24-hour incubation, the culture medium was aspirated from the cells. All solvents were tested in three wells for each dilution by adding 200 µL diluted solvents or 0.01% DMSO diluted with DMEM (control) in each well. The cells were incubated with diluted solvent in the same condition for 30 minutes and 24 hours exposure time as indicated for each experiment group. Next, an MTT assay was performed for an *in vitro* cytotoxicity test.

2. MTT assay

The MTT solution was prepared using 3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, CA, USA) in 10 mg/mL concentration and diluted with DMEM into 10% final concentration. At the end of the exposure period, diluted solvent and culture medium was removed. A total of 200 µL of MTT solution was added. The 96-well plates were incubated for 1 hour in a chamber with 95% humidified atmosphere and 5% CO_2 at 37 ˚C. After incubation, MTT solution was removed, and then 200 µL of DMSO was added to each well to solubilize formazan crystals formed by the survived cells. The plate was shaken for 10 minutes at room temperature before light absorbance was measured at 540 nm using a microplate photometer (Thermo Scientific Multiskan EX, Thermo Fisher Scientific, CA, USA).

The percentage of cell viability in each replicate was calculated by the following formula.

Percentage of cell viability = $(100*OD_{540e})/OD_{540c}$

where OD_{540e} is the absorbance of experiment sample, OD_{540c} is the mean absorbance of controls

Statistical analysis

Normality of data was tested by Kolmogorov Smirnov test, and homogeneity of variance was tested by Levene's test. The data were not normally distributed. To compare the difference of cell viability among experimental groups, Kruskal-Wallis was performed. Multiple comparisons were compared by Dunn's Test.

Statistical analyses were performed using SPSS software version 18 (IBM Corp, Armonk, New York) with a significant level of $\alpha = 0.05$.

Results

The mean percentages of cell viability after 30 minutes and 24 hours exposed to various dilutions of solvents are reported in Table 1. Reduction of cell viability by more than 30% is considered cytotoxic effect according to International standard ISO10993-12:2009 [22]. The HPDLF cells showed viability above 80% at 1:12800 and 1:25600 dilutions of all solvents in both groups of 30 minutes and 24 hours exposure times. The cytotoxicity of each solvent was relatively dose-dependent, as shown in Figures 1 and 2. At the higher concentration of solvent, the cytotoxicity was more prominent. The toxic effect was shown at the dilutions over 1:6400, 1:3200, and 1:800 of GuttaClear, GP-solvent, and xylene, respectively, regardless of the exposure times.

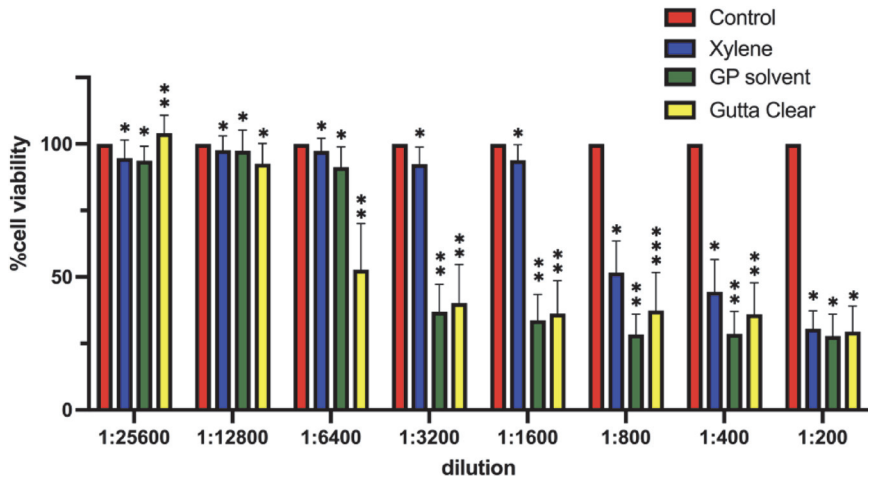

Figure 1 Percentage of cell viability after exposed to solvent for 30 minutes. Different symbols indicate the statistically significant differences among types of solvents in the same concentration ($p < 0.05$).

