Charcoal Toothpaste on the Effects of Fibroblasts- Gingival Cell Survival, Proliferation, and Migration

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Abstract

Introduction: Patients feel having a lighter and brighter smile increase their quality of life. Many whitening products on the market today have unwanted side effects towards the dentition through abrasives and over the counter bleach. The periodontal status has been positively affected by whitening agents in the past. The latest trend in teeth whitening is charcoal toothpaste, but not much evidence has surfaced on its effects on the periodontium. The purpose of this study was to examine the lasting effects charcoal toothpaste has on gingival fibroblast survival, proliferation, and migration, in comparison to no exposure to toothpaste and control toothpastes.

Methods: Gingival fibroblasts were treated for up to 24 hours with various percentages of toothpaste extracts. Eight variations of non-charcoal and charcoal toothpastes underwent testing with observations recorded between the control and test groups. Extracts were made by incubating 1.3g toothpaste in 15mL of growth media containing 10% serum and antibiotics. Cell survival after 5%, 10%, 15%, 20% and 25% extract exposure was assessed fluorometrically using Calcein-AM. Toothpaste solution pH was obtained and analyzed against cell survival. Toothpaste ingredient lists were dissected, organized and compared to cell survival. Cell proliferation was assessed after 3-minute exposure to 25% extracts at 1, 3, and 6 days post exposure via utilization of cyquant dye for quantification of cell DNA. Cell motility was assessed using a scrape assay after 3-minute exposure to 25% extracts at 0, 1, 2, and 3 days post scrape and exposure.

Results: Colgate Optic White Advanced and Colgate Revitalizing White reduced gingival fibroblast survival for >/=20% solution exposures of 3 minute, at 24 hours post exposure. The above-mentioned toothpastes also significantly inhibited cell proliferation and migration. Gum detoxifier also significantly inhibited cell migration, while cavity Protection delayed gingival fibroblast motility. Other toothpastes had no significant effect on fibroblast motility.

Conclusion: The findings of this study conclude, most charcoal toothpastes exhibit no exacerbation of cytotoxicity, inhibition of cell proliferation, or inability for cell migration; but in fact, they may lead to slight increase in cell motility following 3-minute exposure to gingival fibroblasts. Colgate whitening toothpastes, Colgate Optic White Advanced and Colgate Revitalizing White, experienced significant decrease in gingival fibroblast survival, proliferation and motility. Charcoal toothpastes may be a good alternative to whitening for periodontal disease patients and for those recovering from periodontal surgery.

Introduction

In dentistry, one of patients' primary esthetic concern is the whiteness of their teeth. Various forms of dental bleaching have been around since 1898 [4]. Patients feel having light and bright teeth will increase their quality of life[1]. Forms of whitening vary from professional application, such as bleaching via peroxides, and at home products that are available over the counter in forms of whitening toothpastes, mouthwashes, and gels [2]. Common whitening agents in use are H_2O_2 and carbamine peroxides.

Many forms of whitening contain abrasive agents and peroxide which can lead to unwanted outcomes on the oral cavity [1]. Although some are successful, over the counter bleaching systems can have unwanted effects on general health and the periodontium[2].

Past studies have shown evidence in the effectiveness of dental whitening with the use of carbamine peroxide, but few studies have looked at the effects of dental whitening on the soft tissues[5]. According to Ouellet, et al., when examining periodontal pockets, plaque, index, and bleeding index, no adverse effects were seen in use of whitening with ten percent concentration of carbamide peroxide. The gel did not seem to cause gingival issues, but instead induced slight reduction of gingival irritation[4]. Scherer and colleges also determined that the use of carbamide peroxide for effective dental whitening caused an improvement in gingival inflammation causing therapeutic effect [5].

Traditional toothpastes have been shown to have deleterious effects on gingival fibroblasts and high toxicity. In one study it was seen that gingival cytotoxicity was >90% in toothpastes containing sodium lauryl sulfate and cocamidopropyl, and 25%-70% in toothpastes containing sodium fluoride[10]. Traditional whitening toothpastes have been shown to be even more cytotoxic compared to traditional toothpastes. In a study comparing toothpastes, Colgate whitening was seen to be the most cytotoxic[11]

The latest trend in whitening that has become popular is charcoal toothpaste. In the past charcoal has been used medically as antidote for acute poisoning and drug overdose, as well as control for skin infection, and decrease of wound malodor[8]. With its recent use in dentistry, many social media posts have targeted advertisements of the product, as well as multiple celebrities who have endorsed it. The purpose of the product is for extrinsic stain removal on enamel[7]. Numerous brands have followed the trend and created their own charcoal toothpaste for whitening. Since peak of interest in the population has come about with this toothpaste, limited research has been documented on its outcomes[8]. Research on the topic has pertained primarily on its effects with enamel and abrasiveness. In Machla et al's study on charcoal toothpaste, it was determined that charcoal dentifrices are abrasive, but within an expectable limit[9].

Charcoal is created by slow-burning natural materials that are carbon- rich. The source of the charcoal can be a multiple of materials, such as: wood, coal, nut shells, bamboo and peat, or coconut shells and husk[6.7]. Abrasiveness of charcoal depends on the source it was made from.

