The Cytotoxicity Effect of Silver Solder Materials on Human Periodontal Ligament Fibroblast Cells

Joseph Jeffrey Curry
Marquette University

Follow this and additional works at: https://epublications.marquette.edu/theses_open

Part of the Dentistry Commons

Recommended Citation
https://epublications.marquette.edu/theses_open/538
THE CYTOTOXICITY EFFECT OF SILVER SOLDER MATERIALS ON HUMAN PERIODONTAL LIGAMENT FIBROBLAST CELLS

by

Joseph J. Curry, DDS

A Thesis submitted to the Faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the Requirement for
the Degree of Master of Science

Milwaukee, Wisconsin

August 2019
ABSTRACT
THE CYTOTOXICITY EFFECT OF SILVER SOLDER MATERIALS ON HUMAN PERIODONTAL LIGAMENT FIBROBLAST CELLS

Joseph J. Curry, DDS
Marquette University, 2019

Objectives: Orthodontic appliances with silver soldering can be in the oral cavity for several years and very few studies have been done to detect the biosafety of the silver solders. The aim of this in vitro study was to compare the cytotoxicity effects of two different solder materials on Human Periodontal Ligament Fibroblast cells (HPLF).

Methods: Two commonly used solder materials from two companies, (Leone and Summit) with different metal elements and percentages were studied. The solder samples were exposed to HPLF cells for 48 hours and the cytotoxicity effect of soldered materials on HPLF cells was measured via the MTT colorimetric assay (n=10/sample) and morphological microscopic analysis. Furthermore, the mechanism of cytotoxicity of the Summit silver solder was investigated using both a caspase inhibitor Z-VAL-Ala-Asp-flu-oromethylketone (ZVAD) and the free radical scavenger Trolox (n=8/sample). Statistical analysis was performed with a one-way analysis of variance (ANOVA) with a Bonferroni correction. P<0.05 was considered statistically significant.

Results: Compared to control (cells only), both Leone silver solder and Summit silver solder were cytotoxic (p<.001). The 3M Unitek stainless steel bands were significantly cytotoxic compared to control. There was a significant difference in cytotoxicity between the 3M Unitek stainless steel band and the Summit silver solder (P<.001), but not the Leone silver solder. Between the two silver solders, the Summit silver solder was significantly more cytotoxic than the Leone silver solder (p <.05). These MTT results were supported by the microscopic morphological changes of the HPLF cells. Cellular necrosis is likely the method of cytotoxicity as indicated using ZVAD. Furthermore, the addition of Trolox did not prevent cell death.

Conclusions: The stainless steel bands were significantly cytotoxic compared to the control. Furthermore, the two silver solder materials demonstrated different levels of cytotoxicity to HPLF cells. Summit silver solder showed significantly higher cytotoxicity than Leone silver solder.
ACKNOWLEDGMENTS

Joseph J. Curry, DDS

I would like to thank Dr. Ghada Nimeri, Dr. Douglas Lobner, Dr. David Berzins and Dr. Dawei Liu for their expert guidance on this thesis, which could not have been completed without them. Their knowledge and time committed has been indispensible to the completion of this thesis.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: RESULTS

CHAPTER 4: DISCUSSION

CHAPTER 5: CONCLUSION

REFERENCES
LIST OF TABLES

Table 1. Results of the MTT colorimetric assay showing different effect of cytotoxicity on HPLF cells by the various samples.

Table 2. Comparison to control values and intergroup comparison of MTT experiments using Bonferroni t-test.

Table 3. Mean values of MTT calorimetric measurements, testing for protection and mechanism of cell death.

Table 4. Comparison of MTT measurements to control values testing for protection and mechanism of cell death using Bonferroni t-test.
LIST OF FIGURES

Figure 1. HPLF cells cultured in changed MEM medium with no FBS (negative Control) for 48 hours at 40x magnification………………………………………………………………………………16

Figure 2. HPLF cells exposed to calcium ionophore (positive control) for 48 hours at 40x magnification ………………………………………………………………………………………16

Figure 3. HPLF cells exposed to 3M Unitek stainless steel bands for 48 hours at 40x magnification…………………………………………………………………………………………17

Figure 4. HPLF cells exposed to bands with Leone silver solder for 48 hours at 40x magnification…………………………………………………………………………………………17

Figure 5. HPLF cells exposed to bands with Summit silver solder for 48 hours at 40x magnification…………………………………………………………………………………………18
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Several metals are used daily in orthodontic practices. The orthodontist utilizes fabricated extra-oral appliances, and intra-oral appliances consisting of brackets, bands, wires, and different synthetic materials to assist in the completion of different orthodontic treatments. The fabrication of the many appliances used in orthodontics often requires the use of soldering to allow for their intricate design.

