Assessing the Neurotoxicity of Endodontic Sealers

Assessing the Neurotoxicity of Endodontic Sealers

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I. Abstract

Many times the relationship between dental materials and clinical outcomes is poorly understood because so little is known about the biocompatibility of materials and the biological pathways they may affect. In endodontics, there is no doubt a relationship between materials like canal sealers and the tissues they come in contact with, such as dental pulp, nerve, bone, and periodontal ligament—but how this relationship manifests in clinical outcomes such as post-operative pain is unknown. This study seeks to begin to uncover these pathways by examining the toxic effects of bioceramic, resin, zinc-oxide eugenol, and silicone-based endodontic sealers on cells and tissues. An *in vitro* model testing three cell types, human dental pulp stem cells (hDPSCs), neuron-like cells (NBs), and murine peripheral neurons (MPNs), exposed to sealers in a dose-response fashion was used to determine the relative toxicity of sealers. An *in vitro* model testing inflammatory and neurotoxicity biomarker gene expression after murine mental nerve exposure to sealer provided further evidence to the toxicities of these compounds and elucidated some of the biologic pathways they may trigger from the mental nerve to the trigeminal ganglia. Results from both models generally agree that AH Plus is the most toxic and that GuttaFlow 2 exhibits nearly no toxicity, but more importantly the results of this study show how *in vitro* modeling can complement more complex *in vivo* modeling in biomaterials science.

Key Words: Bioceramic, AH Plus, Zinc-Oxide Eugenol, GuttaFlow II, inflammation, neurotoxicity, RTqPCR, biomarkers

II. Introduction

Endodontic therapy contributes to more frequent and severe post-operative pain than does any other dental operative procedures.[1] In fact, the reported incidence of post-operative pain after root canal treatment ranges from 1.5% to 63.8%, depending on its definition.[1, 2] Ranging from mild discomfort, soreness, throbbing aches, to severe pain and swelling, post-operative pain is an unwanted burden for patients and a challenging hurdle for clinicians.

Post-operative pain, whether manifesting as an acute episode requiring a few analgesics or a severe migraine resulting in chronic morbidity, is a procedural complication that is best avoided. Prevention, however, is dependent on the knowledge surrounding the causes and mechanisms of its existence. For root canal therapies, some causes can be linked to mechanical, chemical, and microbial injury to localized tissues such as pulp, periodontal ligament, or bone, or even projected injury to ectopic structures such as peripheral nerves, ganglia, and pain centers in the central nervous system.[3, 4]

For non-microbial causes of post-operative pain, severity relies on the degree of injury, the amount and type of tissue damaged, the toxicity of the chemical factor, and the intensity of the inflammatory reaction.[5] An understanding of the relationship between chemical factors, inflammatory responses, and nerve injury is therefore critical to the prevention and management of post-operative pain and improved outcomes with dental procedures.

Endodontic Therapy

Root canals are complex systems of spaces within the hard tissue of a tooth that house the *dental pulp*—a tissue comprised of connective, vascular, lymphatic, and nervous elements. The nervous element returns proprioceptive and pain information to the central nervous system that is useful in oral function and the detection of impending disease (e.g. caries); the lymphatic element is useful in combating that disease; and the vascular element is required for the functioning of the prior two. Loss of any of these elements can lead to the failure of the dental pulp, manifesting as pulpitis or pulp necrosis that in turn can lead to pain, inflammation, and infection—even into the surrounding tissues of the periodontium, oral cavity, or sinuses.

Loss of the dental pulp in fully developed teeth if treated does not lead to any surmountable adverse consequences for patients in and of itself. In fact, it could be argued that the dental pulp really exists in adults as a vestigial remnant of tooth development, and, weighing out proprioception and the detection of disease, probably causes more morbidity for patients than if it was non-existent.

Endodontic therapy is warranted for situations of pulpal disease or if required for restoration of the tooth. Therapies can include pulpal regeneration (in younger teeth, a topic that occupies much of the current literature in endodontic research), traditional non-surgical root canals, and apical surgeries with root-end resection and filling.

The immediate goal of endodontic therapy is to alleviate pain and clear infection through cleaning of the root canal system. A more long-term goal that underlies the true clinical success of therapy, however, is the sealing and filling of root canal systems to prevent future microbial contamination. This is accomplished through endodontic materials designed with physical and chemical properties that are anti-microbial, hermetic, durable, and especially biocompatible.

During the cleaning process in non-surgical root canal therapy, canal systems are shaped to receive standard core filling material, most commonly gutta percha. Gutta percha has been used in dentistry since 1843, and the thermoplastic latex-related filling material, originally derived from the Malay gum tree (now synthetic), is popular for its clinical ease-of-use and its biocompatibility and well-established as being inert towards oral tissues.[6] To fill the core of the shaped root canal system as completely as possible, gutta percha is manufactured in the shape of a tapered cone. However, the existence of voids, lateral canals, multiple foramen, isthmuses, and other irregularities in the canal system and the inability of gutta percha to chemically bond preclude it from forming a complete seal with walls of the canal system.[7] This necessitates the use of an additional material known as an *endodontic sealer*.

While compensating for the inability of the core filling material to completely seal the canal system, endodontic sealers ultimately are responsible for the prevention of microleakage and bacterial re-contamination of the root canal. They are typically viscous enough so that when placed alongside gutta percha, the pressure of the progressing cone 'flows' the sealer into any spaces around or not occupied by the cone. An unintended side effect of this process is that sealer can flow into spaces and tissues beyond the limits of the root canal system (e.g., "puff"), placing it in contact with periodontal ligament, bone, nervous, and vascular tissues of the periapex.[8] Consequently, the biocompatibility, toxicology, and chemical factors associated with endodontic sealers and the tissues they come in contact with is paramount to understanding endodontic clinical outcomes and post-operative pain.[8]

Endodontic Sealers

A plethora of endodontic sealers exist that attempt to meet the ideal requirements of Grossman (Table 1), but none have succeeded the task. Sealers have unique chemical compositions and setting reactions that place them into different classes, such as glass ionomer, salicylate, zinc oxide-eugenol, fatty acid, epoxy resin, methacrylate resin, calcium hydroxide, silicone, and bioceramic-based.[9] Many factors influence clinician's choice of sealer during endodontic therapy, but some include cost, ease of use, proven success, and availability. Scant literature exists as to which sealers might be the most commonly used, but arguably resin, zinc oxide-eugenol, and bioceramic sealers are the most widely used in clinical practice in the United States today.[11] Newer sealers, such as the silicone-based (i.e., GuttaFlow 2), and new subsets of existing sealers (e.g., country-specific spin-offs of bioceramics such as Ceraseal (Korea), Bio-C Sealer (Brazil), and Endo CPM Sealer (Argentina)) routinely enter the market, many times with little clinical data on their safety. Table 2 is a summary of the composition of sealers used in this study.

Resin: AH PLUS JET

Epoxy resin was invented in 1938 and its use as a sealer, AH 26, began in the 1940s.[11] Issues with the initial formulation were first discovered in 1964, when AH 26 was found to cause more tissue irritation than pure silver implanted in a rabbit.[12] In 1993, it was reported that in its setting reaction AH 26 releases formaldehyde[13], which led to the development of AH Plus, which does not release formaldehyde. AH Plus Jet exists in a paste-paste mixture dispensed in an automix syringe and sets over 8 hours by an addition reaction between epoxide groups attached to epoxy resins and amines to form a polymer.[11] Microleakage, presumably because of polymerization shrinkage, can be a downside of AH Plus.

Table 1: Grossman's Requirements for an Ideal Root Canal Sealer[10]
-Tacky to help adhesion between the canal wall and core filling material when set
-Provide an excellent seal apically and laterally
-Radiopaque
-Non-staining
-Dimensionally stable
-Easily mixed and introduced into the canal
-Soluble in a common solvent
-Insoluble in tissue fluids
-Bacteriostatic
-Slow enough set for placement
-Neither mutagenic or carcinogenic
-Does not provoke an immune response in periapical tissues
-Biocompatible

Zinc Oxide-Eugenol: PULP CANAL SEALER

Zinc oxide-eugenol (ZOE) sealers have set the standard in endodontics because of their long-term success since their development in the 1930's.[11] ZOE is popular among dentists because of its slow set working time (up to 6 hours), low cost, antibacterial properties, and ease of use.[14] When the zinc oxide powder is mixed with the eugenol liquid, it forms an amorphous gel that when set forms a rigid matrix.[15] When compared to other sealers, ZOE has a relatively high rate of microleakage.[11]

Tricalcium Silicate/Bioceramic: ENDO SEQUENCE BC SEALER

Tricalcium silicate sealers were introduced in the 1990's and share similar properties to Portland cement (but more pure, have finer powders, and are radiopaqued).[16] When mixed with water or in contact with tissue fluids (e.g., from dentinal tubules), the calcium silicate powders in these 'ceramics' react, forming a hydrated matrix embedded with calcium hydroxide ions.[17] These ions are continually released for about one month after setting, raising the pH to around 12 and stimulating phosphate ions in body fluids to precipitate hydroxyapatite at the interphase, creating a hermetic seal.[18, 19] Complete setting time, an element of controversy for the bioceramic sealers, has been shown to exceed one month.[20] The bioactivity of the tricalcium silicates is the most unique feature of this material, and has been shown (*in vitro*) to not only induce proliferation of stem cells of the apical papilla but also differentiation into osteogenic cells.[21] In trials comparing microleakage of bioceramic to other sealers, it had the least.[11] Despite their high cost, bioceramic sealers are popular for their bioactive nature and their ease of use—a single paste.[22]

Silicone: GuttaFlow 2

Silicone-based sealers were developed in 1972 and set between addition reactions of vinyl groups and siloxane groups to form a polymer.[11] Alone, they show relatively no leakage, are virtually non-toxic, but also show no antibacterial activity.[23] GuttaFlow 2 integrates gutta percha and silver nanoparticles within the silicone sealer, overcoming the silicone's antimicrobial deficiency while keeping it biocompatible.[24] These nanoparticles range in size from 0.1nm to 100nm and are polycationic and polyanionic in nature, giving them their antibacterial action.[6] Thin films can be produced with this sealer, giving a greater seal with fewer voids and greater adhesion to dentinal walls.[25] Working time for GuttaFlow 2 is 15 minutes, and the sealer sets in 25-30 minutes.[23]

Table 2: Test Sealers[11]

Class	Product Name (manufacturer, country)		Composition
Epoxy Resin	AH Plus Jet (Dentsply Sirona, Germany)	Paste A	Bisphenol A epoxy resin, Zirconium oxide, Bisphenol F epoxy resin, Calcium tungstate, Iron oxide, Silica
		Paste B	N,N-dibenzyl-5-oxanonadiamin-1,9, Amantiameamine, Tricyclodecane-diamine, Calcium tungstate, Zirconium oxide
Tricalcium Silicate (MTA/Bio ceramic)	EndoSequence BC (Brassler, USA)	One Paste	Zirconium oxide, Calcium silicate, Calcium hydroxide, Calcium phosphate, Filler, Thickening agents
Zinc Oxide-Eugenol	Pulp Canal Sealer (Kerr, USA)	Powder	Zinc oxide, Precipitated silver, Oleo resin, Thymol iodide
		Liquid	Oil of cloves, Canada balsam
Silicone	GuttaFlow 2 (Coltene/Whale dent, USA)	Base	Zirconium oxide, Polymethylvinylsiloxane, Polymethylhydrogensiloxane, Micro-silver, Gutta percha
		Catalyst	Zirconium oxide, Polymethylvinylsiloxane, Platinum catalyst

Models for Sealer Testing
Many models for *in vitro* testing of endodontic sealer cytotoxicity and biocompatibility have been developed, ranging from testing human gingival fibroblasts and periodontal ligament (PDL) cells to mesenchymal stem cells.[1] Sealers from all classes in varying combinations have been used in these studies, and they are either used freshly mixed or prepared separately and allowed to set prior to exposure. Outcomes are measured with the MTT assay, LDH assay, and even immunohistochemistry. The *in vitro* model designed in this study, though having many similar aspects to prior studies, is unique in that the cell types chosen are related more to the peripheral nervous system and hint at aspects of neurotoxicity.

