Cox-2 Inhibitors Increase Heart Rate in Daphnia Magna

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I. Abstract

In this investigation, I set out to confirm or possibly disprove the notion that Cyclooxygenase-2 (Cox-2) inhibitors cause increased cardiovascular (CV) risk. To state my objective more precisely, I wanted to find a relationship between heart rate and concentration that would look like a $y = |x|$ (these bars are the absolute value notation) graph where $x > 0$ (“x” being concentration, and “y” being heart rate). All the tests were performed using Daphnia Magna to observe physiological state and also to test heart rates. Essentially, two different types of were tested. The daphnia were given doses of Celebrex ranging from one to three days; and furthermore, a control was tested in range from one to three days in distilled water. The results were essentially that Cox-2 increased heart rate significantly in higher doses; however, in these higher doses, the daphnia did not live longer than two days at the most. This process was repeated with different concentrations however those results were inconclusive.

II. Introduction

Cox-2

It is essential to note that regular non-Non-steroidal Anti-Inflammatory Drugs (NSAIDS: generally Cox-2 Inhibitors and non-Advil, Tylenol type drugs, etc.) work by inhibiting the production of prostaglandins (PGs). Cox-2 is an enzyme that is activated at sites of injury. It manufactures hormone-like substances called prostaglandins, which trigger painful inflammation. Cox-2 Inhibitors, are intended to block this phenomenon. Prostacyclin, a prostaglandin produced by the Cox-2 enzyme in the blood vessels, are fatty-acid derivatives located within the human body. (Simmons et al, 2004). PG’s are involved in as diverse normal processes as ovulation, blood clotting, renal function, wound healing, vasomotor tone, platelet aggregation, differentiation of immune cells, nerve growth, and bone metabolism (Wolf MM et al, 1999).

Common anti-inflammatory drugs like Aspirin, indomethacin (Indocin), ibuprofen (Motrin), naproxen (Naprosyn), piroxicam (Feldene), and nabumetone (Relafen), and other NSAIDs, block the function of the Cox-1 enzyme along with reducing the lining of the stomach and causing vasodilation (blood thinning) as well. But more specifically, NSAIDS block the COX-1 enzyme that the stomach produces to send to certain chemical messengers (called prostaglandins) that ensure the natural mucus lining which protects the inner stomach. When the Cox-1 enzyme is blocked, inflammation is reduced, but this also causes the stomach to be upset, leading to ulceration and internal bleeding from the stomach and intestines. (Farkouh et al, 2004).

Cox-2 inhibitors were discovered later, as a “healthier, more targeted” (US Surgeon General) way of treating the inflammation – without the side effects. While Cox-2 is more specific to inflammation, the side effects can be worse than NSAIDs when used regularly (twice a day) over extended periods of time (more than 4 months or so depending on the individual) (Simmons et al, 2004).
Merck Vioxx is a Cox-2 Inhibitor drug, which was approved by the Food and Drug Administration (FDA) in May 1999 (Time, 2004). Upon its approval, Vioxx was hailed a miracle drug, as it could provide the effects of non-steroidal anti-inflammatory drugs (NSAIDs), without causing the gastrointestinal problems that are commonly associated with NSAIDs. (Farkouh, et al). Vioxx, alongside BEXTRA, Celebrex and others, were able to isolate the Cox-2 enzyme solely. Five years on, research indicated that increased heart risks and cancer effects came with the territory of these drugs. (Wong et al, 2004). See figure 1 below to acquire a better sense of how the Cox-2 Inhibitors function:

What’s interesting to note is that Kaiser Permanente’s APC study revealed that there is a 60-90% risk of MI or sudden death from CV in Celecoxib (Wong et al, 2004). Death from CV causes, MI, stroke, or heart failure was higher in the groups taking Celecoxib; 200 mg twice per day and 400 mg twice per day compared to the placebo group (7.8 and 11.4 vs. 3.4 events per 1000 patient years). Like the APPROVe study, this increased CV risk became apparent only after at least 12 months of treatment. Furthermore, both studies found increased CV risk with increasing doses, which supports findings from previous studies.