Soldering can be defined as joining two or more metal components by using a third component (Willison & Warunek, 2004). A dental flux is used on the metals to be joined to prevent the formation of oxides and to allow the flow of the solder material. Orthodontic solders are often metal alloys composed of silver, copper, zinc, nickel, chromium and iron (Barrat et al., 1993, Wataha et al., 1993). The solder must have a lower melting temperature than the other two metals being joined or damage can be done to the other metals. In most cases, a torch is used as an efficient method to heat the flux, and soldering material and complete the goal of joining the two metals (Willison & Warunek, 2004). The use of orthodontic bands connected to a steel wire via silver solder is a common method of appliance fabrication. As previously stated, some of the metals included in silver soldering are silver, copper, cadmium and zinc, which are known to be potential toxic elements by the United Nations Environment Program (Wataha et al., 1993). However, very few studies have been done to detect the cytotoxic effects of silver soldering on both in vitro and in vivo experiments. One of the initial removable orthodontic appliances used in the United States, the Crozat appliance, was developed in the early 1900s and required the use of soldering for its fabrication (Proffit, Fields &
From the primitive orthodontic appliances developed by Angle, to the appliances used today, the use of soldering to fabricate the appliances is still required.

An orthodontic treatment lasts about 24 months, but some appliances in phase 1 treatment can be in the oral cavity for many years. Maxillary expanders are in place for about 6 months but could remain in place for longer periods depending on the case specifics. Lingual arches, a common appliance used in mixed dentition patients, can be in place for several years. This is why it is important that the orthodontist should have adequate knowledge related to the biocompatibility and cytotoxicity of the numerous appliances used on orthodontic patients.

**Biocompatibility and Intraoral environment**

The biocompatibility of orthodontic materials is a critical issue because of the long-term contact with the oral mucosa and the effect of corrosion of the different materials. Before a discussion of toxicity, and biocompatibility can be introduced, it is important to define biocompatibility. An accepted definition of biocompatibility is the ability of a material to elicit an appropriate biological response in a given application (Brantley & Eliades, 2001). Another definition is the ability of a material to perform with an appropriate host response in a specific situation, in other words, it also refers to optimizing the clinically relevant performance of a given therapeutic intervention and generating the most appropriate beneficial cellular tissue response in a given situation (Williams, 2008).

There are three key points that need to be understood when discussing biocompatibility. It is understood and agreed upon today that there are no truly inert
materials, as when a material is placed in the body, an interaction between the body and the material will occur. A second point to biocompatibility is that it is not static, but dynamic over time. A material may change through corrosion, fatigue, etc., and these changes may alter the conditions that initially accounted for the desired response. Therefore, the biocompatibility and response of the material to the body, and the body’s response to the material are constantly changing. The third key point with biocompatibility is that it is not only the property of the material, but it is the material interacting with its particular environment that it is in. A material may be biocompatible in one area of the body, but not biocompatible in another. Thus, it is important to define the location and function of the material when discussing its biocompatibility (Brantley & Eliades, 2001). The material that is chosen for orthodontic use should meet the criteria for biocompatibility. The material should contain no toxic, leachable, or diffusible substances that can cause adverse systemic effects, or cause an allergic response in a sensitized patient (Imirzalioglu et al., 2012). Corrosion is one of the major concerns related to the issues surrounding the biocompatibility of metals, or materials used (Menezes et al., 2010). Metal ions may be released at different concentrations causing hypersensitivity, or allergic reactions with local or systemic effects (Matos et al., 2008, Menezes et al., 2004).

**Corrosion**

An ideal dental alloy for intraoral use should be easy to melt, cast, braze and polish. The material should also include minimal shrinkage, high wear resistance, high strength, and high corrosion resistance (Imirzalioglu et al., 2012). The oral environment
contains an abundance of saliva, which consists organic and inorganic components. Through the variation in pH values that occur in the oral cavity and in the saliva, intraoral devices, such as orthodontic appliances, will be subject to the stresses of the variations in pH, electrochemical activity, temperature differences and abrasive and mechanical forces. All of these stresses contribute to deterioration through such mechanisms of corrosion and degradation (Brantley & Eliades, 2001).

Corrosion is defined as an electrochemical reaction between a metallic material and its surrounding environment (Brantley & Eliades, 2001). In the oral cavity, corrosion of orthodontic appliances is very common, consisting of positive metal ions being released from orthodontic alloys to become stable. Orthodontic solders are often metal alloys composed of silver, copper, zinc, nickel, chromium and iron. Some of these metal ions, specifically chromium, nickel and copper, are known to be cytotoxic to cells (Barrat et al., 1993, Wataha et al., 1993). Currently, there are concerns regarding the biocompatibility of some of the orthodontic materials, the ions released and the correlation to their toxic effects (Locci et al., 2000, Goncalves et al., 2014, Martin-Camean et al., 2015). The composition of dental alloys is important and elemental release has been researched in relation to the composition of metals. Results from these experiments have shown that elements like zinc and nickel tend to be released easier than elements like gold and palladium (Can et al., 2007). Recent studies have demonstrated that silver, copper, zinc, cadmium and copper are detected in the surrounding environment of alloys and according to the International Register of Potentially Toxic Chemicals of the United Nations Environment Program are listed as potentially dangerous chemical products (Martin-Camean et al., 2015). This stresses the fact that
clinicians need to be aware of the materials that make up the appliances they are using to treat patients.

