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# Pregnancy Associated Breast Cancer: an Analysis of Fetal Treatment Risk Melissa Barnett

## Abstract

There are several viable treatment options for patients with PABC considered un-harmful to fetal development. Trastusumab, or Herceptin, targets HER2 protein and successfully combats aggressive breast cancer. In standard doses, it appears to be safe to the fetus even when administered during the first trimester. A likely side effect of Herceptin is anhydramnios, which can be monitored for throughout the stages of a pregnancy (Shrim et al. 2008). Anthracyclines, commonly used in chemotherapy, appear to be non-toxic, and have been used to successfully cure PABC patients in their second and third trimester. However, first trimester spontaneous abortions are documented to increase dramatically in anthracycline receiving PABC patients (Ring A. E. et al. 2005). Taxanes, such as paclitaxel, docetaxel, and vinorelbine, are microtubule agents that are highly active against breast cancer. As cytotoxic drugs they can possibly disrupt organogenesis and their administration should therefore be delayed until after the first trimester. Taxanes as well as anthracycline treatments should also be halted three weeks prior to delivery to prevent the occurrence of neutropenia, a decrease in white blood cells (neutrophil) that raises the risk of infection, and allow for regeneration of platelet count in the bloodstream (Mir O. et al 2008). Breast surgery can be safely performed at any point of a pregnancy although it is recommended to be postponed until after the first trimester. External beam radiation and hormone therapy are both documented to have negative effects on a developing fetus and therefore should not be considered as possible treatment options for PABC patients. In diagnosing and assessing PABC, ionizing radiation should be replaced by chest x-rays accompanied with proper shielding to protect the fetus. Sonograms appear to be more effective in diagnosing breast cancer than mammograms, because of the changes in pregnancy related breast density. Although PABC treatment is more complicated than standard breast cancer treatment, treatment during pregnancy has been proven to be a feasible and recommended option (Loibl S. et al. 2005).

## Introduction

Pregnancy Associated Breast Cancer (PABC) presents a treatment plan quandary. The diagnosis appears to present a conflict between the mother and fetus' health. The challenge facing the health care provider is how to balance the mother's cancer care, with the fetus' prenatal care. The aim of the physicians is to provide optimal treatment to the mother so as to maximize her chances of survival whilst minimizing risk to the fetus. This will often require an extensive healthcare group consisting of an OBGYN, a medical oncologist, radiation oncologist, pediatricians, and sometimes a breast surgeon. This complex scenario arises in approximately three percent of breast cancer cases. However, this statistic is likely to increase as women are increasingly delaying pregnancy until a later age (Ring A. E. et al 2005). It is therefore crucial to gain a clear understanding of this commonly misconceived situation.

It has generally been the public supposition that a woman with cancer who was found to

Melissa Barnett, BS '09 graduated Touro with a degree in Biology. She is currently in a B.S.N program at Seton Hall University. be pregnant would have to choose: her life or her baby's. However, with the publication of many more case studies it has become evident that, at least in regard to breast cancer, this is not the case. It has been shown through controlled case studies that the prognosis for women who undergo voluntary abortion is not more favorable than for those women who continue their pregnancy (Gottlieb S. 1999). Analysis of the treatment options will support the recommended course of treatment to the highest possible standard despite the difficulties in the diagnosis and treatment of breast cancer.

The major difficulties in diagnosing breast cancer in a pregnant woman arise from the physiological changes that occur in the breast, and from the inability to use certain staging investigative procedures that are normally used in non-pregnant women. Mammograms have been shown to be relatively ineffectual in pregnant women because they are too hard to interpret due to the increased breast density in pre-menopausal and pregnant women. Therefore, sonography is more often the method of choice to diagnose PABC. There are also many routine staging investigations for breast cancer that use ionizing radiation, which should certainly not be used on a pregnant woman within the first trimester. In addition, not enough proof is available to support the safety of this technique for the fetus even during the later stages of pregnancy. Instead, chest X-rays are considered relatively safe in pregnancy when proper shielding is used (Ring A. E. et al 2005a).

Treatment for breast cancer is manifold. There are various options and each case must be individually assessed to design a treatment plan that will work. The basic care recommendations are breast surgery, radiation, systemic chemotherapy and other cytotoxic drugs, hormone therapy, and supportive care. Breast surgery may be performed throughout pregnancy, however, because there is a greater chance of spontaneous abortion within the first trimester. It is recommended that surgery be postponed until the second or third trimesters and that fetal activity be monitored throughout surgery. External beam radiation is contraindicated during pregnancy because of the serious risks associated with fetal exposure, such as mental retardation and other malformations. Hormone therapy should be postponed until after delivery, because defects have been seen in mice studies; there have been reports of Goldenhar's syndrome and of ambiguous genitalia in children born to women exposed to hormone therapies. Supportive care for anthracycline-based chemotherapy is safe after the first trimester (Loibl S. et al 2005). The use of systemic chemotherapy is dependent on the drug type and the trimester in which it is used. Research on cytotoxic drugs is ongoing and is mostly confined to case studies and individual case reports. There are several specific drugs that deserve further analysis. They are: anthracycline-based Trastuzumab Herceptin, drugs such the doxorubicin, or as cyclophosphamide, and fluorouracil combination, and taxanes or anti microtubule agents such as, docetaxel, paclitaxel, and vinorelbine.

## Trastuzumab

Trastuzumab, generically also known as Herceptin, is an IgG1 monoclonal antibody that is directed against the 2(HER2)protein. The HER2 protein is a member of the epidermal growth factor receptor family. In recent years, researchers have found that in certain breast cancer cases this protein can be over expressed. When this occurs it causes increased cell growth and proliferation leading to a much more aggressive breast cancer. This type of cancer was considered one of the more fatal breast cancers until the advent of drugs like trastuzumab that target this protein. The drug has been shown to significantly improve outcomes in patients with HER2-positive breast cancer (Shrim A. et al 2008). Because HER2 over-expression has been found in up to 35% of breast cancer cases in young patients (<35 years), it is probable that it will be concurrent with PABC. Therefore, trastuzumab may be necessary during pregnancy, and the risk factors must be analyzed.

Epidermal growth factor receptors seem to be important in fetal development. HER2 is expressed in embryofetal tissue. Therefore, it might be critical to fetal development. It has also already been demonstrated that transplacental transfer of maternal monoclonal IgG1 antibodies is possible. It is therefore reasonable to expect that trastuzumab might cross the placental barrier (Witzel I. D. et al 2007). In this regard, however, animal research has not exhibited any fetal harm. Reproduction studies were conducted in monkeys at 25 times the weekly human dose, and no decrease in fertility or fetal harm were noted. This study indicates that more research is required on the exact mechanism of action of the HER2 protein in fetal development (Shrim A et al 2008).

There are several reports of case studies where trastuzumab was part of the treatment in a pregnant breast cancer patient. With this particular drug, there does not seem to be increased harm to the fetus if it is administered during the first trimester. This conclusion is drawn from the case of a patient presenting with metastatic breast cancer who was treated with trastuzumab at normally recommended doses throughout the first 24 weeks of her pregnancy. She underwent a caesarean section at 37 weeks and was delivered of a healthy baby. However, in another case a woman received 25 cycles of trastuzumab before becoming pregnant, then nine more cycles during pregnancy, and in this case, the fetus was affected. Because the mother presented at 27 weeks pregnant with severe vaginal bleeding, the baby was delivered preterm at 28 weeks via caesarean section. The baby suffered several disorders and died 21 weeks later due to multiple organ failure. This second case does not specifically indicate that trastuzumab caused the irregularities in the fetus because they could be related purely to prematurity. However, in this case the drug dose to the mother was much higher, which could indicate that despite reassuring findings in other research, higher doses of the drug could affect fetal development and result in an impaired prognosis of the newborn.

Treatment after the first trimester does not appear to result in long term fetal harm. There are two case studies of women with metastatic breast cancer who underwent trastuzumab treatment during the second trimester and had no reports of any disorders. One woman was treated until 24 weeks gestation, and the other started treatment at 27 weeks gestation and delivered a healthy male at 34 weeks. The other case reports of women treated with the drug during the second or third trimesters all document a condition called anhydramnios, a lack of amniotic fluid. One patient was treated with trastuzumab until 23 weeks when anhydramnios was diagnosed, and treatment was discontinued. The anhydramnios resolved itself after discontinuation of the drug and a healthy baby was delivered at 37 weeks. Two other patients underwent trastuzumab treatment in their second and third trimesters and suffered anhydramnios. In one case the mother received treatment from her 26<sup>th</sup> week to her 32<sup>nd</sup> week when a caesarean section was performed. The baby showed signs of several dysfunctions, however these were probably related to its prematurity, and were resolved. The child's further development was normal. In the second case, a mother treated at weekly intervals until 35 weeks gestation when labor was induced, showed persistent anhydramnios throughout treatment. However, she was delivered of a healthy baby. It appears that trastuzumab treatment is possible during pregnancy

with close monitoring of the fetus, especially monitoring of fetal renal function and amniotic fluid volume (Witzel I. D. 2007).

### Anthracycline Based Chemotherapeutic Drugs

Anthracyclines are a drug species that damage DNA through different mechanisms. One of these mechanisms is topoisomerase IIalpha poisoning. Since topoisomerase is over-expressed in rapidly growing tissues, it can lead to poisoning which can cause severe damage to the embryo or fetus. However, only low concentrations of anthracyclines have been found in fetal tissues and their cytotoxic potential is unknown. Also, existing case research appears to prove that anthracycline use during pregnancy is not toxic to the fetus, especially when not used during the first trimester. Anthracyclines are one of the most used and effective types of chemotherapy for breast cancer patients, and it is therefore useful to understand their effect on the fetus. Anthracyclines such as commonly used doxorubicin do not easily cross the placenta because of their high molecular weight. They are also substrates of the P-gp, a placental drug-transporting glycoprotein. This glycoprotein is of great importance in limiting the fetal penetration of potentially harmful compounds. Indeed, it has been noted that after intravenous injection of anthracyclines only barely detectable concentrations can be found in the fetus (German N. et al 2003). It would appear that anthracyclines are not likely to be toxic, however specifically with anthracycline-based chemotherapy, like the combination previously mentioned, the date of administration has an effect on the risks to the fetus.

The first trimester of pregnancy is the time of organogenesis, and the fetus is therefore at its most susceptible. When chemotherapy is administered in the first few weeks of pregnancy there is a very high rate of spontaneous abortion. Throughout the rest of the first trimester there is still a chance of spontaneous abortion and there is the additional risk of fetal malformations. The risk of fetal malformations when chemotherapy is administered within the first trimester is up to 17% as opposed to 1.3% in the two subsequent trimesters. In two different studies three women treated during the first trimester all suffered spontaneous abortions. However, one woman in a study from the University of Texas M.D Anderson Cancer Center was treated at 11 weeks and experienced no apparent adverse effects. As a result of these significant risk factors to the fetus, chemotherapy is usually avoided during the first trimester (Ring A. E. et al 2005b).

Treatment with anthracycline-based chemotherapy in the second or third trimesters does not appear to increase the risk of malformations. There are three large case study series published about this treatment. In a U.K study 16 children of mothers exposed to anthracyclines showed no congenital malformations. In a French survey 18 children were identified as having been exposed and none of them presented malformations. The M.D Anderson series reported 23 children exposed none of whom had malformations. However, when the doxorubicin dose exceeds 70 mg/mg2 per cycle it has been shown that risk of severe fetal toxicity increases 30 fold. There is also a specific risk of cardiotoxicity associated with anthracycline toxicity later in pregnancy, although this has not been observed in any case studies. While chemotherapy appears to be safe during the second and third trimesters, it is advisable to stop treatments at least three weeks prior to delivery, in order to prevent neonatal infection, neutropenia and to ensure that the mother's blood counts are optimal at delivery (German N. et al 2003).

## Taxanes

Among emerging therapeutic options for breast cancer are antimicrotubule agents or taxanes such as paclitaxel, docetaxel, and vinorelbine. These drugs display a high activity against breast cancer, and it is therefore necessary to understand how they may be used in conjunction with pregnancy. Because these drugs are relatively new the available research is confined to case studies, some animal data and knowledge of the nature of the drug.

Paclitaxel, docetaxel, and vinorelbine are mainly metabolized by cytochrome P450 isoforms. Because the maturation of these cytochromes mostly occurs in the first weeks of neonatal life, fetus' cannot metabolize these drugs and would therefore be highly susceptible to their toxic effects if a transplacental transfer were to occur. These drugs would actually be expected to cross the placenta because they have a relatively low molecular weight and are highly lipophilic. However, they are highly bound to plasma proteins and plasma protein binding is increased during pregnancy, which would potentially lead to a decreased active fraction of these drugs. These drugs are also substrates for the Pgp which has been found in high expression in the placental Pgp serves to protect the fetus from a broad range of xenobiotics. The placental Pgp therefore appears to reduce transplacental transfer of antimicrotubule agents, making their clinical use possible in the second and third trimesters (Mir O. et al 2008).

Animal studies have shown paclitaxel and docetaxel to be toxic to the fetus. This has not been evident in human case studies, although it is recommended to postpone the use of these cytotoxic drugs until after the first trimester. This is firstly, to prevent spontaneous abortion, and secondly, to prevent disruption of organogenesis which could lead to malformations (Ring A. E. et al 2005a).

There are four reports of PABC treated with Paclitaxel. The median maternal age in the reports is 36 years. Treatment was started during the second trimester in 3 cases and during the third in one case. There were no documented complications, and three out of the four patients underwent caesarean section with no reported malformations. In all cases there was a follow up with a median of 16 months, where all the children appeared healthy and normally functioning. However, in three cases, women treated with paclitaxel combination therapies in the last three weeks before delivery, exhibited mild anemia in two fetus' and one case of grade four neutropenia.

In a literature review study there were six reports of patients with breast carcinomas who were pregnant and treated with docetaxel. The median maternal age was 34.5 years and docetaxel was only administered by itself in one case. Treatment was initiated during the second trimester in three cases, and in the third trimester in three cases as well. One fetus developed hydrocephalia only before treatment with docetaxel, but remained stable thereafter. The child developed normally and was considered healthy at 28 months. In the other five cases no malformations were reported. All the offspring appeared healthy with a median follow-up of 17.5 months.

There were five identifiable cases of PABC treated with vinorelbine. Vinorelbine is generally associated with other cytotoxic drugs such as fluorouracil, csiplatin,or trastuzumab. The advantage of using vinorelbine is primarily its lower rate of side effects, which is especially a factor in pregnant women. The median maternal age of these five reported women was 32

years. The drug treatment was started in the second trimester in two cases and during the third trimester in three cases. There was no grade three or four maternal toxicity reported, and there were no fetal malformations. All the children were reported healthy at a 23 month median follow up (Mir O. et al 2008).

Researchers and medical practitioners have voiced the safety of using taxanes during the second and third trimesters of pregnancy based on the data available. It is agreed that the use of these cytotoxic drugs should be stopped for three weeks prior to delivery in order to prevent neutropenia, and to allow the mother's blood to regenerate its platelet counts. Further research and analysis is required to investigate the possibility of low birth weight being associated with the use of taxanes during pregnancy.

### Conclusion

This area of study is an ongoing development and does indeed require much more investigative research. One of the principal problems standing in the way of a clearer understanding of placental transfer of drugs is the inability to ethically analyze live placental tissue. In order to observe and document the way in which these drugs react within the placental barrier one would need to inject the drug into the placental tissue: not a feasible option. When the placenta exits the uterus after delivery the tissue dies immediately, and the activity of the drug within its tissues would be totally different then when it is in its living state. This means that essentially all the understanding and chemical analysis of the way drugs pass through the placenta is conjecture. Furthermore, there is no way to test human subjects with increased doses of these drugs to observe their potential toxicity, and animal testing has given results that do not match human experience. Many women abort a pregnancy in the face of a serious diagnosis causes less real life cases to be analyzed.

This last reason is the principal impetus behind this paper. The ability to treat cancer during pregnancy is generally believed to be a modern, groundbreaking concept. In reality, the cases of women successfully cared for while they were pregnant date back to the 1970's. Yet still today, the public is just beginning to understand that one need not make that most painful of choices (Paul P. 2008). The situation presented by a breast cancer patient who is also pregnant and wishes to continue their pregnancy, is still unusually sensitive. The idea of harming a fetus to benefit the mother is difficult. The majority of women who do choose to continue their pregnancy, even today, generally do so because of religious beliefs, not because they think they and their babies will survive and be healthy. It is this public misconception that must be righted. Women should not be allowed to feel self-doubt or nervousness, because they are opting to be treated while continuing their pregnancy. Although research on breast cancer specifically is most extensive, based on its higher rate of correlation with pregnancy, the examples made in this field of cancer also apply to various other less documented cancers. It is crucial that further research and analysis, such as the ongoing study at the MD Anderson Cancer Center, continue and are publicized, so that unborn children need not be lost, and so that mothers should not be forced to make an impossible choice.

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# <u>Clostridium difficile</u> Associated Disease (CDAD) Rivka H. Borger

#### <u>Abstract</u>

*Clostridium difficile* bacteria (*C. difficile*) are a spore-forming species of bacteria that lies dormant in the colon, in the presence of normal intestinal flora. Due to overuse of certain antibiotics,



Figure 1 –Endoscopic view of classic *C difficile–* associated pseudomembranous colitis (Hull and Beck, FMC, 2006)

normal intestinal bacteria may be depleted, and combined with other possible risk factors, allow *C. difficile* bacterial spores to develop into active, infectious, and extremely resistant toxin-producing bacteria. The toxins cause severe damage and inflammation to the intestinal wall that can result in gastrointestinal discomfort and severe pseudomembranous enterocolitis that must be treated with a low-risk *C. difficile* targeting defense.

#### Introduction

*Clostridium difficile* bacteria (*C. difficile*) are a spore-forming species of bacteria that lie dormant in the human colon, in the presence

of normal intestinal flora. It is a commensal bacterium in a minority of the population. Smaller numbers do not develop into significant disease. (Nation Master Encyclopedia, 2005) Most commonly due to overuse of certain antibiotics, normal intestinal bacteria may be depleted, and through nutrient competition, allow *C. difficile* bacterial spores to develop into active, infectious, and extremely resistant toxin-producing bacterial overgrowth. The toxins cause severe damage and inflammation to the intestinal wall resulting in a wide range of disease, but most commonly - severe enterocolitis. (Merck Manual of Diseases, 2006)

The following discourse will detail the morphology, mechanism of infection, causes, diagnoses, treatment, and prevention of *Clostridium difficile* Associated Disease.

## Clostridium difficile Bacteria

*Clostridium difficile* is a Gram-positive anaerobic rod-shaped bacterium (Kunkel, 2006). The species was named 'difficile' because it was initially hard to culture (Schroeder, MD, American Family Physician, 2005). It belongs to the *Clostridiaceae* Family and genus *Clostridium*, which form spores. They are motile bacteria that are numerous through nature, particularly soil. *Clostridium* show optimal growth at human body temperatures, but can exist under stress in the more tolerant spore form. (Nation Master Encyclopedia, 2005). The spores are resilient and resistant to high temperatures. Early studies demonstrated that *C. difficile* could be isolated from the gastrointestinal tracts of most neonates, identifying it as a commensal organism (Schroeder, 2005). It is found as part of the normal

intestinal flora, and passed out with the feces of those with *C. difficile* spores in their intestines. (EMIS and PIP, 2007)

*Clostridium difficile* produce at least two exotoxins: an enterotoxin (Toxin A) and a cytotoxin (Toxin B). They can kill cells by altering the apical membrane permeability of the mucosal cells of the intestinal wall, and are subsequently responsible for severe inflammation possibly leading to enterocolitis (Gerding et al, 1995). Another toxin, binary toxin, has been identified without a role in CDAD infection. (Nation Master Encyclopedia, 2005)

*C. difficile* can be passed through fecal matter and spores can lie almost anywhere in the environment.

*Clostridium difficile* is considered a member of infant intestinal flora as up to 50% of infants carry asymptomatic *C. difficile* in their intestinal tracts. It can grow to high numbers with elevated toxin



(Wiggs, University of Waikato, 2007)

production with no harmful effects to infants. (Bongaerts and Lyerly, 1996)

The normal intestinal flora under typical conditions keep the spores of *C. difficile* inactive by outnumbering them in strength. Upon depletion of normal flora, the dormant inactive spores develop into active, infectious, and resistant bacteria – replacing the lost bacteria due to imbalance (Merck Manual, 2006)

More recently, *C. difficile* infections are more rampant – severe, extremely resistant, and easily recurrable. (Kelly and LaMont, 2008). It is the most frequent etiologic basis for healthcare-associated diarrhea and a common cause of antibiotic-

associated diarrhea (AAD), accounting for 15-25% of all AAD episodes (Center for Disease Control and Prevention (CDC), 2004). There have been increasing rates of CDAD-associated mortality, and this can be due either to elevated risk population or identification of new highly virulent strains of the disease. (Center for Disease Control and Prevention (CDC), 2005).

Additionally, two studies have proven *C. difficile* to be the most frequent cause of nosocomial diarrhea. Multiple studies demonstrate the link between *C. difficile* and noscomial infection – many cases arise from contamination of hospital environment (Gerding, et al, 1995).

*Clostridium difficile* was first linked to disease in 1978 when it was identified as primary causative agent of pseudomembranous colitis. It can cause disease ranging from minor gastrointestinal



discomfort to severe enterocolitis, toxic megacolon, or death. (Sunenshine and McDonald, 2006)

#### **Pathogenesis**

Inflammation develops when environmental spores ingested infect the patient. Spores can survive the low pH of the gastrointestinal tract and survive well in the anaerobic environment of the large intestine, allowing for germination. Upon depletion of normal intestinal flora, C. difficile spores no longer have to compete with other microbes,

allowing them to thrive to full capacity, growing to large numbers – approximately 10<sup>8</sup> bacteria per gram of feces. In the absence of colonic flora depletion, colonization of *C. difficile* will be asymptomatic and harmless (Bongaerts and Lyerly, 1997).

Antibiotic overuse is the most common form of intestinal flora depletion. The inactive spore from of *C. difficile*, once held in check by nutrient competition, becomes the dominant intestinal species. It is transformed to its infectious form, producing toxins that inflame and damage the intestinal mucosa.

Inflammation results in hyperleukocytosis, influxing towards the colon. Colitis is variable in degree, from mild to extremely severe. In pseudomembranous colitis, a more severe form, toxins destroy the tissue of the intestinal epithelia until the tissue falls off, mixes with leukocytes in pus discharge; giving the appearance of a white membranous patch on the inner intestinal lining. Some patients infected with *C. difficile* do not develop colitis, but are carriers of the disease, able to transmit it to other high-risk individuals.