Rofecoxib and Cardiovascular (CV) Disease Risk

Rofecoxib belongs to the group of NSAIDs known as Cox-2 selective inhibitors. Being Cox-2 selective means that these drugs act specifically on one form of the cyclooxygenase enzyme, namely the Cox-2, whereas previous NSAIDs inhibited both Cox-1 and Cox-2. This specificity allows rofecoxib and other Cox-2 inhibitors to reduce inflammation and pain while minimizing undesired gastrointestinal adverse effects - peptic ulcers - that are common with non-selective NSAIDs such as aspirin, naproxen, and ibuprofen (Wie et al, 2005). Rofecoxib was withdrawn from the market following the evidence revealed from the Adenomatous Polyp Prevention On Vioxx (APPROVe) study (Bresalier et al, 2005). This study used a placebo-controlled double blind technique designed to determine whether or not Rofecoxib prevented recurrence of colorectal polyps in patients with a history of colorectal adenomas. Due to increased CV risk, this study was terminated early (though effective results can still be derived nevertheless). The increased risk became apparent after 18 months of treatment. (Wong et al, 2004).
The APPROVe study also found pulmonary oedema to be increased in the Rofecoxib group, yet this took only five months to deduce (Wong et al, 2004). Further studies revealed that chronic use of Rofecoxib yielded to more drastic heart failures. In light of this research, Merck & Co. recommended using the drug in low quantities, and for the shortest duration of time possible. (Farkouh et al, 2004).

**General Risks of Cox-2 Inhibitors**

There is significant evidence that increased CV risk is seen with other Cox-2 inhibitors and may even be apparent with NSAIDs. (Wong et al, 2004). The Adenoma Prevention with Celecoxib (APC) study was similar to the APPROVe study, in the sense that both were trials terminated early because of too much CV disease risk. (Wong et al, 2005). However, it is ironic that Rofecoxib was taken off the market because of the fact that there were more heart failures/defects from groups taking Celecoxib. Celecoxib is still on the market, yet it is more harmful to the heart. (Wong et al, 2005)

There are theoretical mechanisms and some evidence that Cox-2 inhibition may have a beneficial effect on vascular endothelial function. Inhibition of Cox-2 may decrease vascular inflammation, mononuclear cell infiltration, improve nitric oxide availability, enhance plaque stability, and decrease atherosclerosis progression. Improvements in abnormal vascular endothelial function with celecoxib have been shown in patients with hypertension and with coronary artery disease already taking aspirin and statins. Furthermore, in patients with coronary artery disease, high sensitivity C reactive protein (HsCRP) and oxidised-LDL (Ox-LDL) decreased after treatment with celecoxib. Improved vascular endothelial function has not been found in studies using Rofecoxib or parecoxib. However, it is likely that mechanisms which increase thrombogenicity outweigh any beneficial effects on vascular endothelial function. Rofecoxib is associated with increased CV risk. There is increasing evidence to suggest that this may indeed be a class effect, and that risk is associated with chronic use and higher doses. Recent studies have also implicated traditional NSAIDs, possibly via similar mechanisms. (Wie et al, 2005).

**Cox-2 Inhibitors and Cancer Cells**

There is evidence that Cox-2 is upregulated in human cancer cells, but also, significantly increases expression of Cox-2 protein in hepatocellular carcinoma (HCC) cells. (Park et al, 2005). Interesting to note is that expression of Cox-2 was upregulated in both small-sized and well-differentiated HCC, suggesting that this phenomenon is involved in the early stages of hepatocarcinogenesis. In Figure 3 below, the Cox-2 expression is well-differentiated HCC liver.

