Comparison of the Potential Genotoxicities of Resin-, Silicone-, and Bioceramic-based Root Canal Sealers against Human Lymphocytes

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Abstract

Root canal sealer materials leak through the foramens and remain in close contact with the periradicular tissues for prolonged periods of time. Hence, biocompatibility with periradicular tissues is an important requirement for these materials. Genotoxicity is one of the major factors affecting the biocompatibility of a material. The currently used root canal sealers contain chemicals that can cause DNA damage when exposed for long periods of time. There are a wide range of sealers including resin-based, silicone-based, and bioceramic-based sealers that have potential for genotoxicity.

The purpose of this study was to compare the genotoxicity potentials of a resin-based (AH Plus; Dentsply US), silicone-based (GuttaFlow Bioseal; Coltene), and bioceramic-based (iRoot SP; Innovative BioCeramix Inc.) sealers against human lymphocytes. The γ-H2AX assay was used, and the presence of DNA double-strand breaks (DSBs) was evaluated in the cells after incubation with the sealers for one, three, and seven days. Lymphocytes were examined under a fluorescence microscope (100x magnification). Kruskal–Wallis and Mann–Whitney U post hoc tests demonstrated significant differences in γ-H2AX foci among all three sealers. The highest value was found in the resin-based sealer, followed by the silicone- and bioceramic-based sealers.

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Introduction

The purpose of root canal fillings is to fill in the empty space generally occupied by the pulp and to create a hermetic sealing of the root canal. Gutta-percha and root canal sealers are most commonly used for these purposes. In addition, the procedures used during root canal filling should provide optimal conditions for healing and should stimulate the biomineralization process. Root canal sealers serve to fill the gaps between the gutta-percha filling material and the root canal wall. Root canal sealer materials often leak out of the apical or lateral foramens and form sealer puffs that remain in close contact with living periapical tissues over long periods of time. It is important for the tissues to respond favorably to this close proximity with the root canal sealer without the occurrence of any adverse reaction.[1,2] Therefore, an ideal endodontic material should be biocompatible with the tissues surrounding the dental root.[1,3]

The biocompatibility of a materials is its ability to elicit an appropriate biological response without causing any adverse reactions to the surrounding living tissues and it can be determined in terms of the degrees of cytotoxicity, genotoxicity, and mutagenicity caused by the substance.[4] Genotoxicity is one of the important factors influencing biocompatibility. It does not result in cell death, but the DNA can get damaged if exposed to genotoxic materials. DNA damage can occur at any phase during the life cycle-base alterations (basic depuration and deamination of bases), two-base alterations (alkaline oxidation and base methylation); chain breaks (DNA single strand break SSB and double strand breaks DSB); and cross-linkages (DNA–DNA, and DNA-protein.[5,6] DSB refers to heavy DNA damage caused by a break in the

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strand leading to long-term damage. If the repair is inefficient, DNA damage can trigger temporary cell cycle arrest for DNA repair or permanent cell cycle arrest, causing the cell to enter the senescent phase. If the process of DNA repair does not occur the cell undergoes apoptosis. However, in some cases where the DNA repair process fails, the damaged cell continues to proceed with its life cycle; however, further replication of this cell can eventually lead to permanent damage and result in mutagenesis or carcinogenesis.[6]

A wide variety of chemical-based root canal sealers are available for use. Resin-based sealers, such as AH Plus (Dentsply), are commonly used because of their efficient sealing properties. However, the base material of this sealer is composed of epoxy bisphenol A (BPA). According to Jontell et al (1995), the toxicity of BPA is known to be very high in the presence of a nonpolymerizing material and can potentially cause mutagenesis.[7] In addition, AH Plus was found to release formaldehyde in small amounts after setting.[8] High doses of formaldehyde can damage the DNA and demonstrate a carcinogenic activity when it comes into contact with tissues for a long period of time.[9]

According to some studies, resin-based sealers are also genotoxic.[2,10] Darrag and Fayyad (2014) found out that the DNA damage caused by AH Plus was dependent on the dose and exposure time of the sealer.[2] Similarly, resin sealers have been shown to play a role in DNA damage.[10] Conversely, in the study by Silva et al. (2015), only EndoREZ, a methacrylate resin-based sealer, was found to possess genotoxic properties,[11] whereas in another study, AH Plus did not demonstrate any genotoxic or mutagenic properties.[12]