Charcoal is carbon residue dehydration, it is organic material that has been burned, usually from wood. Activated charcoal is carbon residue of dehydration. It is an organic material that has been burned by heating to a very high temperature. Compared to charcoal, activated charcoal is more porous, is burned at higher temperatures and is used in medicine and to remove toxins. Charcoal is used for cooking and filtering[9]. Activated charcoal extracts toxins and can be used in medicine when someone has alcohol poisoning or in a brita filter to filter water. Therefore, activated charcoal in toothpaste should have some beneficial effects on the oral cavity.

According to Brooks et al. there is limited and insufficient scientific evidence to endorse charcoal toothpaste for cosmetic use, healthy benefits, or safety claims[8]. The authors urged practitioners to educate patients about charcoal dentifrices unproven claims of oral benefits and

health risks that accompany the use of these toothpastes. These healthy risks include the use of human carcinogenic polyaromatic hydrocarbons and bentonite clay in the toothpastes[7].

Claims have also been made that charcoal toothpaste helps in treatment of periodontitis, and this is considered opportunistic marketing. A negative impact for patients with periodontal disease who use this dentifrice, can be the possibility of a charcoal particle becoming trapped in deep periodontal defects which can cause grey/black discoloration to the periodontal tissues[7].

Currently no studies exist on exact effects of charcoal toothpaste on the soft tissues. With limited research on charcoal toothpastes, especially on soft tissues, it is important to research the potential harms that the product can cause. The aim of this study is to gather more information on the effects of charcoal toothpaste on the gingiva of the mouth, more specifically its effects on fibroblast cells.

Methods

Cell Lines

Gingival fibroblasts were isolated from a periodontally healthy 13 year old boy with no systemic diseases who underwent periodontal surgery. Cells were maintained in minimum essential medium *alpha* (MEMα) containing 10% fetal bovine serum (FBS) and 200 units/ml of penicillin and 200 µg/ml streptomycin (GIBCO Grand Island, NY). Gingival Fibroblasts between the 14th and 19th passage were used in this study.

Tooth Paste Extracts: Extracts were made by incubating 1.3 g of Non-charcoal/charcoal toothpaste in 15mL of growth media containing 10% serum and antibiotics. Preliminary studies determined a tooth brushed with a “pea” sized amount of tooth paste measures 1.3g of toothpaste. 15mLs is the average volume of paste + saliva produced after 3 minutes.

Table 1: Toothpaste Examined

	Non-Charcoal/Charcoal Toothpastes Examined
C1	Colgate Optic White Advanced
C2	Tom’s Fluoride Free
C3	Colgate Cavity Protection
C4	Colgate Revitalizing White
C5	Tom’s Activated Charcoal
C6	Crest Whitening Therapy- Deep Clean Charcoal
C7	Hello Charcoal
C8	Moon Charcoal

Treatment groups:

Control Groups: no toothpaste exposure, Colgate Optic White Advanced, Tom's Fluoride Free, Colgate Cavity Protection

Test Groups: Colgate Revitalizing White, Tom's Activated Charcoal, Crest Whitening Therapy-Deep Clean Charcoal, Hello Charcoal, Moon Charcoal

Calcein staining of live cells (Cytotoxicity Assay). Cells were allowed to adhere to multiwall pates for 24 hours and exposed to various concentrations of toothpaste extracts. To examine cell survival, cells were treated with Calcein AM for 1 hour. This dye is a non-fluorescent esterase substrate, that is readily absorbed by live cells. Once internalized, this dye is cleaved by endogenous esterases and rendered fluorescent. Therefore, this dye readily labels the cytoplasm of live cells. This allows for cell morphology examination via a Nikon inverted TE2000 microscope equipped with a CoolSNAP cfi camera and metamorph software. Cell survival was quantified using the same cells and a BioTek Synergy2 fluorescent multi-well plate reader with filters appropriate for ~ 480nm excitation and ~520nm emission.

Tooth Paste ingredient Analysis: Ingredients for all toothpastes were grouped and analyzed in comparison to cytotoxicity assay data, for elimination of other toothpaste ingredient as cause of cell death.

***pH*:** Using a calibrated digital pH tester, pH of water as a control and Non-charcoal/charcoal toothpaste extracts were measured and recorded 3 times per solution. In between each measurement, pH tester was dipped into water for recalibration.

Cell Proliferation: Cells were isolated and resuspended after trypsinixation at a concentration of 20,000 cells/ml in MEMα containing 10% fetal bovine serum. Cell will be isolated and resuspended after trypsinization at a concentration of 20,000 cells/ml in MEMα containing 10%

fetal bovine serum. Cells will be plated into 48-well tissue culture plate at 4000 cells in 200µl in MEMα, and allowed to adhere for 1 hour. Eight samples will be prepared for each condition for each experiment. Cells will be incubate from 1-6 days at 37ºC and adherent cells will be quantified fluorometrically using CyQuant fluorescent dye (Molecular Probes, Eugene, OR). The basis for this assay is the use of a proprietary green fluorescent dye, CyQUANT GR dye that exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Under these conditions, this assay has a linear detection range extending from about 50 to 50,000 cells per microplate well. This dye fluoresces at 530 nm when bound to DNA. The sample fluorescence will be measured using a fluorescence microplate reader (Bio – Tek Instruments FL600, Winooski, VT) with filters appropriate for ~ 480nm excitation and ~520nm emission.