![Fig. 3 White circles represent a prevalence of tumors at 200x. (Sakisaka et al, 2004).](image)

Cox-2 inhibitors cause growth inhibition of human hepatocellular carcinoma cells in vitro and in vivo. The related mechanisms remain to be determined (Park et al, 2004). The study was aimed at determining the effect of Celecoxib (NS-398) on growth of hepatocellular carcinoma cells and the related mechanisms. Both low Cox-2
expressing PLC/PRF/5 and high Cox-2 expressing HuH7 cells, and nude mice bearing hepatocellular carcinoma xenografts were used to study the effect and mechanisms of Celecoxib on hepatocellular carcinoma cell growth. (Park et al, 2005). Celecoxib resulted in a comparable growth inhibition of both hepatocellular carcinoma cell lines that was associated with decreased production of prostaglandin. This information is important because the suppression of prostaglandins indicates that prostacyclin (which is produced by Cox-2 enzyme), means that the drug is functions the way that it’s supposed to in the sense that the Cox-2 enzyme has been sufficiently blocked (hence the title “Cox-2 inhibitor”). Addition of prostaglandin E2 only partially counteracted the effect of Celecoxib on both cells. Celecoxib resulted in a significant reduction of retinoblastoma phosphorylation (a protein in HCC cells) and DP1/E2F1 complex in both cells. Celecoxib caused a significant increase of apoptosis (programmed cell death as signaled by the nuclei in normally functioning human and animal cells when age or state of cell health and condition dictates) in vitro and activation of caspase-3 caspase-9 in both cells. In nude mice inoculated with HuH7 cells, Celecoxib resulted in decreased frequency and mean weight of hepatocellular carcinoma xenografts (Park et al, 2005). Park and colleagues concluded that the two human HCC cell lines expressed Cox-2, and that NS-398 inhibited prostaglandin E2 production and cell production and cell proliferation in a concentration-dependent manner. Furthermore, when SNU-387 cells were transfected with Cox-2-specific siRNAs, they found a significant reduction in Cox-2 expression, prostaglandin E2 production and proliferation. (Park et al, 2005)

The point of including this information about Cox-2 Inhibitors and cancer cells is that it is critical to note that more research needs to be done with more oncologists putting out vital information about the full range of Cox-2 inhibitor side effects. So many cancer types when tested with Cox-2 inhibitor drugs reveal that there may in fact be beneficial uses of the drug. The following paragraphs speak to that notion.

Tuynman and colleagues (2005) realized that high Cox-2 and/or MET expression levels are negative prognostic factors for Adenocarcinoma of the esophagus. A common cellular pathway causing tumor cell survival, proliferation, and invasion is mediated by the hepatocyte growth factor (HGF). The receptor of HGF is MET (Tuynman et al, 2005), a proto-oncogene that has been implicated in progression and dissemination of several cancer types, including esophageal cancer. (Tuynman et al, 2005). In experimental models, activation of MET expression causes decreased apoptosis and enhanced proliferation, angiogenesis, and invasion. Prostaglandins have been known to promote MET activation. The inhibition of MET expression may constitute an important factor in explaining the anticarcinogenic effects of NSAIDs, so Tuynman have deductively reasoned. Nonsteroidal anti-inflammatory drugs (NSAIDs) and selective Cox-2 inhibitors exert anticancer mechanisms as is evident from epidemiologic studies and from experimental models for esophageal cancer. (Tuynman et al, 2005)

Esophageal Adenocarcinoma cell lines were used to assess the effects of Cox-2 inhibitors in vitro. To study the clinical effects 12 patients with Esophageal Adenocarcinoma were included for Neoadjuvant treatment (4 weeks) with Celecoxib at 400 mg twice
daily. Fifteen patients not receiving NSAIDs or Celecoxib were included as a control. Effects were evaluated using the MTT-cell viability test, Western blot analysis, immunohistochemistry, and RT-PCR. In vitro Celecoxib administration resulted in decreased cell viability, increased apoptosis, and decreased Cox-2 and MET expression levels. In patients, Neoadjuvant treatment with Celecoxib significantly down-regulated Cox-2 and MET expression in the tumor when compared with the non-treated control group and when compared with pretreatment measurements. (Tuynman et al, 2005) This is the first study to show in vitro and in patients with esophageal Adenocarcinoma that selective Cox-2 inhibition down-regulates Cox-2 and MET expression, both important proteins involved in cancer progression and dissemination. Therefore, (neo) adjuvant therapy with Celecoxib might have clinical potential for patients with esophageal Adenocarcinoma.