GuttaFlow, a silicone-based sealer, is currently available in combination with some bioactive components, such as calcium or silicate (GuttaFlow Bioseal; Coltene).[13] A study evaluating the biocompatibility of materials with the viability of fibroblast cells in the periodontal ligament revealed that the cytotoxicity levels of both GuttaFlow2 and GuttaFlow Bioseal were lower than those of AH plus and MTA Fillapex, a mineral trioxide aggregate-based salicylate resin root canal sealer.[14] Similar results have been reported by Accardo et al. (2014).[15] In another study examining the cytotoxicity of GuttaFlow on human gingiva fibroblast cells using an 8-cell counting kit. The sealer was found to be nontoxic during the first 24 h and minimally toxic after 72 h.[16] The toxicity of GuttaFlow is thought to be caused by silver particles that produce reactive oxygen species (ROS). However, Brzovic et al. (2009) reported that the GuttaFlow sealer has no genotoxic potential against lymphocytes.[17]

Currently bioceramic based sealers, such as IRoot SP (Innovative BioCeramix Inc.), a bioactive biocompatible, osteoinductive sealer, are available.[18] Candeiro et al. (2015) found out that bioceramic sealers were significantly less genotoxic than AH Plus, yet their toxicity levels were significantly higher than that of the control group.[19] The toxicity of bioceramic-based sealers is thought to be due to the production of calcium hydroxide during hydration.[20] High concentrations of calcium ions in the cells can prove toxic and trigger cell damage.[20] However, the nature of its genotoxicity has been examined in few studies.

In the present study, lymphocytes were used as human primary cells. Lymphocytes play a role in the adaptive defense system in response to foreign agents. They are sensitive to DNA damage, and contain large, round nuclei with less plasma, making them easier to isolate and observe.[6]

The methods used to test genotoxicity include the comet assay (DNA fragmentation test) or there are several methods of genotoxicity test such as DNA fragmentation test by Comet assay, or the γ-H2AX assay (observation of γ-H2AX foci). The observation of γ-H2AX foci was first discovered in damaged DNA. The damage process leads to extensive phosphorylation of the H2A histone variant H45, resulting in the formation of γ-H2AX foci, which can be observed under an immunofluorescence microscope. the phosporylation process occurs quickly, many and very well as a reaction due to DNA damage.

The current study, we used the γ-H2AX assay to test the genotoxic effects of the root canal sealers on the DNA of lymphocytes. This assay is the most sensitive biomarker for the detection of DNA damage and stimulates further repair if required.[21] Histone H2A is a type of core protein found in DNA strands. Exposure of cells to radiation or other DNA-damaging agents results in DNA damage.

Materials and methods

The lymphocytes obtained from the blood cells of one woman (aged 37) were used in this study. The root canal sealers AH Plus, GuttaFlow Bioseal, and Iroot SP were used as test materials. The blood samples obtained intravenously and collected in heparin tubes, were divided into 36 samples consisting of nine resin group samples, nine silicone group samples, nine bioceramic group samples, and nine control group samples without sealer materials and were placed in well plates. Each well plate was filled with 2 mL of whole blood and 2 mL of medium (Roswell Park Memorial Institute [RPMI], medium, fetal bovine serum [FBS], or phosphate-buffered saline [PBS]. The well plates were then placed in an incubator for one, three, and seven days. After incubation, lymphocyte isolation was performed using the Histopaque separation technique. The blood samples that were exposed to the different sealer materials were transferred to a centrifuge tube filled with 3 mL of PBS and resuspended. The entire solution was transferred into a centrifuge tube containing 3 mL of histopaque solution and centrifuged at 1,500 rpm for 30 min. Three layers compromising the plasma and PBS, a lymphocytic ring, and blood sediments were formed. The lymphocytic ring was transferred into a centrifuge tube containing 5 mL of PBS (pH=7.4) and centrifuged for 15 min at 1,000 rpm. The resultant supernatant layer was discarded, and the pellet formed was carefully collected, placed inside a centrifuge tube containing 3 mL of RPMI medium, and centrifuged at 1,000 rpm for 15 min. The lymphocytes were reconstituted with 100 µl medium, mixed with ethanol, and stored in a freezer for further ɣ-H2AX analysis.

