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Lander College of
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Table of Contents

Prosopagnosia
By: Leah Fleischman 5

PARP Inhibition: A Method of Treating and Preventing Certain Cancers
By: Chana Tropper 10

It's All in the Mind: Mind Controlled Prosthetics
By: Tziril Joselit 17

How Does Spaceflight Affect the Human Body?
By: Sara Dina Feinzeig 23

Immunotherapy As a Treatment Option for Patients With Pancreatic Cancer
By: Yehuda Lehrfield 29

The Hormones of the Placenta
By: Shaya Oratz 35

Eating Disorders: The Hidden Hormonal Effect On Fertility
By: Esther Tessler-Karfunkel 42

The Efficacy and Safety of the Human Papillomavirus Vaccine
By: Gail Tessler 51

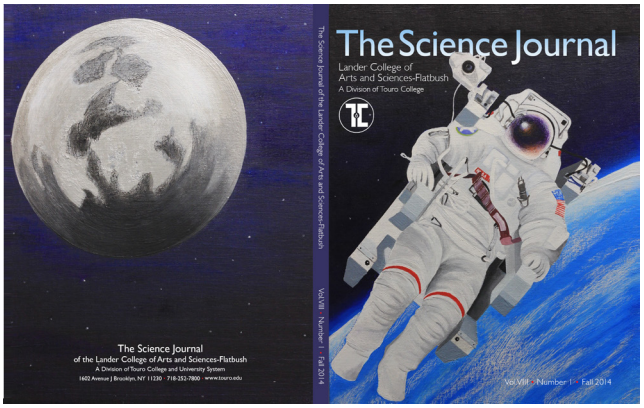
Theories on Varicella Zoster Virus Reactivation Based on Shingles Patterns
By: Ralph Nussbaum 59

The Search For Novel Inhibitors Of The Mycobacterial Enoyl Reductase InhA Through Structure Based Drug Design
By: Esther Saul *67*

Pediatric Gastroesophageal Reflux Disease
By: Adina Mezrahi *81*

Bioengineered Hearts
By: Rivky Loeb *88*

The cover illustrations are original paintings of Karen Bleich.
They are representative of the article "How Does Spaceflight Affect the Human Body?"



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Prosopagnosia

By: Leah Fleischman

Leah graduated in September 2014 with B.S. in biology.

Abstract

Prosopagnosia is a cognitive disorder that affects one's ability to recognize faces. Prosopagnosia can be caused by a congenital defect, or it can be acquired as a result of brain damage. Much research has been devoted to discovering the specific causes and effects of Prosopagnosia. Many case studies have been performed in order to determine the specific effects that each case of Prosopagnosia causes for various individuals suffering from the disease. This article discusses the various aspects of Prosopagnosia; specifically focusing on the behavioral, anatomical, and neurological implications.

Introduction

Prosopagnosia, also called face-blindness, is a cognitive disorder of face perception. A person's ability to recognize faces is impaired, while other aspects of visual processing and intellectual functioning may remain intact. There are two types of Prosopagnosia: Congenital or Developmental Prosopagnosia, and Acquired Prosopagnosia. Congenital Prosopagnosia is a face-recognition deficit that is life long, beginning in early childhood, and is not attributed to brain damage. Acquired Prosopagnosia refers to a condition that follows acute brain damage, usually brain damage specifically to the occipito-temporal lobe. The term Prosopagnosia is derived from the Greek words for "face" and "lack of knowledge." Although individuals with this disorder may be able to see normally, their ability to recognize faces is deficient. This disease can be life altering, as individuals with this disease may have limited social and behavioralinteractions. However, there are some Prosopagnosiacs who are able to lead normal, happy lives despite their inability to recognize faces. Scientists have performed many case studies and much research has been done to discover the underlying causes of Prosopagnosia. Both Congenital and Acquired Prosopagnosia are complex conditions caused by various factors. What are some of the neural, behavioral, and anatomical implications of Prosopagnosia?

Materials and Methods

In order to answer the question proposed above, many research papers and journal articles with relation to this topic have been read. Touro College's library database and the NYU library database were used to search for relevant studies and reviews. The next step taken was to look for articles that were referenced by those obtained through the Touro College Library and NYU search engines that seemed relevant. All of the articles and information that was accumulated through this research have been used in the attempt to conclusively determine the neurological, anatomical, and behavioral results of Prosopagnosia.

Results

Background on Congenital/ Developmental Prosopagnosia

Congenital Prosopagnosia (CP) refers to the impairment in face processing that is evident from the time someone is born. CP is not due to any brain damage and can occur in a person with intact sensory and intellectual functions. Individuals that suffer with CP are usually able to acknowledge that they are looking at a face; however they are unable to identify the specific face. As a result, they have come to rely on other cues such as voices, clothing, or specific accessories (hairlines, eyebrow shapes, etc.) to help recognize a person. CP can even affect the recognition of even the most familiar faces, such as close family members. In extreme cases, one may not even be able to recognize his- or herself. An important fact to understand regarding those with CP is that they have never experienced normal face perception. Therefore, research that is conducted relies largely on self-testimony and the testimony of parents (Behrmann, Avidan, 2005).

Developmental Prosopagnoisa, a more general label, refers to a disorder caused by anomalies occurring at any time during development, whereas the title of CP automatically assumes that prosopagnosia occurred at birth or early infancy (Susilo, Duchaine, 2013). To be categorized as someone suffering with Prosopagnosia, impairment in face recognition is required. However, it is important to note that face recognition is complex and there are multiple types of face recognition and face perception. For example, one needs to know whether a face is present among non-face stimuli, to determine whether two faces are the same or different, and be able to recognize the individual identity of a specific face. Studies show most patients with CP are able to detect faces from among other objects. Additionally, some individuals with CP find it easy to match faces (usually because they are able to identify key features), but when the reaction time or more specific measures of discrimination are measured and the tasks demanded are more difficult, the deficit becomes quite obvious. However, most CP patients fail in the area of identifying specific faces (Behrmann, Avidan, 2005).

Congenital Prosopagnosia: The Thatcher Effect

Case studies have been performed regarding the discussion on whether CP is specific to faces, as opposed to other objects. It is known that face processing typically involves individual identification, where one is shown a face and one responds with the individual's identity. This process is different than that for identifying others objects that are usually recognized at a basic level (for example, as a chair, an apple,or a house); face recognition requires the perception of very detailed features. People are able

to perceive and discriminate between objects based on either a single feature, involving a feature process, or based on the relationship between features, involving configural processing.
Scientists have theorized based on case studies that related configural processing to face perception. Face perception is often described as a configural process and it has been suggested that a loss of configural processing may be a clear indication of Prosopagnosia. In an effort to determine the relationship between configural processing and Prosopagnosia, a case study was performed. The 'Thatcher Effect' is a phenomenon where it becomes more difficult for a non- Prosopagnosiac to detect local changes in an upside down face, despite identical changes being obvious in an upright face. An experiment was performed to demonstrate that prosopagnosiacs, both CP and AP patients, do not suffer from the 'Thatcher Effect'. A control group of people who had no indication of prosopagnosia was formed. Another group of mixed CP and AP sufferers were collected. Then, two pictures of faces were held in the upright position with pictures of the same face flipped upside down next to them.

In order to recognize faces, each brain has developed configural processing to be able to distinguish different details of individual face features such as eyes, nose, etc. When a face is turned upside down, configural processing cannot take place and so those seemingly minor differences are very difficult to detect. Patients suffering with CP however, do not suffer from this effect. This can be because the area of their brain that controls configural processing and face perception is impaired. These results were also found among the patients suffering from AP, and those patients exhibited an even easier time detecting the similar facial features between the right-side-up faces and the upside down ones than the CP patients (Behrmann, Avidan, 2005).

Congenital Prosopagnosia: The Part-Whole Effect

Impairments in configural processing may affect other visual stimuli as well. Recognition of faces depends on the spatial relations between the components which need to be represented to distinguish between individuals. Another experiment was conducted to in order to obtain data regarding configural impairment in other areas. Five CP individuals and five controls were shown four compound letters at global and local levels. Two of the stimuli had identities that were consistent at the local and global level and two had identities that were inconsistent. This means that a large letter is composed of smaller letters, and can either be categorized as consistent or inconsistent. A letter consistent at both global and local levels would be a large letter H made up of small letter H's, while an inconsistent letter would be a large letter H shaped by small letter S's.

CP individuals showed normal speed at recognizing and identifying the local letters, however they were extremely slow at deriving the global whole from the local elements. They were able to identify the small components, but had difficulty in reading the large letter formed by the small parts. This can be another indication of a failure in representing the spatial elements of a display. This demonstrates that CP patients can usually identify or recognize individual aspects and features of a face, but cannot perceive the global picture and recognize the face (Behrmann, Avidan, 2005).

Another case study was performed to demonstrate a similar idea. Unlike most types of objects, faces are represented as a perceptual whole, meaning that there are many individual features that come together to form one image of a face. Therefore, the recognition of a face is referred to as holistic or configural face processing. This raises the possibility that face recognition deficits in CP patients may result from abnormal holistic face processing. To test this theory, a performance test was conducted called the 'Part-Whole Effect'. A control group of 38 non-Prosopagnosiacs were selected along with 38 CP patients. Participants were briefly shown a target face and then asked to choose which of two images placed side by side was the target face that they had been shown. This test was repeated with a picture of a specific facial feature instead of an entire face. Both the control group and the CP patients demonstrated the 'Part-Whole Effect' (Avidan et. al. 2011).

This refers to a greater ability to discriminate in the whole condition than in the part condition. Each group was slower to recognize target features than a target face. Although both groups were affected, the CP group did have a lower average accuracy than the control group. Interestingly, this low accuracy for discriminating in the part condition varied based on the specific feature. The patients with CP were suffered from the 'Part-Whole Effect' only when the target features were the mouth, eyes, and nose. With all other facial features, they were able to identify the target features as accurately as they could identify a target face (Susilo, Duchaine, 2013).

Congenital Prosopagnosia: Anatomical Implications

The ability to recognize faces is so important in humans that the brain appears to have an area solely devoted to that task, the fusiform gyrus. The fusiform gyrus is positioned between two lobes and is therefore a part of the temporal and occipital lobes. This area is believed to comprise the core visual representation system for faces. Specifically, this core is attributed with recognition of facial features. The fusiform gyrus is also responsible for face selectivity, meaning that it responds to images of faces more strongly than to any other objects.

In order to determine the functional relationship between the anatomy of the brain and facial perception, experiments have been performed utilizing fMRI technology. Brain imaging studies consistently find that the fusiform gyrus becomes active when people look at faces. Studies have also shown a strong correlation

between activity in this area and face recognition, leading scientists to believe that the fusiform gyrus performs essential functions for facial perception.

Face selectivity and repetition suppression, both forms of neural processing, were measured using fMRI technology. Their relationship to face recognition ability was then determined in a case study. A control group of 15 non-Prosopagnosiacs was selected along with a group of 15 CP patients. The CP group showed reduced face-selective responses than the control group. In addition, it was demonstrated by these studies and analyses that there is a correlation between high performance on tasks involving facial identity processing and fMRI face selectivity activity in the fusiform gyrus. This brain-behavior relationship is associated with behavioral factors relating to face identification. Further tests were performed that measured quantitatively the relationship between behavioral skill in facial recognition and fMRI test results. The tests and analyses that were performed successfully presented correlations between the anatomy of the brain, specifically the fusiform gyrus, and facial recognition, selection, and perception. Despite this, the scientists who performed these studies admit that they would like to improve their approach to this research. This could be done in a variety of ways, including greater number of participants, a greater number of behavioral measures performed by those participants, and a greater diversity of the behavioral measures. These improvements would help produce more accurate data regarding the brain-behavior relationship (Furl et. al., 2012).

Congenital Prosopagnosia: Behavioral and Social Implications

Face recognition plays an important role in social and behavioral cognition. Therefore, tests have been performed in an effort study face recognition abilities in CP patients. Specifically, scientists were interested in processing of facial stimuli with emotional valence and its differences from non-facial stimuli. This is important in order to understand the impact of emotional facial expression on the success of long-term memory. A group of 49 people were selected; 24 of them were CP patients and the rest were non-Prosopagnosiacs. The participants in this study were shown a series of images including neutral faces, positive and negative faces, and building facades. The pictures were shown for 3.82 seconds each, and the participants were asked to perform certain tasks. For example, for the faces, they had to determine the gender of the face. For the building facades, they had to decide whether it was a public building or a private one. The reaction times were studied, and there was a slightly lower performance by the CP patients than by the non-prosopagnosiacs. Interestingly, the slow response by the CP patients was only present with the images involving faces; for the non-face stimuli (i.e. the building facades) the CP patients had a quicker response time than the control participants.

Once the responses were analyzed, voxel-based morphometry was used to relate anatomical differences to memory success. This revealed that CP patients had a lower grey matter density. This low density spanned a variety of areas, including the right middle temporal gyrus and the left precentral gyrus, which is associated with the Brodmann area. The scientists then used fMRI data to compare the face processing of the CP patients and the control participants. There were three major areas of the brain that correlated with the decreased face recognition activities of the CP patients. These areas are the left fusiform gyrus, the right lateral occipital complex in the core face processing area, and the right DLPFC.

This data conclusively determined that CP patients have impaired long-term memory for faces, and for complex visual stimuli as well, although impaired memory is more severe with regards to faces as opposed to buildings (Dinkelacker et. al., 2010).

Background on Acquired Prosopagnosia

Acquired Prosopagnosia is a neurological syndrome which does not allow a person to recognize faces as a result of brain injury. This is in contrast to CP patients who have never had the experience of face recognition. Patients with AP can have many different types of lesions which cause their acquisition of this disease. Research has shown that the most common injury to the brain that results in AP is damage in the inferior or medial occipito-temporal cortex. This is not surprising, as the core area for face processing is found in that region. The core is comprised of the fusiform gyrus, also called the fusiform face area (FFA), as mentioned above, as well as the occipital face area (OFA) and the posterior superior temporal sulcus (pSTS).

The exact relationship between damage to each of these areas and the acquisition of AP is not very clear, but it has been proven that most cases of AP do result from injury to these areas. As is the case with many other acquired diseases, there are a multitude of possible injuries that can act as the causing factor. Therefore, it is difficult to determine what the extent of damage in Prosopagnosia patients is in these specific areas. For example, in the case of a particular patient with AP, it was determined that Prosopagnosia was caused by a right hemisphere lesion to the inferior occipital gyrus. As a result, there was no activity in the occipital face area, and the patient became unable to carry out face recognition. However, there was still activation of the right fusiform face area, containing the fusiform gyrus, and the pSTS. This phenomenon is hard to explain according to the current understanding of the hierarchical view of face perception. This means that a healthy human brain may be able to form direct pathways to bypass activation in the OFA, as opposed to a hierarchical pathway (Prieto et. al., 2011).

Acquired Prosopagnosia: The N170/M170 Effect

According to the recently discovered view of branched pathways involved in face recognition, there should be an early onset time to preferential activation to faces. This activation time should not be dependent at all on the integrity of the OFA. In order to test the accuracy of this statement, researchers endeavored to determine the time course of brain activation in these areas in both healthy people and in those with AP. Using electroencephalography and magneto-encephalography, the N170 was identified. This is a potential of negative polarity on the human scalp in the occipito-temporal area. The negative polarity appears between 130 and 170 ms after the presentation of the stimulus (i.e. the vision of a face) and continues to increase in amplitude when a face is presented along with another object. This is a marker of the first electrophysiological response to faces by the brain and is referred to as the 'N170/M170 Effect'.

Researchers are not sure which areas of the brain are the sources of this neural event, but they have identified the posterior and middle sections of the fusiform gyrus as primary contributors. A study was performed that reported the electromagnetic recordings on the scalp of patients with AP during stimulation by faces and other objects. During the course of this study, the researchers also wanted to prove definitively whether a lesion in the OFA prevents activation of the FFA or pSTS. This could be determined by testing whether early face-preferential responses, the N170/M170, were observed for those areas.

The patient that participated in this study had extensive brain damage and a severe case of AP. However, she also exhibited the N170 amplitude increase as a response to faces compared to non-face stimuli (i.e. other objects). These results and others demonstrate that the deformations in the patient's skull and scalp caused by her injuries, as well as the lesions in her cortex, did not affect her N170. When presented with a face and other object, a larger response was measured via neuroimaging studied in the fusiform gyrus and pSTS. In addition, fMRI data of this patient appears similar to that of healthy people. This information is understood when presented along with the electromagnetic findings of this study. They demonstrate that the patient's brain is able to differentiate between faces and other objects within 200 ms of seeing the image. This response time is as early as that of normal observers. Therefore, this confirms that the N170 face effect is related to the initial reaction to a stimulus, to the activation of a generic face representation. However, the patient's difficulty in recognizing faces lies in individualizing faces. This would mean that the electrophysiological response to identical faces would be the same as the response to different faces. When healthy people are presented with subsequent images of the same face, the amplitude of the N170 is reduced when compared to the amplitude when shown different faces one after another. However, in a patient with AP, the amplitude of N170 remains the same irrelevant of the faces being shown.

These results also demonstrate that the OFA is not involved, or not essential, in generating the N170. According to fMRI data alone, the FFA, the OFA, and the pSTS are all involved in preferential activation to faces. However, the electrophysiological findings of the N170/M170 help explain the relationship between the activation of these brain regions and face recognition. Because the patient in this study exhibited normal N170/M170 amplitude despite her lesion in the OFA, that area can be viewed as unessential for generating the N170/M170 increases. This supports other claims that have hypothesized that at least two of these three areas must remain functionally intact in order to generate the N170 face effect. Despite these positive findings, the researchers caution that some inconsistencies arose during this study. For example, despite the fact that the N170 face effect is possible without OFA activation, there is evidence of OFA contribution to the generation of N170/M170 in a non-Prosopagnosiac brain. Therefore, more research must be devoted to understanding the relationship between OFA activity and N170 generation (Prieto et. al., 2011).

Acquired Prosopagnosia: Residual Function

The three major areas of the brain that relate to face recognition are the FFA, the OFA, and the pSTS, as previously discussed. Within these regions, many aspects of face recognition take place. For example, perception of facial identity is linked to the fusiform gyrus, while initial perception of facial structures is linked to the OFA, and perception of facial expression is linked to the pSTS. These divisions of activity have been decided based on anatomic models but they may not be completely accurate. Therefore, it is interesting to study the surviving face-selective regions in patient with AP (Sorger et. al. 2007).

A study was performed using fMRI technology to assess the specific functions of cortical regions. The researchers set out to determine whether patients with AP exhibited any residual sensitivity to facial identity or facial expression in their surviving face-selective regions. Three patients with AP were involved in this study, two of whom had uninjured fusiform gyrii. Only one similar study had ever been performed and in that case, the fMRI data on the AP patient found that residual sensitivity to facial identity changes were present in an object-selective region of the ventral lateral occipital cortex. This is not the expected area; one would assume that the residual activity would take place in the fusiform gyrus area that was undamaged and therefore functionally intact. However, the two patients with intact fusiform gyrii involved in this study exhibited residual sensitivity to facial identity in the fusiform gyrus, consistent with its role in identity processing. This study successfully demonstrated that there is residual activity in the surviving face-selective regions of patients with AP. However,

because each patient's injuries are different, and because of the small group of patients involved in this study, it is difficult to determine the overall accuracy of the study (Fox et. al., 2013).

Conclusion

This review has gathered much research and information, concerning the anatomical, behavioral, and neurological aspects of Prosopagnosia. However, this disorder is extremely complex. Each individual with Congenital Prosopagnosia was born with their specific brain malfunction and the individuals with Acquried Prosopagnosia have different brain lesions and damage. As a result, it has become extremely difficult to understand the exact causes and results of Prosopagnoisa due to the specificity of this disorder. Research is continuing, however, to try and improve the understanding and the causes, along with an attempt to find a cure for the individuals with Prosopagnosia.

Abbreviations

CP	Congenital Prosopagnosia
AP	Acquired Prosopagnosia
DP	Developmental Prosopagnosia
OFA	Occipital Face Area
pSTS	Posterior Superior Temporal Sulcus
fMRI	(functional) Magnetic Resonance Imaging

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PARP Inhibition: A Method of Treating and Preventing Certain Cancers

By: Chana Tropper

Chana will graduate July 2015 with a B.S. in biology. Chana is currently in a program for Radiation Therapy at Memorial Sloan Kettering Cancer Center.

Abstract

Breast cancer is one of the largest causes of cancer related deaths in women. Less than 5% of breast cancer cases are genetically inherited and most often develop after menopause. The BRCA gene mutation is a genetic inheritance which increases ones chances of developing breast cancer at a young age tenfold. Recent research has proposed a method of treatment in genetically inherited breast cancers by taking advantage of the impaired DNA repair pathway caused by the BRCA mutation. The combination of a BRCA mutation, which leads to deficient double strand DNA repair, and PARP inhibition, which leads to deficient single strand DNA repair, has proven to be synthetically lethal to tumor cells. Clinical trials are determining if this method should be used as a mono-treatment or as an enhancer to other treatment options. Research has also shown that PARP inhibition may be extended to non-genetic cancers as well by targeting similar proteins involved in DNA repair and cell cycle regulation. The most effective inhibitors, their dosages, and side effects are still being studied in clinical trials. The purpose of this paper is to determine the most effective way to take advantage of the synthetically lethal relationship between PARP inhibition and DNA damage repair deficiencies.

Introduction

Damage to DNA happens on a regular basis due to normal metabolism mishaps and external triggers. DNA breaks are categorized into two types: double strand and single strand. The healthy body has DNA repair pathways in place to repair both kinds of breaks. Both the Homologous Repair pathway and the Non-homologous End Joining repair pathway work to repair double strand breaks. The Base Excision repair pathway repairs single strand breaks. As long as there is damaged DNA in a cell, the cycle should be stopped, preventing the cell from dividing. If the damage cannot be repaired, the cell has systems in place to initiate apoptosis. This paper explores the possibility of taking advantage of the systems in place to initiate apoptosis as a method of killing out tumorous cells.

Methods

The Touro database and PubMed.gov were used to find articles and original research regarding PARP inhibition in genetic cancers as well as cancers resulting from Homologous Recombination deficiencies. Articles referenced in the articles found on the above mentioned databases were used as well.

Double Strand Break Repair Pathways

Although both the Homologous Recombination (HR) and Non-homologous End Joining (NHEJ) repair pathways work to repair double strand breaks, the HR repair pathway is a much more reliable pathway. The HR pathway uses a sister chromatid as a template to repair the damage, which allows the repair to be extremely precise. Because sister chromatids are only available in the S and G2 phases of the cell cycle, HR occurs at those points in the cycle. NHEJ is a less complex process which does not require a template; the two broken ends are joined by ligation, a less precise process which often results in insertion or deletion of nucleotides (Murphy, 2010). (Figure 1)

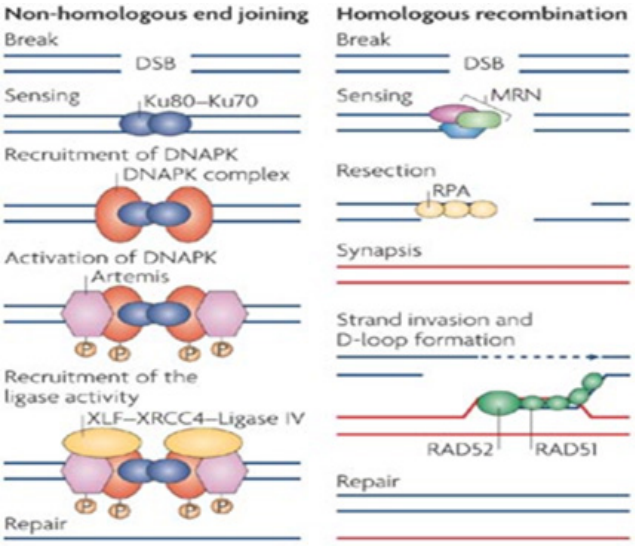

Figure 1

Homologous recombination repair: *A DNA lesion is recognized by the MRN (MRE11–RAD50–NBS1) complex, which is recruited to the DSB to generate single-stranded DNA by resection (see the figure; right panel). The single-stranded ends are bound by replication protein A (RPA), RAD51 and RAD52 and can subsequently invade the homologous template, creating a D-loop and a Holliday junction, to prime DNA synthesis and to copy and ultimately restore genetic information that was disrupted by the DSB.*
HR: Homologous Recombination, NHEJ: Non-homologous End Joining

The BRCA Gene: A Tumor Suppressor

The BRCA gene functions as a tumor suppressor gene, a category of genes which repress the cell cycle and promote apoptosis. Tumor suppressor genes are in place to prevent damaged DNA

from replicating and integrating into the genome. The most established role of the BRCA gene is its regulation of the Homologous Repair pathway. Cells with mutations in the BRCA gene thus lead the cell along the more error prone NHEJ pathway, causing an accumulation of chromosomal abnormalities and instability, which contributes to tumorigenesis. Mutations in the BRCA gene have been shown to cause a significant increase in the probability of developing tumors of the breast and ovaries. Breast cancer resulting from a BRCA mutation, however, is unique in the sense that only the tumor cells are deficient in the HR repair pathway. Healthy cells have fully responsive repair pathways. This allows treatment targeting this deficiency to be specific only to tumor cells (Murphy, 2010).

The Role of the PARP Enzyme in Single Strand Repair

The PARP enzymes' main function is to initiate the Base Excision Repair pathway by recruiting specific proteins to the site of single strand DNA breaks; once the proteins are recruited, the PARPs are released and the resultant protein complex does the actual repair work. After initially binding to the DNA using its zinc finger domains, the PARP begins to transfer ADP ribose units from NAD+ to a variety of acceptor proteins, thus creating a negative charge which recruits the enzymes necessary for base excision repair (Drew, Calvert, 2008). PARP inhibitors work either by trapping the PARP on the DNA, preventing the protein complex from beginning its repair work, or by actually inhibiting the enzymatic activity. Without PARP to initiate the Base Excision Repair process, the single strand breaks accumulate and eventually develop into double strand breaks. (Figure 2)

Inhibition of the PARP Enzyme in BRCA Deficient cells is Synthetically Lethal

Inhibition of PARP causes an increase in DNA single strand breaks, which eventually evolve into double strand breaks at the site of the original damage. Cells with a functional BRCA network can respond to the inhibition of PARP through the use of the double strand repair pathways. Cells with deficient BRCA genes, however, are unable to properly respond to inhibition of PARP, leaving the double strand breaks without a reliable repair method, causing the two deficiencies to become a synthetically lethal combination (Warrener, et al., 2012). Synthetic lethality is a condition by which deletion or inactivation of only one of two genes would not cause cell death, but deletion or inactivation of both of them is lethal (Reinbolt, 2013). Thus, the inhibition of PARP in healthy cells is not necessarily lethal because the double strand break repair pathways can kick in to repair the damage. When paired with homologous repair deficiencies, however, the inhibition of PARP has proven to

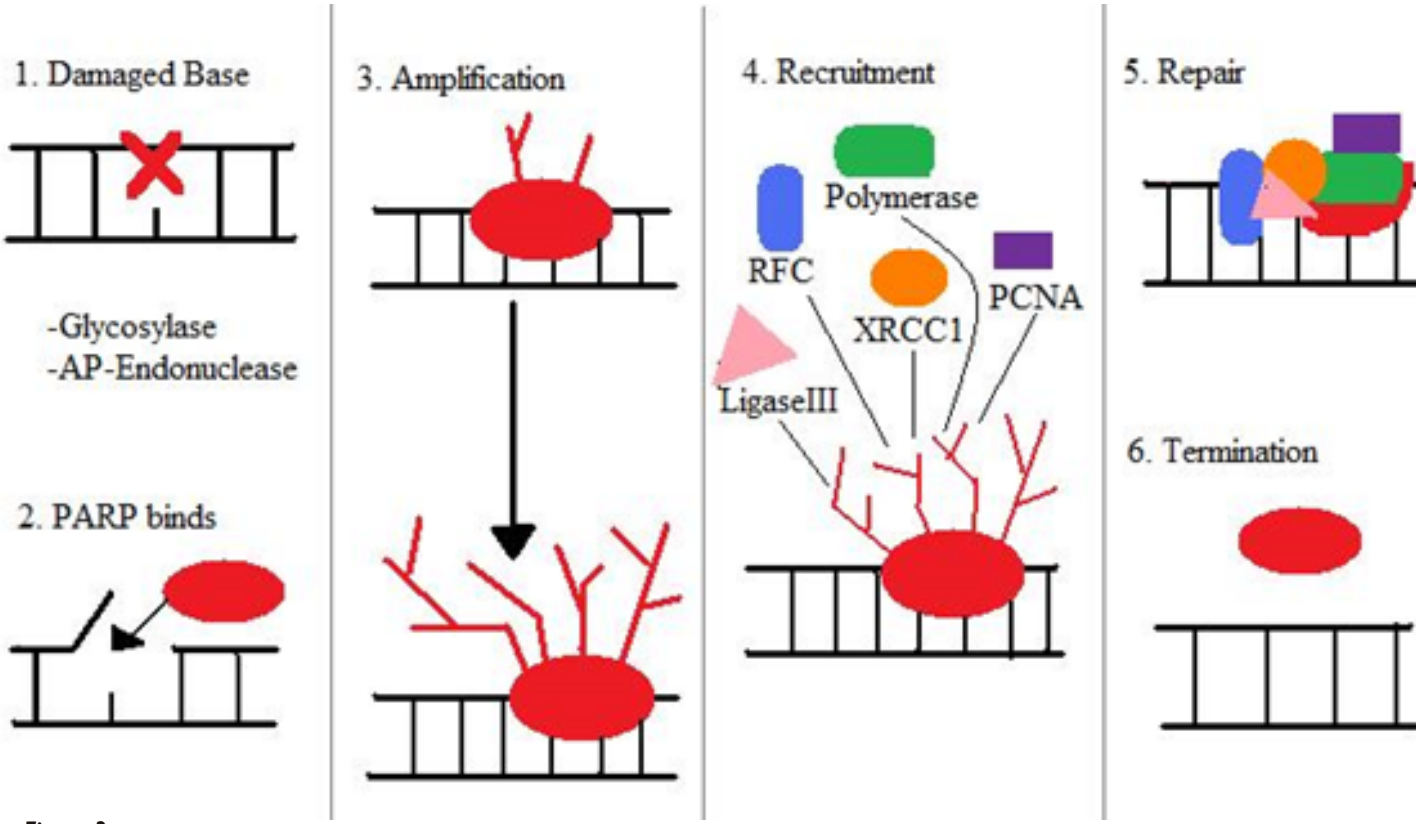

Figure 2:
PARP: Poly-ADP Ribose Polymerase *(intechopen.com)*

be lethal, because there are no efficient repair pathways for single or double strand damage. With no repair pathways in place, the cell is unable to maintain a stable cell cycle and should eventually undergo apoptosis. Inhibition of the PARP enzyme in tumor cells resulting from homologous repair deficiencies inhibits single strand repair mechanisms, initiating a selectively lethal response in the tumor cells, allowing for effective and targeted treatment.

Proteins Involved in the HR Pathway

Homologous recombination is a pathway which involves many different proteins. Identifying the roles these proteins play is a possible way of determining a biomarker to predict if tumor cells will respond to PARP inhibition. RAD51 is a protein which has been found to co-localize with BRCA2, which is suggestive of the interconnected roles they each play in homologous recombination. The HR pathway is initiated in either the S or G2 phases of the cell cycle by a double strand break. The broken ends then need to be resected to expose 3' single stranded DNA tails, which need to be loaded with RAD51 in order to invade the identical homologous strand of the sister chromatid and form new identical DNA (Golmard, et al., 2013). BRCA2 has been shown to have binding sites for both DNA and RAD51 and thus facilitates the localization and binding of RAD51 to the single strand DNA. Without the aid of BRCA2, the RAD51 is unable to bind to the DNA and the HR repair is unable to proceed (Murphy, 2010). BRCA1 is also involved in initiating double strand repair by playing an active role in resecting the broken DNA.

Cyclin-dependent kinases serve as regulators of the HR pathway

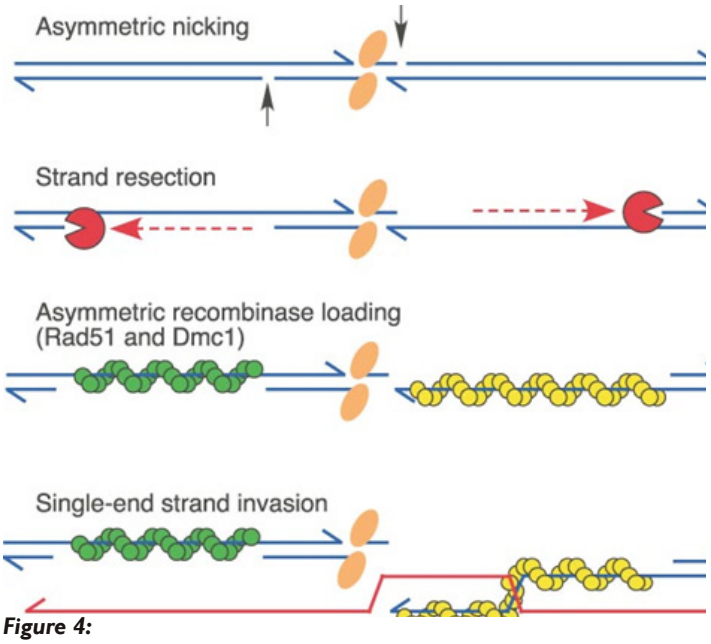

Figure 4:

(Google Images)

through their roles in phosphorylation. They can phosphorylate BRCA2 at the RAD51 binding domain and thereby block the binding of DNA and RAD51. This decreases the rate of HR, and is meant to occur when the cell is exiting the G2 phase of the cycle, and HR can no longer occur. They also phosphorylate the protein CtIP, causing it to bind to a BRCA1 domain, which then becomes activated and is responsible for the resection of the broken strand of DNA to expose a 3' tail, initiating the entire HR pathway. Cells

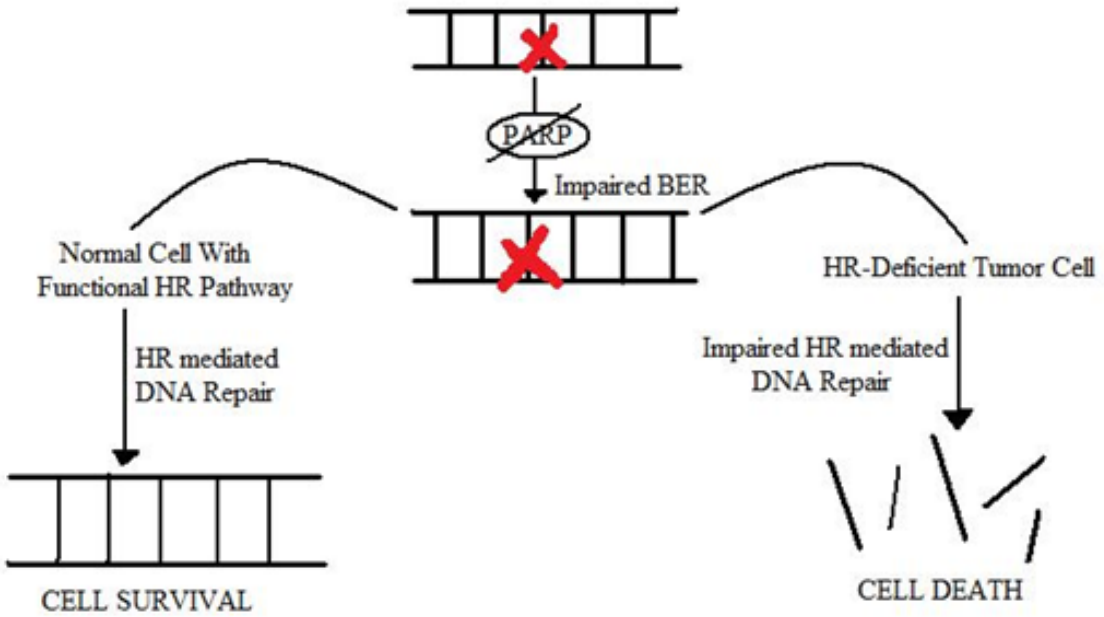

Figure 3:

HR: Homologous Repair, BER: Base Excision Repair

(intechopen.com)

without CtIP have been shown to have defects in the HR pathway, which is indicative of the involved role it plays in Homologous Recombination (Murphy, 2010).

ATM is a kinase involved in phosphorylating many proteins involved in DNA repair and cell cycle check points. Cell cycle checkpoints are in place to ensure that there is no damaged DNA before the cell moves into the next phase. (Dujka, et al., 2010) Studies have been done to determine if lack of ATM induces sensitivity to PARP inhibition, due to the lack of yet another DNA repair mechanism. ATM was genetically repressed in breast cancer cells which were then treated with Olaparib, a PARP inhibitor. The results were compared with a control group of breast cancer cells with active ATM. The control group cells got stuck at the G2/M checkpoint, but only the ATM depleted cells actually initiated apoptosis (Mantani, et al., 2013). These results indicate that ATM deficiency is not necessary for response to PARP inhibition but it does enhance the overall response greatly. Deactivating ATM in a cell is a possible way of increasing the response it will have to PARP inhibition.

P53 is a tumor suppressor protein which is activated in response to DNA damage. P53 is normally held inactive by the mdm2 complex, which it is bound to. In response to DNA damage, p53 disassociates from mdm2, and binds to the damaged DNA (Clegg, et al., 2012). Once activated, p53 activates p21, which then inhibits CDK2, a complex important in transitioning in the cell cycle (Zhao, et al., 2012). The cell cycle is then stopped, giving time for the proper repair proteins to be recruited to repair the damage. If the damage is repaired, then p53 is deactivated and the cell cycle continues. If the damage cannot be repaired, p53 initiates apoptosis. It has been found that p53 is somewhat regulated by the BRCA gene, implying that a mutation in BRCA will cause a similar deficiency in p53 (Murphy, 2010). This correlation explains why BRCA mutated cells are prone to becoming tumorous; the tumor suppression system regulated by p53 is not fully responsive, and is unable to properly respond to the unrepaired DNA damage.

Proteins as a Biomarker to Determine Sensitivity to PARP Inhibition

Identifying if cells are deficient in proteins involved in HR, such as RAD51, CtIP, ATM, CDK's and P53 can be a method of determining if a tumorous cell will respond to PARP inhibition. In addition, inducing mutations in these proteins and other proteins involved in DNA repair can be a way to prompt sensitivity to PARP inhibition, even in cells that are not necessarily deficient in BRCA (Reinbolt, 2013).

Discussions

Trial experiments have been done to study the most effective way to take advantage of the synthetically lethal relationship between PARP inhibition and BRCA gene mutations. Different inhibitors have been identified and tested in varying doses. Various side effects have been identified, and the timing of the therapy has been experimented with. It is questionable whether to use PARP inhibition as a single agent or as an enhancing agent to other treatment options.

PARP inhibition has been shown to enhance the effects of DNA damaging agents, such as ionizing radiation and chemotherapy .The principle driving the success of radiation therapy, for example, is that oxidizing free radicals are induced to produce single and double strand breaks, prompting the tumorous cells to initiate apoptosis. (Abolfath, 2013) The role PARP plays in repairing DNA damage is a potential resistance mechanism to the desired effect of the radiation. Thus inhibition of the PARP enzymes is not only allowing the radiation to produce the desired results, but it enhances the effects by creating even more breaks (Basu, et al., 2012).

Zinc deficiency and arsenic as potential PARP inhibitors

The zinc finger domains on PARP are what allow it to bind to DNA, beginning the whole Base Excision Repair process. Without being bound to DNA, PARP is essentially useless. Thus a deficiency in zinc ultimately leads to PARP inhibition due to the resultant inability of the PARP to bind to DNA. Research has also shown that arsenic can bind to the zinc finger domain in place of zinc, which changes the zinc finger structure, also preventing it from binding to DNA and causing it to lose all functionality (Sun, et al., 2014). Practically, zinc levels can play a dual role: they are a possible biomarker to determine cell sensitivity to PARP inhibition and depletion of the metal is a method of inducing the inhibition. Arsenic, as well, although via a different mechanism than the others, is considered to be a PARP inhibitor.

Olaparib as a monotherapy in BRCA1/2 mutation associated breast cancers

In a study done in 2008, Olaparib, a PARP inhibitor taken orally, was administered to a group of women all possessing mutations in BRCA1 or BRCA2 genes. Olaparib was taken as a monotherapy, but all the patients had been given at least one chemotherapy regimen earlier. The study had 2 cohorts, the first was 400 mg of Olaparib, which was established to be the maximum tolerable dose, taken twice daily. The second was 100 mg of Olaparib, also taken twice daily. Both study schedules lasted for 168 days. In the first cohort, 41% of the patients achieved the objective response, with 4% achieving a complete response and 37% achieving a partial response. Forty four percent still had stable disease and 15% had progressive disease. The second cohort had only 22% who achieved the objective response, with no patients achieving a

complete response. Forty four percent still had stable disease and 33% had progressive disease (Tutt, et al., 2010). The results of this trial show definite response of tumor cells to Olaparib. The higher dosage produced better results compared to the lower dosage, implying an enhanced response when the dosage is raised.

