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Title

The Harm of Harm Reduction Tobacco Products: Reduced Tar Cigarettes Cause Mitochondrial Damage in Developing Cells

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Author
Hanna, Michael

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A capstone project submitted for
Graduation with University Honors

University Honors
University of California, Riverside

APPROVED

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Dr.
Department of

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Dr. Richard Cardullo, Howard H Hays Jr. Chair and Faculty Director, University Honors
Interim Vice Provost, Undergraduate Education

Abstract

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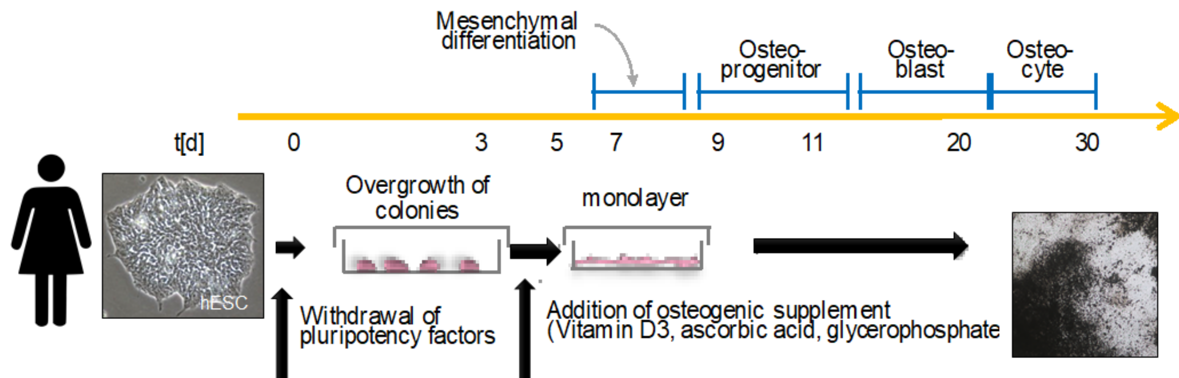
Introduction

Use of tobacco products remains to be the leading cause of preventable disease and death in the United States. Despite awareness of issues regarding tobacco use, many persist in their harmful habits. The tobacco industry has released harm reduction tobacco products (HRTPs) in order to provide consumers with an alternative product to minimize consumer concerns. Because these products are consistently advertised as "less harmful", consumers frequently equate these tobacco products with being safe. As a result of this added sense of security, HRTPs have grown in popularity among tobacco users, including pregnant women. This misconception is of concern as studies have shown that these HRTPs have not reduced health risks associated with tobacco [1,2]. However, there is a lack of evidence of the toxicity mechanisms associated with the use of HRTPs and their impact on development. It is known that tobacco products have adverse effects on osteogenesis, caused by decreasing the expression of bone morphogenetic protein-2 (BMP-2), which is important in the development of bone structures [3,4]. Also, there is evidence in our lab that displays the embryotoxicity of HRTPs. However, there is inadequate understanding of HRTP-specific toxicity mechanism, therefore an incomplete confirmation of their embryotoxicity effects. In light of these understandings and lack thereof, our laboratory has proceeded to research the effects of these HRTPs on *in vitro* osteoblasts. Our lab began this process by developing one of the first *in vitro* osteogenic development models using human embryonic stem cells (hESCs). This model was used to perform the initial screen of both, conventional cigarettes (i.e. Camel Filters) and their harm-reduction counterparts (i.e. Camel Blue), on bone development. Several assays were performed in an attempt to discover the mechanism by which these products impact development.

This was done under the precedent of the embryonic stem cell test (EST), which tests for embryotoxicity. The original EST tests for cytotoxicity among 3T3 fibroblast cells, cytotoxicity among mouse embryonic stem cells (mESCs), and differentiation potential of ESCs [5]. Our laboratory developed a modified version of this procedure that utilized human ESCs and osteoblast differentiation, as opposed to the use of mESCs and cardiomyocyte differentiation in the initial EST procedure. An MTT assay measures the ability of the cell to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, following the notion that only viable cells will be able to metabolize this effectively, as shown by the purple coloration of the product. This assay was used to measure the cytotoxicity of the tobacco products on both, the fibroblast cells and the hESCs.



The hESCs used were differentiated as seen by the schematic above, ultimately resulting in the production of functional osteocytes that are able to display impairments in function based on calcium production. The differentiation potential of the hESCs was assessed through the use of a calcium assay, measuring the concentration of the calcified matrix released by the differentiated osteoblast. All of the assays of the EST were done with a treatment of the mainstream (MS) smoke, the smoke released from the mouthpiece of the cigarette into the lungs of the smoker upon inhalation, as well as sidestream (SS) smoke, the smoke released from the back-end of the

cigarette. Sidestream smoke is typically found to be more harmful because it does not pass through the same filter that the mainstream smoke does. This is a large contributing factor the adverse impact of second-hand smoke as well, as second-hand smoke is composed of both sidestream and mainstream smoke.

