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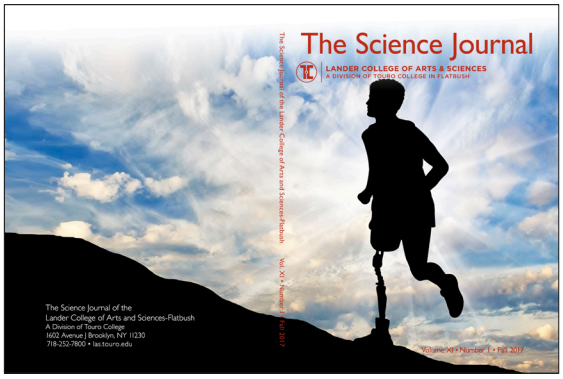

The cover illustration, created by Professor Antony O'Hara of the Digital Multimedia Design Program, represents the application of modern prosthetic technology..

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Using Dental Stem Cells to Regenerate Tooth Tissue and Whole Tooth Replacement

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Abstract

Irreversible dental problems such as dental carries and periodontal disease create a plethora of general and oral health issues. Although there are solutions to treat these different problems, an emphasis has been placed on finding a solution to these points with the help of bioengineering and stem cells. Using stem cells to treat these problems could result in a more permanent fix than the methods dentists employ now. Two novel approaches to fixing irreversible dental problems via stem cell therapy are tooth tissue regeneration and whole tooth replacement. This paper reviews the advantages and disadvantages of using dental pulp stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells and stem cells of the apical papilla for these novel techniques. Ultimately, these methods provide a promising future for dental patients, particularly with the use of stem cells from of the apical papilla.

Introduction

The tooth is a complex ectodermal organ consisting of various hard tissues as well as soft connective tissue. The hard tissues found in the tooth include: enamel, dentin, and cementum. Teeth also contain soft connective tissues like pulp and a periodontal ligament composed of fibrous connective tissue with both vascular and neural sections. Whole tooth loss and oral diseases such as dental carries, oral trauma, periodontal disease create many problems that impact proper oral function. Issues with enunciation, mastication, occlusion and general health issues are known to be induced by tooth loss or the onset of oral disease. (Oshima et. al, 2014)

Currently, there are many procedures using artificial materials to replace missing or damaged teeth. Dental implants, bridges, dentures and crowns all help to replace missing teeth, however, many believe that the optimal replacement is to regrow tooth tissue or an even an entire tooth in place of the missing tooth.

Due to extensive, active research in embryonic development, stem cell biology, and tissue engineering, regenerative therapy is a promising method for the restoration of function in missing or damaged organs including teeth. There are numerous tooth-tissue derived stem cells that have been found to aid in the development of new teeth allowing for the evoution of teeth regeneration techniques. Tooth-tissue regenerative therapy involves transplanting a bioengineered tooth germ grown in vitro via stem cells. At the time of implantation, these bioengineered specimens must contain all the components of a naturally growing tooth in order to be able to function as one in the future. Whole tooth replacement transplants a complete, bioengineered tooth to replace the missing tooth. Many believe that tooth replacement via stem cell therapy will be the treatment of choice for missing or severely damaged teeth. Several other methods have been proposed to replace missing teeth including three-dimensionally bio engineered teeth and the cell-aggregation method. Additional reports have been released regarding fully functional bioengineered teeth with correct tissue orientation and the masticatory capabilities of regular teeth. This review will discuss the most recent findings on tooth tissue engineering and whole tooth regeneration, and will provide the most viable method for the near future.

Methods

This review is based on the analysis of scientific articles and original works found on the internet. Several different internet databases were used including Touro college's online library and PubMed.

Discussion: Tooth Development

To ensure the success of tooth regeneration techniques, it is important to first understand how a biological tooth is formed and attempt to mimic those natural steps in the lab. As a tooth develops, it undergoes four distinct morphological phases; the initiation, bud, cap, and bell stages (Yildrim, 2013). During initiation, support structures such as the alveolar bone and periodontal ligament begin to develop. The tooth germ is first observed as a thickening and propagation of the cells of the oral epithelium and these cells form a bud that extends to the dental mesenchyme. The dental epithelium continues to undergo extensive proliferative activity, eventually forming a cap-like structure and completing tooth development.

During tooth development, the cells differentiate into three different sections: the inner and upper layers and the central cell layer that forms the stratum intermedium and stellate reticulum. As they do so, the enamel knot signaling center can be identified. At this point, ectomesenchymal cells of the dental papilla condense, giving rise to both the dentine and pulp of the tooth. The dental follicle forms around the dental papilla and enamel, giving rise to the periodontal tissues. The final bell stage is heavily defined by the continued proliferation and tissue differentiation. In this process, the inner epithelial cells adopt a cube shape and produce a considerable amount of glycogen, while the cells of the stratum intermedium produce alkaline phosphatase and the stellate reticulum adopts a star shape, encircled by the outer epithelial layer.

As the tooth continues to grow and differentiate, odontoblasts from dental mesenchyme differentiate and build up the dentin matrix, while ameloblasts from epithelial cells produce the enamel matrix. Once the crown of the tooth has formed, tooth root structures start to develop from Hertwig's epithelial root sheath, creating periodontal ligament, dentin, cementum and alveolar bone.

Throughout the entire tooth development, the only portion that does not have regenerative properties is the dental enamel.

Enamel is the only mineralized tooth tissue sourced from the dental epithelium. All other mineralized tooth tissue, such as dentin, periodontal ligament, alveolar tissue and cementum have some form of regenerative properties. These tooth tissues originate from neural crest-derived dental ectomesenchyme. Dental stem cells are believed to play an important role in the regenerative properties of these tissues (Dannan 2009).

Dental Stem Cells

Unlike other cells found in the body, stem cells have unique capabilities. They are unspecialized, yet can produce specialized cell types, even after prolonged periods of inactivity. Stem cells are able to proliferate and can be isolated based on unique cell surface markers. It is important to note that in vitro culture conditions may alter the cells, thus causing the cells to behave in a different manner than they would have in vivo. There are two categories of stem cells that can be used: embryonic or somatic stem cells. Being that the use of embryonic stem cell is ethically controversial, researchers work with strictly somatic, or adult human stem cells for clinical application. Somatic stem cells are easily accessible and without the controversy of their embryonic counterparts, making them the optimal choice for use in dentistry.

There are several types of stem cells that can be found in teeth. Dental stem cells are found in the periodontal ligament, apical papillae, and the pulp of both adult and children's teeth. Although these stem cells differ in their growth rate in culture, cell differentiation and gene expression, they most likely share a common lineage. Dental stem cells are derived from neural crest cells and all have generic mesenchymal stem cell-like properties. (Volponi et. al, 2010)

Dental Pulp Stem Cells

Dental pulp stem cells (DPSCs) are a mesenchymal type of stem cell located in the center of the tooth. Dental pulp stem cells are considered a great candidate for stem cell therapy for many reasons. DPSCs are easily accessible for collection and have a low morbidity once extracted. They are also known to generate more dentin than other stem cells, being easily conserved via cryopreservation (Yan, et. al. 2010). There are various methods to isolate DPSCs from the dental pulp including size-sieved isolation, stem cell colony cultivation, magnetic activated cell sorting (MACS), and fluorescence activated cell sorting (FACS). In addition, DPSCs were found to generate a dentine structure in vivo and differentiate in vitro (Gronthos et.al, 2000). These features make DPSCs a prime source of stem cells for 3-dimensional tooth regeneration.

Stem Cells from Human Exfoliated Deciduous Teeth

Stem cells from the pulp of human baby teeth, also known as stem cells from human exfoliated deciduous teeth (SHED), were found to differentiate into non-dental mesenchymal cell derivatives in vitro, produce dentine, and induce bone formation. SHED differs from DPSCs with respect to their higher rate of proliferation, sphere-like cell clustering and, most importantly, their inability to produce a dentin-pulp-like complex. It is believed that the reason for these differences is due to SHED being a multipotent stem cell that is less mature than DPSCs. SHED were reported to not be able to differentiate into osteoblasts, yet managed to form an osteoinductive template, thus leading to new bone formation. Following transplantation, SHED were found to aid in bone formation and generate dentin. SHED were found to be a highly proliferative cell population, capable of differentiating into various cells such as neural and glial cells as well as odontoblasts. Although there are better types of stem cells for use in dentistry, due to its penchants for widespread differentiation there is still much potential for SHED stem cells in other areas of regenerative medicine, such as in the brain and nervous system. (Miura et. al, 2003).

Periodontal Ligament Stem Cells

The periodontal ligament (PDL) has been viewed as a potential source of stem cells for a long time. The PDL is a connective tissue that acts as a shock absorber during chewing and is found between the cementum and the inner wall of the alveolar socket, anchoring both to each other. These stem cells have characteristics of mesenchymal stem cells and have potential to be used in periodontal regeneration. It is important to note that unlike other stem cells, PDLSCs do not have a unique cell marker that differentiates them from mesenchymal stem cells. Therefore, PDLSCs are not an optimal choice for stem cell research and dental regeneration (Zhu and Liang, 2015).

Root Apical Papilla Stem Cells

Stem cells of the apical papilla (SCAP) are found by the tips of growing tooth roots. These cells are only present during root development, prior to the eruption of the tooth. Stem cells of the apical papilla were reported to differentiate into both adipocytes and odontoblasts. When compared to the proliferative potential of DPSCs in vitro, SCAP were noted to proliferate at a higher rate, making them a very promising source of stem cells for stem cell therapy. Current implant-based methods to replace whole teeth have failed to reproduce the necessary root structure, causing jaw-bone resorption around the implant due to the force of chewing (Volponi et. al, 2013). When combined with periodontal ligament stem cells and transplanted into mini pigs, researchers noted formation of periodontal ligament and dentine. These results suggest that the necessary technology to implant a biological root and place an artificial dental crown on it has been attained.

Another point of advocacy is that SCAP can be harvested without the ethical issues of stem cell harvesting in embryos.

In addition, an important attribute of SCAP is that they are believed to be the source of root dentin, while DPSCs are the source of the replacement odontoblasts. SCAP could be distinguished from other mesenchymal stem cells in a number of ways. The most significant difference is that while all other mesenchymal stem cells test negatively for hTERT (human telomerase reverse transcriptions) activity, SCAP were found to test positive for it. This suggests that there is a difference in the genetic lineage of stem cells from the apical papilla compared to that of other dental stem cells. SCAP seem to come from an early population of progenitor cells and may be a better option for tissue regeneration (Huang et. al, 2008).

Using Dental Stem Cells for Tooth Tissue Regeneration

When stem cells isolated from the apical papilla of human teeth and human periodontal ligament were transplanted into mice and mini pigs, the successful regeneration of the root and periodontal complex capable of supporting a crown was achieved. Being that pigs have an orofacial tissue organization similar to humans, the successful regeneration of a bio root and transplantation into pigs shows the tremendous potential of this method for use in humans. Unlike other experiments to regenerate tooth tissue, this method used human stem cells and not stem cells from mice or pigs, proving that human stem cells were capable of producing the above mentioned successful results (Sonoyama et. al, 2006).

