**Prevention of chronic inflammation induced ovarian cancer using NSAIDs**

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**Abstract**

 Chronic inflammation induced from talcum powder particles travelling to the ovaries causes the increased risk for ovarian cancer. Chronic inflammation induced ovarian cancer involves cytokine signaling pathways that are viable therapy targets, such as the secretion of TNFα and IL-6. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been suggested as a useful target to decrease the chronic inflammation. Utilizing NSAID treatment on the mouse ovarian cancer cell line, ID8, presents to decrease the amount of proliferation, number of cells in G1 and S phases, and secretion of TNFα and IL-6 (p<0.05). There was no significant decrease in amount of cell death, validifying that NSAID treatment does not harm normal cells. Aspirin presents to be the most effective in treatment of chronic inflammation in ovarian cancer cells. Further research should focus on combination of therapies and *in vivo* studies of long-term use.

**Introduction**

Ovarian Cancer is known as a “silent killer” for women across the U.S. In 2013, it was estimated that 22,240 new cases of ovarian cancer would arise and 14,030 deaths would occur due to ovarian cancer in the United States (Houghton 2014). The early symptoms of ovarian cancer for women include, abdominal pains, frequent urination, and bloating. Women who are going through the early stages of ovarian carcinoma have a 70% to 90% chance of living. However, women who are in the advanced stages only have a 20% to 30% chance of survival (Goff 2000). The cause of this disease can arise through mutations, heredity, menstruation, and chronic inflammation (Babic et al 2018).

Research has been conducted regarding what factors assist in epithelial inflammation leading to ovarian carcinogenesis and found that asbestos, talc, endometriosis, and pelvic inflammatory disease were the leading causes (Ness and Cottreau 1999). The research conducted for this experiment focuses on the relation of the chemical talc and inflammatory pathways. Talc was found to pass through the female reproductive organs, such as the cervix and endometrium, and then becomes lodged in the fallopian tubes where it then induces inflammatory pathways (Narod 2016). Other findings suggest that female hormones, estrogen and prolactin, assist in this process by recruiting macrophages which leads to the inflammatory response (Cramer et al 2016).

The natural response of inflammation in the body due to injury or foreign objects, both acute and chronic, induces the release of inflammatory cytokines. When these pathways are activated the cytokines are then constitutively secreted, which ultimately results in a tumorigenic environment (McAlpine et al 2014). It was recently discovered that a network of these inflammatory cytokines, and their interactions, are actually essential for ovarian tumor growth, which leads to the conclusion of this network as a target for therapy (Kulbe et al 2012). TNFα is a family of cytokines that are activated in response to the macrophages recruited to the area of concern. IL-6 is another key component of triggering the same mechanisms that allow for inflammation to occur. As research has progressed, scientists have found that there are more molecular processes that contribute to inflammation being required for successful carcinogenesis. These factors can include over-expression of pro-inflammatory enzymes, abnormal nuclear localization of NF-kB, and suppression of anti-inflammatory transcription factors. All of which, when treated with non-steroidal anti-inflammatory drugs, reduced the inflammatory potential of growing neoplasms (Ghangas et al 2016).

NSAIDs are over the counter medications that are used on a daily basis by a vast majority of the population. As stated in its name, anti-inflammatory agents assist in relieving pain and discomfort in the body and could be applied to reducing risk for cancer formation in targeting cytokines mentioned. It was found that aspirin has high potential to prevent cancer progression and metastasis due to its inhibitory affects on platelet aggregation (Thea et al 2016). One hundred and fifty milligrams of aspirin used long term, and continued use of aspirin, shows pronounced affects in the tumor microenvironment, which presents to be a viable candidate for standard of care (Baandrup et al 2015). Cancer therapies generally focus on proliferating cells as to prevent further neoplasms, but non-proliferating ovarian cancer cells are just as much of a threat for tumor progression. Multiple NSAIDs, such as flufenamic acid, Flurbiprofen, Finasteridem Celocoxib, and Ibuprofen were effective in inducing apoptosis in non-proliferating ovarian cancer cells and had a heightened effect when combined (Duncan et al 2012). Revealing NSAIDs increased effect when combined gave promise for future drugs in treating ovarian cancer. One of these is a dual-threat metallodrug that simultaneously delivers synergistic bioactive drugs (Srivastava et al 2018). Other NSAID conjugates showed potential in the ability to promote cell cycle arrest in S and G2 phases, induce apoptosis, inhibit cell migration, disrupt microtubule production, and regulate proteins of the Bel-7402/5-FU cell line (Zhang et al

2017).