Olaparib in combination with Paclitaxel

Triple negative breast cancer, TNBC, is defined by lack of receptors for estrogen, progesterone, and human epithelial growth factor, and responds to few treatment options. TNBC has been shown to share similarities with BRCA1 associated breast cancers, (Nowsheen, et al., 2012) and a study was done in TNBC patients to determine the effect of combining PARP inhibition with Paclitaxel, a chemotherapy agent which stabilizes microtubules. The combined effect should prevent them from breaking down during cell division, causing the cell cycle to be stopped and apoptosis to be initiated. Patients received 200 mg of Olaparib twice daily and 90 mg of paclitaxel once a week for 6-10 28 day cycles. There was a greater than expected occurrence of neutropenia, a decrease in neutrophils which are responsible for destroying bacteria, within the first two cycles, a side effect also found in other studies done testing the combination of PARP inhibition with chemotherapy agents. Neutropenia is common side effect of Paclitaxel, but its occurrence increased significantly when combined with PARP inhibition. At the point which patients were experiencing neutropenia of grade 2 or higher, PARP inhibition was maintained, Paclitaxel administration was stopped and G-CSF was administered until the absolute neutrophil count (ANC) went up. If the absolute neutrophil count went up, Paclitaxel was continued, if it did not paclitaxel was discontinued. Although the rate of neutropenia occurrence increased, up to 40% of patients in the study showed partial response to the combined treatment, a greater response than has been seen with either treatment on its own (Dent, et al., 2013). Research is being done to understand the molecular reasoning of the increased neutropenia occurrence. Studies are also determining the best treatment schedule to minimize the side effects and maximize results.

Niraparib as a monotherapy in BRCA mutation carriers and sporadic cancers

Niraparib is also an oral PARP inhibitor and it has been studied as a monotherapy not only in carriers of the BRCA mutation carriers, but in patients with sporadic high grade serous ovarian cancer as well, which has a high prevalence of Homologous Recombination dysfunction. The study experimented with escalating doses to determine the maximum tolerable dose, which was found to be 300 mg, taken twice daily. Thrombocytopenia and neutropenia presented as side effects but were easily controlled with dose reductions. Results of this study showed that greater response was seen in cells sensitive to platinum, a common base in chemotherapy agents, as opposed to platinum resistant cells. There was some response seen in platinum resistant cells, however, implying that the resistance mechanisms for PARP inhibition and platinum do not entirely overlap (Sandhu, et al., 2013).

The results of the above studies demonstrate the existence of a relationship between DNA damaging agents, such as chemotherapy, and PARP inhibition. They also establish that PARP inhibition is not only effective in genetic tumors, but in other cancers involving homologous repair deficiencies. The relationship between PARP inhibition and platinum is logical, as they share a common goal of inducing DNA damage. The mechanisms of action, however, are clearly not the same because even the platinum resistant cells showed a response to PARP inhibition. Further understanding of the differences between the two and their resistance mechanisms must be understood in order to maximize the positive effect PARP inhibition can have on platinum resistant tumors. The response shown by the sporadic tumor cells shows promise in expanding PARP inhibition to include not only genetic cancers, but other cancers resulting from HR deficiencies. The key will be in finding a biomarker to determine which cells will respond positively.

Interferon Gamma as an enhancer of PARP inhibition

Because of the role they play in tumor suppression and cell cycle regulation, a study was done to determine if the interferon pathway is affected by or involved in the synthetically lethal combination of PARP inhibition and BRCA gene mutations. The interferon pathway is a crucial response of the immune system to viruses, bacteria, and tumor cells. Interferons are proteins which have various functions, including regulation of the cell cycle, anti-viral responses, and apoptosis. Studies have suggested that interferons serve as regulators of the P53 gene, which is involved in the initiation of apoptosis (Takaoka, et al., 2003). H2AX is a gene which codes for the histone H2A, around which DNA gets wrapped, allowing for organized nucleosome formation. ATM is involved in the phosphorylation of H2A as a reaction to DNA double strand breaks in order to create space for the recruitment of repair proteins. Some of the interferons involved in cell cycle regulation and apoptosis are activated via an ATM dependent pathway, suggesting that an interconnected relationship exists between ATM, H2A, interferons, P53, and PARP (Warrener, et al., 2012).

The above-mentioned study took BRCA silenced cells and studied the effect of PARP inhibition on interferon pathway activation. The PARP inhibited cells showed a three-fold increase in H2A phosphorylation, which is indicative of the increase in double strand breaks which results from the unrepaired single strand breaks. Enhanced activation of the interferon pathway was shown to correspond with the level of response to PARP inhibition, suggesting that the role the pathway plays in promoting apoptosis serves as an enhancer to the effects of PARP inhibition. The study

also determined that when interferon gamma was administered together with the PARP inhibitors, the lethal response increased ten-fold, verifying the involvement of the interferon pathway in initiating apoptosis in PARP inhibited BRCA deficient cells (Warrener, et al., 2012).

Because of the role it plays in initiating double strand break repair, ATM depletion is another possible enhancer of PARP inhibition. When ATM is not active, H2A cannot be phosphorylated, which slows the response of the double strand break repair proteins. A decreased double strand repair response leads to more unrepaired double strand breaks with no method of repairing themselves, thus increasing the amount of cells with sensitivity to PARP inhibition. This relationship was proven in a study which showed increased activation of the interferon pathway in ATM deficient cells, confirming both the dependency of successful PARP inhibition on lack of proper double strand repair mechanisms and the involvement of the interferon pathway in the apoptosis of PARP inhibited BRCA deficient cells (Warrener, et al., 2012).

Possible Resistance Mechanisms to PARP Inhibition

BRCA2 mutated cells have been found to develop resistance to platinum based chemotherapy due to the development of a secondary mutation which corrects the original mutation, restoring BRCA function (Wiedemeyer, et al., 2014). Cells harboring this resistance will also display resistance to PARP inhibition, whose success is dependent on a defective Homologous Recombination deficiency. Not all platinum resistant tumor cells, however, develop as a result of the secondary mutation; those cells are still expected to retain sensitivity to PARP inhibition (Weil, Chen, 2011).

P-gp, P-glycoprotein, is a protein of the cell membrane coded for by the ABCB1 gene that acts as a pump, pumping out foreign substances. This protein is involved in developing drug resistance, and has been shown to be the cause of resistance to some anti-cancer drugs. Up-regulation of the ABCB1 gene has been proven to demonstrate resistance to PARP inhibition due to the increased presence of P-gp. This resistance, however, has been counteracted with the administration of Tariquidar, a P-glycoprotein inhibitor (Weil, Chen, 2011).

53BP1 is a protein attached to DNA which is replaced by BRCA1 during DNA damage repair. If 53BP1 is not replaced by BRCA1, the HR pathway becomes inhibited. If 53BP1 is absent in a cell, even in the case of a BRCA mutation, HR is still able to proceed because there is no 53BP1 which needs to be displaced. Only when 53BP1 is present and there is a BRCA mutation is HR actually impaired. An absence of 53BP1, therefore, is a possible resistance mechanism to PARP inhibition, through its restoration of the HR pathway (Weil, Chen, 2011).

PARP inhibition as a proactive treatment option

Until recently, BRCA1/BRCA2 mutation carriers have been faced with the disheartening knowledge that they will most likely develop breast or ovarian cancer and the only proven preventative measure to be taken was prophylactic surgery. The evolution of PARP inhibition as a treatment option also introduces the possibility of using PARP inhibition as a chemo-preventative measure for BRCA carriers who did not yet develop cancer, a much less drastic measure than prophylactic surgery. There have not been any conclusive studies done regarding using PARP inhibition as a preventative treatment, but the same mechanism it uses to selectively kill tumor cells has been proposed as a way of preventing them from developing in the first place. Tumor cells resulting from BRCA deficiencies are the result of genetic alterations and defective DNA repair. Pre-exposing a BRCA mutation carrier to PARP inhibitors is a proposed method of killing the genetically altered cells, preventing them from developing into full blown tumors. It has also been proposed to use PARP inhibition as a means of preventing relapse, but no conclusive studies have been done at this point to investigate the possibility of success in this approach. Eventualities which must be considered in using PARP inhibition as a long term treatment option are the side effects as well as the danger in maintaining a defective repair pathway long term, especially in already high risk patients (Vinayek, Ford, 2010).

Conclusions

PARP inhibition shows promise on many different levels. The main factors to bear in mind in the development of treatment options are the specific target of only tumorous cells and minimal side effects, both of which are realized in PARP inhibition. The success of PARP inhibition expanded from genetically inherited breast cancers to other cancers resulting from DNA damage repair defects was demonstrated in clinical trials, which expands the network of patients eligible for treatment, making it easier to maintain the funding of research. PARP inhibition has elicited positive responses both as a mono-therapy and as an enhancer of other DNA damaging agents, such as chemotherapy and radiation. The proteins involved in Homologous Recombination show great promise in functioning as predictive biomarkers of sensitivity to PARP inhibition. Further understanding of the roles they play will enable mutations of those proteins to be a method of inducing and/or enhancing sensitivity to PARP inhibition. The hope that PARP inhibition presents not only as a treatment option but also as a precautionary alternative to prophylactic surgery makes the investment needed to make it a reality most worthwhile.

Abbreviations

PARP	Poly ADP-ribose polymerase
HR	Homologous Recombination
NHEJ	Non-homologous End Joining
BER	Base Excision Repair
ATM	Ataxia Telangiectasia Mutated

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It's All in the Mind: Mind Controlled Prosthetics

By: Tziril Joselit

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Abstract

The problem with prosthetics is a longstanding problem that researchers have been working on for many years. They are attempting to create a prosthetic that acts as if it is the original limb or body part. In recent years they have discovered a technology that has assisted many of those who are greatly in need of a prosthetic, such as an amputee or someone who is "locked in". "Locked in" refers to a person who is technically confined in his own body and has no methods of communication with the world. Brain-computer interface (BCI) has opened up a whole new world of prosthetics. It has opened doors for those who have been "locked in". BCI assists those with severe neural disorders. BCI links the brain to a machine, allowing for actions to be performed by circumventing the damaged or missing body parts. It captures the brain signals, interprets them, translates them and transfers them as control signals to the device being used. Using this technology, targeted-muscle reinnervation (TMR) has been designed to create a prosthetic for amputees as well. Altogether, it has been established that prosthetics controlled by the mind is possible.

Introduction

A prosthetic is a device that substitutes for a body part or function that is defective or missing. The aim of a prosthetic is to replace the missing or damaged body part so perfectly that when a function is performed it is as if the original body part performed it. The Brain-Computer Interface (BCI) is a device that captures nerve signals that the brain produces when there is an intention to act, translates the signals through algorithms and then produces the action through a machine; thereby, circumventing the damaged limbs or missing body parts (Leuthardt et al., 2006). The system requires no muscular control and it can consequently liberate someone who is paralyzed, or it can create a prosthetic limb that acts as the original limb. The following paper will discuss BCI and TMR, explaining the basics of how each works, demonstrating how BCI is helpful for those with severe neural disorders and TMR assists amputees.

How BCI works

The BCI works through a basic four step process: signal acquisition, signal processing, device output and operating protocol (Leuthardt et al., 2006).

Signal acquisition is when the brain signals are recorded and amplified, by electrodes. The brain signals can be recorded through many methods: non-invasively through Electroencephalography (EEG) and invasively through Electrocorticography (ECog), Local Field Potentials (LFPs), and Single Units. The non-invasive EEG is the safest method because it records the signals through the scalp and no electrodes penetrate the brain. The EEG mainly measures sensorimotor rhythms, slow cortical potentials (SCPs), and P300 potentials. The Sensorimotor cortex produces Mu (μ) rhythms which are typically 8-12 Hz and are found when the brain is not processing any new information, when it is "idling", and Beta (β) rhythms, which are typically 18-26 Hz. The μ and β rhythms decrease in activity when there is movement or preparation for movement. However, more importantly, these rhythms occur even

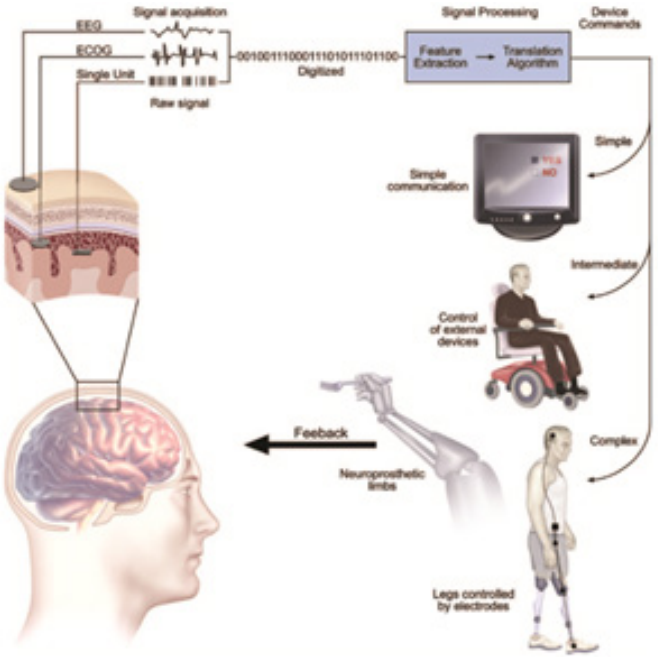

Figure 1:
This is a diagram of the BCI system. It shows the pathway of the signals (Leuthardt et al., 2006).

when there is only imagined movement and actual movements are not necessary for their activation. This makes them useful for the BCI because if someone imagines a movement, the μ and β rhythms will be activated and they can then activate the BCI without muscular contractions (Leuthardt et al., 2006). SCPs are the lowest frequency signals recorded over the scalp. They are slow voltage changes that are generated in the cortex over .5-10 seconds. Negative SCPs are associated with movement and cortical activation, while positive SCPs are associated with a reduction of movement and a reduction of cortical activity (Wolpaw et al., 2002). The SCPs are beneficial to the BCI because they are

directly associated with movement and cortical activity; therefore, when a movement is desired, the SCPs will be activated and can power the BCI. The P300 potentials, otherwise known as "oddball" potentials are produced in the parietal cortex. The P300 is useful for the BCI because it differentiates the brain's response to a significant stimulus from a routine stimulus (Leuthardt et al., 2006). Additionally, unlike sensorimotor rhythms and SCPs, the P300 does not require any training of the user for control; it is a natural response to a preferred choice (Wolpaw et al., 2002). However, the system requires "training" to learn the user's preferences (Leuthardt et al, 2006). The EEG is the most commonly used signal acquisition system for the BCI as of now, because of its non-invasiveness (Leuthardt et al., 2006). However, the invasive methods are more accurate and specific in their recordings.

The next level of recording is through ECog, which measures the signals from beneath the cranium. The ECog was at first assumed to be very similar to the EEG; however, this is not true. The ECog can detect signals to a much higher frequency; up until 200 Hz versus the EEG which measures only up until 40 Hz. when the electrode is placed beneath the skull, as is done in the case of ECog electrodes, a higher frequency can be measured by the electrodes. This then allows for the recorded signals to be more precise and for there to be less other "distracting" signals (or a higher signal to noise ratio) (Leuthardt et al., 2006). Because ECogs are placed on the surface and do not actually penetrate the brain, they are considered more durable than the microelectrodes which measure LFPs, and Single Units (Schwartz et al., 2006).

LFP's are recorded through penetrating electrodes into the parenchyma. There the frequency can be measured typically from 300-5000 Hz, but can record lower frequencies as well (Schwartz et al., 2006). Single Units are recordings of individual neuron action potentials. The microelectrode is place deepest into the brain, and therefore, has the most accurate recordings. As a result, the use of Single Units can produce the most complicated actions. The further into the brain that the electrode is placed, the more accurate the recordings of the signals will be.

After signal acquisition, the signals are digitized and then the more complicated process of signal processing occurs. Signal processing is broken down into two components: feature extraction and signal translation (Wolpaw et al., 2002). Whenever there is signal acquisition, "noise", otherwise known as artifacts, such as other brain signals or even muscular movements, will get mixed in and can even sometimes be thought of as the target signal. Therefore, feature extraction removes the desired signals from the total signal; it identifies the meaningful signal that was produced from the combination of all the signals together (Leuthardt et al., 2006). The purpose of this step is to identify the user's intent, which is identified through the signals that are captured (Wolpaw et al., 2002). Different algorithms are used for feature extraction and artifact

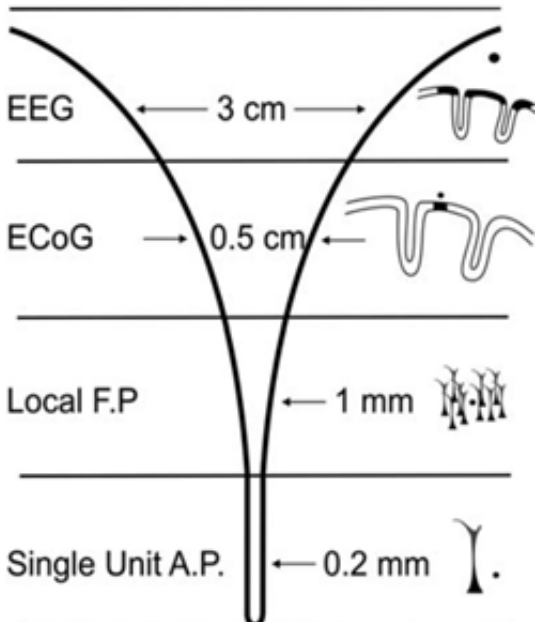

Figure 2:
The different kinds of signal acquisition methods and their distances from the neurons. The black dot in each picture symbolizes the electrode and its distance from the neuron (Schwartz et al., 2006).

removal (Vallabhaneni et al., 2005). The algorithms adapt to the user on three levels: first the algorithm adapts to the signal features that the user uses, for example if the user uses Mu rhythms, the algorithm will adapt to the user's characteristic amplitude of Mu rhythms. The second adaption is periodic adjustments to spontaneous changes, because the user will produce more than just one kind of signal and one intensity level of that signal. The third adaption of an algorithm is to use the brain's adaptive capacities. For example, as feedback occurs, hopefully the brain-computer interface will improve, as each "gets used to each other". This adaptive algorithm will assist the natural adaption of the brain by responding to the user with faster communication or other such "rewards" (Wolpaw et al., 2002). The more specific and exact the method for feature extraction and the better the adaptive algorithms, the more exact the signal to noise ratio will be (Wolpaw et al., 2002). Signal translation converts the signal features (rhythm amplitudes or neuron firing rates) that were extracted, into device commands (Wolpaw et al., 2002). Translational algorithms are used for this conversion. The signals are then converted into a different kind of signal that is appropriate for the device that is being used.

The signals are then sent to the device output section of the BCI which is the actual machine that produces the action. The action can be anything, whether it is controlling a cursor on a screen or the movement of a robotic arm (Leuthardt et al., 2006). The device output then translates the signals into physical control signals that can then power the device (Bashashati et al., 2007).

The operating protocol is how the device is controlled. How it is turned on and off, the feedback that is provided (such as the speed of the reactions), and the timing of the commands and actions. It is the basic operating manual of the prosthetic (Leuthardt et al., 2006).

How Targeted Muscle Reinnervation Works

Another method for a natural acting prosthesis is through Targeted Muscle Reinnervation (TMR). The prosthetics that are used in TMR are called myoelectric prosthetics. A myoelectric prosthesis uses residual muscles after an amputation or other, unrelated muscles, to amplify and supply signals to move the prosthesis. After a conscious thought to move that muscle, sensors relay the information to a controller which then powers the motor to move the arm. A myoelectric prosthesis works well and almost intuitively for an amputation that is below the elbow because Electromyogram (EMG) signals or motor action potentials are recorded from the residual muscles that formerly powered the amputated arm. However, for a shoulder amputation (and for some transhumeral amputations), TMR is performed to make the control of the prosthesis more intuitive. TMR is a process that takes the residual nerves from an amputated limb, the nerves that had innervated the limb before the amputation. They then transfer the residual nerves to another muscle group that had also "worked with" the amputated limb, but is no longer functional because it is no longer attached to the limb. As a result, when there is a thought about movement of a part of the amputated limb, such as a finger, the reinnervated muscle will contract. Nerves that would innervate the "recipient" muscles are denervated so that the muscles can be reinnervated by the transferred nerves and there will not be as much interference. The reinnervated muscles serve as biological amplifiers of the nerve signals that are sent to the limb. Subcutaneous tissue is also removed so that the myoelectric signals or the EMG signals can be recorded with relative ease. In this way TMR provides EMG control signals that are associated with the lost limb. This amplified, natural signal can then be used to power the prosthesis (Kuiken et al., 2007). After a signal is sent, through a mere thought or desire, to "lift a finger" or perform another action, electrodes record the EMG signals non-invasively, from the body surface. The electrodes are placed above the reinnervated muscles. Because the muscles amplify the signals, they are relatively easily recorded. The signals are then sent to a microprocessor chip that is in the prosthetic limb which interprets the signals and then powers the myoelectric arm to do what the signal was asking (Kuiken et al., 2007).

In most of the cases TMR was performed on someone with a shoulder amputation; however, some were performed on transhumeral amputations as well. In the cases of a shoulder amputation, the musculocutaneous nerve, median nerve, radial nerve, and ulnar nerve were transferred to the pectoralis major, pectoralis minor, latissimus muscle, and serratus anterior (each case was slightly different, but overall these were the nerves and the

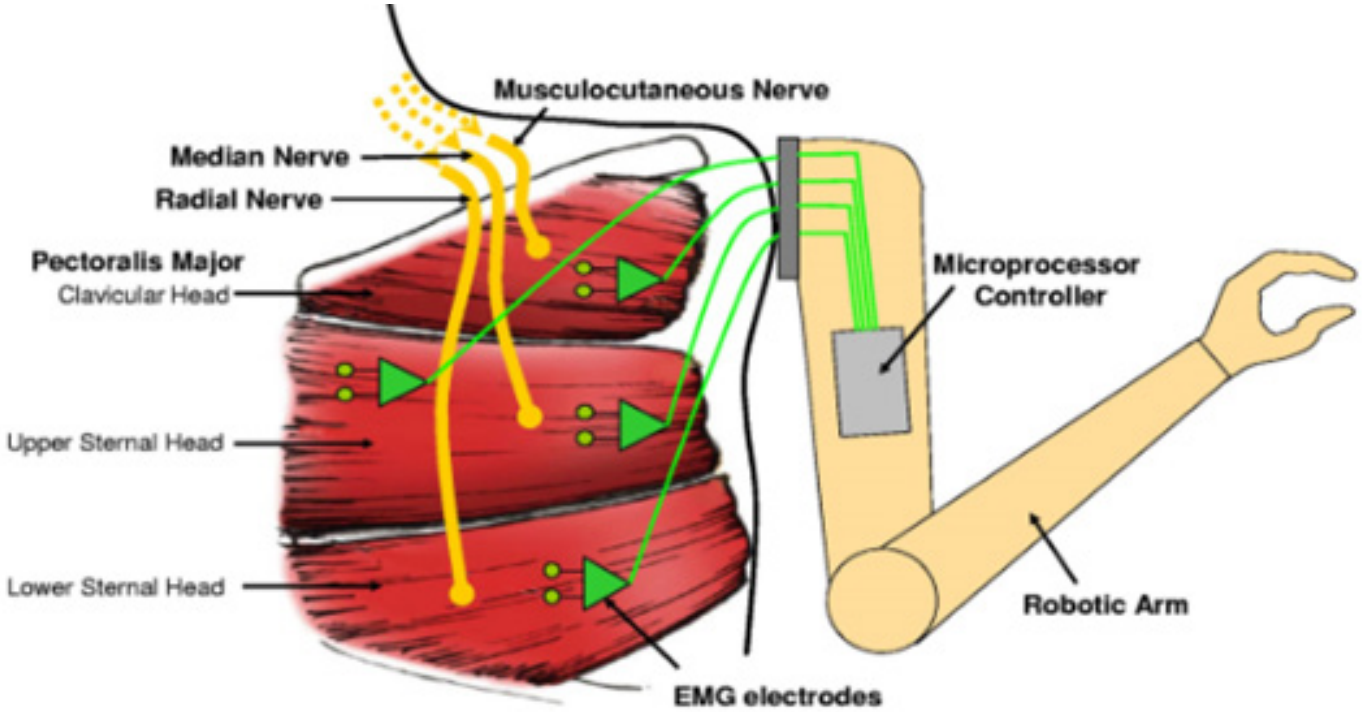

Figure 3.
Diagram of the TMR process. The 3 transferred nerves are yellow, the electrodes are green (and are in reality placed on the body surface, but the diagram places them on the muscles because the body surface was removed for the diagram's sake), and the microprocessor chip is in the prosthetic arm (Zhou et al., 2007).

muscles they were transferred to). In the transhumeral cases, the median and distal radial nerves were transferred to the medial biceps and the brachialis or lateral triceps, respectively (Kuiken et al., 2009). Approximately three months after TMR surgery, muscle reinnervation was felt and at approximately five months, strong contractions could be seen and palpated (Kuiken et al., 2007). At this point the muscle contractions can be used to power the myoelectric arm.

The capture of the EMG signals is the signal acquisition step of the BCI. Therefore, next is the signal processing step. In the case of TMR, there are artifacts that must be eliminated; however, they come from a different source. Most of the artifacts that disturb the signal in TMR originate from electrocardiogram (ECG) signals. Nonetheless, it was found that the ECG interference does not disturb the accuracy of the signals significantly when a pattern recognition algorithm is used for signal processing (Zhou et al., 2007). The microprocessor chip then sends the signals to the part of the arm that the signal was intended for, it powers the arm, performs the motion that was requested, and turns the arm off.

When TMR is done on a leg, the process is very similar. The sciatic nerve is separated into its two smaller branches; tibial and common peroneal nerves. The tibial nerve was then sewn onto the semitendinosus muscle and the common peroneal nerve was sewn onto the long head of the biceps femoris. In this way, the residual nerves reinnervated the hamstring group. The process is the same as by the arm prosthetic; however, there are more obstacles or details that need to be perfected with the leg than with the arm. Such as, the leg is required to bear weight, maintain balance, have the ability to change ambulation modes, and other such functions that the arm does not have to deal with. These functions are of vital importance and therefore, the error must be extremely low, because if not, the person is at risk of falling. Again a pattern recognition algorithm was used and it lowered the percent error from 12.9% to 2.2% (Hargrove et al., 2013).

Methods

Google Scholar and the PubMed database were used to search for information for this paper. Key words such as, "mind-controlled prosthetics" or "prosthetics controlled through thought" were used to find review articles. These articles gave a better idea of other key words to use, such as, "brain-computer interface" and "neural-machine interface" to find original papers. To find out about prosthetics for an amputee, key words like, "prosthetic limbs" were used and then "targeted muscle reinnervation" and "myoelectric prosthesis" were used to narrow down the search.

Discussion

Although each method for intuitive and natural control of prosthesis sounds like it can be a "perfect" prosthesis, each has its pros and cons which lends each prosthesis to a specific function. BCI is, in a technical sense, the perfect prosthesis because it captures the brain signal and performs the action through just a thought. However, there are some distinct issues that make it a useful prosthesis for someone with a severe neural disorder, but not for an amputee. One major concern is the problem and debate with signal acquisition. If the non-invasive EEG is used, the electrode is at a significant distance from the neurons because the scalp is 2-3 cm away from the cortex. Therefore, the signals recorded are limited and are not sufficiently effective to control a more complicated device, such as a device with more than two dimensional control (Schwartz et al., 2006). The information rate is only 5-25 bits/minute, which is so slow that it can take several minutes to insert a word into a computer and the average time for a task to be completed (including signal acquisition, signal processing, and device output) was an approximately 6.20 seconds (Cheng et al., 2002). In a different study performed in 2004, the average time for a cursor to be moved was 1.9 seconds (which is a significant improvement from the study in 2002) and the movement precision or the precision in hitting the target was 92%. Although these percentages are a significant improvement, they can still be improved in accuracy and speed, through better adaptive algorithms and other such reforms (Wolpaw, McFarland, 2004). In addition, EEG signals can only detect lower frequencies, frequencies that are less than or equal to 40 Hz, which again limits the complexity of what the device can accomplish. Furthermore, if sensorimotor rhythms or SCPs are used, only two dimensional control or at times three dimensional control has been proven to be possible, such as the movement of a cursor on a screen or a basic movement of a robotic arm (separate from the body). These rhythms do not have the capability of performing more complicated functions. Regarding the P300 signals it has not yet been determined if gaze fixation is necessary for the system to work. In this case the BCI would only be of assistance to someone with the ability of eye movement. However, with the P300 signals, only a simple word processing program can be used (Leuthardt et al., 2006). An invasive method of signal acquisition (such as, LFPs or Single Units) would solve the above mentioned problems with EEG signal acquisition; however, then the problems with implanted electrodes arise. First of all, surgery is required to place the electrodes in the brain and that in itself causes the risk of damage to the brain. Furthermore, if Single Units are used, the microelectrodes are placed beneath the cranium, which automatically causes blood vessels to be broken in the process. This causes a reactive response from the brain; astrocytes and glial cells will begin to aggregate there and they then basically insulate the microelectrode until no signals can be recorded after a period of time, so signal acquisition can only occur for approximately a year. Repeating the placement of the microelectrodes can cause scarring on the brain which can damage the person's cognitive status and can further damage the person's ability to function properly (Leuthardt et al., 2006). Additionally, the electrodes must remain stable for a long period of time and although algorithms can maintain the stability of an electrode, implanted

electrodes have a limited time that they are functional (Leuthardt et al., 2006). The ECog system was tested and it was successful in many instances. In a stroke patient, for the movement of a robotic arm, the signal acquisition method decoded 61% of the ECog signals, at least one second before the movement was performed by the participant's functional arm. Selection of the movement that was desired (out of three different kinds of movements) was detected with 69.2% accuracy (Yanagisawa et al., 2011). In patients with epilepsy, the ECog signals had classification rates of 70-90% in selection of letters from a spelling system (Birbaumer et al., 2014).

Although the ECog's success rates are good, they are not good enough. In the real world there is not so much room for error, especially with people who cannot help themselves if something goes wrong. Success rate with the patients with epilepsy was high; however, this is only with a simple word program. Additionally, the problems with implanted electrodes still exist. However, they do not exist to the extreme that they exist in Single Units because ECog electrodes are placed on top of the parenchyma and not within. Therefore, the electrodes do not invade the blood brain barrier, which causes the inflammatory response that occurs in Single Units. In addition to the problems with the electrodes, in the BCI system the signals are easily interfered with through slight distractions, such as an eye movement. Because of all the above mentioned issues with BCI, BCI is limited to people with severe neural disorders, to someone whose only method of communication with the world is through their brain, such as someone with high level quadriplegia (Ohnishi et al., 2007). The BCI system is satisfactory for someone with a severe neural disorder because it provides adequate two dimensional control and even at times three dimensional control of a robotic arm. However, for an amputee two dimensions is not enough. Consequently, the problem of creating a "perfect" prosthesis for an amputee still exists. To solve this problem, TMR was designed. TMR unites BCI and existing prosthetics to create the perfect prosthesis. TMR solves most of the problems with BCI; however, it does have its limitations as well. First of all, TMR solves the problems of non-invasive electrodes because the signals are adequately biologically amplified through the muscle, and the signals are clear; however, they are not invasive and therefore overcome the problems of implanted electrodes as well. Compared to participants who used their own limbs as a control group in an experiment, the TMR patients performed exceptionally well, as seen in Table 1.

The motion selection time for arm function was very good, it was an average of less than or equal to 220 milliseconds. For hand grasps it was also pretty good; the motion selection time was an average 380 milliseconds. The average speed of an action was between 90°/second to 120°/second. For elbow and wrist function, the success rates were high. For hand grasps, the success rate was high, but not as high, most probably because this requires more cognitive control of the user (Kuiken et al., 2009). The TMR system does not have as much interference with the signal acquisition, with the exception of ECG signals which are easily taken care of through a pattern recognition algorithm. EMG classification accuracy has been shown to be in the range of 90%-100% (Kuiken et al., 2009). Users of TMR prosthetics have reported intuitive use, as one participant stated "I just think about moving my hand and elbow and they move." (Kuiken et al., 2007). TMR also provides multiple degrees of freedom. Until now, prosthetics

Table 1.

Performance Metrics for Virtual Prosthesis Testing Protocol With a 5.0 Second Time Limit:

	Mean (SD)	
Performance Metric	TMR Patients (n = 5)	Control Participar (n = 5)
Motion selection time, s		
Elbow and wrist[a]	0.22 (0.06)	0.16 (0.03)
Hand grasp[b]	0.38 (0.12)	0.17 (0.09)
Motion completion time, s		
Elbow and wrist[a]	1.29 (0.15)	1.08 (0.05)
Hand grasp[b]	1.54 (0.27)	1.26 (0.18)
Motion completion rate, %		
Elbow and wrist[a]	96.3 (3.8)	100 (0)
Hand grasp[b]	86.9 (13.9)	96.7 (4.7)

Abbreviation: TMR, targeted muscle reinnervation.
[a]For 108 attempted elbow and wrist movements.
[b]For 72 attempted hand grasps.

Table 1 is showing the time it took for each motion to be selected and completed and the motion completion rate, compared to the control group which used their natural arms. (Kuiken et al., 2009)

required concentration for each movement to be performed, the myoelectric arm, without TMR performed, required a distinct thought about a muscle that was unrelated to the arm previously. Now one just thinks about moving his/her arm and it moves. In this way multiple degrees of freedom are possible, for example, now the shoulder can flex from -15° to 185° versus the conventional prosthetic arm that used gravity to swing forward from 0° to 90° (Miller et al., 2008). Additionally, more than one motion can be performed at once with TMR, for example, when grasping an object the wrist can be rotated and the elbow extended in one motion versus each motion having to be thought about and performed separately; however, executing this was not desired by the participants because of the cognitive burden it entailed. With the TMR prosthesis, the participant was almost four times as fast than with the conventional prosthesis (Kuiken et al., 2007). The level of classification accuracy here was found to be very similar to participants "using" their real arms, 95-97% (Zhou et al., 2007).Nevertheless, TMR has limitations as well. First of all, TMR is a surgical procedure which has the same dangers that every surgery has; however, it is not brain surgery which contains higher risks than other surgeries. Other side effects, such as, recurrence of phantom limb pain, permanent paralysis of the targeted muscles, and other painful neuromas are all risks of the TMR surgery (Kuiken et al., 2007). Secondly, TMR requires intensive therapy after recovery from surgery to gain control of the prosthesis. TMR of the arm is much more developed and reliable as of now than the leg. The leg prosthesis is still not available for clinical use because of some withstanding difficulties; such as, the leg must be made quieter, lighter, and more reliable. Additionally, for the leg to work properly the EMG signals must be of very high clarity and the electrodes must remain in contact with the residual limb without causing discomfort to the wearer which is hard to accomplish while movement is occurring, and lastly, improvement of the pattern recognition algorithm is necessary (Hargrove et al., 2013). Despite the limitations, TMR is in essence a perfect prosthesis for an amputee in that a signal meant directly for the limb is recorded through a residual muscle, processed and then used to power a prosthetic. In the case of a "locked in" patient, where TMR would not be of assistance, BCI is used. Through both BCI and TMR, prosthetics controlled through the mind is possible.

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How Does Spaceflight Affect the Human Body?

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Abstract

Spaceflight can impact just about every organ of the human body. The launch into space increases gravitational forces, which may decrease consciousness. Once subjected to the microgravity of outer space, the constant mechanical stress exerted on the body from Earth's gravity decreases enormously, causing bone degeneration to occur. Calcium, a major component of bone is excreted in very high amounts, often leading to the formation of calcium kidney stones. Astronauts perform weight bearing exercise, take osteoporosis drugs and calcium and vitamin D supplements in order to combat bone loss. The vertebrae of the spine are also impacted by the lack of gravity. The spine actually expands up to two inches. On Earth, gravity pulls all the liquid of the body to the lower extremities. Without gravity, the liquids are equally dispersed throughout the body causing faces to appear puffy and increasing pressure on the brain due to excessive cerebrospinal fluid. The body responds to this liquid buildup by eliminating plasma and red blood cells, causing anemia. The increased fluids flatten astronaut's eyeballs, and they often experience farsightedness. The immune system is also impacted during spaceflight as T-cells are not activated properly. Astronauts are also impacted by an excess radiation, which can increase the chances of future occurrences of cancer or cataracts. Muscle atrophy takes place as a result of microgravity as well as other factors. Exercise along with growth hormone, are recommended in order to combat the muscle degeneration. In space, the vestibular system of the ear does not function properly and impairs proper balance which can result in space motion sickness. Although many may not be aware, the benefits of the space program have impacted most everyone on Earth; as there have been dozens, if not hundreds of inventions and discoveries which were only developed because of the space program. Although the space program has benefitted humankind, the risks to the fragile human body are gargantuan. It would be preferable to continue the space exploration program remotely via robotics.

Introduction

Since the middle of the nineteenth century, and perhaps even much earlier, mankind has been enthralled by the idea of space exploration. Back in 1865, Jules Verne envisioned space travel via a projectile shot at high speed out of a gigantic cannon in "From Earth to the Moon." In 1901, H.G. Wells envisioned space travel in a space "sphere" in "The First Men on the Moon." This was closely followed by the very first science fiction movie: Mieles' "A Trip to the Moon", a silent movie based on Jules Verne's earlier tale.

Our unquenchable fascination with space travel continues until this very day. In fact, science fiction movies have been among the top grossing movies of all time. One only has to think of the six Star Wars movies (with more episodes coming), E.T., the numerous Star Trek series and spinoffs and countless other hugely popular fictional productions.

It is even possible that these many popular works of fiction depicting the ease of future space travel may have enabled the reality of Nasa's Project Mercury (1958 – 1963) and the subsequent Apollo space program (1961 - 1975). The dream of space exploration was further attained in the Skylab project (1973 - 1979), the Mir Space Station (1986 – 2001), in the International Space Station (assembled starting from 1998; manned since 2000 - present) and the Space Shuttle Program (1981 - 2011). Our expectation of human space travel to alien worlds continues to this day, as scientists envision establishing a colony on Planet Mars. And Richard Branson of Virgin Atlantic has created Virgin "Galactic", with plans to allow any person who has $250,000 to burn to experience space travel in the private sector.

One thing in common in most of the previously mentioned works of fiction is that they all seem to imply that it will be pretty easy to get anywhere we want to go in the galaxy and even beyond. What the general public fails to understand, however, is the grim danger one faces every moment spent in space. Our bodies are just not created to exist in space. This paper reviews the research conducted regarding the many things that can injure, maim or even kill those who dare to travel into space and recommends a safer alternative via robotics.

Discussion

From the very first moment, travel to space is fraught with acute danger to the human body. The launch of a rocket ship places unique strains on the body. On Earth, normal gravity conditions are termed "1-G". As the rocket accelerates toward outer-space, however, the G-forces increase to 3-G or more. An excessive G-force can have many deleterious effects on the body: The heart loses its ability to pump blood efficiently; circulation of the blood is impaired and oxygen is prevented from reaching the brain or other organs sufficiently. Consequently, a decrease in

consciousness may occur: Colors may begin to fade; vision may be limited to tunnel vision; and a total blackout may even occur. While the G-forces usually do not have such serious side-effects at take-off, the effects can be much more severe upon reentry into the Earth's atmosphere since the astronaut's body is in a much more weakened state after a stay in space. (Harrison, 2001)

On Earth, the average human head weighs approximately ten pounds. However, if one experiences 3-G's, the weight is tripled, placing enormous strain on the neck muscles which can result in severe neck pain. Therefore, astronauts are strapped into specially made seats which provide extra support to sensitive areas, such as the cervical and lumbar regions. Astronauts also sit facing the direction of acceleration, in order to spread out the forces evenly over the body. (Harrison, 2001)

Upon reaching outer space, the hazards continue to multiply, but for the opposite reason. During takeoff, an astronaut is subject to the force of 3-Gs or more. On the other hand, in outer space there is minimal gravity which can also adversely affect many organs of the body.

A major organ which degenerates during space travel is bone. Although bone may seem static, it is actually a dynamic, living tissue. There are three types of cells found in bone dynamics: osteoblasts, osteoclasts and osteocytes. Osteoblasts are responsible for the formation of osteocytes; which are responsible for the deposition of bone. Simultaneously, osteoclasts resorb bone. This coordinated process of bone degeneration and the formation of new bone is intensified by mechanical stress. (Caetano-Lopes et al, 2007) Therefore, perhaps the most serious hazard faced by astronauts is the effect of microgravity (very low forces of gravity) on the bones of their skeleton.

On Earth, the human skeleton is constantly working in opposition to the force of gravity. In outer space, however, the skeleton faces no mechanical load stimulus. As a result of this, the whole process that is natural on Earth does not take place; bone resorption increases while bone formation remains constant or decreases; this causes a loss of bone density. Moreover, bone is made up significantly of calcium. The calcium freed from the bone migrates through the blood and the astronauts may therefore suffer from calcium kidney stones. (Smith et al, 2012)

In addition to the lack of mechanical stimuli, there are several other causes of bone density loss. Microgravity reduces the bodily fluid pressure in the legs which may also contribute to bone density loss in the legs. (Carpenter et al, 2010) Astronauts often experience "gastroparesis", a slowing of the gastric process. The bloated feeling from gastroparesis can cause a reduction in appetite. (Harris et al, 1997) Consequently, if astronauts do not eat properly, they may suffer from a lack of nutrition which can also adversely affect bone density.