**Cytotoxicity**

The use of orthodontic appliances, whether removable or fixed, are always associated with different gingival or soft tissue reactions. Most common reactions are swelling of the gingiva due to gingivitis, while other reactions are overgrowth of gingiva, or oral lesions (Bishara, 1995), and irritations of unknown reasons. Cytotoxicity can be one of the reasons associated with these irritations. The specific effects of the reaction depend on the chemical composition, degradation, absorption and host response. The most common of these types of reactions are lichenoid reactions, hyperplasia of the gingiva and discoloration (Imirzalioglu et al., 2012).

Cytotoxicity is a response to an alteration in cellular function, especially intracellular processes and changes in metabolic pathways (Jacoby et al., 2017). The metal ions that are released from the alloys may attack the surrounding cells at the molecular level, which in turn affects the cells and the organelles inside the cells (Storch, 1988). Messer’s article confirmed this by exposing fibroblasts to different ion concentrations of beryllium, chromium, nickel, and molybdenum. The cells were examined under scanning electron microscopes and transmission electron microscopy. It was found that the cells had irregular shaped nuclei for cells exposed to hexavalent chromium and nickel, pseudopodia for cells exposed to beryllium and molybdenum, and lipid droplet formation in cells exposed to nickel (Messer et al., 1999).
In 1995, Bishara published a case report that demonstrated an association between an orthodontic retainer which was fabricated using silver solder, and an oral lesion. This case report led to additional research on orthodontic materials biocompatibility. Jacoby et al., has shown that under identical experimental and cultural conditions, different cell lines can show different levels of cytotoxicity (Jacoby et al., 2017). Through their experiment exposing different cell lines to orthodontic bands with silver solder joints, it was shown that decreased viability is present in skin keratinocyte cells, kidney epithelial cells and lung fibroblasts, while oral fibroblast were not affected (Jacoby et al., 2017).

Locci in his article showed that stainless steel bands were more biocompatible than the brazing alloy on human gingival fibroblasts, and among the metals tested in the brazing alloys, Ag and Pd showed the highest toxicity as shown using culture cell studies (Locci et al., 2000). Gjerdet and Hero stated that all soldered appliances undergo some amount of corrosion, which facilitates the release of metals that may cause adverse effects (Gjerdet & Herø, 1987).

Goncalves et al. conducted a study that tested the cytotoxicity and genotoxicity of orthodontic bands with and without silver soldered joints. They tested one type of silver solder and one type of stainless steel band on two different human cell lines. They found that stainless steel bands alone are biocompatible, which is well described in other research. They also found that the bands with solder joints induced stronger cytotoxic and genotoxic effects than the bands without silver solder joints. The conclusion from their experiment advised further research, with different cell lines and different silver solders to be tested (Goncalves et al., 2014).
The experimental models used to evaluate the cytotoxicity of orthodontic materials mainly include established cell lines of human and non-human origins; mostly gingival keratinocytes, fibroblast cells, and osteosarcoma cell lines. Furthermore, some of these cells had no relation to orthodontic treatments. The results of these studies were quite variable and contradictory (Martin-Camean et al., 2015).

Cytotoxicity tests constitute an efficient and initial step in biocompatibility experiments before moving into animal experiments (Assad et al., 1994). Considering the previous reported literature and possible toxic effects of silver solder material, it is beneficial to evaluate the cytotoxic effects of different silver solder materials that have different alloy compositions. The aim of this in vitro study was to compare the cytotoxicity effects of two silver solders with different alloy compositions that are commonly used in orthodontics, on human periodontal ligament fibroblast cells (HPLF).
CHAPTER 2
MATERIALS AND METHODS

Samples:

Silver solders and stainless steel bands were purchased from multiple dental supply companies. It was decided to choose two commonly used silver solders with published differences in alloy propositional ingredients, Leone (Solder for Stainless Steel; Firenze, Italy, Ag 49-51%, Cu 26-29%, Zn 20-22%, Mn 1.5-3%, Ni .41-1%), Summit Orthodontic Services and Laboratory Products Co. (Summit Silver Solder; Ag 56%, Cu 22%, Zn 17%, Sn 5%; Munroe Falls, OH). Stainless steel bands from 3M Unitek were used. The soldering flux used was (3M Unitek Potassium Tetraborate 30-40%, Boric acid 20-30%, Potassium bifluoride 20-30%, Water 10-20%, Boron potassium oxide 1-5%, Sodium Lauryl Sulfate <.5%).