 The possibility of NSAIDs in becoming standard of care for chronic inflammation induced ovarian cancer seems a promising opportunity. With any new therapy, extensive research should be conducted to validate its effectiveness. In this research, NSAIDs were utilized as a potential therapy on a mouse ovarian cancer cell line in order to observe if there would be a reduction in proliferation and secretion of TNFα and IL-6. It is hypothesized that NSAIDs will reduce the cytokine levels and proliferation to pose as a therapeutic and preventative option for all chronic inflammation induced cancers.

**Methodology**

*Cell Treatments*

 The mouse endothelial ovarian cancer cell line, ID8, containing 20,000 cells, was obtained from. Aspirin, Acetaminophen, Ibuprofen, and Naproxen were obtained and dissolved in dimethyl sulfoxide (DMSO). Ovarian cancer cells were treated with each medication for 24 hours. The concentrations for each treatment was 5 mM of Aspirin, 1 mM of Acetaminophen, 200 µM of Ibuprofen, and 200 µM of Naproxen. For controls, cells were treated with an equal amount of DMSO or fetal bovine serum (FBS), which was less than 0.1% final concentration.

*Apoptosis Assay*

To quantify the percentage of cells that died after NSAID treatment, an LDH apoptosis assay was conducted. The amount of lactase dehydrogenase that was released from the dead cells was measured and analyzed. The LDH assay was conducted using the Pierce LDH Cytotoxicity Assay Kit with standard procedures from ThermoScientific. Significant statistical difference of the control samples against the treatments of each NSAID was determined by a *t*-test.

*Proliferation Assay*

 To quantify the amount of proliferation of cells after NSAID treatment, an MTT proliferation assay was conducted. Fluorescence was measured after conversion of MTT tetrazolium to a formazan product in the cells. The MTT assay was conducted using the CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit with standard procedures from Promega. Significant statistical difference of the control samples against the treatments of each NSAID was determined by a *t*-test.

*Cell Cycle Analysis*

 To quantify the number of cells that are in G1 and S phases after NSAID treatment, cell cycle analysis was conducted. Incorporation of propidium iodide into the DNA of the cells was measured by the amount of fluorescence present using flow cytometry. A modified protocol from BioLegend was followed, by instruction of Dr. Amorette Barber of Longwood University. Significant statistical difference of the control samples against the treatments of each NSAID was determined by a *t*-test.

*Sandwich ELISA*

 To quantify the amount of TNFα and IL-6 cytokines that were secreted from the cells, a sandwich ELISA was conducted. Cytokines’ concentrations were quantified between the capture and detection antibodies. The sandwich ELISA was conducted from ELISA MAX Standard Sets with standard procedures from BioLegend. Significant statistical difference of the control samples against the treatments of each NSAID was determined by a *t*-test.

**Results**

To ensure that the medications were not killing the cells, an LDH apoptosis assay was conducted. After treatment with medication, it is important to ensure that the medications are not killing the cells. In this case, the goal was to reduce inflammation. Average amount of cytotoxicity for each treatment group was determined by the averages of triplicates

There was no significant difference in cell death between the cells treated with NSAIDs and the controls, as desired. Aspirin and acetaminophen displayed the least amount of cell death (Figure 1).

**Figure 1. NSAIDs do not cause cell death.** Average percent of cytotoxicity of cells was calculated from triplicates. Bars display standard deviation from the mean. Treatments do not show statistical significant difference from the controls.

 To ensure that the NSAIDs were decreasing the amount or proliferation of the ovarian cancer cells to aide in the reduction of chronic inflammation, an MTT proliferation assay was conducted. Average amount of proliferation for each treatment group was determined by the averages of triplicates. There was a significant decrease of proliferation in ID8 cells after treatment with all NSAIDs. Aspirin presented to have the greatest decrease in proliferation, at approximately 0.5 (Figure 2).

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**Figure 2. NSAIDs decrease proliferation of ID8 cells.** Average cell proliferation was calculated from triplicates. Bars display standard deviation from the mean. Significance of p<0.05 is displayed with asterisk.

 To determine how much DNA content was in the ovarian cancer cells after each treatment, propidium iodide was incorporated into the DNA and measured with flow cytometry. This is another way of determining how many cells are growing and preparing to divide after treatment by observing the cells that are in G1 and S phases. Average amount of DNA content for each treatment group was determined by the averages of triplicates. After treatment with NSAIDs, all treatment groups of cells had a significant decrease in amount of DNA content, all approximately half that observed from cells in media and DMSO (Figure 3). These findings further support that observed from the MTT proliferation assay.