Cell Motility Assay: In order to examine cell motility, cells were plated into 12-well tissue culture plates, and allowed to adhere for 24-hours. Using a P-1000 pipette tip, a 1.5 mm wound was made in each well. Cells were immediately treated with 25% toothpaste extracts for 3 minutes, and the width of the wound was measured at 24 hour intervals. Untreated cells served as controls. For each time point, 25 measurements were made for each condition. Cells were treated with Calcein AM for 1 hour to aid in visualization. Cells were examined using a Nikon inverted TE2000 microscope equipped with a CoolSNAP cfi camera and Metamorph software.

Statistical Analysis: For the Calcein AM staining, the mean and standard deviation of eight samples were compared to control values for untreated cells.

For pH the average of the 3 independent pH measurements from each solution was determined and used for data analysis. For the cell proliferation assay, each group of eight samples were averaged, and a mean and standard deviation were compared to the control value according to the following formula:

(Experimental Value / Initial Value (1 hr)) x 100% = % Proliferation)

Paired student t-tests were used for statistical comparisons between TTE and TPE where p values <0.05 will be considered statistically significant. For the cell motility assay, 25 measurements were averaged. The mean and standard deviation were compared to the control values for untreated

Results

Cytotoxicity Assay/ Cell Survival

Non- charcoal and charcoal toothpaste extracts were examined for their effects on the behavior of gingival fibroblasts. Toothpaste extracts made from Colgate Revitalizing White and Colgate Optic White Advanced were statistically significant for reduction of gingival fibroblast cell survival after 24 hours at 25% solution concentration. Crest Whitening Therapy- Deep Clean Charcoal and Hello Charcoal were moderately toxic for gingival fibroblasts at 24 hours post exposure. The largest cell survival 24 hours after initial exposure was observed with Moon Charcoal, Colgate Cavity Protect, Tom's Activated Charcoal, and Tom's Fluoride Free toothpastes. Cell survival was reduced to less than 50 percent for Colgate Revitalizing White and Colgate Optic White Advanced at 20% concentration and Crest Whitening Therapy- Deep Clean Charcoal at 25% concentration.

pH

Toothpaste extract pH differed between the tooth paste types. Tom's Fluoride Free toothpaste was determined to have the most basic pH, pH average of 8.99, followed by Hello Charcoal, pH average of 8.77, and Crest Whitening Therapy- Deep Clean Charcoal, pH average of 8.24. Moon Charcoal was determined to have the most acidic pH, pH average of 6.11. The remaining toothpaste extracts exhibited no significant difference between them for pH (Colgate Revitalizing White, Colgate Optic White Advanced, Colgate Cavity Protect, and Tom's Activated Charcoal), pH average of 7.34. Tom's Fluoride Free toothpaste had the most basic pH and high cell survival, while Moon Charcoal had the most acidic pH and high cell survival. This study determined pH had no statistically significant effect on gingival fibroblast survival.

Cell Proliferation

Cell proliferation of gingival fibroblasts was significantly altered by specific toothpaste extracts at 25% concentrations. Colgate Revitalizing White and Colgate Optic White Advanced appeared to have a statistically significant negative correlation with gingival fibroblast proliferation at days 1,3, and 6. Colgate Cavity Protect was observed to have a statistically significant negative effect with gingival fibroblast proliferation at day 1 but was insignificant at days 3 and 6. Gingival fibroblast proliferation showed no statistically significant effect from exposure to the five remaining solutions at any experimental time point (Moon Charcoal, Hello Charcoal, Crest Whitening Therapy- Deep Clean Charcoal, Tom's Fluoride Free, Tom's activated Charcoal).

Cell Motility

Toothpaste extracts of 25% had statistically different alterations of gingival fibroblast motility compared to each other and the control. Colgate Optic White Advanced and Colgate Revitalizing White exhibited statistically significant decrease in cell motility at 1, 2, and 3 days versus the control and the other toothpaste types. Tom's Fluoride Free and Colgate Cavity Protect toothpastes had no significant effect on gingival fibroblast motility at any experimental time point. No statistically significant difference was observed between the control and remaining groups (Tom's Activated Charcoal, Crest Whitening Therapy- Deep Clean Charcoal, Hello Charcoal, Moon Charcoal) on gingival fibroblast motility at day 1 and 2 post exposure, but by day 3 all 4 charcoal toothpastes exhibited significant increase in motility.

Toothpaste ingredient

Several ingredients were found in common among the cytotoxic group, and absent among the other toothpaste types, including the moderately toxic toothpaste types. The following ingredients were common to Colgate Revitalizing White and Colgate Optic White Advanced: pentasodium triphosphate, PEF-12, and Cocamidopropyl betaine. No specific ingredient was determined to have a statistically significant effect on gingivalfibroblast survival.