*Clostridium difficile* bacteria's capacity to form spores allows the organism to persist in various areas of the environment. Additionally, the spores can be spread through fecal contamination, infecting at-risk individuals. (EMIS and PIP, 2007) Transfer can occur via the hands of healthcare workers as well. (Center for Disease Control, 2008). Infection used to be limited to elderly hospitalized individuals but has grown rampant more recently. (DeNoon, 2006).

After colonic colonization, *C. difficile* bacteria carry out metabolic activities similar to other anaerobic organisms. The bacteria use some monosaccharides (i.e. glucose, fructose, mannitol, mannose, and xylitol), but not disaccharides (i.e. lactose or sucrose), oligosaccharides, or polysaccharides (i.e. starch). *C. difficile* uses substances not found free in nature, such as N-acetyl-glucosamine and N-acetyl-neuraminic acid, by using extracellular hydrolytic enzymes to degrade substrates for nutrition. It can then obtain carbohydrates, amino acids, and related nutrients allowing the bacteria to thrive in the intestine. With the depletion of normal flora, there are more nutrients available for the bacteria to thrive on. Additionally, when a large amount of resident intestinal bacteria are destroyed, it allows *Clostridium difficile* exposure to previously hidden sites of the intestine. They can be then used for microcolony formation or receptor sites for toxins in toxigenic strains. As the organism grows, it becomes harder for normal flora to replace it since the bacteria produces inhibitory metabolic products, such as p-cresol, ammonia, and isocaproic acid – all inhibiting growth of normal flora, and disturbing intestinal epithelial cell membrane function. (Bongaerts and Lyerly, 1997)

Pathogenic strains of *Clostridium difficile* cause enterocolitis primarily through the production of toxin A (enterotoxin) and toxin B (cytotoxin). Both toxins are capable of stimulating proinflammatory cytokine production implicated in pseudomembranous colitis infection. Toxins act by altering regulation of cytoskeletal protein, leading to cell rounding and cell death. (Hull and Beck, College of Family Physicians of Canada, 2004) The toxins cause leukocyte chemotaxis and the upregulations of inflammatory mediators such as cytokines, producing colonic inflammation. Clinical evidence would be a significantly elevated white blood cell count, correlating to presence of infection. Focal ulcerations occur with increasing severity of colitis, and necrotic tissue form a membrane-like material. Thus, it is



Figure 4 – The Potent Effects on Toxin B vs. Toxin A on Intestinal Epithelium

(A) Control, treated with buffer alone – no toxins. (B) treated with 32 nM of toxin A for 5 hours. (C) and (D) treated with 3 nM of toxin B for 5 hours.

(B) Exposure to toxin A shows disruptions of superficial epithelium with crypt epithelium intact. (C) treatment with toxin B shows disruption of superficial epithelium as well. (D) shows higher magnification of colonic damage wrought by toxin B.

(Riegler, et al, Journal of Clinical Investigation, 1995)

called pseudomembranous colitis (Schroeder, 2005).

Animal models show toxin A induction of epithelial desquamation, increased mucosal permeability leading to increased fluid secretion in the intestine. Toxin B has less enterotoxic effects in animals with less correlation as a cause of disease in humans, but recent *in vitro* data based on human intestinal epithelial cell testing suggests that toxin B is ten times more likely to induce colonic damage than does toxin A. In this experiment, human intestinal mucosa was exposed to varying amounts of both *Clostridium difficile* toxins A and B for five hours and subsequent damage was observed. Significant damage was observed afterward, both electrophysiologically and morphologically. Both toxins caused disruption to cellular F-actin and patchy damage and exfoliation was noted on superficial intestinal epithelium. The difference lay in the potency of individual toxins. While both toxin A and toxin B caused severe intestinal damage, toxin B disrupted the colon in a minute concentrated amount, while toxin A's measurement was significantly greater. This suggests the high potency of toxin B in *Clostridium* difficile Associated Diseases. (Riegler et al, 1995)

In a 1999 study hypothesizing how *C. difficile* – induced alterations in intestinal barrier facilitate microbial entry to the intestinal mucosa (thus facilitating microbial pathogenic shift within the intestine), mature enterocytes were deliberately treated with varying concentrations of toxins A and B followed by an hour incubation with an enteric microorganism such as *Escherichia* coli. Effects of toxins A and B were assessed on all aspects of enterocyte function ability. Testing of the effects of toxin A and B on mature enterocytes *in* vitro resulted in damage and alterations in enterocyte actin, increased bacterial adherence and paracellular transmigration, thus correlating that Toxins A and B may facilitate bacterial adherence and penetration of the intestinal epithelium. (Feltis, et al, 1999)

If the infecting strain is toxigenic, the patient is at risk for disease. There are other factors that possibly affect its potency such as fimbriae (enabling bacterial adherence) and glycocalyx (antiphagocytic capsule), both of which are produced in greater numbers in toxigenic strains of *C. difficile*. Some highly virulent strains produce elevated protease levels, linked among other clostridial pathogens to increased virulence. Other extracellular hydrolytic enzymes may play a similar role (Bongaerts and Lyerly, 1997).

*C. difficile* bacteria can also have specific antibiotic-resistant genes, targeting specific antimicrobial agents through unique bacterial physiology and biochemistry. An example of this would be the *ermB* gene, encoding a 23S ribosomal methylase that causes resistance to macrolide-lincosamide-streptogrammin (MLS) antibiotics. This marker was noted in several cases of *C. difficile* after use of Clindamycin, a lincosamide derivative. (Hull and Beck, 2004).

Another strain of *Clostridium difficile* bacteria has been identified relatively recently, named North American pulsed-field gel electrophoresis type 1 (NAP 1), causing several outbreaks in North America and Europe. It is resistant to both gatifloxacin and moxifloxacin antibiotics (both of the fluoroquinolone antibiotic group), unlike other strains previously designated. NAP 1 produces 16 times greater toxin A and 23 times greater toxin B than other strains do, possibly related to a deletion in a negative regulatory gene. It also produces a third toxin, binary toxin, whose purpose has not been deemed significant yet. (Sunenshine and McDonald, 2006). This new strain has become the more dominant strain, due to the high rate of mutation. It is highly virulent and has raised *C. difficile*-related death rates by 35% yearly (DeNoon and Chang, 2008). Additionally, resistance to fluoroquinolone antibiotics gives the NAP-1 strain with the ability to spread more rapidly among healthcare environments where those antimicrobial agents are most frequently used. (Center for Disease Control and Prevention (CDC) 2005)



Figure 5– marked exudates protruding through mucosal ulcerations

(Cleveland Clinic, 2006)

### Causes and Risk Factors

The primary cause of infection is due to antimicrobial therapy, more prevalent in certain antibiotic groups.

Antimicrobial agents that target anaerobic bacteria are potentially more lethal, possibly because they alter intestinal flora and microbial ecology. (Gerding et al, 1995)

Almost all antimicrobial agents except for aminoglycosides are associated with *Clostridium difficile* infection (Sunenshine and McDonald, Cleveland Clinic Journal of Medicine, 2006). Clindamycin has been identified as a

targeting agent, confirmed by *ermB* gene isolation, encoding methylase enzyme causing antibiotic resistance to lancosamides (Clindamycin belonging to that family). (Hull and Beck, College of Family Physicians of Canada, 2005). Other common high-risk antibiotics are broad spectrum penicillins, second and third generation cephalosporins, erythromycin, sulfonamides, chloramphenicol, tetracycline, and fluoroquinolones. Fluoroquinolones in particular were isolated in a cohort study as the predominant risk factor for CDAD in a specific epidemic in Quebec 2003-4 (Pepin et al, 2005). It is most commonly due to oral antibiotics but can also occur due to intravenous or intramuscular antibiotics. (Merck Manual, 2006). Other antimicrobial agents, including antiviral and antifungal drugs increase the risk as well (Mayo Clinic 2006). Risk more than doubles with greater than three days of antimicrobial therapy.

Reduced-risk antibiotics include vancomycin, metronidazole, and antipseudomonals. (Schroeder, 2005).

The critical point of infection is right after normal intestinal flora depletion before replenishing (Bongaerts and Lyerly, 1997).

Other drugs may increase susceptibility as well, such as drugs and conditions that decrease gastric acidity. Over the counter antacids and proton pump inhibitors such as aciphex, prevacid, and related drugs would put a patient at higher risk (Merck Manual, 2006). Proton pump inhibitor (PPI) utilization is associated with upper gastrointestinal tract colonization and altering of intestinal flora. Decreased gastric acidity is a known risk factor for infectious diarrheal illnesses. Since *Clostridium difficile* bacteria thrive on higher gastric pH levels, decreased gastric acidity may also pose a risk factor for CDAD. This is supported by CDAD cases reported from patients receiving *Helicobactor pylori* treatment – combining proton pump inhibitors with antibiotics. A study comparing rate of *C. difficile* infection in patients undergoing gastric acid suppressive therapy with those who did not, with PPI usage increasing the risk significantly. (Dial et al, CMAJ, 2004). Two case-controlled studies conducted over ten years showed the increase in rate of *Clostridium difficile* infection due to PPI usage and H<sub>2</sub> blockers. (Dial, et al, JAMA, 2005).

Additional risk factors included use of non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen. Being methicillin-resistant *Staphylococcus aureus* positive increases susceptibility in hospital patients. (Dial, et al, JAMA, 2005).

Exposure to *Clostridium difficile* can occur through a variety of methods. Transfer of pathogenic organisms is highly prevalent via the hands of health-care workers and is considered the most likely mechanism. (Centers for Disease Control and Prevention (CDC) 2007).

Other risk factors include underlying illness, weakened immune system, recent hospitalization, residency in long-term care facilities, recent surgery – primarily abdominal/gastrointestinal, chronic colon disease such as inflammatory bowel disease or colon cancer. Additionally, previous infection with *Clostridium difficile* increases patient susceptibility to reinfection. (Mayo Clinic, 2006). Highest risk patients are those with recent immunosuppressive therapy or recent surgical procedures, partly due to patients' inability to generate IgG antibody immune response against *Clostridium difficile* toxin A. IgG immune response ability does not protect against colonization but can decrease risk of morbidity, mortality, or recurrence with *C. difficile* infection (Schroeder, 2005).

Cancer chemotherapy and increased age and severity of underlying illness are other possible risk factors (Hull and Beck, 2005)

#### **Clinical Manifestations of Enterocolitis**

More commonly, patients experience colonization rather than disease. Such patients exhibit no clinical symptoms even though they would test positive for *C. difficile* organism and/or toxins. Other times, patients contract *Clostridium* difficile-Associated Disease (CDAD) and exhibit clinical symptoms (Centers for Disease Control and Prevention, 2005).

Common symptoms for *Clostridium* difficile infection are watery diarrhea (characterized by at least three bowel movements daily for more than two days but usually ten or more), abdominal cramping and tenderness, nausea, loss of appetite, and fever of up to 104 - 105 degrees F. (Centers for Disease Control and Prevention, 2004). It is also possible to have an abnormal heartbeat (Healthwise, 2006). Other symptoms include blood or pus in the stool, dehydration, and weight loss (Mayo Clinic, 2006). Watery diarrhea is the most common symptom of CDAD in children (Infectious Diseases and Immunization Committee, 2000). Rarely are symptoms manifested outside the gastrointestinal tract, but can include cellulitis, bacteremia, visceral abscess formation, and reactive arthritis. A common indicator would be leukocytosis with a white blood cell count greater than  $30.0 \times 10^9$ /L (Hull and Beck, 2004).

Symptoms can appear immediately after or during antimicrobial therapy, but can often appear several weeks after completing all antibiotics. This was evidenced in a study of cancer outpatients diagnosed with CDAD where the median interval (range 2-60 days) from discharge to infection was 20.3 days – a 3 week delay (Sunenshine and McDonald, 2006).

Range of disease can vary in different individuals from asymptomatic colonization to severe fulminant pseudomembranous colitis.

Many cases are mild. Such patients develop mild to moderate watery diarrhea with abdominal cramping and nausea, similar to ordinary viral gastroenteritis. They may last a short duration to several weeks but usually improve without treatment (EMIS and PIP, 2007). Systemic symptoms are usually



absent and disease would only be considered due to abdominal tenderness upon physical examination (Sunenshine and McDonald, 2006). They may also include low grade leukocytosis and tenesmus – a sudden urge to evacuate the rectum (Hull and Beck, 2004).

Moderate infectious symptoms include leukemoid reaction – a stress response to disease with an increased leukocyte count of approximately 50.0  $\times 10^9$ /L, fever, dehydration, nausea, vomiting, and abdominal tenderness. Hull and Beck, 2004).

The severest cases present with dehydration and electrolyte disturbance, severe bloody diarrhea, fever, and abdominal pain and cramping (EMIS and PIP, 2007). It may lead to sepsis and shock, acidosis (increased acidity of blood plasma), tachycardia, multisystem organ failure including kidney failure (due to rapid dehydration), hypoalbuminemia, paralytic ileus, ascites (fluid accumulation in the peritoneal cavity), acute abdomen – colonic perforation, toxic megacolon (toxic colitis with dilatation), and death. Endoscopic evaluation of the colon will show pseudomembraneous white patches or lesions



Figure 7 – (L) Image of normal cecum

(R) Image of pseudomembranes formed from *Clostridium difficile* overgrowth of cecum.

with erythema and edema. The lesions are yellow plaques 2-10 mm in diameter with interposed normal intestinal mucosa (Hull and Beck, 2004). Should a patient develop paralytic ileus or toxic megacolon, it may actually lead to a decrease in diarrheal episodes. (Sunenshine and McDonald, 2006). Toxic megacolon is rare but has been documented in several cases, leading to a mortality rate of 33% for those who develop it. A critical factor in survival would be early diagnosis of *Clostridium difficile*-associated pseudomembranous colitis (Cone and Wetzel, 1982).

## Diagnosis

Detailed history is the first step to proper diagnosis. Patients are screened for recent antibiotic usage, abdominal pain, diarrhea, and related symptoms. Doctors may still test without presence of diarrhea since in rare instances *C. difficile* bacteria can cause abdominal pain and tenderness without diarrhea (MedicineNet, 2008). *C. difficile* is also suspected when diarrhea develops during or soon after hospitalization (EMIS and PIP, 2007).

Treating without diagnostic laboratory basis is not indicated, especially since only 30% of hospitalized patients with diarrhea have CDAD, even during epidemics. There are some exceptions for which empiric therapy would be necessary – including severely ill or rapidly deteriorating high-risk patients (Sunenshine and McDonald, 2006).

Patients with CDAD often have leukocytosis with strong elevation in severe enterocolitis. Physicians may screen leukocyte count as well as for white blood cell presence in the stool. Confirmation of those two tests proves positive for colitis and would need more testing to diagnose CDAD (MedicineNet, 2008). Stool leukocyte measurement may have limited diagnostic accuracy (Nation Master Encyclopedia, 2005). A sudden rise in the leukocyte count to between 30-50x10<sup>9</sup>/L cells combined with severe bandemia (immature white blood cells) is an indicator of fulminant colitis. Patients should be monitored for leukemoid reaction as shock can progress very quickly (Schroeder, 2005).

Stool assay for *Clostridium difficile* is a routine method. It is not always reliable as it sometimes produces false-negatives (Mayo Clinic, 2006). Additionally, although the most sensitive test possible, it



Figure 8 – anaerobic culture of *Clostridium difficile* bacteria on Cycloserin-Cefoxitin Fructose Agar (Anaerobe Systems, CA)

can also cause false-positives due to the available nontoxigenic strains. It must grow 48-96 hours anaerobically for proper results. (Centers for Disease Control and Prevention (CDC), 2005). It has an overall sensitivity of 95% but has a low specificity, necessitating toxicity testing (Canadian Paediatric Society, *Paediatrics & Child Health*, 2000). It is not specific for pathogenic toxinproducing strains of *Clostridium difficile* and is not as clinically helpful (Schroeder, *American Family Physician*, 2005). It is the least chosen method of testing in hospitals due to cost and length of procedure. It does however have an advantage of lending itself to molecular typing of strains, useful in a *C. difficile* outbreak (Sunenshine and McDonald, 2006).

Antigen detection for C. difficile are rapid tests

completed in less than an hour, used to detect presence of *C. difficile* antigen by latex agglutination or immunochromatographicassays. It must be combined with toxin testing for diagnostic confirmation (Centers for Disease Control and Prevention (CDC), 2005).

Toxin testing includes both enzyme immunoassay and tissue culture cytotoxicity.

Enzyme immunoassay detects toxins A, B, or both together. It uses monoclonal antibodies to detect toxin (Hull and Beck, 2004). Assay is completed same-day but is less sensitive than tissue culture cytotoxicity assay (Centers for Disease Control and Prevention (CDC), 2005). Enzyme-linked Immunoabsorbant Assay (ELISA) has a sensitivity rate of 63-99% and a specificity of 93-100%. Experts recommend sending as many as 3 samples to rule out disease if patients receive negative result, but

may not be necessary as much with ELISA (Nation Master Encyclopedia, 2005). The majority of combination enzyme immunoassays have a sensitivity of 85-95%. It should not be used as an indicator of response to therapy since results remain positive for extended duration in 25% of successfully treated patients (Schroeder, 2005).

Tissue culture cytotoxicity tests only for presence of toxin B. It is called the 'gold standard' toxin bioassay (Canadian Paediatric Society, *Paediatrics & Child Health*, 2000). It requires 24-48 hours for a final result and is very sensitive for *C. difficile* (Centers for Disease Control and Prevention (CDC), 2005). Organisms are cultured on selective medium and tested for toxin production and cytopathic effect in cell culture. It is the most sensitive and specific test although it is slow and labor-intensive (Nation Master Encyclopedia, 2005). Due to its high specificity and sensitivity, one should be cautious for false results (Canadian Paediatric Society, 2000).

*C. difficile* toxin is very unstable. Toxin degrades at room temperature and may not be detectable 2 hours after collection of stool. False-negative results are prevalent due to delayed testing or lack of refrigeration.

Stool lactoferrin levels can also be a diagnostic test but has limited diagnostic accuracy as well (Nation Master Encyclopedia, 2005). Other rapid testing such as Immunocard (Meridian Diagnostics) is highly specific but has poor sensitivity with up to 20% false negative results (Canadian Paediatric Society, 2000). Latex agglutination-based assays recognize enzyme glutamate dehydrogenase but do not have sensitivity (Hull and Beck, 2004). Polymerase chain reaction (PCR) detects toxigenic *C. difficile*. Amplification of a gene portion of either toxin A, toxin B, or a combination of the two genes is performed. PCR can be conducted on the specimen organisms for presence of toxins that match with the reading of the known toxins. One testing conducted (Kato et al) amplified only toxin A from *C. difficile*. Others had similar or less success. It is not as sensitive as other testing methods (Gerding et al, 1995).

Colon examination is used to confirm diagnosis of Clostridium difficile. Patients undergo



sigmoidoscopy or colonoscopy to screen for presence of inflammation and pseudomembrane appearance, both suggesting CDAD (Mayo Clinic, 2006). It should, however, be reserved for when a patient's condition needs rapid diagnosis, to rule out other diagnoses, or when clinical suspicion is high despite negative results. Colonoscopy can detect more than a sigmoidoscopy since it examines the whole colon where *C. difficile* can encompass, rather than just the sigmoid colon (Hull and Beck, 2004).

Imaging tests such as CT scans provide detailed images of the colon. Scans can show thickening of the colon wall, common in pseudomembranous colitis (Mayo Clinic, 2006). In conjunction with the clinical history, presence of ascites, colon wall thickening, or dilation can predict severity of enterocolitis (Schroeder, 2005).

#### **Treatment**

No treatment is necessary for colonization without symptomology. Should symptoms be present with diagnosis confirmed, there are different treatment options.

If at all possible, the disease-causing antibiotics should be stopped. This alone can allow normal intestinal flora to regenerate. Overgrowth of *C. difficile* would be reduced with less symptoms ensuing. For mild to moderate diarrhea and other symptoms, cessation of antimicrobial therapy may be the only necessary cause of treatment (EMIS and PIP, 2007). CDAD typically resolves in 23% of cases upon removal from the antimicrobial treatment (Centers for Disease Control and Prevention (CDC), 2005).

Fluids may be given to prevent dehydration and restore electrolyte balance in the blood (Healthwise, 2006).

For severe cases of diarrhea or diagnosed colitis, patients will be treated with an antibiotic that eradicates C. difficile organisms, usually Vancomycin (Vancocin) or Metranidazole (Flagyl), for ten days. In a study of 189 patients with CDAD, 97% responded to initial antibiotic therapy (Sunenshine and McDonald, Cleveland Clinic Journal of Medicine, 2006). Symptoms should subside within 2-3 days. The antibiotics can also prevent perforation of the colon if treated in time (EMIS and PIP, 2007). Drugs are usually effective with few side effects (Centers for Disease Control and Prevention (CDC), 2007). In severe cases, intravenous medications may need to be administered (Robert Michael Educational Institute, 2007). Both Vancomycin and Metranidazole are equally effective. Physicians may choose to prescribe Metranidazole first since it is far less expensive than Vancomycin. Vancomycin is reserved for patients who are allergic to Metronidazole, do not respond to it, or have side effects. Other physicians choose Vancomycin primarily for severe colitis since it can achieve higher antibiotic levels in the colon and can theoretically be more effective at eradicating bacteria there with more area specificity (MedicineNet, 2008). It can, however, contribute to the growth of antibiotic-resistant bacteria. Metranidazole can not be used for women who are pregnant or breastfeeding. Both antibiotics kill only the active infectious form of C. difficile, not the tougher spores. Since spores are resistant and remain in the body, infection can return, requiring further treatment (Mayo Clinic, 2006). For those unable to tolerate oral medication, IV Metronidazole is used since it is excreted in the intestine (Canadian Paediatric Society, 2000).

Other drug regimens compared in randomized therapeutic trials for CDAD with good results are Bacitracin, Teicoplanin, and Colestipol. Cure rates in Bacitracin are somewhat lower than Vancomycin, and it should be treated as a secondary agent in treatment. Colestipol is even lower than Bacitracin (Gerding et al, 1995).

Some physicians prescribe supplementary probiotics to restore normal intestinal flora. A natural yeast, *Saccharomyces boulardii*, and *Lactobacillus* species has proven effective in treating *C. difficile* infections together with antibiotic (Sunenshine and McDonald, 2006).

Antidiarrheal medications such as loperamide, diphenoxylate, and bismuth compounds are contraindicated and can worsen the course of pseudomembranous colitis. Slowing of fecal transit tie can possibly extend toxin-associated damage. Cholestyramine, usually used to lower cholesterol, is more effective in slowing bowel motility without causing more damage (Nation Master Encyclopedia, 2005).

