Hypothesis for ocular injury in marine mammals in chlorinated, brominated or ozonated pools.

F.E. Latson MS, DVM
Central Park Aquatic Health
2715 Main St.
Buffalo, NY 14214
cell 716-861-6226
Tel 716-835-3369

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(If you don't feel like wading through this whole thing there is a summary at the end)

Introduction

The eye is amazing in its ability to maintain living tissues in an exposed environment in a transparent state. Despite exposure to irritants, uv light, mechanical trauma, heat and cold the normal eye transmits light directly to the retina to allow visualization of the environment. This is not a simple achievement but represents a constant process of response to factors tending to reduce clarity. Much of the following is from Forrester, John V. et al, The Eye Basic Sciences in Practice, Saunders 2002.

The cornea and lens must not only be clear but also maintain precise shapes to focus images on the retina. For animals living in both aquatic and terrestrial environments the problems are magnified. The cornea uses special structural molecules of precise size and arrangement to keep from scattering light. This structure is maintained by remaining slightly unsaturated with water due to active transport in the cells of the endothelium lining the inside of the cornea. This requires a constant energy supply for the epithelial cells to keep the pumping going. Similarly the lens must maintain a constant state of hydration requiring active processes. Problems in these active processes can be perceived by haziness or opacity of these tissues. The transparency of the cornea represents a very sensitive window into the functional state of the eye.

Another factor increasing the sensitivity of the eye to environmental conditions is the lack of direct blood supply to these active tissues. Movement of glucose and oxygen to these cells and wastes away from them requires diffusion and transport in solution in the aqueous fluid. Aqueous fluid is the fluid in the anterior chamber of the eye, between the cornea and lens produced by the ciliary body behind the iris and flowing out through the iris opening behind the cornea to drain out of the eye at the periphery of the cornea where it meets the iris. These passive transport processes can also carry drugs, damaging environmental compounds and products of metabolism that must be handled by the exposed cells. Aqueous fluid flow is also regulated to maintain the pressure inside the eye so it cannot be changed easily to accommodate changes in needs of the cornea. These tissues are on their own in maintaining homeostasis. In fact one of the signs of healing of penetrating injury to the cornea is growth of

blood vessels to the site of the injury to provide more efficient transport systems to aid the healing process. These cells sometimes need help.

Even the conjunctiva under the eyelids and around the eye is a very specialized tissue. It has minimal keratinization and abundant blood vessels very close to the surface. It more closely resembles mucosal surfaces of the respiratory or digestive systems than skin. It is closely integrated in its circulation with the aqueous flow from the eye.

All cells use similar energy producing pathways and mechanisms to handle damaging intermediates. For example the Cytochrome p450 system enzymes which act to begin the breakdown of drugs or toxins are present in ocular tissues (specifically the ciliary body) at about 5% of the concentration in liver cells. Antioxidant compounds and systems are very important in handling free radicals, peroxide and other damaging intermediates. A reducing environment is important in the cell. Oxidation is the enemy of living tissues. Glutathione is one of the important protective compounds. It binds to free radicals and then in an energy requiring step is regenerated to continue its protective function. "When the intracellular levels of glutathione are reduced in the cornea by one-third, the clarity of the cornea and its ability to pump fluid declines dramatically." From Forrester.

In summary the eye and all its structures are metabolically active. It uses transport mechanisms less efficient and controllable than tissues relying on blood circulation. It contains the normal mechanisms of energy production and utilization, detoxification of free radicals, and production of protective molecules like glutathione. It also contains the enzyme systems that break down drugs and toxins. However if the homeostasis is slightly disrupted the result may be dramatic with loss of clarity and potential damage from uncaptured free radicals or fluid changes from disruption of the normal pumping mechanisms that maintain clarity and shape.

Aquatic mammals in captivity are commonly afflicted by ocular changes ranging from slight loss of corneal clarity to severe inflammation and cataracts. A big question is why? What causes these changes and can the cause be eliminated or ameliorated? Are there many causes or a single thing? Are there general treatment modalities that can reduce the injury? Why are some individuals affected and not others?

A lot of work is done on ocular inflammation mechanisms to explain why human eyes are sometimes injured by such things as contact lenses and laser corneal surgeries. Some individuals undergoing laser corneal surgeries suffer from long-term inflammatory problems that are not seen at all in most people. Research has shown that measurement of glutathione levels in animal models of ocular injury can reflect the severity of the resulting inflammation. As discussed above glutathione is a protective molecule that binds with reactive molecules produced in normal cell processes. Glutathione is a reducing molecule so it can bind with many potentially oxidizing compounds and prevent them from oxidizing important cellular components. Providing similar protection are other molecules

such as ascorbate (vitamin C), vitamin E, vitamin A, Superoxide dismutase, catalase and peroxidase. All these compounds would be quickly used up by normal cellular processes but energy requiring enzymes regenerate them and further process the resulting conjugates.