Lymphocyte staining procedure using ɣ-H2AX biomarker. The RPMI medium containing the isolated lymphocytes was subsequently calibrated for 40 s. The supernatant was removed, and the pellet was placed onto a hydrophobic glass slide using a micropipette, allowing it to dry for approximately 30 min. After drying, a paraformaldehyde solution was added to the pellet for fixation and was allowed to stand for 5 min, after which the solution was discarded. Next, drops of Triton X (0.25%) were added to the pellet and allowed to remain for 5 min. After the removal of the solution, 1% BSA was added to the pellet, allowed to remain for 15 min, and then discarded. Drops of antibodies (BSA, H2AX and 53BPI) in BSA were added to the pellet and stored for 45 min in a dark room inside a moist chamber at a temperature of 30^{o}C. the antibodies were subsequently removed by washing the glass slides with 0.25% BSA (three times, 2 min each). Next, drops of the secondary antibodies (BSA, DAPI, 488, and 512) were added and allowed to remain for 30 min in a closed room above a moist chamber. After 30 min, the antibodies were removed and the slides were washed with PBS (three times, 5 min each). The slides were dried under a fan in a dark room for 15 min, following which drops of vectashield were added and the slide was coverslipped and stored in a freezer for 15 min. After confirming the change in color by visual inspection, the slides were returned to the freezer for 24 h. Finally, the glass slides were sealed with a nail polish and observed under a fluorescence microscope equipped with red, yellow, and blue filters (100x magnification).

For γ-H2AX calculations, the average number of foci or γ-H2AX foci groups was noted as 100 cells. If the number of γ-H2AX Foci was found to be stable until reaching the mark of 100 cells, then 50 cells counted are enough.

Statistical analyses were performed using SPSS ver. 20 (IBM Corp., Armonk, NY, USA). The hapiro Wilk test was used to check for normal distribution. Consequently, the nonparametric Kruskal-Wallis and post hoc Mann-Whitney U tests were used to compare the three test materials and evaluation times (one, three, and seven days) in the current study. The level of significance was set at $P<0.05$.

Results

The results of the foci counts in all the study groups are shown in Table 1. a significant difference ($P<0.05$) in the ɣ-H2AX expression was noted between the control group and the resin-based, silicone-based, and bioceramic-based sealer groups. ɣ-H2AX foci were not seen in the control groups at one, three, and seven days.

The highest expression value of ɣ-H2AX was noted in the resin-based sealer group on day

1 (0.04), but on days 3 and 7, no foci were observed. Similarly, the expression of ɤ-H2AX on day 1 in the bioceramic-based sealer group was 0.01, but no expression was noted on days 3 and 7. However, an increase in the expression values of ɤ-H2Ax was observed in the cells with the silicone-based sealer over time (0.01) on day 1, 0.01 0n day 3, and 0.03 on day 7.

Groups	*N*	Median (min-max)			*P*
		Day 1	Day 3	Day 7	
Control	9	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	**0.04***
Resin	9	0.04 (0.02 - 0.07)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	
Silicon	9	0.00 (0.00-0.03)	0.00 (0.00-0.04)	0.04 (0.00-0.06)	
Bioceramic	9	0.00 (0.00-0.04)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	

Kruskal-Wallis test. N: number of samples, *P*-value <0,05 *: meaningful

Table 1. Mean values and standard deviations of ɤ-H2AX expression levels among the groups on days 1, 3, and 7.

As seen in Fig. 1(a), no ɤ-H2AX foci were noted in the normal lymphocytes. On the other hand, Figs. 1(b), 1(c), and 1(d) show microscopic images showing the ɤ-H2AX expression on the DNA of lymphocytes (bright green).

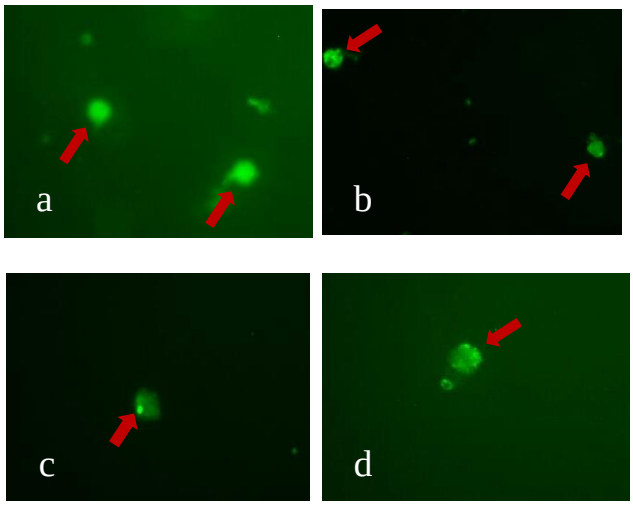

Figure 1. Microscopic images of normal lymphocytes from human blood. (a) no ɤ-H2AX foci in the cells. (b,c,d) ɤ-H2AX foci (red arrows) seen in the DNA of the lymphocytes after exposure to the silicone based sealer.