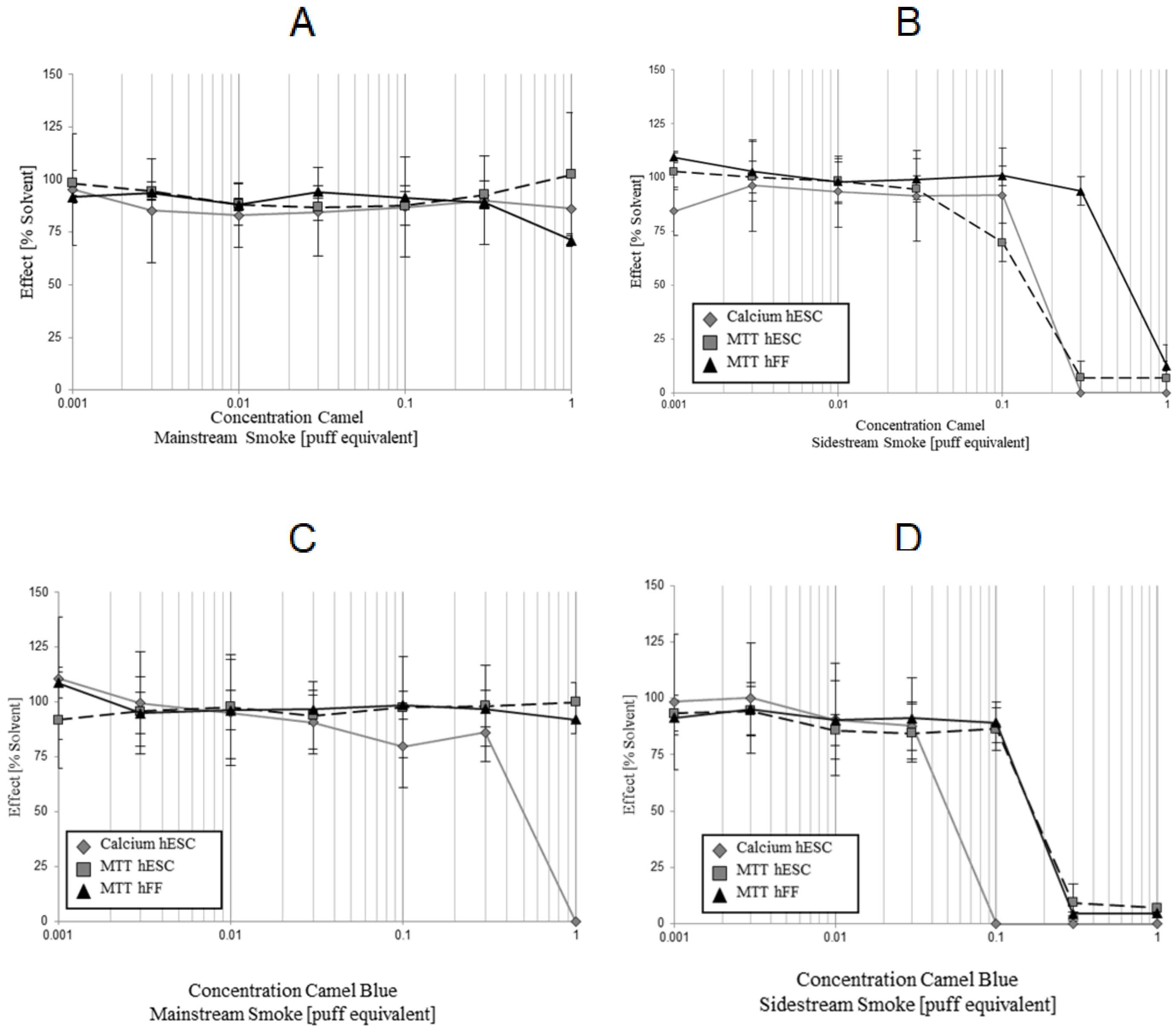
To further assess impacts on cell health, an assessment was performed using the caspase 3/7 assay to identify cells undergoing apoptosis. Additionally, an apoptosis qPCR array was tested, to assess the expression of mRNA of genes that are typically upregulated in response to DNA damage within the cell. The findings of these experiments have led to the quantification of DNA damage, as was measured by the comet assay.

Because the comet assay visually shows the amount of DNA damage in the cell, the assay was used to verify the results of the apoptosis qPCR. This assay works by lysing the nucleus of a cell and using electrophoresis to separate the strands of DNA by size. In the case of frequent DNA repair, caused by a large amount of damage, many small fragments of DNA are excised, creating a comet-shaped streak adjacent to the centralized comet "head". This damage can then be quantified by measuring the length of the streak that is formed from the central comet. This damage may also be quantified by observing other features of the comets such as the amount formed, the diameter of the comet heads, and tail moment.