In vivo models looking at neurotoxicity and endodontic sealers on a gene-expression level are not apparent in the literature. Though studies exist examining neurotoxicity-associated biomarkers upregulated as a result of sealer exposure, they are *in vitro* and only look at a few biomarkers. The *in vivo* model in this study is entirely unique and designed to screen for a large number of both inflammatory (n=84) and neurotoxicity (n=84) biomarkers to help elucidate the mechanisms and pathways that endodontic sealers affect in localized tissue (i.e., the mental nerve and surroundings) to the trigeminal ganglia.

The power of this study is that the *in vitro* and *in vivo* models complement one another, providing a unique research perspective that enables a validation of both techniques.

Formulating a Hypothesis
The hypothesis for this study grew out of an article read about the bioactivity of bioceramic sealers that induces stem cells to proliferate and osteogenically differentiate.[21] Considering that extrusion of bioceramic sealer into areas of apical periodontitis could potentially increase bone regrowth and healing, could it do the same for other tissue types, such as nerves—is bioceramic sealer similarly bioactive for neuronal tissues? The hypothesis generated, that *bioceramic sealer is less neurotoxic than resin, zinc oxide-eugenol, and silicone-based sealers*, stems from this question, and the design of this study grew out of ways to test this hypothesis.

III. *In Vitro* Materials and Methods

Experimental Design
The *in vitro* study design tested the four experimental sealers on three cell types in culture. Exposure of cells to sealer was performed as serial dilutions of freshly[2] mixed sealer in respective culture media for four hours[3] in all trials. Two tests, the MTT and Live/Dead™, quantified toxicity based on spectrophotometry and fluorescent microscopy, respectively.

Human Dental Pulp Stem Cells
hDPSCs were chosen as the basic cell type for this study for their reliability in culturing. These cells proliferate easily on well plates (average population doubling time is less than 24 hours)[26], becoming confluent in only a few days. hDPSCs can be passaged nearly a dozen times without loss of pluripotency, providing a relatively quick and inexpensive *in vitro* line to test.[27, 28] Their potential to regenerate and migrate in association with cell scaffoldings and bioactive materials is just being realized, already proving valuable in dental research.[29]

Pre-purified hDPSCs were obtained from frozen lines previously cultured by B. Michot in the Gibbs Lab and grown in flasks prior to seeding. α-MEM media containing 1% L-glutamine [200 mM], 1% ascorbic acid [10 mM], 10% fetal bovine serum (FBS, S0615), 1% amphotericin B [250 ug/ml], and 1% penicillin [10,000 U/ml]/streptomycin [10mg/ml] was used for all hDPSC culturing. Cells were seeded onto 96 well plates at a density of approximately 5,000 cells/cm^2 and allowed to proliferate at 37°C with 5% CO_2 for 7 days.

Human Peripheral Neuron Equivalents
hDPSCs were differentiated into neuron-like NBs according to a protocol established by Clarke et al.[30] Confirmation of differentiation of hDPSCs into neuron-like cells using this protocol was performed by B. Michot by measuring neuronal biomarkers with immunohistochemistry. After culturing hDPSCs as previously described, α-MEM media was replaced with NB media, and cells were allowed to rest in the new media for an additional 7 days at 37°C with 5% CO_2. Cell density therefore equated approximately to that obtained with pure hDPSCs.

[1] These are discussed later in the conclusion.
[2] The difference in sealer toxicity between freshly mixed and set sealer was tested in murine PDL fibroblasts, finding that sealers have more toxicity when freshly mixed.[38]
[3] Preliminary trials confirmed that there was no measurable difference in cell death between 0.5, 2, 4, and 6 hour exposure time. Preliminary trials also tested the possibility of delivering sealer to cells via a tube, but this proved ineffective due to the tube only exposing a specific area of the well plate floor.

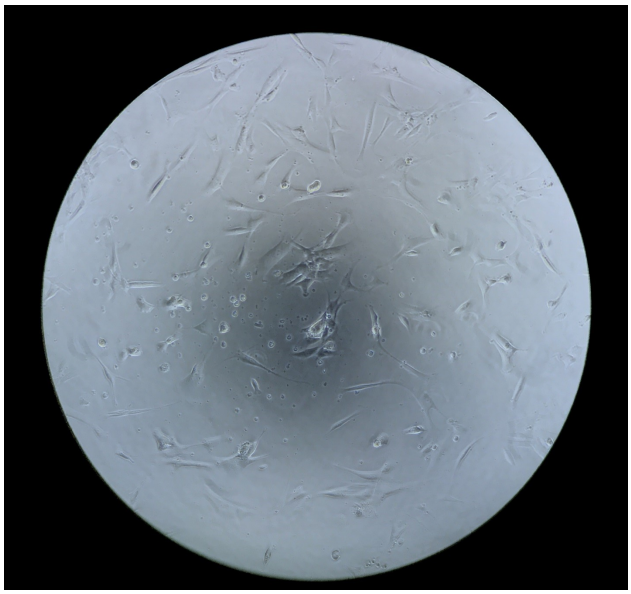

Figure 1: hDPSCs growing in culture; note projections from cell bodies as cells network.

Murine Peripheral Neurons

MPNs were obtained from the dorsal root ganglia (DRGs) and trigeminal ganglia (TGs) of euthanized cage-raised mice. Once removed, cells were isolated and purified according to a protocol[4] of tissue digestion and trituration prior to final plating. MPNs were plated on a 96 well plate to a density of 70-90 cells per well and allowed to rest in culture at 37°C with 5% CO_2 for 72 hours. MPNs will not proliferate in culture, do not live as long as hDPSCs or NBs, and are more sensitive to culturing techniques.

Exposure of Cells to Sealer

Freshly mixed sealer was diluted into each cell types' respective media using a serial dilution of 1.3X.[5] Well plates were partitioned in duplicate rows with decreasing concentrations towards the right; the right most three wells were utilized as controls with media and no sealer. Cells were exposed to media in all trials for four hours.

Quantification of Toxicity

hDPSCs and NBs. Cell counts within each well of these two types warranted the use of the MTT assay to quantify toxicity. This assay works through metabolically active cells (i.e., live) that up-take the MTT dye within their mitochondria. Cells are then washed and lysed to release the dye (purple to the naked eye) into solution read by a spectrophotometer. Higher MTT values imply less toxicity, whereas low MTT values imply cells were not metabolically active (i.e. dead) to store then release the dye.

MPNs. The limited number of neurons obtained from DRGs and TGs and their lack of proliferation in culture disallows the use of the MTT assay to quantify toxicity because of detection thresholds in spectrophotometry. Therefore, the Live/Dead™ assay was used to quantify toxicity in MPNs. This assay utilizes two fluorescent dies, calcein-AM that interacts and stains live cells green, and ethidium homodimer-1 that interacts and stains dead cells red. Following exposure of cells, each well was photographed under fluorescent microscope and images were processed using the freeware ImageJ/FIJI program to produce counts of both live and dead cells.

Confirmation of MTT results with Live/Dead™

To exclude any reactions between sealer, media, and assay reagents that could have occurred using the MTT assay, NBs were subjected to the Live/Dead™ assay and quantified as with the MPNs. Figure 2 is a composite of micrograph images of the results. Figure 3 illustrates the density of NBs achievable with both live (green) and dead (red) cells. Figure 4 is a similar image but with MPNs, note the change in cell density and morphology.

Data Analysis

Data was compiled and statistically analyzed using Graphpad Prism version 9.1.0. Data was baseline corrected using corresponding control samples. Dunnett's multiple comparison test in an ordinary one-way ANOVA was used to determine significance between experimental groups and control for each concentration. Dose-response curves including an EC_{50} (the concentration that gives half-maximal response) were generated using nonlinear asymmetric sigmoidal regression analysis.

[4] Tissue was placed into 1.5 ml of HBSS and an equal volume (1.5 ml) of Papain Solution (60 units of papain, 1 mg L-Cysteine, 3 ul of saturated NaHCO3 [from solution of 1ml sterile water and 0.15g of NaCO3); warmed at 37C for at least 20 minutes and then filter sterilized) was added. The tube was incubated for 20 minutes in a 37C water bath then spun in a Heraeus Megafuge 16R centrifuge for 3 minutes at 200G at 25C to form a pellet in the tube. The Papain Solution was carefully removed with the pellet remaining, and a collagenase solution (10 mg collagenase Type 2, 14 mg Dispase Type II, 3 ml HBSS; warmed at 37C for at least 20 minutes then filter sterilized) was added. The tube was incubated for another 20 minutes in a 37C water bath, then spun in the centrifuge for 6 minutes at 400G at 25C. The collagenase solution was carefully removed from the tube leaving the pellet behind. The cell pellet was then triturated with 750 ul of Ham's F-12 Nutrient Mixture carefully using 1000 ul and 200 ul pipette tips, not introducing air bubbles, and then spun for 6 minutes at 500g at 25C. The solution was removed from the tube again, and 1 ml of 5% FBS with F-12 (5 ml FBS diluted into 95 ml F-12; filter sterilized; 1 ml penicillin/streptomycin and 1 ml amphotericin B added) was added to the tube and triturated with a 200ml pipette, forming a cloudy solution of cells with minimal tissue fragments.

[5] Though the concentrations may seem arbitrary, starting at 1 g/ml concentration and using a 1.3X dilution factor, resulting concentrations in g/ml become 0.444, 0.342, 0.263, 0.202, 0.155, 0.120, 0.092, etc. In preliminary trials, it was determined that concentrations above 0.342 resulted in complete toxicity, so trial data begins at 0.342.

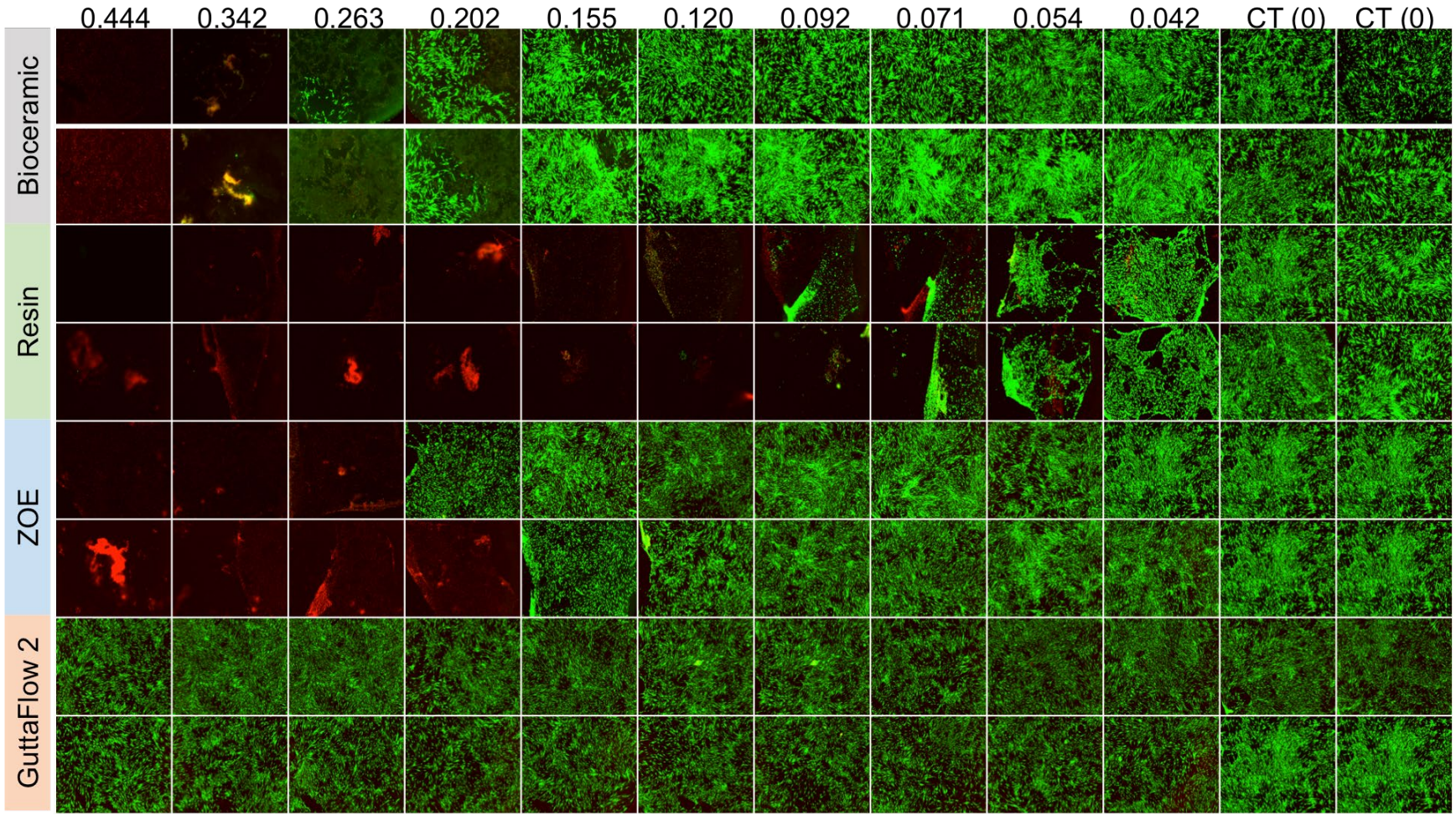

Figure 2: Fluorescent Microsopy of NBs cells exposed to endodontic sealer with the Live/Dead assay. Numbers at top indicate concentration of sealer in g/ml. CT = Control.

