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RESILON™ Toxic to Oral Squamous Carcinoma Cells: A Live/Dead Assay

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Abstract

Background/Purpose: Biologic responses and biomaterial/cell-and tissue-interactions are essential of particular significance for the clinical applicability of developed products.

Purpose: To assess the toxicity of endodontic filling materials on oral cancer cells.

Materials and Methods: A LIVE/DEAD *Bac*Light fluorescent assay was used to stain and detect Cytocompatibility after 24 hr, 48 hr and 72 hr incubation periods with aggressive salivary gland-origin squamous cell carcinoma (SCA-9) and adenocarcinoma (WR-21) lines. Quantitative analysis (in triplicate) was performed using a colorimetric method, MTT, to measure cellular viability.

Results: Resilon is significantly ($\rho < 0.05$) more toxic to SCA-9 cells as well as to WR-21 cells than is gutta-percha, in a time-dependent manner. This can be attributed to its resin/primer content and biodegradability of polycaprolactone and by-products, over time.

Conclusion: Concerns pertaining to safe clinical usage are valid.

Keywords: *Cytocompatibility; Endodontics; Resilon; Root canal filling; Sealer; Cancer*

Introduction

Endodontic obturation materials should not only eliminate or minimize the ingress or egress of bacteria and their by-products. Rather, they are expected to promote healing of peri-apical tissues and encompass a favorable tissue response [1]. Despite the known shortcomings, gutta-percha remains the gold standard core root filling material [2]. With advances in polymer chemistry and dental materials, Resilon™, a FDA-approved thermoplastic synthetic polymer was introduced into the endodontic practice and consequently challenging gutta-percha, robustly, increasingly suggested to replace gutta-percha and become the stand-of-care in root canal therapy [3]. When used along a resin-based sealer, Resilon has been shown to offer improved bonding potential, enhanced resistance to tooth fracture as well as minimal microbial leakage in filled roots, when compared with gutta-percha, among others [4]. Further, Resilon was claimed to possess superior biocompatibility where several investigators concluded that it was a non-cytotoxic and non-mutagenic material [3]. Interestingly, those efforts were recently reviewed with inconclusiveness calling for more research [2-4]. Biological responses in different cell lines have been shown to be beneficial and proposed by others to be even essential [4]. For example, Key., et al. [5] reported that Resilon was as toxic or even less toxic than gutta-percha to human gingival fibroblasts after 1 and 24 hours. On the other hand, a few recent studies have reported that Resilon points were more cytotoxic than gutta-percha cones. Indeed, the cytotoxicity of Resilon to L929 mouse skin fibroblasts as well as RPC-C2A rat pulp cells was shown to significantly increase after 48 hours of exposure using a sulforhodamine-B assay. Authors concluded that the material was more toxic than gutta-percha, more so, it was time-dependant [6]. Although the material is expected to linger within the root canal, theoretically, it is well-known that peri-apical extrusion through the apical constriction or via

iatrogenic perforation is far from rare, thus raising legitimate concerns for endodontists and the general practitioner regarding safety and subsequently, use efficacy [7]. So, how is Resilon compared to gutta-percha in terms of *cytocompatibility*?

Herein, the aim was to evaluate and compare the (a) cytotoxicity and (b) anti-proliferative effects of Resilon points with gutta-percha cones on two *murine* carcinoma cell lines by means of qualitative and quantitative analysis. Those cell lines were selected for their aggressiveness in comparison to others used in previous studies. This pilot *in vitro* investigation predecessors further *in vivo* and clinical studies evaluating the biocompatibility and safety of extruded materials on the condition of peri-apical tissues.