Free radicals is a term describing highly reactive molecules produced by cellular processes, environmental effects such as ultraviolet light, and damaging compounds in the environment. Antioxidants is a term describing any compound that binds with and tames these damaging free radicals. Free radical oxidation damage is thought to be a big part of the cause of injury in aging, arthritis, and any inflammatory condition. Free radicals binding with normal cellular compounds such as phospholipids in cellular membranes produce compounds that act as messengers in the cell that produce secondary messenger chemicals that can stimulate the immune system. One example is arachidonic acid which when formed feeds the arachidonic acid cascade system to produce prostaglandins, leukotrienes and a host of messenger compounds. The resulting process magnifies the original injury many fold. The immune systems response may cause much more damage than the original injury. This is likely what happens to start inflammation in the eye that results in the pain and sensitivity to light seen in uveitis.

Sunlight can cause oxidation injuries which the eye must handle. Sunlight includes a broad spectrum of radiation. The atmosphere absorbs some of this radiation but is transparent to some ultraviolet light, visible light and infrared light. The shorter wavelengths contain the most energy and ultraviolet and blue light have the most potential for injury. Most of the ultraviolet in sunlight is UVA. UVB is most damaging to DNA and causes sunburn but UVA is not harmless. Studies on the Solar Disinfection of Drinking water can provide some insight into what factors may increase injury. See http://almashriq.hiof.no/lebanon/600/610/614/solar-water/unesco/ The authors give an excellent summary of the practical aspects of solar radiation which I will summarize.

First, the shorter wavelengths including UVA and blue light are scattered the most by the atmosphere. That is the reason the sky is blue and the sun looks red at sundown or sunrise when its light has to travel through more of the atmosphere. The scattering means the UVA intensity from indirect sunlight can be significant. Just because one does not look directly at the sun does not mean no UVA exposure. Since looking directly at the sun is uncomfortable most of the UVA exposure would be from indirect radiation. Second, clear water can absorb as little as 5% of UVA radiation per meter so light reflected from a light colored pool bottom could be marginally less injurious that direct sunlight. Third, they found that light blue or green colored containers were almost as good for disinfection as clear containers. Red or brown containers provided no disinfection. That means that white, light blue or green colored surfaces would maximize exposure by increasing that from reflected or transmitted light.

The mechanism of disinfection or biological injury by UVA radiation has

been found to be the production of oxygen free radicals and hydrogen peroxide as well as direct absorption of radiation by biological molecules. Skin injury by UVA has been found to be due the production of these oxidizers and UVA penetrates much deeper into tissues than UVB. In the transparent tissues of the eye UVA radiation could cause oxidative injury that accumulates over a lifetime. (see:http://www.sodis.ch/files/note8.pdf, and Svoboda et al, 2006) This article provides a current review of the mechanisms of injury from UV light in the skin. **http://publib.upol.cz/~obd/fulltext/Biomed/2006/1/25.pdf**

Mechanism of injury In Life Support Systems

In closed water systems oxidizing compounds are used for disinfection, reduction of growth of unwanted bacteria or algae, and reduction of unwanted color or odor. Oxidizing agents act by disrupting molecules and producing new molecules. If carried to completion biological molecules are oxidized to simple molecules such as CO2, H2O, NO3-. As an example Chlorine is often kept at about 1 ppm in marine mammal pools. These compounds are polar or ions that do not penetrate biological membranes so injury they might cause would only be expected on exposed surfaces and not inside the cells.

During this oxidation process intermediate steps may persist if oxidation is not completed. The possible compounds in these intermediate steps are legion. Some of them are more stable than the original molecules and may tend to persist. Consider the possibilities of 1 ppm chlorine in a pool with 5 ppm disssolved organic carbon. The intermediates must predominate, there is not enough chlorine to complete the process.

The original oxidizers in high concentration can cause significant injury, but the theory is that if no ozone can be measured, and if the chlorine or bromine levels are 1 ppm or less there should be no problem.