Discussion

The genotoxicity test is one of the methods used to examine the biocompatibility of a material. It has been widely accepted as an important indicator of the risk for developing carcinogenesis. This test can detect the early onset of tumorigenesis and is therefore very important in evaluating the toxicity potential of the ingredients.[5] Knowledge about the genotoxic properties of root canal sealer will assist clinicians in selecting sealers that will cause no local or systemic harm to the body.

The resin, silicone, and bioceramic-based sealers were found to cause DSB damage to the DNA in the present study, as noted by the formation of ɤ-H2AX foci (Table 1). significant differences in the expression of ɤ-H2AX in the lymphocytes was noted between the three root canal sealer groups and the control group. Furthermore, the expression level of ɤ-H2AX in the resin-based sealer group was slightly higher than those in the silicone and bioceramic-based sealer groups. This is presumably because resin-based sealers, but not silicone and bioceramic-based sealers, contain BPA as the base material. According to Jontell et al (1995), the toxicity of BPA is very high in the unpolymerized state, rendering it potentially mutagenic.[7] In addition, resin-based sealers were also found to contain a small amount of formaldehyde,[8] which is known to damage the DNA and cause carcinogenicity when in contact with tissues in high doses and over extended periods of time.[9] Thus, the presence of both BPA and formaldehyde in resin-based sealers in thought to contribute to the high expression level of ɤ-H2AX in the AH Plus group. Alternatively, the expression of ɤ-H2AX in the silicone- and bioceramic-based sealers is thought to be associated with the presence of silver nanoparticles[16,22] and calcium hydroxide,[20] respectively. High consentration of intracellular calcium ions can prove toxic for the cells and cause damage.[20]

The results of the current study are in line with those reported by Candeiro et al. (2015), who performed micronuclear assays to compare the effects of bioceramic-based sealers with

those of AH Plus on human gingival fibroblasts. The bioceramic-based sealers were less genotoxic when compared to AH Plus, but more toxic when compared to the control group.[19] Several other studies have also confirmed the genotoxic property of resin-based sealers.[2] In another study, micronuclear tests conducted on hamster fibroblasts (V79) for 12, 48, and 72 h revealed that the AH Plus sealer was more genotoxic than the MTA Fillapex sealer.[10]

Significantly different expression level of ɣ-H2AX were noted between incubation days 1 and 3 and incubation days 1 and 7 (P<0.05) with regard to the resin-based sealer (Table 2). the highest expression level of ɣ-H2AX was observed on day 1. This may be attributed to the setting time of the resin-based AH Plus sealer (8h); therefore, the resin- may have been incompletely polymerized on day 1, resulting in high levels of BPA and leading to an increase in the expression of v. The expression level of ɣ-H2AX were lowered or absent on days 3 and 7 in the current study. This is in accordance with a study by Bouillaguet et al. (2006), who reported that the toxicity of the resin-based sealer was highest at the time of setting and decreased over time.[23] A decrease in resin toxicity is thought to reduce the leaching of toxic substances over time.

Material	Incubation	Resin		Silicon		Bioceramic	
		day 1	day 3	day 1	day 3	day 1	day 3
Resin	day 1						
	day 3	**0.04***					
	day 7	**0.04***	-				
Silicon	day 1						
	day 3			0.79			
	day 7			0.25	0.35		
Bioceramic	day 1						
	day 3					0.32	
	day 7					0.32	-

Table 2. *P*-value for ɣ-H2AX expression among the three treatment groups on days 1, 3, and 7 using the Mann-Whitney U post hoc test;*p<0.05

Although no significant difference in the expression levels of ɣ-H2AX in the silicone-based sealer group was noted among the three incubation days. The levels on days 3 and 7 were higher than that on day 1. This is in line with a study by Mandal et al. (2014), where GuttaFlow2 was found to be nontoxic during the first 24 h and minimally toxic after 72 h.[16] The toxicity of the silicone-based sealer (GuttaFlow) was suspected to be due to the presence of silver nanoparticles, which produce ROS.[16,22] ROS are toxic and the degree of toxicity of the silver particles increases over time. In addition, wherein nanosilver particles are more toxic than microsilver particles.[22] This has been attributed to the greater number of atoms present on the surface of the nanosilver, causing larger reactions when compared with the larger microsilver particles.[24]

The expression of ɣ-H2AX in the bioceramic-based sealer was noted on day 1, but not on days 3 and 7. This is thought to be related to the content of calcium hydroxide produced during the hydration reaction.[20] Although the release of calcium ions plays an important role during the tissue repair process, high concentration of ion in the cells can prove toxic and cause damage.[20]

DSB damage is the most common type of DNA damage inducing mutations in the cell. Failures or imperfections during the process of DSB repair may lead to chromosomal aberrations. Other types of DNA damage, such as damage within the nitrogen bases or SSB, have relatively lower potential to cause mutations in genes and are considered less harmful because the damage is relatively repairable.