Whole Tooth Regeneration

Whole tooth regeneration is another approach to fix the issues associated with irreversible tooth damage. This method of tooth regeneration attempts to grow a whole tooth in vitro and transplant it into the mouth. To successfully regenerate a whole tooth, the same process that the tooth undergoes when it is first formed in the developing embryo must be replicated. Organogenesis of the tooth takes place by the interaction of mesenchymal and epithelial progenitor stem cells. Once the bioengineered tooth germ is produced in vitro, it may be transplanted it into the area of tooth loss, thus regenerating a new tooth. The biggest challenge is to develop a technique that will allow for the mesenchymal-epithelial stem cell interaction while in vitro. So far, there are several suggested methods that will allow scientists to use dissociated stem cells to bioengineer a tooth germ (Oshima et. al, 2014).

The Scaffold Method

The first method which allows for the necessary mesenchymal-epithelial stem cell reaction, is using a biodegradable scaffold. Scaffold technology has provided us the with a method to regenerate specific tissue by sowing cells on degradable materials. The scaffold method has been used to generate tissues in cartilage and bone regeneration therapies. Experiments using a collagen sponge as the scaffold material have reported successful formation of small teeth. These lab-grown teeth had all the characteristics found in biological teeth including dentin, enamel and dental pulp as well as forming the correct shape and size (Young et. al, 2002). Despite the scaffold method's ability to regenerate teeth, there are some significant problems with this technique. It has a low frequency of tooth formation. This approach fails to provide the many precise intercellular reactions, causing irregularities in the created tooth tissues, such as in the enamel-dentine complex. For proper tooth structure, there needs to be many precise junctions between the enamel, cementum and dentine for normal tooth development. These complex junctions are the result of very specific mesenchymal-epithelial stem cell tissue interactions, resulting in a very precise combination of ameloblasts, cementoblasts and odontoblasts, a process the scaffold method has yet to mimic perfectly (Thesleff, 2003).

The Cell Aggregation Method

A second approach is known as the cell aggregation method. This procedure is used to reconstruct bioengineered organ germ and has been able to produce the proper mesenchymal-epithelial cell interaction during the developmental process that the scaffold method has failed to produce. Recent studies have reported that transplanted bioengineered mammary gland stem cells successfully caused organ regeneration in vivo with the proper structure and cellular arrangement (Zheng et. al, 2005). The cell aggregation method was also used to successfully regenerate hair follicles (Zheng et. al, 2005). There has been much research done using the cell aggregation method to successfully regenerate teeth with correct structures. Currently, this technique has only been able to create teeth with the proper structure, but to date, only at a low frequency. Although the cell aggregation method replicated the organogenesis of biological teeth partially, a method that has a higher frequency of success is being sought (Nakao and Tsuji, 2008).

The Organ Germ Method

The interaction between the epithelial and mesenchymal stem cells in the embryo gives rise to the organ germ. It can be concluded from this that to properly reproduce organs, one needs to reproduce the steps and conditions that created them in the first place. Both tooth and whisker follicle germs were used as a source of the disassociated epithelial and mesenchymal stem cells. Although there has been successful regeneration of organs in the past, they were all produced in vitro. The organ germ method allows for the regeneration of teeth in vivo as well as in vitro.

Self-reorganization of the mesenchymal and epithelial stem cells is the first step in multicellular aggregation. Self-reorganization is accomplished by these stem cells moving and adhering themselves to select cells until there is the necessary

equilibrium. After the proper cell configuration has been accomplished through self-reorganization, the next step is the initiation of organogenesis by the mesenchymal and epithelial stem cells. These stem cells regulate the morphogenesis and differentiation as well. It is important to note that the frequency of successful self-reorganization and tissue regeneration is dependent on the source of the stem cells used in this procedure.

In research performed by Nakao, the organ germ method successfully produced tooth germ with the correct structure, resulting in transplantation that yielded tooth growth. Similarly, when growing whole teeth in vitro and then transplanting them, the bioengineered teeth were able to attach themselves to the mouth and form both nerve fibers and blood vessels from the implanted stem cells. This method used completely disassociated mesenchymal and epithelial stem cells to generate the necessary tooth germ resulting in successful tooth transplantation both in vivo and in vitro.

When the experiment was first conducted, single cells from the incisor tooth germ at cap stage from the lower jaws of mice were used. These explants failed to produce a complete tooth, yet managed to generate bone or keratinized oral epithelium-like structures. The explants that failed to form cell compartmentalization at high cell density, failed to produce the correct tooth structure as well. To form a tooth with the correct cell compartmentation of epithelial and mesenchymal stem cells, the cells that could compartmentalize at high density were then collected and injected into the adjacent areas via a collagen gel drop. After just one day, there was already evidence of tooth germ formation with the correct compartmentalization of stem cells. This bioengineered tooth germ was then transplanted into sub renal capsules in mice. After transplantation, the formed tissue was observed histologically and was found to contain odontoblasts, dentin, enamel, dental pulp, Tomes' process, root analog, alveolar bone, periodontal ligaments and blood vessels in the same layout as natural teeth. This transplantation produced successful results 100% of the time in 50 different transplants.

Analysis of the regenerated teeth showed mRNA for specific markers for periodontal ligaments and ameloblasts. To find out if tooth germ taken from bioengineered teeth would also produce the same successful results, the origin of the mesenchyme and epithelium cells from the tooth germ of GFP-transgenic mice were analyzed. The mesenchymal cells derived from GFP-transgenic mice were found to contain dental pulp and odontoblasts as well as produce alveolar bone and periodontal follicles, which are normally derived from dental follicles. The epithelial cells from these mice produced ameloblasts. It was also determined that bio engineered tooth germ did not develop into teeth as frequently as natural tooth germ. This is a distinct indication that the developmental stage of the natural tooth germ is vital for successful regeneration of tooth germ.

To prove that bioengineered tooth germ successfully produces teeth in vitro with the correct cell types, time-course images were observed. These images proved that the tooth germ successfully produced the necessary cell types in proper fashion. After successfully growing a tooth in the renal capsule, the next step was to check if it can be successfully transplanted and develop into a fully functioning tooth. Bioengineered teeth were allowed to grow in the sub renal capsule for a duration of two weeks. After transplanting the tooth into the oral cavity of a mouse, there were successful results. There was formation of a correct tooth structure with all the components that natural teeth have including dental pulp, dental root, enamel, dentin, and periodontal ligaments. The size of these regenerated teeth was found to be within a 1.1-fold increase, thus ensuring that we have attained a method for successful whole tooth replication with the proper proportions as well as with the necessary components. (Nakao et. al, 20007)

Although these results provide us with a viable method to regenerate teeth successfully in mice, there are still many things that need to be investigated before this method can be considered for successful whole-tooth regeneration in humans. There are a few more things that this experiment did not determine, such as the hardness of the regenerated tooth and its response to mechanical stress (Ikeda et. al, 2008).

Although there have been no reported cases of transplanting a tooth grown with the organ germ method into a human, there have been studies that prove that teeth grown in this manner and implanted in mice exhibit the appropriate hardness and normal function. To determine whether the regenerated teeth were as hard as natural teeth, the Knoop hardness test was performed. It is used to determine the hardness of brittle materials. By testing multiple times throughout the growth of the bioengineered teeth, the result values showed the same significant increase in dentin and enamel hardness as is found in natural teeth between three and nine weeks, proving that they can successfully take the place of missing teeth.

In order to determine if the regenerated tooth successfully replaces the missing tooth, the regenerated tooth's response to mechanical stress must be examined. It is believed that in order to regrow a fully functioning tooth, the tooth must interact with the oral and maxofacial regions by means of the periodontal ligament. This is because the periodontal ligament is known to induce alveolar bone remodeling when under mechanical stress (Ikeda et. al, 2008). Histochemical analysis of the periodontal ligament revealed a connection between the tooth and alveolar bone, showing the successful interaction between the tooth and the oral and maxofacial regions via the PDL. When the regenerated tooth was subjected to mechanical stress, localization of osteoblasts and osteoclasts occurred. These cells have been found when the PDL of normal teeth was subjected to mechanical stress as well, proving that the

regenerated tooth is able to properly respond to mechanical stress. (Ikeda et. al, 2008)

There are those that believe the successful regeneration of teeth in mice does not translate into successful results in regenerating human teeth. Unlike humans, mice are known to only grow molars and incisors separated by a toothless region known as the diastema. Mice also only possess one set of teeth that grow continuously throughout their life. Thus, mice may not be the best model for tooth regeneration in humans (Huysseune and Thesleff, 2004). Being that whole teeth were successfully regenerated in the diastema as well as in the lower jaw-bone of mice there is evidence to suggest that despite notable differences between human and mouse mouths, the regeneration of teeth in mice is not exclusive to mice and may be done in humans as well.

Conclusion

Although there have been no reports of successful whole tooth regeneration in humans, scientists appear to be on the right track. The research presented above indicate that although the cell aggregation method is a promising technique to regenerate whole teeth, there is still much work to be done before this will be the treatment of choice to replace missing teeth. Being that a bio root composed of human stem cells has been reported to be successfully transplanted into mini pigs, there is strong reason to believe that this method may be applied to humans in the near future. This review highlights differences between mice and humans that may affect the success of regeneration in humans. Despite there being extensive research and roadblocks ahead in the development of tooth regeneration techniques, progress and success with model organisms has introduced an element of hope for science to provide cures and resolutions to damaging oral diseases and encourage improved overall dental health.

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Upper Limb Prosthesis: A Functional Replacement for the Biological Limb?

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Abstract

The 21st century continues to produce major advancements in prosthetic limb technology. Specifically, improvements to the myoelectric prosthesis have helped numerous upper limb amputees, especially transhumeral patients. Targeted muscle reinnervation surgery has allowed for more seamless control of the prosthetic device by creating new control centers for the unused nerves of the residual arm. Additionally, improvements in pattern recognition technology have enabled transhumeral amputees to gain more natural control of the prosthesis and consequently better imitate a biological limb. Another important development is in regards to the configuration and placement of electromyography electrodes on the amputee's body. Using high-density electrodes implanted totally beneath the patient's skin have dramatically improved accuracy and performance of the electromyography readings. One of the most current and promising developments has been targeted sensory reinnervation. Preliminary studies have shown that this surgery can provide a dual flow of both motor and sensory information simultaneously between the patient's residual limb and the prosthesis. Studies also indicate that using osseointegration surgery to connect the prosthetic device directly to the patient's bone has improved its performance and made it more comfortable for the user. Finally, by undergoing extensive training and rehabilitation under the guidance of therapists knowledgeable with upper limb prostheses, transhumeral amputees can gain remarkable skills in prosthetic limb locomotion. Further advancement is required but research continues at a quick pace in improving prosthetic devices so that one day they can truly replace biological limbs.

Introduction

In 2005, there were 1.6 million amputees residing in the US, with that number expected to double by the year 2050. Of that number, 41,000 people live with major upper extremity limb loss (Ziegler-Graham, et. al. 2008). The loss of a limb is a devastating experience but an amputee's quality of life can be dramatically improved with the use of a prosthetic device. Creating a truly functional prosthetic has been, and continues to be, a tremendous challenge. The importance of providing patients with advanced prosthetics and thereby enabling them to function normally can't be understated. This is especially true for upper limb amputees (ULAs) who are severely inhibited in accomplishing even simple everyday activities. For example, transhumeral amputees require a prosthetic device that can provide full function of the elbow, wrist, and hand. The objective of this paper is to familiarize the reader with the current prosthetic technology, as well as discuss the areas where further advancements are required in order to equip transhumeral amputees with the full range of motion, sensory input, and practicality of a true biological limb.

Methods

Research papers and articles were obtained primarily through databases such as ProQuest, Ebsco, and PubMed accessed through the Touro College library. Google Scholar was also used to search for pertinent material. Relevant keywords were used to mine for source material and the references found within them were further retrieved as additional sources. The librarians of Touro College were also extremely helpful in gaining access to articles which aren't publicly available via the web.