Discussion

These experiments display that exposing gingival fibroblast cells to most charcoal toothpaste extracts do not induce cell death, prohibit cell proliferation or impact cell migration at any more of an increased rate than non-charcoal toothpastes. In cases of Colgate Optic White Advanced (control) and Colgate Revitalizing White (charcoal) toothpaste extracts gingival fibroblasts can experience increased cell death, prohibition of cell proliferation and reduction in cell motility. Cell survival was affected at different rates for each toothpaste extract type, regardless of the inclusion or absence of charcoal as an ingredient. Interestingly, Colgate's charcoal toothpaste was on of the solutions with the most deleterious effects to gingival fibroblasts, just behind their traditional whitening toothpaste; indicating a common ingredient in the two toothpastes may be the causative agent, as opposed to charcoal versus non- charcoal containing.

Toothpastes were cross referenced to compare for similar ingredients. Both Colgate Revitalizing White and Colgate Optic White Advanced contain PEG-12, Pentasodium Triphosphate, and cocamidopropyl betaine, which all other toothpaste types observingly lack. Polyethylene glycol, PEG-12, is a thickening agent for toothpastes, whereas cocamidopropyl betaine is a foaming agent. Notably, cocamidopropyl has been linked to gingival cytotoxicity in previous studies, such as one study that displayed >90% gingival cytotoxicity in toothpastes containing sodium lauryl sulfate and cocamidopropyl[2]. The current study further provides evidence on cocamidoproxyl containing toothpastes causing increased gingival cytotoxicity by exhibiting greater than 80% and greater than 60% cell death in the only two cocamidoproxyl containing toothpastes (Colgate Optic White Advanced and Colgate Revitalizing White), at 15% and 20% concentrations, respectively. At 25% concentrations, both extracts showed nearly 100% cell death, whereas the remaining 6 types of extracts showed only 0-60% cell death. Sodium Lauryl sulfate was a common ingredient in both Colgate whitening toothpastes, as well as in Crest Charcoal, Tom's Fluoride Free, and Colgate Cavity Protect toothpastes. This study

lacked evidence to support increased cytotoxicity for sodium lauryl sulfate containing toothpastes due to a large range of 40-100% cell survival for these toothpastes. Moderate cytotoxicity of Hello charcoal and Crest charcoal toothpastes did not have any common links of ingredients to explain the increase in gingival fibroblast death compared to the less cytotoxic counterpart toothpastes.

Change in pH and abrasiveness has been shown to have a combined effect on oral fibroblast disruption[10]. The average pH of the saliva is 7.4 and of blood is 7.4. Charcoal toothpaste extracts had a range of 6.11-8.77 for pH. Due to the large role pH plays in oral homeostasis, pH for the study toothpaste abstracts was recorded and analyzed. The current study was able to determine that pH of charcoal toothpaste is not the primary cause of gingival cell death due to the large range and lack of correlation of pH with cell survival.

A well-established association of toothpaste and delayed wound healing does not currently exist, much less an association of charcoal containing toothpastes and delayed wound healing. A previous study reported whitening toothpastes have increased gingival cytotoxicity compared to non-whitening toothpastes[11]. Since charcoal toothpaste is the latest trend in whitening, its safety on the gingival tissues needs to be determined, especially for the use in patients with periodontal disease or use post periodontal surgery. One study noted that charcoal toothpastes had advantageous effects on periodontitis patients, while another study noted the unwanted greying effect of charcoal particles becoming lodged in periodontal pockets[7]. A 2019 study presented toxic effects on human gingival fibroblasts due to certain toothpaste and mouthwash ingredients[10]. Our study showed a significant negative correlation of Colgate whitening toothpaste extracts with decreased gingival fibroblast proliferation and motility, whereas a positive correlation between the remaining 4 charcoal toothpaste extracts, as well as Tom's Fluoride Free Extract was presented with increased gingival fibroblast proliferation and motility. Future studies are needed to determine a definitive effect of charcoal toothpaste on

wound healing, but our study supports the safe use of charcoal toothpaste, as an alternative to traditional whitening toothpaste, in patients experiencing wound healing of the periodontium.

Conclusion:

The findings of this study conclude, most charcoal toothpastes exhibit no exacerbation of cytotoxicity, inhibition of cell proliferation, or inability for cell migration; but in fact, they may lead to slight increase in cell motility following 3-minute exposure to gingival fibroblasts. Colgate whitening toothpastes, Colgate Optic White Advanced and Colgate Revitalizing White, experienced significant decrease in gingival fibroblast survival, proliferation and motility. Charcoal toothpastes may be a good alternative to whitening for periodontal disease patients and for those recovering from periodontal surgery.