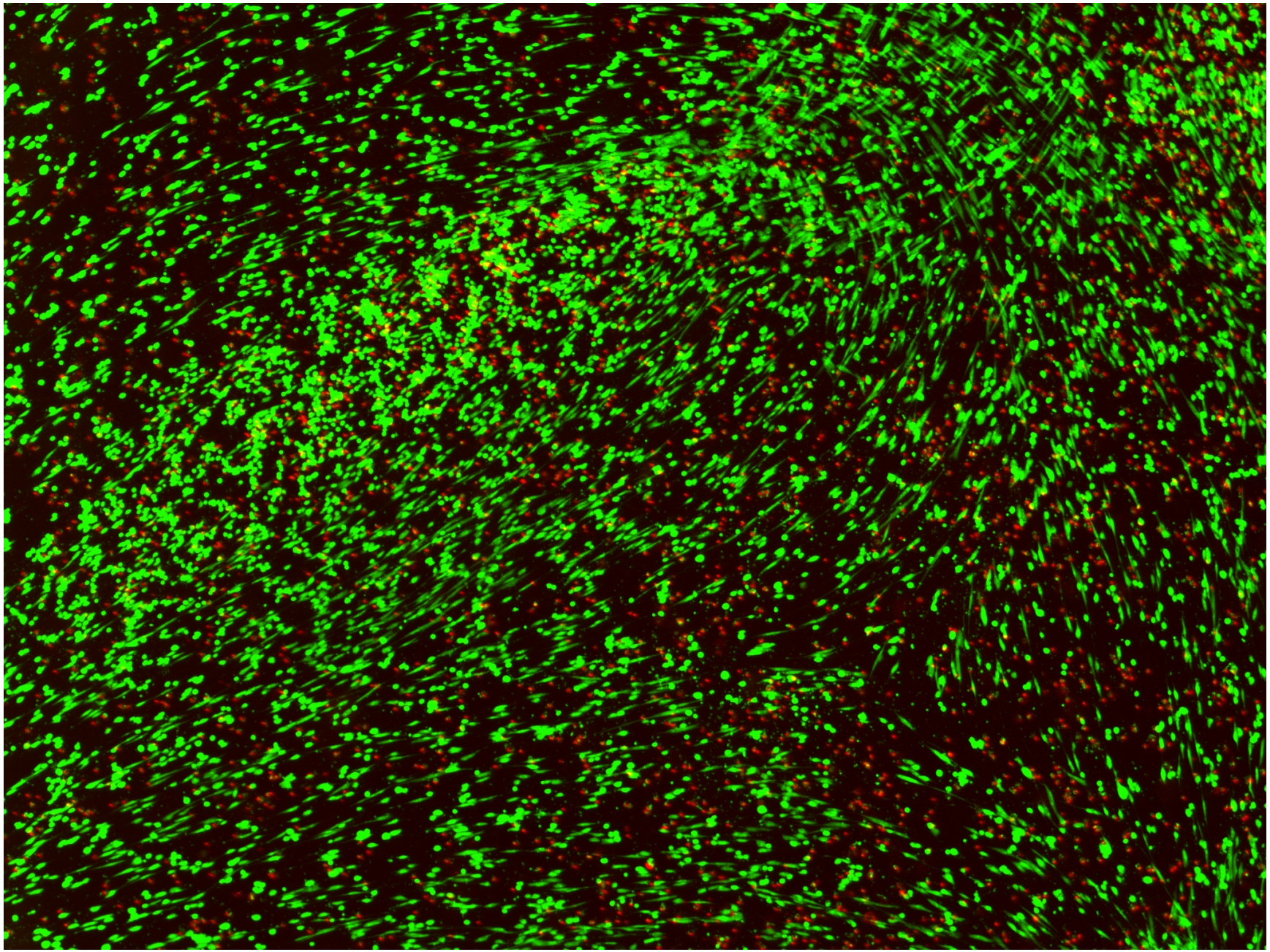

Figure 3: Image of single well, Live/Dead assay with NBs

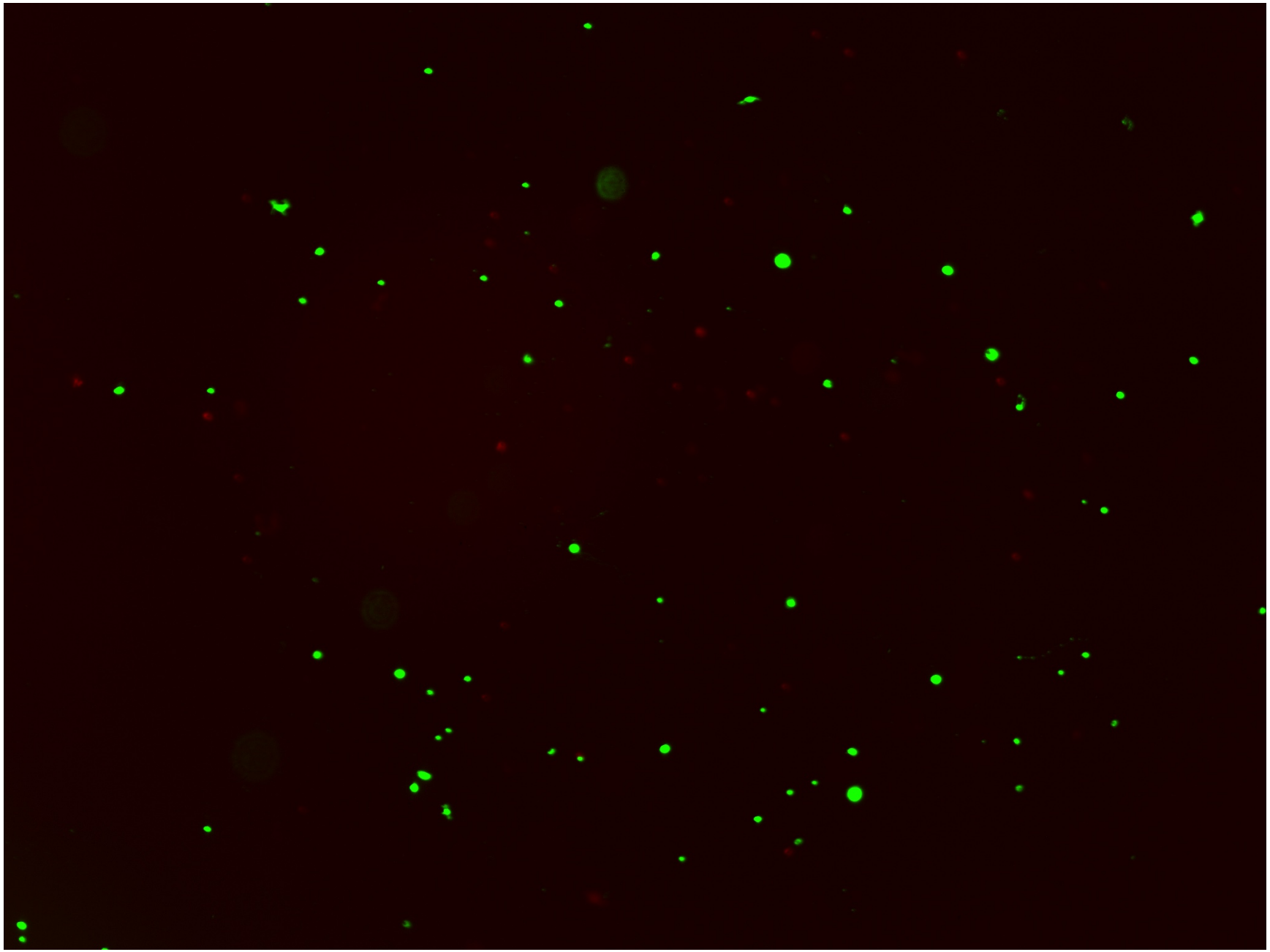

Figure 4: Image of single well, Live/Dead assay with MPNs

IV. *In Vitro* Results

The *in vitro* trials carried out in this study utilized the MTT and Live/Dead™ assays to quantify the dose-response toxicity of the four experimental sealers in three different cell types. hDPSC, NB, and MPN models all proved feasible ways to test toxicity *in vitro.* The hypothesis tested in this part of the study was that bioceramic sealer would show the least toxicity *in vitro* of the four sealers tested.

Statistically Significant Concentrations

Utilizing statistical significance as the threshold for toxic concentrations, resin sealer is the most toxic in all three cell types (Table 3). Variability occurs for the second most toxic sealer when comparing each cell type. For hDPSCs, bioceramic and ZOE have the same lowest significant concentration at 0.120 g/ml. For NBs, bioceramic is more toxic than ZOE by one concentration. For MPNs, ZOE is more toxic by two concentrations. GuttaFLow 2 shows no significant difference from the control at any concentration in any cell type.

When comparing cell types, it would appear as though MPNs exhibit a higher tolerance of sealer toxicity, as the highest significant concentrations of the first three sealers is greater than for both hDPSCs and NBs.

For many concentrations, there is equal significance against the control for each experimental group. To distinguish between the experimental groups themselves, a significance table (Table 4) for a fixed concentration of 0.120 g/ml for hDPSCs and MPNs was tabulated. This data shows that GuttaFlow 2 is significantly different from all the other sealer's toxicities, whereas the toxicity of the remaining three sealers is not significantly different from each other.

Dose-Response Curve and EC_{50}

Fixed-interval concentrations may not differentiate exact limits of toxicity, but utilizing data analysis software to create a 'best fit' curve allows the trend of dose-response to be visualized more effectively. Figures 5 and 6 are the dose-response curves for hDPSCs and NBs, respectively. These curves show similar trends with resin the most toxic, bioceramic and ZOE following, and GuttaFlow 2 showing no toxicity. Figure 7 is the dose-response curve for MPNs, and demonstrates a less clear differentiation between the three most toxic sealers. These curves show that between models though trends are similar there is some variation in results. Differences in the y-axis between the hDPSCs/NBs and MPNs may explain some

Table 3: Significance level per concentration of sealer compared to control

hDPSC				
Concentration, g/ml	Bioceramic	Resin	ZOE	GuttaFlow 2
0.324	****	****	****	ns
0.263	****	****	****	ns
0.202	****	****	****	ns
0.155	****	****	****	ns
0.120	****	****	****	ns
0.092	ns	****	ns	ns
0.071	ns	****	ns	ns
0.054	ns	****	ns	ns
0.042	ns	***	ns	ns
NBs				
	Bioceramic	Resin	ZOE	GuttaFlow 2
0.324	****	****	****	ns
0.263	****	****	****	ns
0.202	****	****	****	ns
0.155	****	****	****	ns
0.120	****	****	****	ns
0.092	****	****	ns	ns
0.071	ns	****	ns	ns
0.054	ns	****	ns	ns
0.042	ns	***	ns	ns
MPNs				
	Bioceramic	Resin	ZOE	GuttaFlow 2
0.324	**	****	****	ns
0.263	**	****	****	ns
0.202	**	****	****	ns
0.155	*	**	***	ns
0.120	ns	*	**	ns
0.092	ns	*	**	ns
0.071	ns	*	ns	ns
0.054	ns	ns	ns	ns
0.042	ns	ns	ns	ns

of the variation, as well as the smaller sample sizes for the MPN model

The EC_{50} values provide perhaps the best way to interpret toxicity from the data collected in these trials. As Table 5 shows, EC_{50} values for hDPSCs and NBs are very similar. Resin has the lowest value (i.e., most toxic) in these two cells types, followed by similar values for bioceramic and ZOE. GuttaFlow 2 has no EC_{50} value as it is no different from the control. As seen in the other data, MPNs exhibit a slightly different trend, with ZOE having the lowest value followed by similar resin and bioceramic values.

