The effects of fluoride on macrophage THP-1 cell apoptosis

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THE EFFECTS OF FLUORIDE
ON MACROPHAGE THP-1
CELL APOPTOSIS

A Thesis Submitted
in Partial Fulfillment
of the Requirements for the Designation
University Honors

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May 2021
This Study by: Jessica Heims

Entitled: The Effects of Fluoride on Macrophage THP-1 Cell Apoptosis

has been approved as meeting the thesis or project requirement for the Designation University Honors.

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Introduction

Fluoride is an earthly mineral that occurs naturally both in the sedimentary earth as well as in the human body. The main biological function of fluoride in the human body is to enhance bone structure, as well as prevent the decay of teeth. This mineral has been used by dentists and public health officials to artificially introduce an extra layer of protection through the means of fluoride-enriched public water supplies and toothpastes. By using extra levels of fluoride, dentists have been able to help patients prevent future cavities and dental diseases. Although most dentists highly encourage strategic consumption of these enriched products, there are health concerns that could potentially overshadow the benefit. Excess fluoride in one’s diet may have the ability to do harm to the body and prevent certain physiological functions.

There exists little research on the effects on specific cells of the human immune system under insufficient, normal, or excessive fluoride consumption. The purpose of this thesis is to study how the apoptosis pathway of macrophage THP-1 cells, a class of human immune response cells, is affected by varying levels of fluoride. Macrophage cells, in a controlled and healthy environment, function mainly to phagocytize foreign bacteria and secrete inflammatory chemicals. This response to invasion of the human body helps to fight off unwanted infection and disease. Macrophage cells are also extremely important in eliminating damaged or infected cells through their specialized pathway of programmed cell death. This programmed death, known as apoptosis, can induce a ‘self killing’ signal in cells that are at the end of their lifespan or in cells showing
foreign and cancerous markings. This pathway is vital to the health and maintenance of the human immune system. Chemicals or foreign agents that can inhibit or alter this apoptosis pathway have the potential to be detrimental, or in some cases fatal, to human health.

**Literature Review**

Fluoride-enhanced toothpastes and water supplies have been prevalent in American cities since the year 1945. Within the first thirty years of the introduction into the mainstream market, fluoride helped create a 70% reduction in the need for cavity fillings and a 50% improvement in dental caries (Birkeland et al., 1977). The correlation between fluoride and oral health can be traced back to the 1800s, but the addition into public consumption aided in cementing the mineral’s use within the medical field. Due to health concerns, the chemical concentrations in public water, including fluoride, have been monitored through the years. The number of cities containing this enhanced water supply have been limited as well. As of 2018, only 63.4% of American citizens had direct access to a fluoridated water supply provided through city water (United States et al., 2018). The majority of dental-related studies in the medical field celebrate the positive effects of fluoride consumption, but studies outside of the dental realm have also published potential negative consequences.

Throughout the fluoridation years, some studies have published contradictory results which claim that chronic overexposure to fluoride can increase the risk of bone fractures (Li et al., 2001). Potential fluoride complications are not only seen in the
skeletal system but in the endocrine system as well. Excess fluoride consumption increases the risk of thyroid gland malfunction and can alter hormone production in both adults and children. In diabetic patients, fluoride in a diet can act as an endocrine disruptor and cause toxic reactions (Sananda Dey et al., 2015). Repeated exposure to fluoride over time has the potential to create an insulin resistance in patients due its hyperglycemic effects. Repressed insulin secretion from Langerhans pancreatic cells causes elevated blood glucose levels and increases the risk of diabetic complications. For many of these patients dealing with fluoride complications, a healthy and working immune system is necessary for healing and fighting off disease-related infections. In order to ensure that fluoride is not disrupting the human immune system, fluoride-based experiments are necessary within all the varying classes of immune cells. The immune system’s ability to carry out important immunological functions under introduction of fluoride should be compared to their normal functions without any chemical interference.

Methodology

The experiment took place through a quantitative lab using a culture of macrophage THP-1 cells. Within the month of November 2020, the macrophage immune cells were cultured and suspended using an ATCC modified RPMI (Rosewell Park Memorial Institute) medium of 500ml. The cells in this culture were maintained within a fresh and weekly rotated medium and were incubated at 37ºC with 5% CO2 until the start of the experiment in December. These cells were monitored under a
microscope before the trials to ensure that there were no abnormalities or contaminations.