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Figure Legends

Figure 1:

Effect of charcoal/non-charcoal toothpaste extracts on cell survival. Gingival fibroblasts were exposed to toothpaste extracts up to 25% concentration for 3 minutes and results were observed after 24 hours. Eight different charcoal/non-charcoal toothpastes were used {C1 (Colgate Optic White Advanced), C2 (Tom's Fluoride Free), C3 (Colgate Cavity Protect), C4 (Colgate Revitalizing White), C5 (Crest Whitening Therapy- Deep Clean Charcoal), C6 (Tom's Activated Charcoal), C7 (Hello Charcoal), C8 (Moon Charcoal)}. Points represent the mean and standard deviation of eight samples. (n=8 *= $P<0.05$)

Figure 2:

Average of 3 measurements of pH of each toothpaste extract. Eight different charcoal/non-charcoal toothpastes were used {C1 (Colgate Optic White Advanced), C2 (Tom's Fluoride Free), C3 (Colgate Cavity Protect), C4 (Colgate Revitalizing White), C5 (Crest Whitening Therapy- Deep Clean Charcoal), C6 (Tom's Activated Charcoal), C7 (Hello Charcoal), C8 (Moon Charcoal)}. Points represent the mean and standard deviation of 3 measurements. Bars of same color are statistically similar. Bars of different colors are statistically different from similar pHs (Moon, Hello, Crest, Tom's Fluoride Free).

Figure 3:

Effect of charcoal/non-charcoal toothpaste extracts on gingival fibroblast. Gingival fibroblasts were exposed to toothpaste extracts up of 25% concentration for 3 minutes and proliferation at 0-, 1-, 3-, and 6-days post exposure were analyzed compared to % of starting gingival fibroblasts and results recorded. Eight different charcoal/non-charcoal toothpastes were used {C1 (Colgate Optic White Advanced), C2 (Tom's Fluoride Free), C3 (Colgate Cavity Protect), C4 (Colgate Revitalizing White), C5 (Crest Whitening Therapy- Deep Clean Charcoal), C6 (Tom's

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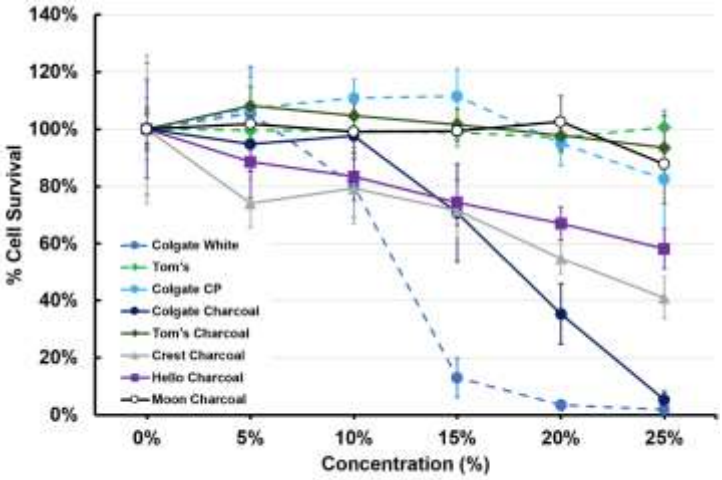
Figure 4:

Effects of toothpaste extracts on gingival fibroblast migration. Gingival fibroblasts were exposed to 25% charcoal/non-charcoal toothpaste extracts for 3 minutes. Eight different charcoal/non-charcoal toothpastes were used {C1 (Colgate Optic White Advanced), C2 (Tom's Fluoride Free), C3 (Colgate Cavity Protect), C4 (Colgate Revitalizing White), C5 (Crest Whitening Therapy- Deep Clean Charcoal), C6 (Tom's Activated Charcoal), C7 (Hello Charcoal), C8 (Moon Charcoal)}. 25 measurements were created for each of the eight charcoal/non-charcoal toothpaste extract wells at each time point. Points represent the mean and standard deviation of 25 measurements. (n=25 *= P<0.05)