Following the comet assay, in order to further observe the pathway of apoptosis within these cells, a measurement of mitochondrial activity was conducted using a JC-1 assay. This was done in order to observe the potential impact of these tobacco products on the mitochondria within the cell, and the correlation to apoptosis, if any.

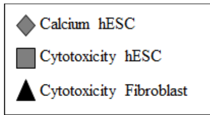
Results

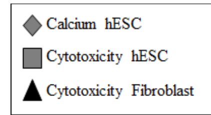
Embryonic Stem Cell Test



The EST for the MS smoke of the conventional tobacco treatment (Camel Filters), as seen in image A, indicates no measurable cytotoxicity. However, when the SS smoke is used (Image B), there is measurable cytotoxicity within the cells, as seen by the decline in cell viability and

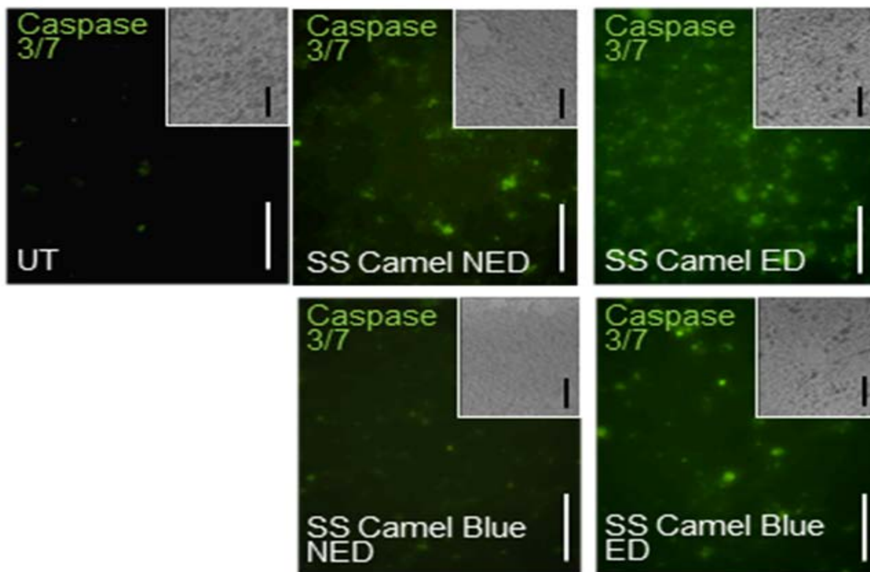
calcium production. Image C displays no impairment in cell viability when using the MS smoke of the HRTP (Camel Blue), however, there is a decline in calcium production. Image D indicates a similar finding to that of image C, however, the SS smoke of the HRTP causes an earlier cessation of calcium production as well as a cytotoxic effect on the hESCs as well as the adult fibroblast.





For further reference, the effective dose of smoke is the concentration at which there is a 50% decrease in calcium production observed, while the non-effective dose is the concentration at which there is no significant decrease in calcium production.