A study was conducted in the year 2000 on the astronauts of the Mir Space Station, as well as on astronauts of the International Space Station (ISS). Researchers utilized a Dual-energy x-ray absorptiometry (a DEXA scan), which uses laser beams to measure bone density. The DEXA scan results showed a one percent decrease in bone density at the lumbar vertebrae. Furthermore, there was a greater loss in bone mass density at the hip (1-1.6%). This decrease in bone density can exacerbate the danger of bone fractures. (Carpenter et al, 2010)

Research conducted on astronauts of Skylab IV who stayed in space for eighty-four days analyzed the calcium loss, which indicates bone loss, from their journey. Increasing amounts of calcium were secreted in the astronauts' urine over the duration of the first 30 days, after which the loss leveled off. Additionally, the loss of calcium excreted in defecation continuously increased throughout the journey. Overall, the astronauts lost about 200 mg of calcium a day throughout the mission. (Rambault et al, 1979)

In order to combat bone mass loss by creating artificial mechanical stress, astronauts exercise strapped down to a treadmill. The up-and-down movement of running may cause the body to build new bone. (Chang, 2014) Although exercise may prevent bone mass loss in most of the body, the bones found in the oral cavity cannot be helped by exercise. In fact, jiggling or clenching of the teeth does not cause bone strengthening, but rather bone deterioration in that area. Furthermore, as opposed to long bone loss, which can possibly be reversed upon return to Earth, tooth loss cannot be. (Stenberg, 2001)

In order to prevent excessive bone loss in the oral cavity, as well as the long bones, astronauts are instructed to consume calcium and vitamin D supplements. However, even with the supplements, studies have still shown bone resorption markers in high levels. (Stenberg, 2001)

The bone loss faced by astronauts is almost identical to that faced by sufferers of osteoporosis, a disease that affects many elderly people on Earth. Therefore, drugs commonly used to combat osteoporosis, can be useful to prevent skeletal atrophy during a spaceflight. These drugs are known as bisphosphonates and are marketed under the names of Fosamax, Boniva, Actonel and other brands. They effectively prevent bone resorption, but do not help bone fracture healing. Therefore, anabolics, such as parathyroid hormone treatment may be advisable since it can improve fracture healing. (Carpenter et al, 2010)

Aside from bone density loss, the spine itself is affected by microgravity. The human spine is composed of vertebrae separated by intervertebral discs. On Earth, the discs are compressed due to the effects of gravity. In space, there is no compression and the vertebral column expands up to two inches causing astronauts to

actually "grow" taller in space. (Kramer, 2013) The spine's expansion, however, affects the muscles and ligaments and often causes backaches. (Carpenter et al, 2010) This height boost is only temporary and reverses itself a few months after returning to Earth. Currently, astronauts on the ISS are studying the effects of microgravity on the spinal column using ultrasound scans. (Kramer, 2013)

The lack of gravity also engenders a different problem in the human body. The body is composed of approximately sixty percent water. Normally, gravity shifts majority of the water to the lower part of the body. In space, however, fluids are dispersed equally throughout the body, causing an irregularly high amount of liquid in the upper part of the body and lower than normal in the lower extremities. Therefore, astronauts' faces appear puffy and their heads actually feel bloated. Internally, the brain is also subjected to higher pressure than normal, due to excessive cerebrospinal fluid. (Chang, 2014) Within the first day in space, an astronaut may experience an upward shift of up to a liter of liquid volume from each leg. (White, 1998)

This upward shift of bodily fluids has both irritating effects and more serious consequences on the human body. The extra fluid in the head blocks the sinuses, causing what is known as "space sniffles." Astronauts often have stuffed noses throughout their stay in space. (White, 1998)

The upward fluid shift has other more serious ramifications: the body recognizes the excess fluids in the head and face, and responds by attempting to eliminate bodily fluids. Therefore, blood plasma and red blood cell mass are reduced. As a result of the hemolysis or destroyed red blood cells, astronauts often suffer from anemia. Anemia is a condition characterized as the lack of erythrocytes or red blood cells which carry oxygen via hemoglobin throughout the body. (De Santo et al, 2005)

A study was conducted on the astronauts of Gemini IV, who spent around 4 days in space and Gemini V where astronauts spent 7 days in microgravity. Data indicated a decrease of red blood cell mass of 12.2% in Gemini IV and 20% in Gemini V. Plasma volume was reduced in Gemini IV by 8.3% and 6.75% in Gemini V. (De Santo et al, 2005)

Erythropoietin is a hormone that controls erythrocyte production. Therefore, in order to combat the anemia that commonly occurs in microgravity, it would sensible to conduct studies on the usage of erythropoietin. (De Santo et al, 2005)

Another strange impact on the body after exposure to microgravity is that the astronaut's eyeballs become flattened. Scientists suppose this is yet another result of the excessive cerebrospinal fluid applying pressure to the back of the eyeballs. As a result of the flattened eyeballs, astronauts have reported difficulty seeing things up close while in space; a condition known commonly as farsightedness. (Kramer et al, 2012)

In 2009, Dr. Michael R. Barratt and Dr. Robert B. Thirsk, both astronauts and physicians, noted a difficulty seeing things up close during their six month stay in the ISS. Consequently, the doctors checked each other's eyes and noticed inflammation in their optic nerves, and scarring on their retinas. NASA is still researching the long term effects of microgravity on the eye, and if the farsightedness is a symptom of even more serious problems. (Chang, 2014)

Another study on astronaut vision problems seems to indicate that the fluid shift from the lower extremities to the upper body is not the sole cause of the vision problems experienced by many astronauts. NASA examined urine and blood samples of twenty astronauts before, during and after their stay in space. The researchers discovered that the astronauts who were experiencing vision problems also displayed lower folate levels preflight and consistently during the flight. This may explain why only certain astronauts face vision problems; the astronauts with a prior deficiency in folate may be more likely to experience a change in eye anatomy and therefore vision changes. This is a theory which requires more study in order to be proven. (Zwart et al, 2012)

It has been long noted that astronauts tend to get sick much more often during and after their spaceflight than normally. In fact, fifteen out of the twenty-nine astronauts who participated in the Apollo program became ill either during or right after their space flight. (Young, 2005) The University of California conducted a study on human immune cells, subjecting them to microgravity. The results proved that T-cells, a vital part of the human immune response, were not activated properly. Normally, when the body discovers an invader, such as a virus or bacteria, it signals ninety-nine genes to turn on which activate T-cells. However, the study found that ninety of the genes did not turn on in the absence of gravity. (Boonyaratanakornkit et al, 2005)

Another major issue faced in space, is radiation and its effects on the body. The Earth is protected from excess radiation by a natural shield known as the atmosphere. The ozone layer, which is found in the stratosphere, absorbs much of the ultraviolet radiation which emanates from the sun. There is also a magnetic field surrounding the Earth which deflects harmful rays. (Setlow, 2003)

In space, where there is no atmosphere, radiation is free to travel. In fact, if an astronaut would stay in space for a year in a low orbiting space vehicle such as the ISS, he/she would receive ten times the amount of radiation that he/she would have received on Earth. And if a solar flare would occur, the radiation risk would be enormously multiplied. An event such as this happened in August of 1972, releasing in just one day one thousand times the radiation

that we are exposed to in an entire year on Earth. (White, 1998)

The danger of radiation is that it can cause mutations in DNA, genetic material present in every cell of the body. DNA damage and mutations are often the first of many steps in the carcinogenic process. Therefore, astronauts have an increased risk for the development of tumors and cancer over their lifetimes. In addition to cancer, alterations in the genetic code can cause infertility and sterility, birth defects and stillbirths. (Durante & Cucinotta, 2008)

There are some actions that can decrease the risk of space radiation induced cancers. A diet rich in antioxidants, such as fruits and vegetables, may help prevent cancer. Vitamins A, C and E along with hormones, such as melatonin, superoxide dismutase and phytochemicals from plants such as green tea, are all effective at preventing tumors. Proper shielding on the spaceship, utilizing materials such as polyethylene, may protect astronauts from the radiation. (Durante & Cucinotta, 2008)

The cosmic rays of space can penetrate the spaceship as if there was no barrier and cause some interesting effects. When cosmic rays pass through the retina, they seem to cause the rods and cones, the photoreceptors of the eye, to be stimulated. Therefore, astronauts perceive flashes of light that aren't actually there. This occurs even when they are sleeping, at approximately three minute intervals. Furthermore, the rays can impact the electronic equipment such as laptops and cameras causing them to malfunction. (Atkinson, 2012)

Radiation found in space is different than that on Earth. Celestial radiation is composed of high energy protons and high energy nuclei; terrestrial radiation is made of Y-rays or X-rays. The different rays affect the body differently. Research based on Earth, such as studies of atomic bomb survivors, cannot be applied to astronauts exposed to space radiation. Therefore, it is imperative to conduct more studies on celestial radiation and its impact on the body. (Durante & Cucinotta, 2008)

Another danger facing astronauts due to their exposure to high levels of radiation and high energy particles is the thickening of the ocular lens, a condition known as cataracts. A study was conducted on twenty-one former astronauts and/or cosmonauts, which compared the opacification of their lenses to a control group of approximately four hundred other people of the same age group. Using a Scheimpflug camera system, the study found that the opacity of the lenses in most of the astronauts and cosmonauts was noticeably increased compared with the control. (Rastegar et al, 2002)

On Earth, muscle is strengthened when the body acts against the gravitational pull. Therefore, when an astronaut travels to space, muscle atrophy can begin within five days, and muscles continue to degenerate during the entire stay in space. The degeneration increases the risk of muscles and ligaments tearing. Along with the weakening of muscles, astronauts have noted an increase in muscle twitches and a reduction in fine motor control. (Harrison, 2002)

A study conducted at Brown University School of Medicine analyzed the effects of space travel on muscle fibers. Researchers placed cultured muscle cells aboard the Space Shuttle for several days. The researchers noted that while in space there was a decrease in protein synthesis, while protein decay levels remained normal; naturally, this would result in significant muscle atrophy. When the muscle tissues were brought back to Earth, however, protein synthesis returned to normal levels. Predictably, the lack of gravity was found to be a major cause of decrease in protein synthesis. Interestingly, the scientists discovered other contributing factors to the muscle attenuation, such as reduced levels of growth hormone, anabolic steroids and glucocorticoids. (Vandenburgh et al, 1999)

As a result of this study, scientists delineated steps to be taken in order to avoid muscle atrophy as much as possible, as a result of space travel. First, it was recommended that astronauts exercise regularly in order to build muscle. However, exercise alone was not deemed effective enough. The study showed that injecting anabolic factors or growth hormones, along with the exercise program, would provide a far stronger defense against muscle deterioration. (Vandenburgh et al, 1999)

Research has shown that the lower extremities of the body suffer especially from microgravity. After six months on the ISS, even with an exercise program of treadmill, bicycling and weight lifting, crew members suffered a thirteen percent loss of calf muscle mass. A second study conducted on the ISS crew, proved that spaceflight caused a four to seven percent decrease in thigh muscle mass. Additionally, astronauts lost up to twenty-four percent muscle strength at the knee and four to twenty-two percent at the ankle. Finally, even elbow strength decreased from eight to seventeen percent. (Carpenter et al, 2010)

Cosmonauts Berezovou and Lebedev, who spent two hundred and eleven days on Mir, were impacted so strongly that they were not able to walk off of the spaceship. In fact, the cosmonauts were not able to walk for a week following their flight and needed massive physical therapy in order to return to their muscle tone level before the flight. (Harrison, 2002)

In microgravity, the positioning of the organs in the human body actually shifts. The diaphragm is elevated, as is the liver and the spleen. The heart is correspondingly shifted upward; therefore, a space physician would have to listen for heart beat in a different area of the chest wall than on Earth. (Harris et al, 1997)

Microgravity also negatively impacts the proper functioning of the ear. The human ear is not only used for hearing, but serves an important purpose in assisting balance. The vestibular system is the primary system of the ear responsible for this. Deep in the inner ear is a pretzel-like structure known as the labyrinth. The labyrinth connects to the semicircular canals and otolithic organs, both important in achieving proper balance. The semicircular canals include three loops filled with liquid which convey to the brain when one's head is rotating or moving from side to side. The otolithic organs are comprised of two liquid filled pouches; the brain uses these organs in order to determine the position of the body, such as: standing, sitting, walking, leaning back or lying down. These organs are extremely attuned to the effects of gravity. Therefore, when a person is in a microgravity environment, the vestibular system cannot function properly. This often results in space motion sickness (SMS); symptoms of SMS include imbalance, vomiting, stomach ache, fatigue and irritability. (White, 1998)

Researchers studied the frequency and severity of SMS on astronauts of the Space Shuttle. The study on eighty-five crew member found that fifty-seven (67%) suffered SMS at various levels of severity. Twenty-six astronauts (30%) had a mild case of SMS, while twenty (24%) suffered moderately and eleven (13%) on a severe level. Fascinatingly, there was a disparity in the effect of SMS between men and women. There also seemed to be a significant decrease in SMS severity when an astronaut experienced a second spaceflight. (Davis et al, 1988) Fortunately, most astronauts acclimate to microgravity and symptoms subside after the first few days in space. (White, 1998)

Although many people may be under the impression that the space program has had little impact on their personal lives, they couldn't be farther from the truth. In fact, se College compiled a list of upward of one hundred discoveries or inventions as a result of the NASA space program that directly enhance the lives of the average individual. This list includes everything from microcomputers to new food packaging techniques. Perhaps the most import impact is pertaining to health and medicine; NASA paved the way toward the inventions of ultrasound scanning, MRI, methods of bone analyses and ocular screening techniques, to name just a few. Astronaut Ron Garan has stated: "the ISS provides a unique environment for scientific discovery that simply cannot be duplicated on Earth… it is leading to countless improvements for life on Eearth… [and] enhances our understanding of the human body." (Garan, 2014)

Conclusion

Although humans have traveled and lived in space, even for weeks at a time, outer space still remains an extraordinarily unforgiving and dangerous environment; an alien world that humans are not capable of adapting to. An unprotected human being accidentally exposed to the vacuum of space, would only be conscious for up to eleven seconds or possibly less. Consequently, the body would begin to suffer from paralysis, followed by convulsions, then followed by paralysis again. Then the water of the human tissues would change to water vapor due to the lack of pressure. This would subsequently cause the body to swell enormously, up to double its normal volume. Heart rate might rise initially, but then would rapidly fall. Arterial blood pressure would decrease and blood circulation would cease. (Parker & West, 1973) Undoubtedly, death would shortly follow.

There are some recorded cases of humans who managed to survive very short term exposure to a hard vacuum. In 1966, a technician in Houston was testing a space suit in a chamber that simulated the vacuum of space. Due to a malfunction, he was exposed to the vacuum. In just seconds, he lost consciousness. Fortunately, he was rescued from the chamber within thirty seconds, and he survived. Scientists have experimented with animals, and discovered that no creature has ever survived decompression if exposed longer than ninety seconds. (Roth, 1968)

Robots can do almost anything humans can do, but they do not require food, oxygen or sleep. Robots do not get space sniffles and do not experience SMS. Vision is not impaired by microgravity, nor can it be afflicted by cataracts. Robots cannot be afflicted by skeletal or bone atrophy. It is true that it takes a long time to transmit directions to robots located huge distances away. But even if robotic space exploration is slower than human exploration of alien worlds, it is safe. Today, many researchers even feel that the Apollo project climaxing with Apollo 11's humans on the moon was: "mainly a geopolitical stunt during the Cold War to show American technological superiority over Russia, with science piggybacking on the ride." (Mann, 2012)

The present research supports this argument. The deadly risks of human space exploration way overshadow the benefits. The countless works of fiction depicting voyaging through space, discovering new planets and establishing colonies should remain just what they have always been: works of fiction.

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Immunotherapy As a Treatment Option for Patients With Pancreatic Cancer

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Abstract

Pancreatic cancer is one of the worst forms of cancer that can develop in an individual. Traditionally, chemotherapy is administered but it has very limited success. Using the immune system to treat the cancer is very enticing and many studies have been conducted to attempt to harness the body's own mechanisms to defeat the cancer. It seems that in order to properly treat the tumor a two pronged approach must be used. First, the immune system must be stimulated to react to the tumor and attack it. A possible cytokine that can be utilized is interferon alpha, which could result in a proliferation of T cells, but also appears to cause severe side effects. This can be overcome by introducing the interferon virally. Second, immune suppression must be overcome. This can be accomplished by using antibodies to destroy regulatory (CD-25) T cells that would ordinarily prevent a T cell response. However, care must be taken to avoid inducing autoimmunity.

Introduction

Pancreatic cancer is one of the worst forms of cancer that can develop in an individual. A growing tumor in the pancreas can grow for fifteen years before it metastasizes, at which point it becomes symptomatic. It is a silent killer growing inside the body. Less than 1 % of patients diagnosed with pancreatic cancer survive even five years, with a median survival of four to six months (Warshaw, Fernandez-del Castillo, 1992). Pancreatic cancer is resistant to traditional chemotherapy treatment and therefore even once the cancer is detected, the treatment currently available to patients is mildly effective at best (Michel, Gress, 2013).

Surgery, as well has proved only mildly effective. Researchers found that less than 10% of patients survived five years after surgical removal of the tumors. This failure has been attributed to small amounts of cells that are left behind that prove to be lethal. In fact, 28% of patients have been found to have circulating tumor cells in the blood and the prevalence increased with the cancer stage (van Heerden, et. al. 1981). This would indicate that surgery alone is not effective and other treatments should be sought.

Recently, a new chemotherapy (FOLFIRINOX) was proposed which showed promising signs, giving patients a longer survival time than that associated with the traditional chemotherapies. However that advantage didn't translate into actually curing the disease, just prolonging the patients' lives by a few months. In addition, this drug showed an increased toxicity, which limited its use to only the few patients that can tolerate it (Conroy, et. al. 2011).

It is therefore obvious that new approaches must be sought towards defeating this cancer. Prolonging the lives of its victims is not an end goal. Rather, a way of ridding them of the disease altogether is required. It is therefore obvious that new approaches must be sought. Recent research has shown promising results in the field of immunology which could indicate some new treatment approaches geared towards eliminating this disease.

Adaptive immunological protection is provided to our bodies via two pathways. B cells provide extracellular or humoral immunity and create antibodies while T cells provide intracellular or cell mediated immunity. Both of these pathways are crucial in order for our bodies to be able to fight off even the "simplest" infections. If these systems are compromised, even a common cold can prove fatal as is evidenced by the HIV virus.

Innate immunity is provided by mechanical barriers such as skin and epithelial linings and cellular protection by macrophages, other phagocytes and natural killer cells. Macrophages phagocytose antigens into a phogosome and upon receiving signals provided by certain cytokines, kill the antigens that are engulfed by combining the phagosome with a lysosome. The lysosome contains toxic chemicals that can degrade the antigens. Natural killer cells respond to infected cells and destroy them before more cells become infected.

Adaptive immunity is provided primarily by B cells and T cells. B cells create a number of antigen receptors. Some of these receptors can be secreted as antibodies and others are attached to the cell surface and function as B cell antigen receptors. The first receptor that the B cell creates is the IgM receptor, which is closely followed by the IgD receptor. These receptors are very specific and can detect sequences of polysaccharides as well as peptide antigens and respond to them. The B cells can then produce different antibodies depending on the nature of the antigen in order to better combat it. The other antibodies that they can produce are IgA, IgE, and IgG. IgA is produced in response to mucosal infections and has the ability to cross mucous membranes. IgE is primarily produced in response to allergens and activates mast cells to release histamine. IgG is one of the primary antibodies used to fight infection due to its utilization of many mechanisms to assist in destruction of pathogens. It can coat antigens (opsonization) in order to assist in their phagocytosis, as well as directly neutralizing them by binding to their surface.

T cells are subdivided into helper T cells and cytotoxic T cells. These cells are distinguished by certain biochemical markers on their surface. The helper T cell has a CD-4 molecule on its surface, while the cytotoxic T cell has a CD-8 molecule on its surface. The CD-4 T cell is further subdivided into Th1 helper T cells and Th2 helper T cells. The Th1 helper T cell is supportive of a CD-8 cytotoxic response and help the cytotoxic T cells lyse infected cells. However the Th2 helper T cell is not supportive of a CD-8 cytotoxic response and promotes immune suppression. All T cells can only recognize antigen by their T cell receptors if the antigen is presented by other cells via a molecule called MHC (Major Histocompatibility Complex) which is present on the other cells. Certain cells are specialized towards presenting antigen to the T cells. This is accomplished by the cell engulfing the foreign material (phagocytosis), degrading it and presenting peptide fragments to the T cell. The cells that are specialized towards presenting antigen are known as antigen presenting cells. These can be dendritic cells, B cells and macrophages. Dendritic cells are especially proficient at antigen presentation therefore, the presence of mature dendritic cells is important in order to present the antigen to the T cells. The CD-4 helper T cells recognize antigen presented via MHC class II which is primarily found only on these specialized antigen presenting cells. On the other hand, the CD-8 cytotoxic T cells recognize antigen presented by MHC class I which is present on almost all cells of the body.

The amazing thing about the immune system is its diversity. Because of the various genes that code for the T cell receptors, the amount of combinations that the T cell can use is astronomical. Therefore, not only can the receptors be incredibly specific, but there can also be a tremendous amount of diversity. The big problem that the T cells and the B cells face is how to "know" how to react to foreign antigen and not react to self-antigen and cause autoimmunity. Autoimmunity is when the immune system targets and starts killing the body's own cells. The way that the body solves this problem is by negative selection. This consists of a rigorous test that T cells undergo in the Thymus and B cells undergo in the bone marrow during development. The cells are exposed to self-antigen. If they recognize self-antigen with high affinity they undergo apoptosis and are not permitted to mature. If however they recognize self-antigen with only low affinity, they are permitted to mature and perform their function.

In addition to regulation at the T cell and B cell development stage, there are certain helper T cells that ensure that T cells do not react to self-antigen. These are known as regulatory T cells and help shut down the T cell response at the end of an adaptive immune response and also ensure that T cells that escaped negative selection in the Thymus can be prevented from attacking healthy tissue and causing auto-immunity. There are also receptors on the T cells themselves that prevent auto-immunity. One of the primary receptors is the CTLA-4 receptor. When it is engaged, even if the T cell is recognizing antigen, the T cell is prevented from mounting a response (Abbas, et. al. 2012).

It has been thought since the 1950s that one of the functions of the immune system is to prevent the occurrence of cancer in the body. This theory is known as immune surveillance. This theory is supported by the high incidence of cancer in immune-compromised hosts such as patients with HIV and AIDS (Goedert, et. al. 1998) as well a high incidence of tumor formation among patients taking immunosuppressive medications (Sanchez, et. al. 2002). This would indicate that there is some role in the prevention of tumors by the immune system. It has therefore been assumed that there must be some antigens that cancerous cells express that are recognized by the immune system. In fact many tumor antigens have been identified. In some cases the antigens are normal proteins that are usually only present during a particular stage of the cell's life but, because of the tumor are now present during other stages as well. They can also be normal proteins that are merely over expressed in the cancer cells. Other antigens that have been identified are mutated proteins that are present in the diseased cell. However, due to the fact that even immune-competent hosts have developed cancer, it would seem that the immune system is not highly adept at fighting these tumors. Can the immune system be induced to fight against pancreatic cancer?

Discussion

Samples of pancreatic tumors from 80 surgically resected tumors were studied for signs of immune cell presence, particularly CD-4 and CD-8 T- cells. Some patients had one and not the other while some had both or none. They noticed that from the patients that had both CD-8 and CD-4 T cell infiltration, the overall survival rate was much higher than those that were missing one or both of these cells. In addition it was apparent that the depth to which the cancerous cells were able to penetrate in the pancreas was much less for those patients with high levels of both CD-4 and CD-8 T cells. It would seem that the presence of the T cells indicates that the T cells are suppressing the cancerous cells and preventing them from infiltrating further. The researchers thus concluded that a high level of T cells in the affected pancreas positively correlates to an increased survival rate (Fukunaga, et. al. 2004).

What can be used to attract the T cells? Pancreatic cancer patients treated with Interferon alpha after undergoing surgery to remove part of the pancreas were studied. Interferon alpha is a cytokine produced by the body and is used in the immune response to infected cells. When a cell is infected it produces various cytokines that cause downstream signaling. This downstream signaling refers to signals that the antigen presenting cells receive by these cytokines and increase the antigen presentation to the T cells. This is an obvious benefit because additional antigen presentation will lead to an increased T cell response. Introducing Interferon alpha in addition to the standard chemotherapy increased the length of

Kaplan-Meier survival statistics

Time	Overall survival (95% CI)	Disease-free survival (95% CI)
One year	95% (91%–98%)	67% (60%–74%)
Two years	64% (56%–72%)	52% (44%–60%)
Three years	64% (56%–72%)	52% (44%–60%)
Five years	55% (46%–65%)	52% (44%–60%)
Follow-up (months)		
Mean ± SD	31.9 ± 24.6	29.7 ± 25.9
Median (range)	21.8 (4–86)	16.0 (3.9–86)

Median survival could not be calculated as 29 of 43 patients (67%) are still alive.

CI = confidence interval.

Table 1:

Survival statistics of patients undergoing interferon alpha therapy (Picozzi, et. al. 2003).

survival for pancreatic cancer patients. 88% percent of patients undergoing the interferon therapy were still alive after two years and 55% survived to the five year mark (Tables 1 and 2). This is a significant increase as the normal 2 year rate of survival for patients being treated with standard chemotherapy alone is 40%. The theory is that as interferon is introduced, tumor antigen presentation is upregulated and therefore more T cells can infiltrate the area and fight the cancer. The study noted however that further research was still needed (Picozzi, et. al. 2003).

However this study was conducted using patients that had already had surgery. Only 20% of pancreatic cancer patients have operable tumors and therefore it is apparent that other methods must be sought out as well.

Some big drawbacks of using interferon-based chemotherapy are the side effects. A recent study of 28 pancreatic cancer patients undergoing this treatment showed an increase in toxicity resulting in a decrease in white blood cells (leukopenia) and a decrease in neutrophils (neutropenia). Although the toxicity was reversible and no patients died, an important consideration is the effect the medication has on overall quality of life. The study found that although the overall quality of life decreased, it wasn't a significantly larger decrease than those patients being treated with the standard chemotherapy. However, it is noteworthy that some of the patients being treated with this therapy needed to be hospitalized with vomiting, abdominal pain and dehydration (Katz, et. al. 2011).

In response to this systemic toxicity, scientists have proposed infecting cancer cells with adenoviruses that express Interferon alpha in order to restrict the effects of the interferon localy rather than systemically. In studies of other cancers, viruses have been shown to exhibit promising effects. The virus can be induced to specifically target cancer cells and not healthy cells by using a tumor specific promoter called cyclooxygenase 2 which is over expressed in cancerous cells. In the case of the adenovirus proposed treatment for pancreatic cancer, after the virally infected cell goes through its replication cycle in the infected host cell, it produces a "death protein" causing cell death so as to release viral particles and infect neighboring cells (Kuruppu, Tanabe, 2005). Although previously pancreatic cancer has shown resistance to adenoviruses, that has been attributed to a lack of coxsackie-adenovirus receptors on the cell surface. The newly developed adenovirus has been coaxed to bind to alternate cell surface receptors namely arginine-glycine-aspartic fiber and Ad3 receptor. When the virally infected cell dies it releases interferon and can thus be contained locally and not cause widespread toxicity and dangerous side effects. This treatment thus combats the cancer in two ways. The virus itself kills cancerous cells, and the interferon

Reported outcomes in patients with resected pancreatic head adenocarcinoma

First Author, year of publication, institution	Adjuvant therapy	No. of patients	Median overall survival (months)	Two-year actuarial survival
GITSG, 1985	EBRT + 5-FU	21	18	43%
GITSG, 1987	EBRT + 5-FU	30	21	43%
Whittington, 1991, Univ Pennsylvania	EBRT + 5FU + mitomycin	20	16	43%
Foo, 1993, Mayo Clinic	EBRT + 5-FU	29	23	48%
Spitz, 1997, M.D. Anderson	EBRT + 5-FU	19	22	44%
Yeo, 1997, Johns Hopkins	EBRT + 5-FU	99	21	44%
EORTC, 1999	EBRT + 5-FU	60	17.1	37%
Sohn, 2000, Johns Hopkins	EBRT + 5-FU (variety)	366	19	39%
Nukui, 2000, Virginia Mason	EBRT + 5-FU, CDDP, IFNα	17	*	84%
Picozzi, 2003, Virginia Mason	EBRT + 5-FU, CDDP, IFNα	43	*	64%

* The median has not been reached for IFNα group survival as 29 of 43 patients (67%) are still alive in the current group.

GITSG = Gastrointestinal Tumor Study Group; EBRT = external beam radiation therapy; 5-FU = 5-fluorouracil; CDDP = cisplatin; IFNα = interferon alpha.

Table 2:

Survival outcomes for pancreatic cancer patients undergoing various treatments (Picozzi, et. al. 2003).

that is released induces immunological resistance to the tumor. (Armstrong, et. al. 2012).

There is another problem that must be dealt with in treating any cancer using immunotherapy; the problem of immunosuppression. As the tumor grows, it changes the local environment from one of immunogenicity (favorable to destruction by the immune system) to one of immunotolerance (unfavorable to destruction by the immune system). As mentioned earlier, CD-8 and CD-4 Th1 T cells are extremely important in proper immune function. However as the disease progresses there is a larger presence of Th2 T cells, ineffective CD-8 T cells and perhaps most significantly, an increase in regulatory T cells which aid in immune suppression by expressing CTLA-4 receptors and secreting immunosuppressive cytokines like TGF-β, IL-4 and IL-5 (Ikemoto, et. al. 2006). There is also an increase in myeloid derived suppressor cells. These cells suppress both the innate and the adaptive immune response (Gabitass, et. al. 2011). In addition, rather than there being an infiltration of M1 - classically activated macrophages - which phagocytose and destroy antigens, there is an infiltration of M2 macrophages which are associated with tissue repair. They therefore remodel the matrix and in fact enhance the tumor and assist in its growth (Schmeider, et. al. 2012) (figure 1).

Therefore, to truly mount an immunological assault on the cancer, these obstacles must be overcome. A way must be found in which to cause an infiltration of Th1 helper T cells, classically activated macrophages and a decrease in the number of regulatory T cells and myeloid derived supressor cells in the area of the tumor. Recent research has been exploring possible options.

Using antibodies that specifically target the CD-25 marker on regulatory T cells, researchers depleted a splenic cell suspension containing T cells, of the regulatory T cells within. They injected

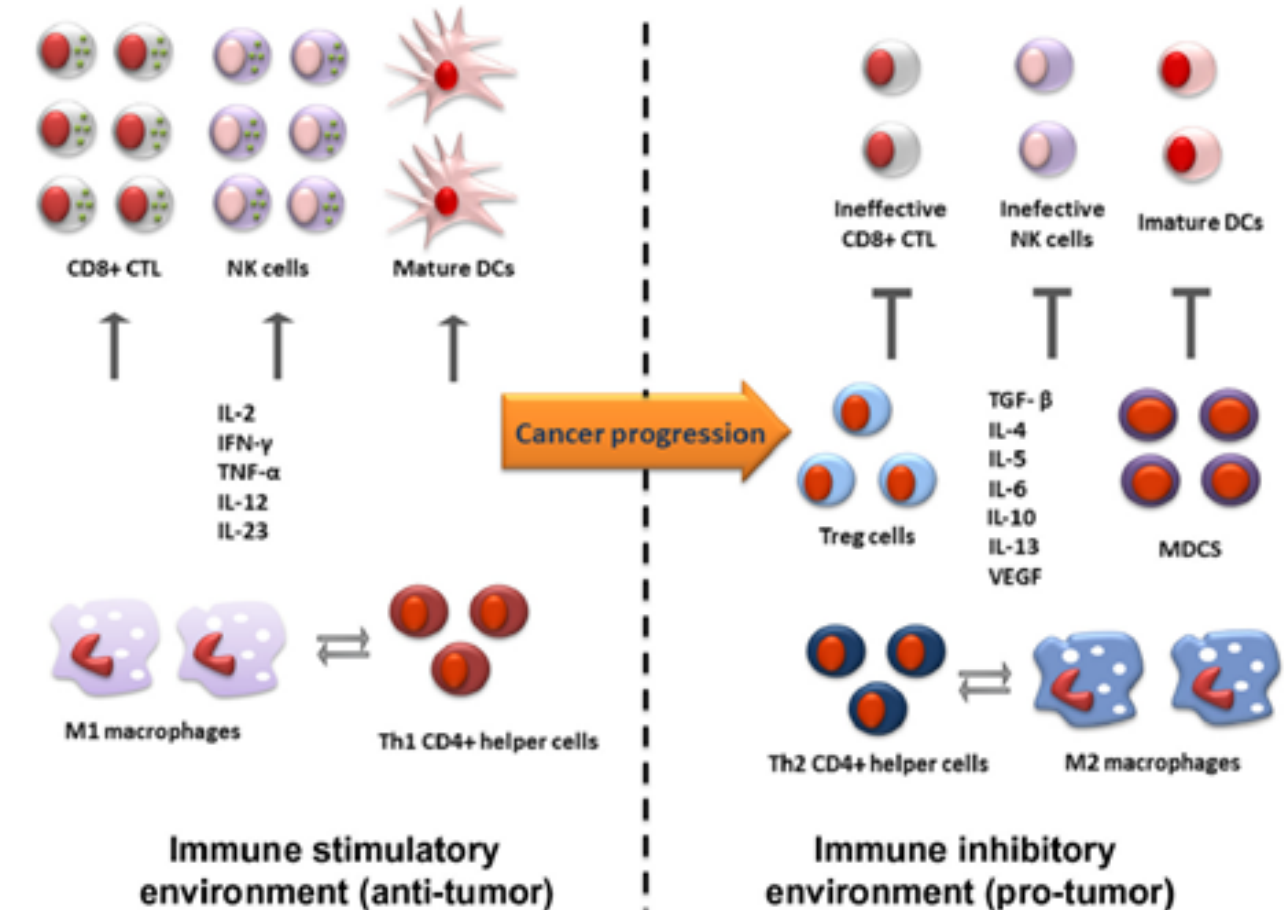

Figure 1.
Changes in the tumor microenvironment during cancer growth (Sideras, et. al. 2014)

nude mice (a strain of mice that due to a genetic mutation have no thymus and therefore have no T cells), with this sample and later with leukemia. They noticed that the tumors first grew and then regressed in most of the mice, allowing the mice to live long term (more than 80 months). Most of the mice that were injected with a non-depleted splenic cell suspension died of the tumor within 40 days. Furthermore, when the mice were reinjected with larger doses of leukemia the immune system mounted an even stronger response and rejected the leukemia much more vigorously. This indicated that the mice had become immune to the cancer. The conclusion of the researchers was that regulatory T cells prevent other T cells from mounting an immune response. They also injected ordinary mice with anti CD-25 antibody and leukemia suspensions. These tumors also grew and then regressed within 1 month in more than 90% of the mice whereas all the control mice, injected with ordinary antibodies (not specific to CD-25), died within 1 month indicating that even normal mice were able to mount a response once the regulatory T cells were depleted (Shimizu, et. al. 1999).

It seems that depletion of the CD-25 T cells allows activated, anti-tumor CD-8 and CD-4 T cells to infiltrate and kill the tumor. In a clinical trial of breast cancer patients treated with Daclizumab, a drug containing anti CD-25 antibodies along with an anti-cancer vaccine a marked decrease in CD-25 T cells was observed (Rech, Vonderheide, 2009). However at the time of this report only three patients were analyzed, and it is therefore premature to declare this treatment a success. In addition, Dr. Vonderheide, one of the researchers in this clinical trial, declared a potential conflict of interest due to his involvement in developing the included cancer vaccine administered along with the Daclizumab. More research on pancreatic cancer as well as other cancer patients still needs to be conducted before this treatment can be assumed to be effective.

However, regulatory T cells have been associated with prevention of autoimmunity. Previously, in studies done where T cells were removed, autoimmunity was generated. It was, however, unclear whether removing T cells caused autoimmunity because of a lack of regulatory T cells or because of a profound lack of any T cells which then allowed microbial infections to occur. Recently, however a study was conducted in which specifically regulatory T cells were removed from mice. Autoimmunity soon developed in various forms such as diabetes, thyroiditis, and autoimmune gastritis (Sakaguchi, et. al. 2001). Any treatment that attempts to eliminate regulatory T cells should therefore try to limit the treatment locally to the site of the tumor or cancerous cells in order to prevent autoimmunity in other parts of the body.

Conclusion

Although pancreatic cancer is one of the most difficult cancers to treat, a multi-pronged approach to treating this disease immunologically would perhaps lengthen the survival time and maybe even cure this disease. A possible approach to treating this disease could include interferon alpha alone, or virally, in combination with anti CD-25 antibodies. This would attract T cells to the site of the cancer while at the same time limiting the amount of regulatory T cells that ordinarily prevent an anti-cancer response. Care must be taken however, to ensure patients do not suffer significant loss of quality of life. In addition a balance must be struck between eliminating regulatory T cells while still avoiding autoimmunity.

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The Hormones of the Placenta

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Abstract

The human pregnancy begins with fertilization and implantation. As the embryo evolves and develops within the uterus of the mother, the placenta is formed. The placenta is a transient organ that develops to meet and accommodate specific needs during pregnancy. Its two major functions are the exchange of nutrients and gases between the mother and fetus and its role as an endocrine unit. Through the production and release of many hormones the placenta works to regulate the many necessary physiological changes in the mother in order to maintain the pregnancy, meet the needs of the developing fetus and prepare the mother's body for birth. The placenta releases both steroid and peptide hormones. Each hormone has specific target tissues and is used to signal a specific response. These responses work to facilitate a healthy pregnancy and a healthy outcome for the newborn.

Introduction

Pregnancy, specifically human gestation, begins even before the release of a mature oocyte and ends with parturition, approximately 9 months later. Pregnancy is an amazing phenomenon that integrates many complex physiological processes and ultimately results in reproduction of the human species. During this vital time the placenta is formed as a transient organ, and it continues to play a critical role in maintaining the health of both the embryo/fetus and the mother up until the time of delivery. It continuously evolves to meet the changing needs of the developing fetus within its maternal physiological environment (Linzer and Fisher, 1999).

The placenta has two major functions. The first function is the exchange of metabolic and gaseous products between maternal and fetal bloodstreams. In this way the placenta is able to provide oxygen and nutrients, as well as remove waste products for the developing fetus. The second function is hormone production (Linzer and Fisher, 1999). The hormones produced and released by the placenta assist in triggering the maternal physiological changes necessary to meet the needs of the fetus, as well as the changes needed to prepare the mother for the specific needs of the baby after birth. These changes are widespread amongst the various systems of the mother and are coordinated largely by circulating hormones. Some of these hormones are hormones that are commonly found circulating within the adult (non-pregnant) female in various concentrations. Others are hormones unique to the pregnant female only and are not otherwise found in the body. This role makes the placenta an important endocrine organ necessary for the successful continuation of a pregnancy (Sadler, 2012) (Linzer and Fisher, 1999).

Background

In order to understand how the placenta functions, it is important to first understand how it is formed, beginning with fertilization. During ovulation, one mature oocyte is usually released by the ovaries and is transported into the uterine tube by the sweeping action of tubal fimbriae. When all biological conditions are suitable, and if a sperm meets the released egg, then fertilization of the embryo generally takes place in the fallopian tubes. The fertilized egg then goes through a series of cell divisions eventually creating a tightly grouped ball of sixteen cells called a morula. On approximately the third or fourth day after fertilization the morula begins to travel down the fallopian tube, eventually reaching the uterus. A fluid filled cavity begins to appear within the morula, and it develops into a blastocyst. The inner cell mass of the blastocyst will form the embryoblast, which gives rise to tissues of the embryo proper, and the rest of the cell mass, which surrounds the inner cells and blastocyst cavity, will form the trophoblast, which later contributes to the placenta (Sadler, 2012).

By this time, the zona pellucida, which is the glycoprotein membrane that surrounded the ovum, has disappeared and implantation into the uterus begins as the blastocyst continues to evolve. This occurs on the sixth day after fertilization, as trophoblastic cells begin to penetrate between the epithelial cells of the uterine mucosa (Sadler, 2012). At this time, the mucosa of the uterus is in the secretory phase of the menstrual cycle, in response to progesterone that is released by the corpus luteum that remains following ovulation. The corpus luteum is maintained for the first few weeks of pregnancy by the release of hCG from the trophoblast and it continues to release specific hormones that affect the uterus and allow implantation to occur. Eventually, however, the corpus luteum degenerates and is replaced by the placenta, which then becomes responsible for continued hormone production (Cole, 2012).

In preparation for implantation, the glands and arteries of the uterine endometrium become coiled and three distinct layers form in the endometrium. The formation of the placenta then results from mutual interaction of the trophoblast and the endometrium. Under normal circumstances, the blastocyst implants along the anterior or posterior wall of uterine endometrium, where it becomes embedded between the openings of the glands and continues to root itself more deeply into the endometrium until it becomes completely embedded and almost entirely covers the uterine wall, on approximately the eleventh or twelfth day. (Sadler, 2012).

At the time of implantation the development of the placenta begins, as the trophoblast continues to develop by differentiating in

to two layers. These layers include the cytotrophoblast and the syncytiotrophoblast (Carlson, 2004). The cytotrophobast, which is the inner layer, consists of mononucleated cells that migrate and fuse to form a multinucleated syncytiotrophoblast, which will function as the endocrine unit of the placenta (Alsat et. al. 1997). The syncytiotrophoblast, which is a highly invasive tissue, soon surrounds the entire blastocyst and begins to insert small projections between uterine epithelial cells. It continues to spread towards the basal lamina underlying the endometrium, forming a flattened trophoblastic plate, eventually eroding maternal tissue, actually penetrating the basal lamina, and making its way into the endometrial stroma. During this time, isolated vesicles, called lacunae form in the trophoblast, and as the syncytiotrophoblast erodes maternal vessel walls, maternal blood begins to fill the lacunae. As this process continues, and as maternal blood flows through the trophoblastic system, a basis for utero-placental circulation is established (Carlson, 2004).