For the orthodontic bands with silver solder joints, a stainless steel band was bent with a cotton plier to create a crescent shape to allow for a more uniform solder application and to allow better fit into the culture inserts. A ruler was used to measure 10mm of the silver solder and a mark was placed on the silver solder with a washable marker. Ten mg of silver solder flux was placed on the band and a flame was regulated on a butane micro-torch (GB 2001, Blazer, Farmingdale, NY, USA) to allow for soldering. The silver solder was then fused to the stainless steel band until soldering was completed. The bands with solder were then weighed to ensure the same amount of silver solder fused to the bands, which was 0.16g total weight of the bands with solder. The samples were allowed to cool to room temperature, placed in a plastic bag with 70%
alcohol and placed in an ultrasonic for 10 minutes to remove excess flux. The bands were rinsed under water and stored in 70% alcohol.

**Cell Culture and Exposure to Experimental Conditions:**

The human periodontal ligament fibroblasts (HPLF) #2630 were purchased from ScienCell Research Laboratories (Carlsbad, Ca). The HPLF were incubated in 75 cm² tissue culture flasks at 37°C with a minimum relative humidity of 95% and an atmosphere of 5% carbon dioxide. The HPLF cell line was cultured in alpha-MEM with 10% fetal bovine serum (FBS) for 48 hours and the culture medium was refreshed every 24 hours. The cells were seeded at a density of $2 \times 10^5$/well in 24-well cell culture dishes for 48 hours, starved with serum-free alpha-MEM overnight and then exposed to the specimens in an insert (Falcon Cell Culture Insert, .4µm pore size, Manufacturer: Corning 353095).

The cells were exposed to stainless steel orthodontic bands with and without silver solder joints. Calcium ionophore, A23187 (10 µmol/L) was used to treat the cells as the positive cytotoxic control. The cells were exposed to the culture medium alone with the cell inserts as the negative control. Two independent experiments were performed (N=5/group for each experiment). The study groups were as follows:

1. Negative control: The cells were exposed to changed culture medium, 750 µL alpha-MEM with 0% FBS.

2. Positive control : The cells were exposed to changed culture medium containing calcium ionophore, A23187 (10 µmol/L) and 750 µL alpha-MEM with 0% FBS.
3. Samples to be tested (B): Stainless steel orthodontic bands from 3M Unitek: the cells were exposed to the culture medium containing the stainless steel orthodontic bands as received from the manufacturer and 750 µL alpha-MEM with 0% FBS.

4. Samples to be tested (BS): Stainless steel orthodontic bands from 3M Unitek with Summit silver solder joints: the cells were exposed to the culture medium containing the stainless steel bands with Summit silver solder joints in the cell insert and 750 µL alpha-MEM with 0% FBS.

5. Samples to be tested (BL): Stainless steel orthodontic bands from 3M Unitek with Leone silver solder joints: the cells were exposed to the culture medium containing the stainless steel bands with Leone silver solder joints in the cell insert and 750 µL alpha-MEM with 0% FBS.

The different samples were exposed to the HPLF cells via tissue culture inserts suspended above the cultured cells for 48 hours. This ensured that the samples were in contact with the media bathing the cultures, but were not in direct contact with the cells; avoiding possible cell damage due to physical contact of the cells (Lobner & Asrari, 2003). The cell culture inserts with the different combinations of bands and solders were placed in the wells. Additional medium (250 µL) was added to the wells to ensure the bands were fully immersed in the medium. The 24 well plates were covered and placed in an incubator for 48 hours with a controlled environment of 37°C with a minimum relative humidity of 95% and an atmosphere of 5% carbon dioxide.

After 48 hours, the inserts with the different bands and solders were removed from the wells. Photographs of the HPLF cells were taken under the different
experimental conditions using a VWR Trinocular Inverted Microscope at 40x magnification. An MTT assay was then completed to assess cytotoxicity.

**Analysis of Cytotoxicity**

Cellular metabolism was measured by means of the MTT colorimetric assay. Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was reduced by mitochondrial succinate dehydrogenase. This reduction forms a blue formazan crystal, which cannot cross plasma membranes and accumulates in the cells (Sjogren, Goran, et al., 2000). The culture inserts with the bands were removed and 50 µL of 10% (V/V) MTT solution was added to each well. A 30 minute incubation period in a 37°C, humidified, 5% CO₂/air incubator was used. The MTT solution was removed via suction and 250 µL of dimethyl sulfoxide (DMSO) was added in each well to dissolve the formed formazan crystals and cells. A fraction of solution (200 µL) was removed from each well and placed on a 96 well plate in the same order and the absorbance was measured by a spectrophotometer (Molecular Devices VERSAmax microplate reader), with a measured absorbance at 570nm. For the detection of the mechanism of cell death or protection by MTT assays, either 100 micromolar of a caspase inhibitor ZVAD (Z-VAL-ALa-Asp-flu-oromethylketone) or 100 micromolar of free radical scavenger Trolox was added respectively.