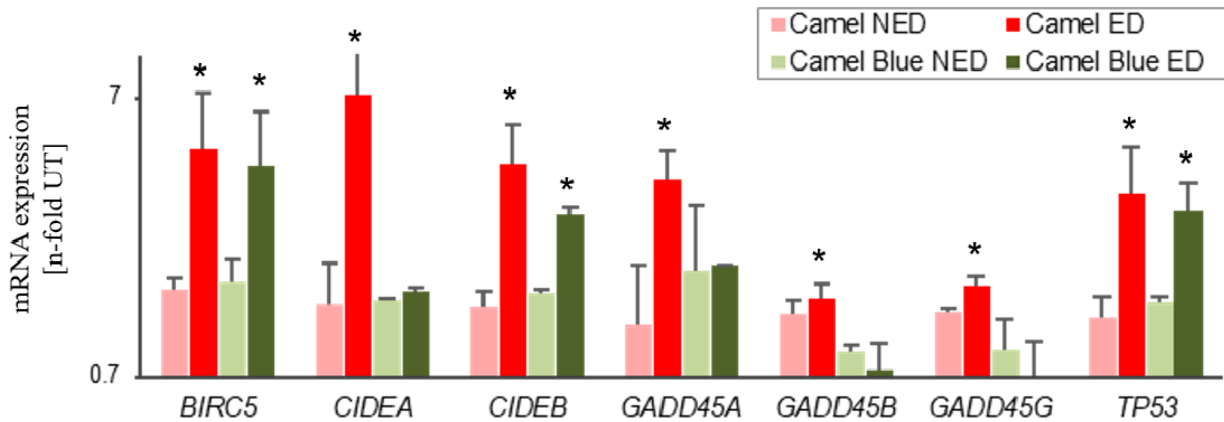
Caspase 3/7 Assay

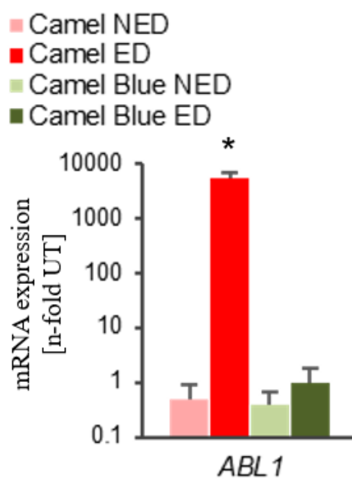


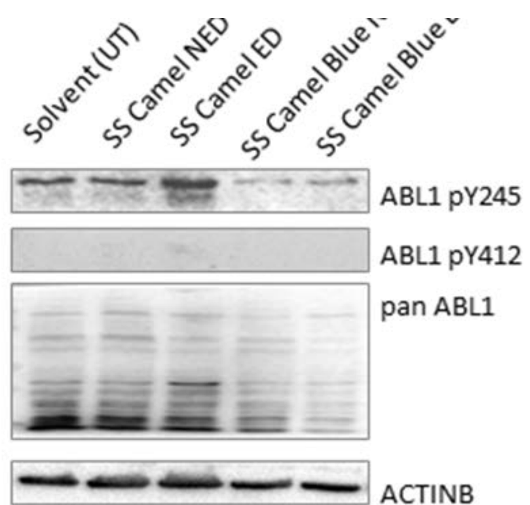
Caspases 3 and 7 are both involved within the signaling of the apoptosis (cell death) pathway. The results of the caspase 3/7 assay display the varying levels of cell death among the different treatments, the conventional (Camel Filter) and the HRTP (Camel Blue). This assay

marks caspase 3 & caspase 7 activity with a green fluorescence. As expected, there is minimal fluorescence within the untreated (UT) cells, indicating normal growth. There is a very high amount of caspase 3 & 7 activity when cells are treated with the effective dose of the conventional tobacco product. The non-effective dose of the conventional tobacco product had a mid-level amount of caspase 3 & 7 activity, much like the effective dose of the HRTP treatment. A very low caspase 3 & 7 activity was seen in the non-effective dose of the HRTP treatment.

qPCR Array







The qPCR above assessed for the mRNA expression of several genes that are upregulated in response to DNA damage, including the growth arrest and DNA damage 45 family (GADD45) and ABL1.The majority of these genes are upregulated significantly in cells treated with the effective dose of conventional tobacco products, but there is a much more infrequent upregulation of these genes in cells treated with the effective dose of the HRTP. The same result is seen in ABL1 in particular, with a significant rise in mRNA expression of these gene with the "Camel ED" treatment. The western blot above also displays the increase in protein levels of ABL1, when treated with the Camel ED.

Comet Assay

The comet assay shows DNA damage by way of the "tail" part of the comet, this occurs due to the difference in size of the DNA fragments that have been removed by means of a repair mechanism. This separation of a long strand of DNA from smaller DNA fragments occurs through electrophoresis as the negatively charged DNA strands are attracted to the positively charged anode. During this process, the shorter DNA fragments move at a faster rate than the larger strands, thereby creating a comet tail. Ten samples have been imaged and analyzed: Control, Positive Control (5-fluorouracil), Camel Non-effective, Camel Effective, Camel Effective + Caspase 9 Inhibitor, Camel Effective + Ascorbic Acid, Camel Blue Non-effective, Camel Blue Effective, Camel Blue Effective + Caspase 9 Inhibitor, Camel Blue Effective + Ascorbic Acid. It was expected that this comet assay would display relatively small amounts of DNA damage in the control and the Camel conventional non-effective treatment groups. However, these were not the results that were observed through the images collected. Several factors were taken into account when looking at these images, these include: comet head diameter, comet tail length, tail moment

(the measure of the center of the comet head to the center of the comet tail to give information on the extent of the DNA damage), as well as percent DNA in the tail. The contrast of the images was adjusted using ImageJ, so that the measurements would be done with greater accuracy.