As the trophoblast continues to grow and develop, so does the embryoblast, as it differentiates into two layers. A thin membrane then lines the inner surface of the cytotrophoblast, forming the exocoelomic cavity or the primitive yolk sac. A new layer of cells appears between the trophoblast and this membrane, and forms a matrix of loose connective tissue known as the extraembryonic mesoderm. It lies immediately internal to the entire trophoblast, as well as around the layers of the embryoblast (including the yolk sac and amniotic cavity). Cavities soon develop and then merge to form a single large cavity that surrounds the yolk sac and amniotic cavity. This cavity is the chorionic cavity. The chorionic plate encompasses the part of the extraembryonic mesoderm that borders the fetal side of the placenta, along with its trophoblast. The connective stalk is then the only place where the chorionic plate traverses through chorionic cavity. With the development of blood vessels as well as other elements, the connective stalk becomes the umbilical cord which will form the connection between placenta and embryo (Sadler, 2012).

By the beginning of the third week, the cells of the cytotrophoblast, which continue to proliferate towards the endometrium, eventually penetrate the syncytiotrophoblast and form column-like structures that are surrounded by a syncytial layer. These are the primary villi. Mesodermal cells then penetrate the core of the primary villi to form secondary villi. Mesodermal cells then differentiate into blood cells and blood vessels, forming a capillary network that makes contact with capillaries developing in the chorionic plate, the connecting stalk, and eventually the intraembryonic circulatory system. The villous system is getting ready for the time that the heart begins to beat, at approximately the fourth week of development (Sadler, 2012).

The villi on the embryonic pole continue to grow and develop, giving rise to the chorion frondosum, which is a bushy chorion area, while the villi on abembryonic pole eventually degenerate. The portion of the endometrium that lies over the embryonic pole becomes abundant with lipids and glycogen and becomes firmly attached to the chorion. This is the decidua basalis. The remaining portion of the endometrium that lies over the abembryonic pole also degenerates. Together, the chorion and the decidua basalis make up the placenta, which becomes the only portion of the chorion where the exchange of maternal and fetal materials takes place. Maternal blood from the lacunae of the syncytiotrophoblast begins to fill the intervilli spaces of the chorion. Septa continue to form from the decidua, protruding into intervilli space, but never actually reaching the chorion. This, in turn, forms compartment-like structures, called cotyledons, that are not fully separated by the septa. It is in these compartments that high pressure arteries bring oxygen-rich blood towards the villous trees that have developed in the chorion and then back towards endometrial veins. The intervillous spaces of a mature placenta contain approximately 150 mL of blood, which is replenished about 3-4 times per minute. At all times there is a layer of syncytium that separates maternal blood from fetal tissue, as part of the placental membrane. The villi serve to increase surface area and are the primary site of placental exchange (Sadler, 2012).

As the pregnancy progresses, the placenta continues to grow, covering approximately fifteen to thirty percent of the internal surface of the uterus (Carlson, 2004). This growth serves to accommodate the ever growing needs and demands of the developing fetus and ensures sufficient transfer of nutrients. Continuous proliferation, differentiation, and fusion of cytotrophblast is what maintains and expands this syncytial interface throughout pregnancy as syncytiotrophblast cells are shed into maternal circulation when they reach the end of their life cycle (Forbes and Westwood, 2010). A clear relationship has been established, with a direct relationship between placental weight and fetal weight throughout pregnancy. This points to the ever important role that the placenta unit plays in fetal growth, through its various functions (Alsat et. al. 1997).

One major function of the placenta at this point, as mentioned, is the exchange of nutrients and wastes. Through the process of diffusion, gases, such as oxygen, carbon dioxide, and carbon monoxide are exchanged between mother and fetus. Additionally, the exchange of nutrients and electrolytes, such as amino acids, fatty acids, carbohydrates, and vitamins are also diffused through the placental barrier. Although immunity is beginning to develop, maternal antibodies are also transported from the mother to the fetus via the placenta (DeChemey et. al. 2013).

The seconds major function of the placenta is the production of hormones which serve to maintain the pregnancy, to uphold an optimal environment within the physiology of the mother, and to prepare the mother for birth. The release of hormones by the placenta is an endocrine communication system that stimulates

specific reactions on target tissue, facilitating changes necessary for the state of the pregnancy. The myometrium, which lies just beneath the endometrium is a vital tissue during the pregnancy period, and it is a critical target for fetal placental communication. The major hormone changes that occur through these placental communications are noted specifically in the increased levels of estrogen, progesterone, and chorionic gonadotropin. However, there are other hormones as well, that participate in this complex system of fetal-maternal interaction, although on a smaller scale, (DeChemey et. al. 2013).

Steroid Hormones

Over the years much research has been done in order to determine the actual role of the placenta in hormone production. One group of hormones produced by the placenta are the steroid hormones estrogen and progesterone. Various studies have been done to verify that it is actually the placenta that produces the elevated levels of these hormones during pregnancy. Studies in which pregnant women underwent surgery to remove the corpus luteum at various stages of pregnancy resulted in a substantial amount of lost pregnancies when the corpus luteum was removed before the eighth week. Removal of the corpus luteum after the eighth week did not result in abortions. These results suggest that after the eight week there are a significant amount of placental secretions (Csapo et. al. 1972). Furthermore, women with ovarian failure who participated in an ovarian assisted reproduction program demonstrated increased peripheral blood progesterone and estradiol levels by the fifth week of pregnancy. These levels were above the background levels achieved by the constant replacement regimen. With the absence of ovarian function, this study also suggests the role of the placenta in maintaining proper hormone levels and sustaining pregnancy (Scott et. al. 1991).

Estrogen

Estrogen is a steroid hormone that is produced in the syncytiotrophoblast of the placenta (Alsat et. al. 1997), with the placenta becoming the primary source of this hormone approximately during the ninth week of pregnancy. Estrogen levels remain low during the first trimester of pregnancy and then progressively increases, remaining elevated, until labor.

An important role of estrogen during pregnancy is the synthesis of contractile proteins in the myometrium, as well as local concentration of contraction associated proteins, such as oxytocin and connexin-43 (Cx-43). It also increases prostaglandin production and regulates the expression of nitrate oxide synthase isoforms. Estrogen, therefore, plays a significant role in preparing the uterus for labor contractions and delivering the baby. Although estrogen assists in the priming of the myometrium for labor, it is important to note that the estrogen alone is not clinically effective in the induction of labor. Furthermore, a pregnancy can continue to exist and labor is still possible, even amongst women with low levels of circulating estrogen (Ticconi et. al. 2006). However, in cases of low placental estrogen synthesis, changes that generally occur in the reproductive tract before parturition, such the ripening of the cervix, generally do not occur (Strauss et. al. 1996). The actions of estrogen are therefore efficient in conjunction with other factors (Ticconi et. al. 2006).

Other significant functions of estrogen are the stimulation of uterine growth, as well as development of the mammary glands, in preparation for birth. The development of the mammary gland begins initially at puberty with estrogen exposure and is completed in the third trimester of pregnancy (Sadler, 2012).

Progesterone

Progesterone is another major steroid hormone that is produced by the placenta. Until approximately ten weeks gestation, progesterone is produced by the corpus luteum that remains after ovulation. In a normal menstrual cycle where pregnancy does not occur, the corpus luteum degenerates soon after ovulation. However, when pregnancy does occur, hCG that is released from the trophoblast stimulates the maintenance of the corpus luteum, which, in turn, releases progesterone. This prevents the shedding of the endometrium lining (Cole, 2012) and allows for proper implantation (Kumar and Magon, 2012). Progesterone continues to be released by the corpus luteum until approximately week eight to ten gestation. It then continues to be produced by the placenta, specifically by the syncytiotrophoblasts of the chorion, in response to the uptake of maternal lipoproteins. The expression of low density lipoproteins appears to be directly related to the presence of progesterone (Ticconi et. al. 2006).

Progesterone acts in multiple ways to decrease the contractility of the myometrium lining of the uterus. By decreasing Cx-43 expression, stimulating nitrate oxide expression, and down-regulating calcium channels and oxytocin receptors, progesterone serves to maintain a state of dormancy during most of the pregnancy. Three progesterone receptor subtypes, PR-A, PR-B, and PR-C have been identified. Only PR-B mediates the expression of progesterone responsive genes, whereas PR-A represses this activity. During most of pregnancy, PR-A levels are low. At the time of labor, however, PR-A levels increase drastically, as do PR-A receptors, thereby suppressing the effects of placental progesterone during this crucial time and allowing uterine contractions to progress, despite maternal circulating progesterone (Ticconi et. al. 2006).

Although progesterone has a quiescent effect on the uterus, another primary function of this hormone is its effect on the cervix. Progesterone has been found to have a direct effect on the length of cervix, and in this way serves to prevent preterm labor. Studies have shown that a shortened cervix in the second trimester is often an early indicator that preterm labor will occur. Both natural and synthetic progesterone have been found to have a

strengthening and lengthening effect on the cervix, and are thereby often effective in postponing preterm labor. Local withdrawal of progesterone at the cervical level appears to have the opposite effect of softening and effacing the cervix in preparation for delivery. Although exact mechanisms are unknown at this time, progesterone has been shown to have an effect on mucus plug formation and possibly contributes to improved antimicrobial activity in this area (Campbell, 2011).

Another important function of progesterone is its contribution to mammary gland development. Although the mammary glands begin to develop at puberty, continued development and preparation for lactation is still necessary. Progesterone had been found to be associated with ductal proliferation and lobuloalveolar differentiation. This enables the mammary glands to produce and secrete milk after delivery (Lydon et. al 2000).

Peptide Hormones

Human Chorionic Gonadotrophin

Human Chorionic Gonadotrophin (hCG) is a pregnancy hormone that is critical to both the establishment and maintenance of the pregnancy, playing a role in multiple facets of the pregnancy process. HCG is a glycoprotein hormone that is similar in structure to luteinizing hormone (LH). It is synthesized and secreted primarily by the syncytiotrophoblasts, and it binds to specific hCG receptors in target tissues (Ticconi et. al. 2007). It is detectable early in pregnancy, as soon as eight to nine days after ovulation and levels continue to rise until the final weeks of pregnancy. Emerging evidence continues to demonstrate a wider range of biological effects that hCG has on various tissues types than was previously recognized.

One of the major functions of hCG during the first trimester is the prevention of luteolysis and stimulation of progesterone production in the corpus luteum (Cole, 2012). As mentioned, this ensures that the uterus, which is generally in the secretory stage of the menstrual cycle at the point of fertilization, maintains an optimal state for implantation to occur (Ticconi et. al. 2007). By sustaining the production of progesterone, hCG indirectly works to maintain the decidual cells of the endometrium, thereby preventing aptosis. At this point, hCG also functions to promote angiogenesis in the uterine vasculature, allowing for improved circulation and maximum blood supply to the area as the placenta forms. HCG has also been shown to control growth and development of the umbilical cord in this way (Cole, 2012). HCG, which is considered to be similar to thyroid stimulating hormone (TSH), mildly stimulates the thyroid gland to produce more thyroid hormone throughout pregnancy. This is especially important, as the thyroid hormone passes through the placenta and is critical for brain and nervous system development in the fetus. At approximately twelve weeks, the fetus begins to produce its own thyroid hormone (Kilby et. al. 2005).

After the first trimester, hCG continues to play a pivotal role in maintaining the pregnancy through various mechanisms. Studies have shown that hCG strongly induces the proliferation of myometrial smooth muscle cells. This contributes to the continuous growth of the uterus throughout pregnancy, providing an optimal environment for the simultaneous continuous growth and development of the fetus. HCG also assists in inhibition of uterine contractility. Just like progesterone, HCG has been found to directly decrease expression of connexin 43. It has also been found to reduce the expression of PR-A, further reducing uterine contractility in this way. HCG also decreases intracellular concentration of free calcium in the smooth muscle cells, thereby inhibiting the amplitude of response to oxcytocin-stimulated contractions. Additionally, hCG is the only pregnancy hormone that has been found to inhibit the expression of phosphodiesterase (PDE) 5 enzyme, which is involved in the hydrolysis of cAMP and cGMP that regulate myometrial relaxation. This further assists in modulating uterine contractility and in inhibition of preterm labor (Ticonni et. al. 2007). During the final weeks of pregnancy, levels of hCG tend to drop, in preparation for delivery. This drop allows for increased responsivity to prostaglandin and oxytocin, enabling contractions to occur (Ticonni et. al. 2006).

Another important function of HCG is its effect on the fetal membranes. A study conducted by Ticconi et al (2007) demonstrated increased expression of the isoform of nitric oxide synthase in response to hCG. This, in turn, may contribute to the relaxed state of the uterus as well as to immune system activity in the fetal membranes, although this has not yet been proven (Ticconi et. al. 2007). Additionally, hCG promotes anti-macrophage inhibitory factor, thereby preventing rejection of the foreign fetal tissue by the mother throughout pregnancy (Cole, 2012). Additionally, new studies have found hCG receptors in many fetal organs and tissues. This may indicate that hCG also plays a role in fetal growth during pregnancy. These receptors are then removed upon partuition. It is also interesting to note the hCG receptors are also found in the hippocampus, hypothalamus, and brain stem of the mother. This may explain the presence of nausea and vomiting that often occurs in pregnancy, as receptors detect this hormone in maternal circulation (Cole, 2012).

Another form of hCG is the sub-type hyperglycosylated hCG. Although it is an autocrine, and not a hormone, hyperglycosylated hCG promotes cytotrophobast cell growth during the early stages of pregnancy. HCG then promotes the fusion and differentiation of these cells to syncytiotrophoblast cells, leading to the formation of the villous system. This, combined with angiogenesis and umbilical cord formation, becomes the fetal maternal interface of the placenta (Cole, 2012).

Chorionic Somatomammotrophin

Chorionic somatomammotrophin (hCS), also known as placental lactogen hormone (LH) is a lactogenic protein that is also produced by the syncytiotrophoblasts of the placenta (Ayala et. al. 1989). It is considered to be similar, immunologically, to pituitary growth hormone, and it effects fetal growth in an indirect manner, according to present studies. It is secreted almost exclusively into maternal circulation, with only small amounts crossing into fetal circulation. It can first be detected in the maternal blood stream at approximately six weeks gestation, and it plays a significant role in maternal metabolism, as maternal hCS concentrations tend to rise throughout pregnancy with irregular fluctuations (Handwerger and Freemark, 1987). During approximately the last month of pregnancy, hCS levels usually level off. It has been found that depressed levels of maternal hCS correlate with intrauterine growth retardation and are associated with high risk pregnancies. It should also be noted that levels of hCS have been noted to increase in direct proportion to increased placental volume (Macmillan et. al. 1976).

HCS works in a manner that is similar to human growth hormone (hGH). It works to effect both carbohydrate and protein metabolism in the mother. Like hGH, hCS enhances insulin secretion and impairs glucose tolerance in the mother, thereby facilitating and increasing the supply of glucose and energy that is available to be delivered to the fetus. HCS also facilitates an increase in lipolysis in adipose tissue of the mother. This helps to ensure that more free fatty acids are available for energy use by the mother instead of glucose. In this way even more glucose is available for use by the fetus. Furthermore, ketones that are formed from the free fatty acids can cross the placenta and can be used by the fetus. Large amounts of hCS released from the second month of pregnancy onward appear to be related to breast, nipple, and alveolar growth, although exact mechanisms are uncertain (Handwerger and Freemar, 1987).

Placental Growth Hormone

During the early stages of pregnancy, until approximately 20 weeks, pituitary growth hormone (GH), which originates in the pituitary gland of the mother, is the primary growth hormone found in maternal circulation. Then, from approximately twelve to twenty weeks, until term, placental growth hormone gradually replaces pituitary growth hormone, which eventually becomes undetectable in maternal circulation. Placenta growth hormone is similar in structure to pituitary growth hormone, but is produced in the syncytiotrophoblasts of the placenta. Additionally, it binds to cell receptors with similar affinity to pituitary GH. PGH has a relatively short half-life and there is a rapid fall in serum concentrations within one hour after removal of the placenta following delivery (Lacroix et. al. 2002).

Placental GH is secreted in a continuous, non-pulsatile fashion into maternal circulation only and is not detected in fetal blood at all. This continuous secretion of placental GH into maternal circulation has critical implications for maternal physiological adaptation to gestation, as it impacts metabolism. Although it does not have a direct impact on fetal growth, this hormone works to stimulate gluconeogenesis, lipolysis, and anabolism in the liver and other organs of the mother. In this way it serves to increase nutrient availability for the fetal-placental unit, indirectly affecting fetal growth. This is especially important during the last few months of pregnancy when fetal growth is most prominent. Furthermore, intrauterine growth restriction has been found to be associated with decreased levels of placental GH, indicating the crucial role this hormone plays for the developing fetus. Both the rate of synthesis, as well as maternal blood levels of this hormone, have been found to be directly related to growth of the placenta (Lacroix et. 2002,) (Alsat et. al. 1997).

Placental GH demonstrates high somatogenic activity, and just like pituitary GH, it has been found to induce weight gain. It has also been found to be one of the key regulators of maternal Insulin Like Growth Factor 1 (IGF1). Additionally, placental GH concentration has been found to decrease in response to high levels of glucose. As the syncytiotrophoblast, which regulates the expression of Glut1, a major glucose transporter, comes into direct contact with maternal blood circulation, elevated glucose levels may be detected by these cells. The sycncitiontrophoblast then respond to variations in maternal blood glucose by modifying placental GH secretion. Recent data on the expression of placenta GH and its receptors in the villi of the trophoblast open new questions as to other roles that this hormone may play (Lacroix et. al. 2002).

Insulin-Like Growth Factor

The presence of various growth factors (GF) in maternal circulation is generally increased throughout gestation and specific growth factors, such as insulin-like growth factor (IGF), have been found to be linked to fetal growth. Two specific IGF types, IGF 1 and IGF 2 have been identified, and both have been found to be positively correlated to fetal birth weight (Forbes and Westwood, 2010). These peptide hormones work by binding to specific receptors on target tissues in order to mediate a variety of metabolic and mitogenic processes. Although IGF 1 and IGF 2 each have individual receptors, both typically bind to IGF 1 receptor (IGF1R) through the aid of binding proteins. This receptor is found in all cell types of the placenta, including trophoblast, villous endothelium, and mesenchymal cells and is thought to regulate the mitogenic effects of IGF 1 and IGF 2. IGF binding proteins that are generally found in abundance in the area of the placenta are also correlated to fetal growth (Hiden et. al. 2009).

Although exact mechanisms remain unclear, it appears that one way in which IGFs affects fetal growth is through its effect on the placenta. Placentally derived IGF2 have been found to have a role in promoting trophoblast invasion, thereby promoting placental growth and function. Studies have also shown that exposure of the syncytial surface to IGFs enhanced cytotrophoblast proliferation and differentiation. Because placenta growth has been closely associated with fetal growth, continuous proliferation of cytotrophblast cells in order to maintain the placenta is crucial. By stimulating placental growth, IGF works to enhance nutrient transport and delivery to the fetus, thereby promoting fetal growth (Forbes and Westwood, 2010)

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is an essential hormone for placental growth, and it has a significant impact on coordinating angiogenesis, blood flow, and the breakdown of the extracellular matrix in the placenta. It is found to be present in the blood vessels of both placenta and the umbilical cord, and it is hypothesized that it acts as a paracrine hormone on fetoplacental circulation. In this way it contributes to the growth and maintenance of the placenta as well as delivery of oxygen and nutrients to the fetus. This hormone further assists in vasodilation, as there is no sympathetic innervation in the spiral arteries and in the placenta. These functions are vital for the growth and development of the fetus, as adequate nutrient supply is crucial. Studies have noted a decreased vascular response to VEGF in cases of intrauterine growth retardation. This indicates the importance of the effects of this hormone, and further research is needed to verify the exact mechanisms of the decreased responsivity in these cases, and how this can be prevented (Krukier and Pogorelova, 2006) (Szukiewicz et. al. 2005).

Relaxin

Relaxin is a peptide hormone that is considered to be similar to insulin. It is produced and secreted during pregnancy, at first by the corpus luteum in the ovary, and then later by the decidua, endometrium, and trophoblast of the placenta. Highest levels of relaxin are detectable in the first trimester of pregnancy. Then, levels tend to drop and stabilize. It mediates its effect both on the reproductive organs, as well as on other organs in the body (Dschietzig and Stangl, 2003).

This peptide hormone originally received its name due to its relaxing and elongating effects on the interpubic ligament. It further assists in softening the cervix and the tissues of the birth canal, thereby facilitating the passage of the fetus during parturition. This was later attributed to the ability of relaxin to reduce cervical collagen concentration and increase collagen solubility during pregnancy. Estrogen was then found to further enhance these effects (Dschietzig and Stangl, 2003).

As years went on, more research has been conducted to try to uncover some other important functions of this hormone. Relaxin has been found to have regulating effects on oxytocin secretion, although data is conflicting in regard to exactly how. Other effects of relaxin include its ability to inhibit uterine contractile activity throughout gestation. However, this effect has been found to be mild in humans. Relaxin also promotes the growth and differentiation of the mammary parenchyma and stroma in preparation for lactation. It is further considered essential for the development of mammary nipples (Dschietzig and Stangl, 2003).

Conclusion

The placenta is a transient organ that is formed during pregnancy for the purpose of nourishing the developing embryo/fetus and for maintaining an optimal physiological environment within the mother in order to sustain the pregnancy. During this approximately ninth month period, the placenta undergoes continuous growth and change in order to accommodate the ever changing needs of the mother and fetus. One of the major roles of this organ is its function as an endocrine unit with its release of many hormones which have widespread effects on the maternal tissues and organs. This is what allows the pregnancy to continue and thrive, ultimately resulting in parturition and birth of the fetus.

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Eating Disorders: The Hidden Hormonal Effect On Fertility

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Abstract

Women who have a history of eating disorders, specifically anorexia nervosa, are more prone to suffer from infertility. There are several hormones which are the driving force in this system and are therefore responsible for this. Fortunately, there are treatments which can help women with a history of eating disorders to reproduce. Using information found on Pubmed and Touro College's database, this paper will discuss why the body cannot reproduce when it is lacking proper nutrition, as well as the various dynamics in the human reproductive system which are compromised when the body is not properly nourished.

Introduction: Eating Disorders

The term "eating disorders" constitutes a range of disorders including anorexia nervosa, bulimia nervosa, binge eating disorder, and other unnamed eating disorders. Overall, these disorders are characterized by a distorted body image and abnormal behaviors pertaining to food intake or weight loss that lead to decreased quality of life and extreme distress.

Up to 24 million people in the United States suffer from an eating disorder (National Association, 2014). For years it was believed that eating disorders typically affect white, wealthy, well-educated females living in major cities. However, new studies are proving otherwise (Mitchison & Hay, 2014). Eating disorders are commonly found in adolescents; 13.4% of female teenagers display signs of a disorder, while 7.1% of males exhibit those signs (Treasure et al., 2010). Overall, eating disorders are slightly more predominant in individuals of lower socioeconomic status, and bulimia is more common among minorities. Education, marital status, and urbanicity, studies find, do not play a significant role in eating disorders. It is also evident that individuals who participate in specific sports, such as aesthetic (e.g. dancing and gymnastics) and leanness- or weight-related (e.g. wrestling), have a higher prevalence of developing an eating disorder. Modeling is associated with an increased incidence of anorexia and subclinical anorexia. Individuals who live with a great amount of stress report higher incidence of eating disorders. While not thoroughly studied, evidence is emerging that even minor stresses such as moving to a new house or a change in family dynamic may lead to the development of an eating disorder. With this new research, scientists have come to a general understanding about the demographics of eating disorders; however, they still admit that there are some limitations to the studies that have been performed on epidemiology, and they may never know the true prevalence of eating disorders (Mitchison & Hay, 2014).

Much of the information about individuals with eating disorders comes from those who are treated. However, many patients are embarrassed about their condition and do not seek treatment. For example, males with eating disorders tend to be ashamed, as it is more accepted for a female to be diagnosed with an eating disorder, and they therefore do not seek help (National Association, 2014). In addition, teenagers living at home may be brought to a doctor by their parents, whereas adults or the elderly who live alone have no one looking after them and forcing them to get help. Therefore, much of the research fails to include those who do not seek treatment, which may skew statistics (Mitchison & Hay, 2014).

There are various factors that cause an individual to develop an eating disorder. Some theories suggest that the root of eating disorders is possibly biological, genetic, and psychological in nature. Treasure et al. in their article explore the biological causes and believe that the structure of the appetite system and the way it functions can cause eating disorders. They believe that the three components of the appetite system can aid in the understanding of eating disorders.

The first component of the appetite system involves a homeostatic system that is primarily situated in the hypothalamus and brainstem. This system utilizes signals from the gastrointestinal tract and specific metabolic markers to affect hunger and satiety. The second component is the drive system that registers the reward value associated with food, such as the pleasure or energy obtained from eating, and also helps motivate a person to want to eat and therefore look for suitable food. This drive system is comprised of striatum with afferent inputs from sense organs, neural structures that are involved in memory and learning, and neural circuitry that is distributed throughout the mesolimbic cortex. The third component is a self-regulation system which controls appetite and lets a person how much he needs to eat. When there are abnormalities in any of these three components, an individual is at risk of developing an eating disorder or of maintaining and exacerbating a pre-existing disorder (Treasure et al., 2010).

Despite the fact that eating disorders are often referred to as a whole, each type is characterized by distinguishing features that differentiate one from another. Since eating disorders are largely classified as psychological in nature, they are included in the

Diagnostic and Statistical Manual of Mental Disorders (DSM), the standard classification of mental disorders used by mental health professionals in the United States (DSM, 2014).

Anorexia Nervosa

The most recent update of the DSM, DSM-5, has published changes to the previously-used classification system of eating disorders. According to DSM-5, a patient must exhibit the following symptoms to be appropriately diagnosed with anorexia:

1. Patient has low body weight relative to age, gender, physical health, and developmental trajectory; this is due to limited energy intake compared to what is actually required.

2. The patient is underweight; however, he/she has constant unrealistic worry about weight gain or becoming fat.

3. Patient denies his/her low body weight as problematic; patient is overly concerned with the way his/her body looks to the point it affects the way he/her thinks about him/herself – he/she has a false perception about the way his/her body looks (The alliance for eating disorders awareness, 2014).

Anorexia has a higher mortality rate than any other psychological disease (National Association, 2014) and is associated with a host of devastating metabolic changes to the body. Patients may experience dangerous arrhythmias due to low potassium levels, seizures due to sodium or fluid depletion from constant vomiting or diarrhea, thyroid problems, increased infections due to decreased white blood cells, dehydration, tooth decay, and reawakening of the bones. An obvious result of anorexia is malnutrition (Anorexia nervosa, 2014).

Approximately 75% of anorexia patients are female making 25% of the cases belong to the male gender, however there is not much known about the anorexic male population. (Wooldridge & Lytle, 2012).

Eating disorders negatively affect the skeletal system; growth retardation, bone loss, and osteoporosis are common. Weight gain will improve the bone density and reduce these effects. In addition, many patients with anorexia suffer from concomitant psychological ailments such as depression and anxiety due to hormonal imbalance (Misra & Klibanski, 2014).

The brain is the most important organ affected by eating disorders. The brain uses approximately 20% of the calorie intake and is also very dependent on the glucose from food. Therefore, when the body has poor nutrition, the brain is not receiving the correct amount of glucose and other nutrients. Most cases of eating disorders occur during adolescence, and this can be a major problem because this is the time of optimum growth and development. When a person starves his brain, his brain is actually shrinking from lack of nutrition. This can lead to several behavioral and psychosocial disturbances. If a person would regain his lost weight and restore the brain mass to its normal level, his condition would most likely improve (Treasure et al., 2010).

Another important body system which is affected by eating disorders is the reproductive system. Since reproduction and nutrition are connected, severe weight loss will inhibit the cycle of reproduction from functioning properly. The records of a number of infertility clinics were reviewed to determine the various sources of these women's infertility. Almost 17% of the patients in the clinics were suffering from eating disorders in general, including 7.6% suffering from anorexia and bulimia (Stewart et al., 1990). In a London-based study, 11,088 pregnant women were questioned. Of those women, 171 (1.5%) said they had a history of anorexia, and 199 (1.8%) had suffered from bulimia at some point. Eighty-two women (0.7%) suffered from both. These women were compared with the remaining 10,636 women (96%). The following statistics were recorded: 39.5% of those who suffered from an eating disorder took over 6 months more to conceive than did the 25% of the general population. Of those women with a history of anorexia or bulimia who did become pregnant, 6.2% said they had undergone treatment to help them get pregnant, while in the general population the number was only 2.7% (Easter et al., 2011).

A hindrance in the reproductive system due to a lack of nutrition can be seen from Holocaust survivors who lived to tell the tale of those terrible times. One lady, who was hiding in Siberia, hardly ate anything for the few years she was there because of the lack of food. This caused her menstrual cycle to stop for those years that she was not receiving proper nutrition. Another lady was in the Auschwitz concentration camp, where she was literally starving; the inmates were given potato peels and moldy bread to eat. This caused her to also experience a cessation of her monthly periods. Both of their menstrual cycles resumed within a few years after the war, and with that, each of them was able to begin a family. This paper will go on to explain in detail why this is so. What is the connection between these two aspects? What are the underlying factors which cause a lack of nutrition to lead to an inability to become pregnant? While infertility caused by eating disorders was once thought to be untreatable, new research is identifying various hormones, such as GnRH, Kisspeptin, and Leptin, which boost LH and FSH to make it possible for women with anorexia to conceive. How do these three hormones regulate the reproductive system?

Methods

The question above will be answered based on the information compiled from various articles and reviews. These references were obtained through Pubmed and Touro College's database which connected further to a variety of medical journals and

publications. The information was narrowed down to those directly relevant to the topic at hand. Once all the information was in place, an attempt could be made to answer the question regarding these specific hormones and their involvement in the reproductive system when adequate nutrition is and is not present.

Discussion

The two hormones, FSH and LH?

Follicle-stimulating hormone, better known as FSH, is a hormone which controls and maintains development, maturation, and all reproductive processes in the human body. In males, a low FSH level can result in a cessation of normal sperm development, while in females it can halt the reproductive cycle. The Luteinizing hormone, or LH, is a hormone that triggers ovulation. When there is an LH surge in the female body, this indicates that ovulation will occur within the next 24-48 hours. (When a woman is pregnant, there is a decreased level of LH since a similar hormone, HCG, takes over. HCG is the hormone which helps preserve the uterine lining in pregnancy and will further produce progesterone.) A deficiency in LH will cause the same effects as the FSH deficiency does in both males and females (Barker et al., 2012).

The menstrual cycle

The menstrual cycle is on average a 28-day cycle of various hormones and activities circulating the female reproductive system. The goal of the menstrual cycle is to produce eggs and to prepare the uterus for pregnancy. In cases where pregnancy is not achieved, the uterus will shed the eggs. The endocrine system is in charge of the cycle because of the various hormonal changes. At the beginning of the cycle, there is an increased level of estrogen and the lining of the uterus thickens, forming what is called the corpus luteum. Various hormones assist with the development of follicles in the ovary. Eventually, only one will become dominant while the others die. At mid-cycle, there is a surge in LH; this occurs when the egg has matured and the estradiol, or estrogen, begins to stimulate the production of LH from the anterior pituitary gland, initiating a surge of LH. This surge causes the dominant follicle to release an egg, an event known as ovulation. Unless it is fertilized to become an embryo, this egg will survive only up to 24 hours. FSH and LH aid the formation of the uterine lining from the remains of the dominant follicle, and this produces progesterone. This increased amount of progesterone leads to a rise in estrogen levels. If pregnancy is achieved, the embryo will implant itself within the uterus for the duration of the pregnancy. If, however, the egg is not fertilized, then approximately two weeks later, the corpus luteum will begin to disintegrate. The uterine lining will atrophy, causing the progesterone and estrogen hormones directly influencing the FSH and LH levels to diminish. As a result, there will be a large drop in the estrogen and progesterone levels, which will then cause the uterus to shed its corpus luteum and egg, bringing about another menstrual cycle (Chrousos, 2009).

GnRH, Gonadotropin-releasing hormone

"In mammals, a sparsely populated and widely dispersed network of hypothalamic neurons, the Gonadotropin-releasing hormone (GnRH) neurons, serve as the pilot light of reproduction via coordinated secretion of GnRH" (Balasubramanian et al., 2010).

A network of approximately 1500 GnRH neurons is found in the hypothalamus. These neurons originate elsewhere in the body and migrate to the brain during embryological development. Scientists tested rodents to investigate the origin of these GnRH neurons and found that they originate in the nose, outside of the CNS, and migrate into the brain, where they scatter throughout the hypothalamus. It is obvious from the neurons' specific path from the olfactory placode to the preoptic area of the hypothalamus that they play a large part in a number of body systems which ultimately transfer data, including body weight and nutritional status, over to the reproductive system. GnRH is initially secreted from early fetal life until a few months of infancy. It is then quiet until the child reaches puberty, when it is once again secreted, resulting in sexual maturation. Since this neural network spurs the reproductive system in mammals, the GnRH neurons will determine, upon receipt of information, whether it is the right time and place and under the right body conditions for the reproductive system to prompt fertility. Once in the hypothalamus, the neurons bundle together to pass chemical signals to each other, causing the release of GnRH. In order for GnRH to be emitted, the neurons must extend their axon projections into the median eminence of the hypothalamus, the part of the hypothalamus that connects it to the pituitary glands (Balasubramanian et al., 2010). GnRH is then secreted in a coordinated, pulsatile manner into the pituitary gland, where it activates its own receptor, GnRHR, to release the gonadotrophin hormones, Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH), from the gonadotrope cells located in these glands (Counis et al., 2009). This unique pulsatile pattern in which the GnRH is released is directly responsible for the degree of LH and FSH produced; low-frequency GnRH pulses lead to FSH release, while high-frequency pulses stimulate LH release (Jayes et al., 1997). In males, the GnRH pulses are constant, but in females, the pulses vary throughout the monthly menstrual cycle, and a large surge of GnRH occurs just before ovulation. Thus, LH is released prior to ovulation due to the large surge (Chrousos, 2009). The fact that the gonadotropes react to the fluctuations in the GnRH pulses demonstrates that these pulses are critical for the reproductive system to function properly. This further proves a strong correlation between a healthy neuroendocrine system and a properly-functioning reproductive system (Counis et al., 2009). Therefore, GnRH is a neurohormone that mediates brain control of the reproductive system; all of human reproductive activity is generated by GnRH and the network of neurons in the hypothalamus.

Kisspeptin

There is a neuropeptide in the hypothalamus known as Kisspeptin, whose role is to regulate the GnRH neurons. In the last decade, studies have been done to prove the role that Kisspeptin plays in the reproductive system. This further enhanced scientists' understanding of neuroendocrine regulation of reproduction (De Roux et al., 2003). Kisspeptin was originally discovered in 1996 in Hershey, Pennsylvania and was amusingly named after the famous Hershey "Kisses" that were produced in this town (Skorupskaite et al., 2014).

"Kisspeptin is now recognized as a crucial regulator of the onset of puberty, the regulation of the sex hormone-mediated secretion of gonadotropins, and the control of fertility" (Pinilla et al., 2012, in Skorupskaite et al., 2014 article).

It does so by stimulating the hypothalamus to secrete GnRH at the appropriate time, thereby maintaining a homeostatic environment in the body. In order for the reproductive system to function properly, there must be a balance of energy, and the Kisspeptin neurons ensure this. They signal to the GnRH neurons to release GnRH, which stimulates the secretion of LH and FSH from the gonadotropes (Clark & Cummins, 1985). The secretion of LH is much greater than that of FSH (Dhillo et al., 2005). In this way, Kisspeptin plays a key role in fertility; it activates the GnRH neurons and locates itself near the GnRH neuron which stimulates LH release. Because Kisspeptin plays a large role in reproduction, it is evident that it can help individuals who are in a state of negative energy. During a study, when Kisspeptin was administered to a group of starving rats, their LH and FSH levels rose (De Bond & Smith, 2013). Kisspeptin accomplishes this by sensing the energy storage and initiating the secretion of GnRH, providing a connection between nutrition and reproductive function. This demonstrates the possibility of using Kisspeptin to restore reproductive activity in individuals who are in a negative state of energy, as in cases of anorexia nervosa (Skorupskaite et al., 2014).

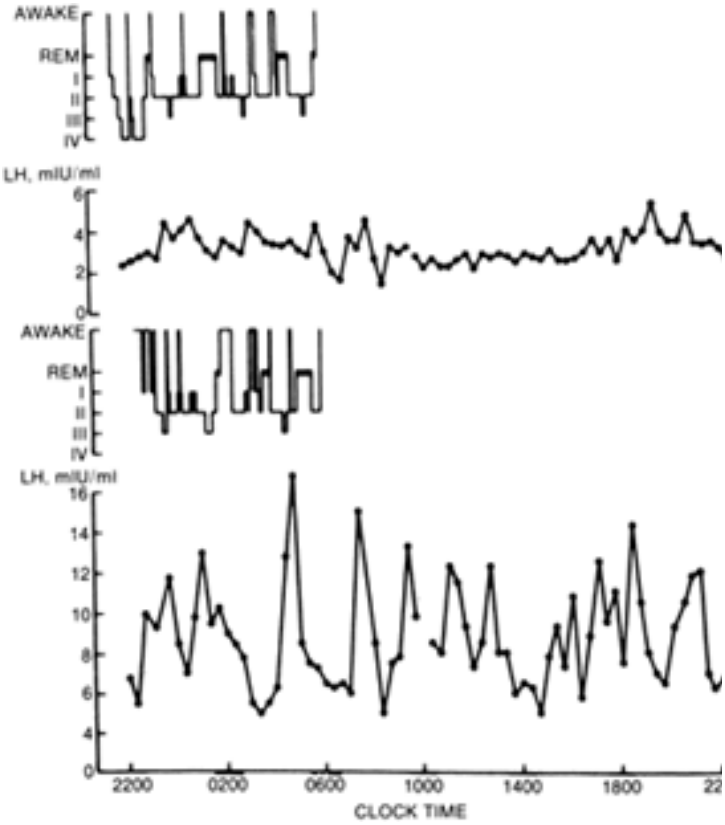
Most GnRH neurons contain a receptor for the Kisspeptin neurons (Kiss1r). However, there are many other Kisspeptin receptors located in different areas of the brain. This suggests that Kisspeptin does not only play a role in controlling the energy balance in reproduction, but has other functions, as well. (De Bond & Smith, 2013).

Leptin

Leptin is a hormone derived from the adipose tissue. It comes from the Greek word "Leptos" meaning thin (Meier & Gressner, 2004). It is a recently-discovered hormone from the OB gene (Grasemann et al., 2004). It regulates energy balance along with hunger and metabolism, and it tells the brain when the body has enough energy and when it needs more food to provide energy. Due to its role in regulating food intake, Leptin is sometimes referred to as the satiety hormone. Each person has a set Leptin threshold that is specific to him, which is believed to be genetically determined. When the Leptin level is above the threshold, such as after a person eats a sufficient amount of food, the brain receives a message that it has enough energy to expend on daily activities, such as exercising and eating, and on expansive metabolic activities, such as pregnancy and puberty. However, the Leptin level is low due to a food deprivation, the brain senses this and goes into starvation mode, and basic metabolic processes are halted due to a lack of energy. The pituitary gonadal axes become suppressed and other critical neuroendocrine axes malfunction. It is believed that Leptin affects the neuroendocrine system because it suppresses neuropeptide Y (NPY) production and prevents its secretion from neurons in the arcuate nucleus. NPY strongly stimulates appetite and regulates many of the hormones that are secreted by the pituitary gland, such as stimulating the pituitary adrenal axes, suppressing growth hormone by stimulating somatostatin, and suppressing gonadotropins (Meier & Gressner, 2004). A decrease in Leptin levels demonstrates a lack of energy; this will lead to a non-operative reproductive system, since it requires a lot of energy to function properly. Women with anorexia generally have low amplitude secretion levels of Luteinizing hormone, similar to those in young women who have not yet reached puberty or just begun it. This may be due to the decreased amount of fat mass and the alterations of the hormones produced by the adipocytes, such as Leptin. Leptin is an important factor in the timing of puberty, and as such, it facilitates the secretion of normal gonadotrophic hormones. Therefore, when an anorexia patient has reduced levels of Leptin due to the lack of necessary energy, her menses can be affected (Misra & Klibanski, 2014). A study done on rodents proved that in a state of starvation they displayed a reduction in LH pulse frequency, and when Leptin was administered, these rodents experienced an LH surge prior to ovulation as well as restored menstrual cycles, which led them to be fertile once again. The lack of energy present in patients with anorexia nervosa resulted in low Leptin and gonadotropin levels. These levels were restored with the aid of administering Leptin and adding nutrition to the patients' diets. There is a hypothesis that Leptin initiates puberty, since studies indicate that a critical amount of body fat is required in order for a person to begin maturation. This suggests a positive correlation between the energy stored up in a human's body and the onset of puberty (Elias & Purohit, 2012).

Leptin concentration is based on BMI. Therefore, in patients with anorexia and bulimia, Leptin levels are low, leading to a starvation state in which menstrual cycles are disrupted and fertility is severely affected (Meier & Gressner, 2004). However, these studies indicate that Leptin is not the only component necessary in puberty. A study in Jackson Laboratories observed Leptin-deficient mice. It was discovered that even though these mices' gonads and gonadotropes were fully developed and ready for puberty, they were GnRH deficient. Based on this piece of information,

Figure 1:
LH pattern in an anorexia case vs LH pattern in a normally-functioning system (Boyar et al., 1974).