If the disease causes fulminant colitis, surgical resection of the colon may be needed, especially with colon perforation (EMIS and PIP, 2007). In e cases causing severe pain, organ failure, or inflammation of abdominal wall lining, surgical removal may be the only option (Mayo Clinic, 2006). Surgery should be considered especially if initial treatment does not resolve the disease and symptoms Still, treatment should not be considered failure before 6-7 days of therapy progress rapidly. (Sunenshine and McDonald, 2006). At times, a patient may relapse with recurring episodes of CDAD. Multiple courses of antibiotics may be needed. Probiotic treatment may be helpful for this (Canadian Paediatric Society, 2000). Approximately 15-35% of patients have recurrent disease. This could be from reinfection or germination from residual spores. The most likely reason for relapse is that C. difficile had not been completely eradicated during treatment (MedicineNet, 2008). There is no evidence that recurring infections cause more severe disease (Hull and Beck, College of Family Physicians of Canada, 2004). Another possible reason leading to relapse is the body's inadequate production of antibodies against the bacterial toxins (MedicineNet, 2008). Fecal enemas, however, are difficult to perform and there is an increased risk of transmitting retroviruses or other infectious diseases (Schroeder, 2005).

Fecal bacteriotherapy, sometimes commonly called a "stool transplant", has its basis in probiotic therapy research. Normal intestinal bacterial flora obtained from the feces of a healthy individual is infused through the intestine of the patient in an effort to restore normal flora balance, decreasing the strength of the *C. difficile* organisms and lessen likelihood of recurrence. This treatment is usually used for people with recurring episodes of disease. It has a success rate of nearly 95% (Nation



Figure 10 – Laboratory preparation of stool for fecal bacteriotherapy

(The Medical Post, 2009)

Master Encyclopedia, 2005). Anaerobic bacteria and fecal rectal enemas are usually obtained from healthy relatives to promote better acceptance by the patient's body. They are instilled rectally and can restore colonic flora (Hull and Beck, 2004).

For patients with multiple relapses possibly due to antibody deficiency, passive immunizations with human gammaglobulin can be administered intravenously. This will grant them large amounts of antibodies to eradicate the disease. Additional work is in progress to promote active vaccination against *C. difficile* toxins, to increase patient levels of antibodies. (MedicineNet, 2008)

#### Prevention

Most importantly, avoid using antibiotics unless absolutely necessary. Antibiotics will not eradicate viral illnesses, yet they are still used for that purpose several times annually. Even some common bacterial ailments like bronchitis and ear infections can be treated without antibiotics (Mayo Clinic, 2006). In particular, restriction of Clindamycin has been shown to decrease incident of CDAD (Schroeder, 2005).

If antibiotics are necessary, have the physician prescribe from a narrow-spectrum range to be taken in the shortest amount of time possible for least likelihood of disrupting intestinal flora (Mayo Clinic, 2006).

Use probiotic supplements – yogurt with live cultures, acidophilus, and similar during the antibiotic course. However, only *Saccharomyces boulardii* is proven effective against *C. difficile* specifically (Mayo Clinic, 2006). *Lactobacilli* have been proven effective against antibiotic-associated diarrhea, but not necessarily that caused by *Clostridium difficile*.

Any patient with CDAD, even asymptomatically colonized, can transmit the disease to others. Only those on antibiotics, hospitalized, or with other prevailing risk factors are most likely to get ill. To reduce transmission, wash hands carefully especially after restroom use and before eating. Regularly clean surfaces used routinely, such as kitchens and bathrooms (Centers for Disease Control and Prevention (CDC), 2007). Ideally, a mixture of bleach and water should be used – with a ratio of 1:10 bleach to water. Patients with diarrhea should try to avoid using the same toilet other family members use unless it can be washed out each time with the bleach and water mixture (Robert Michael 2007).

Hospitalized patients known or suspected to have the disease should be treated using the 1994 Hospital Infection Control Practices Advisory Committee (HICPAC) Guideline for Isolation Precautions in Hospitals recommended contact precautions (Sunenshine and McDonald, *Cleveland Clinic Journal of Medicine*, 2006). Place those patients in private rooms if possible, or with other patients with *C. difficile*associated disease. Use gloves and gowns to prevent transmission and wash hands with alcohol-based hand rubs or soap and water. Soap and water alone is best for direct care of CDAD patients as it is more effective against spore-forming bacteria. Dedicate equipment to them wherever possible. (Division of Healthcare Quality Promotion (DHQP), 2005) Visitors should wash hands with soap and warm water before entering and leaving a CDAD patient's room. (Mayo Clinic, 2006). One hospital reported a 60% decrease in CDAD after instituting more stringent precautions (Sunenshine and McDonald, 2006).

#### Summary

*Clostridium difficile*-Associated Disease (CDAD) may be a slightly rare disease but with devastating effects. Due to depletion of normal intestinal flora in conjunction with other risk factors including immunocompromised state or recent hospitalization, dormant resistant spores transform to virulent possibly toxigenic infectious form of bacteria that can multiply rapidly. Although broad in arrange of symptomology, the possibility of leading to severe pseudomembranous colitis with risk of colonic perforation, toxic megacolon, or death exists. Treatment is possible – with expensive drugs and other measures, but not without the possibility of relapse, even numerous reoccurrences. Prevention must be taken to avoid susceptibility to this virulent and damaging organism altogether, by maintaining proper precautions. Should someone already have the disease, proper care must be implemented to ensure no further transmission as spores can be spread through contact and fecal-oral methods. Research is still preliminary for other treatments, including vaccination against *C. difficile* toxins. With proper prevention and treatment, and increase in patient antibodies to target the organism, even in the absence of normal intestinal flora, *Clostridium difficile*-Associated Disease can be eradicated.

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#### Aspartame: A sweet toxin?

#### Atara Rena Degani

#### Abstract

L-aspartyl-L-phenylalanyl-methyl ester, commonly known as aspartame, is one of the most widely used and controversial sweeteners. Many have questioned the safety of this chemical, concerned that it may be neurotoxic and carcinogenic. Numerous studies have been conducted on the three basic constituents of aspartame: aspartic acid, phenylalanine and methanol; scientists have tried to determine whether the ingestion of aspartame will cause a significant increase in blood plasma levels of these chemicals, and whether such an increase is dangerous. This review analyzes various studies conducted on the health effects of these metabolitic byproducts of aspartame.

#### Introduction

Consumers were thrilled when aspartame was introduced to the market. Since this artificial sweetener's safety was approved, it has found its way into over 6,000 products, including soft drinks, chewing gum, hot chocolate, candy, desserts, sweeteners, and yogurt. Sold commercially under names such as Nutra-Sweet, Equal and Canderel, aspartame is two hundred times sweeter than sucrose. Although it has the same number of calories per gram as sucrose, people generally use less of it, consuming fewer calories (Soffritti et al. 2006).

The discovery of aspartame was accidental. In 1965, James M. Schlatter discovered this chemical as he was trying to produce an anti-ulcer drug candidate for G.D. Searle & Company. Some aspartame spilled on his hand, yet he did not wash it off, believing that it was not toxic. He came to recognize its sweetness when he licked his fingers in order to pick up a weighing paper. Despite its unintentional discovery, aspartame has had a profound impact on the dieting habits, and it is one of the most widely used artificial sweeteners in the world (Sofffritti et al., 2007).

Aspartame's unique formula helps obese maintain their weight loss programs and allows diabetics to enjoy exceptional dishes within their dietary restrictions (Butchko et al. 2002). In the United States, the acceptable daily intake (ADI) of aspartame is 50 mg/kg body weight. Consumption by the general population ranges from 2 to 3 mg/kg body wt, and the average consumption by children and women of child bearing age has been estimated at 2.5-5.0 mg/kg bw/day (Soffriti et al., 2007).

Extensive research was done on the safety of aspartame before it entered the market. Various studies were conducted with a number of human populations in order to determine its safety; research

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exists about its safety. Numerous anecdotal reports were released subsequent to aspartam's approval describing the adverse health affects associated with it. Written complaints include that of dizziness, visual impairment, disorientation, ear buzzing, tunnel vision, loss of equilibrium, severe muscle aches, numbing of extremities, increased blood pressure, retinal hemorrhaging and depression (Monte 1984). Even more serious is the startling rise in brain tumor rates since the introduction of aspartame to the market (Olney et al. 1996).

### FDA Approval

Aspartame was approved by the FDA for use in solid foods in 1981, in soft drinks in 1983, and as a general sweetener in 1996. The FDA denied approval of this sweetener for eight years before allowing it on the market since researchers had discovered that the ingestion of aspartame yielded toxic effects. A Public Board of Inquiry (PBOI) convened in 1980 to review the research of G.D. Searle and Company and they denied approval of the chemical due to lack of sufficient evidence proving that aspartame did not cause cancer. The FDA commissioner, Arthur Hull Hayes, established another PBOI in 1981. This board did not reach a consensus regarding the chemical, yet the commissioner overruled his own board of inquiry and approved the food additive. Conspiracy theories brew because Hayes left the FDA in 1983 to join the public relations department of Searle (Whitmore 1996). The validity of the FDA's approval is further questioned when the research conducted on aspartame is analyzed. One is compelled to question the design efficiency of many studies when one notes that 100% of the 74 industry-funded studies found no problem, while 83 out of 90 non-industry funded studies found one or more problem with aspartame (Warner 2006).

Structure and Components of Aspartame



Aspartame is a dipeptide which has the formula L-aspartyl-L-phenylalanyl-methyl ester (Maher and Wurtman 1987). Upon ingestion, aspartame is metabolized in the gastric tracts to its three constituents: aspartate, phenylalanine, and methanol. These components proceed to be transformed into other products. Aspartate is transformed into alanine and oxaloacetate; phenylalanine is transformed into tyrosine, phenylethylamine and phenylpyruvate, and methanol is transformed into formaldehyde which later forms formic acid (Soffritti et al. 2006). While these components are commonly digested, concerns have been raised about their safety when they are metabolized after aspartame ingestion.

#### Aspartate

Aspartate is an extremely common, non-essential amino acid. It is usually rapidly metabolized by the body and subsequently incorporated into proteins and utilized for energy. This amino acid is found in large concentrations in natural products; 100g of chicken yields 2600 mg of aspartate whereas a 355 ml beverage sweetened 100% with aspartame only provides 70 mg of aspartate. Furthermore, a glass of no-fat milk provides 13 times as much aspartic acid as a beverage sweetened 100% with aspartame (Monte 1984).

While aspartate is not toxic when absorbed, even in large quantities, from natural foods, it may be harmful to the body when it is absorbed after the consumption of aspartame. Blood component levels of amino acids such as aspartate and phenylalanine will not rise significantly after the digestion of natural proteins. Due to their quaternary structures, natural proteins are digested slowly and enzymes must catalyze the protein and release numerous amino acids before phenylalanine or aspartate can be released. On the other hand, the digestion of aspartame can raise component blood levels rapidly since the chemical only requires the breakage of two bonds for absorption (Monte 1984).

When aspartate it is absorbed in excess, it can wreak significant damage on the body. Daabes and co-workers (1985) determined that plasma levels of aspartate and glutamate which exceeded 110 numol/dl, induce hypothalamic neuronal necrosis in neonatal rodents. Later, Olney and Sharpe (1969) conducted a similar experiment on non-human primates. They delivered large boluses of glutamate, a similar dicarboxylic amino acid, and found that it led to hypothalamic neuronal necrosis. Furthermore, scientists have found that high plasma levels of this amino acid cause endocrine disorders in mammals, leading to the release of pituitary gonadotropins and prolactin in the rhesus monkey and noticeably elevated plasma levels of luteinizing hormones and testosterone in rats (Monte 1984).

Experiments have determined that aspartame alone will not elevate plasma aspartate levels for an extended time period. However, concerns have been raised about the ability of aspartame to spike plasma aspartate levels when it is ingested in conjunction with mono sodium glutamate (MSG). The ingestion of MSG has been known to cause adverse symptoms in individuals due to the substance's neuroexcitatory activity (Reif-Lehrer 1976). Since both aspartate and glutamate are structurally similar, and pose a threat of neurotoxicity at high dosages, Olney (1982) has suggested that consuming aspartame along with foods which contain MSG leads to a risk of toxicity and of focal brain lesions. However, Stegink and coworkers (1983) conducted numerous studies and discovered that while MSG consumption will lead to a significant increase in glutamate aspartate concentrations when consumed alone, the addition of aspartame to MSG will not cause any further increase in dicarboxylic amino acid levels. Thus, they concluded that is impossible for humans to ever consume enough MSG and aspartame to raise plasma concentrations to those associated with rat neurotoxicity.

#### Phenylalanine

Phenylalanine is an amino acid which is beneficial for one's health, and can be found in proteincontaining foods such as non-fat milk and fruit juice. This amino acid can be lethal to those who suffer from phenylketonuria (PKU), a rare genetic disease. The diets of phenylketonurics are extremely restricted from shortly after birth in order to avoid the risks of mental retardation or various degrees of cognitive impairment (Butchko et al. 2002). However, phenylalanine is not only dangerous for phenylketonurics; spikes in plasma phenylalanine levels can be toxic due to the body's method of uptake of this amino acid.

Upon ingestion, phenylalanine is absorbed across the gastrointestinal mucosa into portal circulation. Most dietary phenylalanine goes unchanged into systemic circulation and is taken up across the blood-brain barrier and into the central nervous system via a transport system that is specific for large neutral amino acids (LNAA). The amount of amino acids which enter and leave the brain is determined by the concentration of the LNAA and their specific affinity constants to the carrier system (Fernstrom and Wurtman 1997). The danger of spiked plasma levels of phenylalanine lies in the fact that it will interfere with the availability of tyrosine and tryptophan. Consequently, phenylalanine will act as a competitive inhibitor of the enzyme tyrosine hydroxylase. This, subsequently, lowers the concentration of brain catecholamine and serotonin, which, in turn, mediates neurological changes and induces seizures (Maher and Wurtman 1987).

Some researchers suggest that aspartame ingestion poses a risk because it provides phenylalanine without other LNAA. Consequently, upon being metabolized there will be an increased phenylalanine uptake by the brain causing the aforementioned problems (Maher and Wurtman 1987). However, other scientists, such as Stegink and coworkers (1987), found that this is not the case. In their studies, they found that the changes in phenylalanine LNAA in normal subjects was no greater than those occurring under normal dietary conditions. This idea was reinforced in a study conducted by Koeppe and coworkers (1991). In this study, positron emission tomography was used in order to observe the effects of elevated plasma phenylalanine levels after the consumption of large boluses of aspartame. An 11.5% decrease in amino acid transport rate constant was observed along with a 6% decrease in tissue distribution volume of aminocyclohexanecarboxylate. Thus, under normal dietary use, aspartame is unlikely to cause changes in brain amino acid uptake which would be measurable by PET. Despite the fact that many scientists do not believe aspartame will negatively affect the brain's uptake of amino acids, several adverse side effects are observed after phenylalanine's digestion. Many individuals have reported that they have suffered from neurological or behavioral reactions in association with aspartame consumption, a symptom which can be linked to increased phenylalanine levels (Maher and Wurtman 1987). Furthermore, Walton and coworkers (1993) found that individuals with a history of mood disorders, such as depression and bipolar, exhibited stronger symptoms after consuming aspartame. They hypothesize that the disorders are exacerbated by aspartame's phenylalanine component which upsets the balance of neurotransmitters.

Another concern raised regarding phenylalanine is its ability to induce seizures. In one study, mice were given dosages in which phenylalanine levels rose above tyrosine levels, a phenomenon which will occur after any aspartame dose in humans. Subsequently, the mice were introduced to epileptogenic drugs, inhaled fluorotyl or electro convulsive drugs; the frequency of seizures following these treatments was greatly increased due to increased plasma phenylalanine levels (Maher and Wurtman 1987). Some researchers disagree with these findings. In research done by Dailey and coworkers (1989), acute oral doses of aspartame, ranging from 0 - 2500 mg/kg were administered to CD-1 mice. Increases in phenylalanine and tyrosine and modest reduction in brain serotonin and 5-hydroxinol acetic acid were observed. However, these changes were insufficient to cause functional deficits which might have the capacity to facilitate pentyl enterazol-induced seizures. Thus scientists have not been able to conclusively determine whether or not aspartame will induce seizures.

#### Methanol

Methanol, or wood alcohol, is the simplest alcohol with the formula CH<sub>3</sub>OH. Occurring naturally in fruit juices and alcohol, it can be found in considerable quantities in a daily diet; for instance, tomato juice provides six times more methanol than an equivalent amount of beverage sweetened 100% with aspartame (Butchko et al. 2002). Ten percent of aspartame's weight is absorbed as methanol. This chemical is released in the small intestine after chymotrypsin hydrolyzes the methyl group of the dipeptide. It is transformed later into formaldehyde and formic acid., both toxic metabolites. Absorption of methanol increases if it is ingested as free methanol, such as in heated foods and soft drinks (Monte 1984).

The dangers of methanol consumption via aspartame have been raised over the years. Methanol does not cause toxicity when consumed in wines and juices because of the beverages' natural protective features. For instance, juices and wines contain high ethanol to methanol ratios; some neutral spirits contain 200 molecule of ethanol per molecule of methanol and orange juice contains 0.8 mg/L of methanol and 380 mg/L of ethanol. This has a protective effect since ethanol slows the rate of methanol being transformed into formaldehyde, thereby allowing the body to excrete methanol in breath and in urine. Juices have an added protection since they have high osmolality and an average caloric density of 500 kcal/L, which also puts definite limits on consumption level rates of methanol.

Since aspartame is not limited by calories or osmolarity, daily methanol levels may rise to unprecedented levels, and may prove to be a cumulative toxin (Monte 1984).

One of the key concerns about methanol is its production of the methyl alcohol syndrome. This toxicity is found only in humans because man has limited biochemical pathways for detoxification. For twelve to eighteen hours after the methanol consumption there is a latent period, followed by severe acidosis which is caused, in part, by formic acid formation. Patients complain of confusion, lethargy, and impairment of articulation, and may also suffer from back pain, vertigo, abdominal pain, labored breathing, leg cramps, and visual loss. There are also fatal cases in which the liver kidney and heart show parenchymal degeneration and the lungs display desquamation of epithelium, edema, emphysema, congestion, and bronchial pneumonia (Monte 1984).

The danger of methanol is rooted in its production of formaldehyde, a known carcinogen. Formaldehyde has been proven to form squamous-cell carcinomas when inhaled by experimental animals. It reacts with DNA causing irreversible denaturation and can interfere with DNA replication, thus causing mutations (Monte 1984). Studies have verified that formaldehyde accumulates after the consumption of aspartame. Trocho and coworkers (1998) synthesized aspartame using a methanol group which had radioactive C-14 and fed it to mice. They later found that the methanol had accumulated in plasma and in the liver, and was bound to protein, thus determining that aspartame contributes to the formation of formaldehyde, but it even intensifies the toxicity of this chemical. Forty percent of aspartame breaks down into excitotoxic amino acids, and formaldehyde's toxicity increases when it is in the presence of high levels of free radicals (Saito et al. 2005).

One of the dangerous consequences of methanol absorption is the increased risk of developing lymphomas and leukemias. A case-control study in Argentina discovered that urinary tract tumors (UTT) were directly correlated with aspartame consumption. The risk of UTT was significantly increased in long-term aspartame users compared with non-aspartame users (Andreatta et al. 2008). Another long-term study which studied the affect of aspartame consumption on the incidence of tumors took place in Italy. Soffritti and coworkers (2007) observed Sprague Dawley rats from eight weeks of age until natural death. They observed a statistically significant increase in the incidence of malignant tumors among rats that had ingested aspartame. These tumors included lymphomas, leukemia, preplastic, neoplastic, and lesions of the renal pelvis and ureter. Since rodents have been found to be consistent predictors of human cancer risks, they conclude that aspartame is a multipotential carcinogenic compound whose carcinogenic effects are evident even at daily dose of 20 mg/kg bw.

Despite the compelling evidence, some researchers do not believe that methanol will cause toxicity in humans when ingested in the form of aspartame. They assert that the alcohol is absorbed in such minimal levels that it will be unlikely, and even impossible, to reach levels of toxicity associated with cancers and lymphomas (Butchko et al. 2002). Nonetheless, there are still significant concerns with aspartame's production of formaldehyde.

#### Conclusion

The results and conclusions of the different studies about the safety of aspartame are spread over a large spectrum; views range from those who claim that aspartame is absolutely safe to those who claim that it is toxic. Research is centered on the possible risks associated with the ingestion of each of aspartame's constituents. Regarding aspartate, many claim that high levels of this amino acid can induce neuronal necrosis and endocrine disorders. A large group of scientists believe that the ingestion of aspartame can lead to dangerous spikes in aspartate plasma levels when eaten in conjunction with MSG, while many others believe that such a spike is impossible.

The debate about phenylalanine's toxicity revolves around the ability of this amino acid to interfere with LNAA at the blood-brain barrier. Some researchers are concerned that this constituent can exacerbate mood disorders and induce seizures. However, the issue is still debated, and many scientists have determined that aspartame's phenylalanine will not cause adverse effects.

Methanol consumption has raised significant concerns since this alcohol is metabolized as formaldehyde, a known toxin. Long-term studies have proven that the methanol component of aspartame has caused lymphomas and leukemias in rats. Nonetheless, other scientists debate these results and claim that typical ingestions of aspartame will not cause toxic effects.

#### Summary

Aspartame is one of the most controversial sweeteners. The pro-aspartame camp presents data that shows that the ingestion of this sweetener does not lead to significant negative health effect. They claim that the negative studies on lab animals are run with high concentration of materials that will never be present during normal consumption. On the other hand, the anti-aspartame camp believes that the fast digestion of aspartame leads to the concentration of its constituents in the body, causing toxic effects. Clearly, more research must be conducted on the subject in order to conclusively determine whether aspartame is harmful or not. In the interim, it would be advisable that people who are susceptible to metabolic conditions which are possibly affected by aspartame should try to avoid significant consumption of the sweetener.

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# Folic Acid and Neural Tube Defects

# Rachel Leah Feinstein

Neural tube defects (NTD) are the most common types of birth defects. Research shows that folic acid taken periconceptionally greatly reduces the risk of having a NTD affected child. This paper will explain the role that folate plays in the metabolism, specifically in synthesizing methionine. It will bring evidence to show that methionine is crucial for normal neural tube development. In addition, it will explore the genetic factor involved in folate metabolism and possible folate deficiency.