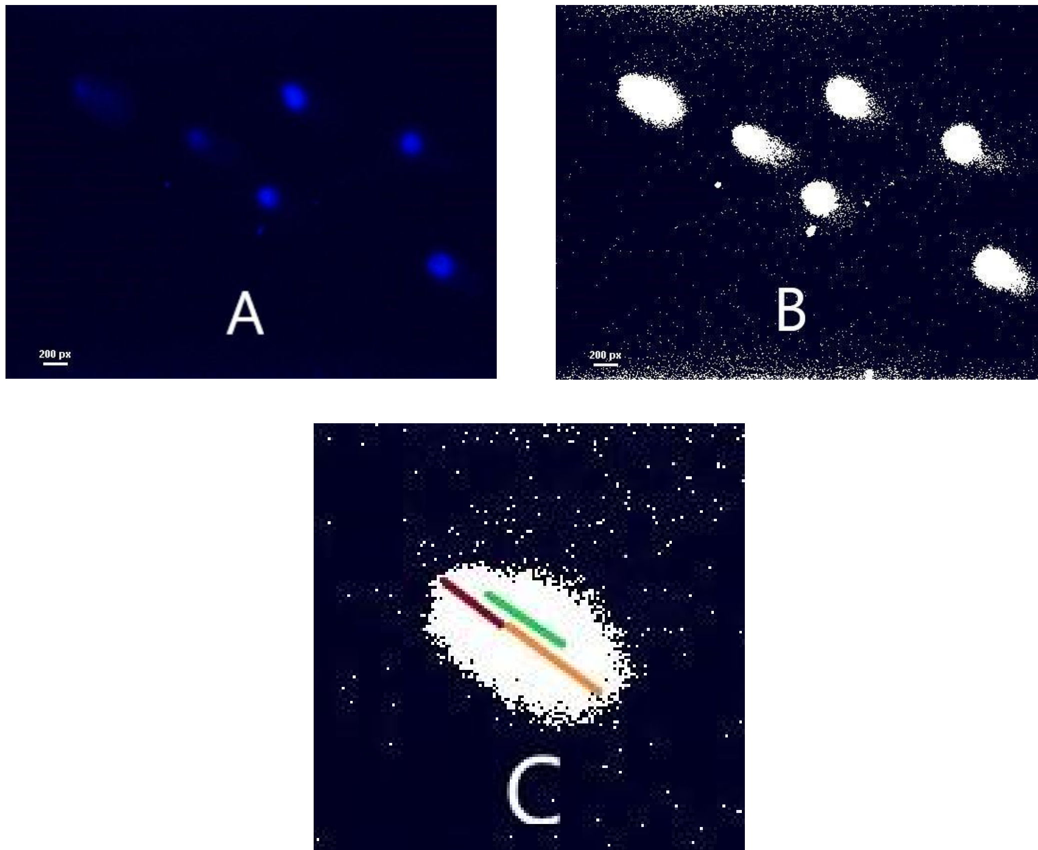
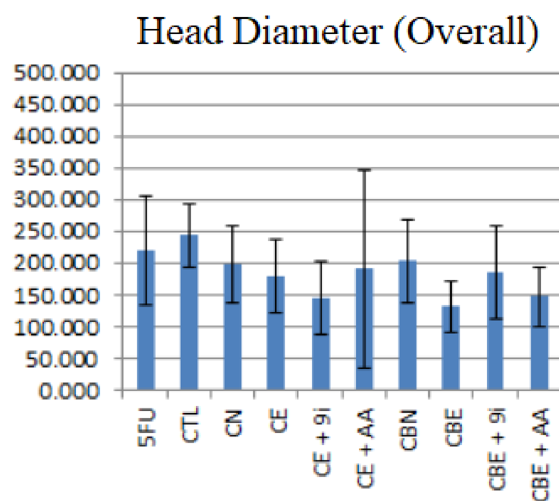
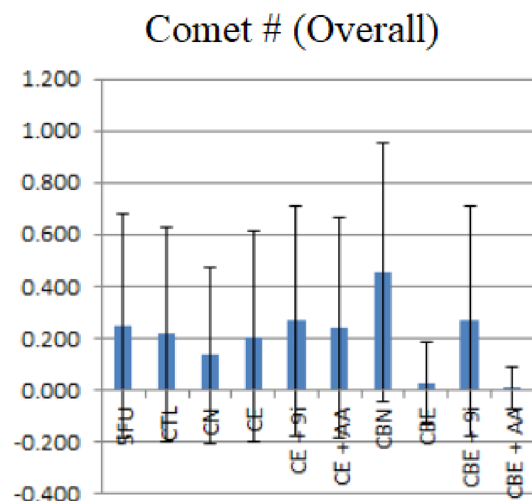
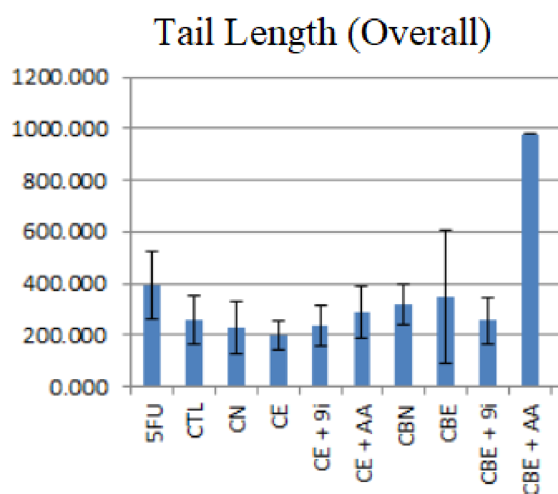
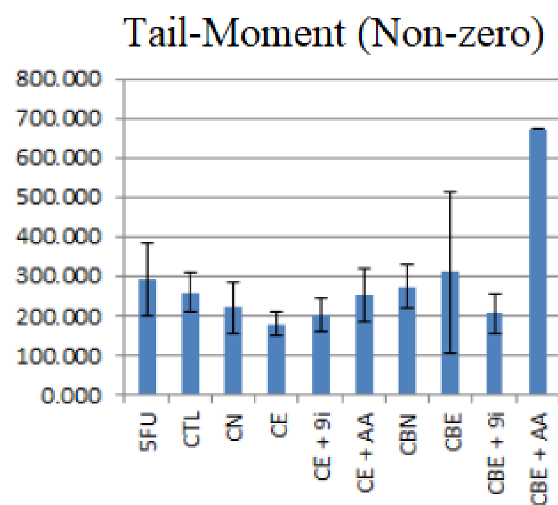