**Statistics**

Calculations were performed with one-way analysis of variance (ANOVA) with a Bonferroni correction, P values less than .05 were considered to be significant statistically. Each well culture was considered to be an individual sample.
CHAPTER 3
RESULTS

From Tables 1 and 2 below, the results of the MTT tests show that there is a statistically significant difference in cytotoxicity between cells only (control) and both Leone silver solder (BL) and Summit silver solder (BS). The 3M Unitek stainless steel bands (B) were also significantly cytotoxic compared to cells only (control). The results also show that there is a significant difference in cytotoxicity between the 3M Unitek stainless steel bands (B) and the Summit silver solder (BS). However, there is no significant difference between the 3M Unitek stainless steel bands (B) and the Leone silver solder (BL). When comparing the cytotoxicity between the two silver solders, the Summit silver solder (BS) is significantly more cytotoxic than the Leone silver solder (BL).

Each cultures well was considered as an individual sample (n=10/sample tested). The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P≤0.001). The statistical power for all the data was greater than 80%, which confirms that there is a large enough sample size to be significant.
Table 1. Results of the MTT colorimetric assay showing different effect of cytotoxicity on HPLF cells by the various samples.

<table>
<thead>
<tr>
<th>Group Name *</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>3</td>
<td>100.000</td>
<td>13.528</td>
<td>5.113</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>2</td>
<td>69.000</td>
<td>9.227</td>
<td>3.262</td>
</tr>
<tr>
<td>BL</td>
<td>10</td>
<td>0</td>
<td>51.900</td>
<td>17.978</td>
<td>5.685</td>
</tr>
<tr>
<td>BS</td>
<td>10</td>
<td>0</td>
<td>28.800</td>
<td>10.685</td>
<td>3.379</td>
</tr>
</tbody>
</table>

* Control = cells only, B = Bands only, BL = Bands + Leone silver solder, BS = Bands + Summit silver solder

Table 2. Comparison to control values and intergroup comparison of MTT experiments using Bonferroni t-test

<table>
<thead>
<tr>
<th>Comparison *</th>
<th>Diff of Means</th>
<th>t</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. BS</td>
<td>71.2</td>
<td>10.72</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Control vs. BL</td>
<td>48.1</td>
<td>7.242</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Control vs. B</td>
<td>31</td>
<td>4.444</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>B vs. BS</td>
<td>40.2</td>
<td>6.288</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>B vs. BL</td>
<td>17.1</td>
<td>2.675</td>
<td>0.071</td>
<td>No</td>
</tr>
<tr>
<td>BL vs. BS</td>
<td>23.1</td>
<td>3.83</td>
<td>0.003</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Control = cells only, B = Bands only, BL = Bands + Leone silver solder, BS = Bands + Summit silver solder

These tables show the mean values among the treatment groups, the standard deviations of each and multiple comparisons versus control group using the Bonferroni t-test. The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.006).
Tables 3 and 4 show the results of the MTT experiments testing for cytotoxicity protection and the mechanism of cytotoxicity using only the Summit silver solder (B+S) since it showed the highest cytotoxicity effect compared to the others.

An attempt to determine the mechanism of cytotoxicity of the Summit silver solder (BS) was completed by testing the effects of a free radical scavenger, Trolox, and the addition of a caspase inhibitor, ZVAD (Z-VAL-Ala-Asp-flu-oromethylketone). As seen in Table 4, the results of adding the caspase inhibitor, ZVAD (BS+ZVAD), still show significant cell death; meaning it was not significantly protective against the Summit silver solder (BS). This result suggests that under the testing conditions, Summit solder does not induce apoptosis, but that cellular necrosis is likely the method of toxicity.