The central nervous system in an embryo begins as a flat region, which then rolls into a tube known as the neural tube. The development of the neural tube is completed 28 days after the baby is conceived. When the neural tube fails to close completely, it is known as a neural tube defect. The nerves that are exposed to the environment due to the failure of the tube to close may become damaged causing the affected baby to have a disability, specifically some measure of paralysis. Neural tube defects are one of the most common birth defects, and affects 1.3- 2.0 babies in every 1,000 live births in the United States. There are two main types of NTDs, depending on whether the cranial or caudal end of the neural tube fails to fuse properly. The foremost cranial defect is known as anaencephaly which is usually fatal. The main caudal defect is spina bifida (Pitkin, 2007).

The exact causes of NTDs are not clear. It is believed that a combination of environmental factors, genetics, and nutrition contribute to the development of a NTD. However, as early as the 1970 scientists have suspected that folic acid taken by the pregnant mother helps prevent NTDs. In the United Kingdom, R. W. Smithells noticed that a disproportionate amount of babies affected with NTD were born to mothers of a lower socioeconomic class. He hypothesized that the birth defects may have been caused by the mothers' poor nutrition. He conducted tests and in 1976 reported that women who had a child with an NTD had much lower red cell folate and vitamin C levels than mothers who had an unaffected child (Smithells, Sheppard, & Schorah, 1976). He then conducted a placebo-controlled study in which he gathered women who had already given birth to a NTD affected child. He gave one group of women a multivitamin and an iron tablet containing 360 ug of folic acid. The group of women who were given the vitamin pill only had a .5% occurrence of NTD births, while the control group had a 4% NTD birth rate incidence (Smithells et al, 1981). Smithells results were published, however his experiment was criticized because his subjects had not been randomized.

K. M. Laurence in Whales also performed experiments with folic acid based on Smithells' works. He performed a double blind randomized controlled trial of folate treatment prior to conception. Among the group that received the folate, there were no NTDs in the group's 44 births. However, in the control group there was 6 NTDs in 67 births. Laurence's work was not considered statistically significant since it was performed on such a small population (Mills et al. 1996).

Smithells' findings were eventually proved definitively by the Medical Research Council in 1991. The MRC conducted a double-blind randomized study on women who had a previous NTD pregnancy. Women received 4 mg of folic acid, a multivitamin, both, or iron and calcium. There was a 74% reduction of NTD births in the group taking folic acid, but no effect in the groups that did not receive folic acid. Another study, conducted just one year later also helped to validate Smithell's works. Czeizel and Dudas in Hungary enrolled women who had never had an affected pregnancy. They were randomized and given a multivitamin, some with .8 mg folic acid, and some without. The group of 2394 women who were given folic acid had no affected offspring, and the group of 2310 women who did not receive folic acid had 6 babies with NTD (Mills et al. 1996).

Due to the solid evidence of the protective role of folic acid, public health officials recommend women of childbearing age to take 400 ug of folic acid at least 4 weeks before conception, and through the first trimester of pregnancy. Folic acid is the oxidized and most active form of folate. They may do this by taking a folic acid supplement. They are also encouraged to eat folic acid enriched grain products and large amounts of folate rich foods such as green leafy vegetables, or liver. The United States, as well as other countries, have implemented a mandatory folic acid food fortification and have seen about a 50% reduction in the occurrence of NTDs (Lamers, Prinz-Langenohl, Bramswig, Pietrzik, 2006).

Despite the awareness of the role that folic acid plays in preventing NTDs, the exact mechanism used to achieve this is not so clear and is still being studied. However, based on various experiments scientists have come to a basic understanding as to how a folate deficiency would contribute to an NTD development.

Folate is a term to describe a water- soluble B-complex vitamin and it serves many important roles in the body. It is vital for cell division and homeostasis due to the essential role of folate coenzymes in nucleic acid synthesis, methionine regeneration, and in the shuttling, oxidation and reduction of one carbon units required for normal metabolism and regulation. There is an increase in folate requirement in pregnant women to support the rapid growth of the embryo and uteroplacental organs. There has been much focus on the role folate plays in methionine regeneration in regard to studies of NTDs. In this process folate is reduced in the body to dihydrofolate and then to tetrahydrofolate. Tetrahydrofolate acts as an acceptor molecule that accepts and donates one carbon units in metabolic pathways. A one carbon unit is transferred from serine to THF by serine hydroxymethyltransferase to form 5,10 methylene tetrahydrofolate. This compound is then reduced by methylene tetrahydrofolate reductase (MTHFR) to form 5 methyl tetrahydrofolate. The 5 methyl tetrahydrofolate is acted upon by methionine synthase together with a B-12 cofactor which facilitates the removal of the N-5 methyl group from the 5 methyl tetrahydrofolate and deposits it onto homocysteine. The homocysteine with the extra methyl group is known as methionine. A mother who is lacking in folate would not be able to carry out this metabolic pathway, since 5 methyl tetrahydrofolate is the only compound capable of this one carbon transfer (Bailey, & Gregory, 1999). It would therefore seem plausible to say that mothers lacking in folate, and therefore at risk for an NTD baby, would have elevated homocysteine levels and low methionine levels. Subsequent studies have indeed supported this theory.

Steegers- Theunissen et al. (1995) was one of the first to report elevated amniotic fluid homocysteine in women who were carrying an NTD fetus. Since then there have been many reports that

plasma or amniotic fluid homocysteine is higher in NTD infants and their mothers than non-NTD infants and their mothers. One such study was conducted by Mills in which blood samples were collected during pregnancy from women carrying affected fetuses, and random women carrying healthy fetuses. The homocysteine levels in the blood were measured, and the mothers if NTD fetuses had significantly higher levels. These studies pointed to the relative inability of mothers with NTD babies to metabolize homocysteine (Mills et al. 1996).

Since it has been established that NTD mothers have higher levels of homocysteine, research turned to try to establish if too much homocysteine is what actually inhibits the neural tube closure. In order to test this hypothesis Greene, Dunlevy and Copps (2003) cultured mouse embryos in the presence of homocysteine thiolactone during the periods of cranial neural tube closure. While the homocysteine thiolactone did cause growth retardation and other negative effects, it did not increase the incidence of neural tube defects. Another study conducted by Afman, Blom, Van Der Put, and Van Straaten (2003) and his colleagues administered homocysteine to chick embryos in ova. This resulted in several malformations, but did not increase the number of NTDs. These results suggest that too much homocysteine is unlikely to be the direct cause of NTD.

Since the overabundance of homocysteine in a mother of an NTD child does not seem to cause the NTD, perhaps the lack of methionine that should have been made from the homocysteine plays a role in the development of an NTD. Methionine is an essential amino acid that cannot be obtained sufficiently through dietary intake and therefore must be synthesized by the body. Methionine formed from homocysteine is converted to S- adenosylmethionine, which is a methyl donor for many reactions including DNA methylation (Friso et al. 2002). This theory was tested in a few different ways. Coelho, Weber, Klein, Daniels, and Thomas (1989) conducted a study in which he grew rat embryos in cow serum. Cow serum has a much lower level of methionine than rat serum. They supplemented some cow serum with methionine, and did not supplement others. There was a significant decrease in the occurrence of NTD in the embryos that were grown in the serum that was supplemented with methionine. Essein and Wannberg (1993) conducted a study involving pregnant mice with an Axd (axial defect) mutation which is known to cause NTDs. They injected these pregnant mice with methionine on days 8 and 9 of the pregnancy. At a dose of 180 mg/ kg body weight the methionine produced a 47% reduction of NTDs. They also conducted studies with mice with the Axd mutation in which they supplemented the mice with folinic acid, but it had no effect on the incidence of NTDs. This seems to suggest that folate, or lack of it, is an indirect cause of NTD, and methionine is the needed product of the folate for normal neural tube development. Perhaps the Axd mutation codes for a mutation in an enzyme that is crucial to make methionine, or a vital product of methionine, but is not involved with folate at all.

Shaw, Velie, Schaffer (1997) conducted a study concerning the effect of methionine on humans. His study involved 424 mothers of NTD children, and 440 mothers of unaffected children. Each mother was interviewed in which they answered a 100 item food frequency questionnaire. The data was then established into quartiles of average daily maternal dietary intake of methionine in the 3 months before conception. There was a 30- 40 % reduction in NTD affected pregnancies among women whose average daily intake of methionine was above the lowest quartile. These observations were unrelated to the maternal level of folate intake, which supports the theory that methionine, or what methioine is converted to, is what is actually crucial for normal neural tube development.

Assuming that methionine is vital for normal embryonic development, and the synthesis of methionine is formed by homocysteine and 5 methyl tetrahydrofolate interaction, then it is clear how a folate deficiency would result in a NTD. However, many studies since Smithells have reported that NTD mothers are not necessarily folate deficient. One such study was conducted in Molloy and Kirke, Hillary, Weir, and Scott (1985). They studied the serum samples taken during pregnancy from 32 mothers with pregnancies affected by NTD and 395 randomly selected unaffected pregnant women. The serum folate levels and vitamin B-12 levels (vitamin B-12 is the coenzyme that facilitates the removal of a methyl group from the 5 methyl tetrahydrofolate onto the homocysteine) were measured and analyzed. To analyze the data the information was sub- classified into folate deficient, possible deficient, and sufficient ranges. The ranges were 0-2 ng/ml, 2.01- 2.7 ng/ml, and 2.71-20 ng/ml respectively. Surprisingly, only 21.9% of the NTD group was deficient in either folate or vitamin B-12 while 22.8% of the control group samples were. 43.8% of the NTD group, and 35.7% of the control group showed sufficient serum concentrations of both folate and vitamin B-12. These results show that a significant percent of women who had folate sufficient levels had a pregnancy affected by NTD. This seems to indicate that folate deficiency may not be the sole or main cause of neural tube defects. There may be a more subtle problem among women who gave birth to NTD babies than simply not consuming enough folate as part of their diet. These results do not contradict Smithells' findings, since he reported that NTD mothers have lower red cell folate levels. This study was only able to measure the serum folate levels, and this difference seems to be significant.

Red cell folate levels and serum folate levels were further investigated in other studies. Once such study was conducted by Yates et al. (1986) measured vitamin levels in twenty women less than 35 years of age who had two or more NTD pregnancies. Each case was compared to a control female who was matched for age, obstetric history, and social class. The red cell folate levels were measured and showed a linear relationship with the number of NTD pregnancies. However, there was no significant difference between the subjects and controls in relation to serum folate levels. The diets of the study and control women were ascertained through a questionnaire. There was no statistically significant difference between the folate dietary intakes of the two groups of women. However, regression analysis showed a difference between the two groups in regard to the relationship of red cell folate to dietary folate. This study provides additional evidence that low red cell folate is linked to NTD, but also demonstrates that low red cell folate is not necessarily due to a folate deficient diet. The difference between the red cell folate levels among women with similar folate intake supported the increasingly popular idea that NTD may be linked to a disorder in folate metabolism, and not exclusively to a folate deficient diet.

More recently, in 1992 Mills et al. conducted a study which measured the maternal serum folate of 89 NTD pregnancies and 178 control pregnancies. This study also demonstrated no relationship between maternal serum folate during pregnancy and the risk of NTDs (Mills et al. 1996).

There is also a strong hereditary link for neural tube defects. The chance of having a NTD child is about 0.15 percent. However, once a woman has a child with a NTD the chance of having a second child with NTD is increased to about 2-5%. Furthermore, if the women herself has a NTD the chances of having an affected baby increases. This supports the idea that there may be a genetic mutation that predisposes someone for NTD among those who are affected by it (Genetics and Neural Tube Defects, 2005).

Due to the evidence provided by studies such as the ones mentioned above, there has been much speculation that there is possibly a genetic defect that affects the metabolism of folate. To prove this hypothesis researchers have identified several common polymorphisms of genes that code for folate metabolizing enzymes, including the 677C / 677T and the 1298A/ 1298C alleles of 5, 10 methylenetetrahydrofolate reductase (MTHFR). These polymorphisms are common, and their frequency varies by race and ethnicity. A recent study by Yang et al. (2008) tried to determine the role that these different alleles play in the metabolism of folate and homocysteine levels.

Data for this study was taken from the third National Health and Nutrition Examination Survey (NHANES III). The NHANES III endeavored to obtain a nationally representative sample of the civilian United States population. Each survey participant was given an interview, a physical examination, and gave a sample of their blood. The researchers genotyped selected polymorphisms of folate metabolizing enzymes in DNA samples (obtained from the blood specimen) for 7159 individuals. They also measured the serum folate levels and homocysteine levels in each blood sample. Based on the interviews of the participants their average daily intake of folate was determined. The effects of MTHFR 677C/677T genotype and the MTHFR 1298A/1298C genotype on the inverse relationship between folate intake and serum homocysteine concentrations were then examined.

The researchers observed significant differences in the serum folate and homocysteine concentrations for individuals with the MTHFR 677 TT genotype. The adjusted geometric mean of serum folate concentration was 24.83 % lower if they had the TT genotype that if they had the CC genotype. The adjusted geometric mean of serum total homocysteine concentration was 29.06% higher if they had the TT genotype than if they had the CC genotype. The other polymorphism, 1298A/1298C, was not significantly associated with serum folate or homocysteine concentrations.

Folic acid consumption also played an important part in determining serum folate levels. Those with the 677 TT genotype that took 400 ug of folic acid per day had higher serum folate concentrations than those with the MTHFR 677 CC who did not take folic acid supplements. As the folic acid consumption increased, the difference between the levels of serum folate of the TT and CC genotypes decreased significantly. The difference actually became non significant among the group who took 400ug or more of folic acid per day. However, homocysteine levels among the MTHFR 677TT only decreased by 11-14% with the supplemental folic acid.

These results seem to indicate that having 677 TT alleles on the gene that codes for MTHFR results in some sort of defect on the enzyme which causes lower folate levels than in someone who has

the same folate dietary intake but has the 677 CC alleles. However, consuming a lot of folic acid, specifically 400 ug per day, can overcome the problem and bring the serum folate to sufficient levels.

The exact explanation for what the polymorphism codes for, and how the folate is able to overcome the defect can be explained. The 677T polymorphism occurs in exon 4 of the genetic code and results in a valine substitution of alanine at codon 222. This valine lies on the binding site for the MTHFR enzyme's cofactor flavin adenine dinucleotide (FAD). This affects the binding of the FAD to the enzyme, making the binding site exposed instead of imbedded in a barrel-like structure. The exposure results in a weakened, thermolabile MTHFR/FAD complex and the MTHFR 677TT enzyme has been shown to dissociate with the FAD cofactor more readily than the MTHFR 677CC enzyme. This results in decreased enzymatic activity. The MTHFR enzymes with the 677TT genotype have a 30% in vitro MTHFR enzyme activity as compared to the MTHFR 677CC enzymes. However, an abundance of 5-methyltetrahydrofolate substrates have been shown to strengthen the complex and protect the MTHFR from losing the FAD cofactor. This explains how consuming an abundance of folic acid overcomes a natural genetic deficiency (Robien, & Ulrich, 2003).

The results of the study by Yang et al. explains the findings in the studies which show that people of similar folate intake may have different red cell folate levels. However, in the those studies, the women who had given birth to NTD children did have sufficient serum folate levels, while in the study by Yang the MTHFR reduced activity was specifically reflected in low serum levels. It is possible that this can be explained simply by the different states of the subjects of the studies. The studies that showed similar serum levels were conducted on pregnant women. It has already been established that pregnant women are in high need of folate. The plasma is the provider of folate during pregnancy and therefore the body does al it can to ensure sufficient serum folate. When the maternal serum folate reaches deficient levels, folate is taken from the red cells that serve as folate storage tissue (Lamers, Prinz-Langenhol, Bramswig, & Pietrzik, 2006). That would explain the consistent findings of low red cell folate levels, but sufficient serum folate levels of the NTD mothers. However, the study of polymorphisms was conducted on a representational population of United States residents, and therefore presumably only has a small percent of pregnant women. Since folate is not as desperately needed among non-pregnant people, the body has no urgent need to bring up the serum folate levels, and therefore they remain deficient.

If the 677TT MTHFR polymorphism is actually what leads to low serum folate and high total homocysteine levels, perhaps it would be more efficient to simply supplement the body with what the MTHFR should be synthesizing rather than with folic acid which stabilizes the enzyme. This speculation was proven true in a study by Lamers et al. (2006). The study involved 144 women aged 19-33 who were not pregnant or lactating. The study was a 24 week double-blind placebo-controlled trial. The women received either 400ug of folic acid, 416 ug [6S] 5 methyltetrahydrofolate, 208 ug [6S] 5 methyltetrahydrofolate, or a placebo daily. Blood samples were collected every 4 weeks of the study. At baseline, the 4 groups did not differ significantly with respect to red cell and serum folate concentration. No change in dietary intake was observed throughout the study period. Although all 3 groups receiving supplements showed significant red cell and serum folate increases, the increase in red cell folate and

serum folate was significantly greater in the group receiving 416 ug [6S] 5 methyltetrahydrofolate than the other 2 groups. The group that received 400 ug of folic acid had the second highest increse.

This study proves that supplemental [6S] 5 methyltetrahydrofolate at approximately the same dosage as supplemental folic acid is more efficient than folic acid at raising folate levels. This supports the idea that the lag in the folate metabolism present in some people occurs at the MTHFR enzyme which is supposed to synthesize 5 methyltetrahydrofolate. Therefore providing the body with the product of the enzyme overcomes the defect more efficiently than trying to stabilize the enzyme with additional folic acid.

The role that folic acid plays in the prevention of the occurrence of a NTD is well documented and proven. Folic acid aids in the deposition of a methyl group on homocysteine, thereby creating methionine. Methionine is then converted to other substances, such as S- adenosylmethionine, which is essential for normal embryonic development. Pregnant women who are folate deficient may therefore not have sufficient methionine levels to support a growing embryo. This could lead to birth defects, such as the failure of the neural tube to close properly. Pregnant women may be folate deficient due to a poor diet. However, even women who do have a folate sufficient diet may have low red cell folate serum levels if they have the 677TT MTHFR polymorphism. This genetic defect can be overcome by consuming an abundance of folic acid, beyond what is normally considered sufficient. Today there is much effort expended on educating the public about the beneficial role of folic acid, and encouraging women of child bearing age to take folic acid supplements.

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# Cholera: An Overview of a Disease

# Ezriel Leifer

### Abstract

Although the disease, cholera, has been recognized since antiquity, the bacteria responsible for causing it was only discovered in the mid-19<sup>th</sup> century. Since 1817, cholera has spread on a global basis to cause seven pandemics. According to information reported to the World Health Organization in 1999, almost 8,500 people died and another 223,000 became sick with cholera worldwide. During the period between full outbreaks, the cholera organism, Vibrio cholerae, thrives in brackish waters, in harmless as well as disease-causing forms. Vibrio cholerae is just one of a variety of ocean-borne microbes that can sicken humans via seafood, drinking water, and swimming. Location, time, and intensity of cholera epidemics can now be accurately predicted from satellite observations of sea surface temperature, sea surface height, and chlorophyll in the water. Bacteria such as Vibrio cholerae have been found to be able to communicate with members of their own species and others to coordinate their behavior in response to cell density in a process known as quorum sensing, which relies on the production of and sensitivity to one or more secreted signal molecules. A growing body of scientific studies has identified a complex quorum sensing network in the human pathogen Vibrio cholerae. To gain a better understanding of this pathogen, this study provides an overview of the bacterium Vibrio cholerae, the mechanism of its virulence, a discussion concerning the symptomatology of the bacterium and its epidemiology. An analysis of how quorum sensing influences the virulence of the bacterium is followed by a discussion of diagnostic and treatment considerations. A discussion of ongoing preventative measures is followed by a summary of the research and salient findings in the conclusion.

## Introduction

As the current swine flu epidemic reaches pandemic proportions, attention has been drawn away from yet another continuing threat to global health in the form of *Vibrio cholerae* and the cholera disease it can cause. Despite this inattention, the threat of cholera remains, and researchers are actively involved in investigating its etiology to identify the best practices in its prevention and treatment. To gain a snapshot of the historic and current efforts to combat this disease, this study provides an overview of the bacterium, *Vibrio cholerae*, the mechanism of its virulence, a discussion concerning the symptomatology of the bacterium and its epidemiology. An analysis of how quorum sensing influences the virulence of the bacterium is followed by a discussion of diagnostic and treatment considerations. A discussion of ongoing preventative measures is followed by a summary of the research and salient findings in the conclusion.

# **Review and Analysis**

# Overview of the Bacterium, Vibrio Cholerae

The cholera disease that is caused by *Vibrio cholerae* has been a source of dread for mankind since the first recorded pandemic in 1817 because of the high death rate associated with it and the rapidity with which it spreads. To date, about 200 O antigens have been distinguished serologically; however, just O1 and O139 have been found in epidemic and pandemic cholera isolates (Salim, Lan & Reeves, 2005). One of the preeminent researchers into the spread of cholera, Colwell, reports that, "Cholera, a diarrheal disease, has been with us for a very long time, even being mentioned in ancient Sanskrit writings. A medical textbook published in 1875 reported cholera to be a global pandemic, consistently appearing in

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India, Bangladesh, Latin America, and Africa. Today, cholera remains a serious problem" (2006, p. 753). The bacterium that causes cholera was first identified in 1854 and has continued to be investigated since that time (Faruque & Nair, 2008).

This scientific interest has been fueled in large part by the fact that the disease has become prevalent worldwide rather than being restricted to the Indian subcontinent as in years past. In this regard, Colwell (2006) reports that, "Until the nineteenth century, cholera was generally confined to the Indian subcontinent, but it then began to appear in Europe and the Americas as well. Since 1817, Western medical history describes seven global pandemics of cholera that have spread illness and death around the world. "The second of these seven pandemics reached the United States in 1832, traveling from New York to Philadelphia in a couple of weeks, and then cases appeared along the Atlantic coast all the way to the Gulf of Mexico" (Colwell, 2006). Likewise, according to Osborne (2008), "The pandemic that swept North American cities in 1832 was caused by a comma-shaped bacterium, Vibro cholerae, known by various names including Asiatic cholera and Cholera morbus. It is now generally accepted that Vibro cholerae originated in India, where cholera was endemic, and first reached the West in the early nineteenth century".

In their account of historic efforts to combat the spread of cholera, Albertine, Persily and Riegilman (2007) repeat a well-known story thusly: "In 1855, pioneering epidemiologist John Snow traced the emergence of cholera in London to the public water supply. He persuaded authorities to remove the pump handle on the Broad Street well and thereby slowed and then stopped an outbreak of cholera". It should be noted that Colwell (2006), though, suggests that the outbreak was stopped as a result of the operation of natural forces more than this

straightforward public health tactic, but the fact remains that the bacterium has been with mankind for millennia and efforts to stop its spread remain an ongoing source of investigation throughout the world.