The problem is the intermediate compounds. Some of them are injurious directly but others require further processing to be dangerous. Carbon containing compounds and Nitrogen containing compounds can be produced. A good summary of byproducts of disinfection can be found at: http://www.greenfacts.org/en/water-disinfectants/index.htm#4 . Another interesting discussion is a powerpoint presentation by Dr. Steve Hrudey www.waterquality.crc.org.au/workshops/DBP_hrudey.pdf. Some of the "by-products of disinfection" are non-polar compounds that can penetrate epithelial surfaces and are easily absorbed through the skin or by inhaling them in the air as they escape into the atmosphere. Some of the compounds are directly irritating.

The simplest nitrogen containing compounds are chloramines. Monochloramine is used as a disinfectant in some drinking water systems. It is a fairly stable compound and in low levels probably not too irritating. Dichloramine is the next step in chloramination and may cause some irritation. The worst chloramine is nitrogen trichloride. It is not very soluble in water, unstable and reactive with organic material. Recent studies suggest asthma in children may be related to this compound with exposure occurring at swimming pools. It forms

more in acid environments which may occur in thick biofilm layers or deep in bio-filters or in sand filters. The "chlorine" odor noted in some chlorine pool environments may be from this compound. It is severely irritating to eyes and mucus membranes. It breaks down within 24 hours in water solution but that is long enough for it to cause injury to mucus membranes or respiratory surfaces exposed to it.

An even simpler appearing compound is cyanogen chloride, which can arise from chlorination of histidine containing proteins. Histidine is an amino acid which could be found in organic wastes. It is also a very reactive and irritating compound. See www.aquaticsintl.com/2007/oct/0710_techtalk.html

The simplest carbon containing by-products are methane compounds. Trihalomethanes is a term that includes Chloroform, bromodichloromethane, dibromochloromethane, and bromoform . Two representative compounds are trichloromethane (chloroform) and tribromomethane (bromoform). These are known to cause liver injury and the mechanism has been worked out. A study of human swimmers in chlorinated indoor swimming pools found chloroform in blood plasma samples at about 4% of the concentration in the pool water. In the discussion they say that more active swimmers had higher levels presumably due to more respiratory absorption. Of course none of these swimmers stayed in or near the water as long as animals living in the water. Skip Young at Vancouver Aquarium communicated to me that when they measured bromoform in their beluga pool water, They could also measure it in the air over the pool and in significant amounts in the animals. This was in an outdoor pool. Indoor pools might be worse. These compounds are heavier than air and in still conditions will stay near the water surface.

The possible mechanism of injury from these trihalomethane compounds starts with initial oxidation by Cytochrome p450 enzyme systems in the cells. In the eye this would occur in the ciliary body which is located around the periphery of the lens and pupil. For chloroform the initial oxidation produces an unstable product that breaks down to Carbonyl chloride (CCl_2O) and HCl. Carbonyl chloride can react directly with other molecules or even with water to produce CO_2 and 2 HCl. Carbonyl chloride is also known as phosgene and has been associated with industrial accidents and has been used as a poison gas. Damage comes from either the phosgene or the resulting 3 molecules of HCl. When everything in the cell is normal glutathione will bind with the phosgene and reduce it and pH regulating processes will counteract the HCl (hydrochloric acid).

If the amounts of these compounds are low enough the damage is negligible and no effects would be seen, but if the dose is too high, if the cell is already under stress or if the individual is more sensitive to these compounds the result is oxidation and damage of cellular components. This brings us back to the stimulation of the immune system that can result from products of oxidation of things like arachidonic acid as mentioned above. Studies of chloroform metabolism in mice show marked differences in different strains of mice so

individual susceptibility or previous insult to the cells could easily explain why not all animals are affected.

More complex carbon by-products can also be produced. Some of these are not volatile so if not removed they could build up to high levels. See Hrudey's presentation for some of these. The volatile compounds can be partially controlled by good aeration of the water and good ventilation over the water. The studies on Vancouver Aquarium's Beluga pool were done on an outdoor pool so even with an outdoor environment the volatile compounds can be a problem.

Other organs

I have mainly been interested in the eye but any organ or tissue containing Cytochrome p450 enzymes could activate these compounds. The organ most affected in research studies of chloroform on different species seems to correspond with the levels of cytochrome enzymes. Liver and kidney are most commonly reported as injured. Even slight injury in the eye can be seen when the cornea becomes edematous. Injury in internal organs would be much less apparent. A recent article on skin injury by ultraviolet radiation mentioned above describes stimulation of Cytochrome p450 systems by exposing keratinocytes in tissue culture to Ultraviolet light and mentions that this might increase bioactivation of polycyclic aromatic hydrocarbons and other environmental pollutants. (Svoboda, 2006.) Could this also happen in the eye?