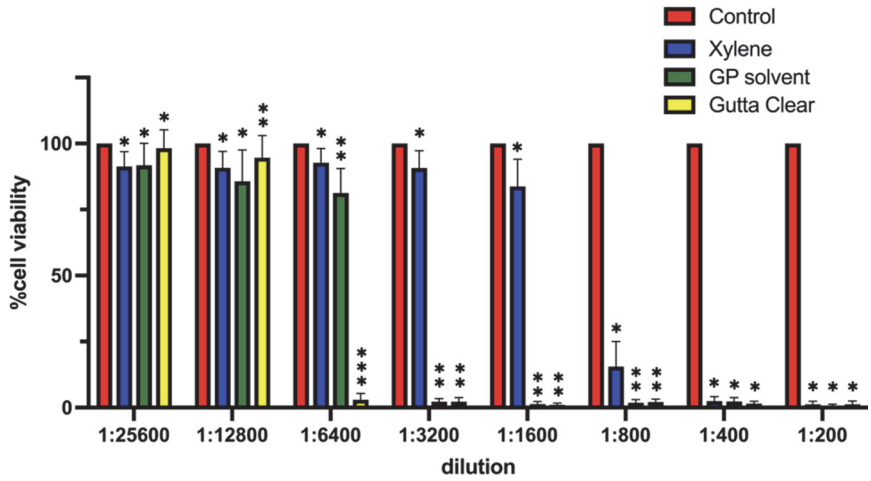

Figure 2 Percentage of cell viability after exposed to solvent for 24 hours. Different symbols indicate the statistically significant differences among types of solvents in the same concentration ($p < 0.05$).

Table 1 The mean percentage of cell viability after exposed to solvents for 30 minutes and 24 hours

Solvent	Dilution	30 minutes exposure		24 hours exposure	
		% cell viability (mean ± SD)	N	% cell viability (mean ± SD)	N
Xylene	1:200	30.527 ± 6.756 [a]	12	1.255 ± 1.136 [A]	12
	1:400	44.424 ± 12.104 [b]	12	2.508 ± 1.643 [A]	12
	1:800	51.617 ± 11.876 [b]	12	15.495 ± 9.596 [B]	12
	1:1600	93.887 ± 5.787 [c]	12	83.739 ± 10.277 [C]	12
	1:3200	92.435 ± 6.360 [c]	12	90.744 ± 6.551 [C]	12
	1:6400	97.388 ± 4.760 [c]	12	92.752 ± 5.381 [C]	12
	1:12800	97.682 ± 5.352 [c]	12	90.762 ± 6.304 [C]	12
	1:25600	94.651 ± 6.828 [c]	12	91.298 ± 5.665 [C]	12
GP-solvent	1:200	27.790 ± 8.202 [a]	12	0.766 ± 0.486 [A]	12
	1:400	28.642 ± 8.384 [a]	12	2.326 ± 1.450 [A]	12
	1:800	28.377 ± 7.639 [a]	12	1.850 ± 1.200 [A]	12
	1:1600	33.723 ± 9.716 [a]	12	1.373 ± 0.953 [A]	12
	1:3200	36.919 ± 10.352 [a]	12	2.198 ± 1.248 [A]	12
	1:6400	91.276 ± 7.655 [b]	12	81.253 ± 9.293 [B]	12
	1:12800	97.403 ± 7.810 [b]	12	85.691 ± 11.901 [B]	12
	1:25600	93.703 ± 5.467 [b]	12	91.739 ± 8.377 [B]	12
GuttaClear	1:200	29.427 ± 9.618 [a]	12	1.248 ± 1.240 [A]	12
	1:400	35.957 ± 11.868 [a]	12	1.528 ± 0.822 [A]	12
	1:800	37.364 ± 14.301 [a]	12	2.043 ± 1.156 [A]	12
	1:1600	36.229 ± 12.426 [a]	12	0.988 ± 0.634 [A]	12
	1:3200	40.211 ± 14.482 [b]	12	2.261 ± 1.469 [A]	12
	1:6400	52.711 ± 17.342 [c]	12	2.969 ± 2.381 [A]	12
	1:12800	92.608 ± 7.564 [d]	12	94.608 ± 8.416 [B]	12
	1:25600	104.110 ± 6.692 [d]	12	98.230 ± 6.956 [B]	12

Different lowercase letters indicate the statistically significant differences among dilutions in the same group of solvent with 30 minutes exposure time ($p < 0.05$).

Different uppercase letters indicate the statistically significant differences among dilutions in the same group of solvent with 24 hours exposure time ($p < 0.05$).

For 30 minutes exposure time, 1:6400 dilution of GuttaClear showed the percentage of cell viability less than 50%, significantly lower than those of xylene and GP-solvent at the same dilution ($p<0.05$). At dilution of 1:3200, the cell viability of GP-solvent and GuttaClear was less than 50% without a significant difference between the groups ($p>0.05$). At 1:800 dilution, xylene showed about 50% cell viability. All solvents at 1:200 dilution showed less than 30% cell viability without significant difference between the solvents.

After 24 hours exposed to solvent, the toxicity of solvents was more obviously detected. The mean percentage of cell viability in the high concentrations dropped to the level of less than 5%. The cytotoxicity to cells of each solvent was found at the 1:6400 dilution of GuttaClear, 1:3200 of GP-solvent, and 1:800 of xylene, which was significantly different ($p<0.05$). Toxic effects of all solvents were very high without a significant difference between the solvents at 1:200 and 1:400 dilutions.