Available Upper Limb Prosthesis

Prosthetic limbs can be classified into the following two categories: body-powered and myoelectric. The former uses harnesses and straps attached to the patient's residual limb and body to move the artificial prosthesis. In the case of a transhumeral amputee, manually locking the joints would be necessary to switch between functions, such as movement of the elbow, wrist, and hand. The terminal device is usually a mechanical hook, but can be interchanged for many useful tools to fit the patient's specific task. The approximate cost of such a device is about $7,000 (Resnik, et. al. 2012). This form of prosthetic, while inexpensive and simple to use, is limited by the patient's own strength. Additional disadvantages include limited ability to perform relatively simple movements, moving only one joint at a time, and having an inhuman-like appearance. Even so, body-powered prostheses are still widely used by about 30% of ULAs today (Ostile, et. al. 2012). Most patients, however, will choose to use the more advanced, battery powered myoelectric prosthesis. One of the great advantages of this prosthesis is its ability to bypass the amputee's limited strength and instead use an external, artificial power source to enable movement. Additionally, the myoelectric prosthesis uses electromyography (EMG) signals to control the operation of the artificial limb, unlike the manual body-powered prosthetic. This is possible due to the neuro-muscular system which remains intact in the ULA's residual arm even after amputation (Sudarsan, Sekaran, 2012). It's this EMG signaling which allows the myoelectric prosthesis to interact so seamlessly with its wearer. Unfortunately, the cost of a transhumeral myoelectric prosthesis can be as high as $100,000 (Resnik, et. al. 2012).

EMG Signaling in Myoelectric Prostheses

The nervous system is made up of billions of neurons, all connected throughout the body. These neurons act as a communications pathway through the means of electrical signals and neurotransmitters at synapses. An electromyograph measures the strength of the action potentials with the use of an electrode placed at target skeletal muscle. Skeletal muscle is composed of contractible bundles of cells attached to bone.

The muscle's contractions control skeletal movement, usually induced voluntarily by impulses transmitted through the neuronal network to the motor neurons. The transmission occurs through the depolarization of the muscle fibers to generate an electric current, thus allowing the EMG signals to be measured (Raez, et. al. 2006). When an amputee is fitted with a myoelectric prosthetic, electrodes that measure EMG activity are placed at a single pair of flexion and extension muscles on the residual limb. A ULA's myoelectric prosthesis then retrieves the EMG signals generated by these two muscles to promote locomotion. However, the prosthesis is limited to controlling only one joint at a time. To switch from one joint to another (e.g. elbow to wrist), the patient must co-contract a pair of muscles, thereby signaling to the prosthesis to switch joints. A common pair used by a transhumeral amputee is the biceps brachii and triceps brachii. Alternatively, a patient may also use a remote controlled by shoulder or foot movement to switch joints. The strength of the EMG signal produced at these sites determines the prosthesis's velocity. For example, the amputee may use a biceps contraction to close the prosthetic hand, and a triceps contraction to open it, thereby promoting movement singularly controlled by the patient's nervous system. However, this becomes increasingly less intuitive with higher levels of amputation due to diminished levels of residual muscles available in the arm. For example, transhumeral amputees must use the biceps/triceps to control all distal prosthetic functions, as depicted in figure 1 (Cheesborough, et. al. 2015). Operation of the terminal device, wrist, and elbow needs to be done sequentially which besides being a very slow and cumbersome process, also requires much cognitive oversight because the same biceps/triceps need to move even the arm muscles not natively controlled by them (Kuiken, et. al. 2004, Carlsen, et. al. 2014). To allow for more seamless use of the myoelectric prostheses, an innovative surgery termed targeted muscle reinnervation (TMR) was introduced.

Targeted Muscle Reinnervation

The concept of targeted muscle reinnervation surgery was first introduced in the year 2004 to help a patient with bilateral amputations of the shoulders gain further control of the prosthesis (Kuiken, et. al. 2004). TMR surgery utilizes nonfunctional muscles as amplifiers for the patient's dormant nerves that were originally attached to the amputated limb. This enables the nerves to produce signals in those muscles, thus providing a source for intuitive prosthetic locomotion. For example, conventional control of a myoelectric prosthesis for a ULA with a transhumeral amputation is with EMG signals from the biceps brachii, as well as from the triceps brachii. Granted, this may provide intuitive control of elbow flexion and extension, but not forearm supination/pronation, nor hand opening/closing. These movements require cumbersome mode switches because the limited residual muscles must control physiologically unrelated movements. (Cheesborough, et. al. 2015, Carlsen, et. al. 2014,

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Kuiken, et. al. 2009, Zhou, et. al. 2007). Luckily, however, the median, ulnar, and radial nerves of the upper arm remain intact even after amputation (see figure 2), and consequently motor commands for the missing limb continue to travel through the residual nerves. Through TMR surgery, these nerves can be transferred to innervate other muscles in the body. Specifically, the median nerve is transferred to the short head of the biceps muscle to allow for hand closing (or pronation), while the distal radial nerve innervates the lateral head of the triceps to provide signaling for hand opening (or supination), as depicted in figure 3 (Carlsen, et. al. 2014). Ergo, through the use of TMR surgery patients will have gained four myoelectric electric sites in place of the standard two (figure 4). This allows for very intuitive control of the prosthesis; attempting to flex/extend the elbow causes the native contraction of the long head of biceps/

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triceps, while attempting to close/open the hand activates the short head of the biceps/lateral head of triceps which have been newly innervated by the median/radial nerve, respectively. Since there are more than one head to the biceps and triceps, one of each can be sacrificed as a recipient signal amplifier. This would not sacrifice the intuitive elbow flexion/extension signals that's provided by the remaining biceps/triceps muscles with native innervation via the musculocutaneous and radial nerves, respectively. Additionally, the four distinct EMG signals can be used to simultaneously control multiple joints, thus avoiding the traditional and tedious mode switching which is normally required (Cheesborough, et. al. 2015). While this use of TMR greatly increases a patient's control of the prosthesis, it's still limited to two simultaneous degrees of freedom: elbow flexion/extension and hand opening/closing. Further movements like wrist rotation still require cumbersome and unintuitive mode switches. However, by applying pattern-recognition techniques together with TMR surgery, substantially more motor control can be extracted from the reinnervated muscles (Zhou, et. al. 2007). Unfortunately, a difficulty that remains even post TMR surgery is separating the distinct amplitudes of the surface EMG signals on different muscles so that the prosthesis can recognize the intended movements of the amputee (Schultz, Kuiken, 2011). Additionally, further study is required to perfect the safety and long-term efficacy of TMR surgery, as well as making it more widely available to the masses.

Pattern-Recognition Techniques

Targeted muscle reinnervation provides a rich source of motor control information. In traditional TMR surgery, the median nerve is transferred to control hand closing, and the radial nerve to control hand opening. In contrast, a non-amputee's body will normally use these nerves to innervate dozens of muscles in the forearm, wrist, hand, and fingers. The goal of pattern-recognition techniques is to enable an amputee to control all these movements in the myoelectric prosthesis simply and dexterously. High-density electrode arrays are placed over the patients reinnervated target muscles as the muscles attempt many different motions involving the elbow, wrist, hand, and fingers. The specific patterns produced by the combined EMG signals are processed by software which

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deciphers which specific movement is being performed, hence the name "pattern-recognition". Detailed mappings of EMG amplitudes across the reinnervated muscle site of a TMR patient show distinctive patterns of activity as the subject attempts a variety of thumb, finger, and wrist movements. The pattern-recognition software has been shown to be 95% accurate in predicting a patient's 16 intended arm movements (8 degrees of freedom). To highlight, without pattern-recognition the conventional myoelectric control methods can only use EMG amplitude at specific myoelectric control sites, and ergo can't take advantage of the true complexity of information. Accordingly, the muscles reinnervated by the median and radial nerves operate only hand opening/closing. However, by using pattern-recognition software, information can be extracted about the subject's desire to perform wrist flexion/extension/rotation, and even movement of the thumb, index finger, or digits (Zhou, et. al. 2007). Further investigation was accomplished in 2009 that demonstrated pattern-recognition control for real-time use of myoelectric prostheses (Kuiken, et. al. 2009). A very brief explanation of how pattern-recognition techniques works is as follows: patients provide example contractions for each of the prosthesis movements (e.g., elbow supination, hand close, etc.), and the algorithm learns which EMG patterns correlate to each intended movement. Thus, when a patient later desires to move the prosthesis, the algorithm predicts the desired movement from the previously learned pattern of EMG signals (Cheesborough, et. al. 2015). This starkly contrasts to conventional EMG readings in that pattern-recognition doesn't simply use EMG signal amplitudes, but rather utilizes numerous EMG recordings to globally classify the patient's intended movements. The use of pattern recognition enables TMR patients to significantly outperform conventional nonpattern-recognition in the following two tasks: 1) a box and blocks task, and 2) a clothespin relocation task. Both of these tasks are validated and standardized measures of gross hand function first developed by occupational therapists. The box and blocks task requires the patient to move one inch blocks from one compartment to another during an allotted time period, while the clothespin task requires the movement of clothespins from a horizontal bar to a vertical bar also during an allotted time period (Mathiowetz, et. al. 1985, Kuiken, et. al. 2004, Miller, et. al. 2008). Patients moved 40% more blocks and completed clothespin relocation in 25% less time when using pattern-recognition over conventional myoelectric control. Additionally, patients' personal preference favored pattern-recognition over conventional control (Hargrove, et. al. 2013).

Electromyography Electrode Configuration

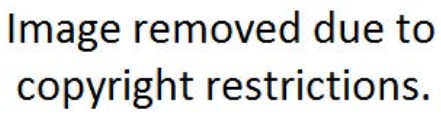
Though TMR surgery, along with advanced pattern-recognition, can provide vast amounts of neural information, it requires the use of a high density (HD) electrode array with over 125 electrodes placed over the reinnervated muscles. The placement of these electrodes is clinically challenging, and even after placement, the input of electromyography signals is slow. Preliminary studies show that by using advanced electrode selection algorithms, the number of electrode placements can be radically reduced to just a dozen. These electrodes can provide sufficient neural information to classify the user's intended movements with 99.1% accuracy in regards to 8 basic joint movements (elbow, wrist rotation, wrist flexion/extension, hand open/close). In a 16-class analysis which includes 8 additional finger movements, the classification accuracy was 93%. It's important to note that as the number of applied electrodes decreases, exact electrode placement becomes increasingly more integral. Thereupon, it's important to keep electrodes stably located over muscles, while being cautious to place them in the most accurate positions possible (Huang, et. al. 2008). Further studies show that by increasing electrode distance, as well as their orientation in regard to muscle fibers, pattern-recognition performance can be improved in the event of electrode shift (Young, et. al. 2011, Young, et. al. 2012). EMG signals are an integral mechanism for helping ULAs to gain full use of their myoelectric prosthesis. Therefore, ensuring that accurate readings can be obtained repeatedly and accurately is essential.