When the free radical scavenger, Trolox, was added with the Summit solder (BS+Trolox), the results still showed significant cell death, showing that it does not significantly block cytotoxicity. There is slight protection from the addition of Trolox, but it is not statistically significant.
Table 3. Mean values of MTT calorimetric measurements, testing for protection and mechanism of cell death

<table>
<thead>
<tr>
<th>Group Name *</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>100000</td>
<td>24.199</td>
<td>9.879</td>
</tr>
<tr>
<td>BS</td>
<td>8</td>
<td>0</td>
<td>62500</td>
<td>15.675</td>
<td>5.542</td>
</tr>
<tr>
<td>BS+Trolox</td>
<td>8</td>
<td>0</td>
<td>71.125</td>
<td>16.444</td>
<td>5.814</td>
</tr>
<tr>
<td>BS+ZVAD</td>
<td>8</td>
<td>0</td>
<td>72.375</td>
<td>17.525</td>
<td>6.196</td>
</tr>
</tbody>
</table>

* Control = cells only, BS = Bands + Summit silver solder, BS+Trolox = Bands + Summit solder when Trolox was added, BS+ZVAD = Bands + Summit solder when ZVAD was added

Table 4. Comparison of MTT measurements to control values testing for protection and mechanism of cell death using Bonferroni t-test

<table>
<thead>
<tr>
<th>Comparison *</th>
<th>Diff of Means</th>
<th>t</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. BS</td>
<td>37.5</td>
<td>3.798</td>
<td>0.002</td>
<td>Yes</td>
</tr>
<tr>
<td>Control vs. BS+Trolox</td>
<td>28.875</td>
<td>2.924</td>
<td>0.021</td>
<td>Yes</td>
</tr>
<tr>
<td>Control vs. BS+ZVAD</td>
<td>27.625</td>
<td>2.798</td>
<td>0.029</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Control = cells only, BS = Bands + Summit silver solder, BS+Trolox = Bands + Summit solder when Trolox was added, BS+ZVAD = Bands + Summit solder when ZVAD was added

Figures 1-5 below are microscopic photographs taken of the HPLF cells under the different experimental conditions using a VWR Trinocular Inverted Microscope at (40x magnification).

HPLF cells were exposed to MEM medium with no FBS for 48 hours (negative control group) (Fig 1.), HPLF cells exposed to MEM medium with no FBS plus the addition of Calcium Ionophore (A23187), for 48 hours (positive control group) (Fig 2.), HPLF cells exposed to MEM medium with no FBS plus the addition of 3M Unitek stainless steel bands in culture inserts for 48 hours (Fig. 3), HPLF cells exposed to MEM medium with no FBS plus the addition of bands with Leone silver solder in culture inserts for 48 hours (Fig. 4), and HPLF cells exposed MEM medium with no FBS plus the addition of bands with Summit silver solder in culture inserts for 48 hours (Fig 5).
Figure 1 shows normal fibroblasts appearing confluent and fusiform. Figures 4-5 show the effect of the silver solder on the HPLF cells with the presence of more round cells, mostly with darkened and granular aspects suggesting lysis with cell death. Note the similarity between Figure 2, HPLF cells exposed to calcium ionophore, A23187 (positive control group) for 48 hours and Figure 5 HPLF cells exposed to Summit silver solder for 48 hours.

Figure 1. HPLF cells cultured in changed MEM medium with no FBS (negative Control) for 48 hours at 40x magnification
Figure 2. HPLF cells exposed to calcium ionophore (positive control) for 48 hours at 40x magnification

Figure 3. HPLF cells exposed to 3M Unitek stainless steel bands for 48 hours at 40x magnification
Figure 3. HPLF cells exposed to bands with Leone silver solder for 48 hours at 40x magnification

Figure 5. HPLF cells exposed to bands with Summit silver solder for 48 hours at 40x magnification
CHAPTER 4
DISCUSSION

The use of orthodontic appliances is always associated with increased gingival irritation presenting as edema, erythema, and sometimes overgrowth of the soft tissue surrounding the appliances. These localized inflammatory reactions cannot be distinguished from gingivitis of bacterial etiology, which is mainly due to improper oral hygiene. However, some of these inflammatory reactions can be due to the corrosion of intraoral appliances and the effect of the released metal ions on the surrounding tissue. Numerous in-vivo and in-vitro investigations related to the toxic effects of the released metal ions on different cell lines or on animals have been completed. However, contradictory results are presented, mainly resulting from the different cell lines, experimental conditions, and the variable materials tested (Mikulewicz & Chojnacka 2011a, 2011b).

Silver solder is routinely used in orthodontics for the fabrication of intraoral appliances. Previous studies have shown that silver solder is cytotoxic to cells depending on which cells were used. However, there is no study that compares different soldering materials on the same cell line. The aim of this study was to evaluate the in-vitro cytotoxic effects of two different silver solder materials that are commonly used in fabricating orthodontic appliances on human periodontal ligament fibroblast cells (HPLF).

From our results in Table 2, stainless steel bands were found to be significantly cytotoxic to the HPLF cells. This result is in agreement with other studies e.g. (Goncalves et al 2014) showing that stainless steel bands release chromium, nickel and iron into the
culture medium tested. The concentrations of these metal ions were able to induce cytotoxicity and genotoxicity to cells (Goncalves, Macedo de Menezes, Trindade, Machado, Thomas, Fenech & Henriques, 2014). One of the components of stainless steel bands is nickel, and numerous studies have elaborated on the cytotoxicity of nickel. Nickel ions have been proven to be released by stainless steel bands, brackets and ligatures (Mikulewicz & Chojnacka, 2011b). One study recently demonstrated in a three-dimensional human-derived oral mucosal model that Ni-based alloys are cytotoxic to cells, and are capable of inducing oxidative stress and inflammatory cytokine expression (McGinley, Moran, & Fleming, 2013).