Figure 1- Effect of Toothpaste Extracts on Cell Survival at 24 Hours



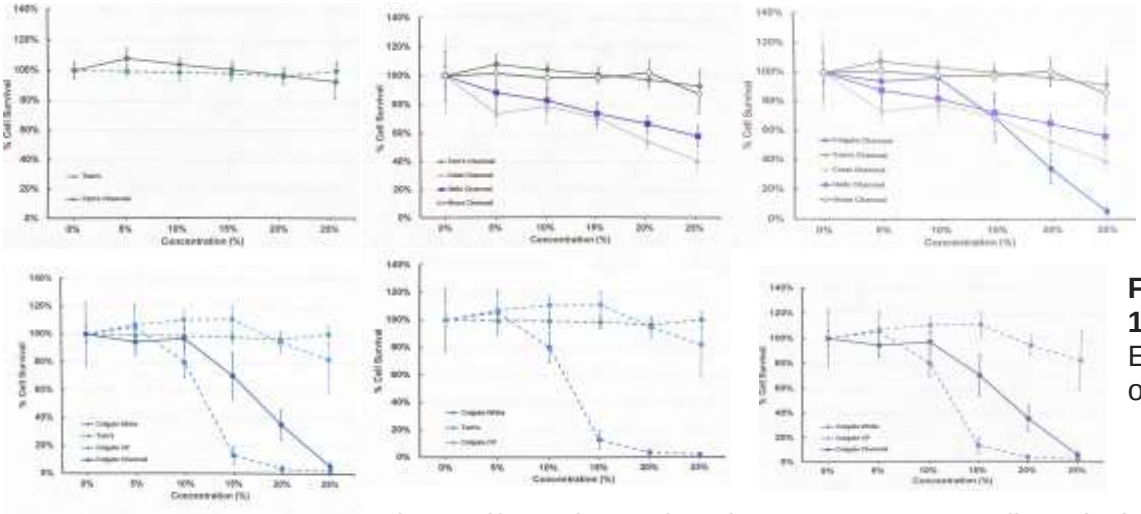


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Figure 2- pH of Toothpaste Extracts

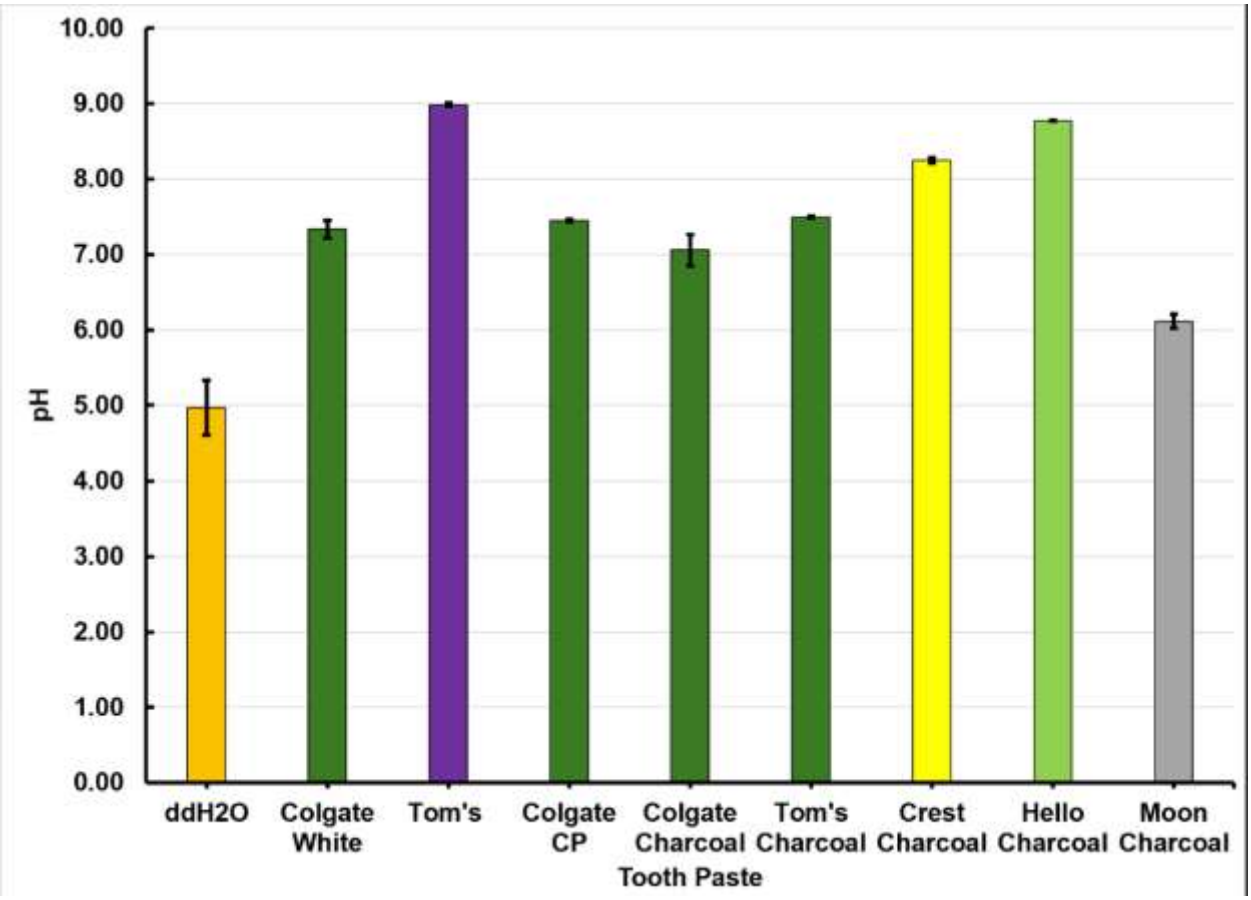

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Figure 3- Effects of Toothpaste Extracts on Cell Proliferation at 0, 1, 3, 6 days