The seventh pandemic (1961-present) is still widespread and has a severe impact on three different continents. The sixth pandemic ended in 1923, but the clone persisted at least until the 1990's (Safa, Bhuiyan, Alam, Sack & Nair, 2005). In addition, a number of cholera outbreaks were reported after the sixth pandemic retreated, but before the start of the seventh pandemic. Isolates from these outbreaks were recognized as different from those of the sixth pandemic and were allocated to the El Tor biotype, while the sixth and fifth pandemics, both of which had been studied microbiologically, were referred to as the classical biotype (Safa et al.).

The outbreaks of the El Tor biotype took place in Indonesia and the Middle East (1926-1960) and are frequently described as being prepandemic isolates because they were later viewed as predecessors of the subsequent seventh pandemic, an episode that is also of the E1 Tor biotype (Safa et al.). Today, the environmental aspects of *V. cholerae* have been the focus of a considerable amount of research, and the major components of the El Tor phenotype have been shown to be present in most environmental isolates; the classical biotype is thought to have emerged through the loss of characters that are otherwise widespread in the species (Safa et al.). In addition, instances of sporadic indigenous cholera have been detected in Australia as well as the United States, both of which have been of the O1 El Tor biotype (Safa et al.). These are generally referred to as the US Gulf and Australian clones. All of the pathogenic forms discussed above had the O1 serotype, but in 1992 a variant of the seventh pandemic appeared with a new O antigen, O139; this variant is known as *V. cholerae* O139 Bengal (Safa et al.).

## Mechanism of Virulence for Vibrio Cholerae.

Adherence to intestinal cells. The infectivity of V. cholerae is related to its ability to defeat the protective qualities that are typically present in the intestinal cells (Sparling, 1985). Microbial adherence to epithelial cell surfaces has been implicated as the first step in the initiation of several infectious diseases. Subinhibitory concentrations of antibiotics affect the adherence properties of microorganisms in various ways. They can inhibit the expression of fimbriae and the synthesis of other surface components, and they may also cause the release of constituents from the cell. The ability of antibiotics to affect the properties of microbial adherence to cell surfaces may be an important criterion in selecting an antibiotic for therapy. The relationships of V. cholerae have been studied in several ways, but the most useful insights have come from multilocus enzyme electrophoresis and more recently by multilocus sequence analyses (Salim et al.). According to Paz and Broza, "Vibrio cholerae bacteria are common hitchhikers attached to the surface of adult nonbiting midges (observed mainly with Chironomus sp., family Chironomidae). Both males and females have been reported to carry Vibrio cholerae strains that remain viable and culturable even after 14 days". In fact, researchers have been investigating the ability of Vibrio cholerae to adhere to animal cells and different ligands involved in intestinal colonization for some time, including virulence-associated toxin co-regulated pilus (TCP), outer membrane proteins, and lipopolysaccharides (Zampini et al. 2006). According to these researchers, "Its interactions with substrates present in the aquatic environment have been described more recently. V. cholerae O1 El Tor does not use TCP to form a biofilm on abiotic surfaces (borosilicate and cellulose), but rather the mannose-sensitive hemagglutinin (MSHA) pilus, which has no role in pathogenicity" (p. 267). The ability of *V. cholerae* to attach to chitin

has been demonstrated to be independent of the MSHA pilus, a finding that indicates divergent pathways for biofilm formation on nutritive and nonnutritive abiotic surfaces (Zampini et al.). Researchers have also determined that MSHA is involved in *V. cholerae* O1 El Tor and O139 adhesion to the exoskeleton of the planktonic crustacean Daphnia pulex and other ligands are believed to be used by V. cholerae O1 classical strains for zooplankton adhesion (Zampini et al.). In addition, V. cholerae O1 classical strain membrane proteins have been found to mediate N-acetyl glucosamine (GlcNAc)-sensitive attachment to chitin particles in vitro (Zampini et al.).

Release of toxins and their operation. The fatal effects of the disease are mainly due to the cholera toxin produced by specific strains of *Vibrio cholerae* (Paz & Broza). According to DNA researcher Lang, "Instead of having one circular chromosome, like most bacteria, the organism has two. The larger chromosome, comprising nearly three million base pairs, contains most of the organism's critical genes, including those coding for the disease-causing toxins and proteins that carry out essential cell functions" (2000, p. 39). The ability of *V. cholerae* to adhere to epithelial cell surfaces and release these disease-causing toxins has been cited as the first step in its infectivity (Shibl, 1985). In this regard, Todar (2009) reports that, "Cholera toxin activates the adenylate cyclase enzyme in cells of the intestinal mucosa leading to increased levels of intracellular cAMP, and the secretion of H<sub>2</sub>O, Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, and HCO<sub>3</sub> into the lumen of the small intestine. The effect is dependent on a specific receptor, monosialosyl ganglioside (GM1 ganglioside), present on the surface of intestinal mucosal cells" (p. 3). The specific manner in which *V. cholerae* operates is described by this clinician thusly: "The bacterium produces an invasin, neuraminidase, during the colonization stage which has the interesting property of degrading gangliosides to the monosialosyl form, which is the specific receptor for the toxin" (Todar, p. 3). The toxin released by *V. cholerae* contains (a) 5 binding (B) subunits of 11,500

daltons, (b) an active (A1) subunit of 23,500 daltons, and (c) a bridging piece (A2) of 5,500 daltons; the latter links A1 to the 5 binding subunits (Todar). After *V. cholerae* has penetrated the cell, the active subunit transfers ADP ribose from NAD<sup>+</sup> to a protein (called Gs or Ns) enzymatically; this process controls the adenylate cyclase system situated on the interior of the plasma membrane of mammalian cells (Todar). According to Todar, "Enzymatically, fragment A1 catalyzes the transfer of the ADP-ribosyl

moiety of  $NAD^+$  to a component of the adenylate cyclase system. The process is complex. Adenylate cyclase (AC) is activated normally by a regulatory protein (GS) and GTP; however activation is normally brief because another regulatory protein (Gi), hydrolyzes GTP".

Typically, the active subunit fragment changes the attachment of ADP-Ribose (ADPR) to the regulatory protein thereby creating Gs-ADPR, a protein from which GTP is unable to be hydrolyzed. Because GTP hydrolysis serves to shut down the adenylate cyclase process, the enzyme continues to be active in the cell (Todar). As a result, the bottom-line impact of the toxins released by *V. cholerae* are to cause cAMP to be produced at an inordinately high rate, a process than in turn stimulates mucosal cells to discharge large amounts of Cl<sup>-</sup> into the contents of the intestine (Todar). Thereafter, H<sub>2</sub>O, Na<sup>+</sup> and other electrolytes are produced as a result of the osmotic and electrical gradients that are the result of the loss of Cl<sup>-</sup> (Todar). In sum, then, "The lost H<sub>2</sub>O and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing the diarrhea, loss of electrolytes, and dehydration that are characteristic of cholera" (Todar, p. 3). Neither *Vibrio cholerae*, though, nor the toxins it releases are capable of passing through the gut wall (Gibbs, 2005).

## Symptomology of Vibrio Cholerae.

Accounts of the symptoms associated with the 1832 North American pandemic were grisly in their descriptions. For instance, Osborne reports that "Victims of the disease were stricken with severe diarrhea and vomiting, accompanied by excruciatingly painful cramps through the trunk and legs. As dehydration continued, the bodily fluids were excreted as 'rice water' and the victim quickly collapsed and turned blue. "Often, fifty per cent or more of those who caught cholera died, with death coming to the more fortunate victims in as little as four to six hours "(p. 30). Empirical accounts from the 1832 North American pandemic described one victim as being: "... a young woman of apparently twenty-five ... absolutely convulsed with agony. Her eyes were started from their sockets, her mouth foamed, and her face was a frightful livid purple. She had been taken in perfect health only three hours before, but her features looked to me marked with a year of pain. The first attempt to lift her produced violent vomiting, and I thought she must die instantly" (A walk through a cholera hospital, 1832, p. 37).

## Epidemiology of Vibrio Cholerae.

One of the first aspects that researchers learned about cholera was about how the disease was transmitted: "The disease was transmitted through human feces, generally ingested in drinking water. It first escaped the Indian subcontinent in 1817, reaching Moscow in September 1830 and thence westward across Europe. "The mysterious origins and terrifying nature of the disease added to the sense of dread it created in Europe and North America" (Osborne, p. 30). In 2000, scientists identified the entire order of paired chemical building blocks that constitute the DNA of the deadly cholera bacterium, *Vibrio cholerae*, and found that it is "a comma-shaped microbe [that] causes a severe diarrheal disease that has been endemic in southern Asia for at least 1,000 years" (Lang, 2000). Cholera continues to represent an important cause of morbidity and

mortality in many regions of the world, and there is currently a high incidence of fresh outbreaks in Africa (Paz & Broza, 2007).

The transmission of cholera epidemics may also be related to the dominant wind direction over land (Paz & Broca, 2007). These researchers examined the geographic diffusion of three cholera outbreaks through their linkage with the wind direction: a) the progress of *Vibrio cholerae* O1 biotype El Tor in Africa during 1970-1971 and b) again in 2005-2006; and c) the rapid spread of Vibrio cholerae

O139 over India during 1992-1993. In addition, it is possible that the influence of the wind direction on windborne dissemination by flying insects, which may serve as vectors, plays a role. The analysis of air pressure data at sea level and at several altitudes over Africa, India, and Bangladesh by Paz and Broca found a correspondence between the dominant wind direction and the intracontinental spread of cholera. According to Holzman (2007), *Vibrio cholerae* is just one of a variety of ocean-borne microbes which can sicken humans via seafood, drinking water, and swimming. Location, time, and intensity of cholera epidemics can now be accurately predicted from satellite observations of sea surface temperature, sea surface height, and chlorophyll in the water (Foster, 2000). Likewise, Colwell (2006) notes that, "In 1977, my coworkers and I reported that *Vibrio cholerae*, the causative agent of cholera, could be cultured from Chesapeake Bay water samples. It was the first report of the isolation of the *V. cholerae* from noncholera-endemic geographical areas; cholera had not been reported in Maryland since the 1900s. "It was difficult for us to make our case, namely that the cholera vibrio was a native inhabitant of the Chesapeake Bay, since cholera had not occurred in the region.

Despite this lack of evidence, though, these researchers did in fact identify *Vibrio cholerae* in the Chesapeake Bay and thereafter applied molecular techniques to demonstrate that

the bacterium is naturally occurring in the aquatic environment, with annual peaks in the spring and fall. In addition, Colwell and her colleagues found that the *V. cholerae* is associated with plankton. We now know that river, estuary, and coastal waters are reservoirs of these bacteria globally, but their data showing an environmental source of the cholera bacteria implied a paradigm shift for the medical community. "It has taken about 20 years for the paradigm change of cholera being transmitted only by person-to-person contact to the recognition that the cholera vibrio exists in the environment as a natural inhabitant (p. 753). In addition, Colwell and her associates determined that the bacterium experiences a dormant phase between outbreaks and epidemics and through the use of gene probe molecular techniques were able to prove its year-round presence in the environment.

As noted above, *V. cholerae* O1 classical strains adhere to zooplankton, and Colwell and her colleagues determined that this relationship with zooplankton was particularly important. According to these researchers, "In the spring, when the water warms, phytoplankton become abundant; using sunlight for energy, the population of phytoplankton increases significantly. That population increase is followed by blooms of zooplankton, the miniature 'cattle' of the sea, which graze on the phytoplankton. We were able to show a relationship of sea surface temperature increase with onset of cholera epidemics because of the fact that vibrios comprise the natural microbial flora of zooplankton, the populations of which increase spring and fall in annual cycles. The seasonal pattern of cholera follows the seasonal rise and fall in sea surface temperature and height (Colwell,).

In 1991-1992, a massive cholera epidemic took place in Peru and about 200,000 cases and 5,000 deaths were reported as a result of this epidemic; this was an unprecedented occurrence because cholera had not been reported in South America for almost a century

(Colwell). Moreover, the epidemic took place during a period of a strong El Nino climatic event. Based on climatologists' prediction concerning another comparable El Nino event in 1997-1998, Colwell and her associates hypothesized that there was a linkage of the 1991-1992 cholera epidemic with El Nino and predicted additional cholera outbreaks would occur in 1997-1998. As Colwell notes, "With colleagues from Peru, Chile, Ecuador, Brazil, and Mexico, we conducted a training session on molecular techniques for direct detection of the *V. cholerae* in water and plankton. As the sea surface temperatures in these Latin American countries increased in 1997 because of El Nino, the team was able to detect the presence of the cholera bacteria associated with plankton, with numbers of the bacteria increasing from spring to summer (September 1977 to March 1998) and cases of cholera occurring in late November through the summer of 1998" (p. 753).

Colwell and her colleagues were able to conclude that E1 Nino is another important climate factor related to cholera, notably in cholera-endemic countries. The cases of cholera in Peru in 1997-1998 were directly correlated with sea surface temperature. The relationship of the disease with this climate factor was statistically significant. In Bangladesh and other countries where severe cholera epidemics occur, such as Peru, Indonesia, and India, the influence of monsoons or severe weather is important. Matlab, near Dhaka, Bangladesh, comprises a "hotspot" of cholera. The villages are constructed around bodies of water, and specific locations of epidemics have been determined. Research on cholera has been conducted in Matlab by Colwell et al. since 1975. The influence of the Himalayas on the weather in Bangladesh is significant, because the monsoon rains wash nutrients into the rivers and ponds. Typically, houses in Bangladesh are located at the edge of a pond, from which villagers draw their water for household use. A definable relationship between sea surface temperatures, sea surface height, and cholera epidemics was established and published in the proceedings of the National Academy of Science (Lobitz et al., 2000). Taken together, the complex factors of sea surface temperature, sea surface height, and zooplankton populations provide a predictive capacity for cholera epidemics in developing countries that derives from climate monitoring through satellite sensors (Colwell, 2006).

Finally, with the assistance of sociology researchers working in Bangladesh, they were able to test the hypothesis they constructed: that if they could remove zooplankton from the water the villagers used to meet household needs, the incidence of cholera could be reduced. With a very simple filtration technique that Colwell et al. devised using sari cloth folded in 4 layers, the researchers were able to reduce cholera by approximately 50 percent in villages where families had been instructed in the filtration method. The complex of plankton, people, and climate, together with a simple solution based on science (under the electron microscope, the folded sari cloth could be seen to provide a 20 micron filter--and the zooplankton range in size roughly 50 to 200 microns), provides the interrelationship that allows an understanding of a global infectious disease--an understanding that would not otherwise be possible.

Moreover, studies conducted by the Smithsonian Environmental Research Center in recent years have also determined that cholera is far more prevalent in ballast water than previously thought; a sampling of ballast water from fifteen vessels entering Chesapeake Bay identified one strain of cholera, Vibrio cholerae O1, on every ship surveyed. In additional, a different strain of cholera, Vibrio cholerae O139, which had been previously unidentified in the United States, was identified in the ballast water of fourteen of the fifteen vessels surveyed (Foster, 2000).

## How Quorum Sensing Influences the Virulence of the Bacteria Vibrio Cholerae.

According to Cámara, Hardman, Williams and Milton (2002), "Bacteria can communicate with members of their own species and others to coordinate their behavior in response to cell density. This phenomenon, known as quorum sensing, relies on the production and sensing of one or more secreted signal molecules. A recent study identifies a complex quorum sensing network in the human pathogen Vibrio cholerae (p. 217). In this regard, Tsou, Cai, Liu, Zhu and Kulkarni (2009) report that, "The quorum-sensing pathway in Vibrio cholerae controls the expression of the master regulator HapR, which in turn regulates several important processes such as virulence factor production and biofilm formation. While HapR is known to control several important phenotypes, there are only a few target genes known to be transcriptionally regulated by HapR (p. 2747). In this study, Tsou and her colleagues combined bioinformatic analysis with experimental validation to discover a set of novel direct targets of HapR. The findings of the Tsou et al. study provide evidence for two distinct binding motifs for HapR-regulated genes in V. cholerae as follows. The first binding motif is similar to the motifs recently discovered for orthologs of HapR in V. harveyi and V. vulnificus; these results also demonstrate that this binding motif can vary in length in V. cholerae; the second binding motif shares common elements with the first motif,

but in contrast to the first binding motif, the second is fixed in length and does not have dyad symmetry at the terminal points (Tsou et al.).



## Figure 1. Quorom sensing in Vibrio harveyi.

Source: Camara et al.

In V. harveyi, a harmless marine bacterium closely related to V. cholerae, quorum sensing serves to activate bioluminescence (luxCDABE) at high cell density levels; to accomplish this, two parallel signaling systems are used to control light production. Likewise, as shown in Figure 2 below, quorum sensing in V. cholerae functions by restricting virulence gene expression at high cell density through the use of three parallel signaling systems as follows:

- 1. System 1 includes CqsA, a putative synthase for the unidentified CAI-1 signal molecule, and CqsS, a hybrid sensor/kinase that responds to the CAI-1 signal.
- 2. System 2, LuxSPQ, is identical to that of V. harveyi (see above).
- 3. System 3 has not been characterized; however, this system is believed to respond to an intracellular signal and is therefore thought to be unrepresentative of a true quorum sensing system. System 3 does not seem to transmit its signal through LuxU but rather

directly through LuxO, the pivotal regulator for all three systems. Like the V. harveyi system, LuxO is activated via phosphorylation at low cell density and, together with  $\sigma$ 54, activates the expression of

an unidentified repressor that blocks the production of HapR, which acts as a repressor of virulence gene expression. As a result, at low cell density, virulence genes are expressed; at high cell density, though, LuxO remains inactive and cannot repress HapR. As HapR is now expressed, virulence gene expression is repressed (Camara et al.).



Figure 2. Quorum sensing in Vibrio cholerae.

Source: Camara et al.

The quorum sensing responses in V. harvevi and V. cholerae represent instances of complex cellto-cell communication techniques that focus multiple cell-density-dependent, and perhaps independent, signals via an alternate regulatory pathway to regulate bacterial behavior (Camara et al.). According to these researchers, "Variations on the complexity or organization of these multiple-channel signaling systems may be forthcoming, as components of the V, harveyi-like signaling systems have been found in V. fischeri as well as the fish pathogen Vibrio anguillarum11-13; however, adding yet more complexity to the process, both of these organisms also possess LuxI/LuxR-type quorum sensing signaling systems" (Camara et al., p. 218). The coordination of a multi-cellular response to rapidly adapt to changes that take place in the surrounding environment is a useful survival strategy for pathogenic and non-pathogenic bacteria alike (Camara et al.). Just as other forms of life respond to the density levels of their populations in different ways, so too does the V, cholerae. In this regard, Camara and his associates report that, "Cell density is just one of a multitude of signaling parameters that a bacterium faces in any given environment, and coordination of all signals into a physiological response will require intersystem cross-talk. Incorporation of quorum sensing into a global network of regulatory responses may be required to increase selectivity and efficiency of a bacterium's response to the stresses it encounters in its growth environment" (Camara et al., p. 218).

One unexpected outcome of a study by Miller et al. (2002) was that, in contrast to many other bacterial pathogens, quorum sensing in V. cholerae serves to repress, rather than activate, virulence gene

expression at high cell density levels in vitro (Camara et al.). A speculative model was advanced that indicated that following initial colonization (low cell density), LuxO represses HapR, which is also a repressor of virulence gene expression, thereby permitting colonization of the intestines and cholera toxin production. In this regard, Camara et al. add that, "As the bacterium multiplies, the quorum sensing signal molecules accumulate and activate the two quorum sensing systems, which inactivate LuxO. HapR is then produced, repressing virulence gene expression while activating the production of the protease, HapA". (Camara et al., p. 218).

HapA is believed by scientists to promote the detachment of the bacteria from intestinal tissue, thus facilitating the spread of the pathogen. As there is still a third unidentified signaling system influencing gene regulation through the LuxO regulatory cascade, and additional regulators of virulence gene expression to be considered, the actual role of signaling systems 1 and 2 in the virulence of V. cholerae in vivo remains unclear. According to these researchers, "Nonetheless, the model proposed for V. cholerae should provide an exciting twist on the role of quorum sensing in virulence" (Camara et al., p. 218).

Finally, a study by Zhu, Miller, Vance, Dziejman, Bassler and Mekalanos (2002) notes that the production of virulence factors including cholera toxin and the toxin-coregulated pilus in the human pathogen Vibrio cholerae is strongly influenced by environmental conditions. These researchers report that, "The well-characterized ToxR signal transduction cascade is responsible for sensing and integrating the environmental information and controlling the virulence regulon" (p. 3129). In their timely study, "Quorum-sensing regulators control virulence gene expression in Vibrio cholerae," Zhu et al. demonstrate that besides the known components of the ToxR signaling circuit, quorum-sensing regulators are involved in regulation of V. cholerae virulence. These researchers concentrated on the regulators LuxO and HapR because homologues of these two proteins control quorum sensing in the closely related luminous marine bacterium Vibrio harveyi in this study. According to Zhu and his associates, "Using an infant mouse model, we found that a LuxO mutant is severely defective in colonization of the small intestine. Gene arrays were used to profile transcription in the V. cholerae wild type and the LuxO mutant. These studies revealed that the ToxR regulon is repressed in the LuxO mutant, and that this effect is mediated by another negative regulator, HapR. We show that LuxO represses HapR expression early in log-phase growth, and constitutive expression of HapR blocks ToxR-regulon expression.

Additionally, LuxO and HapR regulate a variety of other cellular processes including motility, protease production, and biofilm formation. Together these data suggest a role for quorum sensing in modulating expression of blocks of virulence genes in a reciprocal fashion in vivo" (p. 3130).

# Diagnostic and Treatment Considerations.

In many ways, Vibrio cholerae is an elusive bacterium that can elude ready detection that confounds diagnosis. In this regard, Henrickson, Wong, Allen, Ford and Epstein (2001) report that some gram-negative organisms such as V. cholerae are capable of adapting to low-nutrient environments through reductive division; in other words, although there is no change in total biomass, more organisms develop but they develop at a significantly reduced metabolic rate. When there are unfavorable environmental conditions, such as cold or reduced nutrients, Vibrio cholerae bacteria can shrink to 1/300<sup>th</sup> of its original size and enter a dormant state; remaining in the dormant state for extended periods of time, increasing in size when environmental conditions once again become favorable. Moreover, V. cholerae can become nonculturable on routine culture plates, but remain viable, and could regrow under appropriate conditions (Henrickson et al.).