LH concentration in a patient with anorexia nervosa (upper) is drastically lower than it is in a normal pattern when the patient is in remission (lower) (Boyar et al., 1974).

scientists theorize that Leptin affects the hypothalamic part of the brain by stimulating GnRH secretion (Elias & Purohit, 2012).

"It is now well-accepted that Leptin is a key metabolic cue that signals energy sufficiency to control adequacy and timing of reproductive function" (Elias & Purohit, 2012).

Reproductive issues that anorexia can cause

In the DSM IV, amenorrhea is listed as a criterion for the diagnosis of anorexia nervosa; however, this obviously does not include the male population who make up 10% of the anorexic patients, which is the reason that amenorrhea is not included as a criterion in the DSM V. (Misra & Klibanski, 2014).

Hypothalamic oligoamennhorhea, a symptom of anorexia nervosa in which a woman's menstrual cycle is infrequent due to the decreased available energy, can lead to infertility. However, fertility can be restored once the body weight is stabilized and a normal period cycle resumes (Misra & Klibanski, 2014).

Hypothalamic Amenorrhoea: Amenorrhea is defined as the cessation of a regular monthly period for more than six months. 68-89% of women with anorexia reported an absence of their monthly period for at least 3 months (Hoffman et al., 2011). This signifies that there is a lack of normal ovarian activity, probably due to a disturbance in the proper secretion of hormones. This condition occurs when the GnRH pulse is low because the hypothalamus is not functioning as it should be and cannot produce GnRH, thereby causing a decline in LH and FSH secretion and follicular activity. This demonstrates the importance of a normally-operating hypothalamus, as all ovarian activity and hence the menstrual cycle is completely dependent on its smooth functioning. Some functional defects which can interfere with the normal operating system can include eating disorders, stress, and exercise, since they can all potentially decrease GnRH secretion, which will lead to reduction of the other hormones (Baird, 1997).

Kallman's Syndrome: a genetic condition which affects three to five times more males than females and is associated with the inability to conceive. This hypogonadotropic hypogonadism disorder occurs because there is a low amount of sex hormones (testosterone in males, and estrogen and progesterone in females) circulating in the body. The low levels of these hormones are due to a low level of FSH and LH which is caused by the hypothalamus not releasing GnRH properly. This could be a result of a defect in any part of the GnRH neurons' migration from the olfactory placode to the hypothalamus, or it may be a failure of the pituitary gland to secrete GnRH. Without GnRH secretion, LH and FSH will not be secreted and therefore they will not "turn on" the ovaries or the testes, and eggs and sperm will not be produced (Mitchell et al., 2011). This concert of events is illustrated in Figures 2 and 3.

Although this paper mainly discusses the effects of malnutrition and fertility in females, the instabilities in the reproductive system in males with anorexia have also shown to affect male fertility. This is an effect of the low leptin, gonadotropin (LH and FSH), and testosterone secretion levels. A study done to demonstrate the levels of fertility in anorexic males and females shows that from 140 women with anorexia, 50 women had a total of 86 children, and none of 11 anorexic men had children. As shown, anorexia can affect male fertility, but the extent is not documented thoroughly (Misra & Klibanski, 2014).

Treatments

An increase in fat mass has proven to be a key role in restoring the menstrual cycle. Figure 4 shows a study done on adolescents suffering from anorexia nervosa which demonstrates that the menses returned to girls with body fat greater than 24%, while in those with less than 18% body fat, it did not (Misra & Klibanski, 2014).

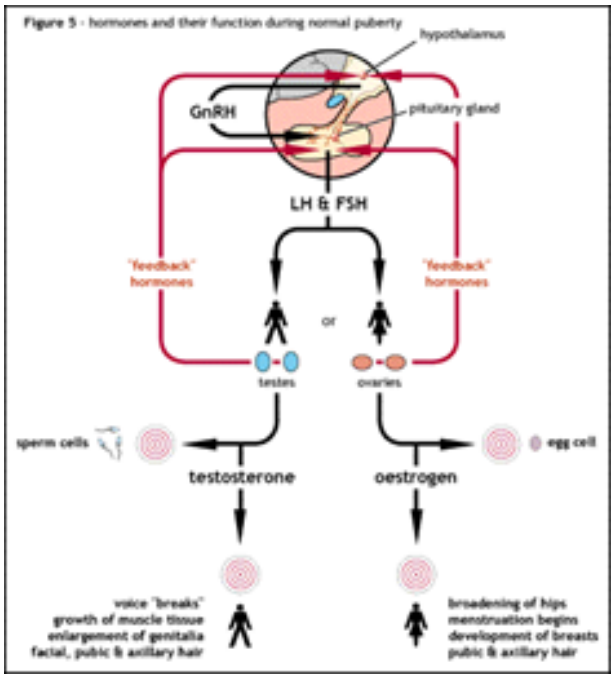


Figure 2:
Hormones and their function during normal puberty; the release of GnRH, LH, and FSH from the hypothalamus and pituitary glands and their effect on the ovaries and testes (Smith, 1995).

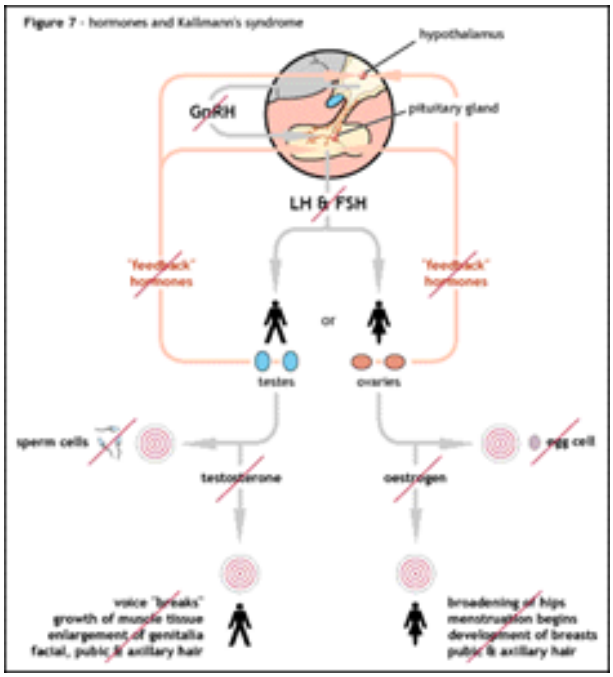
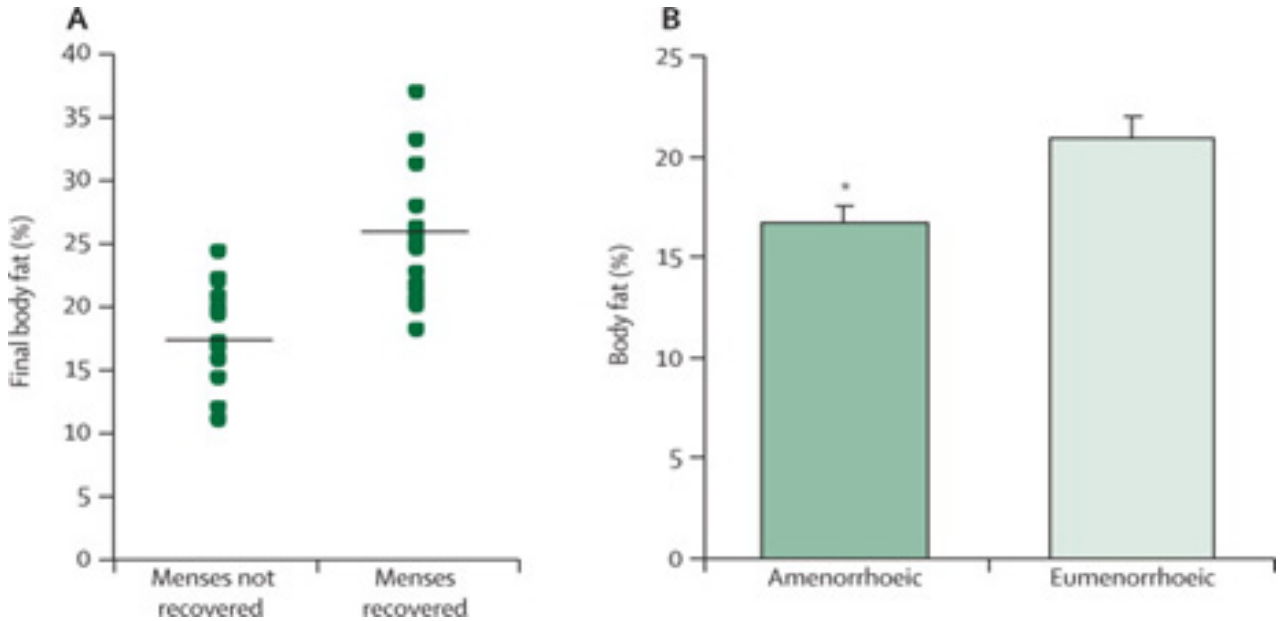


Figure 3:
Hormones and the Kallmann's syndrome; the failure of the hyopothalamus to release GnRH and this will eventually result in a non- functioning testes and ovaries (Smith, 1995).

Figure 4:
Proportion of body fat and relation to menstrual function (Misra, MD & Klibanski, MD, 2014)



Menstrual cycles resume in individuals with an increase in body fat. For individuals with a low body fat (<18%) menses did not resume to its normal state (Misra, MD & Klibanski, MD, 2014).

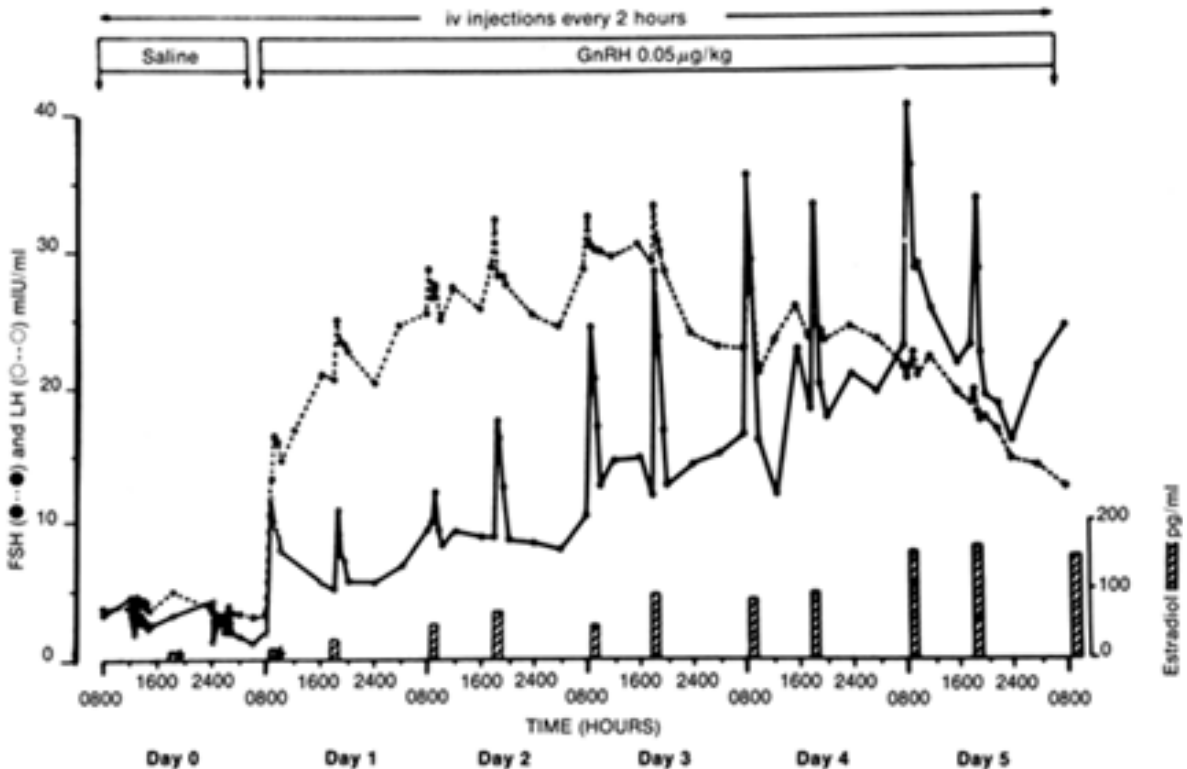
GnRH pumps

The Southern Ontario Fertility Technologies (S.O.F.T.) was faced with several cases of hypothalamic amenorrhea, and since treatments with gonadotropins are expensive, they sought a more affordable option. In 2011, they purchased two pumps to help alleviate the agony of these childless couples. Because hypothalamic amenorrhea is caused by a lack of GnRH, reinstating GnRH seemed like the most practical solution. The only catch is that GnRH has to be released in pulses because a constant flow will desensitize the hypothalamus and pituitary gland. Pulses of GnRH will cause a release of LH and FSH which will then target the ovaries and testes and achieve the desired results of producing eggs and sperm. The pulses are emitted through a device called a GnRH pump, which is a small battery-operated machine. There is a programmable timer attached in order to emit the GnRH in pulses every 90-120 minutes. The pump is worn around the waist or thigh the entire time that the drug is being administered, usually starting from day three of the menstrual cycle until the time the woman ovulates. This form of treatment has been proven to be very successful, with a success rate of 90% in people with Kallmann's syndrome. These fortunate women became pregnant within six months of beginning this treatment. For women with hypothalamic amenorrhea, their success rate of achieving pregnancy per cycle with this treatment is 25% (Martin J., 2011). In women suffering from hypothalamic amenorrhea, this therapy resembles the beginning of puberty and these women will begin to experience normal menstrual cycles with the follicular maturation, ovulation, and corpus luteum formation. Once this is achieved, these women have a pregnancy rate comparable to those with healthy and normal cycles. The side effects of this pulse therapy are mild. Multiple pregnancies are a common occurrence as a result of the GnRH pulse level being too high for the required amount of that specific ovulation (Martin J., M.D., 2011). The GnRH pulse generator accomplishes its goal because it imitates the dynamics of a normal menstrual cycle; it releases GnRH at various frequencies throughout the period over which it is performed (Santoro et al., 1986).

Hypothalamic amenorrhoea can be corrected by injecting Kisspeptin-54 twice a day for two weeks at a dosage of 6.4 nmol/kg. This will result in a tenfold increase of LH secretion and a 2.5-fold increase in FSH secretion. However, ovarian activity is not necessarily restored (Jayasena et al., 2009). Kisspeptin has proven to be a very useful therapy in restoring a normal LH level from a relatively high or low LH pulse (Skorupskaite et al., 2014).

When Leptin was administered to females suffering from hypothalamic amenorrhea due to weight loss and excessive exercise, it was found that Leptin increased the LH volume and created a more favorable environment for reproduction (Elias & Purohit, 2012).

Figure 5: LH and FSH response to injections of GnRH every two hours (Marshall & Kelch, 1979).



The FSH (dotted line) and LH (solid line) patterns increase significantly when administered with injections of GnRH; these pulses are much greater with GnRH than they are with a saline (Marshall & Kelch, 1979).

There is more pertinent information available on the connection of infertility with specifically anorexia nervosa than with any other eating disorder. Whether bulimia nervosa has an effect on fertility is unknown, even though patients tend to display various menstrual irregularities. A study done on 173 women with bulimia provides information on this area. At first, 38.2% of these women were experiencing regular menstruation, while 4.6% had amenorrhea. 10-15 years later, the rate of amenorrhea was much higher at 13.9%. However, when the study was first conducted, 34.7% of the women had been pregnant at least once, while 10-15 years later this number rose to 74.6%, with only 1.7% of the women experiencing infertility. This demonstrates that while bulimia may be associated with menstrual dysfunction, it does not seem to have much of an effect on ability to conceive (Crow et al., 2002). For those who have struggled with infertility, the cause is most probably menstrual irregularities, because infertility from anorexia is a result of a dysfunctional menstrual cycle. The same treatments which help alleviate the anxiety caused by anorexia would likely be appropriate for these individuals who are suffering from infertility due to bulimia.

Conclusion

This paper describes the importance of a proper nutrition to encourage a healthy reproductive system. Whenever the body is lacking necessary energy, as it is in cases of anorexia nervosa, there are alterations in the endocrine axis to help divert the energy that is present to perform the key body functions instead (Misra & Klibanski, 2014). There are various hormones present in every female body which will help boost LH, the chief hormone in a healthy menstrual cycle, in order to enable a malnourished body to conceive. GnRH, Kisspeptin, and Leptin are such hormones which will work together to help achieve a healthy pregnancy in individuals suffering from eating disorders.

If people knew that there are treatments for fertility affected by eating disorders, they would not be so embarrassed and ashamed to seek help. Since the success rate of the treatments has been proven to be quite high, people should be made aware of the various treatment options. Unfortunately, in today's society, the prevalence of eating disorders is very great, and there is no real way to stop this trend. Therefore, it is important that people become aware of the ways they can improve their situation to ensure themselves a healthier future. Once people are diagnosed with an eating disorder, their physician should administer Kisspeptin, GnRH, and Leptin to prevent the damage that eating disorders can cause. Based on research which highlights the importance of these hormones on the reproductive system, the additional dosage of these can only be beneficial for them.

Abbreviations

DSM	Diagnostic and Statistical Manual of Mental Disorders
CNS	Central Nervous System
GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
FSH	Follicle- stimulating hormone
BMI	Body Mass Index
HCG	Human chorionic gonadotropin

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The Efficacy and Safety of the Human Papillomavirus Vaccine

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Abstract

The HPV virus is said to be the cause of many infections, warts, and cancers. In addition to the fact that the treatment for HPV is not always successful, not every individual knows that s/he is infected and is very likely to infect his or her partner, too. This is one factor that explains why 14 million people each year in America alone contract HPV and makes cervical cancer so threatening to many people worldwide. In light of this and the known dangers of cervical, anal, vaginal and penile cancers, the HPV vaccine was created to prevent an infection of HPV from developing into a cancer. Within the past decade, two vaccines, Gardasil, manufactured by Merck, and Cervarix, manufactured by GSK, have been produced to help prevent 70 percent of the Human Papillomavirus strains in order to reduce the chances of contracting the various cancers and infections that are connected to HPV. (However, since the idea of cancer vaccines is so new to the health world, questions and wariness remain: Are Gardasil and Cervarix effective? Are they safe and not counterproductive? Should everybody in the age bracket of 10 until 25 be vaccinated? If so, because it is unusual for there to be two vaccines targeting the same goal, which company's make is preferred?) Many studies have been conducted on the new HPV vaccines within the past decade. The results are positive and the vaccines seem to get a 100% efficacy rate when administered correctly to the proper population. The difference of the vaccines is noted in their targeted strains. Therefore it is the patient's choice for which vaccine she would want to be administered.

Introduction

Prevalence of cervical cancer

Cervical cancer is the third-most common cancer in the world and the second-most fatal in women, causing about 274,000 deaths annually (Vici, 2014; de Noronha, 2013). Cervical cancer is a resilient cancer that is hard to eliminate. Screening programs are beneficial, even in young to middle-adult ages, when it leads to early detection and treatment, increasing chances of survival (de Noronha, 2013). Possible but unpleasant and not always successful treatments are monotherapy, radiation, chemotherapy, and drugs like Benacizumab (Vici, 2014). Researchers are also concerned with quality of life, since the pelvic floor is affected after cancer has developed (de Noronha, 2013). For these reasons, researchers decided to focus on the preventive aspect so fewer treatment options will be necessary. An individual can check for cervical cancer with a Pananicola test which detects precancerous lesions that may eventually develop into cancer. Because of the increase of Pananicola testing recommended and performed, more people are aware of their cervical wellbeing (Markowitz et al., 2007). Many researchers noted that when they studied the pathogenesis of cervical cancer, 99% of cases and over 90% of squamous intra-epithelial lesions that appear before the cancer fully appears are actually caused by a virus, Human Papillomavirus (HPV) (Monie et al., 2008). In fact, the biggest widespread sexually transmitted infection (STI) in the world is HPV; About 79 million people in the US currently are infected with HPV and about 14 million people become infected each year (Markowitz et al., 2007). The cases increase with age until the mid-thirties age group, where the number of cases of HPV begins to decrease. HPV can cure itself, as it actually does in about 90% of cases. However, if an individual's cervix does not heal, then she becomes at high risk for developing cervical cancer (Basu et al., 2013). Besides cervical cancer, HPV can also cause vaginal and vulvar cancer in women, penile cancer in men, and some oropharyngeal cancers and genital warts in both genders. Every year, about 26,200 new cancers are linked to HPV, and two-thirds of them affect women (Markowitz et al., 2007).

Pathogenesis of HPV

Of the 200 HPV genotypes, the majority of them can cause infections that may result in "benign or self-limited tumors (warts) in the skin or in the genitals." Those genotypes of HPV that do form warts are the oncogenic category of HPV (Wang & Roden, 2013). There are about 13 high-risk HPV strains and another seven probable high-risk strains. The most common HPV strains worldwide are HPV 16, 18, 31, 52 and 58 (Tachezy et al., 2013). The HPV virus can lead to cervical, vaginal, anal, pelvis, vulvar and oropharyngeal cancers (Wang & Roden, 2013). The two most common HPV strains that are related to cervical cancer are HPV 16 (50% of all cases) and HPV 18 (20% of all cases). However, in anatomic locations other than the cervix that can be infected by HPV, such as the head and neck centers, HPV 16 is the cause of cancer 90% of the time (Monie et al., 2008). During intercourse, the epithelial cells of the cervix and vagina might acquire some abrasions, which raise the possibility of undifferentiated basal cells being exposed to a strain of HPV. If HPV is present, the cells might differentiate with the viral genome and begin to replicate (Wang & Roden, 2013).

Replication of HPV

HPV's genome "encodes two classes of genes – early and late. The early genes control replication (E1, E2), transcription (E2), reorganization (E4), and transformation (E5, E6, E7). The late proteins are structural components of the viral capsid." However, the expression is controlled by the differentiation of the host - now infected - cell. When development occurs, the HPV genome mixes into the host's genome, causing E2, which is the main regulator of the virus's genes, and L1 and L2 to be suppressed (Monie et al., 2008). That allows E6 and E7 to overregulate. E7 would lead the cell into the S-phase of uncontrollable replication and the cell cycle will be disrupted. Eventually a thickened epithelial lesion will form. Its cells will flow from the epithelium and a virus will spread. HPV will then evolve into an HPV-related neoplasia and develop into warts or cancer if the immune system fails to protect the body (Basu et al., 2013).

Genital warts are the largest cause of sexually transmitted infection in the Western world. Their treatment is excruciating and also not permanent, as the warts commonly recur. Besides the high costs of treatments for genital warts, the recurrence is also nerve-wracking and frustrating to a patient's mental state. Genital warts generally come about from HPV 6 and 11 – two low risk HPV strains – but more research is needed to test for the presence of other strains, like the high risk HPV 16 and 18 (Szarewski et al., 2013).

Development of a vaccine

Because it takes years for an HPV infection to become a cancer, it is probably going to take years before researchers will be able to see the full results of the vaccines (Markowitz et al., 2007). Two vaccines over the past decade were created to prevent the "grave outcomes of a long-lasting HPV infection and HPV-related anogenital maliganancies, high grade cervical intraephithelial neoplasia (CIN), VIN, VaIN, and AIN" (Wang & Roden, 2013). The development of vaginal, vulvar, and anal cancer is not yet fully understood. Unlike cervical cancer, there is no routine screening developed yet for vaginal and vulvar cancer. Vaginal and vulvar are not common cancers; 1,070 and 3,507 cases were reported in the US, respectively (Markowitz et al., 2007).

The vaccines to prevent HPV are created by the use of virus-like particles (VLP) as the antigens needed to combat in the vaccines which come from the L1 surface protein of the precise types of HPV used in that specific vaccine (Basu et al., 2013). These VLPs are assisted by monoclonal antibodies (mAbs) for correctly folding into epitopes, virus typing and activating the proper immune response (Vidyasagar et al., 2014). The vaccine is considered dead as the VLPs are non-pathogenic and cannot cause other cells to become infected. Most importantly, the VLPs lack the viral genome. When a person is injected with the vaccine, the body responds and develops a high concentration of serum immunoglobin G antibody against those specific HPV types. Those antibodies are released in the cervico-vaginal secretion, and are also released when the epithelium acquires micro-abrasions. In this case, the antibodies will fight off any hint of infection before the virus has a chance to penetrate the basal keratinocytes in the vagina and cervix and infect them with HPV (Basu et al., 2013).

CIN usually precedes HPV and can be graded from CIN 1 through CIN 3, with CIN 3 being the most severe. CIN 2 and CIN 3 are dangerous and are believed to be "pre-malignant lesions, as over time CIN 3 has a 30 to 50 percent chance of becoming cancerous." Screening for cancerous cells in the cervix is generally observed during the CIN 2 and CIN 3 stages and with treatment, the cells may be stopped from developing into a cancer. Vaccines can stop CIN 2 and CIN 3 from developing altogether by preventing the infection of the common HPVs which eliminates the chance of cancer growth of those specific strains. The goal of the HPV vaccine is to prevent at least CIN 2 (Basu et al., 2013).

Because the vaccine is made from VLPs, it is considered a dead vaccine, since no live HPV ever enters the body in order to produce antibodies. Cervarix and Garsdasil are based on insect and yeast cells, yielding a high cost for the vaccinations compared to those that are based on Escherichia Coli. The vaccines are actually from virus-like particles (VLPs) that can be created by expressing the recombinant L1 from mammals, insects, yeast, and bacteria. VLPs are similar to the virions in structure and immunology. Studies have shown that these VLPs can cause high concentrations of serum antibodies (IgG) and can also protect against papillomavirus in the outside body layers. When trials began for the L1 vaccines, it was noted that they caused a 40 times increase in concentration of antibodies in the serum in the average person (Basu et al., 2013).

Vaccine coverage

Both vaccines prevent the two most common HPV strains, HPV 16 and HPV 18. Gardasil is a quadrivalent vaccine, preventing against four different types of HPV – HPV 6, 11, 16, and 18. However, HPV 6 and HPV 11 are low-risk HPV strains which are the main causes of "genital warts and laryngeal papillomas." This creates Gardasil's ability to prevent cervical cancer and genital warts. Gardasil is prepared using VLPs from recombinant yeast (Basu et al., 2013).

Cervarix is bivalent, meaning it can only inhibit two types of HPV, which are the most potent types, HPV 16 and HPV 18. The vaccinated population remain at risk of developing cervical cancer that is caused by other strains of HPV, yet the chances of this happening are much lower. Nevertheless, recent studies show that Cervarix also protects against HPV 31 and HPV 45 because of its close genotype, making Cervarix's protection rate of all HPVs close to 80 percent (Basu et al., 2013). It has also been proven that these vaccinations "inhibit HPV-associated neoplasia in the vagina,

vulvar, anus, in addition to HPV 16 detection in oral rinses" (Wang & Roden, 2013). Cervarix is also different from Gardasil in that it is made from insect cells, allowing those with yeast allergies to be vaccinated.

Another important difference between the two vaccines is the adjuvant used. Because synthetic antigens and pure recombinant do not yield sufficient antigens in the body to build a strong response of antibodies, a new idea of adjuvants, something added to a vaccine to promote the antibodies' response to the antigen, began to spread in the immunological world. Merck uses an alum-based adjuvant for Gardasil. Alum-based adjuvant is actually the most accepted adjuvant worldwide because of its effective Th2 response and its side effects – local and systemic effects like myofascitis and eosinophilia are atypical and infrequent (Petrovsky & Aguilar, 2004). Vaccines also have an adjuvant to help expose the antigen to the body for long periods of time so that the body can build a complete adaptive response for future use. The adjuvant used for Cervarix is ASO4, but not much is known its effects (Monie et al., 2008).

Who should be vaccinated

Cervarix and Gardasil are the first vaccine pair in history that focus on the same objective the same way just made by different companies. However, they are not interchangeable. Besides that Gardasil is a quadrivalent vaccine and prevents more than cervical cancer, it can also be used on the male population, while Cervarix cannot.

Gardasil is approved for males and females ages nine through twenty-six. However, the recommended age of HPV vaccination is ages 10-25. Based on studies performed on various ages, the preteens (10-15 years old) contained a higher "anti-HPV neutralizing antibodies response" than the group of 16- to 23-year-old females. Also, for maximum vaccine effect, it should be administered before the person engages in sexual activity (Basu et al., 2013). Pregnant women should not be vaccinated (Gardasil.com, 2013; Cervarix.com, 2013). It is recommended that boys also receive the vaccine during their teenage years. The vaccine seems to be less effective for boys than for girls. The fact that boys may depend on the girls being vaccinated also downplays the urgency of all boys getting vaccinated. Gardasil recommends boys and men to be vaccinated from ages nine through twenty-six to prevent anal cancer caused by HPV 16 and 18, genital warts caused by HPV 6 and 11, and anal intraepithelial grades 1, 2, and 3. Cervarix has not yet been approved in the US for males as more studies are needed to decide whether it is potent.

Administration

Gardasil is an intramuscular injection; is administered three times in either the deltoid muscle or the upper thigh region. The second dose is given two months after the first, and the third dose is administered four months later. Clinical studies have shown that three doses administered within one year create maximum efficacy for the patient (Gardasil.com, 2013).

Cervarix's instruction label has similar directives as Gardasil's. Cervarix is for females ranging from ages 9-25, and it is an

Table 1: Characteristics of the HPV vaccines Gardasil and Cervarix

Characteristic	Gardasil	Cervarix
Manufacturer	Merck Frosst Canada Ltd.	GlaxoSmithKline Inc.
Type	Prophylactic vaccine consisting of virus-like particles containing L1 capsid proteins	Prophylactic vaccine consisting of virus-like particles containing L1 capsid proteins
Antigens	Quadrivalent vaccine: HPV types 6 (20 µg/dose), 11 (40 µg/dose), 16 (40 µg/dose) and 18 (20 µg/dose)	Bivalent vaccine: HPV types 16 (20 µg/dose) and 18 (20 µg/dose)
Antigen expression system	Yeast	Baculovirus
Adjuvant	Alum: 225 µg aluminum hydroxyphosphate sulfate	ASO4: 500 µg aluminum hydroxide and 50 µg 3-deacylated monophosphoryl lipid A
Dose and schedule	0.5 mL intramuscular injection at 0, 2 and 6 months	0.5 mL intramuscular injection at 0, 1 and 6 months
Availability in Canada	Approved for sale	Not yet available

Table 1-
The similarities and differences of the two available vaccines for HPV prevention: Some countries only have one vaccine available, like Canada, and others have both vaccines available, like the United States. (Source: Dawar et al., 2007)

intramuscular injection –preferably in the deltoid – that should be administered three times: the second a month after the first injection and the third six months after the original injection. The common side effects are swelling and redness in the area of where the vaccine was administered, headaches, fatigue, syncope, myalgia, and gastrointestinal symptoms. Because of the dangers of syncope, as with a Gardasil injection, it is necessary for all patients to wait fifteen minutes in the medical office before leaving for observation (Cervarix.com 2013).

One major concern about Cervarix and Gardasil is their limitations in preventing cervical cancer. They do not guarantee a 100% success rate against cervical cancer, as Cervarix only fully protects against two strains of HPV and partially cross-protects against another two. HPV 33, HPV 52 and HPV 58 are other highly potent strains that are not protected and may cause cervical cancer. Therefore, a broader vaccine is still needed to protect fully against around 90 percent of all cancer-causing strains. Some researchers believe that the future plans of cervical cancer vaccines should implement L2 as the antigen to use to cultivate a broad spectrum of antibodies (Basu et al., 2013).

Another major concern which Basu, Banerjee, Singh et al., raise is the unknown knowledge of how long the vaccine is viable and active. Because these vaccines are new, studies regarding their efficacy are not abundant. Perhaps a booster is needed after a few years. Banerjee and his fellow researchers state in their report that studies show that immunity seems to last for about 5.5 years. However, that is not long enough, as pediatricians believe that preteens are the ideal age for the vaccines and as aforementioned, the manufacturers' recommended age for receiving these vaccines is between ages 10-25. Therefore, if a child is 11 when she receives the vaccine, she will be 16.5 when the vaccine loses its effect, while she is still a growing and developing teenager. In addition, not enough years have passed to fully observe the long-term effect this vaccine has on people.

Basu says that around the age of thirty people begin to develop their own antibodies against the virus. However, there is a 12.5-year gap between the time when the vaccine diminished and when an individual became naturally immune to it. On the other hand, more research is needed on the exact length of protection against HPV in order to conclude whether a longer length of vaccine protection is needed, a booster should be administered, or the current vaccines Cervarix and Gardasil are sufficient (Basu et al., 2013).

Prevention, not treatment

The vaccine does not mitigate or affect pre-existing HPV in any way. The basal epithelial cells and cervical cancer cells do not seem to exhibit a considerable amount of their capsid antigen, L1 or L2. If a vaccine targets the L1 or L2, these cells would remain untouched. The true need is to rid all HPV from the world; however, there are too many people who are already infected and are infecting those who are unprotected. (Basu et al., 2013). Therefore, it is crucial to remember that for an individual already infected by HPV, the best course of action is to consider treatment with medications, as the vaccine would be ineffective at this point. New research is being done to perhaps target the E6 and E7 proteins to help those who are already infected and therefore cannot use the vaccine as a solution. In the meantime, infected patients should resort to chemotherapy, radiation, surgery, and antibody therapy as possible treatments (Han & Sin, 2013).

Side effects of Gardasil

According to Gardasil's website, the vaccine has a few minor side effects: redness or swelling around the injection site, headaches, dizziness, nausea, fainting and fever. In 2009, due to an increase of fainting and syncope, the FDA took initiative and ordered the manufacturer of Gardasil, Merck, to add on to the instruction label that it is mandatory for every patient to sit in the office for 15 minutes upon injection. The FDA says that by having the patient lie or sit in one position for fifteen minutes, the medical supervisor can monitor for the initial symptoms that generally develop into syncope, such as paleness, dizziness, sweating, changes in vision and ringing in the ears (Sullivan, 2009). According to Gardasil, the most common side effect is headaches. However, Sullivan states that syncope following injection should not be taken lightly, as about 40 percent of syncope cases as a side effect develop into a tonic-clonic seizure-like activity which necessitates hospitalization. If a syncope or seizure-like activity occurs, the health professional should have the patient lie down to allow blood to continue circulating throughout the body. (Merck points out that fainting is not a side effect only to Gardasil, as it is also common after donating blood, receiving other injections, and is a typical response to pain.) (Sullivan, 2009)

From January 2005 until July 2007, 70 cases of syncope resulting from a Gardasil injection were reported. According to Sullivan in her Pediatricnews.com article, about five percent of the cases were considered serious, 38 cases occurred on the vaccination day, and 37 cases required hospitalization. As of May 2009, out of 24 million vaccinations, 13,758 VAERS reports were filed. 93 percent of those reports were considered not serious, including symptoms such as fainting, swelling, fever, headaches, and nausea. However, seven percent of those events were considered serious (Sullivan, 2009).

Immune response difference

Throughout studies, it has become evident that Cervarix produces a larger antibody response than Gardasil. In one study performed by GSK testing the two vaccines, "geometric mean titers of serum neutralizing antibodies ranged from 2.3- to 4.8-fold higher for HPV-16 and 6.8- to 9.1-fold higher for HPV-18 after vaccination

with Cervarix, compared with Gardasil, across all ages" (Einstein et al., 2009). One major possibility which researchers focus on is the ASO4 adjuvant, as both vaccines use VLPs. Although it is unknown for how long each vaccine is viable, one study claims Cervarix lasts up to 6.4 years. However, both manufacturers believe that they will know when the vaccine becomes irrelevant before the people who were already vaccinated will lose their protection.

How do most healthcare professionals decide which of the two to give? It will usually depend on the cost. However, patients should be notified of the benefits and differences of the two vaccines because they are not interchangeable in their protection (Pichichero, 2009).

HPV infections cannot be treated; only their lesions may undergo treatment. Treatment options for the precursors of genital wart, vaginal and vulvar lesions have different options of removing the lesion and therapy.

Methods

The information in this research paper was obtained from many journals, studies and research papers from the national website of Pubmed and Touro College's Online Library and Database. This paper's purpose is to educate the reader about what HPV is, how it develops into a cancer, why a vaccine is necessary, and how the vaccine is assembled. In addition, this paper will point out the different treatments for HPV, cancer, and genital warts and how a vaccine will make an imprint on the world. Using the manufacturers' (Merck's and GSK's) websites the reader will develop a vast knowledge of every aspect of the vaccines, ranging from what they cover to who should be vaccinated. Also, studies conducted on the vaccines will be examined to find any inaccuracy that exists which might lead those who are not knowledgeable in this area to be misinformed, and to help the reader develop his/her own opinion on the matter. Lastly, the effects of the vaccine on the world will be analyzed to see what the future has in store for its future of HPV and all that it may cause.

Discussion

A study was done in 2003 by the Future II study group of the New England Journal of Medicine on a newly developed quadrivalent vaccine, which eventually became Gardasil, on the recommendations of the World Health Organization (WHO) and the FDA. The goal of the study was to determine the efficiency of the vaccine against HPV 6, 11, 16, and 18 and lesions. The study consisted of 12,167 non-pregnant women from 131 different countries who had normal Papanicolaou smear results and had not had more than four partners in their lifetimes. After the subjects were vaccinated, they were evaluated by gynecologists for the average of the three years for which they were observed. At the conclusion of the study, it appeared that the vaccine is 98% effective in the population that was never exposed to HPV 16 and 18. However, the vaccine was only 44% effective in the pre-exposed population to HPV and cervical lesions. In other words, 42 subjects of this study were infected within the first three years. Once again, the idea that researchers do not know what to expect past the years of research is alarming. Also, the pharmaceutical company Merck, which is currently the supplier of Gardasil, sponsored this study. There might be a risk of bias because of the financial backer (Future II Study Group, 2007).

Prophylactic vaccines that fight against HPV 16 and 18 have a high success rate against CIN 2 and 3 and some external genital lesions. However, because much is needed to persuade the population of the success of the vaccine to prevent CIN, a study was done to create a baseline before the creation and availability of HPV vaccines to the world.

The subjects of this study were from population-based registries from Denmark, Iceland, Norway and Sweden who were "diagnosed with incident cervical vulvar and vaginal cancer and pre-invasive neoplasia from January 1, 2004 until December 31, 2006, with only the primary tumors allowed to be included yielding to over 100,000 subjects in the number." According to the data collected in all countries, the age range of 20 through 29 years old experienced an immense increase of cases and a peak in the thirties age range for cervical cancer, yielding about 10 percent of the cases. However, cervical pre-invasive neoplasia was most commonly found in people in their twenties in all four countries. Nevertheless, vulvar and vaginal cancer peak past the age of 40 and peak in women over 70. These researchers were able to mathematically predict the impact of vaccination by including the fact that only 30% of CIN3 would develop into cancer if not treated. Besides the fact that overtreatment of CIN3 is costly, it is dangerous for those in their reproductive years, as it increases the risk of preterm births. Therefore, it is in the best interests of all involved to prevent neoplasia in women. The researchers state that if the vaccine is really close to 100% efficacy, based on literature review, the cases of pre-invasive neoplasia should decrease by 52 to 67 percent, totaling about 2,471 to 2,911 fewer cases of diagnosed and treated cervical cancer in these four countries (Nygard et al., 2014).

Another study was done in England on the effects of Cervarix and its ASO4 adjuvant. The study was a PATRICIA (Papilloma Trial against Cancer in Young Adults) trial which was "phase III, double-blind, randomized and using the Hepatitis A vaccine as a control." The study did not exclude anyone based on previous or current history of genital warts. The subjects had a cervical sampling done every six months for HPV DNA typing and tested for 14 cancerous HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and another 11 non-cancerous HPV genotypes. In addition, all women were examined every twelve

months and had a colposcopy if necessary. In total, 18,644 women received at least one dose and were included in this study from May 2004 until November 2009. The average monitoring time was 43.7-47.4 months. The results of this study are quite interesting: although the bivalent vaccine only officially covers HPV 16 and 18, a cross-protection was found that prevents HPV 6, 11, 31 and 45. The reason for cross-protection may be from the similarities in structure and homology within the L1 VLP. However, when comparing the response from the L1-specific T helper cell that are caused by HPV 6 and 11 in the bivalent and the quadrivalent vaccines, the numbers of cells were analogous to each other. The study also proves that there was a greater helper T cells response for HPV 31 and 45 in the bivalent vaccine. The reason for cross protection may be from the similarities in structure and homology within the L1 VLP. The study concluded that the risks of persistent effects decreased with the use of vaccines (Szarewski et al., 2013).

England was the first country to try a national immunization program involving Cervarix. As a result, researchers were able to study the records from the general practices and the genitourinary medicine clinics of the general public to observe the trends of genital warts. Although it has been proven that Gardasil prevents HPV 6 and 11, the main causes for genital warts, Cervarix seems to cross-protect them, which also results in a prevention of genital warts. This was proven when the study conducted in England noticed the large decrease in genital warts in the immunized female teenage population. The teenage male population also had a decreasing rate in reports of genital warts, but it was not as significant as the female's population. However, there are many reasons that may have contributed to the decline; one big factor is that the population became more aware of the dangers of HPV and genital warts and therefore realized the need to protect themselves by abstaining from unsafe intercourse. More studies like this one are needed in order to conclusively state that it is the vaccine lowering the cases of genital warts in England (Howell-Jones et al., 2013).