Discussion

The favorable technique for initial screening of biocompatibility of dental materials is *in vitro* cytotoxicity tests. Cell culture assays provide a convenient, controllable, and repeatable method to assess biocompatibility. The MTT colorimetric assay was used to evaluate the cytotoxic effect in this study. The method is a quantitative assay to compare the cytotoxicity between tested materials. The MTT assay reflects the number of cells at any growth cycle stage with simplicity, rapidity, and precision [23].

Cells used for culture assays can be permanent cell lines or primary cells from organ tissues. Many studies used permanent cell lines such as L929 mouse fibroblasts [17, 24]. However, the primary cells are more clinically relevant for biocompatibility studies than the permanent cell lines [25]. For example, L929 mouse fibroblasts have a shorter period of cell division than HPDLF cells and may not have the specific metabolic potential like HPDLF cells [26].

The ISO 10993-5/2009 [22] indicates that a sample in liquid form shall be tested by either direct contact or indirect contact embedded in a biologically inert absorbent matrix. In this study, the direct contact of diluted solvents was used because the toxic elements (if any) can directly reach the cultured cells, which is more clinically relevant than the indirect contact [25].

The solvents tested in this study were evaluated at the dilutions of 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, and 1:25600. These dilutions were chosen from our pilot studies on the range of dilutions allowing cell growth and appropriate for detecting the difference between the solvents.

The contact time of materials to cells was set at 30 minutes to simulate the clinical working time and at 24 hours to represent a short-term contact to periapical tissue. The previous studies maintained solvents in contact with cells in various periods such as 30 minutes, 3 hours, or 4 hours [17, 20]. The longer the contact time is, the more cytotoxicity is expected.

Xylene, GP-solvent, and GuttaClear are oily and not completely miscible in the culture media used for diluting the substance. Thus, DMSO was used to help in the dilution of the solvents. Da Violante G *et al.* [27] concluded that DMSO could be used safely at concentrations up to 10%. This study used less than 0.1% of DMSO per well. This concentration had been proved to be nontoxic in the pilot study. Additionally, we noticed that the mixture of solvents and DMSO still separated from the cell culture media and left a greasy layer on the top. Therefore, the sonication technique was used to facilitate and allow the mixed solvent to disperse homogenously with the media.

The concentrations over 1:6400, 1:3200 and 1:800 of GuttaClear, GP-solvent, and xylene, respectively, were shown to be cytotoxic. The toxicity of the solvents was more prominent after 24 hours exposed to solvent than 30 minutes exposure. However, the results are inconsistent with previous studies which tested with L929 cells. The study found that solvents showed toxicity at the dilution of 1:1600 of xylene and 1:800 of GuttaClear and GP-solvent [15]. Another study evaluated the cytotoxicity of GP-solvent and chloroform on L929 cells. The result showed that GP-solvent is toxic to L929 cells at the dilution of 1:400 [17]. The difficulty in dispersing the solvent homogenously in the media, the different types of cells (primary cell vs. permanent cell line), and contact time may be responsible for the difference in the results.

The findings of this study indicate that GuttaClear was more cytotoxic than GP-solvent and xylene. The toxicity occurs in a dose-dependent manner. All the solvents in higher concentrations showed severe toxic effects without statistically significant differences between types of solvent. Clinically, an undiluted solvent which supposed to have severe toxicity is used. However, the *in vitro* toxicity test cannot be directly implied to *in vivo* situation. A recent study evaluated postoperative pain after non-surgical root canal retreatment when using solvent compared with no solvent used revealed that pain incidence was not significantly different among groups [28]. Thus, the cells in culture media, *in vitro*, lack of extracellular matrix protein or serum that acts as a barrier or diluting agent to any toxic substances [20]. *In vivo*, toxic ingredients can be diluted by tissue fluid and then eliminated from the periapical area by local clearance such as blood flow [29]. However, the toxicity of solvents should not be neglected. Clinicians should be aware of apical extrusion of the solvent during retreatment procedures to avoid unpleasant situations. Further studies of the clinical treatment outcome should be performed to evaluate the effect of solvent toxicity.

Conclusion

All tested gutta percha solvents showed cytotoxicity to HPDLFs in a dose-dependent manner, arranged in descending orders: GuttaClear, GP-solvent, and xylene. At high concentrations, all of the solvents showed similar severe toxic effects.

Acknowledgement

We would like to express our special thanks of gratitude to Miss Wassana Wichai for additional helps in research methodology and Dr.Sittichoke Osiri for comments and suggestions in statistical analysis.

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