Safe levels

For human domestic water supplies WHO recommends less than 100 ug/l. EPA sets a MCL (Maximum concentration level) for trihalomethanes of 0.08 mg/l. (see www.epa.gov/OGWDW/mdbp/qrg_st1.pdf) The WHO levels are based on the assumptions of a person drinking 2 liters of water and taking one shower or bath per day. The amount absorbed or inhaled while bathing is about the same as in 2 liters of water. That would yield a daily dose of 400 ug. For a person my size (95 kg) that would be 4.2 ug/kg. Aquatic animals have a higher exposure because of contact with the water and respiration of the compounds as they move into the air from the water. Trihalomethanes are more soluble in lipid than water so they may concentrate in the bodies of animals. The Studies in Vancouver suggest that the animals could have significant concentration compared to the concentration in the water. "The concentration of chloroform in fish tissues is expected to be somewhat higher than the average concentration of chloroform in the water from which the fish was taken." (Vincoli 1997) Recent measurements of our local tap water gave a measurement of 31.5 ug/l, well within the required limits but if the animals equilibrated with this concentration they would have about 8 times the allowed amount per kilogram. In a recent measurement of a mammal pool 160 ug/l trihalomethanes was found which would be 38 times the allowed amount per kilogram.

This still does not give an accurate idea of how much might be harmful. Eliminating exposure and seeing improvement or regularly measuring the amount in the water and correlating that with observed problems would help to

determine safe levels.

A complicating factor could be amounts in the food. When these compounds are measured in food stuffs, the highest levels reported are for cod fillet from Norwegian waters downstream from paper plants. Ofstad 1981 found chloroform of up to 2200ug/kg of fat. This led them to estimate a concentration factor of 200 to 500 times when comparing fish concentration to seawater concentration.

Measuring these compounds in water, air, blood or foodstuffs requires specialized equipment and is not commonly done in captive animal pools or foodstuffs. The compounds in question are also volatile and careful handling of samples sent for analysis is required for accurate results.

Summary:

1. Chlorine, ozone or bromine used to disinfect water oxidize compounds in the water. (Hrudey lists some by-products from ultraviolet treatement as well)
2. If there is a lot of dissolved organic material in the water, by-products of disinfection are produced.
3. Some of these compounds are non-polar, stable compounds including chloroform and bromoform which can vaporize into the air above the water.
4. The non-polar nature of these compounds mean they can penetrate epithelial surfaces directly or through respiratory surfaces when inhaled. The conjunctival surface resembles respiratory epithelium in its lack of keratinization and close proximity of blood vessels to the surface and is probably similar in its ability to absorb these compounds.
5. Activation of these compounds to harmful intermediates occurs when the normal cell processes metabolize them. The eye does contain the enzymes necessary to activate these compounds.
6. Observable damage is related to concentration of these compounds, sensitivity of the individual, and current conditions in the organs of the affected organism.
7. Constant presence of these compounds means a constant metabolism and production of potentially harmful compounds that even in the absence of observed injury may hasten normal aging and degradation processes.
8. Ultraviolet light also has damaging effects on the eye and may actually magnify the effects of these compounds.

Conclusion. These compounds can be harmful and their presence should be monitored and minimized.

Implications for treatment of ocular problems

I am working on a literature review concerning possible treatments suggested by this mechanism of injury. Ensuring adequate amounts of Vitamins A, C, and E seems rational. Aniti-oxidants that might help maintain the normal reducing environment in the cell might be helpful. Plants also face oxidative damage and many plant derived anti-oxidants have been described. A recent article describes the effect of grape seed polyphenols on canine lens epithelial

cells. Barden, 2008. Compounds that block steps in the arachidonic acid cascade might be expected to be helpful. Corticosteroids have been seen to be effective in alleviating symptoms but attacking the problems at an earlier stage might be better. Some drugs such as cimetidine inhibit the cytochrome p450 system enzymes and might be helpful if a temporary spike of chlorine or by-products of disinfection were seen. The mechanism would be to reduce activation of chloroform and bromoform. This does not seem rational for long term use. A recent article describes the benefit of silymarin in a tissue culture system for studying uv injury. Silymarin markedly reduced oxidative injury and inflammation. (Svoboda 2007)

All these are speculations but I can say that After the pool water change a year ago at Aquarium of NIagara keeping the phosphorus lower using lanthanum which allows decreasing the maintenance levels of chlorine, and increasing the maintenance dosage of Vitamin C and Vitamin E seems to have helped. The sea lions corneas are much clearer, it is easier to appreciate the flattened area on the cornea and they are more comfortable. The prednisolone acetate dosage frequency on Squirt a female sea lion with keratitis and recurrent uveits has been reduced from three times daily to once every other day.