Though data is collected from different cell types by different assays, its trends in these *in vitro* trials seem apparent: resin appears to be the most toxic, followed closely by both bioceramic and ZOE. Some debate would exist whether bioceramic is more toxic than ZOE or vice-versa, but further trials with smaller concentrations differences would likely be necessary to distinguish the two. One trend that has appeared consistently across all trials is that GuttaFlow 2 shows no measurable toxicity compared to the control groups.

Table 4: Statistical Comparison Between Experimental Groups at 0.12 g/ml

hDPSCs				
	BC	Resin	ZOE	GF2
BC		ns	ns	****
Resin	ns		ns	****
ZOE	ns	ns		****
GF2	****	****	****	
NBs				
	BC	Resin	ZOE	GF2
BC		****	*	****
Resin	****		****	****
ZOE	*	****		****
GF2	****	****	****	

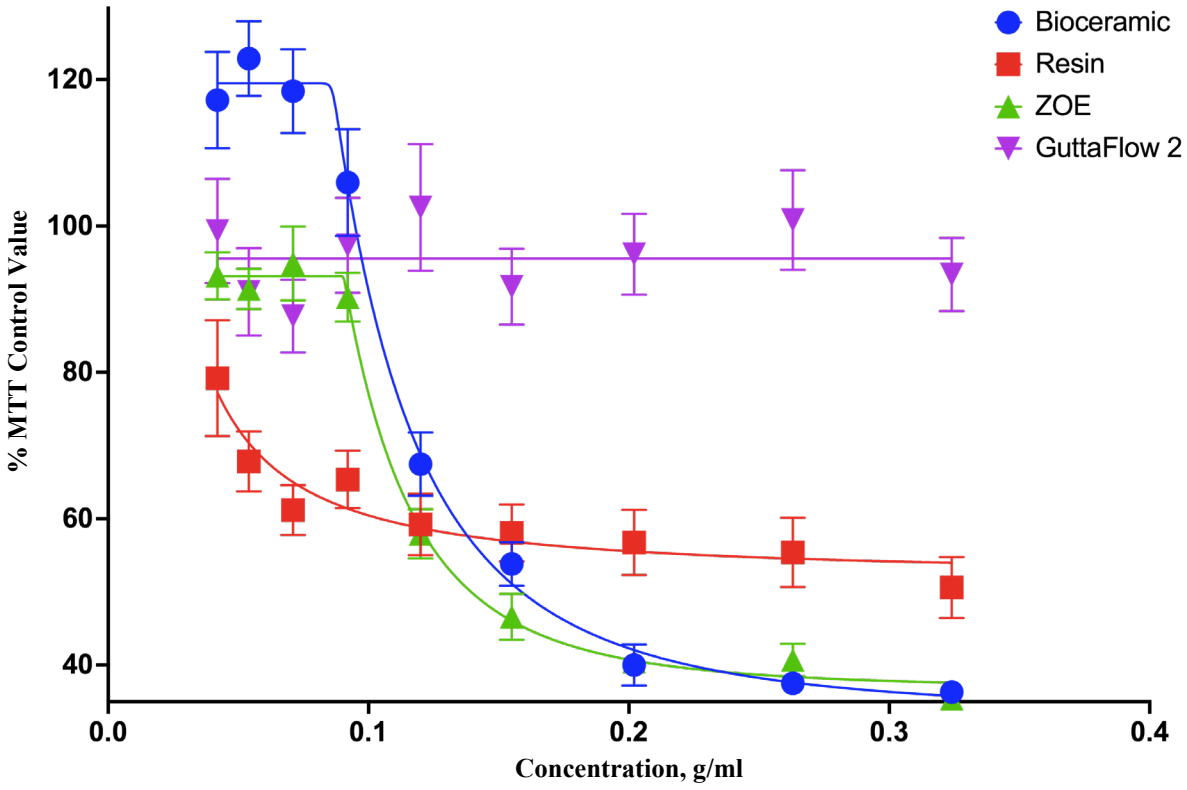

Figure 5: Dose-response curve of hDPSCs to endodontic sealers

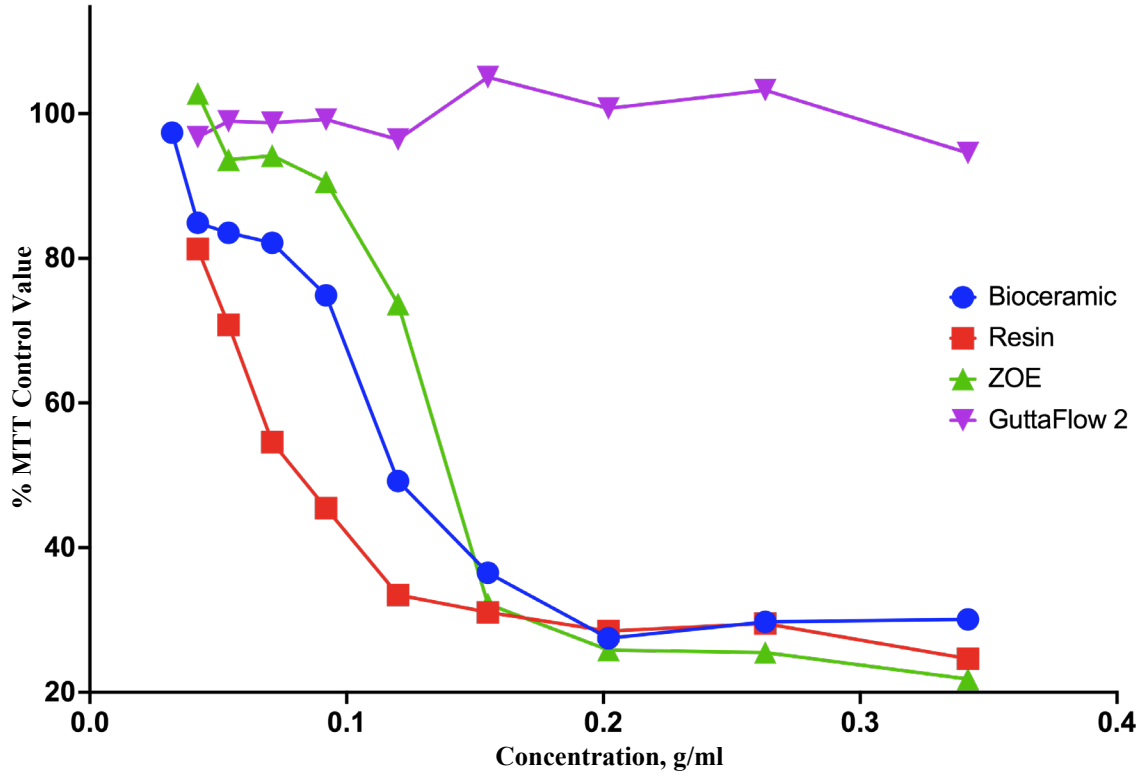

Figure 6: Dose-response of NBs to endodontic sealers

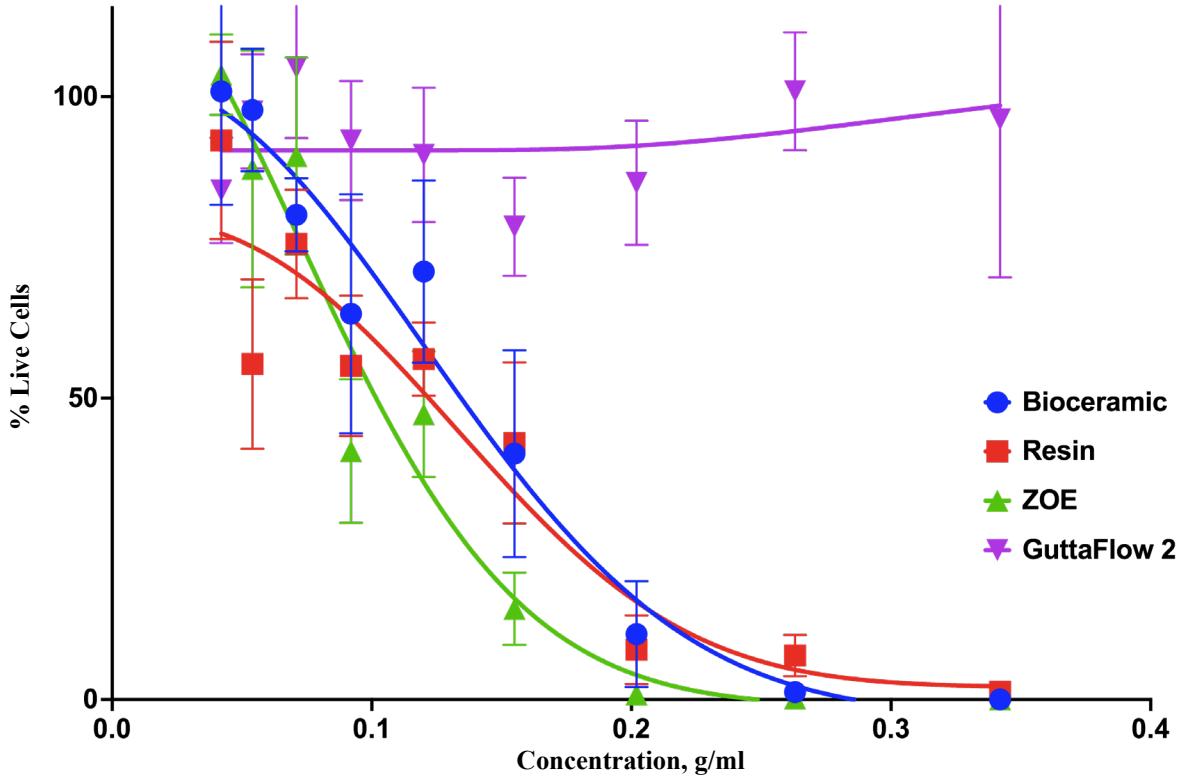

Figure 7: Dose-response of MPNs to endodontic sealers

Table 5: Statistical Summary for *In Vitro* Data												
	DPSC				**NBs**				**MPNs**			
	BC	Resin	ZOE	GF2	BC	Resin	ZOE	GF2	BC	Resin	ZOE	GF2
Test Type	MTT	MTT	MTT	MTT	MTT	MTT	MTT	MTT	L/D	L/D	L/D	L/D
Replicates	34	34	34	34	70	44	44	44	6	6	6	6
Controls per Concentration	3	3	3	3	3	3	3	3	1	1	1	1
EC50	**0.1116**	**0.0469**	**0.1112**	**N/A**	**0.112**	**0.0666**	**0.1301**	**N/A**	**0.1337**	**0.1407**	**0.0894**	**N/A**
Lowest Significant Concentration (g/ml)	0.120	0.088	0.120	N/A	0.092	0.042	0.120	N/A	0.155	0.071	0.092	N/A
P Value	0.0001	<0.0001	0.0052	>0.99	<0.0001	0.0006	0.0052	>0.91	0.0484	0.0327	0.0051	>0.6115

V. *In Vivo* Materials and Methods

Experimental Design
This study was designed to test the four experimental sealers *in vivo* utilizing a total of 25 mice. The method of exposure was direct placement of freshly mixed sealer onto the mental nerve with routine wound closure. Mice were sacrificed 72 hours after surgery for collection of ipsilateral mental nerve and trigeminal ganglia tissue. RNA extracted from the tissue was analyzed using RTqPCR using 84-gene screening arrays for inflammation and neurotoxicity to produce quantifiable gene expression for both experimental and control groups.

Murine Survival Surgery
Adult female mice were housed in a temperature-controlled environment with a 12-h light cycle, and given a standard diet and water ad libitum. Experiments were approved by the Harvard Medical Area Institutional Animal Care and Use Committee (HMA IACUC). Mice were divided into six groups: Bioceramic (n=4), Resin (n=4), ZOE (n=4), GuttaFlow 2 (n=4), Positive Control (mental nerve transection, n=4), and Control (mental nerve exposure, n=4). Surgical intervention was performed on the left mental nerve only.