Image A shows the original photo of the slide that was later thresholded according to the brightness of the DAPI signal to better observe the qualities of the comet and obtain quantitative data. Image C shows the diameter of the comet head (red line), the length of the tail (orange line) and the tail moment (green line).

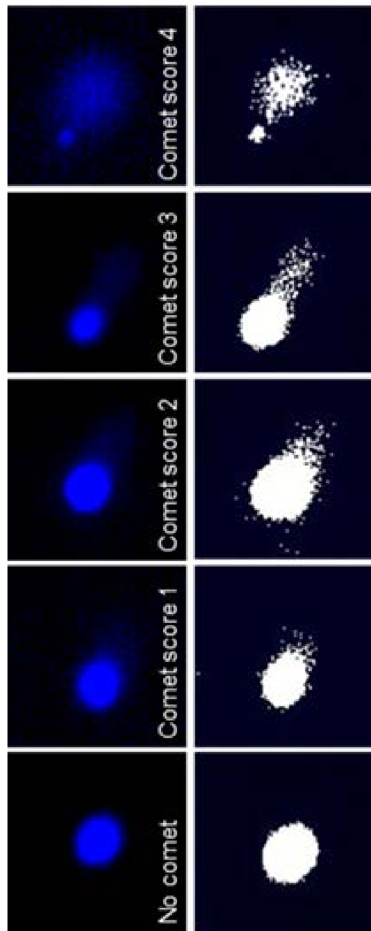


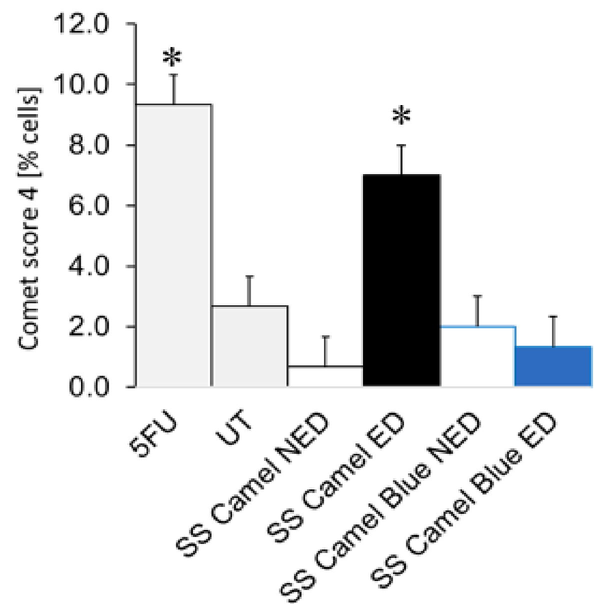






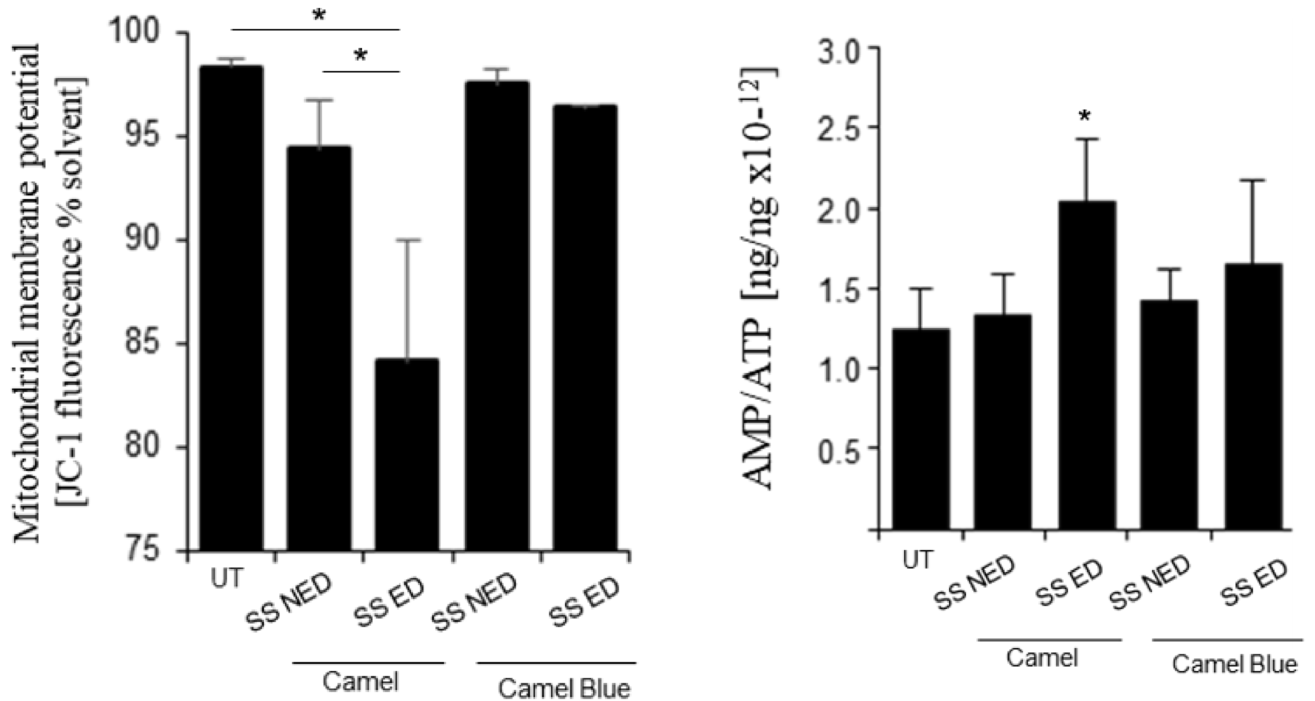
The data collected showed no significant difference in the comet head diameter as well as comet tail length and tail moment (in images that displayed tails) among all of the samples. However, there were more comets in camel effective dose samples that had also been treated with caspase 9 inhibitor or ascorbic acid.





The comet score describes the size of the comet relative to the size of the comet tail. The higher the score, the increased amount of fragmented DNA as seen in the image above. The data displays a significant increase in frequency of cells with comet score 4 in the Positive Control (5FU) and SS Camel ED treatments. There are no other significant differences from the control across all other treatments.

Mitochondrial Data



The JC-1 assay is used to determine the mitochondrial depolarization within the cell. This mitochondrial depolarization is in integral step within the sequence of events that occur during the early stages of the apoptosis pathway. As indicated by graph A above, there is significant decrease in mitochondrial membrane potential in both the Camel SS NED and SS ED treatments, relative to the control. The Camel SS ED treatment also shows a significant decrease from that of SS NED treatment. The Camel Blue treatments indicate no significant difference from the control. When observing the AMP/ATP fraction, there is a significant increase in the Camel SS ED treatment, however, there are no other significant differences.

Discussion

The Embryonic Stem Cell Test (EST) data indicated a substantial effect of the sidestream smoke of the conventional tobacco product (Camel Filter) on the viability of the hESCs. This decrease in viability led to a corresponding decrease in differentiation potential as cells that are no longer functional, have lost the ability to differentiate. However, when cells were treated with the mainstream smoke of the HRTP (Camel Blue), there was a decrease in the differentiation potential, prior