As noted above, cholera has a rapid onset and most frequently occurs in epidemics that are spread through contaminated water. According to Long, "Oral rehydration is an effective treatment, but left untreated, cholera causes severe diarrhea that has a high mortality rate, particularly in young children" (p. 39). Even some simple precautionary methods can reduce the incidence of infection by Vibrio cholera,

though. For instance, Holzman (2007) emphasizes that if people in the least stable situations--for example, in war-torn developing countries—are provided with sufficient warning, they could filter drinking water using low-tech devices, cutting

the infection rate by 50%. Researchers have successfully monitored and predicted cholera epidemics in Bangladesh through the use of satellites, as reported in the 15 February 2000 issue of *Proceedings of the National Academy of Sciences* (Holzman). In this regard, Dremeaux (2003) reports that, "Ingesting a high dose of the waterborne bacteria Vibrio cholerae O1 produces cholera, an infection that causes severe dehydration brought on by acute diarrhea and vomiting. Left untreated, cholera can kill a person in 24 hours. Filtering water with a folded piece of old cloth before drinking it cuts the rate of cholera contraction by half, according to a three-year study in 65 Bangladeshi villages published in January 2003 in the U.S. journal *Proceedings of the National Academy of Sciences*" (p. 9). According to Gibbs (2005), "Up to 90 per cent of cases of cholera will have only mild diarrhea. The remainder may exhibit severe watery diarrhea leading to dehydration. Since neither the bacteria nor its toxins can pass through the gut wall and into the blood, cholera does not increase the sufferer's temperature. In severe cases, a simple antibiotic such as Tetracycline will decrease the diarrhea. Rehydration is imperative. "Oral rehydration salts are usually sufficient, but severe cases will need intravenous fluids" (p. 101).

### Discussion.

As noted above, simply filtering contaminated water with a folded piece of old cloth before drinking it reduces the rate of cholera contraction by fully 50 percent. According to Dremeaux (2003), "The finding has the potential to save thousands of lives annually. Fabric from saris, the flowing, colorful garments South Asian women often wear, was cheap and readily available to the 133,000 people who participated in the study, and comparable fabrics could function as filters for populations at risk for cholera around the world" (p. 9). The sari cloth does not trap V. cholerae per se, but rather the copepods, a type of zooplankton onto whose mouths, surfaces, and egg cases the vibrios attach (Dremeaux). While a majority of the vibrios did remain

free in the filtered water, their numbers were frequently diminished sufficiently to fall short of an infective dose, estimated at  $[10^4]$  to  $[10^6]$  V. cholerae; in fact, the dilution lowered the rate of cholera infection by almost half (48%). According to Dremeaux, "For those who did contract cholera via filtered water, the severity of the disease appears to have lessened, the report says. Electron microscopy had revealed that sari cloth, when folded four to eight times, would create a filter of approximately 20 mm pore size, removing all copepods--and the cholera-causing bacteria attached to them--from the water. The old saris used in the experiment were expected to be more effective than new ones, their laundered fabric resulting in a smaller pore size" (p. 9). Although clean potable water would be preferable of course, and boiled water would be an acceptable alternative, there remains a paucity of fuel for many villagers to use for this purpose. In this regard, Dremeaux reports, "Rivers and ponds are a common source of drinking water for the villages in rural Matlab, Bangladesh. Boiling water, which kills all waterborne microorganisms, is often impossible for the villagers, who are hard-pressed to find dry wood for fuel or the money to buy it. High concentrations of arsenic in the groundwater make well sources a poor alternative (Dremeaux, p. 9). Although nylon mesh filter was nearly as effective as the sari cloth in reducing cholera, that material is more costly and harder to find in rural Bangladesh than material from saris (Dremeaux). In sum, Colwell (2006) emphasizes that, "The interaction of humans, cholera bacteria, the zooplankton host of the bacterium (the copepod), and the environment in the case of cholera can be employed to make reasonable predictions about this climate-driven disease. The issues are truly international and represent those that comprise a global scientific enterprise and encompass many other infectious diseases" (p. 754).

## Conclusion

The research showed that the disease caused by Vibrio cholerae has plagued mankind for thousands of years, but the pathogen that was responsible for causing cholera was only discovered a century and a half ago. Over the past two centuries, cholera has caused seven pandemics and has been responsible for tens of thousands of deaths. The research also showed that even during periods when it is dormant, Vibrio cholerae, continues to thrive in brackish waters in both harmless and disease-causing forms. Researchers have discovered that it is possible to predict with some accuracy future outbreaks of cholera epidemics using satellite observations of sea surface temperature, sea surface height, and chlorophyll in the water. Finally, the research also showed that bacteria such as Vibrio cholerae are capable of communicating with other members of their species in order to coordinate their behavior in response to cell density in a process known as quorum sensing that relies on the production and sensing of one or more secreted signal molecules. A growing body of scientific studies has identified a complex quorum sensing network in the Vibrio cholerae that may help researchers identify superior diagnostic and treatment protocols in the future, but in the meantime, there have been some commonsense approaches to treating water contaminated by Vibrio cholerae that reduce its incidence by almost half.

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# Atherosclerosis and Antioxidants

# Yehoshua Lewis

# Abstract:

Cellular lipid oxidation is a known cause for the cascade leading to the formation of lipid laden foam cells, which can cause of atherosclerosis. While statins and antioxidants have recently come under question in the amelioration of atherosclerosis, Flavonoids have recently been touted as a powerful antioxidant and suppresser of atherosclerosis. This paper will attempt to show why statins and vitamin E have come under scrutiny, and how the desired effects of Flavonoids can be attributed to the role it plays in increased paraoxonase-1 activity (a known anti inflammatory associated with HDL), decreased C- Reactive protein activity, and increased nitric oxide (NO) in endothelial cells among other factors.

# Introduction:

Coronary artery disease (CAD) is the leading cause of death in the United States every year. According to the Center for Disease Control and the National Center for Health Statistics, data for the year 2004 indicate that heart disease killed 652,486 people (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, 2008). This is nearly 100,000 more than the second leading cause of death in the United States, cancer. Atherosclerotic plaque build-up in the arteries is the cause of CAD, and has been the primary focus of scientists in search of a cure for CAD. Needless to say, much research has been done on finding the causes of heart disease and developing drugs that inhibit its development. Other then pharmacological products, there is a very large body of research into more natural remedies for atherosclerosis, including antioxidants such as vitamin E that have been touted as providing protection against lipid oxidation. Recently, however, there is a growing body of evidence that statins and antioxidant supplementation may not be beneficial as thought. In contrast, Flavonoids, by operating under mechanisms different then antioxidants and statins, offers new hope in the treatment of atherosclerosis.

# Coronary Artery Disease:

While there are many factors that contribute to CAD, there are a number of factors specifically related to atherosclerosis that play a key role in its development. Atherosclerosis is a progressive disease of the



Figure 1. Dissected aorta with atherosclerotic lesions (Ewing, 1972)

arteries and is the result of plaque buildup in the arteries driven by the uptake of cholesterol in artery walls (Medline Plus, 2007). Lipids are not water soluble, and must rely on lipoproteins, produced in the liver and small intestines, in order to be transported in the blood. By combining lipids with lipoproteins, the lipids are able to be transported either to the liver for elimination, or from the liver for the production of steroid hormones, bile salts, and for cell membrane repair. The two major lipoproteins involved in these functions are low-density lipoproteins (LDL), and high-density lipoprotein (HDL); with LDL being responsible lipid transport from the liver, and HDL being responsible for transport of lipids to the liver for elimination (Tortora & Derrickson, 2006).

Plaque formation begins with the aggregation of excess LDL in the inner layer of arterial walls where the lipoproteins are subject to oxidation. In response, substances released by endothelial and smooth muscle cells attract macrophages that ingest the oxidized LDL forming plaque. So much so, that the macrophages take on a foamy appearance under the microscope (foam cells). Additionally, lymphocytes (T cells) follow the macrophages into the arterial wall and increase the streaks in the arterial walls. Subsequently, the macrophages cause the migration of the middle layer of the artery to the surface of the plaque thus separating it from the bloodstream, but also narrowing the arterial lumen. In the event that the cap over the plaque bursts, the T cells stimulate the foam cells to produce tissue factor (TF), which ultimately leads to blood clot formation and possible obstruction of the coronary artery, resulting in myocardial infarction (MI). Other factors currently being considered by researchers include C-reactive proteins, which bind to damaged cells and assist in phagocytosis, clotting factors, and the amino acid homocysteine that promotes platelet aggregation (Tortora & Derrickson, 2006).

### Statins:

In order to treat atherosclerosis, scientists have focused their attention on reducing the amount of cholesterol available in the bloodstream for oxidation with the use of statin drugs.

In the 1960's (Endo, 1992) scientists discovered that when cholesterol is completely eliminated from the diet, the liver takes over and produces up to 82% percent of the required cholesterol. However, when cholesterol is added to the diet the synthesis of cholesterol is almost completely suppressed. They discovered that the production or suppression of cholesterol was in response to modulation by HMG-CoA reductase which modulates the inhibition of the Mevalonate pathway of cholesterol synthesis (Figure 2). Through modulating this enzyme they were able to alter the production of cholesterol. Thus was born the first statin drugs. However,



are medical researchers who challenge the claim that statin drugs actually lower the risk factors for myocardial infarction (MI). In a scathing review of studies purporting to show that by lowering

cholesterol with statin drugs mortality rates are decreased, Kauffman (Kauffman, 2007) cites a study carried out in New York City of a group of 2,277 people with the median age of 76 who were studied for a period of ten years. What he found was that "The chance of dying was twice as great in subjects with the *lowest* quartile of total cholesterol (TC) or LDL-C levels, compared with those in the highest quartile". Kauffman goes on to cite another study which shows that for men aged 35-57 "all cause" mortality rates increased for those with a TC of <170 mg/dL and the risk increased further as TC decreased to <140 mg/dL to the same levels seen for subjects with extremely high cholesterol >300mg/dL. Kaufmann flatly states "Serum TC level is not even predictive of cardiovascular disease (CVD) in men over the age of 47". Kauffman is not alone in challenging the use of statins; the British Medical Journal (Ravnskov et al., 2006) also challenges its widespread use, safety and efficacy. This raises serious questions about how the medical community is facing the challenge of CAD.

#### Oxidation:

While statins focus on reducing TC as a remedy to the formation of atherosclerosis, there is another very important juncture in the formation of atherosclerosis that has been the focus of research, namely oxidative stress caused by free radicals and reactive oxygen species (ROS). ROS and free radicals are injurious to lipids, DNA, and proteins if left unchecked by the bodies' native defense mechanisms (Davis, 1995). What are ROS and free radicals and why are they so important?

Examples of ROS include hydrogen peroxide ( $H_2O_2$ ), hypochlorous acid (HClO). Free radicals include superoxide anion ( $\cdot O_2^{-}$ ) and hydroxyl radical ( $\cdot OH$ ) (Davis, 1995). One source of the free radical superoxide anion ( $\cdot O_2^{-}$ ) is that produced by the NADH/NADPH oxidase in macrophages during the uptake of LDL (Runge, 1999). The superoxide is formed during the electron transport chain (ETS) when the reduction of coenzyme Q in complex III forms the unstable Q $\cdot$ - radical which can leak electrons directly to oxygen instead of cascading down the rest of the ETS thus forming superoxide radicals (Holbrook, 2000).

Considering that 1% - 15% of O<sub>2</sub> respired by mammals undergoes the superoxide anion  $(\cdot O_2^{-})$  state (McGraw-Hill's Access Science), there is also ample opportunity to form undesired side reactions. An example of a side reaction causing oxidative stress results when superoxide reacts with nitric oxide (NO) to form peroxynitrite  $(\cdot O_2 - + \cdot NO \rightarrow ONO_2 -)$ . Peroxynitrite then reacts as a strong oxidizing agent with lipids, DNA, and proteins. Superoxide and NO individually can both be efficiently removed from the body. Superoxide reacts with hydrogen in the presence superoxide dismutase (SOD) to form oxygen and hydrogen peroxide  $(2\cdot O_2 - + 2H^+ \rightarrow O_2 + H_2O_2)$ , which can then be converted by catalase to water and oxygen. NO is rapidly removed by its diffusion through tissues into red blood cells. However, when compared with SOD, the kinetics and thermodynamics during a reaction of NO and superoxide favor the formation of peroxynitrite - resulting in the inevitable formation of a highly reactive oxidizing agent. Peroxynitrite acts as a potent oxidizing agent towards LDL which then becomes the target for scavenger cells and the formation of foam cells. Various studies have also shown that LDL can be oxidized by peroxynitrite even in the presence of endogenous lipophilic antioxidants (Pacher et al., 2007).

Another source of oxidation is the Hydroxyl radicals (•OH) that are produced in the iron catalyzed Haber-Weiss reaction. The Haber-Weiss reaction (Koppenol, 2001) was first discovered in the

early 1930's by Fritz Haber and Josef Weiss and proceeds as follows: Recalling that 1%-15% of  $O_2$  respired by mammals undergoes the superoxide anion ( $\cdot O2-$ ) state, we can propose the following reactions:

$$\operatorname{Fe_3}^+ + \bullet \operatorname{O_2}^- \rightarrow \operatorname{Fe_2}^+ + \operatorname{O_2}$$

Followed by the Fenton reaction:

 $Fe_2 + H_2O_2 \rightarrow Fe_3^+ + OH^- + \bullet OH$ ,

For a net reaction of:

 $\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + HO^- + O_2.$ 

Hydroxyl radicals are short lived in vivo, (Approx  $10^{-9}$  s) and are highly reactive (Yan et al., 2005). They also cannot be enzymatically removed because of its short half life and tends to react extremely quickly with whatever is in its vicinity including: carbohydrates, nucleic acids, lipids, and amino acids.

The limitations of this process are inherent in that it requires Iron III in order to proceed, and the blood plasma protein Transferrin is very effective at scavenging free iron, making it unavailable for reduction. However, if there is a buildup of iron either due to dietary Iron overload or any other multiple of diseases, it can outstrip the ability of Transferrin to perform properly and results in the production of free radicals (Fouad, 2008).

#### Antioxidants:

As the oxidation of lipids form a key step in the formation of atherosclerosis, it should also lead to the conclusion that "antioxidants" offer protection against lipid oxidation. However, the more research that is being done, the more it becomes clear that exogenous sources of antioxidants have questionable effects in vivo. The field of antioxidants is very broad and can be the subject of many papers, but just to scratch the surface; antioxidants can be broken down into metabolite and enzymatic categories as well as lipid soluble and water soluble. The difference between them is their mechanism as well as site of action. For example: lipid soluble antioxidants act in cell membranes, and water soluble antioxidants act in the blood plasma and cytoplasm. Lipid soluble antioxidants include vitamin E and coenzyme Q (Q<sub>10</sub>). Water soluble antioxidants include ascorbic acid (vitamin C), glutathione, lipoic acid, uric acid, and polyphenols (Flavonoids, tannins). Enzymatic antioxidants include the previously mentioned SOD, catalase, as well as glutathione among others (Sies, 1997).

Vitamin E is a potent antioxidant and some research has shown that it can regulate HMG-CoA reductase (Pal et al., 2003; Parker, 1993). Vitamin E is the name for a group of four tocopherols and four tocotrienols, of which the most interesting to scientists is the  $\alpha$ -tocopherol variety because of its high bioavailability. Vitamin E is the main lipid-soluble antioxidant in the body and it works in cell membranes where it prevents the propagation of free radical reactions (Herrera & Barbas, 2001).

Because vitamin E is lipid soluble, when absorbed vitamin E is packaged in Chylomicrons (large lipoprotein particles) that are produced in the absorptive cells in the small intestine. They are then

secreted via exocytosis into lacteals of the lymphatic system and delivered into the circulatory system at the juncture of the thoracic duct and the left subclavian where they are transported to the liver. At the liver, lipoprotein lipase (LPL) induces the unloading of some of the chylomicrons into the extrahepatic tissue and the remainder chylomicrons transports the tocopherols into the liver. Here, " $\alpha$ -tocopherol transfer protein" incorporates  $\alpha$ -tocopherol into very low density lipoprotein (VLDL) the excess of which is excreted into the bile duct. The  $\alpha$ -tocopherol binded VLDL is then transported into circulation where it is converted to LDL again by the action of LPL. The  $\alpha$ -tocopherol is then taken up by the endothelial cell membrane by the uptake of LDL via cell receptors (Herrera & Barbas, 2001).

Once in the cell membrane,  $\alpha$ -tocopherol can act as a terminator to lipoprotein oxidation chain reaction as follows:

Where "I" is the initiator and "LH" is the fatty and L• is formed from the fatty acid

 $I + LH \rightarrow L \bullet + IH$  [1]

Propagation continues as follows:

 $L \bullet + O_2 \rightarrow LOO \bullet [2]$ 

 $LOO \bullet + LH \rightarrow LOOH + L \bullet [3]$ 

(See Figure 3)

Termination occurs when tocopherol "TO" breaks the chain reaction:

 $LOO \bullet + TOH \rightarrow LOOH + TO \bullet [4]$ 

The tocopheroxyl radical then reacts with another peroxyl radical to form tocopheryl quinine  $(Q_{10})$  and thereby arresting the chain reaction (Wolf, 2005).

Figure 3. The free radical mechanism of lipid peroxidation

As with almost every system in the body, vitamin E does not function in isolation but is part of an antioxidant network in which vitamin E can be synthesized from vitamin C and thiol redox cycles involving glutathione and lipoic acid. (See attachment). Vitamin C has pro-oxidant properties as well because of its ability to reduce metal ions via the Fenton reaction which can be a source of oxidative stress. However, this is thought to be of minor significance as compared with its antioxidant properties (FREI, 1999).

On the other front, research has shown that vitamin E, specifically tocotrienols, can modulate HMG-CoA reductase similar way to that done by statins as discussed previously. (Parker et al., 1993)

A number of studies showed this effect in mammals. In one study hyperlipidemic pigs given a diet rich in tocotrienols showed a marked decrease in plasma concentrations of cholesterol, apolipoprotein B (required in the formation of LDL), thromboxane B2 and platelet factor 4(both required for blood coagulation), indicating a protective effect on endothelium and platelet aggregation (Qureshi et al., 1991). There have been some conflicting results in similar trials in humans; however, those may be attributed to plasma levels of tocotrienols and not to the efficacy of the compound (Packer et al., 2001). It would thus seem that by ingesting vitamin E one would be able to take concrete measures in arresting the propagation of atherosclerosis.

However, a recent Meta analysis by The Cochrane Collaboration - of studies proving the remedial effects of vitamin E as well as other antioxidants - has not supported those results. Not only did the ingestion of exogenous antioxidants not prove to be beneficial but has also shown to be detrimental. The study included 232,550 participants who were "randomised to antioxidant supplements (beta-carotene, vitamin A, vitamin C, vitamin E, and selenium) versus placebo or no intervention". "A total of 17,880 of 136,023 participants (13.1%) randomised to antioxidant supplements and 10,136 of 96,527 participants (10.5%) randomised to placebo or no intervention died. In the analyses of the trials with low risk of bias, beta-carotene, vitamin A, and vitamin E *significantly increased mortality*." The study flatly states that there is *no evidence* to support antioxidant supplementation. This study brings into question the widespread use of antioxidants as a remedial and/or prophylactic compound for oxidative diseases (Bjelakovic et al., 2008).

## Flavonoids:

Increased consumption of fruits and vegetables is associated with reduced incidences of CAD and other disease. The cause of this has been attributed in part, to antioxidant Flavonoids present in these foods. The hypothesis in this case is that because after the consumption of Flavonoids there is a marked increase in the antioxidant properties of blood plasma and it can play a direct role in the prevention of lipid oxidation (Lotitoa & Frei, 2006). A clear example of this can be seen in the French paradox, where despite the French consuming large amounts of red meat that are rich in unsaturated fats they still develop lower instances of death from CAD then Americans. This has been attributed to their consumption of red wine which is rich in Flavonoids (Ferrières, 2004).

Michael Aviram of the Technion faculty of medicine has done extensive research in the field of pomegranate polyphenols (Flavonoids), and has made a very important contribution to the field. Among the points studied three will be focused on: Antioxidant properties of blood plasma, paraoxonase-1 (PON-1)<sup>1</sup> levels, and aortic stenosis - all after the ingestion of PJ. In one study (Aviram, et al., 2000), both healthy human males and atherosclerotic E-deficient (E<sup>0</sup>) mouse subjects were fed PJ for variable periods of time. The objective of the study was to determine what effect PJ consumption had on lipoprotein oxidation, aggregation and retention; macrophage atherogenicity; platelet aggregation; and atherosclerosis. The results of the study were striking. In humans, PJ consumption resulted in a decrease in LDL susceptibility to aggregation (-11% ex vivo) and retention, also observed was serum PON-1 increased activity by 20% and that it also had a protective effect against LDL oxidation. Human plasma in

<sup>&</sup>lt;sup>1</sup> Paraoxonase1 (PON1) is an anti-inflammatory enzyme located on HDL.

one case showed a decrease in lipid plasma peroxidation of  $\leq$ 33%. In E<sup>0</sup> mice, a 90% decrease in LDL oxidation was observed as well as a 20% decrease in the uptake of LDL by peritoneal macrophages. Also observed was a 44% decrease in atherosclerotic lesions and foam cells as compared with water fed mice. Aviram et al., attributes the protective antioxidant effect of PJ to increased cellular glutathione content, and states that the reduction of atherosclerotic lesions is directly related to the antioxidant properties of PJ. Another study by Aviram of the long term effects of PJ consumption - in human subjects with carotid artery stenosis (CAS) - on carotid lesions and changes in oxidative stress and blood pressure had remarkable results. After 1 year control subjects showed a 9% increase in intima-media thickness (IMT) as opposed to the PJ group that showed a 30% decrease in IMT. PON-1 activity increased by 83%, serum LDL basal oxidative state was reduced by 90%, copper ion induced oxidation was reduced by 59% and total antioxidant status (TAS) increased by 130%. Again, Aviram attributes these results to the antioxidant characteristics of PJ polyphenols (Aviram, et al., 2004).

Jane Higdon, Ph.D. of The Linus Pauling Institute at Oregon State University takes a different tack and claims that because the bioavailability of Flavonoids is very low, its antioxidant properties cannot be attributed to the antioxidant properties proved in vitro. For example, the bioavailability of Flavonoids in vivo is 100-1000 times lower than the bioavailability of vitamin C or glutathione and thus has a very small antioxidant effect. However, it is available in concentrations that effect cell signaling proteins. "Numerous studies in cell culture suggest that flavonoids may affect chronic disease by selectively inhibiting kinases" including those related to growth factors. Higdon hypothesizes that it is this property that protects against CAD by decreasing inflammation, decreasing vascular cell adhesion molecule expression, increasing endothelial nitric oxide synthase (eNOS) activity, and decreasing platelet aggregation (Higdon, 2005). Another study (Lotito & Frei, 2006), attributes the protective effect of Flavonoids not directly to its antioxidant activity, but rather to the consequence of increased uric acid.<sup>2</sup> What is clear from all these studies is that it is not the antioxidant properties of Flavonoids that directly protect against CAD but rather to a whole host of activities including: increased native antioxidants such as glutathione, uric acid, modified cell signaling pathways, increased PON-1 activity, and increased NO levels.