Primay human cells (lymphocytes) were used in this study because the results produced by these cells are more representative (karyotypically normal human cells) when compared with secondary cells (cell lines) despite having a short lifespan.[25] Lymphocyte are more sensitive in the event of DNA damage when compared to other cells.[25] In a study by Brzovic et al. (2009), human lymphocytes were also used ato compare the genotoxicity of root canal sealers.[17] Whereas other studies have used secondary cells such as fibroblasts from humans[12,15,16,19.]

Lymphocytes play a role in the adaptive defense system in the body. In periradicular tissues, humoral (B-lymphocytes) and sellular (T-lymphocytes) cells respond to foreign. Furthermore, lymphocytes have large and rounded nuclei with less plasma, making them easier to isolate and observe.[6] The genotoxic effects of materials on lymphocytes can be

applied on normal cells, thus, if the component of a given material is not potentially genotoxic to lymphocytes, it can be concluded that it is safe for normal cells as well. In this study, lymphocytes were obtained from the same individual to avoid biased measurements that may arise from variations in different individuals.

ɣ-H2AX has been widely used as biomarker to detect DNA damage. The H2A histone protein H2AX is important for the DNA repair process, where in the case of DSB, the H2AX protein will be directed to the location of the damage DNA to initiate the process of repair and stimulate other repair proteins.[21] Three exposure times (one, three, and seven days) were selected in this study on the basis of the setting times of the materials; the final setting time for bioceramic-based sealers ranged from 160 to 240 h.[1,3,26] In some studies, it has been reported that the genotoxicity of root canal sealers increases in a time-dependent manner.[2]

Unlike in previous studies, where significant DNA damage has been reported in the treatment groups when compared with controls, the amount of DNA damage in the treatment groups in the present study was relatively low.[2,10,19] This is probably because the genotoxicity of the materials was tested using ɣ-H2AX, wherein DSBs in the DNS were evaluated. DNA DSB is a form of heavy DNA damage that can occur as a result of exposure to highly toxic agents. On the other hand, other studies used the comet assay,[2] micronuclei assay,[10,19] and Ames assay,[27] wherein other types of DNA damage were also evaluated.

Assay because ɣ-H2AX has been widely used as a biomarker to detect DNA damage. H2A histon protein H2AX varian is an important protein in the DNA repair process where in case of DNA damage, DSB H2AX protein will be directed to the location of damaged DNA to start the process of repair and to stimulate other repair proteins to make improvements.[21] Reasons for using material exposure time for lymphocyte cells at 1, 3, and 7 days are based on the setting time of each material that is not more than 1 day even according to Louishine et al (2016) final setting time in the bioceramic siler that is between 160 - 240 hours.[1,3, 26] Some journals also mention that the genotoxicity of root canal rooters will increase with time or time dependent.[2]

The results of this study, found the amount of DNA damage in the treatment groups are relatively small. Where in the previous studies obtained DNA damage results with significant amounts when compared with the control group.[2,10,19,27] This is probably because the method used in this study is a test of genotoxicity with ɣ-H2AX, where the type of damage that can be seen in this test is a form of DNA double strand breaks (DSB). DNA DSB is a form of heavy DNA damage that can occur due to exposure by highly toxic agents. While in other research, the method used is comet assay,[2] micronuklei assay,[10,19] and Ames assay,[27] where in that assay, the damage seen is besides DNA DSB.

Conclusions

Resin, silicone, and bioceramic-based sealers demonstrated a genotoxic activity against the DNA of human lymphocyte. The highest value of genotoxicity was found with the resin-based sealer (AH Plus) after incubation for one day, whereas the lowest genotoxicity was observed with the bioceramic-based sealer after incubation for three and seven days. The potential for genotoxicity in the silicone-based sealer was lower than that in the resin-based sealer, but higher than that in the bioceramic-based sealer. Furthermore, the genotoxicity of the silicone-based sealer was increased after three and seven days of incubation but remained lower than that of the resin-based sealer.

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Declaration of Interest

The authors report no conflict of interest.

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