Mice were anesthetized with isofluorane (3% for induction and 1.5% for maintenance) until unresponsive to toe pinch, and anesthesia was maintained during surgery. The surgical exposure site of the murine mental nerve was cleared of fur using potassium hydroxide [Nair] and decontaminated with alcohol wipes. A six- to eight-millimeter incision was made and blunt dissection was performed to expose the mental nerve with as little disruption of surrounding tissue as possible. For animals in the experimental groups, 20 microliters of sealer was placed over the exposed mental nerve; for the positive control group the mental nerve was cut, or 'transected'; and for the negative control group the mental nerve was exposed but no other alterations were performed in the area. Immediately following exposure, all tissue was re-approximated with 5.0 silk sutures and cyanoacrylate. Buprenorphine SR was administered intramuscularly for pain, and each animal was allowed post-surgical recovery alone on a heating pad. Once full anesthetic recovery was achieved, animals were returned to their respective cages.

Tissue Collection
Animals were allowed normal function for 72 hours post-surgery with daily monitoring for signs of pain or behavioral changes. Following this period, animals were euthanized with carbon dioxide and cervical dislocation. (Figure 8 displays photographs of the surgical sight in each group.) Left trigeminal ganglia and mental nerves were carefully dissected and biopsied to produce two tissue samples from each animal. Collected tissue was immediately frozen in liquid nitrogen prior to RNA extraction.

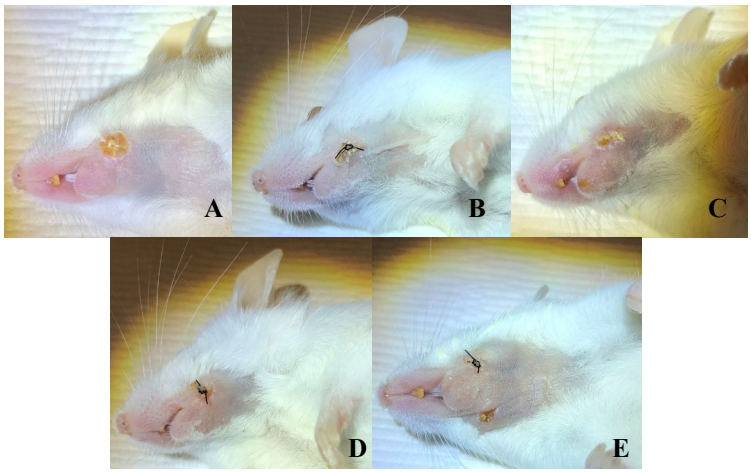

Figure 8: 72 hour post-surgery wound healing. A. Resin B. Bioceramic, C. ZOE, D. GuttaFlow 2, E. Transection/Control

Gene Expression Analysis
RNA from each sample was prepared and gDNA was eliminated using the RN Easy mini kit (Qiagen). 1[ug] total RNA was used to prepare cDNA with the appropriate first strand kit from SABiosciences. The cDNA was characterized on the StepOnePlus™ Real-Time PCR system in a 96-well format using two different RT^2 Profiler PCR arrays: the Mouse Neurotoxicity RT^2 Profile PCR array system for left trigeminal ganglia samples, and the Mouse Inflammatory Response & Autoimmunity RT^2 Profile PCR array system for left mental nerve samples (Qiagen). Each array contained a panel of primers for 84 key genes involved in drug and chemical-induced neurotoxic responses and inflammatory immune responses, respectively. The resulting raw data was then analyzed using the GeneGlobe RT^2 Profiler PCR Data Analysis software (Qiagen). Relative gene expression was determined using the $\Delta\Delta C_T$ method normalized to four housekeeping genes preset within each array. Fold-change [6] and fold-regulation [7] in gene expression between the experimental groups and control were also calculated using the $\Delta\Delta C_T$ method.

Statistical Considerations
Statistical calculations were performed using a Student *t* test in Graphpad Prism version 9.1.0. *P* values were calculated based on a Student's t-test of the normalized

[6] Fold-change is the normalized gene expression in the test sample divided by the normalized gene expression in the control sample.
[7] Fold-Regulation is the negative inverse of the fold-change and values greater than one indicate a positive up-regulation in gene expression.

gene expression $2^{(-\Delta C_T)}$ replicate values for each gene in the treatment and control groups, and $P < 0.05$ was considered statistically significant.

VI. *In Vivo* Results

Using the Mouse Inflammatory Response & Autoimmunity and Mouse Neurotoxicity RT^2 Profiler PCR Array Systems, this section examined the immune response of four endodontic sealers placed on mental nerves and their neurotoxic effects projected to the trigeminal ganglia. Based on the *in vitro* results of Part 1, this study hypothesized that the *in vivo* gene expression profile of inflammatory and neurotoxic genes would be greatest with resin sealer, followed by bioceramic and ZOE sealers, and least with GuttaFlow 2.

Gene Expression: Inflammatory Response & Autoimmunity in the Mental Nerve

Visualization of C_T values[8] in Figure 9 illustrates that the resin sealer experimental group exhibited the most expression of the 84 genes. The bioceramic and ZOE experimental groups appeared to show identical expression between themselves, but overall less expression than resin. The transection control group falls between the bioceramic/ZOE and GuttaFlow 2 groups. GuttaFlow 2 resulted in less expression of the 84 genes overall relative to the other three experimental groups.

Scatter plots (Figure 11) of each experimental group identify up-regulated genes above the top line in yellow that meet a fold-change threshold of 2.0 or greater. The Resin group demonstrates the greatest amount of these genes (n=56), followed by ZOE (n=52), Bioceramic (n=51), and GuttaFlow 2 (n=23). Genes that fall below the bottom line represent down-regulation at a fold-change of 2.0 or greater, and the most down regulation occurs with GuttaFlow 2 (n=35), followed by Bioceramic (n=14), ZOE (n=12), and Resin (n=12).

Volcano plots (Figure 12) are similar to the scatter plots in that they identify 2-fold up-regulated genes in yellow, but genes above the horizontal line represent genes that have a statistically significant ($p<0.05$) up-regulation compared to the control group. The Resin group contains the highest number of statistically significant up-regulated genes (n=25), followed by ZOE (n=22), Bioceramic (n=15), and GuttaFlow 2 (n=5). Significant down-regulated genes are Bioceramic (n=1), Resin (n=0), ZOE (n=0), and GuttaFlow 2 (n=0). This data is summarized in Tables 6 and 7.

Gene Expression: Neurotoxicity in the Trigeminal Ganglia

Compared with Figure 9, neurotoxic gene expression in Figure 10 is not as clearly demarcated nor does it have the same trends previously seen. ZOE and Resin appear to have similar neurotoxic expressions to each other, whereas Bioceramic and GuttaFlow 2 have similar expressions but slightly less than the other two groups.

Scatter plots (Figure 13) of each experimental group identify up-regulated genes above the top line in yellow that meet a fold-change threshold of 2.0 or greater. The Resin group demonstrates the greatest amount of these genes (n=56), followed by ZOE (n=52), Bioceramic (n=51), and GuttaFlow 2 (n=23). Genes that fall below the bottom line represent down-regulation at a fold-change of 2.0 or greater, and the most down regulation occurs with GuttaFlow 2 (n=35), followed by Bioceramic (n=14), ZOE (n=12), and Resin (n=12).

Volcano plots (Figure 14) are similar to the scatter plots in that they identify 2-fold up-regulated genes in yellow, but genes above the horizontal line represent genes that have a statistically significant ($p<0.05$) up-regulation compared to the control group. The Resin group contains the highest number of statistically significant up-regulated genes (n=25), followed by ZOE (n=22), Bioceramic (n=15), and GuttaFlow 2 (n=5). Significant down-regulated genes are Bioceramic (n=1), Resin (n=0), ZOE (n=0), and GuttaFlow 2 (n=0). This data is summarized in Table 6.

Tracking Specific Genes

In order to explore the possibility of certain pathways of inflammation and neurotoxicity, and to more easily follow a subset of the data, select genes that were both of interest (i.e., knowingly should up-regulate) and showed significant variance from the control were identified and tracked (specifics of each gene are discussed in the conclusion). Figure 17 is a fold regulation chart for 6 selected inflammatory genes, and shows that in all these genes resin resulted in the most up-regulation when compared to the control, followed by a close grouping of ZOE, bioceramic, and GuttaFlow 2, except for Il1r1, Tlr2 and Tlr 9, which actually showed a down-regulation for the transection group and GuttaFlow 2. Figure 18 is the scatter plot of these genes, and most noted is the scattered distribution of significance. Figure 16 is the identical chart for 6 neurotoxicity genes, and shows that ZOE caused the most up-regulation followed by another similar grouping of resin, bioceramic, and GuttaFlow 2. Only the ligation group showed down-regulation in Hspa5 and Ldha. Figure

[8] The cycle threshold (C_T) value is the cycle number when the fluorescence of a PCR product can be detected above the background signal. Lower C_T values mean more PCR product present, and thus higher gene expression. Values are first normalized to their respective housekeeping genes (Actin-beta, Beta-2 microglobulin, Gluceraldehyde-3-phosphate dehydrogenase, and Heat shock protein 90 alpha class B member 1) and then inverted and scaled to a y-axis of 100 so that greater gene expression is represented higher on the y-axis.

15 is the volcano plot for these genes, and unlike the volcano plot for the inflammatory genes this plot shows a clustering of genes with similar significance values.

Table 6: Number of expressed inflammation genes for each experimental group

INFLAMMATION	Resin	ZOE	Bioceramic	GuttaFlow 2
Total Up-Regulated Genes	**56**	**52**	**51**	**23**
Total Up-Regulated Genes, $p < 0.05$	**25**	**22**	**15**	**5**
Total Down-Regulated Genes	**12**	**12**	**14**	**35**
Total Down-Regulated Genes, $p < 0.05$	**0**	**0**	**1**	**0**

Table 7: Number of expressed neurotoxicity genes for each experimental group

NEUROTOXICITY	Resin	ZOE	Bioceramic	GuttaFlow 2
Total Up-Regulated Genes	**45**	**48**	**36**	**25**
Total Up-Regulated Genes, $p < 0.05$	**21**	**23**	**16**	**13**
Total Down-Regulated Genes	**19**	**15**	**20**	**26**
Total Down-Regulated Genes, $p < 0.05$	**0**	**0**	**1**	**2**

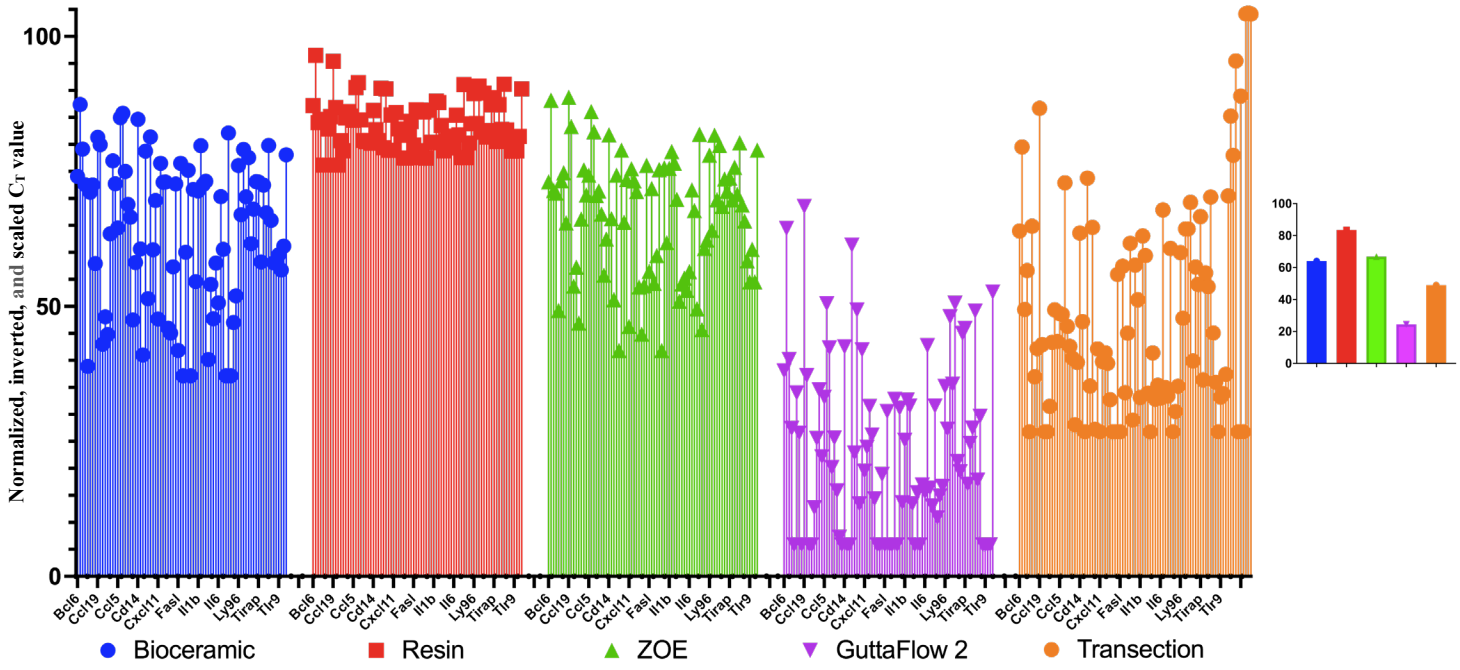

Figure 9: Total gene expression for the Mouse Inflammatory Response & Autoimmunity RT^2 Profile PCR Array. (Note: Only 11 genes are labeled of the 84 in each set.)