Before the start of the trials, the cell cultures were incubated without disruption. At the start of each trial, the cells were added to seven individual vials and placed in a 12-well plate. The cells were then treated with varying amounts of sodium fluoride: 0 ppm for negative control, 500 ppm, 1,000 ppm, 1,250 ppm, 1,500 ppm, 2,000 ppm, and 5,000 ppm. The cell vials were then incubated for a 12-hour period.

After the incubation period was completed, an annexin apoptosis kit was used. The cells were harvested from their respective incubation vials and washed in a cold phosphate-buffered saline (PBS). After the wash, 1X annexin-binding buffer was prepared, consisting of 1 mL 5X annexin-binding buffer within 4 mL of deionized water. After the buffer was prepared, a 100 µg/mL working solution of PI was prepared. This solution was made by diluting 5 µL of a 1 mg/mL PI stock solution in a 45 µL 1X annexin-binding buffer.

After the solutions were complete, the harvested cells from the culture were centrifuged. The supernatant residue was discarded, and the cells were resuspended in the previously prepared 1X annexin-binding buffer. After the resuspension, 5 µL of the previously prepared Alexa Fluor 488 annexin V and 1 µL 100 µg/mL PI working solution were added to each volume of 100 µL of cells. The cells were then incubated at room temperature for 15 minutes.

After the incubation period, the samples were placed on ice. Next, 400 µL of 1X annexin-binding buffer was added and gently mixed with the samples. As soon as the
mixing was finished, the stained cells were analyzed by flow cytometry. The fluorescence emission was measured at 530nm and 575nm using 488nm excitation.

The results of each of the wells was documented. A total of 6 trials were conducted. At the conclusion of each trial, the averages for each treatment were analyzed using ANOVA and a significant difference p-value was calculated. This p-value demonstrated if there was a statistically significant increase or decrease in apoptosis or necrosis in treated samples as compared to the control. The analysis of the experiment took place throughout the month of March 2021. A p-value of ≤0.05 was used to calculate significance. If a treatment group(s) demonstrated a significant change in its mean apoptosis levels, post hoc analysis was used to determine which of the groups were involved.

Cellular changes to the macrophage THP-1 samples were documented through numerical shifts in the flow cytometer data into, or out of, 3 separate quadrants marked by PI+ (Propidium Iodide), PI+ + Annexin+, and Annexin+. The original hypothesis for this experiment stated that there was no expected significant change in the mean apoptosis levels between fluoride concentrations.

**Results**

Two of the three data quadrants, marked as the independent variables in the graphs, had no significant difference between treatment samples. Both the PI+ quadrant and the Annexin+ quadrant had high p-values, measured at 0.8605 and 0.7504 respectively. However, the PI+ + Annexin+ quadrant had an incredibly low p-
value at 0.0008. The standard p-value used for this experiment marked result equal to, or less than, 0.05 as a significant difference.

The PI$^+$ quadrant was a measure of the amount of cell necrosis, and a large influx would indicate that significant amounts of cells were killed off by the fluoride used in the treatment. If necrosis occurred, the cells would become leaky and allow the large PI molecules to enter. As seen in Figure 1, the levels of fluorescence in this quadrant did trend slightly upward, however the high p-value indicates that this is not statistically significant. Since there was no causational shift of treated cells into or out of this quadrant as compared to the non-treated cells, it can be concluded that cellular necrosis was not a result of the fluoride treatment.

![PI$^+$ Quadrant](image)

**Figure 1.** The mean fluorescence of the macrophage THP-1 cells following the treatment of 0 (negative control), 500, 1000, 1,250, 1,500, 2,000, and 5,000ppm of sodium fluoride. This trend’s p-value to 0.8605 was too high to conclude that the increased fluorescence was a product of the sodium fluoride.
The Annexin+ quadrant was a measure of the amount of apoptosis induced in the experiment. Annexin captures the apoptotic cells by seeking out cells whose phosphatidylserine phospholipids flip from inside the cell to the outside. This flip occurs early in the apoptotic event and is therefore a good indicator of the process. Since this quadrant did not show significant increases or decreases in treated cells compared to non-treated cells, it can be concluded that apoptosis did not occur as a result of the fluoride treatment.

![Annexin+ Quadrant](image)

**Figure 2.** The mean fluorescence of the macrophage THP-1 cells following the treatment of 0 (negative control), 500, 1000, 1,250, 1,500, 2,000, and 5,000ppm of sodium fluoride. This data set’s p-value of 0.7504 was too high to conclude that any increases or decreases in fluorescence was caused by sodium fluoride.