Materials and Methods

Experimental materials were RESILON™ points (Pentron Clinical Technologies, Wallingford, CT, USA) and ACEONE-ENDO® gutta-percha cones (ACEONEDENT Korea Industrial Company, Bucheon, South Korea). Only 0.2g of each material was placed in sterile vials containing 6 mL of Dulbecco's Modified Eagle's Medium (DMEM), incubated at 37°C for a total period of 72 hours. The two cell lines were: (a) SCA-9 clone 15 (*Mus musculus* Submandibular Salivary Gland Carcinoma) cells and (b) WR-21 (*Mus musculus* Submandibular Salivary Gland Adenocarcinoma) cells [8,9]. Both (CRL-1734™ and CRL-2189™, respectively) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and used for cytotoxicity evaluation. ATCC reports that both lines are adherent (growth properties) with a fibroblast-like morphology. *Cell culture materials* include DMEM, fetal bovine serum (FBS), Dulbecco's Phosphate Sodium Buffer (DPBS) and TrypLE™ Antibiotic-Antimycotic; were all purchased from Gibco® BRL (Carlsbad, CA, USA). The quantitative cytocompatibility assay was performed via a colorimetric method, using MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (Sigma-Aldrich Chemical, South Korea). Ultra-pure water (UPW) and all other materials and chemicals used were of analytical grade. Briefly, cells were seeded (mono-layer) in 12-well tissue culture plates, at an initial density of 2×10^4 viable cells/mL. MTT was added after 24h to a final concentration of 0.5 mg/mL and incubated for 4h at 37°C. The measurement of formazan absorbance was then carried out using a spectrophotometer (μQuant, Bio-Tek Instruments)-microplate reader-at 570 nm. Control wells were treated with 100 μL of DMEM only (10 μL MTT stock solution was added). Alternatively, cytotoxicity was studied using a nucleic acid staining dye, Hoechst 33342 and propidium iodide. Cells/Resilon and cells/gutta-percha(at 24 hr, 48 hr and 72 hr) were washed twice with PBS and incubated with the dye for 10 min at room temperature. For qualitative purposes, attached cells were imaged following incubation with fluorescent stains (calcein AM and EthD-1) using a fluorescence microscope (Olympus, Japan) for LIVE/DEAD real-time viewing. Access to microscopy was kindly provided by CIBRO (Centro de Investigación en Biología y Regeneración Oral) of the Universidad de los Andes in Santiago de Chile. Unless otherwise mentioned, all experiments were done in triplicates. Colorimetric assays and statistical analysis of obtained data were performed at the BioMAT'X Laboratory, part of CIB (Centro de Investigación Biomédica), Faculty of Dentistry, Universidad de los Andes. Results are reported as mean ± standard deviations. Multiplet-tests (unpaired/paired) were performed to assess for statistical significance at the 95% confidence level where ρ-values ≤ 0.05 were considered statistically significant.

Results and Discussion

The present study evaluated the cytotoxicity of two common endodontic obturation materials on two cancerous cell lines, *in vitro*. Such simple, rapid, reproducible and inexpensive methods provide valuable information and help predict the biocompatibility of materials in pre-clinical and clinical models. Furthermore, *in vivo* and clinical testing of dental materials may be influenced by the skill of the dentist, technical properties of the material and uncontrollable patient factors. To the best of knowledge, no studies exposed *cancer* cells to such materials, thus far. SCA-9 and WR-21 cell lines were used to measure alterations in cell numbers as well as morphology over a period of 3 days. The protocols used herein were optimized previously to investigate anti-cancer drugs [10] hence the cell density used was sufficient for exponential growth throughout the duration of the experiment. Also, WR-21 cells are more aggressive than SCA-9 cells. Figure 1 displays the effect of Resilon and conventional gutta-percha points on SCA-9 cells. Evidently, after 2 days of incubation, the number of cells exposed to Resilon was much less than those exposed to gutta-percha. Likewise, the morphology of cells in the latter group seems to be preserved and more fibroblast-like than the cells in the former group. Quantitative date, plotted in Figure 2, confirmed this microscopic observation. At 72 hrs, Resilon is significantly ($\rho < 0.05$) more toxic to SCA-9 cells as well as to WR-21 cells than is gutta-percha. Several studies have suggested the cytotoxicity of Resilon compared to gutta-percha exhibited in rat pulp cells and mouse skin fibroblasts, especially so with set sealers [6,11]. Interestingly, gutta-percha cones showed a slight increased toxicity after a 48 hr