# Possible Risks of Antioxidants vs. Flavonoids:

So then what really does account for the differences in Flavonoids and for example vitamin E? What may be at play is as follows:

Vitamin E as well as having antioxidant properties is a pro-oxidant as well (Bowry et al., 1992). For example: instead of the termination reaction by tocopherol to the oxidative chain reaction of lipids as a final step:

$$LOO \bullet + TOH \rightarrow LOOH + TO \bullet [4]$$

We instead form

<sup>&</sup>lt;sup>2</sup> Recent studies have implicated hyperuricemia as being a true risk factor in development of cardiovascular disease (Jing Fang & Michael H. Alderman, 2000). However, nearly half of the bodies' antioxidant activity is from uric acid and in moderation may play a positive role.

#### $TO \bullet + LH \rightarrow TOH + L \bullet [5]$

This will then feed into the chain reaction

 $L\bullet + O_2 \rightarrow LOO\bullet [2]$ 

 $LOO \bullet + LH \rightarrow LOOH + L \bullet [3]$ 

One study (Bowry, Ingold, & Stocker, 1992) has found that in aqueous solution, strikes between the stable TO• and other radicals were found to take upward of 17 minutes, a time long enough that even the most stable radical will find "`something to do'" i.e. propagate the chain reaction. The termination of this reaction must then rely on two endogenous antioxidants vitamin C, as demonstrated by the thiol redox cycles (Appendix 1), and ubiquinol ( $Q_{10}H_{2}$ , a reduced form of  $Q_{10}$ ) in the electron transport chain. What can be inferred from this is that by ingesting vitamin E the body sets off a cascade of endogenous antioxidants, in order to minimize the *damage* being incurred by vitamin E. While the body does have the mechanisms of scavenging TO• how can making more of them by ingesting vitamin E be advantageous? Essentially there is a delicate balance of electron radicals that are passed down a cascade until they can be neutralized to a non reactive end product, and by adding more electron radicals in the form of  $\alpha$ -tocopherol it may have the effect of being counterproductive.

#### C - reactive protein and PON-1

By contrast, Flavonoids are effective not by acting as antioxidants but through modulating the inflammatory response of oxidized cells.

The relationship between C - reactive protein (CRP) and Flavonoids illustrates this point. CRP is found to be elevated during inflammatory response by the body, specifically, macrophages and T cells release Interleukin-6 (IL-6) as a pro inflammatory to stimulate a response to tissue damage. CRP production is then part of the acute-phase response to most forms of inflammation, infection, and tissue damage. CRP levels by themselves cannot be used as marker for atherosclerosis, but they have been positively correlated with CAD (Pepys & Hirschfield, 2003). Flavonoids have an inverse relationship with CRP. One study using demographic data (Chun et al., 2008) found that the greater consumption of Flavonoid rich foods, the lower the concentrations of serum CRP thus clearly indicating that Flavonoids affect inflammatory response. Another study has also found an inverse relationship between levels of CRP and levels of PON-1 expression. They found that "Higher levels of CRP seem to be generally associated with low levels of PON1 activity" (Macknessa et al., 2006). Which should lead to the conclusion that the atherosclerotic protection associated with Flavonoids is not related to an inherent antioxidant activity but rather to other factors as mentioned.

Another study also found that polyphenols (i.e. Flavonoids) acutely enhanced nitric oxide bioactivity. NO is necessary to maintain vasodilatation - the lack of which is implicated in increased risk of cardiovascular disease (Anter et al., 2004).

# Conclusions:

In comparing the protective effects on atherosclerosis associated with statins, vitamin E, and Flavonoids, there are many questions as to the ability of statins and vitamin E to afford real protection against atherosclerosis. The data in many cases implicates these very compounds increased mortality rates. By contrast, Flavonoids have shown themselves able to boost the bodies' native antioxidants including uric acid and glutathione, while at the same time reducing the inflammatory response to the uptake of oxidized lipids. It could be that mother really knew best all along "an apple a day – really does – keep the doctor away". (Apple peals have been found to consist of 40% flavonols by weight (Łata & Tomala, 2007 )).

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# Excitoxicity in Retinal Ischemia and Treatment Using Non-Competitive Receptor Antagonists

# Jacob Rube

Ischemia is defined as an inadequacy of blood flow to tissue. Ischemia can deprive tissue of oxygen and metabolic substrates and it can also prevent the removal of waste products. If the ischemia is maintained over enough time the tissue will lose its homeostasis and eventually die causing an infarct. Retinal ischemia occurs when the blood supply to the retina does not meet the metabolic needs that are required to sustain the retina. This can lead to retinal damage and severe vision loss. Ischemia is caused by occluded blood vessels.

The retinal blood supply originates in the ophthalmic artery and branches into the different sections of the retina. The external retina, which contains the cell bodies of the photoreceptors, is provided with nutrients by the choroid blood vessels. The inner layer and ganglion layer, which contains the ganglion bipolar and amacrine cell bodies, is nourished by the central retinal artery (CRA) (Brown, 1991).

Complete retinal ischemia occurs when the opthalamic artery is occluded. Major causes of retinal ischemia are central retinal artery occlusion (CRAO), branch retinal artery occlusion (BRAO), central retinal vein occlusion (CRVO), and branch retinal vein occlusion (BRVO). CRAO is caused by either a thrombosis of the CRA or an embolisation of an artherosclerotic internal carotid artery (Brown, 1991). Ischemia caused by the CRAO leads to an infarction and loss of function of all the inner layers of the retina while the outer layers of the retina including a portion of the inner nuclear layer remain intact (Kincaid et al, 1998).

The CRA branches off into several different branches in order to perfuse the inner layer of the retina. BRAO occurs when one or some of these vessels are occluded. BRAO has the same pathology and risk factors of CRAO but affects more of a specific area of the inner retina depending on which branch is occluded (Brown, 1991).

Venous occlusive diseases are more common clinically than arterial occlusions. The risk factors for CRVO are similar to those of CRAO but also include an elevated intraocular pressure (IOP) or an underlying hematological disease (Mitchell et al., 1996).

# **Ionotropic Receptors**

Ionotropic receptors are a group of <u>transmembrane</u> ion channels that are opened or closed in response to the binding of a chemical messenger. NMDA (N-methyl-D-aspartic acid), AMPA ( $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), and kainite receptors are ionotropic receptors in neurons that are activated by glutamate which is a main neuron neurotransmitter (Digledine et al 1999). NMDA receptors are responsible for regulating the influx of calcium and other ions in the cell. Normally the channel is blocked by Mg<sup>2+</sup> ions (Eby and Eby, 2006). Under normal circumstances a nearby AMPA receptor is activated during resting polarization by glutamate, which starts depolarization. This depolarization spreads to the NMDA receptor and it releases the Mg<sup>2+</sup> ions. Once the NMDA receptor is open glutamate will activate the receptor, which causes an influx of Ca<sup>2+</sup> and Na<sup>+</sup>. Ca<sup>2+</sup> is used by the cell

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to activate metabolic pathways and reactions in the cell.

Kainate receptors are not as well understood as other receptors but its conductance is very similar to that of AMPA receptors.

### Metabotropic Glutamate Receptors

Metabotropic receptors differ from ionotropic receptors, like NMDA and AMPA, in that instead of activating an integral ion channel they activate secondary messengers usually G-proteins, which activate ion channels on the plasma membrane. To date eight distinct subtypes of metabotropic glutamate receptors (mGluRs) have been identified and separated into three groups based on function and sequence homology (Thoreson and Witkovsky, 1999). Group 1 mGluRs (mGlu1 and mGlu5) are usually located postsynaptically and enhance the cell's excitability by mobilizing intracellular Ca2+ and activating protein kinase C, which leads to several signal transduction pathways.

Group II (mGlu2 and mGlu3) and Group III (mGlu4 and mGlu6-8) are typically found presynaptically. The transduction process of these receptors involve a negative coupling with adenylate cyclase and their activation causes a reduction in glutamate release which lowers synaptic excitability (Nicoletti et al, 1996).

### Ischemic Cascade

On the cellular level death from ischemic attack is brought about by many factors. The start of the cascade comes from the lack of oxygen and glucose supply. This leads to decreased rates of glycolysis and oxidative phosphorylation, which causes levels of ATP to fall (Lipton, 1999).

The main impact of the decrease of ATP in the neuron is the inhibition of the Na<sup>+</sup>/K<sup>+</sup>-ATPase transporter, which is a transporting enzyme on the cell membrane. The main function of this enzyme is to restore the resting potential of the neuron after it has been hyperpolarized from the release of excess potassium. When the neuron becomes hyperpolarized the Na<sup>+</sup>/K<sup>+</sup>-ATPase actively transports sodium ions out of the cell and potassium ions inside the cell to restore the neuron to its resting potential so that it can depolarize and make an action potential. Once this enzyme is inhibited the voltage dependent Mg<sup>2+</sup> on the NMDA (N-methyl-D-aspartic acid) receptors becomes loose which allows for an excess uptake of calcium by the cell (Zeevalk and Nicklas, 1992). Ionotropic AMPA ( $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate)/kainite type glutamate receptors also become more activated which leads to an influx of ions and osmotically obligated water which can lead to cell lysis. The inhibition of the Na<sup>+</sup>/K<sup>+</sup>-ATPase is the cause of the hyper activation of these receptors.

These receptors are activated by glutamate, which is an extracellular neurotransmitter in the brain. During ischemia, through some proposed mechanisms, which will be elaborated later, there is an increase in extracellular glutamate levels, which in turn activate the now active receptors during reperfusion, which leads to cell death as will be explained later on in this paper. This rise in glutamate levels has been documented in several studies (Neal et al, 1994; Shimada et al, 1993).

The early mechanism proposed for the increase in extracellular glutamate levels was that after the opening of the Ca<sup>2+</sup> channels there would be an increase in exocytotic neurotransmitter release through normal metabolic pathways. This is because an increase in Ca<sup>2+</sup> in a cell will cause an increase in cell metabolism. However, due to the depletion of ATP in the cell, this mechanism cannot really account the very high levels of glutamate because of the lack of energy available for this process to occur (Nishazawa, 2001).
The main candidate for an alternative mechanism is the reverse transport of glutamate transporter proteins. The transport of glutamate into the cell in order to prevent excessive depolarization is not driven by ATP but rather by the Na<sup>+</sup> gradient inside the cell and the concentration of K<sup>+</sup> and pH-changing anions out of the cell (Barbour et al 1998). When ischemia occurs and Na<sup>+</sup>/K<sup>+</sup>-ATPase begins to reduce in activity, the Na<sup>+</sup> gradient is decreased and the K<sup>+</sup> accumulates in the extracellular space, which may reverse glutamate uptake (Attwell et al, 1993). This mechanism was proposed when it was noticed that there was an increase in extracellular glutamate in anoxic conditions (David et al, 1988). Other studies have shown that glutamate levels are increased when there is either an increase in extracellular K<sup>+</sup> on intracellular Na<sup>+</sup> (Barbour et al, 1991).

Normally glutamate is taken up by glia and converted by glutamine synthetase into glutamine and returned back into the neural soma (Thoreson and Witkovsky, 1999). Ischemia lowers the ATP levels in glia and causes a decrease in the conversion of glutamate. This causes an increase in the glutamate/glutamine ratio, which may also lead to extracellular glutamate (Oliver et al, 1990).

Once the cell constantly becomes depolarized excess calcium enters the cell and sets off many cascades that lead to cell death. Among the most critical are free radical production and nitric oxide (NO) synthesis, activation of phospholipase A<sub>2</sub>, DNA cleavage, activation of proteases, and subsequent damage to cytoskeleton. One of the main influences of the high calcium level is the formation of superoxide radicals (Bonne et al, 1998). Calcium activates a dependent protease calpain, which converts xanthine dehydrogenase to xanthine oxidase. Upon reperfusion, this enzyme converts hypoxanthine to uric acid, which results in the release of superoxide radicals (Chan, 1996). One of the major ways that superoxide damages the cell is that it attacks unsaturated fatty acids which lead to lipid perioxidation of membranes (Doly et al, 1984). This will result in loss of membrane fluidity, cell swelling, oedema, and feed forward production of more oxygen-derived radicals. There are many other pathways that cause cell damage that stem from the increase in calcium ions, which can lead to the activation of procaspases which leads to apoptosis.

The following figure illustrates the different steps in retinal ischemia.



Fig. 1. Summary of hypothesised key events in the process of ischemic retinal neurodegeneration. An interruption in the supply of blood to the retina leads to tissue ischemia which causes rapid failure of energy production and subsequent events as outlined in the figure. Key steps include the failure of the  $Na^+/K^+$ –ATPase pump, membrane depolarisation, cytoplasmic accumulation of sodium and calcium ions and the formation of destructive free radical species. The summed cellular response of these processes, if left unchecked, is cell death. This can occur by the classical and rapid necrotic process or by longer-duration apoptosis (Osborne et al., 2004).

# Retina Damage in Ischemia

Glutamate is the major excitatory retinal neurotransmitter and is released in vivo, by photoreceptors, bipolar cells, and ganglion cells. The photoreceptors have only shown susceptibility to be damaged after the rest of the retina has shut down and studies have shown that ischemia has less effect on the outer layer of the retina than the inner layer of the retina (Peachey et al, 1993). The reason that photoreceptors are less sensitive remains unknown. One possibility is that their mitochondria have a high-density inner segment and is maintained on a smaller  $pO_2$ . In addition there is more neuroglobin in photoreceptors (Schmidt et al, 2003) which helps maintain oxygen levels inside cells in the cerebral and peripheral nervous system. Neuroglobin has a high affinity for oxygen and provides extra oxygen for cells under ischemic attack or in hypoxic conditions.

Ganglion cells are highly susceptible to ischemic attack and all known retinal ischemic paradigms have been described as a loss of ganglion cells (Akiyama et al, 2002; Goto et al., 2002). Ganglion cells express both the NMDA receptor and kainite type receptor (Fletcher et al., 2000), which are activated by the excessive glutamate levels, which lead to chronic depolarization. Amacrine cells also contain these receptors and are susceptible to ischemic attack through the same mechanism. Both amacrine and ganglion cells have many metabotropic glutamate receptors which also may be the reason for their susceptibility to ischemic attack (Osborne et al., 1991).

What sets ischemic attack on the retina different from the brain, is the fact that the retina has a much longer tolerance time. A few minutes of cerebral ischemia in the human results in widespread injury, but it has been demonstrated that a primate retina can suffer up to 100 minutes of CRAO without permanent injury (Hayreh and Weingeist, 1980). This can be explained by the fact that retina contains about 100 times more neuroglobin and other energy reserves that can be used up during the hypoxic conditions of ischemia (Schmidt et al., 2003).

Alternatively there is a "no flow" phenomenon that may explain this difference (Fischer et al., 1977). This phenomenon is involved in where the rigid cranium compresses the microvasculature in a swollen brain, which causes an ongoing ischemic attack even though macroscopic blood flow is restored. In retina ischemia oedematous retina does not compress the microvasculature since there is a vitreous cavity to expand into. This large amount of time before irreversible retina destruction has allowed for many studies to be done on how to reverse ischemia or minimize ischemic damage.

# Treatment Using NMDA Antagonists

It has been shown in vivo that with the addition of NMDA antagonist one can protect a neuron from ischemic attack (Lombardi et al., 1994). Competitive antagonists although shown to be effective, will only likely be used in experimental studies. These receptor antagonists may not be useful in man due to long lasting actions, which can affect neural processes. However, major interest has arisen in the use of non-competitive NMDA antagonists. This is because these compounds have shown the ability to cross blood retinal barrier and it is believed that such drugs will block the toxic actions NMDA receptors in ischemia and yet sustain NMDA receptor functions in the brain to maintain cognitive and memory processes.

This evidence supports the idea that using a NMDA antagonist one can slow down or even stop ischemic attack. One particular antagonist that has been showing great results is dextromethorphan. Dextromethorphan is an antiussive drug and is used in over the counter cough and cold medicines and it has been shown to have uses in pain relief (Bem and Peck, 1992).

Evidence on retina health after ischemic attacks were obtained through Electroretinography (ERG), which is a test in which electrodes are attached to the eye and it measures the activity of the retina by measuring the electrical activity of the retina in response to light. The ERG will contain an a-wave (initial negative deflection) followed by a b-wave (positive deflection).

In studies done using dextromethorphan while invoking a retinal ischemic attack, results show that retinas that were treated with dextromethorphan were able to maintain function better after the restoration of blood flow than retina that were not treated with dextromethorphan. In 30 and 60 minute strokes, rats that were treated with dextromethorphan had a better recovery time and less retinal damage than the mice not treated with the drug (Cao et al., 1994). However, caution is necessary when studying this drug because it may be that dextromethorphan increases cerebral blood flow and/or decrease cerebral metabolic requirements instead of blocking the ischemic stroke pathology (Osborne et al., 2004). Also it could be that dextromethorphan was only protecting the rat retinas from the increased pressure brought on by the induced stroke in the rat and therefore may not be potent for clinical use (George et al., 1988).

Dizocilpine (MK-801), another NMDA antagonist has shown better results in stopping retinal ischemia (Lam et al., 1997). However, it's neurotoxic side affects causes reduced brain function and make it almost impossible for clinical use. Also, the lowing of ischemic affects may be due to other metabolic side affects of MK-801 in that it lowers CNS temperature, which lowers the oxygen consumption during ischemia (Chi et al., 1991).

Other NMDA antagonists have been used in studies for retinal diseases. They include eliprodil, which blocks at the polyamine site of the NMDA receptor (Biton et al., 1994), fliurpitine, and memantine. Even though it has been well noted that NMDA receptor antagonists block excitoxicity, the therapeutic use should be questioned because of the negative affects that they can have on the patient. It has been suggested that memantine may be better tolerated than other NMDA antagonists because of it low affinity for the NMDA channel (Parsons et al., 1993).

#### Conclusion

In conclusion, ischemia in the retina, if left untreated, is almost certain to cause permanent damage to the retina and eventually to severe vision loss. Ischemia unleashes a cascade starting with the lack of oxygen, which causes the  $Na^+/K^+$  ATPase to lose function, which leads to hyper-activation of glutamate receptors. The lack of oxygen also leads to an increase in extracellular glutamate, which can cause two forms of death. Glutamate can lead to an influx of ions, which will be followed by water under osmosis and cause cell lysis. Also, glutamate allows an increase in the uptake of calcium ions, which activates mechanisms to form radicals that can activate apoptotic mechanisms in the cell.

One proposed way of treating retinal ischemia is to administer non-competitive NMDA antagonists, which should block the entrance of calcium into the cell. The interest in using these chemicals has been big because they can cross the blood retinal barrier and are believed to not severely impair cognitive processes. Also, they can be administered after the ischemic attack has already begun. However, clinically there have been no real breakthroughs because of concerns that in vivo experiments only exhibit positive results because of other mechanisms involved and the concern of damaging normal receptor function.

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#### The Nerve Cells of the Retina

Penina Winkler

#### Abstract

The visual pathway in the retina consists of a chain of different nerve cells. Light first travels through all the layers until it reaches the photoreceptor layer, the rod and cone layer. Rods and cones use photopigments, which contain opsin and a chromophore, to help them convert light into energy. This energy is then passed on to the horizontal and bipolar cells. Horizontal cells prevent the hyperpolarization of peripheral rods and cones if needed, and they receive color-coded signals from cones that they then continue along the optic pathway. Bipolar cells can be divided into rod bipolar cells and cone bipolar cells. Cone bipolar cells can be further subdivided into midget cone bipolar cells, which only contact one cone cell and one ganglion, and diffuse cone bipolar cells, which can contact several cone and ganglion cells. Bipolar cells can either hyperpolarize or depolarize with light, and they pass their signal on to amacrine cells or ganglion cells. Amacrine cells provide inhibition to the visual pathway, either through feedback inhibition on the bipolar cells or feedforward inhibition on ganglion cells. A1 amacrine cells provide long-range inhibition of ganglion cells. Indoleamine-accumulating amacrine cells provide a reciprocal response to bipolar cells, preventing them from hyperpolarizing so that their signal does not continue. All amacrine cells receive input from bipolar cells and output the signal to different bipolar cells to be transmitted to ganglion cells. Ganglion cells, the last nerve cells in the visual pathway, only receive input when light hits the part of the receptive zone that produces a discharge when stimulated by a light source. Ganglion cells are specialized to detect movement in a specific direction. This prevents the brain from receiving unnecessary information. EphrinA and EphrinB are two molecules that form gradients that lead the ganglion cells to their specific destination in the brain. Magnocellular (MC) ganglion cells and intrinsically photosentive retinal ganglion cells (ipRGCs) are specific types of ganglion cells, with MC cells dominating during fixational eye movements, and ipRGCs working as circadian photoreceptors like rods and cones, contributing to light-stimulated effects on the body. Müller cells are not part of the visual pathway, but they provide support for the retina and



regulation of nutrients and molecules, as well as perform many other functions in the retina.

(Kolb H. et. al. 1996)

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#### Introduction

The eye is a complex sensory organ which receives visual images and carries them to the brain. When a light stimulus is initiated, the light coming into the eye touches the three tunics, or layers, of the eye; the outer tunic, the middle tunic, and the inner tunic. The first tunic is the outer tunic (colored white in diagram above), which consists of the sclera and the cornea. This layer provides protection to the inner structures of the eye, and maintains the shape and consistency of the eyeball. The sclera is the part of the outer tunic that covers the back of the eye, while the transparent cornea, a clear dome, covers the front of the eye. (Szaflarski, D.M. 1999) The aqueous humor is a thin, watery fluid that fills the space between the cornea and the iris of the middle tunic. The middle tunic is also called the uvea (colored blue in diagram above), which reduces the reflection of light and contains blood vessels. The back of the uvea, located under the sclera, is called the choroid. The ciliary bodies of the uvea cause the iris to constrict or dilate to control the amount of light entering the pupil, the hole through which light enters the lens. After light moves through the lens, it enters the vitreous humor and then the inner tunic. The inner tunic is the retina (colored red in diagram above), the most important part of the eye in terms of sight. The retina is the part of the eye that receives light impulses and converts them into energy that can be passed on to the brain. (Montgomery, T., 1998) Therefore, to understand how a human being is able to see, one must look into the inner workings of the retina, the most vital part of the visual process. The purpose of this paper is to describe the functions of the different types of cells found in the retina, and how each contributes to sight.