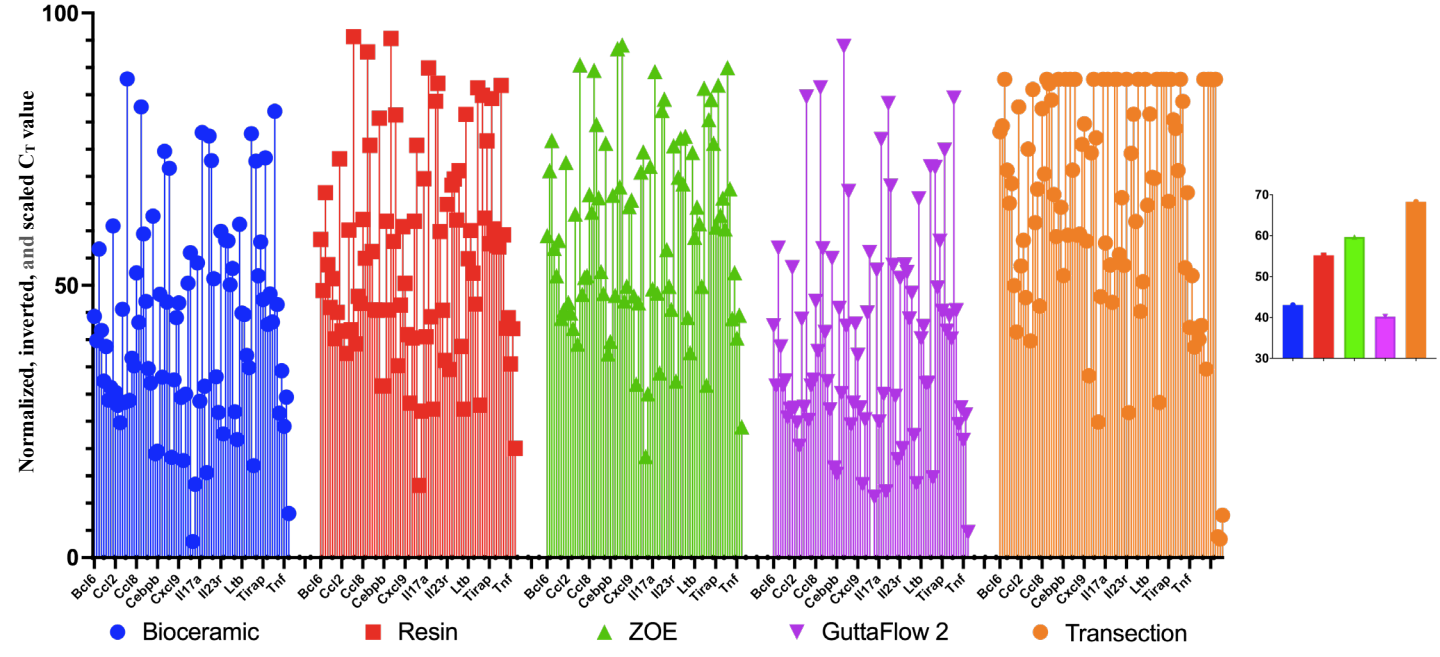

Figure 10: Gene expression in treatment groups for the Mouse Neurotoxicity RT^2 Profile PCR Array.

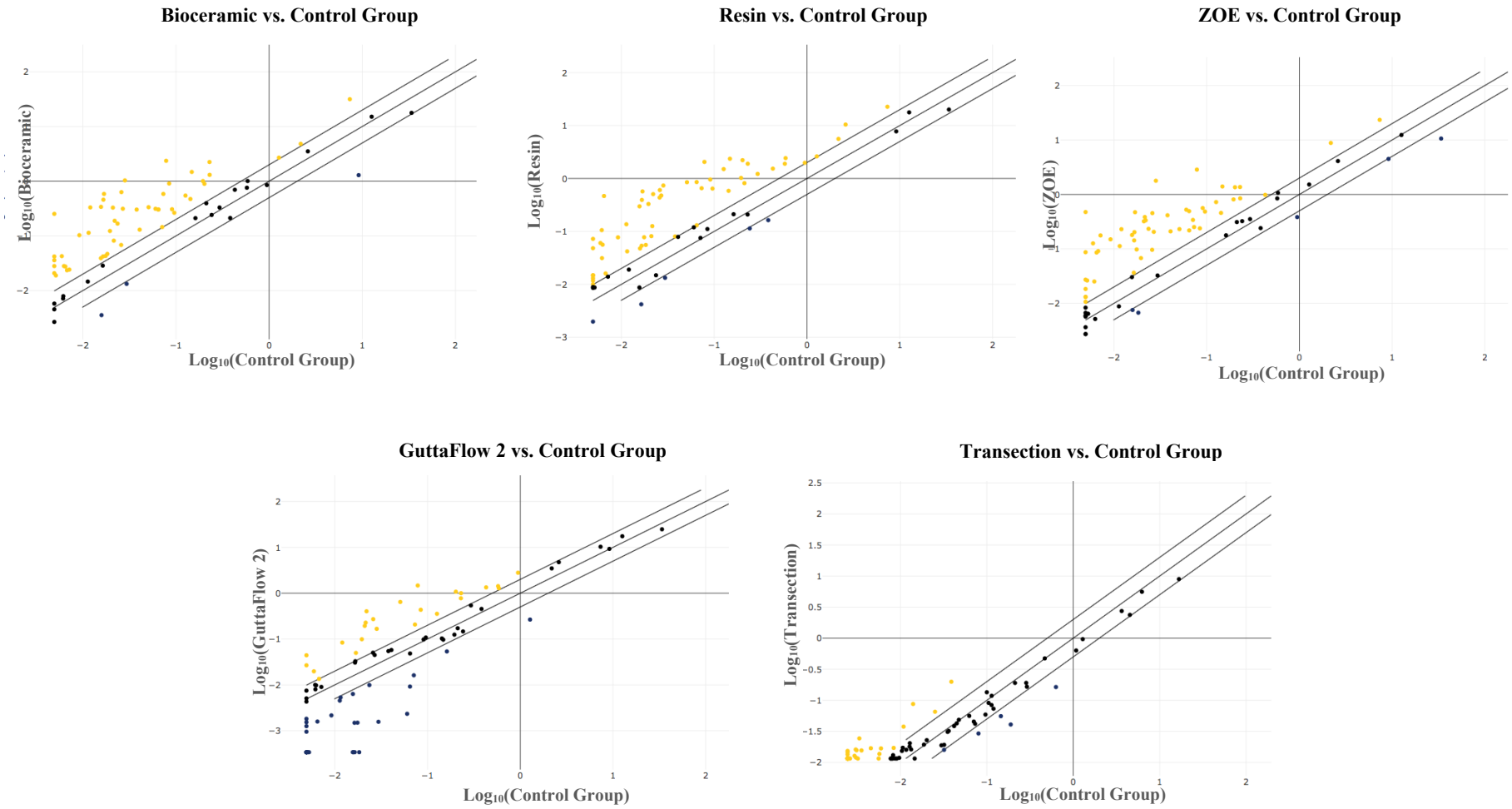

Figure 11: Scatter plots representing gene expression in experimental sealer groups versus control for the Mouse Inflammatory Response & Autoimmunity RT^2 Profile PCR array

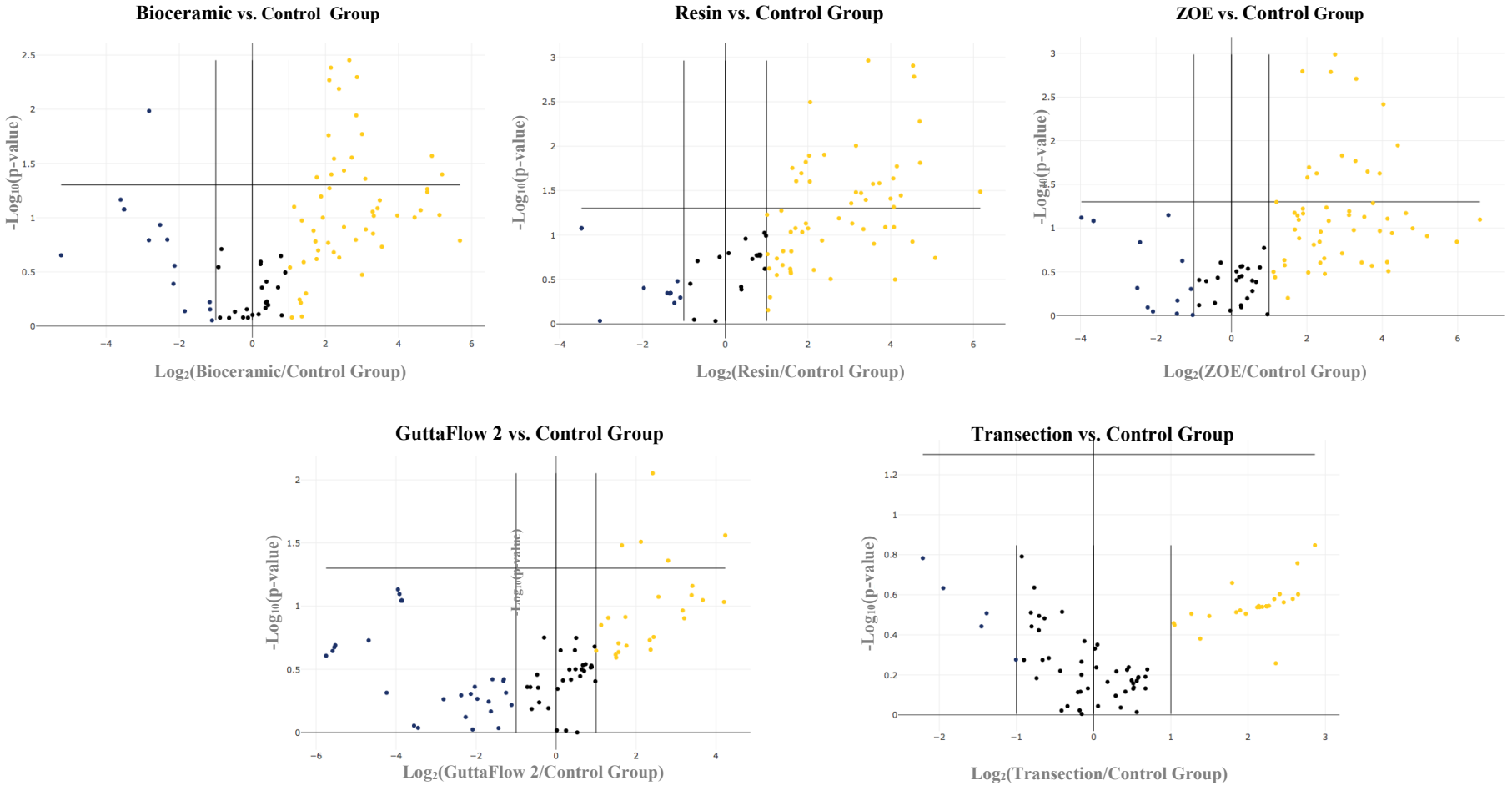

Figure 12: Volcano Plots representing gene expression in experimental sealer groups versus control for the Mouse Inflammatory Response & Autoimmunity RT^2 Profile PCR array