Increased amounts of Vitamin C can increase absorption of iron from the diet. (Hallberg et al 1989) Some marine mammals have very high levels of iron in liver tissue at post mortem so vitamin C supplementation for long periods may not be advised.

Research implications.

Measurement of glutathione levels would be informative but is not that easy in the living eye. Possibly measurement in tears could be informative. If eyes required surgical intervention saving tissues for glutathione measurement would be of interest. Isoprostanes are produced by reaction of arachidonic acid products with free radicals and can be measured in vivo. Measurement of these in blood or tears might provide a measurement of injury.

Simple correlation of clinical signs with levels of by-products of disinfection might provide epidemiological evidence of presumptive injury. Measurement and identification of the exact compounds is not a trivial thing and focusing on preventing their formation seems more appropriate

Determination of the exact compounds in the water seems like a good idea. This is not as easy as it sounds. The water in a life support system for marine mammals who are fed a large amount of food and with various filtration and disinfection processes represents a very complex matrix of compounds. A standard water test for purgeable organic compounds will measure Trihalomethanes and is used in commercial water labs but it only measures volatile compounds. That is compounds that will go off in a carrier gas. Non-volatile compounds would be missed by this test. see Zweiner etal 2007 for a discussion of the variety of techniques used to try to determine the compounds in swimming pool water.

There are a lot of studies of chlorinated swimming pools with a wide range of reported by-products found. Several of these studies used simulated pools with precise addition of specific compounds to see what would be produced. These are much simpler systems than those we deal with having feces, urine and other body wastes as a normal materials entering the pool. I have listed some references for these studies in the references.

Developing a less expensive instrument for monitoring TOC (see below) would allow better control of life support systems to minimize by-product formation.

Management implications

Minimizing the concentrations of by-products of disinfection can be approached in several ways including prevention of formation, aiding removal, and achieving destruction of them. Preventing formation would require either carrying oxidation to completion, reducing the oxidation, or reducing the compounds being oxidized. In domestic water systems measurements of the total organic carbon (TOC) is done before adding oxidizers. TOC is a good measure of material in the water. Levels of TOC should ideally be less than 2 mg/l before chlorination or ozonation to prevent exceeding the allowable limits for trihalomethanes. Measuring TOC in fresh water can be done fairly easily but chloride in salt water interferes with the measurement. A discussion of the measurement of TOC by William Lipps can be found at (http://ezinearticles.com/?Total-Organic-Carbon-Analysis&id=1155673) Measuring TOC to see how effective the life support system was at removing these compounds would be very helpful but equipment for measuring this parameter in salt water is expensive. A simple instrument to measure TOC using recently available CO2 monitoring chips might be a big help.

Reduction of TOC is possible with efficient foam fractionation. Flocculants may also be helpful and are commonly used in domestic water treatment systems. Flocculants require increased backwashing of filters and proper design for use to prevent clouding of water or material settling on the bottom of pools.

Zweiner et al,2007 discusses membrane filtration as a way to remove some of these compounds or precursors from swimming pool water.

Measuring of nitrogenous wastes is another way to see how much is in the water that could lead to by-product formation. Biological filtration, denitrification, and water changes can control nitrogen levels.

Reducing levels of phosphates can be helpful. Phosphates represent a limiting nutrient in the growth of algae and bacteria so reduction reduces the need for chlorine or ozonation to reduce algae or bacterial blooms. Phosphorus can be reduced by precipitation with lanthanum chloride and subsequent filtration but can markedly increase backwash requirements.

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Personal communication
Skip Young, Vancouver Aquarium, Vancouver, BC Canada

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Trihalomethane in a Pinniped Rehabilitation Facility

Alex Chow (1), **Joelle Sweeney** (2), **Ronald Smith** (2), **Jonna Mazet** (3)
(1) Department of Land, Air, and Water Resources, University of California, Davis, CA 95616, USA, atchow@ucdavis.edu
(2) The Marine Mammal Center, Marin Headlands, 1065 Fort Cronkhite, Sausalito, CA 94965, USA, SweeneyJ@TMMC.org, SmithR@TMMC.org
(3) Wildlife Health Center, University of California, Davis, CA 95616, USA
jkmazet@ucdavis.edu