Implanted EMG Sensors

Though advancements in electrode configuration have greatly enhanced the accuracy of readings, they still have severe limitations such as poor skin contact (thus causing electrode liftoff), skin impedance changes due to sweating, lack of repeatable electrode placement due to day-to-day donning, as well as wire breakages (Weir, et. al. 2009, Pasquina, et. al. 2015). Hence, developing methods to solve these multitudes of issues are of the upmost importance. One method showing great promise is the use of fully implanted sensors beneath a patient's skin to ensure accurate EMG readings. These sensors are cylindrically shaped, and about 16 mm long (Figure 5). Each of these tiny sensors are capable of magnetically transmitting vast amounts of data directly to the retrofitted prosthesis. Furthermore, the ability to place these electrodes directly within residual limb muscles allows for stronger and more reliable signals that remain static despite body movement and sweating. Additionally, in the case of a transradial amputee these implanted electrodes present the possibility to record data from superficial and deep muscles simultaneously, allowing for more intuitive control by providing signals from the actual muscles responsible for hand and wrist locomotion prior to amputation (Pasquina, et. al. 2015). Unfortunately, a major technological issue with this system that still requires further research is reducing power consumption, and thus enabling the patient to have a portable prosthesis which can last a full day of use without the need for recharging (Schultz, Kuiken, 2011).

Targeted Sensory Reinnervation

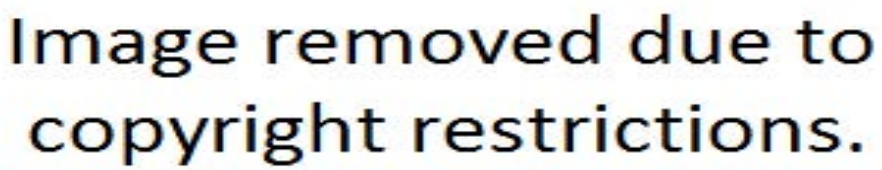
No prosthesis can be called a true replacement of a biological limb without providing sensory feedback to its user. Tactile sensation is one of the earliest developed and basic human senses, providing a rich source of information about environmental stimuli. However, even when a patient uses an advanced myoelectric prosthesis they're still reliant on their limited visual feedback, wherefore hindering natural control of the prosthesis. Surprisingly, one of the reasons why body-powered prostheses continue to see widespread use is because the wearer can sense its movement through the cables attached to his body (Marasco, et.al. 2009). Therefore, proving tactile sensory information to the amputee is integral in providing a lifelike prosthesis. Unexpectedly, a potential source for proving this feedback was first discovered accidentally, as an unintended result of TMR surgery on a bilateral shoulder disarticulation amputee. After undergoing TMR surgery to transfer his residual brachial plexus nerves to the pectoralis muscles, touching the skin overlying the reinnervated muscles produced sensation in different areas of his phantom limb (Kuiken, et. al. 2004). With this knowledge, the targeted reinnervation surgery was extended to include reattaching sensory nerves as well, dubbed targeted sensory reinnervation (TSR). In the reinnervation surgery of a short-transhumeral amputee, the distal ends of the patient's supraclavicular and intercostobrachial cutaneous nerves were attached to the ulnar and median nerves, respectively (see figure 6). Six months after the operation, the patient reported sensation in her phantom hand when her reinnervated pectoralis muscles were stimulated. All modalities of cutaneous sensation were present including graded pressure, thermal feedback, vibration, and edge detection (i.e. sharp vs dull). Stimulation of each of these sensations within the reinnervated region was interpreted by the patient as occurring in her missing hand (Kuiken, et. al. 2007a). Further studies have similarly shown how sensory feedback can be felt by an amputee post targeted reinnervation surgery (Kuiken, et. al. 2007b, Schultz, et. al. 2009). However, the above mentioned studies have been limited mainly to patients with shoulder-level amputations who underwent targeted reinnervation surgery to reinnervate the brachial plexus to the pectoralis muscle group. Since a transhumeral amputee still needs the residual nerves to innervate the upper arm it's unfeasible to simply transfer the brachial plexus to reinnervate the pectoralis muscle group. To help solve this issue, a recent study has attempted to extend the scope of TSR surgery to a transhumeral patient. By using an improved variation of the TSR surgery, along with an incorporated sensory feedback device, a patient can now feel, and accordingly coordinate, the strength of force applied by the myoelectric prosthesis when handling an object, without auditory or visual stimuli. Previously, TMR patients simply obtained sensory reinnervation directly on top of the reinnervated muscle sites, which can lead to over-crowding when placing all the necessary hardware and EMG sensors. Hence, the ability to place the reinnervated sensory site distant from the reinnervated muscle site, but close enough to be viable, is a tremendous advancement in creating a viable sensory feed-back system for myoelectric prosthesis users. During the TSR surgery, the transhumeral patient's median and ulnar nerve are located and their high sensory nerve content fascicles isolated. These



fascicles are dissected out of the main nerve trunk, transected distally, and coapted to the interocostobrachial cutaneous nerve and axillary nerve, respectively (see figure 7). The remainder of the nerve trunks are used to reinnervate portions of biceps and triceps as done in a classical TMR surgery. In addition to creating two spatially separated wide spread areas with discrete sensation for individual digits in the two nerve territories, called hand maps (see figure 8), the patient is able to utilize this sensory feedback to execute tasks while operating a myoelectric training arm, without having to rely on visual guidance or auditory cues. Furthermore, the above mentioned over-crowding problem is alleviated with this innovative surgical technique, as the sensory fascicles are directed to cutaneous areas distant from the muscle electrode sites. In the aforementioned study, the patient showed sensory feedback when gripping and releasing a ball, detected the difference between small and large blocks, as well as their difference in stiffness. Additionally, the patient discriminated between levels of applied force to the reinnervated area. Finally, the study demonstrated the ability to have a dual flow of motor and sensory information simultaneously between the patient's residual limb and the prosthesis. Additionally, further research is required to create a portable and wearable device that can viably transfer all the sensory and motor information between the prosthesis and its wearer. This was a single case study of a transhumeral patient and further investigation is required to test the efficacy of TSR with a real myoelectric prosthesis, as well as whether the sensory pathways will remain long-term (Herbert, et. al. 2014, Zuo, Olson, 2014).

Osseointegration

An unfortunate occurrence amongst amputees is prosthesis



abandonment which can occur when patients refrain from wearing their prosthetic device due to problems including lack of comfort, stability, and durability. Mean rejection rates as high as 45% and 35% were observed for body-powered and electric prostheses respectively in pediatric populations, while adult populations had somewhat lower rates of 26% and 23%, respectively (Biddiss, Chau, 2007). However, by performing an osseointegration surgery which directly attaches the prosthesis to the patient's bone, one avoids the need for an unwieldly socket. This enables the prosthesis to always fit firmly, comfortably, and correctly, consequently giving the patient a more natural prosthetic limb to mimic the amputated biological one. The implant system includes a threaded titanium implant, which is inserted intramedullary into the transhumeral amputee's humerus bone, as depicted in figure 9 (Jönsson, et. al. 2011). As of yet, the FDA has not approved osteointegration trials for ULAs in the US, though one European group has successfully implanted devices in over 100 patients around the world (Cheesborough, et. al. 2015). Important to note is that after the osseointegration surgery, the patient must adapt to having a permanent appendage abutting from the limb.

Rehabilitation and Prosthesis Training

Prosthesis training during rehabilitation directly affects an individual's level of function, and thus providing post-op amputees with proper instruction on how to use their prosthetic limb is vital. Training with therapists knowledgeable in upper limb prosthetic components and control is a significant portion of prosthetic rehabilitation leading to functional success of the ULA (Carlsen, et. al. 2014). Important to note is that fitting a patient with a congenital upper limb absence within 11 months of age leads to a greater acceptance than when fitted at an older age. Similarly, individuals fitted with a prosthetic 3 months after injury are less likely to reject the prosthetic device than patients fitted 6 months after surgery (Biddiss, Chau, 2007). Consequently, it's important to fit the patient with a prosthetic device and start the rehabilitation process as soon as possible. The rehabilitation process can be classified into a three-phase process. Phase one promotes healing of the residual limb wound, starting at the time of injury and continuing until all wounds have successfully closed and are infection free. The length of time spent in this phase varies depending on the extent of the patient's injury, but approximately three weeks after injury phase two begins and introduces pre-prosthetic training. The rehabilitation goal of pre-prosthetic training is to prepare the patient to receive a correctly fitting and functional prosthesis. This begins upon wound closure and ends with procurement of a preparatory training prosthesis. Patients will receive physical therapy to achieve improved flexibility and strength, as well as to educate them in avoiding incorrect postures that may lead to overuse injury of the upper body. Additionally, they learn how to perform activities of daily living, like writing, with just one hand when the prosthesis is unavailable. The third and final phase begins prosthetic training with the goal of the prosthesis becoming an integrated part of the patient's life. The therapy's focus is to help the amputee master the mechanical actions required for prosthetic control and eventually achieve independence in all activities of daily living. To help further this goal, the patient is fitted with varied types of prosthetic devices to gain experience accomplishing many different tasks so as to gauge and refine his skill sets when operating diverse types of prostheses. Hence, many patients will own multiple prosthetic devices and will use them accordingly for different tasks. The amputees are also trained in how to care for their prosthetic devices, to put on and remove them by themselves, as well as perform even complicated activities of daily living (Smurr, et. al. 2008). The rehabilitation for a post targeted muscle reinnervation surgery patient is somewhat more complicated, since each nerve that's being used for reinnervation contains numerous motor neurons. These neurons control many muscle fibers that work in conjunction to create nerve actions and thereupon move the body. For example, the radial nerve innervates hand extensor muscles, wrist extensor muscles, and supination muscles. However, a surgeon can't be certain exactly which nerve fibers will reinnervate the new target muscle. Hence, the patient must first attempt to perform all the actions controlled by the transferred nerves in order to see which will actually develop and innervate the muscle. The first noticeable reinnervation will usually occur at about 3 months after surgery. At this point, the patient can finally begin exercises to strengthen the reinnervated muscle over the next few months, followed by the patient learning to elicit strong, reliable EMG signals for the myoelectric prosthesis to receive. Whenever possible, the most intuitive movements that yield strong and reliable EMG signals will be used to move the prosthesis. As an example, the patient's attempt at extension of the phantom hand (radial nerve) hopefully will produce the strongest and most physiological appropriate signal for prosthetic hand opening, therefore making prosthesis movement intuitive for the subject. Over the long rehabilitation process, it's integral for the success of the patient that the occupational

therapists, physical therapists, and prosthetists understand the principles of TMR, peripheral nerve distribution, specifics of the surgery, as well as the different possible post-op outcomes for each patient (Stubblefield, et. al. 2009). This will enable an amputee to truly gain the most from his prosthetic device and thereby return to functioning as fully and naturally as possible.

Conclusion

Science continues to make great strides in advancing prosthesis technology and thus increasing the quality of life of countless amputees. Electromyography signaling and targeted muscle reinnervation surgery continues to pave the way for leading edge prosthetic limbs. Advanced pattern recognition software is constantly being improved and perfected, while targeted sensory reinnervation surgery stands at the forefront of current scientific research in providing upper limb amputees with truly advanced replacements for their amputated biological limbs. Of course, additional work is needed to improve the control algorithms so that the amputee may interact seamlessly with his myoelectric prosthesis. Additionally, further study is needed to test the efficacy of wireless, implantable EMG electrodes. Osseointegration surgery remains a promising new method to comfortably suspend a transhumeral amputee's prosthesis. Additionally, targeted sensory reinnervation helps produce a real-time, dual flow of mechanical and sensory information between the prosthesis and its wearer. One of the biggest challenges remaining is to create a way to minimize power consumption of the prosthesis, while still providing its wearer with all the technology needed to provide the full range of motor output and sensory input of a real limb. Additionally, the life-time cost of providing a patient with a prosthesis can be astronomical and further advancements are needed to lessen the cost so that it can be a viable clinical option. The human body is a wonder and even a simple task like typing on a keyboard is truly a wonderment of seamless interaction of neurons and muscles. Hence, though a current myoelectric prosthesis is indeed an advanced appendage, it's still far less advanced than one's natural biological limb. Overall, however, the future seems extremely hopeful in one day providing a transhumeral amputee with a fully functional prosthetic limb to truly replace a lost biological one.