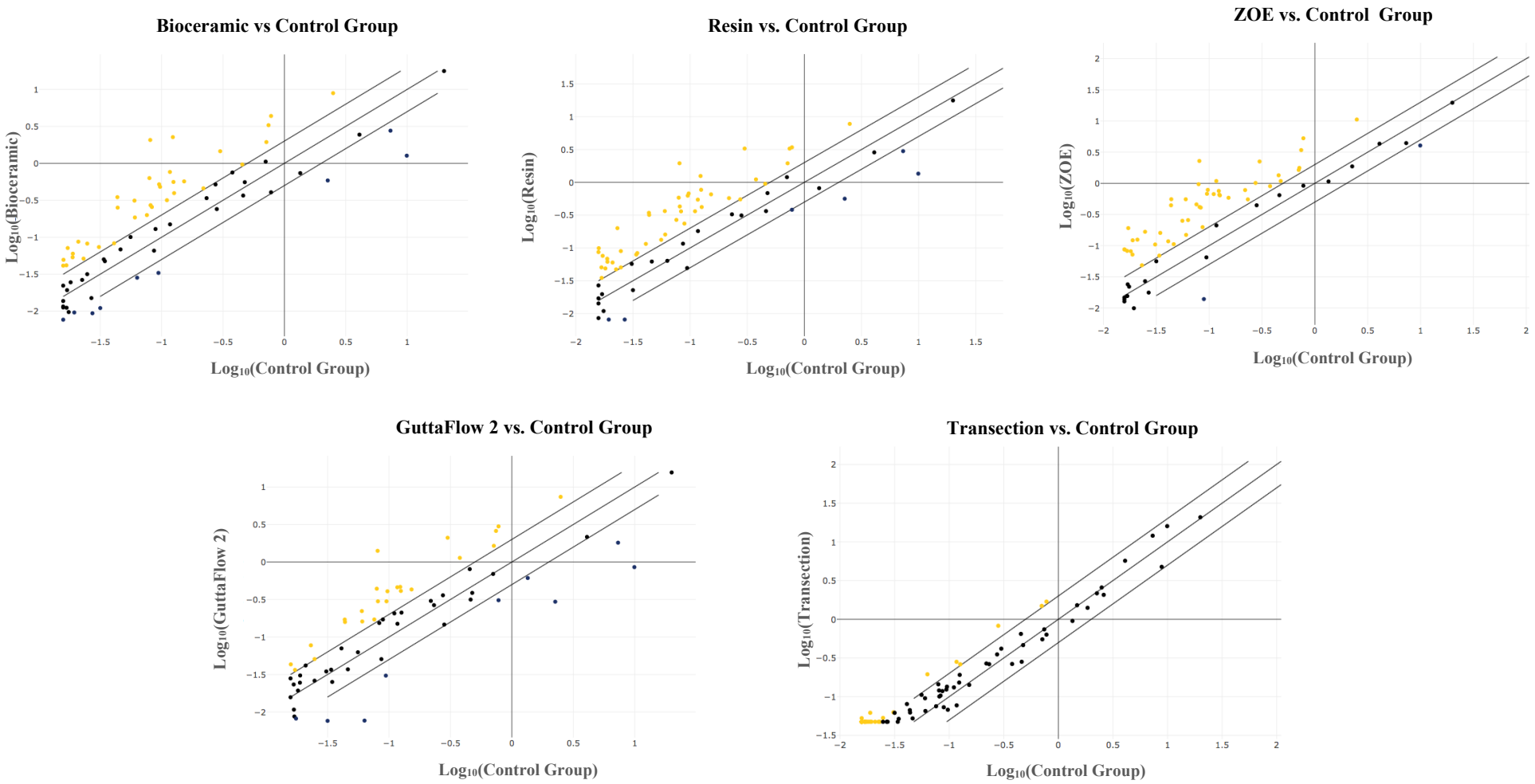

Figure 13: Scatter plots representing gene expression in experimental sealer groups versus control for the Mouse Neurotoxicity RT^2 Profile PCR array

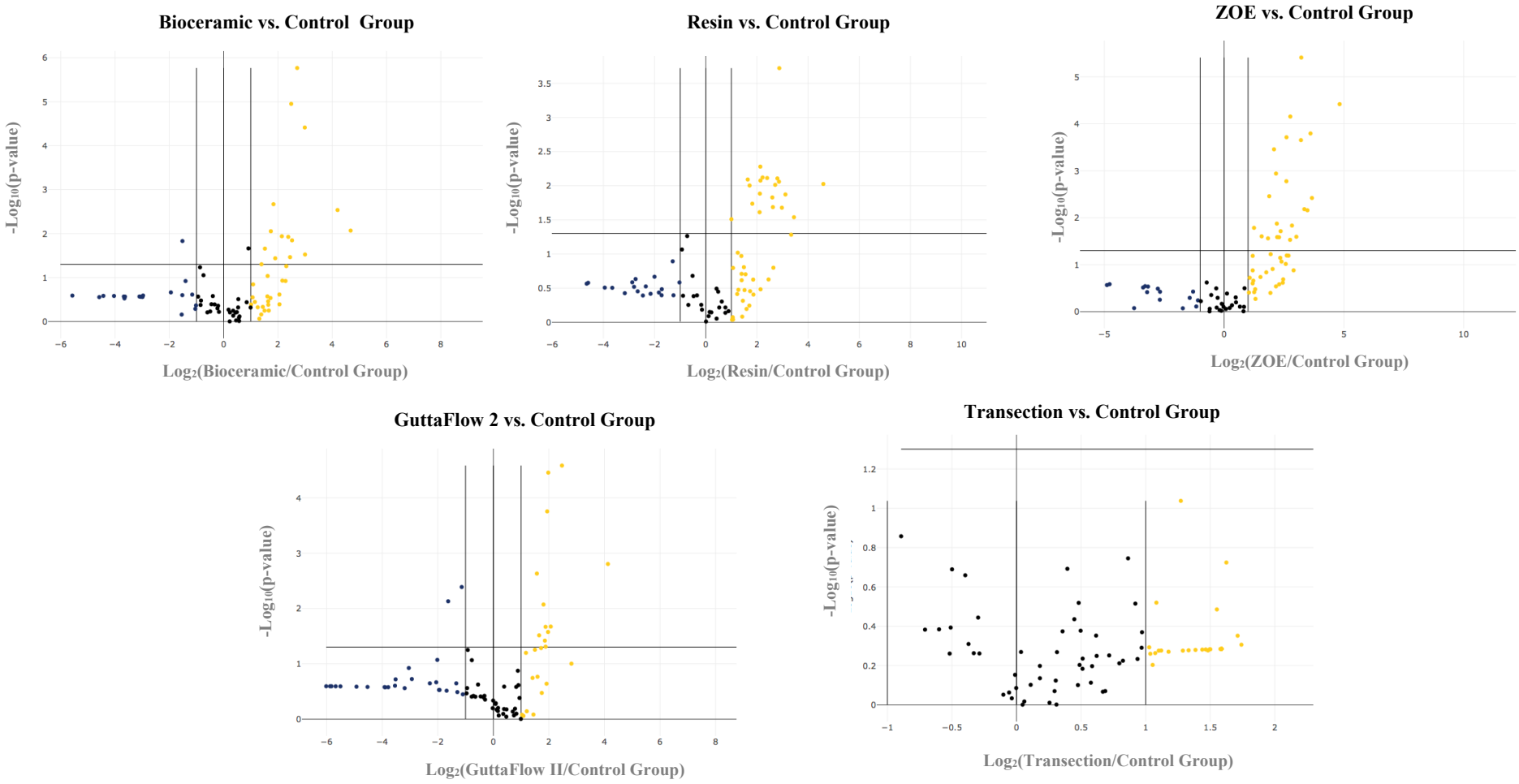

Figure 14: Volcano Plots representing gene expression in experimental sealer groups versus control for the Mouse Neurotoxicity RT^2 Profile PCR array

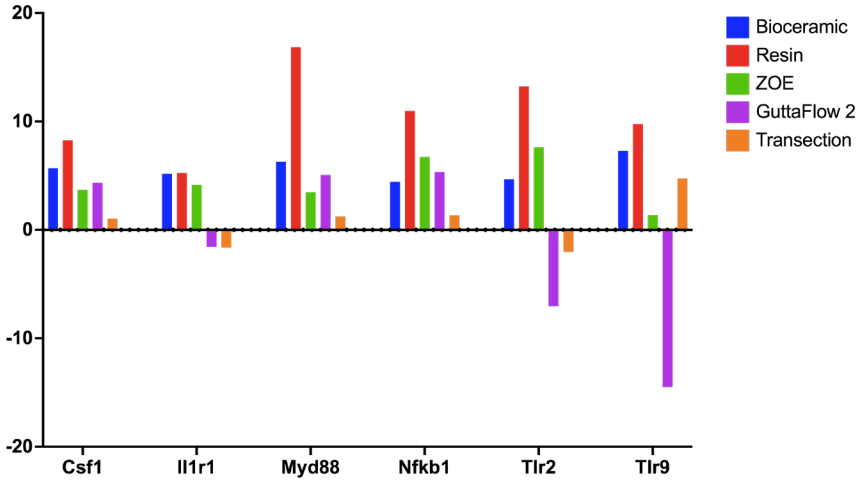

Figure 17: Fold regulation of selected inflammatory biomarkers

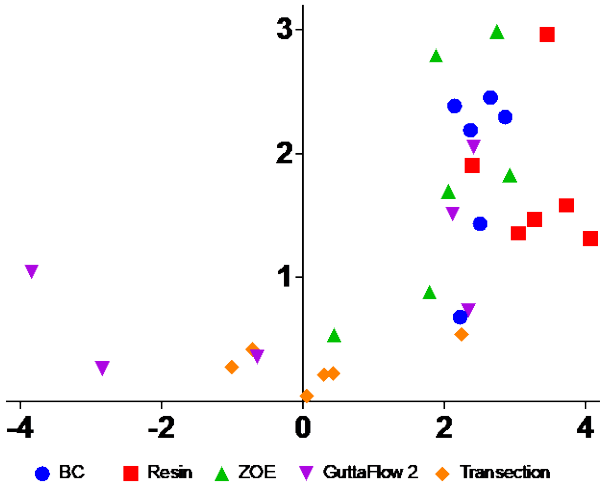

Figure 18: Volcano plot for selected inflammatory biomarkers

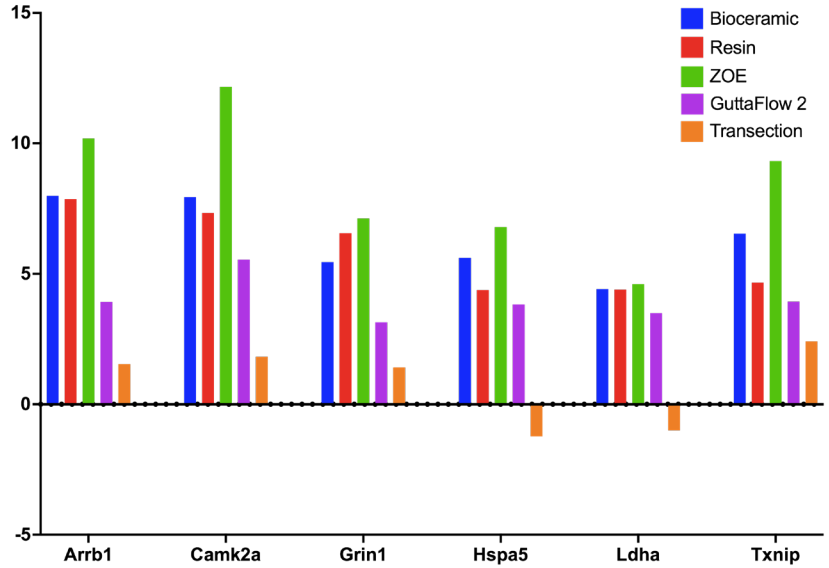

Figure 16: Fold regulation of selected neurotoxicity biomarkers

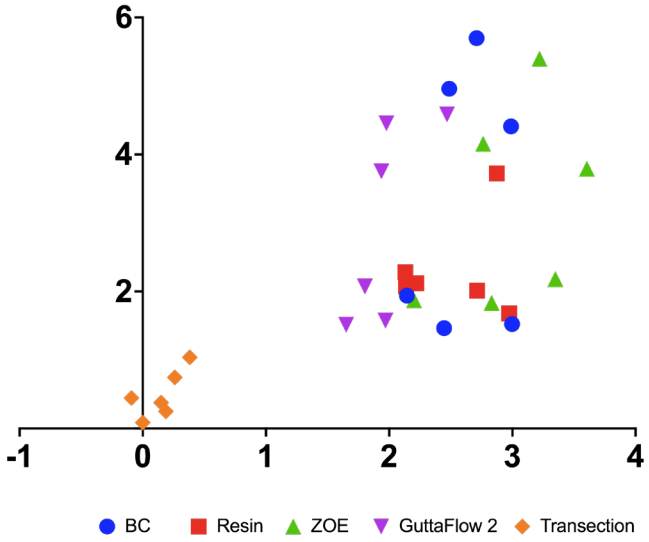

Figure 15: Volcano plot for selected neurotoxicity biomarkers

VII. Conclusions

This study has investigated the toxicities of four endodontic sealers using both *in vitro* and *in vivo* models, determining overall that AH Plus resin sealer is the most toxic and that GuttaFlow 2 silicone sealer is the least toxic. Endosequence BC sealer, which shows bioactivity in many other studies and was hypothesized here to be the least toxic, did not perform as expected, showing a similar toxicity to Kerr Dental Pulp zinc oxide-eugenol sealer, resulting in a rejection of the original hypothesis.