to any adverse effects on cell viability of both the hESC and adult fibroblast cell types. This effect was only further exacerbated when the sidestream smoke of the HRTP was used, displaying an earlier effect on the differentiation potential, followed by an impact on cell viability. This decrease in formation of calcification, signifying improper differentiation, occurs prior to impacts on cell viability, indicating that the effect on differentiation potential is independent of cell viability. The results of the HRTP differ from that of the conventional tobacco treatment, indicating that these products function through varying mechanisms.

In order to quantify the variation in cell viability and further discover differences between these two mechanisms, a caspase 3/7 assay was conducted. The results from this assay confirmed the previous results of the EST, displaying greater frequency of apoptosis within the cells treated with the SS Camel Conventional effective treatment. This further confirms the decrease in differentiation capacity of the Camel Blue treatments, despite the lack of apoptosis. A potential cause for this variance in caspase 3 & 7 is levels of DNA damage, causing the cells to undergo apoptosis.

The qPCR array was then conducted in an attempt to observe the outcome of the hypothesized DNA damage. What was found correlated with the prior assessment, a significant increase in mRNA expression of many of the genes that are upregulated in response to DNA

damage. All of the genes tested displayed significant upregulation when treated with the conventional tobacco product, especially ABL1, which showed nearly 1,000-fold increased expression. Only three of these genes were overexpressed in the Camel Blue treatment, which may account for the decreased levels of apoptosis in the cells with this treatment. In order to further verify these results, a western blot was conducted to confirm the translation of the increased mRNA levels. The western blot verified these results as seen by the much thicker band that was observed in the ABL1 pY245 location when treated with the SS Camel ED. Likewise, a light band is seen in the ABL1 pY412 location in this same treatment, the activating phosphorylation location for this protein. With further confirmation that DNA damage is a significant role player within this pathway, the comet assay was conducted as a way to quantify this data.