The PI+ + Annexin+ quadrant showed a decreasing trend of treated cells as compared to the no treatment cells. As seen in **Figure 3**, the change was immediate.
and consistent throughout the trials. This data piece was unexpected and does not have one concise explanation. One possible reason as to why this trend developed may be due to the older apoptotic cells, which can sometimes be confused as necrotic cells in data collection. Both appear very similarly, and the confusion may have shifted the data. In order to detect if late-stage apoptosis caused a shift, the cells would have to be measured at multiple points in time, as opposed to just one. This would allow the cells to be tracked and differentiated into early apoptosis cells and late apoptosis cells. Experiment-caused apoptosis would be measured early in the experiment, and any additional shifts into the necrosis quadrant after that initial time period would be marked off as late-stage apoptosis. Since the trial cells were only measured at one point in time there was no detectable difference.

Another explanation of the decreasing PI$^+$ + Annexin$^+$ quadrant trend would be the presence of naturally necrotic cells. These cells would have naturally finished their life cycle and been dead due to factors other than the fluoride treatment. Necrosis can occur in cells due to factors such as injury, cancer, or old age. The macrophage THP-1 cells had a weekly media change and were continually incubated to avoid as much human-caused necrosis as possible. The same number of cells were also used in each trial to avoid the possibility of low cell counts altering the data. Despite the consistency in cell culture upkeep, there is still a possibility of unexpected necrosis.
Figure 3. The mean fluorescence of the macrophage THP-1 cells following the treatment of 0 (negative control), 500, 1000, 1,250, 1,500, 2,000, and 5,000ppm of sodium fluoride. This stark decrease in cells, along with the p-value of 0.0008, indicated that the trend was caused by the sodium fluoride.

Another proposed cause of the PI$^+$ + Annexin$^+$ quadrant decrease is the possibility of fluoride becoming a protective agent. When certain types of toxins enter the body in low doses, they have the ability to become ‘protective’ and cause a biphasic response. This biphasic response is seen when there is not a linear progression in cellular response from low doses of chemical agents to high doses of chemical agents. When low doses of biphasic toxins enter the body, a small amount of damage occurs to the cells. The cells detect these damages and become actively involved in repair, and in turn the cells become healthier as they correct mutations and damage. When high-enough doses of biphasic toxins enter the body, the toxin levels become deadly as the cells can no longer keep up with repairs. At this point the cells
are too damaged to repair back to health and they die. This type of response is often seen in cancer-related agents. If the fluoride does indeed have a biphasic property with THP-1 macrophage cells, then the ‘no treatment’ cells would have higher amounts of cell deaths than the treated cells, as is observed in the data, and the shift out of the PI+ Annexin+ quadrant would be expected.

Conclusion

Fluoride is a mineral that can be beneficial to the health and maintenance of human teeth and enamel. Since it is found in many water and dental products, it is imperative that the potential health consequences are understood in the event that a person ingests a large amount of fluoride within a short amount of time. Experimentation with THP-1 macrophage cells helps shed some light on the potential effects of fluoride on human immune cells and immune function. After these cells were treated with varying levels of fluoride, it was concluded that the fluoride baths altered the function and longevity of the cells. These cells may have the potential for a biphasic response, which might indicate harm in large doses.

Experimental data showed that increased doses of fluoride correlates with decreases in necrosis and apoptosis. These decreases are not inherently problematic but may lead to problems with cell mutations and long-term damage. The specific cause of this alteration is not known, therefore more tests would need to be run to better understand how it functions. A larger range of trials, such as sets of 10 trials, may yield a better range of results, as well as running the cells through the flow cytometer at multiple time points. Fluoride should also be studied in regard to other immune system
functions and expressions, including MHC molecule expression and cytokine communication.

Fluoride experiments reveal the health consequences and limitations that should be yielded to by city water management and dentistry offices. Human immune systems are key to the health and longevity of modern societies and should be maintained with caution. If large fluoride doses change the functionality of immune systems, it opens up the possibility for foreign molecules and bacteria to enter the body and replicate. Weakened or altered immune systems have the ability to cause swift epidemics of infectious diseases and may decrease the quality of life for immunocompromised individuals. Many normal bodily functions rely on the immune system to keep foreign invasions at bay in order to maintain homeostatic conditions for health and prosperity. Fluoride should be used in moderation in order to balance oral health with immune efficiency.
References


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