Nickel has also been shown to be a carcinogen, one of the most common allergens, and the most potent sensitizer among all metals (Hensten-Pettersen, 1984). Messer exposed fibroblasts to different ion concentrations of beryllium, chromium, nickel, and molybdenum and the cells were examined under scanning electron microscopes and transmission electron microscopy. It was found that the cells had irregularly shaped nuclei when exposed to hexavalent chromium and nickel, pseudopodia when exposed to beryllium and molybdenum, and lipid-droplet formation when exposed to nickel (Messer et al., 1999). Given the previously stated information, a possible cause of the cytotoxicity from the stainless steel bands could be the release of chromium, nickel and iron into the culture medium.

However, there are studies stating stainless steel bands were not cytotoxic to cells. Jacoby et al., found that extracts from orthodontic bands alone did not decrease viability of the different cell lineages tested (Jacoby, Junior, Campos & Menezes, 2017). Mockers
et al., also found that stainless steel bands can be considered non-cytotoxic to cells (Mockers, Deroze & Camps, 2002)

As shown in Table 2, both silver solders from Leone and Summit were significantly toxic compared to the control group. These results were supported by the different morphological patterns of the cells shown by the microscopic photographs. As previously stated, silver solder cytotoxicity has been supported by other researchers. Freitas et al., found that silver soldering used in orthodontics exhibits severe cytotoxicity. In their study, they found that mice fibroblasts exposed to silver solder resulted in the inhibition of proliferation, growth, and development of the cells that were analyzed (Freitas, Oshima, Menezes, Machado & Viezzer, 2009). Goncalves et al., has also proven that silver solder is both cytotoxic and genotoxic to two different human cell lines (Goncalves, Macedo de Menezes, Trindade, Machado, Thomas, Fenech & Henriques, 2014).

As seen in Table 2, Summit silver solder had the greatest amount of cytotoxicity of the materials tested. One possible reason for the difference in cytotoxicity is the higher silver concentration in the Summit silver solder (56% Ag) versus Leone silver solder (49%-51% Ag). In previous studies, it has been found that silver is known to be cytotoxic to different cell lineages (Jacoby, Junior, Camplas & Menezes, 2017). It has also been shown that a change in the metal concentrations of an alloy will cause a change in their release into the medium in which they were immersed in (Elshahawy et al., 2009). It has been shown that at low concentrations (<2.0 µmol/mL) of silver ion, cellular mitochondrial activity is essentially unchanged from normal (Wataha, 2000). However, as the concentration of silver ion increases in the medium, cellular activity decreases
exponentially. When concentrations exceed 10 µmol/mL, cellular activity is practically zero (Wataha, 2000). It has been concluded that cytotoxicity of the cell line increases with increased concentration of silver nanoparticles (Halkai, Mudda, Shivanna, Patil, Rathod & Halkai, 2019). Thus, as the concentration of silver is higher in the Summit silver solder (56% Ag) versus the Leone silver solder (49-51% Ag), and their alloy concentrations of other metals differ, it is likely that there is an increase in silver release into the surrounding medium with Summit silver solder, supporting the hypothesis that as the concentration of silver increases, so does cytotoxicity.

It is important to note that the amount of metal ions that are released in a solution is not comparable to the makeup of the alloy itself. This was shown by Elshahawy et al 2009, who found that even minor elements of intraoral appliances can be released into the solution they are immersed in, and the abundance of the elements in the material is not proportional to the release of elements. The release of elements is due to the complex behavior of the alloy and the material system, plus the solutions in which they were immersed in. The Summit silver solder contains tin, whereas the Leone silver solder does not contain tin. Wataha et al. has shown that mouse fibroblasts exposed to metal cations, including tin, suppress cell activity by 50%. They have also shown that tin is mutagenic to mouse fibroblasts (Wataha, 2000). Therefore, it is important to determine which metal ions were released into solution from the two silver solders tested in this experiment. It was also stated by Hafez et al. who claimed that it is important to isolate each orthodontic material to evaluate its toxicity as different appliance combinations can lead to varying responses (Hafez, Selim, Kamel, Tawfik, Al-Ashkar & Mostafa, 2011).
There could also be other hidden elements that can cause a cytotoxic effect but are not listed by the manufacturing companies. Goncalves et al., tested a silver solder with the composition of 55-57% Ag, 21-23% Cu, 15-19% Zn, and 4-6% Sn. This composition closely matches the Summit silver solder tested in our experiment, which has a composition listed by the manufacturer of 56% Ag, 22% Cu, 17% Zn, and 5% Sn. They found that cadmium, although not listed as a component of the silver alloy, was detected as an ion that was eluted from all samples of the bands with silver solder. Decades ago, cadmium was routinely added to silver solder to lower the fusion temperature of the alloy (Berge Gjerdet & Erichsen, 1982).