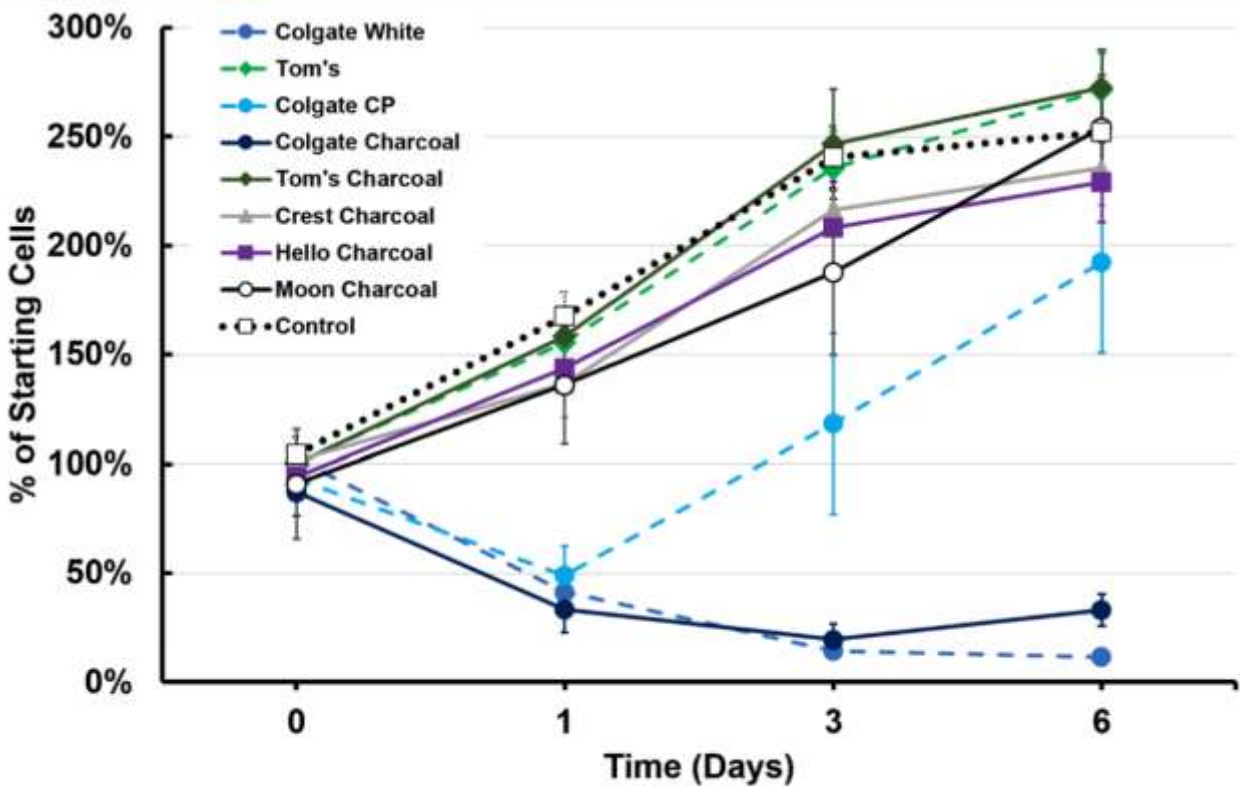

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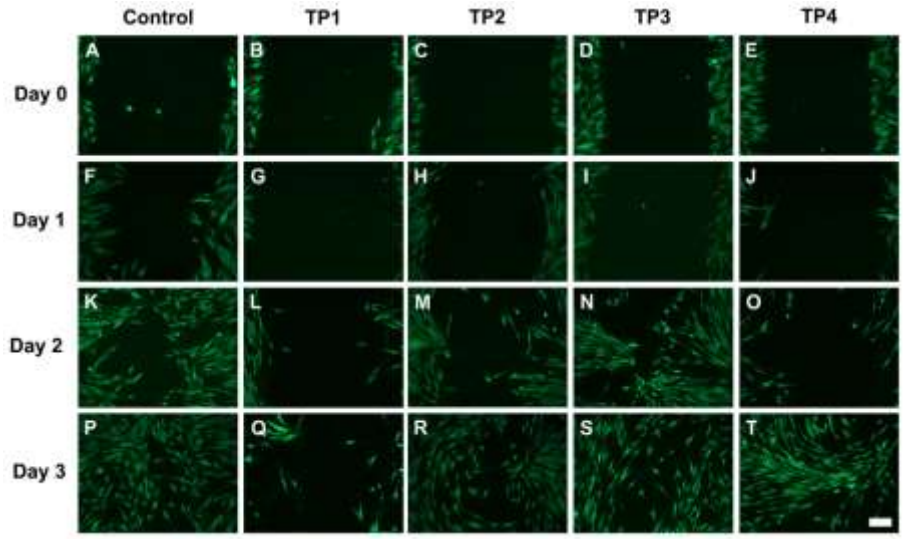
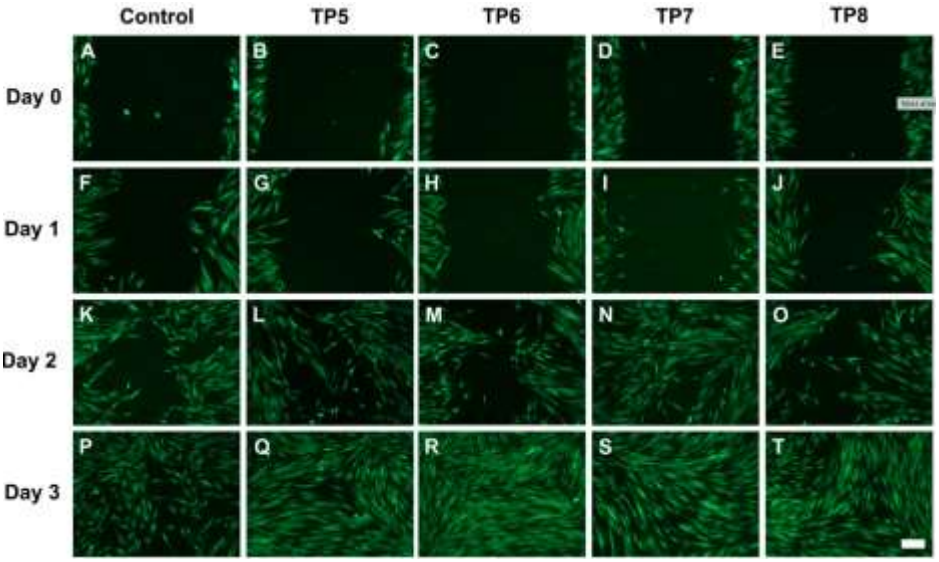