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Silver Nanoparticles and Drug Resistant Bacteria

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Abstract

The scientist who discovered penicillin and its use as an antibiotic, Alexander Fleming, also raised concerns about bacterial resistance. As he predicted, in the twenty first century, the overwhelming use of antibiotics has led to both drug- and multi-drug resistant bacteria. This paper attempts to investigate the antibacterial potential of silver nanoparticles against drug resistant bacteria. By using Touro's online library database, the efficacy of silver nanoparticles as a potential antibacterial agent was comprehensively researched. Using transmission electron microscopy and the disk diffusion method, silver nanoparticles have been found to exert bactericidal effects by adsorbing to the cell surface and by entering the cell. The small size of the particles confers it with a high surface area which thus enables the silver nanoparticles to effectively interact with the cell membrane and thereafter enter into the cell. Moreover, the dose and shape of silver nanoparticles affects their antibacterial properties. While it has been found to be dose dependent, there is controversy regarding which shaped particle, sphere or triangular, has the greatest ability to damage the cell membrane, transport systems, DNA, and proteins, in addition to generating reactive oxygen species. Most studies have found the particles to be nontoxic at low levels, but some uncertainty still exists. In addition, silver nanoparticles seem to have a synergistic effect with the simultaneous use of antibiotics. Further research must be done before silver nanoparticles can be used as a new and effective antimicrobial agent.

Introduction and Background

On February 27th 2017, the World Health Organization (WHO) published a list of the top twelve resistant bacteria that greatly endanger human health (Press Association, 2017). Drug resistant and multi-drug resistant bacteria are one of the most serious public health threats the world faces today. Antibacterial resistance is the ability of bacteria to survive even in the presence of antibiotics. The development of antibiotics has made a great impact on the medical field by enabling doctors and health professionals to successfully combat many diseases. However, with the rise of the use of antibiotics, drug resistance has evolved, and according to WHO statistics, 700,000 people die annually from infections and diseases that have resulted from such resistant bacteria (Press Association, 2017).

Method

The research discussed in this paper was compiled from various published articles obtained from Touro's online database including Proquest as well as PubMed's database to research the actions of antibiotics, drug resistant bacteria, and the use of silver nanoparticles against drug resistant bacteria.

A. Antibiotics and their mechanism of action

Antibiotics are natural or synthetic agents that fight and inhibit the growth of bacteria. Antibiotics are grouped into different classes based on their mechanism of action, and they can either disrupt the cell membrane, or they can inhibit cell wall synthesis, protein synthesis, DNA replication and repair, RNA synthesis, or various metabolic pathways.

1. Inhibition of Cell Wall Synthesis

Bacterial cell walls are comprised of peptidoglycan, the cytoplasmic membrane, and in gram negative bacteria, the outer membrane. The peptidoglycan, the strongest layer of the cell wall, is a netlike arrangement of glycan and peptide strands. The biosynthesis of the peptidoglycan is catalyzed by thirty different enzymes (Shah, 2015). Transglycosylases and transpeptidases are two enzymes which aid in the final steps of the cell wall synthesis by adding new peptidoglycan units to extend the sugar chain and by linking the amides of the peptide strands, respectively (Walsh, 2000). Numerous antibiotics, like the B-lactam class of antibiotics, target different steps in the synthesis of the peptidoglycan. Penicillins and cephalosporins act as pseudo-substrates for the penicillin binding proteins (PBPs), the active site of the transpeptidases, in order to prevent the cross-linking of the PG. The cross-linking action of the transpeptidases is responsible for the strength of the PG, and without it, the bacterial cell wall is significantly weaker and therefore more prone to lysis. When B-lactam antibiotics bind to the PBPs, the oxygen from the serine residue located near the PBP attacks the B-lactam ring and forms a penicilloyl-enzyme complex. The serine is then acylated by the b-lactam which thus inactivates transpeptidases (Walsh, 2000). Consequently, transpeptidases can no longer bind to the substrate, and therefore the enzyme cannot complete the cross linking action (Andersson, et. al. 2001) (Bockstael, Aerschot, 2009).

Vancomycin is a drug that belongs to the glycopeptide class of antibiotics. Like the B-lactams, vancomycin targets the synthesis of the cell wall. However, rather than targeting the enzymes involved in the production of the PG, vancomycin targets the substrate by making five hydrogen bonds with the D-ala-D-ala terminus of each uncross-linked peptidoglycan (Lange et. al. 2007). By blocking the substrate of both the transpeptidases and transglycosylases, vancomycin and other glycopeptides inhibit the cross linking of the peptidoglycan, resulting in a weaker cell wall that is subject to osmotic lysis (Walsh, 2000)

2. Disruption of the Cell Membrane

The cell membrane of bacterial cells are semipermeable membranes that are comprised of phospholipids, carbohydrates, and proteins. Antibiotics like polymyxins and lipopeptides have the ability to disrupt the bacterial cell membrane. Polymyxins like colistin are cationic cyclic peptides that bind to the phospholipids in the bi-layer. By interacting with the negatively charged cell membrane (Taneja, Kaur, 2016), colistin disrupts the bacterial cell

membrane by displacing divalent ions, like calcium and magnesium, from the lipids present on surface of the cell. The disruption of the cell membrane causes cell leakage and ultimately cell death (Biswas, et. al, 2012) (Bockstael, Aerschot, 2009). Similarly, aminoglycosides can also displace these divalent ions to increase the membrane's permeability, resulting in the leakage of intracellular content and cell death (Lange, et. al. 2007).

Furthermore, daptomycin, a lipopeptide, can also disrupt the cell membrane. However, rather than displacing calcium and magnesium ions, daptomycin forms pores in the membrane by inserting its tail into the membrane. Consequently, there is a potassium efflux, and as potassium ions leave the cell, cell depolarization occurs (Shah, 2015) (Bockstael, Aerschot, 2009). Besides for causing cell depolarization, daptomycin can also inhibit the production of lipoteichoic acid which is responsible for regulating both cell division and cell shape (Bockstael, Aerschot, 2009).

3. Inhibition of Protein Synthesis

In addition to targeting the synthesis of the cell wall and cell membrane, other antibiotics exert their effects by inhibiting bacterial protein synthesis. Ribosomes are essential to the synthesis of proteins as they translate the genes from mRNA into proteins through the three steps of initiation, elongation, and termination. The bacterial 70S ribosome has two subunits: the 50s subunit and the 30s subunit. While the large 50s subunit contains a 5S rRNA, a 23S rRNA and 36 proteins, the smaller 30S subunit is comprised of 16S rRNA and 21 proteins. Antibiotics can exert their effects by targeting either the 30S or the 50S subunit (Bockstael, Aerschot, 2009).

Targeting the 30S Subunit

The 30S subunit contains the site where the codons are recognized by their corresponding tRNA anticodons. Transfer RNAs help with the process of translation by acting at either the A site, the P site, or the E site (Lange, et. al. 2007).

Aminoglycosides like gentamicin and tobramycin interact with the 16s RNA located in the 30S subunit. By hydrogen bonding with the substituents on the aminoglycoside cyclitol ring in the 16s RNA located at the A site, mistranslation occurs, and as a result abnormal proteins are produced. These aberrant proteins are then incorporated into the bacterial cell wall, ultimately resulting in a weak cell wall which is associated with cell leakage and further penetration of the drug into the cell (Lange, et. al. 2007) (Bockstael, Aerschot, 2009).

Tetracyclines bind to the 30s subunit (Lange, et. al. 2007) to prevent the elongation step of protein synthesis. By blocking the substrate of the incoming tRNAs, these antibiotics prevent new amino acids from being added to the growing amino acid chain (Shah, 2015).

Targeting the 50S Subunit

While the antibiotics discussed above inhibit protein synthesis via the 30S subunit, many other drugs have the ability to affect protein synthesis through the 50S subunit. The 50S subunit is associated with peptidyl transferase activity as well as the formation of peptide bonds (Bockstael, Aerschot, 2009).

Chloramphenicol, a broad spectrum antibiotic that is used against both gram positive and negative bacteria, binds to the 23s RNA on the 50S subunit. This class of drugs inhibits the formation of peptide bonds by preventing the tRNAs from binding to the A site (Bockstael, Aerschot, 2009).

The antibiotic class of macrolides binds to the 23S rRNA. Consequently, the exit tunnel that helps transport the peptide away from the peptidyl transferase center is blocked.

Lincosamides, like clindamycin, attack both the A site and P site located in the peptidyl-transferase center (Lange, et. al.2007). As a result, lincosamides inhibit the initiation of peptide chain synthesis and detach tRNAs from the ribosome (Bockstael, Aerschot, 2009).

Streptogramins affect protein synthesis and the action of the peptidyl transferase center activity by binding to the 23S subunit on the 50S ribosome. There are two types of streptogramins, Type A and Type B. While type A prevents the step of elongation by blocking the substrate of the peptidyl-transferase center, type B stimulates the premature release of incomplete peptide bonds by inhibiting peptide bond synthesis (Bockstael, Aerschot, 2009).

Oxazolidinones act by targeting the 50S subunit. The drug linezolid binds to the 23S subunit located on the 50S ribosome. By binding to this subunit, the formation of the complex between tRNA, mRNA, and the ribosome is blocked which thus inhibits the formation of the first peptide bond. If the complex is already formed, oxazolidinones can exert their effects by preventing the translocation of the peptidyl RNA from the A site to the P site (Bockstael, Aerschot, 2009).

4. Inhibition of Metabolic Processes

Some antibiotics can interfere with various metabolic processes that are vital to the survival of bacteria. Folate and folic acid are essential for the synthesis of purines, thymidines, and some amino acids (Lange, et.al. 2007). The folic acid pathway is catalyzed by dihydropteroate synthetase and dihydrofolate reductase, two enzymes which aid in the production of 7, 8 dihydropteroate and tetrahydrofolate, respectively (Shledon Jr., 2005). Drugs like sulphonamide and trimethoprim block different steps in this folic acid pathway, and while sulphonamide competitively binds to p-aminobenozic acid in order to prevent the actions of dihydropteroate synthetase, trimethoprim binds to the enzyme dihydrofolate reductase to prevent the reduction of dihydrofolic acid to tetrahydrofolic acid (Bockstael, Aerschot, 2009) (Sheldon Jr., 2005).

5. Inhibition of DNA Replication

Topoisomerases I, II, III, and IV are enzymes that are essential for DNA replication in bacterial species (Walsh, 2000). Bacterial DNA is negatively supercoiled, and during DNA replication, topoisomerase II, DNA gyrase, removes positive supercoils, breaks the double bond, and decreases the linking number by two. Furthermore, during DNA replication, topoisomerase IV unlinks the daughter chromosomes (Bockstael, Aerschot, 2009). Topoisomerases II and IV are vital for DNA topology, replication, and decatenation, and quinolone antibiotics target these enzymes. By interacting with the complex formed between DNA and DNA gyrase, quinolones make conformational changes that affect the activity of both topoisomerases II and IV, and as a result, DNA replication is blocked (Bockstael, Aerschot, 2009).