Beginning in 2006, the National Immunization Survey-Teen in the United States conducted a study by calling random landlines and cellular phones for its sample. In total, 14,133 adolescents (with their guardians' consent) submitted their vaccination history. The results showed that the number of adolescents receiving the vaccine for each dosage increased each year until the number stagnated in 2012. However, in 2012, questionnaires began to ask why people opted not to vaccinate their daughters. The most common answers were that the vaccine is not needed, the vaccine is not recommended, there are questions about the safety of the vaccine, there is a lack of knowledge about the vaccine or disease, and that their daughter is not sexually active (Centers for Disease Control and Prevention [CDC], 2013).

According to the CDC, about 56 million dosages of Gardasil were administered from June 2006 through March 2013, and from October 2009 through May 2013, about 611,000 dosages of Cervarix were administered in the United States. Analysis of vaccine safety by the CDC is limited to Gardasil because it covers 99% of the vaccinated population. Of the 54 million vaccines administered, 21,194 unusual cases were reported in females, and 92.1% of those cases were considered non-serious. The main bulk of these reports were from 2008. Yet, the remaining 7.9% of those cases were considered serious, with the highest total from 2009. However, these symptoms that occurred after patients received the dose are usual symptoms that may occur for most vaccines (CDC, 2013).

The ACIP conducted its own trials and safety studies on Cervarix. Each Cervarix vaccine administration was observed for any symptoms upon injection, like development of a new autoimmune disease or chronic diseases, injection-site reaction, systemic symptoms, and death. Of the 23,713 females studied in the study, 92% complained of injection-site swelling, 48% had redness, and 44% had swelling. Other common side-effects were myalgia, fatigue and headaches. Only about 5.3% complained of serious results; however, 5.9% in the control group also complained of serious results (CDC, 2010).

Gardasil also performed its own studies on a variable population with all different types of ethnicities, ages, gender, and previous history. In total, 28,413 people participated in this study, the majority of whom were women. In the 16-26 age range for females, the efficacy, which was marked by almost one hundred development of CIN, VAIN, AIS, VIN, and genital warts, was close to 100 percent in all categories, with the lowest rate at 96.9%. The rate in older women was not as high as the younger population, where the

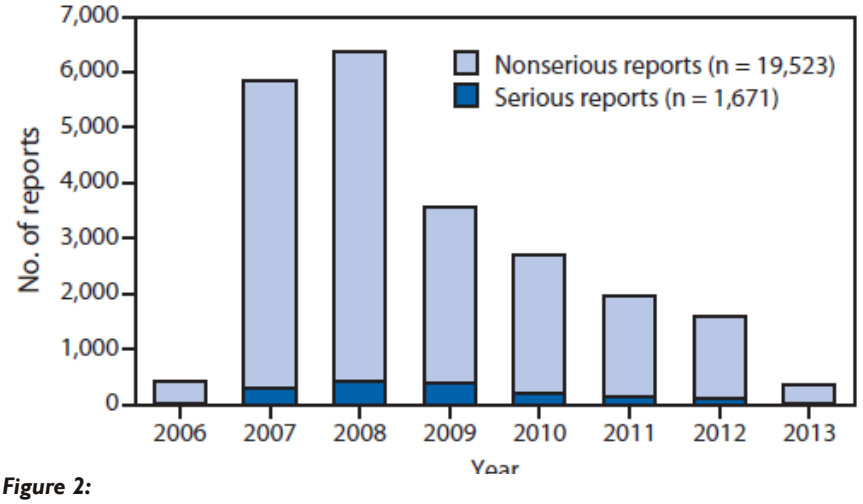

Figure 2:
The number of serious and non-serious reports of side effects of Gardasil by year from June 2006-March 2013 (CDC, 2013).

efficacy rate was approximately 85%. Men ranging from 16-26 had about a 90% efficacy rate. However, the numbers dipped, even at some points close to 60% efficacy, in the prior or currently HPV-infected population. The immunological tests showed good results for geometric mean titers and a high percentage of antibodies that are anti-HPV 6, 11, 16 and 18 in all subjects. During clinical trials, safety was a major area of observation; very few abnormal cases and mainly all typical vaccines reactions, like headache, pyrexia, diarrhea, nausea, and vomiting were noted. In addition, 258 out of 29,323 subjects complained of serious reactions like headache, appendicitis, gastroenteritis, urinary tract infection, and pneumonia. Forty people were healthy and died from motor vehicle accidents, overdose, cancer, gunshot wounds and pulmonary embolus. Those people reflect the all deaths notified in their report so no person actually died from the vaccine directly.

Many more studies like the ones mentioned above have been conducted and analyzed. The common thread among all of them is that the vaccine causes an increase titer of antibodies in close to 100% of the subjects, subjects' side effects include the regular side effects common to all vaccines with the additional of chance of syncope, and only less than 10 percent may have had serious implications afterwards, but nobody ever came close to death because of vaccination (Gardasil.com, 2013).

Based on all of these studies, the vaccine seems to enable the production of antibodies against HPV in all those vaccinated to prevent future cancer. The side effects seem reasonable in comparison to all other vaccines. No serious ailments are connected to the vaccine directly and the vaccine is deemed to be safe. The biggest concern that still remains is how long the vaccine's immunity will last. Since Gardasil has only been distributed to the population for eight years and has been studied for a bit over a decade, not much is known as to the long-term effects, both positive and negative. Cervarix is even less studied because its worldwide distribution began only five years ago. Questions do remain on the preference of the two vaccines: Cervarix leads to more than double the number of antibodies, whereas Gardasil prevents more genotypes and genital warts. As mentioned before, it should be the patient's choice as to which vaccine should be administered to him/her. Realistically, the fact that 54 million people had received Gardasil while only 600,000 had received Cervarix through 2013 sheds light on the fact that doctors only have one vaccine in the office. This might be because of costs or health insurance coverage. Nevertheless, patients should be advised about both vaccines and should have the right to ask for whichever one they think is properly suited for them.

Conclusion

In 2006, a new era of science began with the development of a cancer vaccine – the HPV vaccine. If an individual has persistent HPV infections, he/she has a chance of that HPV virus growing into an abnormal growing stage which will eventually become cancerous. The newly discovered vaccine has been proven to prevent any further growth of lesions from the HPV virus and to prevent these lesions from becoming cancerous. The vaccine also gives the vaccinated community anti-HPV antibodies. Gardasil is a quadrivalent vaccine and protects against HPV 6,11,16,18 and genital warts. Cervarix is a bivalent vaccine that only protects against HPV 16 and 18. HPV 16 and 18 are the most significant strains as it causes about 70% of all HPV cases. Furthermore, each vaccine also cross-protects against other HPV genotypes. Both vaccines have an unusual and yet close to 100% efficacy rate and the sides-effect for each vaccine are minimal. Very few cases of serious side-effects have been reported, which none of them led near death or death situation. The vaccine is catered for the 10- to 25-year-old age range and best results occur when given to individuals who are not yet active or pre-exposed, because the vaccine does not cure HPV infections whatsoever.. Although most people think HPV is not "catchy," it is in fact highly contagious and is similar to influenza and chicken pox in that it spreads through direct contact. HPV can spread from person to person in people ten years and older, with the help of the fact not everybody knows they have it. Therefore, HPV is a serious matter and the number of cases must lessen. If fewer individuals are infected, there will be fewer cancers in the world. Similarly, just like the polio vaccine fewer to less outbreaks of polio, hopefully one day fewer people will contract anal, vaginal, penile and cervical cancer because of the influence of the HPV vaccine. It is important for the public to be educated about the harms of HPV, the cancers HPV cause, and the benefits and risks of preventive HPV vaccines, Gardasil and Cervarix, in order to make the world a healthier place.

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Theories on Varicella Zoster Virus Reactivation Based on Shingles Patterns

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Abstract

Herpes zoster, a disease also known as shingles or as zoster, infects the sensory nerve ganglion and the peripheral nerve and its branches, resulting in pain to the affected dermatomes. Infection results from reactivation of the varicella-zoster virus, the same virus which causes varicella, or chickenpox. The varicella-zoster virus usually causes chickenpox to its host at an early age and then withdraws to the dorsal root ganglia where it enters a latency stage. The virus may reemerge at any time and infect its host with shingles. As shingles is most common in ages 50 and above, it is assumed that cell-mediated immunity plays a role in suppressing the virus, and, therefore, a decline in this immunity allows the virus to reemerge from latency. Shingles also appears to be more common in temperate regions than in tropical regions, leading to a suggestion that certain genotypes of the varicella-zoster virus are more prone to reactivation than others. Decreased herpes zoster incidence in the African American population and the detection of increased presence of the ATA and GCC haplotypes in herpes zoster patients may point to a genetic predisposition to reactivation of varicella-zoster virus. Evidence of increased female shingles incidence has lead to numerous hypotheses, some of which may shed some light on the mechanism of varicella-zoster reactivation, a phenomenon which is still poorly understood.

Introduction

"Chicken pox is most commonly an annoying illness lasting three to seven days, and happily never seen again" (qtd. in Link 2005). This statement is by and large true about the actual chickenpox illness; in response to a chickenpox disease, the body builds immunity against future occurrences, and rarely does a second case of chickenpox occur. However, the virus which caused the chickenpox may be seen again; it may resurface as a disease called shingles after years of lying dormant.

Chickenpox, the popular name for varicella, was a common childhood rash until the varicella vaccine was licensed for use in the United States in 1995. Prior to the introduction of the varicella vaccine, over 90% of Americans contracted chickenpox by age 15 (Chickenpox 2013). Chickenpox symptoms typically last up to seven days (Simon 2012). However, even after recovery from chickenpox, the causative agent—varicella-zoster virus (VZV)—remains dormant inside the body in sensory nerves. Reactivation of the virus later in life results in shingles, or herpes zoster, and presents differently than its predecessor. Chickenpox appears as an itchy rash and blisters all over the body, and shingles appears as a blistery rash restricted to one side of the body (Figures 1 and 2). While chickenpox is generally considered a mild, unpleasant disease and is short lived, shingles is a more severe, painful disease which can have lasting effects. Generally, shingles symptoms clear in seven to ten days; however, it is not uncommon for it to take as long as a month to clear. The most common debilitating complication related to shingles is postherpetic neuralgia (PHN), defined as pain that persists longer than a month after the onset of shingles and lasts at least 90 days (Sampathkumar et al. 2009). The risk for PHN can reach 25-50% for shingles patients over the age of 50 and up 75% for patients over the age of 70 (Giménez-Milà et al. 2014). Other complications that may arise due to shingles include zoster ophthalmicus (reactivation of VZV in the trigeminal ganglia involving the ophthalmic division of the nerve, potentially damaging the eye and surrounding structures), bacterial superinfections, and neurological manifestations such as meningitis, encephalitis, myelitis, and complex regional pain syndromes I and II (Studahl et al. 2013 and Giménez-Milà et al. 2014).

Methods

In researching the background of varicella and herpes zoster, particularly theories regarding the reactivation of varicella-zoster virus, articles were gathered using various databases of scientific literature. These include PubMed,a service of the United States government, and various databases of health science journals made available through access to the Touro College and Ursuline College libraries.

Pathogenesis

Varicella zoster virus, the causative agent of varicella (or chickenpox), is a member of the herpesvirus family and alphaherpesvirus subclass. After the primary varicella infection, VZV withdraws to the dorsal root ganglia and trigeminal ganglia where it remains latent. In fact, VZV in its latent stage can be found in multiple ganglia along the entire neuraxis (Liesegang 1999). It may remain dormant for the remainder of the life of its host, never causing any viral symptoms, or it may reactivate at any time as herpes zoster (HZ), more commonly known as shingles (Figure 3). However, according to Greg Bennett (qtd. in Polansky 2013), even during the latency period, which may last the entire lifetime of its host, VZV does not

Figure 1:
Chickenpox appears as an itchy rash and blisters all over the body. Source: Chickenpox Photos 2013

Figure 2:
Shingles appears as a blistery rash restricted to one side of the body. Source: Shingles Pictures 2014

remain inactive; transcription of the VZV has been detected even during this period. Reverse transcription polymerase chain reaction has identified multiple VZV transcripts in latently infected human ganglia (Baird et al. 2013). "Thus," claims Bennet, "the idea that the virus is inactive and presents no risk during the so-called inactive period is a misconception. Rather, the latent viruses are active and can be dangerous."

The dermatological signs (i.e. rash and blisters) of herpes zoster are similar in their centripetal distribution to dermatological signs of varicella (Figure 4). This is suggestive that during primary varicella infection, the virus (VZV) spreads from infected skin cells to sensory nerve endings and then to the ganglia where it remains dormant. Subsequently, when VZV reactivates, it appears with the same centripetal distribution (Liesegang 1999). However, writes Liesegang, despite the similar dermatological distribution, it does not necessarily have to be so. It may be that the ganglia are directly infected hematogenously during the viremic phase of the initial varicella infection, and the path VZV takes when it reactivates as herpes zoster is simply indicative of the ganglia most exposed to reactivation stimuli.

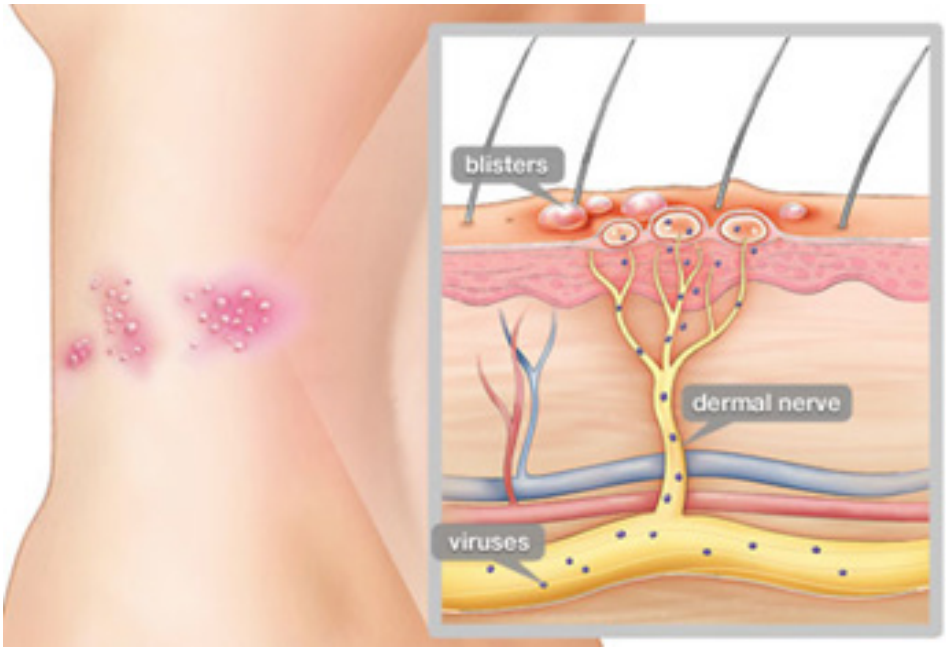

Figure 3:
The varicella zoster virus is the causative agent for chickenpox and shingles. After chickenpox, the virus never goes away. Instead, it settles in nerve cells and may reactivate years later, causing shingles, also called herpes zoster. Source: Smith 2014

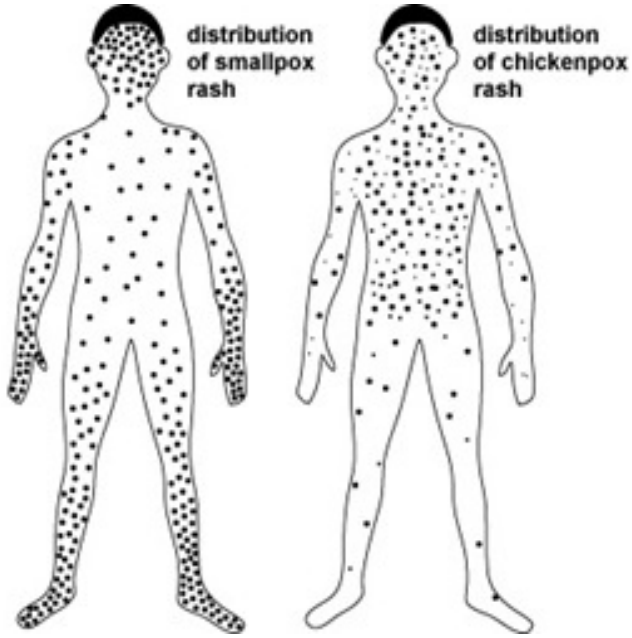

Figure 4:
The chickenpox rash exhibits centripetal distribution, meaning that the greatest concentration of lesions is on the trunk with fewer on distal extremities. This is in contrast to the smallpox rash which exhibits centrifugal distribution, meaning that the greatest concentration of lesions is on the face and extremities with fewer on the trunk. Source: Antipuesto 2008

Epidemiology

Regardless of the path taken by VZV from initial varicella infection through latency and finally reactivation as herpes zoster, the cause of VZV reactivation remains unknown. There are numerous hypotheses as to what causes reactivation of VZV, although there is no concrete evidence supporting any one hypothesis and certainly no consensus among researchers. It is quite clear, however, that risk of herpes zoster increases with age. This is widely attributed to the decline of cell-mediated immunity (CMI) that progresses with aging. It is assumed that the dormant VZV is held in check by the cell-mediated immunity, not humoral immunity, so when CMI declines, the virus can flare up. Humoral immunity is assumed not to play a role in suppressing VZV, for herpes zoster has been shown to occur even in the presence of high levels of antibody titer, and antibody titer is a measure of humoral activity (Liesegang 1999).

Genetic Correlation

Philip S. Rice (2011) suggests a genetic correlation as governing the latency and reactivation of herpes zoster virus. He hypothesizes that exposure to the sun, or more particularly to ultra-violet radiation (UVR), plays a significant role in determining the genotype of the VZV, and, thus, the virus has evolved into different genotypes based on climate. He builds his hypothesis on the fact that incidence of varicella is significantly lower in tropical regions than in temperate regions and that, even in temperate regions, varicella incidence is at its lowest in the summer. (In regions near the equator, i.e. tropical regions, the sun's rays arrive almost perpendicular to them, thus higher levels of radiation. Near the poles, i.e. temperate regions, the angle of the sun's rays spreads them out over a much greater area, providing less energy per unit of area (Raven et al. 2005).) He posits that in the tropics, the high levels of ultra-violet radiation may inactivate varicella virus in the vesicular fluid either before or after rupture of the vesicles. According to Asano et al. (1999), vesicular virus contributes more to the spread of the virus than the shedding of the virus from the respiratory tract. Consequently, in the tropics, transmission of varicella virus is significantly diminished due to ultra-violet radiation. As early humans lived in Africa—a tropical region— the virus evolved into a genotype resistant to ultra-violet radiation as a survival strategy. When humankind spread to temperate regions, however, the virus shed its selective advantage of UVR resistance. However, as an evolutionary tradeoff for its lost advantage and, thus, reduced transmissibility (during the summer months when UVR is strongest), the virus increased its propensity to reactivate as herpes zoster in order to ensure its continued survival in temperate regions. Accordingly, the temperate genotype of VZV is prone to reactivation while the tropical genotype is not (except in severely immunosuppressed individuals). This explains why data of herpes zoster incidence in tropical countries is virtually absent. Rice, thus, suggests that the same mechanism responsible for UVR resistance in tropical VZV genotypes may also play a role in the latency and reactivation of temperate genotypes.

Quinlivan et al. (2013) also mention findings of various genotypes of VZV according to country. They write that VZV genotyping has identified five clades (a clade is a group consisting of an ancestor and all its descendants) of which clades 1 and 3 predominate in Europe, clade 2 in Japan, and clades 4 and 5 in Asia and Africa. Liesegang (1999) also notes the significant variation of VZV between tropical and temperate regions and insists that it must be due to agent specificity.

A point one can take issue with and not addressed by Rice is that if the tropical genotype has acquired UVR resistance in order to avoid inactivation by ultra-violet radiation, then why is incidence of varicella lower in tropical regions than in temperate regions; shouldn't the tropical genotype be able to thrive in constant sunlight just as well as the temperate genotype does in decreased sunlight? The answer to this question may lie in that varicella is easily spread by respiratory route via airborne transmission, and this form of transmission is aided by winter weather. In addition to increased coughing during the winter months and the tendency to remain indoors due to the cold temperatures outside, the cold, wet weather may aid the survival of the virus outside a host (Zak-Prelich et al. 2002). In the tropics, despite the advantage of UVR resistance, the virus lacks the advantage of winter weather aiding in spreading it, and this may explain the lower incidence of

varicella there. Although, as Rice quoted from Asano et al. (1999), vesicular virus contributes more to spreading of the virus than does airborne transmission from the respiratory tract, and the tropics have the advantage of vesicular transmission even in presence of ultra-violet radiation, the lack of increased airborne transmission may still be a viable explanation for the significantly lower incidence rate in the tropics.

Another setback to Rice's hypothesis is a suggestion from Gallerani and Manfredini (2000) that ultra-violet radiation might be responsible for increased incidence of herpes zoster in the summer months (in temperate regions) due to its ability to suppress cellular immune response (Figure 5). When immunity to VZV is reduced, the latent virus is then able to reactivate as herpes zoster. This would suggest that ultra-violet radiation aids in virus activation, while Rice suggests that ultra-violet radiation is an inactivator of VZV. However, this is easily answered by the different methods of activation in both diseases. Varicella is transmitted from person to person by vesicular route or respiratory route. Thus, because the virus in the vesicles (in the temperate genotype) is inactivated by ultra-violet radiation, spread of the virus is decreased. Herpes zoster, on the other hand, can only develop from reactivation of a latent VZV; it is never transmitted from one person to another (Simon 2012). Reactivation of the latent VZV is largely due to immunosuppression. Thus, ultra-violet radiation does not actually reactivate the virus, it merely suppresses the immunity that is keeping VZV dormant, and this allows VZV to reactivate. In other words, ultra-violet radiation may, in fact, inactivate vesicular varicella virus as Rice posits, and the fact that ultra-violet radiation is also responsible for increased herpes zoster incidence is not contradictory, because ultra-violet radiation does not actually activate herpes zoster.

While Rice suggests that genetic variations in the VZV itself result in prevalence or scarcity of VZV reactivation, others suggest that genetic variations in human subjects may be responsible. Schmader et al. (1998), in a study of communities in North Carolina over a six-year period, found significantly fewer herpes zoster cases among black subjects than among white subjects. To explain this phenomenon, they suggest that decline in cellular immunity due to aging may be less in black people than in white people, although they offer no explanation as to why. However Thomas and Hall (2004) take a novel approach in explaining why this may be so. They suggest that a less significant decline in cellular immunity among aging black subjects may be due to differential survival, or natural selection. "In populations with higher mortality rates, those who survive to old age may have robust immune systems (and therefore lower susceptibility to zoster)," they write. (They may be referring to the high mortality rate of even young children in Africa. They may also be referring to the increased mortality rate, even in the US, among African American children [Infant Mortality and African Americans 2013] Another approach Thomas and Hall take is that it may be caused by genetics; genetic differences between black subjects and white subjects may result in decreased or increased incidence of VZV reactivation, respectively. This approach suggests that genetic variations among the human race may be behind the propensity toward VZV reactivation.

One can also argue that the difference in herpes zoster incidence between black subjects and white subjects is due to variations of the VZV genotypes rather than genetic variations among humans, based on the findings of five different clades of VZV, with clade 5 predominant in Africa and Asia (Quinlivan et al. 2013). This argument adds credence to Rice's position that tropical VZV genotypes are less prone to reactivation than temperate genotypes. Although

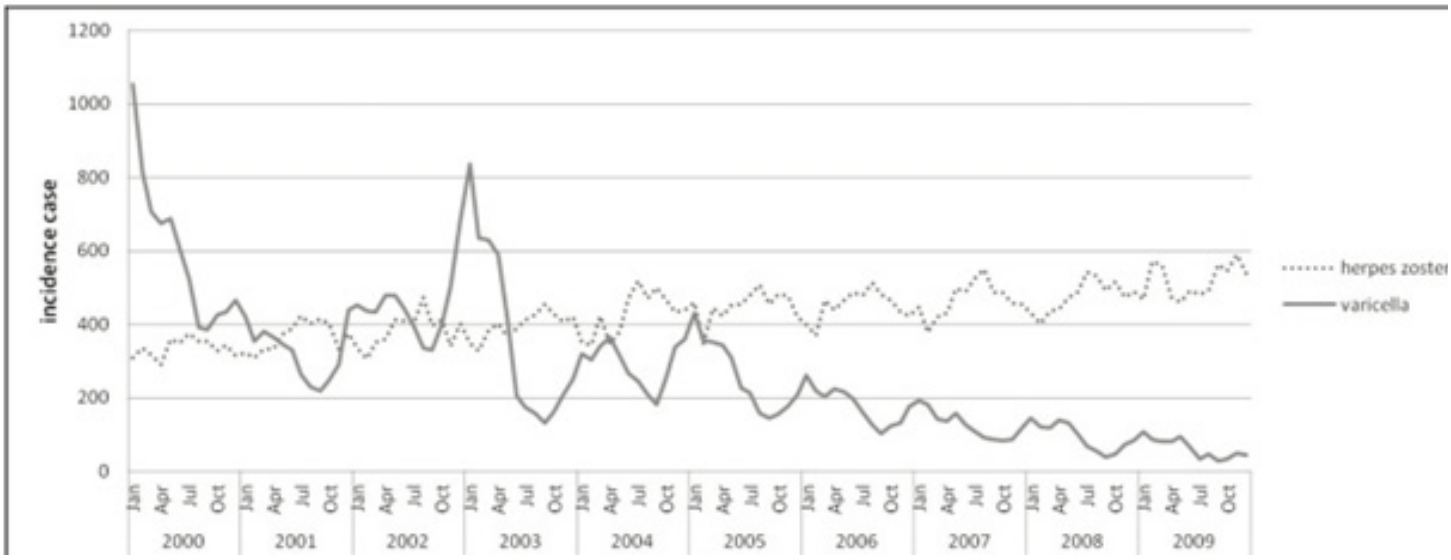

Figure 5:
Annual trend of monthly incident cases of herpes zoster and varicella in 2000–2009. Notice that herpes zoster spikes during the warmer months. Source: Wu et al. 2013

Rice writes that immigration from Africa to temperate regions gave way to the temperate VZV genotype, such a transition might take some time, and recent immigrants and their offspring may still harbor the tropical genotype. This is especially plausible when taking into account that the reactivating strain of VZV is identical to the strain that had caused the primary varicella infection (Sengupta et al. 2007). Thus, the tropical VZV genotype can persist and still be widespread in aging African American populations long after immigration from Africa.

Another genetic correlation is suggested Haanpää et al. (2002). They suggest that susceptibility to herpes zoster may be genetically predetermined. Interleukin-10 (IL-10) is an anti-inflammatory cytokine; it downregulates the production of the proinflammatory cytokines, thus decreasing cell-mediated immunity. The promoter region of the gene for IL-10 is polymorphic, producing three different haplotypes (combinations of DNA sequences on one chromosome that are inherited together): GCC, ACC and ATA. Blood samples taken from herpes zoster patients and from controls not infected with herpes zoster showed a significantly higher presence of the ATA haplotype in the blood of herpes zoster patients. This suggests that IL-10, its presence detected by detection of the ATA haplotype, may play a role in VZV reactivation. The explanation of this is that VZV reactivation is largely due to decreased cell-mediated immunity, and this immunity is decreased by IL-10. Consequently, inheritance of the ATA haplotype may predispose one to herpes zoster. This idea is echoed by Cho (2007) who writes that in Korea, the GCC haplotype is significantly higher in herpes zoster patients than in controls not infected with herpes zoster, and this may point to the role of IL-10 in reactivation of VZV and a possible genetic predisposition to herpes zoster. Cho attributes the differences in increased haplotype findings between the previous study conducted in Finland and his own study conducted in Korea simply to ethnic differences between European and Asian populations. Their differences notwithstanding, the ideas of both studies are the same—that IL-10 may be a factor in VZV reactivation leading to herpes zoster, and genetic inheritance of a specific haplotype of the IL-10 promoter gene may predispose carriers to herpes zoster. This suggestion, too, assumes genetic variations in human hosts, rather than genetic variations in the VZV itself, to be behind increased or decreased incidence of herpes zoster.

Gender Correlation

Numerous studies and trials report a higher incidence of herpes zoster in females than in males (Figure 6). In one such report, Fleming et al. (2004) investigate the gender differences in incidence of herpes zoster by dividing patients into age groups: 0-14, 15-24, 25-44, 45-64, 65-74, and 75 and older. Over the eight-year study, there were 14,532 cases of herpes zoster, of which 59.3% were female patients and 40.7% male patients. Female herpes zoster incidence exceeded males in all age groups except for the 15-24 age group in which the rates were almost equal for both genders. The greatest female risk appeared in the 45-64 age group followed by the 0-14 age group.

There are numerous attempts to determine what is behind the female predisposition to herpes zoster incidence. Possibly, resolving what is behind increased female incidence can shed some light on the mechanism of VZV reactivation in general.

Various reasons for the female excess are given. One suggestion is that there are more herpes zoster cases among females due to their longevity; because women live longer than men, there are more occasions for them to contract herpes zoster. Fleming et al. (2004) write, however, that their analysis disproves the theory, for it indicates female excess in nearly all age groups. If increased female incidence were due to female longevity alone, an increase in female incidence would only be evident in older age groups—age groups in which females generally outlive males. Another suggestion given is that women tend to seek medical advice more readily than men, and, thus, there are more reported cases of females with herpes zoster than of males (Thomas and Hall 2004). This

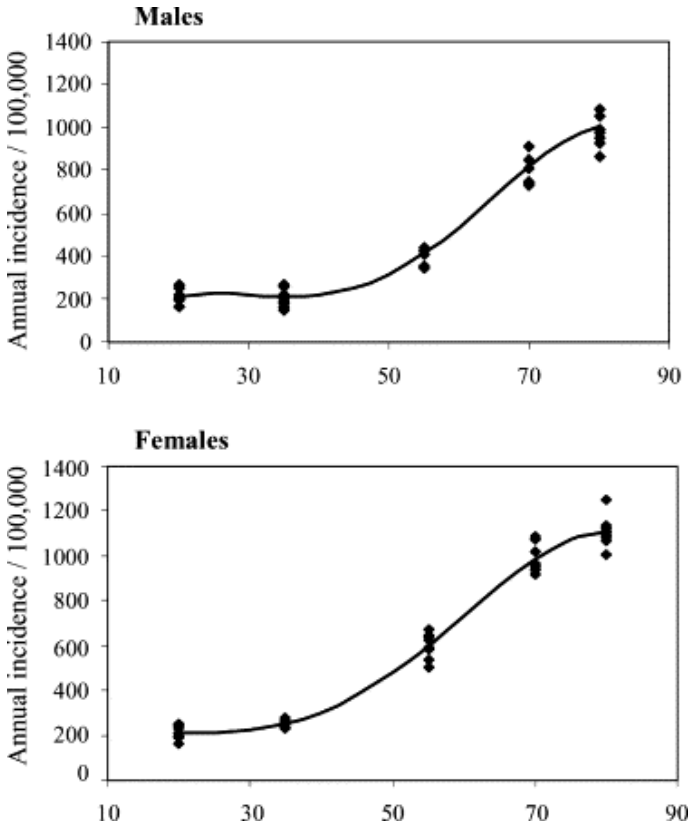

Figure 6:
Annual incidence per 100,000 of shingles by age group and gender in years 1994–2001 with superimposed regression line. Source: Chapman et al. 2003

would suggest that, in truth, females are not more susceptible to herpes zoster than males; it only appears that there is higher herpes zoster incidence in females, because they are quicker to see a doctor for symptoms, and, thus there are more female herpes zoster patients recorded in the system. (In one case, a 60 year old man waited ten days before making an appointment to see a doctor despite pain rated 6 out of 10 on the pain scale and a rash which appeared within a week of the onset of the pain! (Martić 2014)) This theory, however, would seem to be disproved by the data provided by Fleming et al. (2004) which finds that in the age group of 15-24, female rates of herpes zoster did not exceed male rates and by data provided by Ragozzino et al. (1982) which actually finds lower incidence of herpes zoster in females aged 35-44 than in males of the same age group.

A more sophisticated approach to explain the increased herpes zoster incidence in females is taken by Fleming et al. (2004). They suggest that there may be a true gender difference in the way the body responds to reactivation of a latent virus. They base this theory on the fact that there is also a significant female excess in incidence of herpes simplex virus, another member of the herpesvirus family and alphaherpesvirus subclass, which is also a virus that reactivates after a long latency period in ganglionic tissue. Although they do not specify what might be behind the difference in reactivation response based on gender, the suggestion of Studahl et al. (2013) may shed some light on it. Studahl et al. suggest that the difference in herpes zoster incidence between genders may be due to hormonal differences between men and women. It may be the hormonal differences between the genders that affect the way the body responds to virus reactivation.

In a similar vein, the menopause transition period females go through is also suspected to be responsible for increased female herpes zoster incidence due to hormonal changes to their immune response (Saunders 2014). This theory is supported by the aforementioned findings of Fleming at al. that females aged 15-24 did not exceed males in herpes zoster incidence and the findings of Ragozzino et al. that females aged 35-44 actually have lower herpes zoster incidence than males, for they have not yet reached the menopausal stage. This theory, however, fails to address increased herpes zoster incidence in females past the menopausal stage, when hormone levels are static.

It should be pointed out that, even according to hypotheses correlating increased herpes zoster incidence in females with their hormones or menopausal shifts, they do not claim these to be the sole causes of VZV reactivation, for herpes zoster occurs in males too, albeit not as prevalent as in females. They simply suggest that among other factors influencing VZV activity, these contribute on greater levels to its reactivation. Thus, while even those lacking these factors may get herpes zoster, those that have these factors are at a greater risk of getting herpes zoster.

Still, there does not seem to be a clear understanding as to the exact role hormones play in increasing herpes zoster incidence, and no explanation has been offered as to which hormones might be involved. Are female hormones responsible for the increased herpes zoster risk in females, or are male hormones responsible for decreased herpes zoster risk in males? After all, males have a higher risk for a severe case of chickenpox than females (Simon 2013). Maybe this points to some type of male hormone influence on VZV. On the other hand, there is more fluctuation in female herpes zoster incidence between the different age groups, suggesting that female hormones more likely play a role in VZV reactivation and that fluctuation in their levels contributes to an increase or decrease in herpes zoster incidence. In addition, as previously mentioned, the latent VZV is held dormant by cell-mediated immunity, not humoral immunity. In pregnant women, "the fetoplacental unit redirects maternal immunity away from cell-mediated immunity towards enhanced humoral responsiveness" (Wegmann et al. 1993). This would lead to the assumption that women are at a high risk for VZV reactivation during pregnancy. This, however, is not the case. According to Simon (2012), herpes zoster is extremely rare in pregnant women. While this phenomenon is not well understood, it does strengthen the case for female hormone involvement in increased female incidence as opposed to male hormone involvement in decreased male incidence.

Conclusion

While the particular method of VZV reactivation remains unclear, numerous hypotheses are offered based on various presentation patterns of herpes zoster. Some suggest that genetics plays a role. One such study focuses on regions where herpes zoster is or is not prevalent and suggests that VZV genotypes differ according to region with temperate genotypes prone to VZV reactivation while tropical genotypes are not. Another study focuses on the decreased herpes zoster incidence among African Americans and suggests that genetic differences between white subjects and subjects of color may increase or decrease susceptibility to VZV reactivation. Yet another study suggests that inheritance of certain genetic haplotypes may predispose one to herpes zoster. Others focus on the difference in herpes zoster incidence by gender. Instead of citing genetics as possibly influencing increased female incidence, some suggest that female hormones may be largely responsible. Similarly, others suggest that menopausal instability may prompt VZV reactivation.

"The peculiar pathogenetic mechanism of herpes zoster infection, its capacity to migrate after the primary infection (varicella) to the dorsal root ganglia and remain silent throughout life, makes it somewhat difficult to put forward convincing hypothetical explanations" (Gallerani and Manfredini 2000). Nonetheless, each of these observations of herpes zoster patterns and diverse suggestions as to what is behind the patterns and what they mean sheds some light on the mechanism or mechanisms of VZV reactivation. Each insight offers a unique angle at which the phenomenon of VZV reactivation can be further studied.

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The Search For Novel Inhibitors Of The Mycobacterial Enoyl Reductase InhA Through Structure Based Drug Design

By: Esther Saul

Esther graduated in June 2014 with a B.S. in biology.

Abstract

Isoniazid (INH), one of two first-line drugs used to treat tuberculosis (TB), has been shown to be a potent inhibitor of InhA, the mycobacterial enoyl reductase. However, the increasing resistance to INH makes it imperative to find alternative drugs that are as effective as the first-line drugs, yet active against INH-resistant strains. Since InhA has been validated as an excellent target of TB, there have been attempts to find novel inhibitors of InhA. Through rational drug design, a variety of high affinity InhA inhibitors were synthesized. Triclosan itself was observed to be a suboptimal inhibitor of InhA with a K of .22 μM, but with modifications to the 5-position of the A ring, the new diphenyl ether compounds demonstrated higher affinities with nanomolar constants. A particularly potent triclosan derivative, compound PT70, showed slow, tight-binding inhibition with a K value of 22 pM. In addition, arylamides showed moderate to high inhibition of InhA. They did not show sufficient anti-tubercular activity, and therefore future modifications are needed before they can be considered seriously for use against TB. Lastly, pyridomycin, a natural anti-tubercular drug was rediscovered and confirmed to have high activity against TB. It was found to be the first compound ever published to bind to both binding sites of InhA simultaneously. Current research is ongoing in the synthesis and testing of pyridomycin analogs to further increase their anti-tubercular properties.

Introduction

Drug resistance in Mycobacterium tuberculosis (TB) has become a severe global health threat. The fight against TB faces major challenges due to the appearance of Multi-Drug Resistant Tuberculosis (MDR-TB) and more recently, the virtually untreatable Extensively Drug Resistant Tuberculosis (XDR-TB). MDR-TB are strains that are resistant to both top first-line drugs, Isoniazid and Rifampin, while XDR-TB are MDR-TB strains that are also resistant to any fluoroquinolone and one or more of 3 injectable drugs. With this new resistance there emerges a need to find new drugs that are as effective yet bypass the problem of resistance. One method of research is to find new vulnerabilities of M. tuberculosis to use as new target sites of drugs. This method is highly expensive and requires intense and lengthy research just to implicate a new target site (Scheffler et al., 2013). An alternative is to develop new drugs that work on the same known targets as the first-line drugs but by different mechanisms thereby bypassing the resistance of TB to the drug.

Isoniazid (INH) is a powerful anti-TB drug that works by inhibiting the action of InhA, a crucial enzyme in the process of manufacturing the cell wall. Instead of searching for new targets on TB, there is a goal to expand on this target and develop drugs that inhibit InhA by different mechanisms and thus show anti-tubercular activity against MDR-TB strains.

The virulence of tuberculosis is due to its greatly complex cell envelope. Mycobacteria contain cell walls with unusually high lipid content due mainly to the presence of mycolic acids which are alpha-alkyl, beta-hydroxy fatty acids with atypically long alpha alkyl chains (up to C). The existence of mycolic acids in the cell wall makes the cell impervious to common antibiotics (Niederweis et al., 2010). Mycolic acids were shown to be essential for the survival of tuberculosis thereby making them a great target for drug therapy. InhA (NADH-dependent-2-trans-enoyl-ACP-reductase)'s role in mycolic acid biosynthesis is catalyzing the final step in a cycle of elongation by reducing 2-trans-enoyl chains to their saturated forms (Takayama et al., 2005).

Isoniazid (INH) has been used to treat tuberculosis since 1952. It is a prodrug that enters cells via passive diffusion where is it then converted from an inactive nontoxic form to its active toxic form. This conversion is mediated by KatG, a mycobacterial multi-functional catalase-peroxidase that activates INH by peroxidation to form a variety of intracellular INH-derived radical species. The key intermediate in this reaction is the isonicotinoyl radical (Figure 1, compound 3) which then covalently binds to coenzyme NAD+ to generate an isonicotinoyl-NAD adduct (INH-NAD) (Timmins, Deretic, 2006). The addition of the isonicotinoyl radical to NAD+ creates a stereocenter in the INH-NAD adduct, and it is the acyclic 4S isomer that selectively binds to InhA as a slow tight-binding competitive inhibitor (K = 0.75 nM) (Rozwarski et al., 1998; Rawat et al., 2003). The binding of the INH-NAD adduct inhibits InhA from taking part in mycolic acid biosynthesis leading to impaired cell wall integrity and eventual cell death.

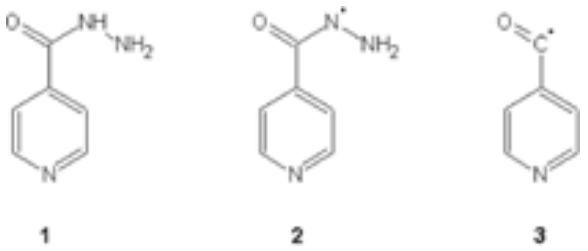


Figure 1
Structures of isoniazid (1), the isonicotinic hydrazyl radical (2) and the isonicotinoyl radical (3) Timmins, Deretic, 2006

Despite its powerful anti-TB potential, the foremost problem with INH is the increasing resistance to the drug. In fact, INH resistance is the most common type of drug-resistant TB. The predominant mechanism of such resistance involves a single point mutation in the gene that codes for the KatG enzyme which results in an amino acid change from serine to asparagine at position 315 in the heme binding catalytic domain of KatG (Zhang, Yew, 2009). This S315T mutant is observed to contain a narrower heme access channel on KatG (4.7 Å compared to the wild type 6 Å) suggesting that the loss of INH access to the oxidizing site of KatG might be the key to INH-resistance (Timmins, Deretic, 2006). While isolates with this mutation completely lose the ability to form the INH-NAD adducts, most retain good catalase activity and are therefore able to survive despite the mutation (Gumbo, 2011). Less commonly, resistance is also seen to occur by mutations in the promoter region of the InhA gene, leading to overexpression of InhA; or by mutations at the active site of InhA, resulting in lower InhA affinity to the INH-NAD adduct (Zhang, Yew, 2009).