Given the previous results, we expected to find a significant increase in comet population and tail length in the treatment with the effective strain of Camel conventional tobacco which confirm indicate DNA damage. We also expected a decrease in comet frequency when cells treated with the camel conventional effective dose were treated with caspase 9 inhibitor or ascorbic acid (vitamin C), suggesting the biochemical mechanism(s) responsible for possible DNA damage associated with use of this tobacco product. Caspase 9 is an enzyme involved in the process of apoptosis, specifically from mitochondrial issues within the cell. Inhibiting this enzyme prevents apoptosis of the cell, therefore changing the features of the comets observed in this treatment. Although there was no difference in head diameter or tail length, there was an increase in the population of comets. An increase in comet quantity also took place for Camel effective + ascorbic acid (vitamin C), an antioxidant that would rescue the cell from oxidative stress by preventing free radicals from causing damage to the DNA strand. Had oxidative stress been the leading cause of

DNA damage within the cell, we expected to have observed the result of this treatment to be like that of the control or the Camel non-effective.

There are several possibilities as to why the results that were observed are highly dissimilar to those that were expected. DNA fragments are formed as a result of the excision of a dimer in the DNA strand, however, if this repair mechanism is being inhibited by the Camel effective treatment, there will not be a significant amount of fragments observed in the comet tail, resulting in a shorter and fainter tail equivalent to that seen in the control. A treatment supplemented with enzymes required for DNA repair may be able to provide results as to whether the DNA repair mechanism is also affected by this camel effective exposure.

When the same data was collected for the Camel Blue strain, we found a significantly lower number of comets overall, providing further understanding for the decrease in apoptosis among these cells. However, the comets that were observed appeared to have similar comet head diameters as the rest of the previous trials. The tail length and tail moment of the Camel Blue strains are not significantly different from those of the camel treatments, with the exception of the Camel Blue Effective + Ascorbic Acid, where we observe a very large increase in the size of the tail length and tail moment.

It appears that the Camel Blue strain of HRTPs may have had less DNA damage than the standard Camel Effective, as seen by the significant decrease in the number of comets observed in this strain. The large increase in tail length and tail moment observed in the CBE + AA strain may be a result of these cells are being sensitive to concentrations of Vitamin C, therefore resulting in an increased amount of DNA repair. This increased level of repair may appear as the tail of the comet, and with increased levels of DNA repair, the tail would become much longer as observed.

As a result of the scoring of these comets, it was further reinforced that the cells treated with the conventional tobacco treatment accumulated increased DNA damage. The cells with a comet score 4 display the greatest amount of DNA damage as the comet head is relatively small, while the tail is broad and long. The frequency of this particular scoring increased significantly in the cells treated with SS Camel ED, indicating that this treatment results in the greatest amount of DNA damage. This data corresponds with the previous results, and further refines the understanding of the varying patterns in apoptosis among the different treatments.

To further comprehension of the apoptosis pathway that is occurring in these cells and how it may be impacted downstream, a JC-1 assay was conducted to observe the effects on the mitochondria. It appears that there is the correlation between the mitochondrial impacts and the levels of apoptosis. In cells treated with the SS Camel ED, there is a highly significant decrease in mitochondrial membrane potential. This mitochondrial depolarization indicates that the mitochondria is no longer functional as the gradient which powers the formation of ATP begins to lose efficacy. To further verify these results, the fraction of AMP/ATP was quantified as well. If the mitochondria are severely impacted, then this should also be observed within the amount of ATP within the cell. These implications are confirmed with this fraction, as there as a significant increase in this value among the cells treated with SS Camel ED, further displaying the dysfunction of the mitochondria. There are no significant impacts on the cells with the HRTP treatment, providing further understanding as to why there may be decreased levels of apoptosis within this cell population.

There is now a greater understanding of these HRTP and their cytotoxic mechanisms and how those vary from that of the conventional tobacco products. This data displays adverse effects of both of the tobacco products; however, the variation in effects reinforces the theory that these

products act on different mechanisms entirely, despite the advertised difference being decreased nicotine and tar content. Although they each act on different mechanisms, it is not clear that one is significantly less harmful than the other, although previous claims, advertisements and present connotations may lead one to believe otherwise. Therefore, regardless of the appeal of a decreased tar and nicotine content, it is observed that these HRTPs have their own adverse effects that are carried out by a unique mechanism. It is likely that other HRTPs may act through various mechanisms because of their differing compositions, however, this remains unknown. The long-term impact of these tobacco products also remains to be of interest and a potential area of further study.

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