The process of transcription, the transferring of genes from DNA to mRNA, is mediated by the multi-subunit enzyme RNA polymerase. The antibiotic rifamycin targets DNA transcription by binding to the beta subunit of RNA polymerase. Rifamycin blocks the entry of the first nucleotide, thereby blocking the action of RNA polymerase and inhibiting mRNA synthesis (Bockstael, Aerschot, 2009).

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B. Mechanism of Resistance

Resistance can be classified as either intrinsic or extrinsic. Intrinsic resistance is resistance that is inherent to a specific species of bacteria. For example, the bacteria belonging to the genus of Enterobacter, Klebsiella, and Escherichia coli are all resistant to methicillin, clindamycin, and vancomycin (James, 1999). Various species of bacteria all differ in the variation of their cell walls, efflux pumps, and biofilms, all of which lends itself to different bacteria's innate resistance to certain drugs (Sheldon Jr., 2005).

Extrinsic or acquired resistance, on the other hand, arises when bacteria acquire new resistance to numerous drugs via different mechanisms. Resistant bacteria can degrade and modify enzymes, alter the targets of antibiotics, change the permeability of their cell wall, or alter metabolic pathways to prevent drugs from penetrating and affecting their cells (Sheldon Jr., 2005).

1. Enzymatic Degradation or Alteration

Some bacterial species can resist a wide array of drugs by enzymatic degradation and alteration. By producing an enzyme that destroys an antibiotic, bacterial species can cause various antibiotics to become ineffective. Antibiotics like penicillins, carbapenems, and cephalosporins all contain a B-lactam ring which binds to PBPs on the peptidoglycan to prevent cross linkage of the cell wall (James, 1999). In response, various bacteria produce different classes of B-lactamases to hydrolyze the four membered B-lactam rings and thereby inactivate these antibiotics (Sibanda, Okoh, 2007).

Similar to the B-lactamases, the aminoglycoside modifying enzymes (AMEs) are enzymes that cause bacterial resistance to aminoglycosides. Some microorganisms can produce enzymes that modify drugs. Aminoglycosides like kanamycin, gentamicin, streptomycin, and neomycin can either be acylated, adenylated, or phosphorylated by aminoglycoside acetyltransferase, adenyltransferase, and phosphoryltransferase. Consequently, the modified antibiotics can no longer exert their antibacterial effects (James, 1999) (Gabani, et. al 2012).

2. Alteration of Targets

In order to have a combative effect on bacteria, antibiotics must bind to their intended receptors. Therefore, in response to various antibiotics, bacteria can reduce the affinity of the antibiotics by modifying the structure of the drugs' active site. Different bacterial species like methicillin-resistant Staphylococcus aureus and Streptococcus pneumoniae produce new penicillin binding proteins, PBP2a and PBP2b respectively, in the presence of antibiotics. These modified active sites have a lower affinity for B-lactams which, in turn, prevent the drugs from properly binding and having an antibacterial effect (James, 1999). Furthermore, substituting at least one amino acid in the PBP can result in a lower affinity of the drug, and as a result the bacterial cell wall is not destroyed by the antibiotic (Gabani, et. al. 2012) (Sibanda, Okoh, 2007).

In different species of bacteria, the N^6 amino group of an adenine residue located in 23S rRNA is methylated, and as a result bacterial resistance to macrolides, lincosamides, and streptogramin B arises. The mechanism behind this resistance is associated with the reduced affinity of the binding sites that results from conformational changes from the methylation (Sibanda, Okoh, 2007).

3. Alteration of Permeability

In addition to altering the targets of antibiotics, drug resistant bacteria can modify the permeability of their cell wall in order to prevent or decrease the entrance of various drugs into the cell. A drug's concentration in the bacterial cell determines the efficacy of the drug on the pathogen, and it is through both porins and efflux pumps that drug resistant bacteria have the ability to decrease the amount of drug that reaches the cell (Lange, et.al. 2007) (James, 1999) (Sheldon Jr., 2005).

Porins

Porins are protein channels that exist solely in the outer membranes of gram negative bacteria. These channels are highly specific, and depending on size, shape, and charge, certain molecules can pass through to the inside of the cell. Being hydrophilic molecules, antibiotics easily enter the cell through these protein channels. Research has shown that when bacteria lose porins in their outer membrane, drug resistance emerges since less of the antibiotic can enter into the cell. For example, when the amount of OPrD porins were decreased in Pseudomonas, the imipenem class of antibiotics could not enter the bacterial cell. Similarly, resistance to imipenem and meropenem occurred after the amount of 29-kDa Porins was reduced in Acinetobacter baumannii. Multi-drug resistant bacteria like Klebsiella pneumoniae also display resistance to cephalosporins and carbapenems after losing OmpK35 and OmpK36, outer membrane proteins (Santajit, Indrawattana, 2016).

Efflux Pumps

Efflux pumps actively transport drugs out of the bacterial cell thereby decreasing the intracellular concentration of different antibiotics. There are five different categories of efflux pumps that exist in either gram positive or gram negative bacteria. The ABC, RND, MFS, SMR, and Multidrug and toxic compound extrusion family transporter efflux pumps all bind to the drug in the phospholipid bilayer, and thereafter export it out of the cell to different locations. While gram positive bacteria transporters work by pumping the drug out of the cell across the cytoplasmic membrane, gram negative efflux pumps can either extrude the antibiotic across the membrane and into the periplasmic space, or directly into the external medium. As these efflux pumps quickly extrude the antibiotics out, the concentration of antibiotics cannot accumulate to a high enough level to have an antibacterial effect (Zgurskaya, 2002) (Lomovskaya, Watkins, 2001).

4. Altered Metabolic Pathway

Resistant bacteria have come up with alternate routes to obtain metabolic products that are blocked by antibiotics. The folic acid pathway produces pyridine thymidylate, an essential molecule in the synthesis of DNA. In order to circumvent antibiotics that target the folate pathway, Enterococcus either uses folinic acid from its host cell, or mutates to have the ability to produce thymine (Mambrio-Jones, Hoek, 2010) (Sheldon Jr., 2005).

Discussion:

C. Combating Drug Resistance

According to statistics, it is predicted that an estimated 10 million people will die from drug-resistant bacterial infections by 2050 if no viable solution is discovered (Press Association, 2017). Therefore, finding effective treatments for drug resistant bacteria is of extreme importance. Over the last few years, much research has been done to determine proper treatments for such deadly infections. This paper will discuss the effectiveness of silver nanoparticles against drug resistant bacteria.

1. Silver Nanoparticles

Silver is widely known for its antimicrobial properties, and therefore, silver nanoparticles (Ag NPs) have been widely studied as a potential antibacterial agent against drug-resistant bacteria. Various studies on different strains of bacteria were performed to uncover the antibacterial effects of Ag NPs. More specifically, the studies focused on the size, dose, and shape of these silver particles coupled with the potential toxicity they pose to human cells.

There are various methods to create nanoparticles. Ag NPs can be synthesized into different shapes and sizes via physical processes like laser ablation, evaporation, and condensation, or through chemical processes like hydrazine, sodium borohydride, and green synthesis (Nurani, et.al. 2015).

1. Mode of Action of Silver Nanoparticles

After determining the bactericidal properties of silver nanoparticles, the mechanism behind their antimicrobial effects was studied. Various studies using transmission electron microscopy (TEM) and other methods verified that Ag NPs have the ability to cause damage to bacterial cells through a wide array of different mechanisms.

A Kirby-Bauer sensitivity test was performed, and by using various antibiotic discs with different silver resistant bacterial strains, the zone of inhibition was measured. It was found that the bacteria showed modified susceptibility to cephalosporins, glycopeptides, aminoglycosides, and fluoroquinolones, thereby indicating the mechanism of action of Ag NPs. Based on the action of these antibiotics and the bacteria's altered zone of inhibitions, this study showed that these particles interact with the cell wall, proteins, and DNA (Lara et. al. 2010).

Because of their small size, silver nanoparticles have the ability to attach to the surface of the cell membrane and disrupt its function. The positive charge of the Ag NPs allows them to electrostatically interact with the negatively charged membrane (Lara, et.al. 2011). As transmission electron microscopy has shown with E. coli cell membranes, silver nanoparticles disrupt the cell membrane, increase the cell's permeability, and ultimately cause cell death (Dakal, et. al. 2016) (Mambrio-Jones, Hoek, 2010).

Furthermore, silver has a high affinity for sulfur and phosphorus (Nurani, et.al. 2015). Therefore, Ag NPs can also adsorb to the cell membrane by interacting with thiol (SH) groups on the cell membrane. As a result of this interaction, a new bond between sulfur and silver (S-Ag) is formed, and the creation of this new bond blocks both respiration and the electron transfer. Ultimately, this results in the collapse of the proton motive force. Without the proton motive force, the cell membrane

is disrupted and cell leakage followed by cell death ensues (Mijnendonckx, et. al. 2013).

Moreover, after weakening the cell membrane, these particles have the ability to penetrate the bacteria (Lara, et.al. 2010) (Mijnendonckx, et.al. 2013). Additionally, in gram negative bacteria, porins help facilitate the entry of Ag NPs into the cell. Once inside, these Ag NPs interact with various molecules resulting in further cell damage (Dakal, et. al. 2016).

Ag NPs have a significant effect on DNA replication. Silver particles interact with thiol groups resulting in conformational changes that ultimately inhibit the activity of various enzymes (Mijnendonckx, et.al. 2013). Furthermore, when silver particles bind to the guanine base, pyrimidine dimerization occurs, and DNA replication is inhibited. Silver particles can also affect protein production and translation by interacting with and subsequently denaturing ribosomes (Dakal, et.al. 2016).

Moreover, silver nanoparticles are oxidized into silver ions upon entering the bacterial cell. Ag ions cause damage and cell death by interacting with lipids, proteins, and DNA. Silver ions also interact with nucleosides in addition to forming complexes with nucleic acids. Furthermore, these ions bind and dimerize DNA and RNA, block the expression of proteins and enzymes involved in ATP production, and generate free radicals (Lara, et.al. 2010) (Mijnendonckx, et.al. 2013).

Reactive oxygen species can be endogenously produced from natural metabolic processes like aerobic respiration. Metal ions, like silver, have the ability to catalyze the generation of free radicals in the presence of oxygen (Mambrio-Jones,Hoek, 2010), and spin resonance measurements have shown that silver ions increase the production of reactive oxygen species (Mijnendonckx, et.al. 2013) (Mambrio-Jones, Hoek, 2010) (Kim, et. al. 2007). These free radicals then act upon the mitochondrial membranes to induce necrosis and cell death. Furthermore, ROS oxidize lipids, nucleic acids, and proteins, thus disrupting the level of homeostasis by inducing oxidative stress and cell damage.

In addition to increasing the production of ROS, silver particles also decrease the levels of glutathione, an antioxidant, by reducing it into glutathione disulfide. Moreover, NPs inhibit the action of NADPH-dependent flavoenzyme, catalase, glutathione peroxidase, and superoxide dismutase, anti-oxidative enzymes that quench free radicals. These enzymes are dependent on thiol groups, but since silver ions interact with thiol groups, these enzymes cannot properly quench the reactive oxygen species (Mijnendonckx, et.al. 2013) (Dakal, et.al. 2016).