The retina has ten layers; the pigmented epithelium, the inner and outer segments of rods and cones, the outer limiting membrane, the outer nuclear layer, the outer plexiform layer, the inner nuclear layer, the inner plexiform layer, the ganglion cell layer, the nerve fiber layer, and inner limiting membrane. By following the nerve cells through these layers, one can see the pathway that the converted energy of light impulses takes to reach the optic nerve, which leads to the brain.

The pigmented epithelium is located on the external retinal wall, providing vitamin A, or retinal to the photoreceptors that it contacts. The layer of the rods and cones is where the conversion of light to energy takes place. The junctions between the rods and cones and the Müller cells make up the outer limiting membrane, which forms a blood retina barrier, isolating the inner layers of the retina from harmful substances in blood. The outer nuclear layer contains the nuclei of rods and cones. The outer plexiform layer is the layer in which the axons of the cones and rods synapse with the dendrites of the bipolar, horizontal, and amacrine cells. In the inner plexiform layer, the axons of bipolar cells synapse with the neurites of amacrine cells and the dendrites of ganglion cells. In this layer, the neurites of amacrine cells and the dendrites. The ganglion cell layer contains the nuclei of the ganglion cells, which then converge in the nerve fiber layer. The inner limiting membrane is made up of Müller cells, which form a barrier between the retinal nerve cells and the potentially harmful substances in the

vitreous humor. (Caceci T., 2001) Each type of cell in the pathway has its own functions which help to increase the accuracy and speed of vision.



**Rods and Cones** 

When light hits the retina, it first goes through all the layers of the retina before the cells begin to respond. This is because the photoreceptor cells, the rods and cones, are located on the outer region of the retina, furthest from the lens.



(Juelich F., 2004)

Both rods and cones are made up of two segments, the outer segment and the inner segment. The outer segment exhibits flat, membranous disks containing a photo pigment. The disks are infoldings of the plasma membrane that become narrower as they move away from the modified cilium, the connecting region between the inner and outer segments. These disks are produced in the inner segment and are transported by kinesins and cytoplasmic dyneins along microtubules toward the outer segment across the bridge of modified cilium. The disks are continuously being renewed; the used disks are phagocytosed by cells of pigmented epithelium. The inner segment is abundant in mitochondria; mitochondria are involved in the synthesis of adenosine triphosphate, the Golgi apparatus, and endoplasmic reticulum. (Caceci T., 2001)

Rods contain the pigment rhodopsin, which functions during night vision, while cones contain iodopsin, which helps to see more accurate detail and color. There are three types of cone cells, which respond to different regions of the spectrum, discriminating the colors blue, green, and red. There are other differences between the two cells. In the cone cell the outer segment is conical, while in the rod cell it is cylindrical. The cone cell axon ends in a cone pedicle, a large, conical, flat end, which is thicker than the rod spherule, small round enlargements of the axon. Both rod and cone synapse with the cytoplasmic processes of bipolar and horizontal cells. (Kolb H. et. al. 1996) Rods and cones are transducers; they convert light into electrical or neural energy. In order for this to occur, the rods and cones need photopigments. The protein opsin and a chromophore, retinal, constitute the photopigments of the rods and cones. The chromophore is needed so that color will be absorbed; without the chromophore, the photoreceptor would just absorb UV light. The photopigments are needed to provide the energy required to cause an electrical change in the membrane of the rod or cone that will be propagated from the point of absorption of the light to the rod spherule. The electrical change occurs when the photoreceptor is bleached, the process in which the photopigment absorbs a photon and changes chemically into its constituents, which are less sensitive to light. In darkness, the rod and cone cells are depolarized, and they continuously release the neurotransmitter glutamate to the bipolar cell; this inhibition of the bipolar cell is known as the "dark current." As light hits the photopigment, retinal is activated. This causes the opsin to activate transducin, which activates phosphodiesterase. Phosphodiesterase breaks cyclic guanosine monophosphate down into guanosine monophosphate. The cell membrane becomes hyperpolarized, and glutamate is no longer released to the bipolar cell. This causes the bipolar and horizontal cells to hyperpolarize as well, and the message continues along the chain. (Stockman A. et. al. 2007)



# Horizontal Cells

Horizontal cells are one of the intermediaries between photoreceptors and ganglion cells. Their nuclei are found in the inner nuclear layer, and their neurites, their cytoplasmic processes (they do not

have axons or dendrites), attach to the rod spherules, cone pedicles, the dendrites of bipolar cells, and the neurites of other horizontal cells in the outer plexiform layer. One horizontal cell synapses with more than one type of cell at the same time, because each neurite can have many branches. Because the horizontal cell has many branches and can reach many cells horizontally across the outer plexiform layer, it allows one part of the retina to influence the other part's activity. Horizontal processes are in close proximity to retinal capillaries, and there may be neurovascular coupling between the processes and the capillaries. Such intimate contact with the capillaries was previously thought to be a characteristic of Müller glial cells and astrocytes; some species of horizontal cells have glial proteins (glial fibrillary acidic protein or vimentin), and both horizontal cells and glial cells develop from the same precursors during retinal development. (Mojumder D.K. 2009)

The membrane of the horizontal cell contains ionotropic glutamate receptors which need direct activation by glutamate. The receptors are ionotropic because the receptor and the ion channel form one complex, so they directly gate ion channels; they open ion channels when receiving glutamate. Glutamate release from rods and cones causes glutamate to be received by the glutamate receptor, which then causes the cell to be depolarized; a decrease in glutamate, a response to light, causes hyperpolarization of the cell. The horizontal cells provide feedback control of photoreceptor synaptic output. They perform negative feedback to photoreceptor synaptic terminals at the first synapse of the visual pathway. The cell exerts a restraining influence on the junction by stopping the response from continuing to the bipolar cells. It is widely believed that these restraining influences only occur on cones, not rods. The role of horizontal cells on the rod pathway is unclear. (Bloomfield S.A. et al 2001)

The three different types of cones transmit their effects to bipolar and horizontal cells. In many experiments conducted on horizontal cells, the cells showed two types of potentials when recorded, color and luminosity. The color types of cell give an opponent type of response; the colors blue and yellow have opposing effects in some cells, while red and green have opposing effects in other cells. The blue-yellow cell has connections with blue and red and green cones, while the red-green cell has connections with only red and green cones. The transmission of different wavelengths to different types of horizontal cells leads to a detailed color-coded message that is passed on to the optic nerve. (Burkhart D.A. et. al., 1978)

#### **Bipolar Cells**

Bipolar cells, like horizontal cells, are intermediaries between photoreceptors and ganglion cells. Their nuclei are found in the inner nuclear layer, their dendrites contact photoreceptor cell terminals in the outer plexiform layer, and their axons stretch into the inner plexiform layer to contact ganglion cells. Bipolar cells can be subdivided based on the photoreceptor cells they synapse with. Therefore, there are rod bipolar cells, which synapse with rod spherules only, and there are cone bipolar cells, which synapse with cone pedicles only. The cone bipolar cells can be further divided into the midget cone bipolar cell and the diffuse cone bipolar cell. The midget bipolar cell synapses with a single cone cell, and has only one axon which contacts a single ganglion cell, which is continuous with the optic nerve. In reality, a true one to one connection in the retina does not exist, because although one midget bipolar cell can contact one cone pedicle, a diffuse bipolar cell can attach to the same cone as well. Also, horizontal and amacrine cells contact midget bipolar cell, ganglion cells, and cones laterally. Because of the interconnections between the nerve cells, no optic nerve fiber actually carries messages from one cone, but because of the midget bipolar cell pathway, an increased level of acuity is reached when a less dilute message reaches the optic nerve. It is this pathway that causes cone vision to be more accurate than rod vision. The diffuse cone bipolar cell contacts up to seven cone cells at one time and synapses with many ganglion at a time. The diffuse cone bipolar cell has wider input and output pathways than the midget bipolar cell. (Kolb H. et. al. 1996) Cone bipolar cells can also be divided into invaginating cone bipolar cells, whose dendrites penetrate the synaptic invaginations of the cone pedicles, and flat cone bipolar cells, whose dendrites remain superficial and establish basal contacts. (Bloomfield S.A. et al 2001)

One rod bipolar cell can synapse with up to fifty rods. This causes the same effect if there are one hundred quanta falling on a single rod or one quanta falling on one hundred rods. There is no difference, because ultimately the same signal will reach the bipolar cell. Also, if a high density of rods converges on a single bipolar cell, and a high density of bipolar cells converges on one ganglion cell, there are many signals falling on one ganglion cell. During night vision, the power of bipolar and ganglion cells to collect impulses increases; the impulses were previously inhibited by higher illumination of the retina. This causes an increase in the amount of signals on the ganglion cell; it is because of this large amount of converging that during night vision, visual acuity is decreased. With so many impulses entering the ganglion cell, the cell can not interpret all the impulses to the degree where vision would be as accurate as it is during normal vision. (Kolb H. et. al. 1996)

When there is a decrease in glutamate levels as a response to light, the photoreceptor cells depolarize, which causes the bipolar cell to depolarize or hyperpolarize depending on whether the cell is an ON-bipolar cell or an OFF-bipolar cell. With decreased glutamate, an ON-bipolar cell depolarizes and an OFF-bipolar cell hyperpolarizes. In the rod vision pathway, rod bipolar cells contact two types of amacrine cells, which serve as interneurons in the transmission of light signals in night vision. These amacrine cells, All amacrine cells, then transmit the signal to cone bipolar cells, the final conduit to the ganglion cell during rod vision. Cone bipolar cells are used during rod vision, revealing that the rod and cone circuits are intertwined. (Bloomfield S.A. et al 2001)

#### Amacrine Cells

Amacrine cells are interneurons with nuclei in the inner nuclear layer. They have one neuritic process which branches and connects to the axons of the bipolar cells and the dendrites of the ganglion cells in the inner plexiform layer. Like horizontal cells, amacrine cells connect to bipolar and ganglion cells horizontally, so that one area of the retina can impact another area. All amacrine cells display dendritic and somatic impulse activity. This impulse activity is important, as it performs feedforward inhibition on ganglion cells and feedback inhibition on bipolar cells via action potentials. The amacrine cell exerts a restraining influence on certain junctions of the retina, reducing the spread of messages. In

relation to the ganglion dendritic tree, bipolar cells make up the center of the receptive field, while amacrine cells make up the periphery. When the amacrine cells exert an inhibitory action, the bipolar cells in the central zone of the field are prevented from responding to the receptors, and the message is not sent to the ganglion cell. (Kolb H. 2003) The inhibition caused by the amacrine cell has two distinct modes, local and global. If the action potential can not be propagated down the cell to the dendrites then they provide local inhibition. If, however, the action potential invades the entire dendritic tree, it provides global inhibition. Amacrine cells can be divided into different types based on the size of their dendritic spread, dendritic sublamination patterns, neurotransmitter subtypes, and variations in the morphological features. (Royer A.S. et al 2007) There are three specific types of amacrine cells discussed in this paper; A1 amacrine cells, indoleamine-accumulating cells (IAC), and AII amacrine cells.

The A1 amacrine cell is a spiking, axon-bearing interneuron in the retina, with thick, spiny, and highly branched dendrites in the inner plexiform layer. A1 cells receive inputs to their dendrites that cause depolarization, which initiates an action potential. The action potential then propagates down the axon and crosses the retina. The A1 cells have extensive branching, indicating that they serve a global function in vision processing. A1 cells are mediators of long-range inhibition of ganglion cells in response to global image motion. Their spiking output structures may be a key element in long range lateral inhibition of the retina. The A1 cells have center-surround receptive fields which combine inputs from ON bipolar cells and OFF surrounds and OFF bipolar cells and ON surrounds. Because amacrine cells are stimulated by both ON-center and OFF-surround, and OFF-center and ON-surround, the inhibition of amacrine cells is postsynaptic to all bipolar cells, regardless of their responses to stimulation. A1 amacrine cells provide inhibitory feedback on all bipolar cells. (Davenport C.M et al 2007)

IAC amacrine cells, also known as A17 amacrine cells, account for half of the rod bipolar cell output. IAC cells receive input from rod bipolar cells and multiple types of amacrine cells in the inner plexiform layer. The sole postsynaptic target of IAC cells is rod bipolar cells; IAC cells return reciprocal synapse back onto rod bipolar cells. This arrangement causes an interconnecting of rod bipolar cells in the inner plexiform layer. IAC amacrine cells contain GABA, and there are GABA receptors present on the axons of the rod bipolar cells; this indicates that the IAC cell has an inhibitory feedback function in the reciprocal synapse. (Bloomfield S.A. et al 2001)



Fig. 11. A Golgi stained example of an All amacrine cell in cat retina

(Kolb H. et. al. 1996)

All amacrine cells function during rod and cone vision. In scotopic vision, night or dim-lit vision, All cells are major carriers of rod signals to ganglion cells. They receive input from rod bipolar cells, but do not return the signal back to rod bipolar cells. Instead, the cells form homogenous gap junctions with other All cells near the site of the synapse of the rod bipolar cell. The All cells then synapse with the axon terminals of cone bipolar cells. All cells also receive input from cone bipolar cells, indicating that the cells are also part of the photopic, normal light vision. All amacrine cells are in dense population in most of the retina because they are a key element in the visual process. (Bloomfield S.A. et al 2001)

#### **Ganglion Cells**

Ganglion cells are the last retinal cells in the visual pathway; their axons form part of the optic nerve. The nuclei of the ganglion cells are in the ganglion cell layer, and their dendrites extend into the inner plexiform layer and synapse with bipolar cells. There are two types of ganglion cells; midget ganglion cells, which synapse with only one bipolar cell, and diffuse ganglion cells, which synapse with several bipolar cells. The midget ganglion cells receive impulses from midget cone bipolar cells, so they are part of the cone visual pathway only. (Kolb H. et. al. 1996) The ganglion cell receives information from all stages in the visual pathway. The ganglion cell can convey signals that drive the circadian and pupillomotor systems by combining the rod and cone photoresponses, as well as its own photoresponses. (Dacey D. M. et al 2005)

The ganglion cell response is not switched on and off as the light source is switched on or off. When the light source is emitting light to the central zone of the receptive field, a discharge is produced. The periphery zone of the receptive field has the opposing response, and will only produce discharge if there is no light. The center and the periphery usually have opposite responses in terms of color; for example, an off-response to green in the surrounding area and an on-response to red in the center of the field. A simultaneous stimulation of red in the center and green in the periphery would elicit no response, because the on-response of the center is cancelled out by the off-response of the periphery. This opponent organization serves to enable the retina to emphasize different colors; the involuntary movements of the eye in normal vision keeps shifting the boundaries of the center and the periphery, causing different colors to be seen. Another effect of this inhibitory organization is to prevent unneeded information from reaching the brain. There are about one million optic nerve fibers, and if all of them were discharging information at once, the brain would have too many messages to decode. This mechanism helps to cut down the amount of information the brain needs to process. (*Encyclopædia Britannica* 2009) Another method that some ganglion cells use to decrease the amount of action potentials it sends to the optic nerve is to respond to light moving only in a specific direction. When an object moves in the ganglion's preferred direction an excitatory impulse occurs. When the object moves in the opposite direction, however, both excitatory and inhibitory impulses occur, effectively canceling each other out. (Barinaga M. 2000)

Each ganglion cell carries a chemical tag that corresponds to chemicals in the tectum, the dorsal part of the midbrain; these chemicals convey positional information to the cell. The ganglion cell interprets this information and responds by connecting to a specific point in the brain. There are two gradients in the ganglion cell pathway to help the axons of the ganglion cells hit their target position exactly. The gradient made up of molecules in the tectum called EphrinA specifies where the ganglion axons will end up in terms of the anterior-posterior axis. The gradient made up of molecules called EphrinB specifies where along the medial-lateral axis the axon will end up. If an axon is moving in a direction that is either medial or lateral to its destination, it projects branches towards the destination. The EphrinB regulates this branching. The Wnt3 protein counteracts the pull of EphrinB towards the most medial tectum, so the lateral tectum is not empty. The gradient of Wnt3 is along the same direction as EphrinB's gradient, but is a repellent gradient. Ryk, the receptor for Wnt3 on the ganglion on cell, is expressed in ganglion cells in the same direction as EphrinB, proving that it is Wnt3 that provides the repellent gradient. (Liqun Luo 2006)

A specific type of ganglion cell, the magnocellular (MC) ganglion cell, was experimented on and found to be responsible for the visual illusions that are seen during fixational eye movements. When one stares at a stimulus, such a star or an optical illusion, for a period of time, there is an apparent splitting of the lines, as well as a fading of lines or whole wedges of the stimulus. These characteristics are not found in parvocellular (PC) ganglion cells, the other type of ganglion cell. This indicates that MC cells dominate during fixational eye movements, as their properties are such that this would happen. In the diagram below, it is shown that in fixational eye movements, such as staring at the star shaped stimulus (A), MC cells exhibit more line fading and splitting than PC cells. (Hennig M.H. et al 2007)



(Hennig M.H. et al, 2007)

Intrinsically photosensitive retinal ganglion cells (ipRGCs), or melanopsin-positive ganglion cells, are ganglion cells that function as a circadian photoreceptor. Mice with severe degeneration of rods and cones, or with no rods and cones at all, still follow circadian rhythms based on light. Light affects a circadian phase by activating the retinohypothalamic tract, which connects ipRGCs to the superchiasmatic nucleus, a part of the hypothalamus. The ipRGCs are the only ganglion cells found to contain melanopsin, an opsin which is photosensitive, indicating that this is the part of the ipRGC that makes it a photoreceptor. IpRGCs are different than the classical photoreceptors in that they depolarize when stimulated by light, while rods and cones hyperpolarize. IpRGCs are also less sensitive and more sluggish than the classic photoreceptors. The melanopsin-positive ganglion cells contribute to light-stimulated effects on sleep, heart rate, cortisol levels, alertness and other effects of the circadian cycle. The pupillary light reflex, where light causes the size of the pupil to decrease and darkness causes the pupil to dilate, is controlled by ipRGCs as well. The cells also perform the normal function of ganglion cells, receiving synaptic input from bipolar and amacrine cells. (Berson D. M. 2003)

# Müller Cells

Müller cells, a specific type of glial cell, fill the spaces between the photoreceptors, bipolar cells, ganglion cells, and other cells in the visual pathway. Müller cells are not part of the visual pathway; they do not synapse with any cells, and they do not receive or send signals. Their function is mainly to support and regulate the retina. The nuclei of Müller cells are found in the inner nuclear layer, and their cytoplasmic processes extend to the outer and the inner limiting membranes. The inner limiting membrane, which separates the retina from the vitreous body, is made up of the basal lamina of Müller cells. The outer limiting membrane is located at the contact site where zonula adherens and

microvilli extending from Müller cells connect the photoreceptors with the glial Müller cells. (Kierszenbaum A.L. 2007)



Nature Reviews | Neu (Dyer, M.A. et. al., 2001)

Before reaching the photoreceptors, light has to go through each layer of the retina. At each layer, the light is refracted by the cells in that layer, ultimately causing less light to fall on the photoreceptors. The Müller cells have a higher refractive index than the other cells, which means that light can be channeled through the cells with little loss, so most of the light hitting the retina is received by the photoreceptors. This high refractive index is caused by the tight bundles of polymer fibers that extend along their lengths. (Castelvecchi, D. 2007)

Müller cells have many different functions in the retina. They regulate extracellular space, and ion and water levels. They maintain the blood-retinal barrier, and retinal blood flow. They provide trophic substances to the nerve cells, and they remove neural waste, such as carbon dioxide and ammonia. (Kolb H. et al 1996) They also modulate neuronal excitability and transmission by sending gliotransmitters to the neurons. They also supply the end product of anaerobic metabolism, they break down glycogen, so that the nerve cells in the retina can perform aerobic metabolism. Müller cells and astrocytes, another type of glial cell found in the retina, remove glutamate, an excitatory neurotransmitter, and gamma-Aminobutyric acid (GABA), an inhibitory neurotransmitter from the extracellular sites in the retina. Müller cells take up glutamate by utilizing glutamate transporters like GLAST, glutamate-aspartate transporter, and convert it into glutamine with the enzyme glutamine synthetase. If the glutamate transporter is malfunctioning, the extracellular glutamate levels rise to excitotoxic levels, causing damage to the retina. Müller cells uptake GABA using GABA transporters (GATs), and convert it to glutamate using GABA transaminase. (Bringmann A. et. al. 2009)



(Kolb H. et. al. 1996)

The *fovea centralis* is a unique portion of the retina where an abundance of light is absorbed and as such contains no rods; only cones. These Müller cells provide the primary structural support for the fovea centralis, binding the photoreceptor cells together. The cells are also a reservoir for retinal xanthophyll, yellow accessory pigment, which is partly responsible for the low density of the cell cytoplasm in the fovea. Müller cells in the *fovea centralis* also have a primary role in age related macular hole formation, providing an anatomical substrate for schisis to occur. X-linked juvenile retinoschisis, the abnormal splitting of the retina's neurosensory layers, is thought to be caused by degeneration of the inner portion of the Müller cells in the *fovea centralis*. (Gass J.D.M. 1999)

In addition to Müller cells and astrocytes, there are also other glial cells found in the retina. Microglial cells are blood-derived immune cells which reside in the retina. They play important roles in host defense against invading microorganisms and the initiation of inflammatory processes and tissue repair. They are present in all layers of the retina. (Bringmann A. et. al. 2009)

# **Conclusion**

At first glance, the reason for the retina's interbranching visual pathway is hard to understand. But, after putting together the functions of each type of retinal nerve cell, one sees that each cell is essential. The complexity of the pathway serves to clarify the messages coming into the eye, and to prevent unnecessary information from being transmitted to the brain. Understanding how the retina functions is important for doctors and scientists who are diagnosing and treating patients who have nerve damage in the retina. Knowing the function of each area and cell type in the retina can help to diagnose what area is damaged when seeing certain symptoms present, and treatment can then be administered. We know that Müller cells in the *fovea centralis* have roles in X-linked juvenile retinoschisis and age related macular hole formation. Further research is being done by foundations such as the Retina Research Foundation in Houston, Texas, in order to further understand the function of each nerve cell in the eye and how each cell can lead to ocular disease.

The intricacy of the eye, and especially the retina, which is such a small part of our body, is aweinspiring. It is amazing to learn the workings of vision, something that is done almost every waking second, and to actually understand what is happening each moment in one's eye.

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