There are many methods of drug development. Older methods rely on trial and error testing of chemical substances on cells or whole organisms. This method is extremely lengthy and expensive as it requires the screening of hundreds of thousands of compounds for activity. A newer method called rational drug design employs prior detailed knowledge of a vulnerable target and its three-dimensional structure to synthesize biologically active compounds that specifically interact with the target. One manner of rational drug design is structure-based drug design, which employs the principle of SAR (Structure-Activity Relationship) that theorizes that similarly structured molecules have similar activities. Thus, if a compound is known to possess a desired activity, the essential functional groups and moieties responsible for this activity can be identified and a variety of new compounds containing those groups can be synthesized. The new compounds will then be tested for efficacy and functional similarity to the parent compound. SAR commonly employs the use of molecular mechanics/dynamics software to analyze and predict the conformations of known molecules and to subsequently model the conformational changes that occur in a bound target molecule. This information is then used to predict how similar synthesized compounds may interact with the target. Additionally, the 3D structure of a target molecule both alone and in complex with the synthesized compounds is frequently obtained through X-ray crystallography and NMR spectroscopy to determine the underlying basis for the efficacy (or lack thereof) of the newly synthesized compounds.

In the following literature review, a variety of SAR studies that attempted to find novel InhA inhibitors are summarized. The majority of the compounds discussed used a backbone of the biocide triclosan to create triclosan derivatives and substituted diphenyl ethers that exhibit InhA inhibition as well as efficacy against a variety of tuberculosis strains. Additionally, arylamide moieties have been confirmed as good InhA inhibitors although additional research is still needed to improve their anti-tubercular activity. Lastly, pyridomycin, a natural anti-Mycobacterial drug was recently rediscovered and shown to be effective against multiple strains of TB, as well as showing inhibition of InhA in a unique manner.

Methods

Literature searches were performed using the Touro College Online Library. In particular, the Health Sciences related databases (Medline, PubMed, Proquest Medical Library (Health and Medical Complete), and EBSCO Multisearch were employed. Additionally, Google Scholar and the Touro Quicksearch option proved to be very helpful in finding the necessary relevant articles. The following keywords were searched: Mycobacteria, tuberculosis, InhA inhibitors, triclosan, triclosan derivatives, high affinity InhA inhibitors, SAR, rational drug design, arylamides, pyridomycin. As a final point, only articles published in scholarly peer-reviewed journals after the year 2005 were included in the search.

Results and Discussion

Triclosan derivatives and diphenyl ethers

The biocide triclosan (Figure 2), originally thought to have non-specific antimicrobial activity, has been shown to target and uncompetitively inhibit enoyl reductases, the family of enzymes that includes InhA (Heath et al., 2001). Consistent with uncompetitive inhibition, triclosan binds to the enoyl reductases in the presence of NAD+. Triclosan is an excellent inhibitor of the E. coli enoyl reductase FabI with a picomolar inhibition constant (K = 7 pM) but is shown to be only a submicromolar inhibitor (K = 0.2 μM) of InhA (Ward et al., 1999). To understand the 30,000 fold difference in triclosan's affinity for FabI versus InhA, crystal structures of FabI in complex with NAD+ and triclosan were compared to structures of InhA in complex with NAD+ and triclosan, so as to compare the ligand bindings and use the information to develop new inhibitors with high affinity for InhA (Sullivan et al., 2006).

The structures of triclosan bound to FabI and to InhA were generally very similar yet an important difference was seen with respect to the ordering of the amino acid loop that covers the substrate

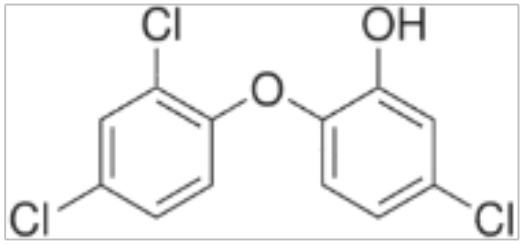


Figure 2:
Molecular structure of triclosan Sullivan et al., 2006

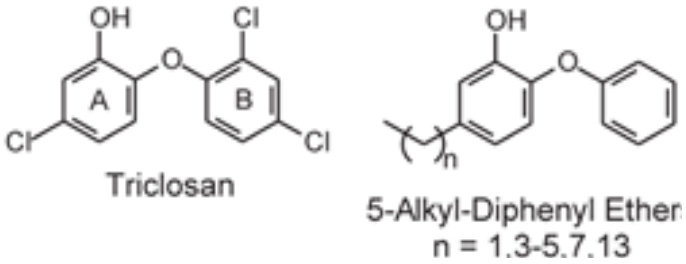


Figure 3
Modified diphenyl ether inhibitors Sullivan et al., 2006

binding site of each enzyme. In the FabI:NAD:triclosan molecule, the substrate binding loop (residues 195-200) is ordered when triclosan is bound to it. In contrast, the substrate binding loop of InhA (residues 197-210) is disordered when triclosan binds to it. It has been suggested that ordering of the loop is indicative of slow, tight binding inhibition (Heath et al., 2001). When the substrate binding loop becomes structured, additional Van der Waals interactions strengthen the binding. A key feature of slow-binding inhibitors is a conformational change in the enzyme upon drug binding. This is likely the slow step in the reaction, a hypothesis that is supported by the fact that triclosan has been identified as a slow, tight binding inhibitor of FabI but not InhA. Since slow-order kinetics has not been determined in the inhibition of InhA by triclosan, the loop is disordered in InhA (Sullivan et al., 2006). Ordering of the loop has been observed in the inhibition of InhA by the INH-NAD adduct, which has also been identified as a tight-binding inhibitor of InhA (Rawat et al., 2003), further supporting the hypothesis that connects slow, tight-binding inhibition to ordering of the active site loop.

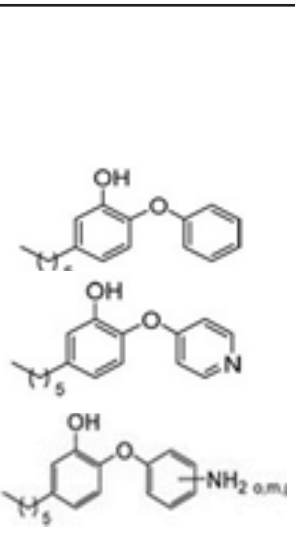
Comparison of FabI and InhA crystal structures showed that InhA contains a larger substrate binding loop than FabI (14 residues vs. 6 residues), making it harder to order the loop in InhA. Sullivan et al. hypothesized that longer substituents on the diphenyl ether A ring of triclosan might result in additional hydrophobic contacts between triclosan and the substrate binding pocket residues, and thereby increase the affinity for InhA. They removed the chlorine atoms from the B ring and substituted the chlorine atom on the A ring with alkyl groups of varying length (Figure 3).

As the length of the alkyl substituents increased from 2 carbons to 8 carbons, the IC value (the concentration of inhibitor needed to inhibit 50% of the enzymes) decreased significantly from 2 μM to 5 nM, indicating considerably higher affinity for InhA. In each case, the compounds were rapid, reversible uncompetitive inhibitors like the parent molecule triclosan. The most potent diphenyl ether, which was identified as 5-octyl-2-phenoxyphenol (8PP), displayed an IC of 5 nM (K =1.1 nM). The octyl chain in this molecule adopted a linear confirmation and burrowed into the pocket, forming numerous hydrophobic interactions with residues in the substrate binding loop which led to the observed significant increase in inhibitory activity. In addition to lower IC values, the alkyl diphenyl ethers showed significantly lower MIC (minimum inhibitory concentration) values than triclosan (MIC 6.5μM vs. 43.1μM) against drug sensitive TB (H37Rv strain) as well as 5 INH resistant strains, thus demonstrating their potential for use against MDR-TB and validating the hypothesis that compounds that do not require activation by KatG will be active against most INH resistant strains of TB. The results of this study revealed that the reason for the differences in triclosan's inhibition of FabI and InhA is that InhA has a large substrate binding loop that triclosan cannot fill as entirely as it can the smaller active site of FabI. Substituting alkyl chains on the triclosan molecule thus allowed for more hydrophobic interactions deeper into the binding site and consequently higher affinity was demonstrated for InhA. Testing of the alkyl diphenyl ethers on H37Rv and 5 INH resistant strains confirmed that this higher affinity also led to greater anti-tubercular activity (Sullivan et al., 2006).

Treatment of TB is lengthy (6-24 months), so toxicity is a significant concern in the development of potential anti-tubercular drugs. The two most potent inhibitors identified by Sullivan et al., 6PP and 8PP, were tested using a Vero cell line and shown to be five times less toxic than triclosan with a therapeutic index of 5-6 compared to <1 for triclosan. Treatment with triclosan at 1xMIC for one day proved lethal to the cells. Furthermore, TB-infected macrophages treated with 2xMIC of 6PP and 8PP demonstrated high level growth inhibition, indicating an ability of these drugs to enter macrophages and remain effective against intracellular tuberculosis growth. Oral administration of 6PP and 8PP in mice also showed no adverse effects, substantiating a lower toxicity for these molecules. These studies demonstrated that alkyl substituted diphenyl ethers exhibited lower toxicity towards cells and TB-infected macrophages than did triclosan (Boyne et al., 2007).

While 6PP and 8PP showed high potency against InhA (nM inhibition constants) and improved efficacy against both drug-sensitive and drug-resistant strains, they were observed to have low bioavailability with significantly high cLogP (partition coefficient) values (6PP= 6.47). The clogP value is important for predicting oral

Table 1

Inhibition and solubility data for modified B ring heterocycles					
Compound	**Structure**	**$IC_{5(}$(nM)**	**MIC (µg/ml)**	**ClogP**	**LogP**
19 (6PP)		11 ± 1	2.1 ± 0.9	6.47	5.76
3c		160 ± 16	3.13	4.97	4.93
14a		62 ± 5	3.13	5.24	
14b		1090 ± 90	100 ± 0	5.24	5.27
14c		55 ± 6	12.50	5.24	4.93

amEnde et al., 2008

absorption as it relates to a compound's solubility and ability to permeate through cell membranes. Compounds that are too hydrophilic (low cLogP) are not able to pass through the hydrophobic membranes, while compounds that are too hydrophobic (high cLogP) tend to be insoluble and have trouble permeating through membranes because they can get stuck in the hydrophobic bilayer. In general, a cLogP greater than 5 predicts poor absorption or permeation (Lipinski et al., 2001).

To lower the cLogP values, modifications were made to the B ring of the diphenyl ether backbone of 6PP. A series of analogs that incorporated different functional groups on the ring, designed to increase the polarity of the alkyl diphenyl ethers, were synthesized. Two series were made: one series replaced the B ring with isosteric heterocycles that incorporated nitrogen atoms into the ring to increase polarity without added steric strain while the other series incorporated polar bulky substituents at different positions on the B ring. The results were primarily stereoselective. Overall, the addition of bulky substituents at either ortho, meta, or para positions of the B ring and the incorporation of most nitrogen heterocycles caused significantly higher MIC values and reduced inhibitory activity. However, the addition of nitro or amino substituents at ortho/para positions only slightly reduced inhibitory activity. While none of the newly synthesized compounds showed IC or MIC values lower than the parent compound 6PP, compounds 3c and 14a (Table 1) had inhibitory values similar to 6PP but also had lower cLogP values (4.97 and 5.24 respectively). The results demonstrate that the addition of nitrogen containing compounds at certain positions on the B ring can lower the hydrophobicity of the compounds. Additional studies are needed to test whether the modifications result in increased bioavailability and improved in vivo antibacterial activity (amEnde et al., 2008).

InhA has two hydrophobic cavities that are capable of being filled: the hydrophobic substrate binding pocket in which the substrate of InhA (2-trans-dodecenoyl-CoA) binds, and the NADH binding

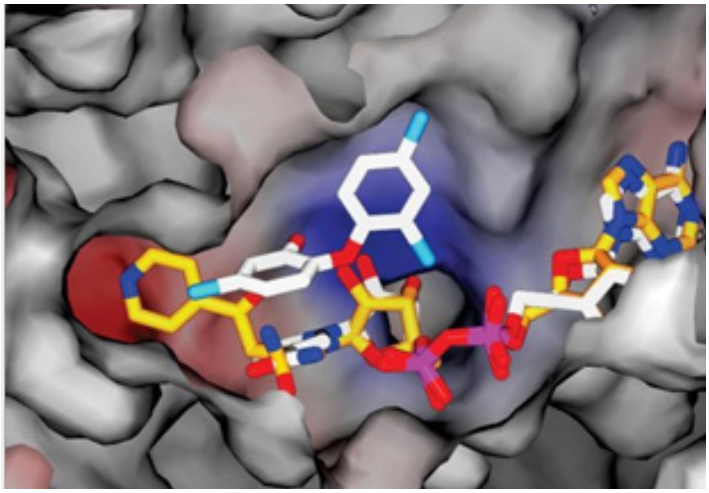


Figure 4:
Cross-section of the InhA active site with superimposed structures of InhA in complex with the INH-NAD adduct (colored in gold) and triclosan (white). The A ring chlorine of triclosan (cyan) is observed to be very close to the isonicotinoyl binding site. Freundlich et al., 2009

active site. The NADH active site sits underneath the substrate binding pocket and is lined primarily with hydrophobic residues. Most InhA inhibitors, including triclosan and its derivatives, inhibit InhA through binding to the substrate binding pocket. The competitive inhibitor INH-NAD adduct, is one of the few inhibitors that binds directly to the NADH active site. Freundlich et al., (2009) observed the structure of InhA:NAD+:triclosan superimposed on InhA:INH:NAD and saw that the chlorine atom at position 5 of the triclosan A ring was only 2 Å from the binding pocket of the isonicotinoyl moiety of the INH-NAD adduct (Figure 4). They hypothesized that triclosan derivatives can be synthesized that can extend into the hydrophobic isonicotinoyl binding pocket and increase inhibition of InhA by occupying both the substrate binding pocket and the NADH binding site. This hypothesis was different than Sullivan et al. (2006) who created analogs to increase interactions with the substrate binding loop, mimicking the structure of the substrate.

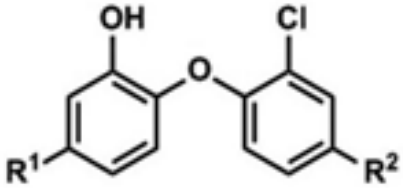
The analogs of Freundlich et al., were similar to those of Sullivan et al., but differed in that a variety of both hydrophobic and hydrophilic substituents were added to position 5 on the A ring. Additionally, contrary to the previous studies of Sullivan et al., it was suggested that the two chlorine atoms on the B ring do in fact participate in hydrophobic interactions with Phe97 and Met103 residues and so they remained in the compounds. The results showed that the compounds with alkyl (hydrophobic) substituents were the more potent inhibitors. As seen with Sullivan et al., inhibition of InhA increased with increasing alkyl chain length. The highest inhibitory potencies for the alkyl chain additions were observed in the 4 carbon chain compounds (Table 2), namely compound 10 (Freundlich et al., 2009).

Surprisingly, while most hydrophobic substituents were observed to show lower IC values, the addition of a phenyl group at the 5 position (Compound 6) showed a much greater IC (IC >10,000 nM) than even triclosan (IC =1,100 nM). The same values were obtained for a pyridyl group at the 5 position. Modeling the inhibitors in the active site showed that the aryl group was too far from the isonicotinoyl binding pocket by 2.5 Å and was also positioned too close to Phe149 which led to steric clashes. Therefore, to better fit the phenyl group into the hydrophobic pocket, a carbon linker chain consisting of 1-3 carbons was added to the 5 position (Compounds 24-26) between the phenyl group and the A ring. This resulted in significantly increased inhibitory potency of the phenyl substituted compounds (IC of 21-51 nM). Compound 25, with a 2 carbon linker chain showed the lowest IC of all the compounds tested with a measured potency 50 times that of triclosan. Adding linkers to the pyridyl substituents (Compunds 17-19) also proved effective in producing powerful inhibitors (IC of 29-75 nM).

Through analysis of crystal structures, it became apparent that the goal of mimicking the isonicotinoyl moiety and reaching the isonicotinoyl binding site was not possible. The triclosan derivatives did reach the isonicotinoyl binding site but they did not occupy the same volume as the INH-NAD adduct. Instead, their actions mimicked the substrate analogs like Sullivan et al.'s compounds did. Attempts to increase the volume of the triclosan analogs in order to mimic the isonicotinoyl moiety would cause a rotation of Phe149, which in turn would displace Tyr158 and result in the loss of a crucial hydrogen bond, leading to the complete loss of inhibitor binding. Therefore, it was concluded that triclosan derivatives can only attempt to place their substituents near the binding pocket and increase hydrophobic interactions in a way that mimics the substrate rather than the isonicotinoyl-NAD adduct (Freundlich et al., 2009).

Six of Freundlich et al.'s compounds (three alkyl and three aryl with IC < 110 nM) were tested for anti-tubercular activity. All of the compounds were more active than triclosan against H37Rv strain (wild type). Additionally, all showed high inhibition against five INH resistant strains demonstrating their potential for use in treatment against MDR-TB. These results revealed that while triclosan analogs cannot be used to mimic the INH-NAD adduct in the isonicotinoyl binding pocket they can act as substrate analogs. The aryl and pyridyl substituents with carbon chain linkers as well as many straight chain substituents showed considerably lower IC values than triclosan, displaying higher affinity for InhA. They also showed greater anti-tubercular activity than triclosan when tested against both drug-sensitive and drug-resistant tuberculosis (Freundlich et al., 2009).

Table 2

In vitro activities of select triclosan derivatives against InhA			
			
Compound	R^1	R^2	InhA IC_5(nM)
Triclosan	Cl	Cl	1100 ± 180
6	Ph	Cl	>10000
10	$(CH_2)_3$ CH_3	Cl	55 ± 20
14	2-pyridyl	CN	>10000
15	3-pyridyl	Cl	>10000
16	4-pyridyl	CN	>10000
17	CH_2 (2-pyridyl)	Cl	29 ± 11
18	CH_2 (3-pyridyl)	Cl	42 ± 10
19	CH_2 (4-pyridyl)	CN	75 ± 16
24	CH_2 Ph	Cl	51 ± 6
25	$(CH_2)_2$ Ph	Cl	21 ± 8
26	(CH_2)3 Ph	Cl	50 ± 14

Feundlich et al., 2009

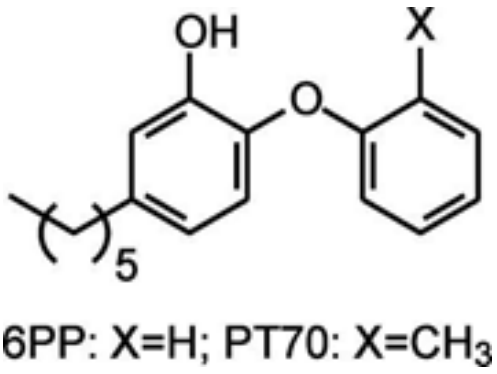


Figure 5
Addition of a methyl group on the ortho position of the B ring of 6PP led to PT70, a slow onset, tight binding diphenyl ether inhibitor. Luckner et al., 2010

One problem with the triclosan derivatives and diphenyl ethers thus far is that they are shown to be rapid reversible inhibitors of InhA. An important and vital factor of in vivo drug activity is long residence times on the targets. Furthermore, structures of the previous compounds had indicated a disordered substrate binding loop. It had earlier been theorized that slow onset inhibition is coupled with ordering of an active site loop, which leads to the closure of the substrate binding pocket. Luckner et al., (2010) attempted to create a slow onset triclosan-derived inhibitor of InhA using the hypothesis that compounds with the ability to order the loop will be slow tight-binding inhibitors of InhA. They used Sullivan et al.'s 6PP analog as a backbone and introduced a variety of groups onto the B ring in the hopes of promoting increased hydrophobic interactions between the inhibitor and the loop residues.

One resulting compound, PT70 (Figure 5), contained a methyl group ortho to the diphenyl ether linkage and showed a substantially higher affinity for InhA, with a K of 22 pM, a value 430-fold lower than that of 6PP (K =9.4 nM). Additionally, PT70 displayed slow-onset inhibition which is essential for in vivo antibacterial activity. Crystal structures of InhA:NAD+:PT70 showed that PT70 binds to InhA in almost the same way that triclosan and its derivatives do. The main difference in the binding is that the methyl group on PT70 generates additional hydrophobic interactions with Ala198, Met199, Ile202 and Val203 residues on the substrate binding loop, leading to a defined loop structure (Figure 6). The

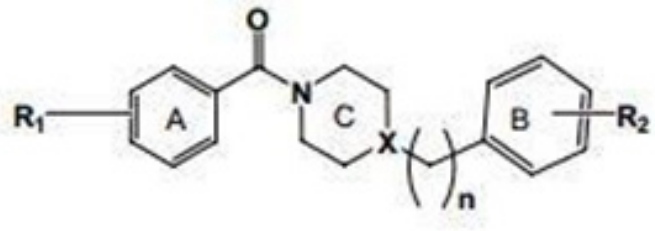


Figure 7
Scaffold of He et al.'s arylamides He et al., 2007

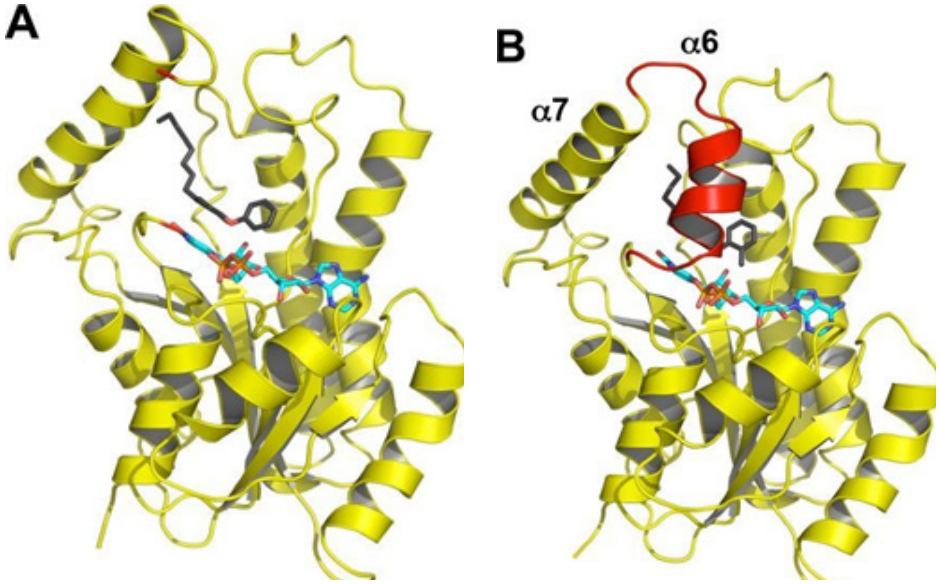


Figure 6
Loop ordering is observed upon slow binding inhibition. A) InhA•NAD+•8PP complex in the binding of 8PP (Sullivan et al.'s most potent inhibitor) to InhA. NAD+ is displayed in cyan and the 8PP molecule is in black. The substrate-binding loop is disordered in the 8PP structure, and the loop ends are depicted in red. B) InhA•NAD+•PT70 complex. The PT70 compound is depicted in black. The substrate-binding loop (shown in red) is ordered in this structure and covers the binding pocket. Luckner et al., 2010

ordered binding loop covers the entrance to the binding pocket locking PT70 inside and increases the residence time on the target. Kinetic data show that PT70 has a residence time of 24 minutes on the target, a 14,000 fold increase over the residence time of its parent compound 6PP (0.1 sec). This study also confirmed the hypothesis that ordering of the active site loop is coupled to slow-onset inhibition. Further research is needed to test PT70 on tuberculosis strains to confirm that the increase in affinity of PT70 for InhA does indeed correlate with enhanced anti-bacterial activity (Luckner et al., 2010).

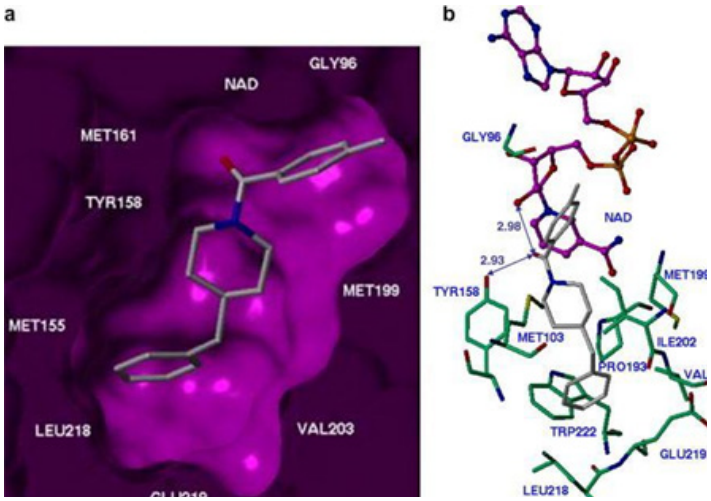


Figure 8

a) Inhibitor b3 bound to the active site of InhA. As illustrated, extra space is observed in the area below the B ring of the inhibitor b) Details of the InhA-b3 interactions. Relevant residues of the binding pocket are shown. The amide oxygen hydrogen-bonds with the 2`-hydroxyl moiety of the nicotinamide ribose and the hydroxyl group of Tyr158 (blue arrows). He et al., 2007

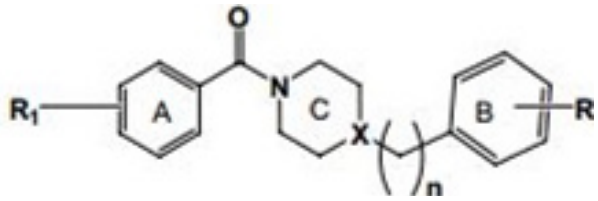
Triclosan has been recognized as a great molecular scaffold for InhA inhibition. The previous studies all employed the diphenyl ether backbone of triclosan. Modifications to both the A and B ring demonstrated increased affinity for InhA which was also coupled to greater anti-tubercular activity. The compounds show tremendous promise for rational drug design and SAR use in the treatment of TB. Significantly, it was confirmed that these compounds do not require KatG activation affording them great potential against the most common strains of drug-resistant TB. Further research is still needed to test the compounds in vivo to confirm their anti-tubercular capacity.

Arylamides

Arylamides (Figure 7) have also been identified as a potential scaffold of InhA inhibitors. Using High Throughput Screening (HTS), He et al., (2007) identified a variety of arylamides all of which contained either a piperazine or piperidine compound as the core structure. Compounds with the piperazine scaffold exhibited the best inhibitory activity among the initial compounds tested, with compound a4 being the most potent inhibitor (IC =3.07 μM) (Table 3). In order to enhance inhibition, He et al. crystallized Compound b3 (IC = 5.16 μM), containing a piperidine with InhA and NADH and that structure was used as a reference for future compounds. The key hydrogen bond seen in all enoyl reductase inhibition reactions, involving the catalytic residue Tyr158 was observed in the structure. The carbonyl oxygen of the arylamide was hydrogen bonded to both the 2'-hydroxyl moiety of the nicotinamide ribose and to the hydroxyl group of Tyr158. The unsubstituted B ring fit into the substrate binding pocket and interacted with many hydrophobic residues there. However, underneath the B ring, extra space was observed (Figure 8). He et al. hypothesized that addition of hydrophobic substituents to the B ring could form more interactions and lead to higher affinity (He et al., 2007).

Table 3

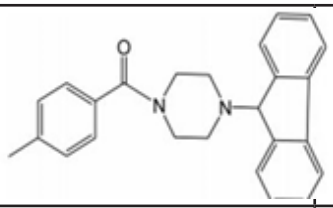
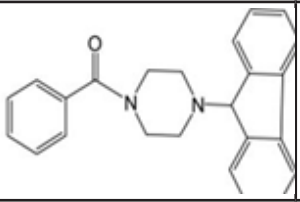
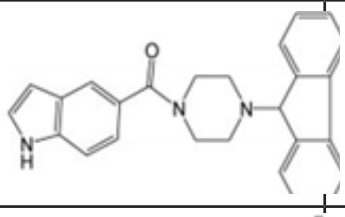
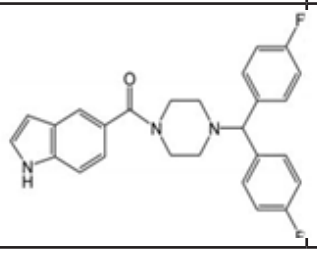
The most potent inhibitors found by He et al. using High Throughput Screening



Compound	*X*	*N*	*R¹*	*R²*	*IC $_{50}$ (μM)*
a4	*N*	*0*	*4-CH3*	*3-Cl*	*3.07 ± 0.48*
b3	*C*	*1*	*4-CH3*	*H*	*5.16 ± 0.45*

He et al., 2007

Table 4

Inhibition activity of newly synthesized arylamides			
	p1	99	0.4±0.02
	p2	97	0.09±0.00
	p3	94	0.2±0.01
	p4	84	1.04±0.04

He et al., 2007

A variety of arylamides containing the piperazine scaffold were synthesized. 12 of the 60 compounds synthesized showed high InhA inhibitory activity. All 12 contained large polyaromatic moieties on the B ring, substantiating the hypothesis that larger substituents on the B ring form more interactions and lead to higher affinity. Compound p2 (Table 4) of the newly synthesized molecules showed the highest inhibitory activity (IC = 90 nM), a 34 fold decrease in IC when compared to compound a4, the most potent of the HTS compounds (He et al., 2007).

Unfortunately, when the new compounds were tested against the H37Rv strain for anti-tubercular activity, most had MIC values >125 μM (He et al., 2007). This data suggests that the inhibitors do not possess optimal membrane permeability, likely due to their amphiphilicity, caused by the hydrophobicity of the B ring substituents and simultaneous hydrophilicity of the potentially protonated C ring piperazine.

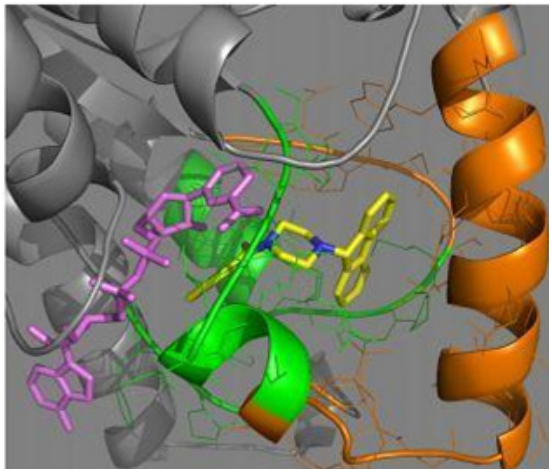


Figure 9

Compound p2, the most potent arylamide inhibitor synthesized by He et al. (yellow) with NADH (purple) in the InhA substrate binding site. The rigid residues around the binding site are displayed in green, while the flexible residues are displayed in orange. As discussed, the A ring of the arylamide is situated near the rigid residues, while the B ring is near the flexible residues. Punkvang et al., 2010

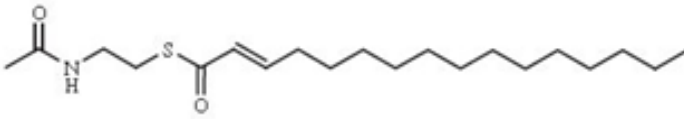


Figure 10a
Structure of the substrate analog, trans-2-hexadecenoyl-(N-acetylcysteamine)-thioester Punkvang et al., 2010

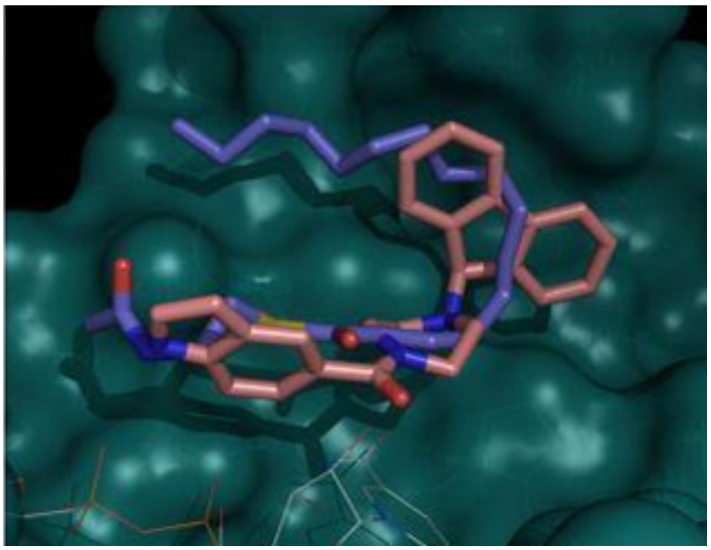


Figure 10b
Superimposition of x-ray structures of compound p3, an arylamide inhibitor synthesized by He et al. (pink) and fatty acid substrate (purple) in the InhA substrate binding site. As discussed, the A ring binds to the hydrophilic part of the binding site where the acetylcysteamine moiety of the substrate binds. The B ring is shown to bind to the hydrophobic part of the binding site where the fatty acid chain of the substrate binds. Punkvang et al., 2010

Punkvang et al. (2010) attempted to identify the reasons behind the suboptimal anti-tubercular activity of the arylamides and to propose modifications to the compounds to increase both their affinity for InhA and anti-tubercular activity. Using molecular dynamics, a computer simulation that mathematically predicts the physical movements of atoms and molecules, they investigated the structural features and dynamic behavior of the InhA-inhibitor interactions providing detailed information about the molecule's flexibility and conformation. RMSF (root mean square fluctuation) of residues around the substrate binding site of InhA was calculated to reveal the mobile flexibility of the residues. Residues 96-99, 155-165, and 192-200 (located around the binding pocket of the aryl A ring of the arylamide) were shown to be too rigid to bind the arylamide inhibitors (Figure 9). In contrast, most of the residues around the aryl B ring binding pocket (100-104, 149-154, 201-223) indicated greater flexibility consistent with He et al.'s proposal that additional large substituents can be added to the B ring to increase the number of hydrophobic interactions (Punkvang et al., 2010).

Crystal structures and subsequent kinetic studies identify arylamides as competitive inhibitors of the substrate binding site of InhA. Consequently, arylamides should mimic the binding of the substrate. Superimposition of arylamide inhibitors bound to InhA and a substrate analog (trans-2-hexadecenoyl-(N-acetylcysteamine)-thioester) (Figure 10a) bound to InhA shows the conformational change that occurs in the enzyme when the arylamide or the substrate bind. It was observed that the A ring of the arylamide binds to the hydrophilic part of the pocket where the hydrophilic acetylcysteamine moiety of the substrate binds. In contrast, the B ring lies in the same site as the fatty acyl chain of the substrate surrounded by flexible hydrophobic residues (Figure 10b). The hydrophilic A ring is favored in the hydrophilic site and the hydrophobic B ring substituent is favored in the area with the hydrophobic residues (Punkvang et al., 2010).

Based on this data, Punkvang et al. proposed a series of modifications to the arylamides to mimic substrate binding and increase inhibitory activity. Introduction of small hydrophilic substituents such as an NH moiety or an acetyl oxygen on the A ring can be used to mimic the analogous part of the substrate and increase the hydrophilicity of the ring. Care must be taken, however, to ensure that the substituents are not too large and do not lead to steric interactions with the inflexible A ring residues and preclude proper binding as discussed. Additionally, binding affinity of arylamides can be increased with the addition of bulky hydrophobic substituents to the B ring (Punkvang et al., 2010).

These results explained the findings of He et al. and led to suggested future modifications of arylamides that may increase their inhibitory potency. Molecular dynamic simulations showed that only the hydrophobic part of the substrate binding pocket is flexible enough to bind arylamide inhibitors. Therefore, the addition

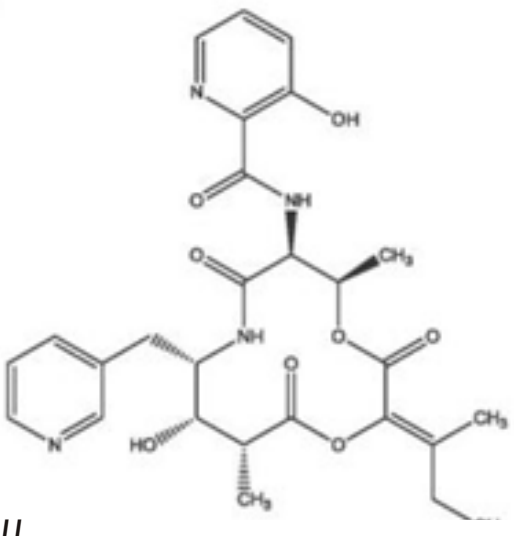


Figure 11
Pyridomycin structure Hartkoorn et al., 2012

of bulky hydrophobic substituents on the B ring can increase the binding affinity for the arylamides and also potentially increase membrane permeability. More research is needed to synthesize these proposed compounds and to measure their InhA inhibitory properties as well as their anti-tubercular properties and to see if membrane permeability indeed increased.

Pyridomycin

Discovered in 1953, pyridomycin (Figure 11) is a natural antibiotic produced by the bacteria Streptomyces pyridomyceticus and Dactylosporangium fulvum that exhibits specific activity against Mycobacteria (Maeda et al., 1953). Nevertheless, since it was only discovered a short time after the introduction of isoniazid as an exceedingly effective treatment for tuberculosis, pyridomycin was neglected and research into the drug ceased. Presently, with the rise of INH-resistant strains of TB, it is imperative to reexamine effective alternatives to INH. Despite earlier reports of pyridomycin's effectiveness against Mycobacteria as a genus, its efficacy against M. tuberculosis specifically had not yet been completely demonstrated. Additionally, pyridomycin's target and mechanism of action had also not yet been elucidated. Hartkoorn et al. (2012) ascertained the drug's target and specificity, and assessed its efficacy against TB.

Pyridomycin was tested to confirm earlier reports about its specificity, efficacy, and low toxicity. The new research showed pyridomycin to be a competent inhibitor of all species of Mycobacteria including tuberculosis (MIC= 0.62-1.25 µg/ml). It remained ineffective against other genera of both gram positive and gram negative classification (MIC > 100 µg/ml), confirming its specificity to a feature unique to Mycobacteria. It effectiveness against TB-infected THP-1 derived macrophages was verified which indicated its ability to physically enter macrophages and inhibit growth. Toxicity testing demonstrated pyridomycin's higher selectivity for tuberculosis than for human cells with a selectivity index of >100-fold. (Hartkoorn et al., 2012)

The identity of the target of pyridomycin was obtained by selecting for mutant pyridomycin-resistant strains (PYR7) on solid medium containing pyridomycin at 10xMIC (3µg/ml). The genome of mutant PYR7 was referenced to H37Rv (wild type strain) and an a443g point mutation in InhA was isolated in PYR7 which resulted in replacement of the aspartic acid at position 148 by a glycine (D148G). Asp148 was previously identified in the binding pocket of InhA thus validating this as pyridomycins's target. Subsequent experiments involving overexpression of InhA and the resulting increase in pyridomycin resistance further confirmed these findings (Hartkoorn et al., 2012).

Since the primary goal of research into pyridomycin was to determine its potential as an alternative to isoniazid, pyridomycin was tested against INH-resistant strains of TB. KatG mutants

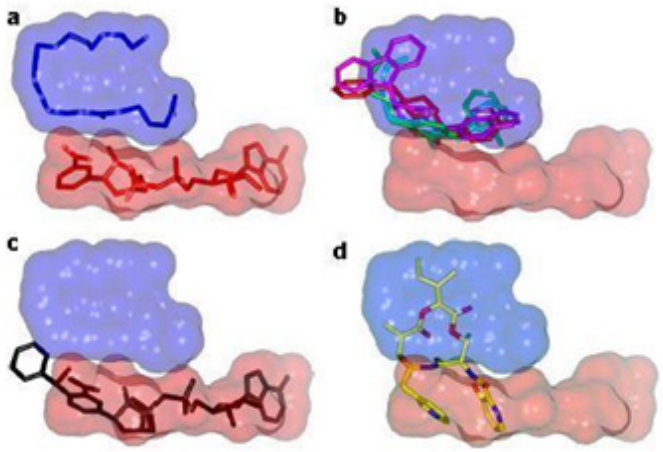


Figure 12

Binding comparison of various InhA inhibitors. The blue area represents the lipid substrate binding pocket, while the red area represents the NADH binding site. a) The lipid substrate is bound to the substrate binding site. b) Triclosan derivatives have all been shown to bind solely to the substrate binding site. c) The INH-NAD adduct binds solely to the NADH binding site. d) Pyridomycin binds to the NADH binding site and, because of its large size, extends into the lipid substrate binding site as well. Hartkoorn et al., 2014

(the majority of the resistant strains), despite being highly resistant to INH (MIC >10 µg/ml), showed no resistance to pyridomycin. This demonstrated that pyridomycin does not depend on catalase-peroxidase bioactivation and therefore holds increased potential for use against the more common KatG INH-resistant strain (Hartkoorn et al., 2012). The results of this research confirmed previous reports of pyridomycin's selectivity for and efficacy against tuberculosis including H37Rv and INH-resistant strains.