Phosphorylation and dephosphorylation are important signaling processes in bacterial growth. Phosphorylated proteins guide DNA replication and recombination, metabolism, and bacterial cell cycle. Silver nanoparticles block this signaling pathway to prevent the production and action of phosphorylated proteins. Furthermore, phosphorylated tyrosine kinases aid in the transport of exopolysaccharide and capsular polysaccharides. Therefore, to prevent bacterial growth, Ag NPs can also dephosphorylate these tyrosine residues (Dakal, et. al. 2016) (Shirvastava, et.al. 2007).

Effects of Silver Nanoparticles on Gram Positive and Gram Negative Bacteria

A great deal of controversy exists regarding the efficacy of silver nanoparticles in both gram negative and gram positive bacteria. In some studies, the Ag NPs had an equal effect in both types of bacteria, rendering silver as a potential broad antibacterial agent (Lara, et. al. 2010). However, other studies found that these particles are more effective in gram negative bacteria. This phenomenon is attributed to the differences in the composition of the cell wall in both gram positive and gram negative bacteria. The peptidoglycan in gram positive bacteria is very thick, averaging around 20-80 nm, and rigidly cross linked. Because of this thick, rigid membrane, there are fewer sites for the silver to adhere to, making it harder for the particles to adsorb and penetrate the cell. On the other hand, the cell wall of gram negative bacteria is composed of a much thinner, roughly 7-8 nm, and weaker PG. In addition, the membrane of gram negative bacteria contains the lipopolysaccharide (LPS). Because of its negative charge, the LPS increases the silver nanoparticles' ability to successfully bind to the cell's surface. The thinner and weaker PG coupled with the negatively charged LPS makes gram negative bacteria more susceptible to silver nanoparticles (Feng, et.al. 2000) (Shirvastava, et.al. 2007) (Dakal. et.al. 2016).

2. Size of Silver Nanoparticles

Particle size, shape, and dose all contribute to the efficacy and toxicity of silver particles. Extensive research has been done in attempt to uncover the antibacterial effects of different sized Ag NPs. Using the disk diffusion method, one study compared the efficacy of different sized nanoparticles against both gram positive and gram negative bacteria. The relationship between size and efficacy of Ag NPs has been extensively researched, and it was concluded that the two are inversely related; the smaller the diameter of the particle, the more effective it is against drug-resistant bacteria. The efficacy of smaller nanoparticles compared to larger NPs can be attributed to the relationship between surface area and size. Surface area and particle size is inversely related; the smaller the particles are, the larger their surface area is (Rai, et. al. 2014). As a result of the increased surface area, the smaller particles can interact with the bacterial cell more efficiently (Rai, et.al. 2009) (Haider, Kang, 2015).

3. Dose of Silver Nanoparticles

After determining the mode of action of Ag NPs, researchers began to study the dose required to have a bactericidal effect. By using various doses, 0.0, 6.25, 12.5, 25.0, and 50.0 mM, against different strains of bacteria, it was found that dose and antibacterial effect were directly related; the higher the dose of the Ag NPs,

the more effective they were. The highest dose, 50.0 mM, was the most successful at killing bacteria (Lara, et.al. 2010). Furthermore, the dose required to have a combative effect was found to be bacteria specific for each genus (Morones, et. al. 2005).

4. Shape of Silver Nanoparticles

In addition to amount and size, shape also plays a role in the efficacy of the silver nanoparticles against drug-resistant bacteria. Facets are flat surfaces that are present on geometric shapes. Because of their large densities, facets have a higher surface reactivity, and therefore Ag NPs with more {111} facets, facets that are cut through the x, y, and z planes, have a greater effect against bacteria (Mambrio-Jones, Hoek, 2010). Some research has shown that triangular shaped particles are more effective than both sphere and rod shaped particles at combating drug resistant bacteria. The increased surface area and antibacterial effect of triangle shaped particles can be due to their geometric structure which contains numerous facets (Mambrio-Jones, Hoek, 2010) (Raza, et.al. 2016) (Pal, et.al. 2007) (Mijnendonckx, et.al. 2013).

However, other research found contradictory results. Using the disk diffusion method, the zone of inhibition of Pseudomonas aeruginosa was measured with the use of five different sized and shaped particles: S1, S2, S3, S4, and S5, was measured. While S1, S2, S3, S5 were all sphere shaped varying in size, S4 was triangular shaped. After placing the particles in the center of the sampled bacteria on an agar plate, the zone of inhibition of S2, the smallest sphere particle, was 1.5 mm, while the zone of inhibition for the triangular shaped particles was 1.4 mm. This demonstrated that the smallest sphere particle had a greater effect than the triangular shaped one (Raza, et.al. 2016). Using x-ray diffraction, it was determined that the smaller spherical shaped particles also contained high atomic density facets that are found in triangular shaped Ag NPs which evidently contributed to the sphere's greater bactericidal effect. Furthermore, the sphere shaped Ag NPs acted as acids and interacted with bases, sulfur and phosphorus containing compounds, to cause damage to both the cell membrane and DNA (Raza, et.al. 2016).

5. Toxicity of Silver Nanoparticles

The toxicity of any antimicrobial agent is a major issue of concern, and despite silver particles' potential to act as an antimicrobial agent, at certain levels, silver nanoparticles can be toxic to all mammals, including humans. Research has shown that Ag NPs stimulate an immune response and induce apoptosis via the JNK and ROS signaling pathways. As indicated by the levels of glutathione, silver particles induce oxidative stress by decreasing the amount of the glutathione in the body. Additionally, due to their high surface area, Ag NPs can also generate free radicals from the respiratory chain. This imbalance between ROS and antioxidant levels results in damage to both lipids and proteins (Arora et.al. 2008) (Mambrio-Jones, Hoek, 2010). Silver NPs also interact with the mitochondria to increase oxidative stress and to disrupt ATP production, ultimately causing damage to DNA. Ag NPs also affects protein folding. This increased stress in the cell leads to cytotoxicity and apoptosis. Furthermore, Ag NPs can enter the nucleus and induce genotoxicity by causing DNA mutations, base damages, and strand breaks. Finally, these particles induce carcinogenesis by activating different signaling cascades and inflammatory responses (Dakal, et.al 2016).

In a few studies, it was found that the dose of Ag NPs needed to induce apoptosis was different from the dose required to stimulate necrosis, 0.78-1.56 vs. 12.5 µg. Therefore, it was concluded that a safe dosing range of silver can be determined (Mambrio-Jones, Hoek, 2010). However, a contradictory conclusion was reached by AshaRani et. al who found silver nanoparticles can be toxic to humans at any dose (AshaRani et.al. 2009) (Mambrio-Jones, Hoek, 2010). Therefore, due to this discrepancy, further research in regards to the toxicity of Ag NPs must be conducted.

6. Resistance to Silver Nanoparticles

As is the case with antibiotics, bacterial resistance to silver is a major concern. When bacteria are exposed to silver nanoparticles, it results in the natural selection of bacteria, thus causing bacterial resistance to silver particles.

Bacterial resistance to silver NPs can be encoded in the plasmid or in the chromosome as it is seen in both Salmonella and E. coli respectively (Mambrio-Jones, Hoek, 2010). In Salmonella, the resistance to silver ions is attributed to nine genes on the plasmid. Furthermore, resistance is also associated with the SilCBA and SilP efflux pumps, the SilE and SilF periplasmic binding proteins, and in Escherichia coli porin loss. However, resistance to silver nanoparticles as a result of a one point mutation is not very common because of the complexity of silver's actions (Chopra, 2007).

Additionally, since silver nanoparticles have a high surface area, they can aggregate and combine together, causing them to lose their antibacterial effect (Nurani, et.al. 2015) (Beyth, et.al. 2015). Furthermore, because of its high surface energy, Ag NPs can become contaminated by the air, and therefore Ag NPs are synthesized with either chitosan, alginate, or gelatin, biodegradable polymer matrixes, to prevent their oxidation (Nurani, et.al. 2015).

7. Combination of antibiotics and AG NPs

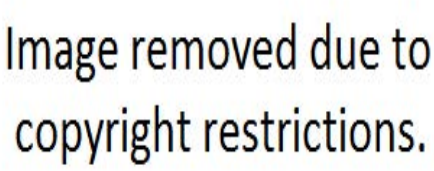
The synergy of two drugs or compounds has the potential to successfully combat drug-resistant bacteria by acting through different mechanisms. Therefore, after determining the bactericidal effect of silver nanoparticles, many researchers attempted to determine the effects of the use of Ag NPs in conjunction with the use of antibiotics against different strains of bacteria.
To determine the synergy of Ag NPs with drugs, silver particles were used with amoxicillin against samples of E. coli. By comparing the minimum inhibitory concentration of various doses of silver nanoparticles alone, the use of different amounts of amoxicillin by itself, in addition to the combined effects of both silver NPs and amoxicillin, the advantages of combination therapy was identified.

The combined effect of amoxicillin and Ag NPs was seen to have a greater bactericidal effect than each of them alone. Furthermore, when the two were administered together, a lower dose of both amoxicillin and silver nanoparticles were needed to stimulate an antibacterial effect as opposed to when each one was given alone (Allahverdiyev, et. al. 2011) (Li, et. al 2005).

The success for this synergy can be attributed to a few different theories. With combination therapy, the two compounds or drugs that are used usually target different steps, pathways, or enzymes. Therefore, if bacteria resist the action of one antibiotic, the other compound can still exert its antimicrobial effects through a different non-resistant mechanism.

Additionally, in non-resistant bacteria, the synergy of the two can be attributed to the chelation reaction that occurs between both the hydroxy and amino groups of the B-lactam and the Ag NPs. The binding between the silver and the amoxicillin in addition to the binding between the antibiotic with other drug particles resulted in the formation of a new compound. This newly synthesized antimicrobial agent, containing silver on the inside and amoxicillin on the outside, attaches to the surface of the cell membrane and causes more damage because of the synergy of the two compounds. Additionally, the amoxicillin disrupted the cell wall which increased the penetration of the Ag NPs into the cell. Moreover, from this chelation reaction, silver nanoparticles prevent DNA from unwinding, thus resulting in further damage to bacterial DNA (Li, et. al. 2005) (Allahverdiyev, et. al. 2011).

Furthermore, silver nanoparticles can be used as a drug carrier. While antibiotics are usually hydrophilic, silver nanoparticles are hydrophobic. Therefore, these nanoparticles can interact with the hydrophobic bacterial cell membrane more easily than antibiotics, enabling the transport of hydrophilic antibiotics to the bacterial cell surface (Li, et. al. 2005).



Conclusions

This paper attempted to explain the mechanism of action and mechanism of resistance of antibiotics and drug resistant bacteria, respectively. As antibiotic resistance continues to emerge, researchers are constantly searching for new antimicrobial agents. Silver nanoparticles have gained much attention as a possible tool in combating drug resistant bacteria, and studies have proven the efficacy of Ag NPs to induce cell damage and cell death. However, despite the growing potential of silver nanoparticles,

further research must be conducted before implementing silver nanoparticles into clinical trials as a promising way of replacing or supplementing the currently used antibiotics.