**Figure 3. NSAIDs cause a decrease in cells in G1 and S phases.** Average fluorescence of propidium iodide incorporation into the cells after was calculated from triplicates. Bars display standard deviation from the mean. Significance of p<0.05 is displayed with asterisk.

 A sandwich ELISA was conducted to determine the secretion of TNFα and IL-6 from the ID8 cells. Average secretion amounts for both cytokines for each treatment group was determined by the averages of triplicates. Since these cytokines are important in the response to inflammation, the goal of NSAIDs is to decrease the secretion amounts. In fact, both cytokines’ secretions were significantly decreased after treatment with NSAIDs. After treatment with aspirin, the amount of TNFα secretion was decreased the most, by approximately 200 pg/mL less than media (Figure 4). After treatment with acetaminophen, the amount of IL-6 secretion was decreased the most, by almost 200 pg/mL less than media, closely followed by naproxen and aspirin (Figure 5).

**Figure 4. NSAIDs reduce the amount of TNFα secretion in ID8 cells.** Average secretion was calculated from triplicates. Bars display standard deviation from the mean. Significance of p<0.05 is displayed with asterisk.

**Figure 5. NSAIDs reduce the amount of IL-6 secretion in ID8 cells.** Average secretion was calculated from triplicates. Bars display standard deviation from the mean. Significance of p<0.05 is displayed with asterisk.

**Discussion**

In conclusion, proliferation, DNA content, and secretion of cytokines, TNFα and IL-6, were decreased with the administration of NSAIDs. Aspirin is suggested to be the best therapeutic agent for the treatment of chronic inflammation induced ovarian cancer. Aspirin was the best medication for its abilities to reduce the amount of proliferation, DNA content from cells, and TNFα secretion. It was also a front runner in reducing the amount of IL-6 secretion. Aspirin was also the least cytotoxic to the cells and caused the least amount of death to the cells, even compared to media.

It comes to no surprise that aspirin has the best therapeutic effects. A questionnaire survey conducted on women’s occurrence of breast, colon, ovarian, and pancreatic cancers was significantly reduced in the women that were on a long-term aspirin regiment (Vaughan et al. This study found a correlation between aspirin and reduced cancer incidence, but this research supports the biological network of how this powerful NSAID functions on cells.

Research shows that nonsteroidal anti-inflammatory drugs (NSAIDs) decrease the cytokine secretion of TNFα and IL-6, which may decrease the risk of ovarian cancer. NSAIDs are known to reduce carcinogenesis that was caused by chronic inflammation. In parallel with the results displayed from this research, Rodriguez-Burford determined that the administration of NSAIDs caused a decrease in cell count compared to the controls. The research conducted and the observed study both displayed similar results, by a statistically significant decrease in cell count when acetaminophen and aspirin were administered. In comparison, COX-2 inhibitor presented to be the most powerful NSAID in the observed study, by causing a significant decrease in proliferation of cells, posing to be even more powerful than aspirin was from this research conducted (Rodríguez-Burford 2002).

Cytokines are important for controlling the immune response in the body. Proinflammatory cytokines, such as TNFα and IL-6, are often constitutively secreted when chronic inflammation is present, ultimately inducing ovarian cancer. NSAIDs function by reducing that inflammation. In another study, scientists determined that combining NSAIDs present to even induce apoptosis in nonproliferating cancer cells (Duncan 2012). This combination therapy could be localized to a specific tumor to kill the cancer cells.

This research should encourage the use of over-the-counter NSAIDs as a long-term preventative option to decrease the onset of cancer incidence. Other long-term effects of previously researched powerful NSAIDs, such as COX-2 inhibitor, should be combined with low-dose over-the-counter NSAIDs. These combinations, such as COX-2 inhibitor and aspirin, should test the efficacy of decreasing cancer cell count located in the ovaries. The results from this experiment, using easy access, over-the-counter medication proves that even a low dose of NSAIDs can have a positive effect, thus providing a therapy to reduce the presence of cancer cells. Further research should focus on the effects of other cytokines in the inflammatory transduction pathways. Since this research was conducted *in vitro*, *in vivo* future studies may define the abilities of this medication to interact with other cells of the body, providing a more realistic approach.

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