As discussed earlier, InhA contains two essential sites: the NADH binding site and the lipid substrate binding pocket. Crystal structures and kinetic experimentation show that pyridomycin is a direct competitive inhibitor of NADH binding in InhA and occupies the NADH binding site. Pyridomycin forms several interactions with residues in the NADH binding site including the crucial hydrogen bond between the side chain of Tyr158 and the C7 carbonyl oxygen of pyridomycin. Loss of this hydrogen bond and consequent lack of the crucial enoyl reductase-inhibitor interaction in D148G mutants (pyridomycin-resistant InhA) resulted in markedly decreased affinity for pyridomycin. Crystal structures show that, as a result of its unusually large size, pyridomycin not only occupies the NADH binding site, but its upper hemisphere also extends into the substrate binding pocket forming a hydrophobic interaction with Phe149. While previously identified InhA inhibitors exclusively inhibited only one of the sites, pyridomycin is the first published compound to bridge the gap between both sites and simultaneously inhibit the lipid substrate binding pocket as well as the NADH binding site (Figure 12) (Hartkoorn et al., 2014).

Pyridomycin is a naturally produced antibiotic and efforts to synthesize it have been hampered by the inability to form the double bond of the enol ester moiety between C1 and C2. The compound was instead synthesized with a saturation in that position (and a replacement of the sec-butyl group with a symmetrical isopropyl group) to identify if the double bond was indeed necessary for anti-tubercular activity. Saturation generated a new stereocenter, producing two stereoisomers: the R isomer (OH141) and the S isomer (OH139) (Figure 13). Both compounds, as well as natural pyridomycin were then tested for anti-tubercular activity. Pyridomycin was the most effective against TB (MIC = 0.39 µg/ml). OH139 had a MIC value 32-fold higher than pyridomycin while OH141 measured only 4-fold higher, indicating that the enol ester moiety is not actually a critical requirement for anti-TB activity (Horlacher et al., 2013).

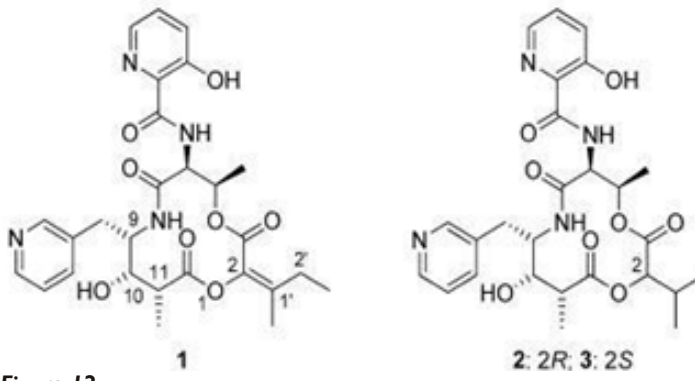


Figure 13

Pyridomycin (1) and the newly synthesized compounds (2,3) with a saturation in the C1-C2 bond and replacement of the sec-butyl group with an isopropyl group. A new stereocenter led to the production of two stereoisomers: the R isomer (OH141) and the S isomer (OH139). Horlacher et al., 2013

X-ray crystallography and in silico modeling highlight the reasoning behind the difference in efficacy between the two stereoisomers. Superposition of InhA:pyr on InhA:OH141 show pyridomycin and OH141 occupying the same position in the active site. The isopropyl group of OH141 was found in the equatorial position avoiding any steric clashes with residues in the active site. OH139 however, was shown with its isopropyl group in a steric interaction with the side chain of Met199 in the substrate binding loop, indicating why OH139 has a lower affinity for InhA than does OH141 (Horlacher et al., 2013). The slight decrease in the activity of OH141 compared to pyridomycin could be due to the lower hydrophobicity of the isopropyl group compared to the 2-butan-2-ylidene substituent. The results of this research demonstrate that pyridomycin can be synthesized with a saturation in the C1-C2 bond without significantly impacting its anti-tubercular activity. This establishes the potential for future SAR work in the development of pyridomycin-derived drug candidates for TB treatment. Current research is now focused on using OH141 as a scaffold for synthesis of new pyridomycin variants.

Conclusion

In this review, SAR has been shown to be an effective means to structurally design and modify drugs to increase their InhA inhibition abilities. The underlying assumption of the research was that compounds that do not require KatG activation would be effective against most INH-resistant strains. This hypothesis proved correct in the case of triclosan and pyridomycin derivatives. Additional modifications to arylamides are needed to optimize their activity. Through analysis of crystal structures, along with computer-assisted molecular dynamics and modeling, a variety of high affinity compounds were synthesized. Current efforts are now concentrated on optimizing the in vivo properties of these novel compounds.

Abbreviations

TB	Tuberculosis
MDR-TB	Multi Drug Resistant Tuberculosis
XDR-TB	Extensively Drug Resistant Tuberculosis
INH	Isoniazid
SAR	Structure Activity Relationship
IC_{50}	Half maximal inhibitory concentration (the concentration of inhibitor at which half of the enzymes are inhibited)
K_i	Inhibitory constant
MIC	Minimum Inhibitory Concentration
H37Rv	Wild type strain of TB
cLogP	Calculated partition coefficient

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Pediatric Gastroesophageal Reflux Disease

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Abstract

This objective of this review is to present the known data in regards to Gastroesophageal Reflux Disease (GERD) in infants. Articles with relevant definitions, diagnosis and treatment options were evaluated. It is evident that much controversy exists in the diagnosis and treatment of this disease, and there is the question as whether this disease can be called GERD. Current ability to attribute the symptoms infants present with the disease is still difficult to clarify, despite the fact that as many as 60% of infants show symptoms of this disease. The current testing options have proven to be insufficient in concretely diagnosing infants. Treatment for GERD has proven to be controversial as well. The medications for acid suppression are not a guaranteed cure and are being proven to have a lack of efficacy, show adverse effects and other negative aspects. The invasive options are not always ideal either. Altering changes in lifestyle helps, but is usually not the cause or cure when it comes to infants. Research is being done to come up with a test that is a definitive diagnosis, as well as a treatment option that is completely effective. Research has shown that despite the fact that acid suppression therapy is the most common answer by physicians today, it does not effectively work to eliminate all the symptoms. This does suggest that either GERD is not the condition, or that modern therapies and treatments for reflux are not effective. Further research is needed on the subject.

Introduction

Does Pediatric Gastroesophageal Reflux Disease (GERD), also known as acid reflux, exist? GERD is a condition in which over 60 million Americans suffer from at least once a month (Maddox 2012); this condition is commonly called heartburn. Acid reflux is not the typical pain felt after eating a heavy meal or the wrong kinds of foods. It is a much stronger and severe condition. Reflux is defined as the passage of gastric contents into the esophagus and is a physiologic process. (Vandenplas, et al. 2009) Doctors have questioned whether it is a possibility for infants and children to suffer from such a condition, which is often believed to come with age or bad eating habits. The NIH and many other prestigious organizations and hospitals in the United States and around the world are doing extensive research on this condition (Nelson, et al. 1997). This review will attempt to present the known research on the acid reflux-like symptoms in infants and children and what evidence there is to prove if it is indeed pediatric GERD, as well as present the possible medical ways of alleviating their suffering.

Methods

General websites like NIH.gov, and Webmd.com, with definitions of what GERD is were accessed first and reviewed to gain a general knowledge about GERD. The NIH's scholarly website, Pubmed, with original research and review articles from all types of medical and research journals was accessed. Information about GERD in infants was evaluated. General data regarding GERD, GERD in infants and any diagnostic testing and treatment of the disease were researched and evaluated for relevant information. In Touro College's library, the Touro Ebsco search engine was accessed and utilized to locate useful articles and original research papers as well.

Discussion

Many people feel the sensation of food "coming up" on them after eating heavy foods. This sensation may indeed be food escaping the lower esophageal sphincter and rising upward into the esophagus (fig.1). There is no cause for alarm when the person does not feel any troubling symptoms as a result of this; at this stage the condition is not called GERD and is not labeled a disease (Rudolph, et al., 2001). It is simply called GER, Gastroesophageal Reflux. Once the condition crosses over to the point of symptoms with major side effects, it is called GERD, Gastroesophageal Reflux Disease. The same parameters of diagnosis are applied for infants. When a child is thriving and growing healthily, despite showing symptoms for GERD, it is not regarded as a disease (Vandenplas, et al. 2009). The symptoms for this condition in infants include spitting up, vomiting, refusal to eat, feeding difficulties, colic, incessant crying, arching the back and neck, hoarseness and cough (Brodsky, et al. 2000). Some say that there are even a wide range of respiratory problems as a result of acid reflux as well. The problem with

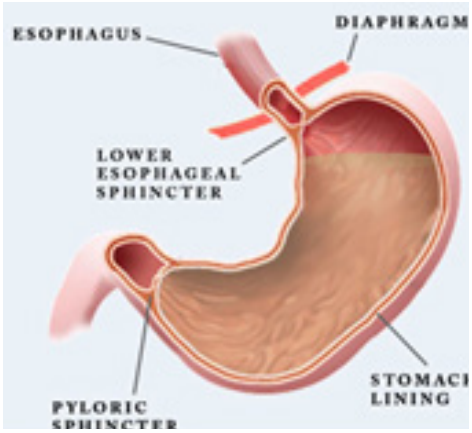

Figure 1
Reflux Defense 2009

diagnosing GERD based solely on an infant's symptoms is that these are symptoms for many other conditions as well (Ghezzi, et al. 2010). Guaranteeing that they are a result of reflux is very hard to prove.

Many infants may show signs for some or all of these symptoms. When it comes to diagnosing adults, doctors can rely on asking the patient about the symptoms they feel. The patient would be able to describe when, how, what time of day, before or after or even during meals, what specifically they feel regarding their symptoms. However, it is very different with an infant. They do not have the ability to specifically communicate what they are feeling. Infants have one means of communication for all of their necessities and issues, good or bad. Infants cry as their means of indicating something is not right with them. Parents are asked many questions to help doctors understand what exactly their child may be feeling and what symptoms they may have (Deal, et al. 2005). Figures 2 and Table 1 have a list of some of the questions a doctor might ask the parents. Doctors have to rely on the parents of the infants to relate what they presume is going on with their child. This hinders the ability to properly diagnose the disease tremendously.

Site No.	Patient No.	Patient Initials ☐☐☐	Visit ID		
Date Completed ☐☐ ☐☐☐ ☐☐ D D M M M Y Y					
Relationship to Patient ☐ Mother ☐ Step Mother ☐ Grandmother ☐ Guardian ☐ Father ☐ Step Father ☐ Grandfather ☐ Other, *specify* _____					
SYMPTOMS			**QUESTION A** How many times did each symptom occur in the past 7 days? *(such as 0, 1, 2, 3, etc)*		
1. **VOMITING / REGURGITATION** Throwing-up / swallowing food, or liquids that have come back up into the infant's mouth.			☐☐ Times in the past 7 days **(Do not leave blank)**		
2. **CHOKING / GAGGING**			☐☐ Times in the past 7 days **(Do not leave blank)**		
3. **ARCHING BACK**			☐☐ Times in the past 7 days **(Do not leave blank)**		
4. **IRRITABILITY / FUSSINESS** Episodes of crying during feeding or inconsolable.			☐☐ Times in the past 7 days **(Do not leave blank)**		
5. **REFUSAL TO FEED**			☐☐ Times in the past 7 days **(Do not leave blank)**		
TOTAL			☐☐ **Total should be > 16**		

Figure 2
Cohn, et al. 1996

GERD is considered a very complicated disease to diagnose. As of now there is no one test that has received the "gold star" as the perfect test to diagnose the disease. (Arko, et al. 2009) When it comes to children, the process of diagnosing is even harder. Much of the research on the subject begun because of parents coming forward with their infants because they did not understand what is wrong with their children. Parents have come forward when a child refuses to eat as an infant (from as early as four weeks old.) Other parents came forward because of incessant vomiting;the infant would not stop vomiting and presented no signs of infection or virus (Omari, et al. 2002). These are just two of the more common symptoms that cause parents to approach a doctor about their child's issue. Without parents taking the initiative to ask the doctor what issue their child has, research may not exist.

One wonders if the parents themselves are a blind spot in the diagnosis. If the parents have GERD themselves, it may hinder their ability to objectively see what is going on with their child and relay all pertinent information to the doctor. Another aspect that may need to be brought for further research is whether there is a genetic component to GERD. If the parents have it as adults, could their children have it as infants? Do doctors and researchers need to take this into account when setting up parameters for diagnosis and treatment?

There exists a few means of testing patients for GERD. The problem with many of them is that they are not perfect and by no means perfect for infants. Another issue that arises is that many doctors suspect that reflux is just a cover for a real condition that is going on in the body, like anatomical abnormalities or hernias, for example (Rudolph, et al., 2001). The doctors questioned whether they should begin invasive testing for severe conditions and possible surgeries, or to first try out the known protocols for testing for reflux and the treatments done on adults . As many of these doctors do not believe reflux exists in infants, they begin intense procedures to discover what is really wrong with the child. One theory was that the H. Pylori bacteria was causing over production of acid in infants. This theory was soon dismissed as eradicating the bacteria made no change in the patients' condition (Maris, et al. 2013). Other doctors who do not believe it exists either,take the approach of ignoring the matter and waiting until the child outgrows the condition, which is often the case with infants (Carroll, et al. 2002). Most research indicates that infants do outgrow this condition by 24 months of age. A third approach is to follow the protocol done on an adult, with the future risks to the infant still unknown.

Testing

The first test commonly done when reflux is suspected is called the Barium Study Swallow Test. (Arko, et al. 2009). This test is considered to be the least invasive test of all the reflux diagnostic tests. This test is done to determine if there are any blockages in the anatomy of the patient or anomalies or conditions that do not belong there. The infant is given Barium Sulfate, a white substance, to drink; Barium Sulfate is opaque to x-rays causing a contrast in the body. X-rays are taken immediately and during swallowing of the liquid to highlight the upper gastrointestinal tract and show if anything is out of the ordinary. This test does not indicate any form of acid levels in the body or if reflux is the diagnosis. It simply rules out other issues like pyloric stenosis, tracheoesophageal fistulae as well as other abnormalities associated with the upper gastrointestinal tract.

Figure 3: Questionairre

1.a	1	*Does your child vomit or regurgitate > 1x / day
1.b	2	*Does your child vomit or regurgitate > 3x / day
1.c	3	*Does your child vomit or regurgitate > 5x / day
2.a	4	*Is the mean volume > 1 coffee spoon?
2.b	5	*Is the mean more than 1 soupspoon?
2.c	6	*Is the mean more than 3 soupspoons?
	7	Is the vomit/regurgitation projectile (with force)?
3	8	*Is the regurgitation painful?
4	9	*Does your child, according to your opinion, cry "too much"?
5.a	10	*Does your child cry more than 1 hour a day?
5.b	11	*Does your child cry more than 3 hours a day?
6	12	*Does your child cry during and after feeding?
7	13	*Does your child refuse feeding even when hungry?
	14	Does your child burp difficulty?
8	15	*Is the weight gain OK?
	16	Does respiration make a lot of noise?
9.a	17	†Did your child have an apnea with cyanosis?
9.b	18	†Did your child have an apnea with "tossing"?
9.c	19	†Did your child have an apnea with cyanosis and tossing?
	20	Did your child have a pneumonia?
	21	Did your child have a bronchitis?
	22	Does your child have chronic coughing?
10	23	*Does your child have hiccups more frequently than normal?
	24	Does your child have hiccups more than once a day?
	25	Does your child have hiccups more than 5 min a day?
	26	Are the stools normal?
	27	Does your child suffer "chronic diarrhea"?
	28	Does your child have "chronic constipation"?
	29	Does your child not have daily stools?
	30	Is there more than 2 days between 2 defecations?
	31	Is there a reflux pathology in your family?
	32	Do you have other children?
	33	Did a brother/sister have a reflux pathology?
	34	Is there allergy in your family?
	35	Do you think your baby has a reflux pathology?

Figure 3
Hauser, et al. 2005

Endoscopy with pH probe testing is another means of testing for GERD. This method was the focus of testing for many years. This method looks to see what the acid levels within the infant are and shows any signs of damage to the esophagus (Hauser, Novario, Salvatore, Vandemaele, & Vandenplas, 2005). Research indicates

that when the pH testing showed the child having an esophageal pH of less than 4, reflux was the main cause. This test uses an endoscope probe that has a camera attached at the end, as well as an LED light to aid in visualization. It is inserted down the patient's throat (some up the nose and down the throat) and lowered down the esophagus. (Ferreira, et al. 2012) This test is considered invasive for infants and children and does require general anesthesia. The pH probe is attached to the endoscope, so both are lowered down the esophagus together.

If the readings of the pH levels indicate lower than 4, acid is present and reflux is suspected. The endoscope will indicate how much damage has been done to the esophagus, if any. Most children did have less damage to the esophagus when the symptoms they experienced did start to get alleviated. This test is not considered perfect because many times the lower esophageal sphincter may have just opened and released a little acid into the esophagus, altering the validity of the pH probe's results (Ferreira, et al. 2012). Another issue is that the pH probe missed acid levels in some childrenwho showed signs of severe esophageal damage, which clearly indicated that acid was present. The endoscopy test also is not a clear indicator for acid reflux. Infants/Children may have damage to their esophagus for other reasons, and not everyone with GERD has damage to their esophagus. Both of these indicated that the means of diagnosing at that time was terribly insufficient. The logical conclusion is that further research was needed to come up with a new, more accurate test. (Badriul, et al. 2004)

Another option for diagnosis is to do pH-probe tests over an extended 24-hour period. This tested for pH dropping to levels below 4 and infants having the symptoms during or after the drop. This test data did show that some patients have a correlation between reflux like symptoms and the test scores. However, the research tests do not don't show a big difference in acid reflux levels between those undergoing the 24-hour test and those being evaluated for reflux-like symptoms. (Badriul, et al. 2004). This has to mean that the test is not perfect as well.

The next test available is called the pH-MII test, Multichannel Intraluminal Impedance Test. It is similar to the pH probe. It also goes through the nose and down the throat to the esophagus where it stays for 24 hours. This test measures acid levels, esophageal flow and bolus presence. It has small detection devices at 6 levels along the catheter within the esophagus. It can test for acid or non-acid reflux and whether it is liquid or gas. When researchers define non-acid reflux, as having a pH of greater than 4, this test was able to detect reflux in infants, that the standard pH test missed 89% of the time. (Omari, et al. 2002)

This test helps infants that were being diagnosed simply by evaluation of symptoms. If the patient was treated solely based on symptoms, they might have been misdiagnosed. This test shows that the regurgitation may have nothing to do with pH levels. It greatly proved that the old pH test was not enough. The need to come up with a better means of diagnosis was clearly evident. The pH-MII test does provide more accuracy. However, it also causes more questions. Even if the pH is less than 4, and is recorded by the probe, how close in time does the reflux event need to happen next to the appearance of a symptom to be considered associated? And how many events and symptoms need to occur together in a day, for example, for it to be considered problematic and relevant for diagnostic purposes? Another question is why is there a correlation between the non-acid reflux and symptoms? If it is all an indication of the presence of acid in the infant's esophagus, how could the test show that acid levels were above pH of 4, and yet there was a bolus present at the same time as the symptoms? One has to wonder if there is a third entirely different option going on that just has not been recognized yet.

The pH-MII test was good for eliminating GERD as a diagnosis in some patients, however, for those that didn't fit into the category, all it did was raise more questions. Another study questioned if raising the pH level to between 4.5-5.5 in different positions was the answer. (Chiou, et al. 2011) Many more infants were diagnosed with GERD when this was the new parameter for acid reflux. Parents that need some reprieve were provided with an answer and some form of treatment. Indeed, many did respond to treatment. As of yet this is the best test there is. It is not perfect but it does provide some answers.

Treatment

An interesting factor in treatment for GERD is that it can also be diagnostic. If the patient gets better with the treatment, doctors presume they had the disease. When it comes to infants, the first treatment tried is called nonpharmacological therapies (Carroll, et al. 2002). This includes positioning the infant differently, thickening the child's formula, changing the formula and modifying how many times a day the infant has a meal. The positioning changes only worked on some patients and only in some positions. Thickening the formula helped with caloric intake, but it did not reduce the symptoms or reflux episodes in infants. (Khoshoo, et al. 2000) Changing the formula from a milk protein to a different amino acid basis did help some of the symptoms. Symptoms of milk allergy or intolerance are very similar to reflux (for example: regurgitation, fussiness, colic, etc.); however the research doesn't show that it limited reflux episodes (Nielsen, et al. 2004). Infants that did show improvement with the switched formula usually did so as a result of not having reflux to begin with. Their symptoms were mistaken for reflux because the symptoms of a milk allergy or intolerance do overlap with reflux in many areas. Research indicates that these therapies of altered lifestyle tend to help a little, but most do not last very long.

The most common treatment done for infants with reflux is acid suppression. This is done through medications. They are divided into histamine 2 (H2) antagonists and proton pump inhibitors. (Bachmaier, et al. 2014) Both of these reduce gastric acidity and help heal damage to the esophagus. Very often the medication is prescribed without doing a test like an endoscopy or pH probe. So there is no way of knowing what caused the symptoms to be alleviated or if the acid content and production in the stomach was ever there. (Comer, et al. 2010)

H2 blockers work by decreasing the amount of acid produced by the stomach. Brand name examples of these medications are Pepcid AC, Tagament and Zantac. The symptoms in infants did tend to improve. However, a double blind research study indicated that infants with the medication did not show improvement in symptoms greater than those with the placebo (Comer, et al., 2010). So the question remains, what made the child get better and why isn't another child getting better? Figure 4 shows a list of common H2 receptor drugs as well as proton pump inhibitor drugs.

Proton Pump Inhibitors work by suppressing acid production as well. However, the mechanism is different and they target different types of acid and different means of acid production. They seemed to work a little better than H2 antagonists for those with more severe GERD symptoms. The issue arises though, that with both types of medication acid is being minimized in the gastric area. The infants do become more prone to GI tract and pulmonary infections. The question remains, what is worse- reflux or infection? Many doctors will not give medication for those infants who seem to be growing well, despite the other symptoms. There have been some adverse effects by some of these medications too. One has been removed from the market because of the possible neurological damage as a side effect (Chen, et al. 2012). Despite all this, medication does seem to be the most common form of treatment for infants with GERD.

One other means of treatment is surgical; the most common being inserting a feeding tube. (Kuwata, et al. 2013) This did alleviate the symptoms to a great extent, however at a great price. These children did tend to develop issues with eating orally later on; they had trouble with chewing, swallowing and sensory issues, to name a few. The children needed physical and occupational therapy to help them learn how to properly eat, chew and not have an aversion to the different textures of their food. Most physicians deem this option to be a last resort because of the developmental issues that can arise later on, as well as the invasiveness of the procedure. Doctors generally have seen that infants do outgrow the condition with age. When evaluating treatment options, this fact has to be taken into account.

Type of medication	Recommended oral dosage	Adverse effects/precautions
Histamine$_2$ receptor antagonists		
Cimetidine	40mg/kg/day divided TID or QID (adult dose: 800–1200 mg/dose BID or TID)	rash, bradycardia, dizziness, nausea, vomiting, hypotension, gynecomastia, reduces hepatic metabolism of theophylline and other medications, neutropenia, thrombocytopenia, agranulocytosis, doses should be decreased with renal insufficiency
Nizatidine	10 mg/kg/day divided BID. (adult dose: 150 mg BID or 300 mg qhs)	headaches, dizziness, constipation, diarrhea, nausea, anemia, urticaria, doses should be decreased with renal insufficiency
Ranitidine	5 to 10 mg/kg/day divided TID (Adult dose: 300mg BID)	headache, dizziness, fatigue, irritability, rash, constipation, diarrhea, thrombocytopenia, elevated transaminases, doses should be decreased with renal insufficiency
Famotidine	1 mg/kg/day divided BID (adult dose: 20 mg BID)	headaches, dizziness, constipation, diarrhea, nausea, doses should be decrease with renal insufficiency
Proton pump inhibitors		
Omeprazole	1.0 mg/kg/day divided qd or BID (adult dose 20 mg qd)	headache, diarrhea, abdominal pain, nausea, rash, constipation, vitamin B12 deficiency
Lanzoprazole	No pediatric dose available (adult dose: 15–30 mg qd)	headache, diarrhea, abdominal pain, nausea, elevated transaminase, proteinuria, angina, hypotension
Pantoprazole	No pediatric dose available. (adult dose: 40 mg qd)	headache, diarrhea, abdominal pain, nausea
Rabeprazole	No pediatric dose available (adult dose: 20 mg qd)	headache, diarrhea, abdominal pain, nausea
Prokinetic		
Cisapride	0.8 mg/kg/day divided QID. (adult dose: 10–20 mg QID)	rare cases of serious cardiac arrhythmia (FDA recommends ECG before administration) beware of drug interactions do not use in patients with liver, cardiac or electrolyte abnormalities (FDA recommends K+, Ca++, Mg++ and creatinine before administration)

Figure 4:
Drugs demonstrated to be effective in gastroesophageal reflux disease (Rudolph, et al. 2001)

Conclusion

As of yet there is no way to conclude with absolute certainty that GERD exists in infants. All the evidence linking the two is not definite. Most infants did tend to get better over time as they aged, or with symptom alleviation due to one form of treatment or another. (Or unfortunately death caused the subject to no longer be in the research data.) None of these indicate with absolute certainty that the GERD was ever there to begin with. Thorough evaluations are needed to properly diagnose GERD in infants. Further research is needed in order to come up with a diagnostic test that clearly identifies GERD as the condition. Physicians should take a conservative approach in treating patients whom they suspect have this condition, because more damage than good can be done to these infants in the process of trying to alleviate their symptoms.

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Bioengineered Hearts

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Abstract

Heart disease is one of the highest causes for fatality in the world. Although many such diseases can be treated by a heart transplant, this in itself can cause countless problems. Aside from the high demand for donor hearts, there is the risk of the patient's immune system rejecting the transplanted heart. A bioengineered heart would reduce the need for donor hearts, and thus save countless lives. Finding a suitable scaffold, obtaining appropriate cells, and ensuring that the tissue will function properly are the main focuses in creating an artificial heart. While most of the studies done have been concentrated on creating cardiac tissue rather than the full organ, with the integration of these aspects scientists are getting closer to the goal of engineering a fully functioning artificial heart.

Introduction

Heart failure is one of the most prevalent causes of death in the world. There are many different diseases affecting the heart. For many of them, the only answer is a heart transplant. Although this has saved countless lives, transplants remain an imperfect solution. Firstly, the list of people requiring new hearts is a long one, but the amount of donor hearts available is few. Once a patient is approved for a heart transplant, s/he is added to a waiting list, which is part of a national allocation system run by the Organ Procurement and Transplantation Network (OPTN). There are approximately 3,500 people on the list, and waiting times can range from about six months to more than a year.

Even once a heart is found for the patient, and the transplant is successful, the patient is still not out of danger. Although the heart itself is healthy, it may not function properly in the recipient, causing a number of complications. One such concern is Primary Graft Dysfunction, which is the most frequent cause of death in the first 30 days after a transplant (National Institutes of Health, 2012).

According to the OPTN, the one year survival rate for patients in the U.S. aged 18-34 is almost 85%, with 75% 3 year survival rate, and 66% five year. The 1, 3 and 5 year survival rate for recipients age 35-50 is slightly higher, at 88%, 81% and 74% respectively (OPTN). Although these numbers are relatively high, one of the main causes for heart transplant failure in the first year after the transplant is due to the patient's immune system rejecting the foreign organ. To prevent this, immunosuppressant drugs are administered; however, this can cause damage to other organs such as the kidneys and liver. High cholesterol, diabetes and cancer are also some risks of the anti-rejection drugs (Bhimji, 2011). The gap between the supply and demand for donor organs, as well as the lifelong consequences for the patient, make the creation and implantation of a bioartificial heart a desirable alternative. While the construction of a functional whole organ has not yet been accomplished, tissue engineering and regenerative medicine research have obtained promising results for heart regeneration.

Bioengineers have been working on creating fully functioning organs that would eliminate the need for donors. This would solve the issue of patients not receiving a heart in time, in addition to getting rid of any concerns of rejection. There have been many studies done all in the hope of answering the question, is growing a new heart for a patient a foreseeable goal of the near future?

When trying to solve such a complicated issue there are numerous factors that must be taken into account, and many different angles that the problem can be studied through. With the case of the engineered heart, some of those factors include finding a suitable base, or scaffold for the organ, deciding what kind of cells would be appropriate for actually building the organ, and maturing the construct to develop some form of contractile and pump function.

Methods

The information in this paper was obtained by analysis of scientific articles and research papers. Various online databases and medical journals were used, accessed mostly via PubMed or the Touro College database. Most of the information is based on experiments done using rat cells since this is the most practical way of obtaining the large quantity of cells needed. Although the data is not completely applicable to human cardiac cells, researchers hope that these studies will provide insight into bioengineering of human hearts.

Discussion

The first major issue in organ engineering is creating a scaffold with enough elasticity, porosity, and strength to enable the cells to grow correctly, thus resulting in a functioning organ or tissue. The makeup of the scaffold is vital to the process because native tissues contain different cell types, with each cell type having its own unique three-dimensional (3-D) extracellular matrix environment, and mechanical properties. In addition, there are many other structures that must be taken into account, such as blood vessels. An ideal construct should display the functional and structural properties of natural heart muscle, and therefore should be contractile, vascularized, and electrophysiologically stable. To recreate such complexity in engineered tissues various approaches have been used.

Much of the research on cardiac tissue engineering that has been done was focused more on small sections of tissue than on the whole heart. However, this can be an important start to full organ engineering, as the results of such studies can be incorporated into studies dedicated to heart engineering.

Scientists have approached the issue of scaffolds from many different angles. In one such approach, cells are seeded on a degradable scaffold on which they reorganize into engineered tissues.

A group of bioengineers set out to research different designs for scaffolds to determine which would be most conducive to cardiac tissue growth. They were able to create two layer scaffolds, with fully interconnected pore networks, which aided in guiding the pattern of cell growth. The material used was a synthetic elastomer poly(glycerol sebacate), known as PGS. This polymer was chosen because of its ability to reproduce the mechanical stiffness and elasticity of the extracellular matrix. Additionally, PGS is supportive of blood vessel formation, and cardiogenesis. Different scaffolds were created with different properties, such as pore size, and thickness. Two layer scaffolds 200μm in total thickness, with interconnected pores were found to be the most effective for allowing heart cells to survive and form functional connections. PGS scaffolds provided a platform for patterned cell distribution while maintaining the geometric and mechanical properties of normal heart muscle (Neal, et al., 2013). A similar study done at Duke University used the flexible material PDMS (polyDimethylsiloxane) to create molds with elliptical pores. Such pores enabled the enhanced diffusion of oxygen and nutrients to the cells (Liau, et al., 2011).

In addition to the design for the scaffold, researchers must determine what kind of material should be used. One material, called Poly(N-isopropylacrylamide) or PNIPAAm, is a polymer recently emerging as a possibility due to its favorable properties. PNIPAAm has controllable features and switchable surface properties, making it an ideal option for scaffold formation. For example, PNIPAAm has a lower critical solution temperature (LCST) of 32oC. This is important because it will shrink and become hydrophobic at temperatures above the LCST, and hydrophilic at temperatures below. Whereas hydrophobic surfaces are attractive for cell attachment, detachment of cells and tissues from these templates requires either an enzymatic reaction or physical scraping, both of which can damage the cells. The use of PNIPAAm enabled controlled cell detachment by adjusting the temperature (Tekin, et al., 2011).

Another advantage to using PNIPAAm is its dynamic properties. Previously, micromolds were used to create microgels. However, these scaffolds had static structures, meaning the surface properties and patterns could not be changed after fabrication. PNIPAAm gel molds were able to be engineered to control 3-D organization of the cells by mimicking the complex native tissue architecture. Additionally, the polymer is a hydrogel, which would enable blood vessels to form in the organ. Coating surfaces in PNIPAAm served to induce capillary network formation. This is especially important for tissues to function properly (Tekin, et al., 2011).

Recently, a group of scientists experimented with adding single-wall carbon nanotubes (SWCNTs) to PNIPAAm to improve the function of the base gel for use in myocardial repair. SWCNTs are sheets of graphene rolled into a seamless cylinder. They have remarkable electrical properties, which may even exceed the best metals or semiconductors known, as experiments have shown (McEuen, et al., 2002). They theorized that the SWCNTs would improve the bioactivites and adhesion of the hydrogel to the cells. After the cells were applied to the gel, it was observed that as expected, cell adhesion and proliferation was about 1.71 times greater in the PNIPAAm/SWCNT hydrogel than in the PNIPAAm. The results indicated that the incorporation of SWCNTs greatly enhanced the bioactivity of the PNIPAAm and aided in its functioning as a scaffold (Li, et al., 2014). Finding a suitable material for the scaffold is one main step in the process of tissue engineering.

Another option recently being explored for use as scaffolds, are decellularized organs. As mentioned previously, even if donor organs were not in short supply, the transplant recipient would still be at risk of immune rejections and lifelong immunosuppression treatment. To date, although numerous modern technologies have been employed to fabricate new tissues, the creation of a functioning whole organ is still in progress. The use of decellularized matrices would be a step in the right direction, as it would overcome the need for the bioengineer to artificially recreate the conditions for cell deposition. It would offer a microenvironment with preserved natural geometry and vascular networks, which would enable cells to grow in the correct patterns (Moroni , Mirabella, 2014).

In one such study, hearts were decellularized with detergents, which preserved the underlying ECM, providing acellular, perfusable vascular architecture, and intact chamber geometry. All cellular material which would induce an immune response, such as cardiac and smooth muscle cells, DNA, RNA and soluble proteins, were removed. This left behind only the collagen, laminins and other structural proteins as a scaffold. Fiber orientation and composition remained intact, as did the arterial and venous basement membranes. As shown in figure 1, the detergent SDS was successful in removing all cells, while the Triton detergent left cells behind (Ott, et al., 2008).

The scaffold or decellularized organ now must be seeded with cells. This leads to the next issue regarding tissue engineering: what kind of cells should be used. Many researchers in the field use a mixture of two or more cell types, such as endothelial precursor cells to line blood vessels, and muscle progenitors, which are

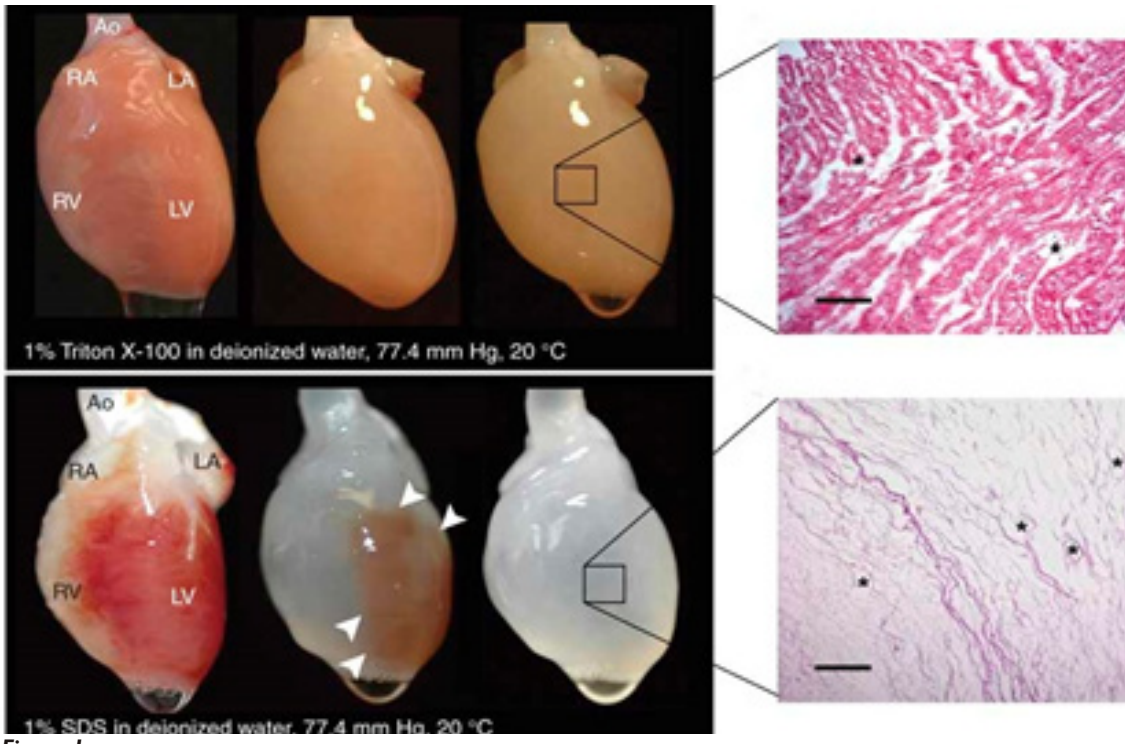

Figure 1:
Hearts decellularized using different detergents. Asterisks indicate intact vascular network.
Source: Ott HC, et al. 2008

similar to stem cells, to seed the walls of the chambers. These can be derived from induced pluripotent stem (iPS) cells- adult cells genetically reprogrammed to an embryonic stem cell-like state, which can be coaxed by scientists to become any kind of cell. This is useful because these can be taken from the patient, and used to make immunologically matched tissues (Maher, 2013). A team from the University of Pittsburgh recently used iPS cells generated from human skin cells to create precursor heart cells called MCPs. The cells were placed on a decellularized scaffold, and grew and developed into heart muscle. After 20 days, the tissue began showing cardiac muscle function (Discovery News, 2013).

Alternatively, as in a different study, heart cells were isolated from neonatal rats and digested in a trypsin solution until dissociated into a single cell suspension. Other steps were done to isolate the cells, and at the end of this method 63% cardiomyocytes, 33% cardiac fibroblasts, 3-4% smooth muscle cells and 2-3% endothelial cells were collected to be used to seed the scaffold (Neal, et at., 2013). Tissues constructed from these heart cell mixtures showed advanced structure, determining that creation of optimal cardiac tissue constructs depends on a mix of non-monocytes and cardiac monocytes (Zimmermann, et al., 2004).

A group of scientists using similar neonatal cells performed experiments to determine the effect of different factors on the growth of the cells in cultures and on the scaffold. For example, by analyzing the gas and CO2 levels in different vessels, they were able to determine what environment would be best for the cell growth. Additionally, by analyzing the density of the seeded cells, they determined that the minimal cell seeding density necessary to maintain construct structural integrity was achieved by using 1.4x106 cells per scaffold. For interconnected tissue structure, 8x106 cells per scaffold were required. From all the collected data, the researchers concluded that structural and functional properties of constructs were improved by seeding polymer scaffolds at high densities using rotating vessels. This study demonstrated that dissociated cells cultured on 3D scaffolds under favorable conditions were capable of forming engineered constructs with features resembling those of native tissues (Carrier, et al., 1998).

Other favorable properties for cell growth were determined in another study. Various factors were tested for their role in engineered heart tissue. High collagen content in the mixture yielded tissue with higher stiffness, but less contractile force development. Decreasing the collagen content yielded tissue with soft matrix structure, but improved contractile properties. Additionally, the inclusion of horse serum was found to be beneficial for engineered heart tissue development (Zimmermann, et al., 2004).

Once the scaffold is prepared and the cells cultured and seeded, the next step is to observe and determine whether the cells will behave as cardiac muscle cells. This includes being able to contract, and electrically conduct signals. While full engineered heart transplants have not been perfected, scientists have been successful on smaller scales. Biomedical engineers at Duke University were able to grow a three dimensional human heart muscle that acted like

natural tissue, in that it conducted electricity at about the same speed as natural heart cells, and contracted appropriately (Duke University, 2013). Using pluripotent embryonic stem cells permeated with fibroblasts, cardiac tissue was created with the capabilities of generating fast, uniform action potential propagation with velocities ranging from 17.8cm/s to 24.1 cm/s, and contractile force of 2mN (Liau, et al., 2011).

With the decellularization method, the recellularized hearts were placed in bioreactors that mimicked the sensation of beating. A combination of electrical signals was used to help synchronize the cardiocytes seeded on the scaffold, along with physical beating motions induced by a pump. After eight to ten days in the bioreactor the hearts were able to beat by themselves. However, when the team implanted the engineered hearts into rats, none of the hearts were able to perform the blood-pumping function at the degree that is required of the heart (Maher, 2013).

In a study done in Tokyo, layered cell sheets coated with PNIPAAm were created and implanted into the hearts of rats. At first the beating of engineered tissue, at 96bpm, was relatively slow in comparison to host hearts, 332bpm; after four weeks it had gotten stronger, though not as fast as the host tissue. A positive factor was that neovascularization occurred in the tissue, which aided it in functioning properly (Shimizu, et al., 2002).

In a different experiment, engineered heart tissue was created and implanted onto an infarcted area in rat hearts. When evaluated later, the engineered tissue showed electrical impulses in synch with the native tissue. It was also evident that the implanted tissue had prevented further dilation and induced wall thickening of infarcted myocardial segments. The epicardial activation of hearts with the tissue graft was normal, indicating undelayed coupling of the grafted tissue to the host tissue. The tissue was also able to propagate electrical potentials (Zimmermann, et al., 2006). This shows that tissue created in vitro can possibly function as natural cardiac tissue in vivo.

Conclusion

Although a completely functioning heart has not yet been engineered, scientists are headed in the right direction. Using scaffolds made of different materials and with different designs, they have been able to stimulate cell growth in the right patterns for tissue construction. Under the right conditions, the constructs behaved in a manner similar to native cardiac tissue. Such engineered tissues have even been shown to function almost as well as natural tissue in vivo. With the integration of these different aspects, and advances in bioengineering, scientists are getting closer to the goal of creating a fully functioning artificial heart.

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