***In vitro* versus *in vivo* models**

The *in vitro* model utilized three cell types and direct exposure of purified cell lines to freshly mixed sealer, allowing consistent reproducibility of results. This model is inexpensive to run, can have a high number of replicate samples simultaneously, and fault at any point does not result in catastrophic failure down the line. Moreover, the simplicity of this model removes many of the confounding factors associated with more complex models.

The *in vivo* model is one of these more complex models, giving a larger picture of the interactions of materials and tissues within a functioning mammalian system. This model is expensive and time consuming, requires more oversight approvals, and relies on a series of stages each dependent on the previous. The compensating advantage, however, is that it gives a more realistic picture of what happens clinically, despite the fact that results are harder to interpret and causality harder to distinguish.

Both models in this study were in general agreement with each other, showing validation of the *in vitro* model with the *in vivo* model. All results showed that resin, bioceramic, and ZOE were far more toxic than GuttaFlow 2, although some minor disagreement existed ranking the toxicity of the former three. It is debatable whether or not one model's results are more powerful than the other, but both models show benefit in this kind of study. In future studies, the *in vitro* model would likely be more useful looking at individual components of sealer, whereas the *in vivo* model would be more useful when looking at specific pain pathways.

Modeling Limitations

Comparison of the three cells types used in the *in vivo* models shows almost identical toxicities when measured by the EC_{50}. Toxicities based on statistical difference from the control did result in some variation among bioceramic and ZOE sealers, which can be explained by the limits set by the tested serial concentration amounts. To further dichotomize the exact toxic levels of these sealers on cells, it would be necessary to hone-in more to the critical range where sealer toxicity becomes statistically significant.

Though this study was focused on neurotoxicity, the use of hDPSCs proved valuable as a validation of MPN and NB results, and opens up the possibility that they are just as reliable as the other two cell types. The use of NBs is limited because these cells are not true neurons but stem cells differentiated towards neurons; however, in a research setting these cells are more practical than *ex vivo* collection of neurons. MPNs, as actual peripheral neurons, have their own set of complications that precludes their widespread use in *in vivo* modeling, such as their short life span, lack of proliferation, and low harvesting quantity.

Results interpretation aside, the *in vivo* model produced a phenomenal amount of data that creates more questions than answers. However, these questions are the start of a foundation to interpret many of the unknowns about pain signaling pathways in future studies.

***In vitro* Interpretations**

Data from the three cells types makes a clear distinction that resin is the most toxic and GuttaFlow 2 is the least. However, there is variation in which is more toxic between bioceramic and ZOE. ZOE is more toxic in MPNs, and bioceramic is more toxic in NBs. For hDPSCs, the two are near equal in toxicity. Comparing them statistically, there is no difference in toxicities at the tested concentrations, but there is also no significant statistical difference between them and resin, either. These variations could be explained by certain cell types being more susceptible to different components in either bioceramic or ZOE sealers. For bioceramic, it may be related to changes in pH induced by calcium hydroxide release. For ZOE, it may be eugenol. Component toxicity, however, is beyond the scope of this study. For variations seen in the MPN cell data, the small sample size for each group may be the contributing factor in incomplete determination of trends and variation from hDPSCs and NBs.

Comparisons with other Studies

Cytotoxicity

Resin sealers, with their limited biocompatibility and potential to induce heavy immune responses, have been the gold standard for cytotoxicity in endodontic sealer research.[20, 31] Particularly toxic before fully setting, resins contain residual monomers (and formaldehyde in some) that are well established as cyto- and genotoxic in mammalian cells.[32] AH Plus and ZOE cytotoxicity on human gingival fibroblasts has been shown to appear as early as one hour after placement and last up to five

weeks, hinting at the temporal effects of sealer toxicity.[33, 34] Of sealers tested in these studies, resin nearly always ranks as the most cytotoxic, which is in agreement with the findings of the present study.

Biocompatability and toxicity of bioceramic sealers has been shown in various studies to be both beneficial and harmful.[11] *In vitro* studies on mouse and human osteoblast cells, human stem cells of the apical papilla, and human periodontal ligament cells all show that bioceramic sealers are highly biocompatible and bioactive.[21, 22, 35-38] Compared to AH Plus, the dose-dependent cytotoxicity of BC sealer was found to be less with serial dilutions of freshly mixed sealers were placed in contact with human bone marrow mesenchymal stem cells.[39] However, it was also found that bioceramic sealer remained toxic over 6 weeks (taking 168 hours to set) compared to AH Plus, whose cytoxocitity was high initially but gradually decreased to a non-toxic state.[22] When compared to ZOE, bioceramic sealer show less toxic effects on hPDL cells and were more bioactive.[40] In rats, bioceramics were cytotoxic to pulp tissue *in vivo*, and caused moderate inflammation subcutaneously.[41, 42] However, BC sealer has been shown to decrease LPS-mediated inflammation *in* vitro.[43]

Studies performed in the 1980's and 1990's established that ZOE is cytotoxic and induces an inflammatory response.[44, 45] When placed in rat connective tissue, ZOE induced irritation and an immune response that showed that its toxicity decreased with time over 42 days.[46] Compared to bioceramics, the eugenol in ZOE was shown to inhibit the adhesion of immune cells and be more cytotoxic to human periodontal ligament.[47] In 1992, freshly mixed ZOE sealer implanted into the mandibles of rabbits showed that tissue reactions improved from 4 to 12 weeks after implantation.[48] ZOE and resin sealers were implanted into the dorsal regions of rats and the subsequent inflammatory response was measured after 15, 30, and 60 days showing that the ZOE had a more severe inflammatory response than resin.[49] Pigmentation and tissue response in the gingival sulcus of rabbits injected with resin, ZOE, and calcium-hydroxide was analyzed after 30, 60, and 90 days of exposure to show that tissue response varied greatly, and that calcium hydroxide-containing sealer actually enhanced healing.[50]

Silicone-based sealers have a record of biocompatibility and low toxicity. In human gingival fibroblast suspension cultures, set silicone (Lee Endo-Fill) was found to be significantly less toxic than set AH Plus over a 48-hour time period.[51] The cytotoxicity of GuttaFlow 2 and AH Plus were compared using 3T3 fibroblasts using the MTT, LDH assays, and MMP-2 and MMP-9 zymography, showing that AH Plus was significantly more cytotoxic and that GuttaFlow 2 showed no cytotoxic effects at all.[52] The present study is in agreement with previous studies that GuttaFlow 2 shows minimal toxicity compared to other sealers.

Neurotoxicity

The effects of sealers on neurotoxicity has been examined by testing their effects on rat phrenic nerves *in vitro*.[53] AH 26 and Roth's ZOE sealer have been tested for neurotoxicity by measuring the duration and amplitude of action potentials recorded with an intracellular clamp on the Iranian garden snail.[54]

Several *in vitro* studies using ELISA exist that look at inflammatory biomarkers involved with cytotoxicity, genotoxicity, and neurotoxicity of endodontic sealers.[55] Another study looking at the neurotoxicity of endodontic sealers utilized an intracellular current clamp technique to measure action potentials with AH 26 and Roth's sealers.[54] All of these prior studies find that resin and ZOE sealers are either neurotoxic or affect neuronal functioning, but do not attempt to elucidate mechanism or pathways. This study has added to the body of knowledge quantifiable levels of neurotoxicity for each sealer group, and once again is in agreement that resin, ZOE, and bioceramic sealers are neurotoxic.

Inflammation, Neurotoxicity, and Gene Expression

The inflammation array of the *in vivo model* was used on peripheral mental nerve tissue because it was originally believed that the threshold levels for many of the genes in the neurotoxicity assay would not be met (i.e., not enough RNA in the harvested mental nerve, which is axon and not cell body). Coincidentally, inflammation induced by biomaterials plays a larger role in neurotoxicity and neurodegeneration than originally expected.

Much of what is known about inflammation and neurotoxicity comes from an examination of inflammatory demyelinating diseases of the peripheral and central nervous system.[56] Diabetic neuropathy, for instance, is highly correlated with increased inflammatory biomarker expression, such as C-reactive protein, interleukin (IL)-6, IL-1, soluble intracellular adhesion molecule-1, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)- β.[57-59] Macrophage colony stimulating factor 1 (Csf1) is correlated with multiple sclerosis and myasthenia gravis, and has a suspected role in the activation of microglia.[60, 61] Toll-like receptor (Tlr)-2, Tlr-9, and myeliod differentiation primary response gene 88 (Myd88)—an innate immune adapter protein for toll-like receptor signaling—are correlated with Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and experimental autoimmune encephalomyelitis.[62-64]

Many pharmacological studies exist that relate neurotoxicity biomarkers to compounds that cause

peripheral neuropathies. Beta 1 Arrestin (Arrb1) is shown to be a key biomarker in sevoflurane neurotocity.[65] Calcium/calmodulin-dependent protein kinase II alpha (Camk2a), the target of tissue plasminogen activator used in hemolytic therapy, dysregulates glutamate and calcium signaling in neurons leading to neuron apoptosis.[66, 67] Epilepsy and the transmission of synapse signals is related to the NMDA1 Glutamate Receptor (Grin1).[68] The compound FLZ, a treatment for Parkinson's, lead to the discovery of the neurotoxic biomarker Heat Shock Protein 5 (Hspa5).[69] Degrees of amyloid beta neurotoxicity have been recorded with Lactate Dehydrogenase A (Ldha) expression, and its expression has also been used to investigate the neurotoxic effects of the malarial drug artemisinin.[70, 71] Verapamil, a calcium-channel blocker, inhibits Thioredoxin Interacting Protein (Txnip), a neurotoxic biomarker show to cause retinal neurotoxicity.[72]

This study has shown that some of these same biomarkers of inflammation and neurotoxicity play a role in mental nerve exposure to endodontic sealer, and has established that there are cellular and molecular pathways to elucidate in order understand their neurotoxic effects on outcomes of endodontic, namely post-operative pain.

Clinical Implications

Studies find that *in vitro* and *in vivo* cytotoxicity data has little significance with clinical practice.[73, 74] Perhaps this is the case because clinicians tend to chose biomaterials more because of cost or ease of use, or perhaps the data available on cytotoxicity or biocompatibility is unavailable or unknown. In any case, when choosing a sealer, clinicians must be well informed of their biomaterial properties and likely clinical outcomes, and decide what is best for each patient.[9]

Based on the findings of this study, if sealer toxicity did relate to post-operative pain, a recommendation could be made that resins, as the most toxic, would likely cause the most or most severe clinical outcomes. GuttaFlow 2, which has shown no cytotoxicity *in vitro* and little neurotoxicity *in vivo*, would be the safest sealer to use to prevent post-operative pain.

[9] Because post-operative pain is an inconsistent outcome and not much data exists on its relationship to a particular sealer in clinical use, the best recommendation for choice of sealer at this point should actually come from whether or not a good seal or a therapeutic effect is preferred.[10] For example, in necrotic cases with apical periodontitis, a choice like bioceramic is better because of the beneficial therapeutic effects of continued calcium hydroxide release and its osteogenic potential. In vital cases where a post-core will be done immediately, a faster-setting sealer with minimal microleakage, would be more suitable.

As with any topic in research, more studies are always needed to delve closer to the truth in order to provide the best evidence-based applications. This study, though meek in form, contributes its piece to the overall knowledge base of the relationship between endodontic sealers and neurotoxicity, and hopefully is an impetus for further research into this topic.

IX. Acknowledgements

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