incubation period, possible explained by its zinc oxide content. However with no statistically significant differences between cell lines or the 48 hr and 72 hr values were detected. This has been shown previously using a radio chromium release assay with toxicity at longer incubation periods attributed to the leakage of zinc ions into the culture medium, according to Pascon and Spangberg [12]. Quantifying zinc oxide content in commercially-available gutta-percha products remains a matter of investigation although it is the responsibility of manufacturers to provide such relevant information [2,6]. Regarding Resilon, the observed cytotoxicity can be attributed to its resin/primer content and material (polycaprolactone) biodegradability over time [13]. It is noteworthy that contradicting results are available in the literature. For instance, in a similar rat connective tissue model, *satisfactory* tissue reactions were reported with the Epiphany/Resilon root canal filling system [14]. Hence, attention to differences in cell types/lines and followed experimental methodologies are necessary. Nonetheless, a systematic review of the available literature reveals more studies (not limited to *in vitro*) reporting the cytotoxicity of Resilon in comparison to other materials [2,15-17]. The LIVE/DEAD assay offers easy and sensitive determination of cell viability, cell vitality and compound cytotoxicity [16,17]. Figures 3 and 4 illustrate the LIVE/DEAD auto-fluorescence images captured in real-time (24 hr and 72 hr) comparing Resilon and gutta-percha in both cell lines, respectively. It is noteworthy here in that in this type of assay, green cells are *alive* and red cells are *dead*. Evidently, Resilon points were found to be significantly more toxic than gutta-percha, irrespective of the cell line. A statistically significant difference can be clearly detected between the 24 hr and 72 hr periods, warranting time-dependency. Inserts (magnification) detects light morphological changes; indicating *latent* cell uptake post-72 hr of incubation.

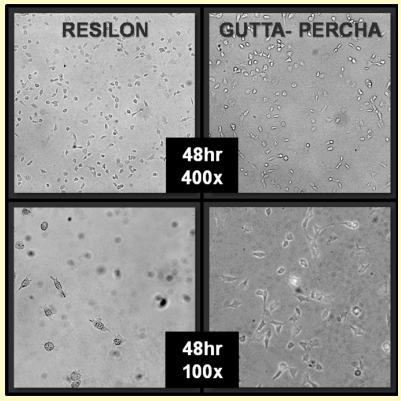

Figure 1: *Effect of Resilon and gutta-percha points on SCA-9 cells after 48 hr exposure.*

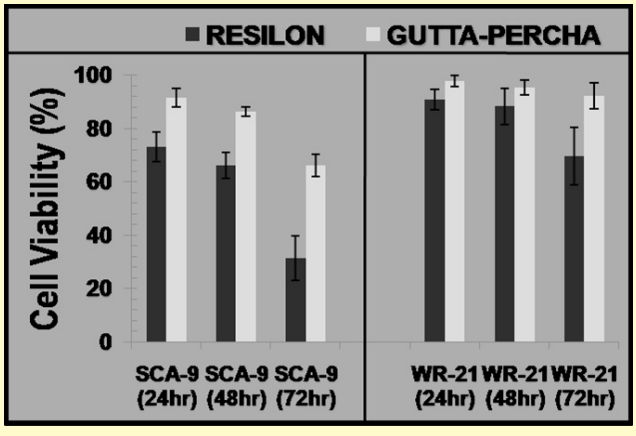

Figure 2: *Comparison of cell viability values over time for SCA-9 and WR-21 cells.*

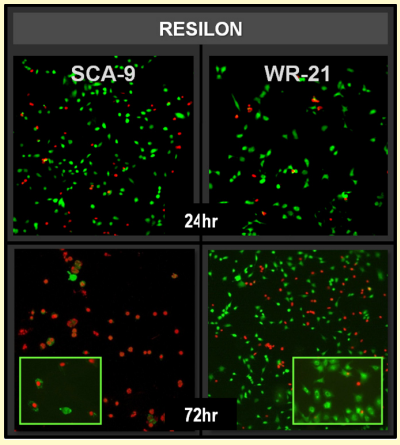

Figure 3: *LIVE/DEAD images captured at 24 hr and 72 hr for cells exposed to Resilon points.*

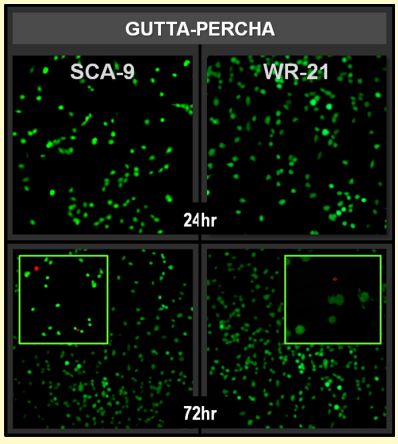

Figure 4: *LIVE/DEAD images captured at 24 hr and 72 hr for cells exposed to gutta-percha cones.*

Conclusions

Overall, the hypothesis was accepted, revealing an alarming cytotoxic potential of Resilon points. This in turn calls for serious pre- and -clinical research investigating its safety and biocompatibility, especially, for the leaked by-products, and over extended periods of time.

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