Σ

Cox-2 Inhibitors exploded onto the market as pain killers and have shown to have serious side effects when used to treat pain. However, there are convincing arguments that the drugs may have beneficial effects in preventing life-threatening illnesses. And so, assessing the side effects of the drugs is imperative to learn if Cox-2 inhibitors such as Vioxx must be permanently off the market. My experiment was essentially designed to determine whether or not the alleged serious side effects of Cox-2 Inhibitors could be found in other organisms unlike humans.

**III. Results**

The most conclusive results were that the range of $3.14 \times 10^{-13}$ to $3.14 \times 10^{-4}$ yielded the most consistent data. That data is that higher concentration of NS-398 resulted in faster heart rates, which is a cardiovascular risk. This trend applies to both white and red daphnia. The other two trials of different ranges were just so inconsistent and yield no conclusive data. Look at figures four through seven to see the conclusive data. For all of these graphs, the y-axis measures the average heart rate in beats per second and the x-axis measures the concentration of NS-398.

![Fig. 4](image) This graph shows the increase in average heart rate of white daphnia as concentrations of NS-398 becomes higher over a one-day period of time.

![Fig. 5](image) This graph shows the increase in average heart rate of white daphnia as concentrations of NS-398 become higher over a three-day period of time. In the highest concentrations, the white daphnia died off after three days because the NS-398 was so detrimental to their survival.
Fig. 6 This graph shows the increase in average heart rate of red daphnia as concentrations of NS-398 becomes higher over a one-day period of time.

Fig. 7 This graph shows the increase in average heart rate of red daphnia as concentrations of NS-398 become higher over a three-day period of time. In the highest concentrations, the red daphnia died off after three days because the NS-398 was so detrimental to their survival.

**Purpose of Using Daphnia for Testing**

Daphnia are very transparent creatures that with a heart that is very visible to count through a microscope. As you can see in figure five below, the daphnia heart is easy to find. More importantly, the daphnia were very responsive to the Cox-2, which is evident in the heart rates.

**IV. Discussion**

The daphnia were extremely hard to work with primarily because of the complications of using methylcellulose to prevent the daphnia from moving in the microscope. Essentially, the reason why methylcellulose created so many problems is that the daphnia’s reaction to the substance was inconsistent. Too much methylcellulose, and the daphnia die. Too little methylcellulose, and the daphnia move around so much that it was literally impossible to track them.

Daphnia are extremely sensitive to disturbances of the ionic composition of their environment. They become immobile and eventually die with the addition of salts like sodium, potassium, fluoride, magnesium, and calcium. Moreover, daphnia are extremely sensitive to changes in pH level (>9 and <6), metal ions like copper and zinc, pesticides, detergents, bleaches and other dissolved toxins. Fact is, is that there was fluoride in the tap water, and the pH level of the daphnia culture was not monitored. The status of the culture may have contributed to the many deaths of daphnia.

After culturing the daphnia magna, they exhibited a dark, dorsal streak. This was the food accumulating in the gut.
The daphnia all appeared to be dying and then suddenly would reproduce in great numbers within one night. Ultimately, metabolic waste accumulated in the culture, causing adverse pH changes in the water and other harmful effects. I suspect that this dying and reproducing cycle is credited to changes in possibly the level of phosphorous in the tank, if there was any in the tank to begin with in the first place. I say this because low concentrations of phosphorus (less than 0.5 ppm) will stimulate reproduction, but concentrations higher than 1.0 are lethal to the young.

The results were very conclusive in the sense that the daphnia heart rate went up with higher concentrations after multiple trials. This data alone confirms a CV risk. The only whole in this experiment was that the daphnia’s blood pressure was not measured to perhaps test for hypertension.