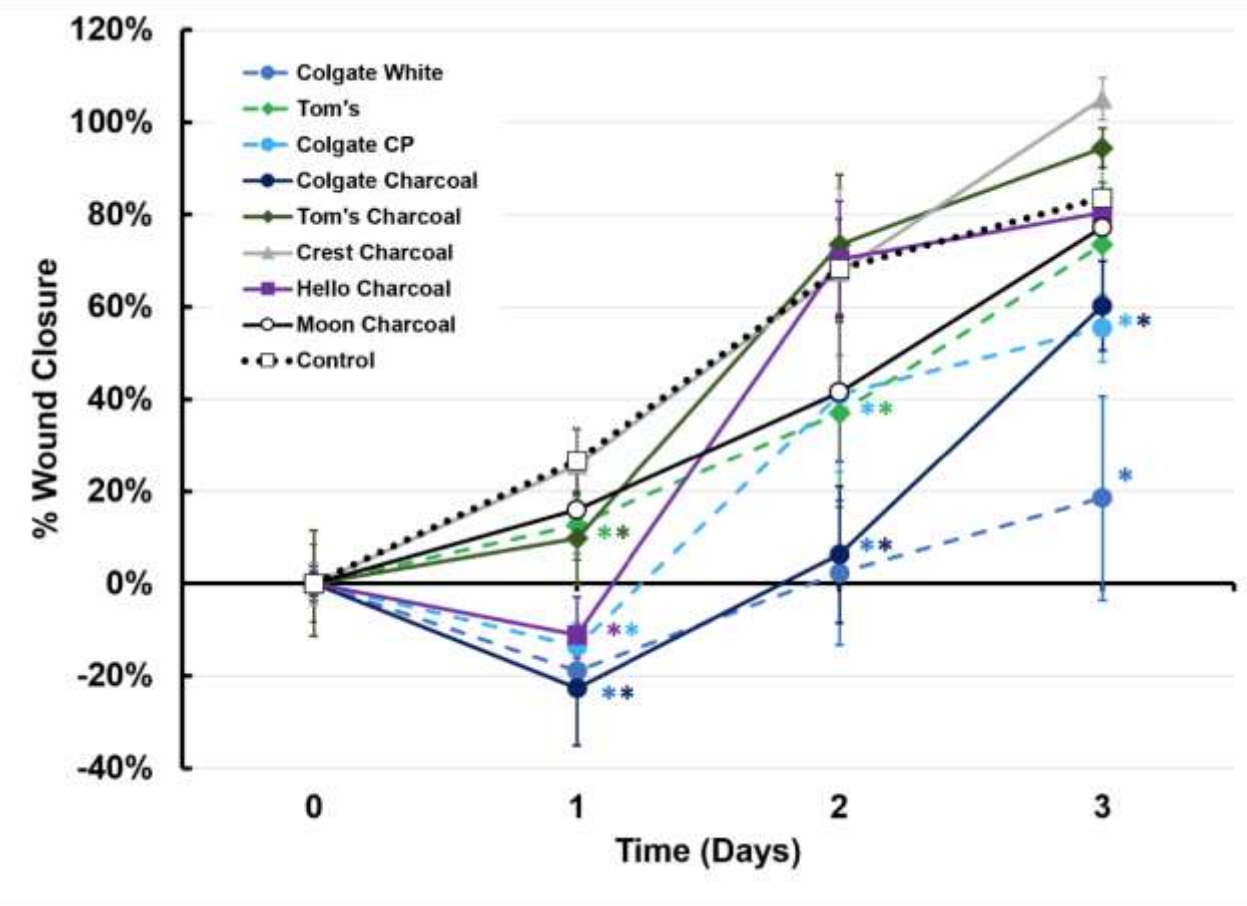
Figure 4- Effects of Toothpaste Extracts on Cell Migration at 0, 1, 2, 3 days







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