Cadmium has been shown to be responsible for damage to the liver, kidneys and heart (Novelli, Hernandes, Novelli Filho & Barbosa, 1998), and has been associated with dental pathologies such as periodontitis (Arora, Weuve, Schwartz & Wright, 2009). Li et al., found that cadmium may cause mitochondrial dysfunction in respiration, loss of membrane potential, and release of c-cytochromes (Li, Xia, Chun-Sun, Li, Fu & Zou, 2003). Thus, without knowing which metals are released from both solders in this experiment, it is possible that there is cadmium released from the Summit silver solder that is responsible for an increase in cytotoxicity.

We attempted to determine the mechanism of cytotoxicity of the Summit silver solder by testing the effects of a free radical scavenger, Trolox, and the addition of a caspase inhibitor, ZVAD (Z-VAL-Ala-Asp-flu-oromethylketone). ZVAD has been shown to be a consistent marker of apoptotic cell death (Gottron, Ying, & Choi, 1997). Neither Trolox nor ZVAD showed significant protection against cytotoxicity. There is, however, some evidence of protection as shown in Tables 3 and 4. The MTT test has also
been shown to be not the most accurate way to quantify apoptotic cell death, but to be better at determining at what point in the death pathway the anti-apoptotic agents exert effects (Lobner, 2000). Based on the evidence from this one experiment using the MTT test, with one concentration, it would not be accurate to say that apoptosis is not involved.

Trolox is a water-soluble vitamin E derivative that is known to be a strong antioxidant and is commonly used in antioxidant assays and oxidative injury studies (Jang, Hwang, Cho, et al., 2008). As result, Trolox did not show statistically significant protection, suggesting that death is not induced by free radicals. However, Trolox does not scavenge all types of free radicals, and it is also possible that we did not use a high enough concentration of Trolox. We may need to try a higher concentration of Trolox, or additional free radical scavengers to better conclude that cell death is not induced by free radicals. Thus, from our preliminary results, we can infer that cell death is not caused by free radicals or apoptosis until further investigation is completed.

The MTT assay was chosen for this study because it is one of the most commonly used assays for testing biocompatibility and cytotoxicity, and it allows for easy determination of the percentage of viable cells (Lobner, 2000, 2003, Jacoby et al., 2017). The assay quantifies mitochondrial activity by measuring the production of formazan crystals formed when the mitochondria reduce the tetrazolium ring of MTT. The reduction of MTT can only take place in living cells, which allows for the quantification of living cells (Halkai et al., 2019).

Although we made every attempt to complete the experiment at the best of our ability, there are limitations. For this experiment, one limitation is the lack of standardization of the silver solder surfaces. In clinical practice, solder surfaces are
polished so they are less irritating to the gingiva. It is known that a smooth homogenous surface makes orthodontic appliances less prone to corrosion (Brantley & Eliades, 2001). Thus, standardizing e.g. polishing the soldered surfaces can potentially lead to less corrosion and different cytotoxic results. It would also be suggested to use testing conditions that mimic the oral environment. The oral cavity varies in pH, microbial activity, temperature differences, and abrasive and mechanical forces; which can all effect material deterioration and corrosion of materials (Brantley & Eliades, 2001). Thus, using a different medium that is somewhat acidic, or completing the experiment with some form of abrasive testing, could give rise to different results.

The continuation of these experiments will involve the detection of the metal ions released, and their concentrations. Moreover, the dose dependent effects of these ions on the cells concentrations also must be analyzed. Our preliminary results regarding the mechanism of cytotoxicity need to be carefully investigated due to the importance and the lack of information in the literature. It is also recommended that the cytotoxicity of other brands of silver solder should be tested, and alternate means of joining metals (e.g. laser welding) should be evaluated.
CHAPTER 5
CONCLUSION

Under the conditions and within the limitations of this study, stainless steel bands show cytotoxic effect to human periodontal ligament fibroblast cells (HPLF). However, the addition of silver solder to the stainless steel bands is significantly more cytotoxic to HPLF cells. Based on the MTT tests and the microscopic examinations, Summit silver solder was proven to be significantly more cytotoxic to the cells tested than Leone silver solder. The findings from this study call orthodontists to pay attention to the composition of the solder material and choose the materials that are less cytotoxic. In practice, minimal amount of solder material should be used in the fabrication of orthodontic appliances.
REFERENCES