All together, the experiment was repeated three times. And each time, there was a different range of concentrations. The most successful concentration range was from $3.14 \times 10^{-13}$ to $3.14 \times 10^{-4}$. The range from $3.3 \times 10^{-15}$ to $3.3 \times 10^{-4}$ resulted in too many deaths with no clear trend in reactions to NS-398. The range from $2.98 \times 10^{-13}$ to $2.98 \times 10^{-4}$ resulted in no clear results. This is to say that there was no conclusive evidence of increase or decrease in heart—all the results were purely inconsistent. The reason for differentiating the ranges is credited to tweaking the volume of daphnia for creating a proportion for the concentration equations.

V. Materials and Methods

Culturing the Daphnia Magna
Some living settled to the bottom of the shipping container, but nevertheless, appeared to be active.

The culture of daphnia was placed in a shielded location from direct sunlight that was maintained at around room temperature. Immediately following, the 1-gal plastic aquarium approximately was filled of hot tap water. Water conditioner was then added to container. The aquarium water was cooled overnight so that the temperature of the water in the aquarium matched the ambient room temperature. In addition to preventing the daphnia from experiencing thermal shock, this cooling period allowed most of the chlorine and/or chloramine commonly found in municipal drinking water to dissipate.

Once the water temperature in the aquarium was close to the ambient room temperature, the Daphnia magna were submerged into aquarium. This method of submersion prevented air bubbles from becoming trapped under their carapaces, which would lift them to the surface where they would die.

Daphnia were given food. The food floated on the surface of the container where it was consumed by the daphnia.

Designing the Experiment
The lab set up was very basic. All together, the general design of the experiment was repeated three times. There were twelve Petri dishes with two white daphnia and two red daphnia within them. Within each Petri dish, there was exactly 20 ml of solely NS-398. The twelve Petri dishes ranged in concentrations of $3.14 \times 10^{-13}$ to $3.14 \times 10^{-4}$ or $3.3 \times 10^{-15}$ to $3.3 \times 10^{-4}$ or $2.98 \times 10^{-13}$ to $2.98 \times 10^{-4}$. The three sets of ranges correspond to three different trials of the experiment.

The daphnia were tested after being exposed to NS-398 overnight and for a three-day period. Different daphnia were used for the
overnight test versus the three-day tests, which explains why this experiment took so long in general.

To count baselines, daphnia were given methylcellulose and then placed under a microscope for recording of data. Heart rate was determined by using a stopwatch.

In retrospect, the reasoning behind making the solutions was relatively simple. A .25g capsule of Celebrex is intended for a 70 kg human being. All that had to be done, was create a proportion for Celebrex capsule: human weight vs. x: daphnia weight. Here is the math in detail in Figure 10:

\[
\text{.25g (whole capsule of Celebrex)} \\
70,000 \text{ g (human weight)}
\]

\[
6.75 \text{ mm}^3 (\text{daphnia}) = \frac{1 \text{ cm}^3}{1000 \text{ mm}^3}
\]

\[
> 0.00675 \text{ cm}^3
\]

\[
\text{.25g (Celebrex)} = \frac{X \text{ amount}}{70,000 \text{ g (human)}} \times 0.00675 \text{ g (daphnia)}
\]

\[X = 3.4 \times 10^{-11} \text{ g of Celebrex in 50ml of water}
\]

The value of X ended up being the low-end concentration, while \(3.14 \times 10^{-4}\) was the high-end concentration. The challenge behind creating the concentrations was figuring a logical amount of Celebrex that the daphnia could take up. Even more importantly, the daphnia needed to be able to respond to the Celebrex in such a way that show a trend in exposure. This is to say that lesser concentrations should display a reaction that reciprocates the reaction of higher concentration exposures to the drug.

VI. Acknowledgments

I would like to thank Dr. Strong for ordering all the equipment. This project was a great way for me to explore the full socio-economic-political effects of Cox-2 Inhibitors. It was such a great opportunity to incorporate my passion for medicine with this Biotechnology independent study. I thank you Dr. Strong for providing me with opportunity to challenge myself in ways I hadn’t before. With myself as my only source of support, I truly taught my self more than I thought I would absorb in a single semester about the complete Cox-2 inhibitor issue. I’d also like to thank Stanford University for providing free access to their multimedia access. Go Cardinals! Hopefully I’ll see you guys for Medical school in 2010!!

VII. References