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Cyclodextrin as a Drug Carrier Increasing Drug Solubility

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Abstract

The development of a new drug requires research and evaluation before the drug is approved to enter the market. One of the factors determining the efficacy of a drug is the aqueous solubility of the drug. A current problem in today's pharmaceutical industry is the low aqueous solubility of many useful drugs. A drug with a low aqueous solubility will not readily be absorbed by the body. The low aqueous solubility of a drug is often due to the drug's hydrophobic character. Drug enhancement methods are necessary to avoid the obstacle of drug insolubility and many methods have been developed. This paper focuses on the use of cyclodextrins and their derivatives as a drug carrier to increase the solubility of poorly soluble drugs. Cyclodextrins have both hydrophobic and hydrophilic character and are capable of encapsulating a hydrophobic drug molecule and forming guest-host complexes. The cyclodextrin's cylindrical shape allows the guest molecule, the drug, to be kept within the hydrophobic interior while the exterior of the cyclodextrin is hydrophilic and soluble in aqueous solution. This complex improves the drug solubility and ultimately the bioavailability of insoluble drugs. In this paper the complexation of four drugs with cyclodextrin was studied; ibuprofen, imatinib, praziquantel and camptothecin. In each case the aqueous solubility of the poorly soluble drug was increased with the use of cyclodextrins.

Introduction

The development of a drug is a process of converting a biologically active compound into a product that is safe and effective, it is a long and expensive process. The performance of a drug, the efficiency of the drug in carrying out the desired action, is dependent on the drug's properties. Chemical and physical properties of a drug determine the drug's ability to react with the necessary components along the path to achieving the desired effect. One of the factors determining a drug's performance and effectiveness is the aqueous solubility of the drug (Gareth, 2007).

Solubility of a Compound

IUPAC defines solubility as the composition of a saturated solution in terms of the amount of solute in proportion to solvent (McNaught, Wilkinson, 2006). Solubility is the term used to describe a solid, liquid, or a gas's ability to dissolve in a solvent. Aqueous solubility of a molecule changes depending on the electrostatic charge of the molecule and the degree of ionization. The dipoles in the water molecules are attracted to the charged molecules forming a shell around the molecule and solvating it (Trevor et.al. 2015).

Solubility of a compound depends also on its structure and polarity. Polar groups can hydrogen bond with water molecules and be solvated, nonpolar groups are insoluble in aqueous solution. Therefore, the aqueous solubility of a compound increases as the number of polar groups on the compound increases. In addition, if the polar groups on a compound have the ability to ionize in water, the compound will be further soluble. Lipid soluble compounds are those with more nonpolar groups and they may contain functional groups such as benzene rings, ethers and esters. Amphipathic compounds with polar and non-polar parts are soluble in both aqueous and lipid solvents (Gareth, 2007).

Drug Solubility

Solubility is a fundamental property that must be considered when evaluating a drug. The solubility of a drug can range from fully soluble, like ethanol in water, to poorly soluble, often referred to as insoluble (Clugston, Fleming, 2000). The Biopharmaceutics Classification System (BCS), is a guide from the U.S. Food and Drug Administration and is used to regulate and classify drugs based on aqueous solubility and membrane permeability. Class I drugs have high aqueous solubility and high membrane permeability, Class II drugs have low aqueous solubility and high membrane permeability, Class III have high aqueous solubility and low membrane permeability, and Class IV drugs have low aqueous solubility and low membrane permeability. Solubility of a drug is determined based on the drug's solubility over a pH range of 1 to 7.5 and is considered very soluble if the highest dose strength is soluble in <250 ml of water. The membrane permeability of a drug is measured by the extent of absorption and a drug is considered highly permeable when the absorption in humans is > 90% of an administered dose (FDA, BCS, 2016). Since cells typically contain 65% water, a drug must be water soluble to be effective. A drug must be absorbed before entering the bloodstream in order for the drug to be transported via the systemic circulation to its site of action. The more soluble the drug the greater amount of absorbed drug in the bloodstream. More drug in the bloodstream increases the gradient between the bloodstream and the extracellular fluid, in this way diffusion of the drug from the blood to the extracellular fluid will be facilitated (Trevor et. al. 2015). In addition, the more water soluble the drug the higher the bioavailability (Gareth, 2007). The amount of drug that is absorbed by the bloodstream, divided by the amount that was administered, is the bioavailability of that drug administered in that specific manner. Drug absorbed / drug administered = bioavailability of the drug (Trevor et. al. 2015).

Oral drugs are the most common form of drug administration. They are easy to administer, are less specific in their sterility requirements, they are most cost effective, have a high patient compliance, and there is flexibility in the dosage form. For this reason many pharmaceutical companies prefer to produce bioequivalent oral drugs. The difficulty with oral bioequivalent drugs is their low aqueous solubility and therefore low bioavailability. Oral drugs must first dissolve in the aqueous gastric fluid in order to be transported to the site of action. Therefore, solubility of a drug is an important factor in determining the concentration of a drug

that is required to achieve a desired response. More than 40% of pharmaceutical developments are poorly soluble in water. Low solubility of a drug slows the absorption rate, decreasing the bioavailability. Thus, solubility enhancement methods are necessary. Increasing a drug's solubility is a challenge faced by the drug development industry, especially the solubility of oral drugs (Savjani et. al. 2012). Increasing the solubility of Class II drugs that have low solubility and high permeability would be very productive. The rate limiting step for Class II drugs, according to the BCS, is the insolubility of the drug, therefore increasing the solubility will increase the bioavailability of those drugs.

There are many methods that are currently used to increase the solubility of new chemical entities (NCE's). There are physical and chemical modifications that can be made to a drug to increase its solubility. Particle size reduction can be employed, as well as solid dispersion, nanosuspension, colloidal particles, and changes to the crystal form. Additionally, chemical changes to pH as well as complexation and salt formation affect the solubility (Savjani et. al. 2012).

Other methods include the use of surfactants, micelles, liposomes and inclusion complexes (Gareth, 2007). These methods utilize the hydrophobic effect in increasing the solubility of a molecule. Cyclodextrins and cyclodextrin derivatives form inclusion complexes with insoluble drugs. Inclusion complexes apply guest-host chemistry in the formation of the complex. This paper will focus on cyclodextrins and cyclodextrin derivatives as a method to increase the solubility of a poorly soluble drug.

Methods

This study was completed by analyzing various articles collected from databases including Touro Library and PubMed. The research collected mainly explored cyclodextrins and their ability to form guest-host complexes.

Discussion:

Cyclodextrin and Cyclodextrin Derivatives

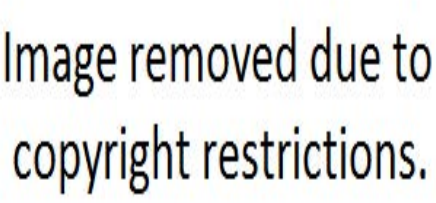
Cyclodextrins (CD's), and their derivatives are amphipathic molecules capable of forming guest-host complexes with drug molecules. The complex improves the drug solubility and ultimately the bioavailability of insoluble drugs. Cyclodextrins are used as a drug delivery system because of their potential to change physical, chemical and biological properties of a drug by forming a guest-host complex (Gareth, 2007). From a microscopial point of view, each guest molecule is micro-encapsulated leading to changes in the chemical and physical properties of the molecule. Cyclodextrins and their derivatives can improve the solubility of a molecule, modify a liquid substance to a powder, and mask a bad taste, smell or color of a drug (Chaudhary, Patel, 2013).

Cyclodextrin Structure

Cyclodextrins are cylindrical oligosaccharides typically made up of six, seven, or eight glucose units. The cyclodextrin ring form is composed of α-D-glucopyranoside units with 1→4 linkage like in amylose (Gareth, 2007). When glucose is degraded by the enzyme glucosyltransferase, a product of the chain splitting can undergo an intramolecular reaction to form cyclic molecules, cyclodextrins. Each glucose molecule in the cyclodextrin contains two secondary alcohols and a primary alcohol. Alpha (α), beta (β), or gamma (γ) cyclodextrins, depending on whether they contain six, seven, or eight glycosyl units respectively, are classified as natural cyclodextrins. Chemically derived cyclodextrins are hydroxypropyl-β-cyclodextrin, randomly methylated-β-cyclodextrin, and sulfobutylether-β-cyclodextrin. These derivatives are often preferred due to their enhanced physiochemical and biopharmaceutical properties (Gidwani, Vyas, 2015).

Cyclodextrin Synthesis

B. macerans is the microbe responsible for the formation of cyclodextrins. A method to synthesize cyclodextrins is to treat starch with amylase from Bacillus macerans. A crude product of cyclodextrin is then obtained with about 60% αCD, 20% βCD, and 20% γCD. The product also contains small amounts of other materials including proteins. To purify the different



cyclodextrin derivatives, various forms of glucosyltransferases are genetically engineered. These glucosyltransferases are active towards the production of specific cyclodextrin derivatives. The glucosyltransferase enzymes distinguish the six, seven, or eight glucopyranose units from the non-reducing end of an amylose and alter the linkage to produce a specific cyclodextrin derivative (Brewster, Loftsson, 2007).

Hydropathy of Cyclodextrin

Cyclodextrins are amphipathic molecules. They have hydrophobic and hydrophilic character. Cyclodextrins have a hydrophobic interior and hydrophilic exterior. The polar exterior of the cyclodextrin forms hydrogen bonds with the aqueous solution. The hydrophobic effect drives the non-polar portion of the cyclodextrin inwards, away from the aqueous solution, forming a hydrophobic cavity. This property enables the cyclodextrin to form guest-host inclusion complexes with hydrophobic molecules (Chaudhary, Patel, 2013).

Cyclodextrins can encapsulate many molecules. The nonpolar hydrophobic molecule interacts with the nonpolar groups in the interior of the cyclodextrin, while the polar hydroxyl groups on the exterior surface of the cyclodextrin are hydrophilic. Hydrophobic molecules the size of one or two benzene rings can fit into the cyclodextrin cavity. The cyclodextrins in the complex increase the aqueous solubility of the guest molecule, often a drug. The interactions between the cyclodextrin and the drug form the inclusion complex, the cyclodextrin host molecule with the guest drug (Tiwari et. al. 2010).

Cyclodextrin-Drug Complex

A number of forces are responsible for the complex formation; hydrogen bonding, van der Waals interactions, release of conformational strain, and exclusion of high energy water bonds in the cyclodextrin cavity. Thermodynamic interactions are the main drive in the formation of the cyclodextrin-guest complex. Thermodynamically, the interaction between the cyclodextrin host, the guest molecule, and the solvent, must overall be favorable in order for the reaction to proceed (Chaudhary, Patel, 2013). The cyclodextrin complex formation is usually enthalpy driven. In an aqueous solution the cyclodextrin cavity contains polar water molecules that are readily exchanged for non-polar hydrophobic guest molecules. The water molecules situated inside the non-polar environment of the cyclodextrin cavity do not have a full complement of hydrogen bonds and are higher in energy than the water molecules outside the cyclodextrin. Liberating the water molecules that are enthalpy-rich, high in energy, is a driving force for the complexation. Sterically, the size and certain functional groups of the guest molecule determine the ability of the guest to fit into the cyclodextrin cavity. Additionally, the complex formation is dependent on the chemical properties of the guest molecule (Brewster, Loftsson, 2007).

The formation of the cyclodextrin-drug complex can be achieved through several methods. The kneading method is a simple and inexpensive method and is therefore the most commonly used method for preparing the complex (Savjani et. al. 2012). This method converts the cyclodextrin into a paste by saturating the cyclodextrin or cyclodextrin derivative with water or hydroalcoholic solution. The drug is then added to the paste and the mixture is kneaded. The kneading can be done either in the laboratory or using machines for large scale achievement of the complex. An additional method, the lyophilization/freeze-drying technique, works with a solution of cyclodextrin and drug. The solvent in the solution is removed by freezing the solution and then drying it by rapidly reducing the pressure, generating a powder. The increased surface area of the powder enables considerable interactions between the cyclodextrin and the drug (Savjani et. al. 2012).

Cyclodextrins can increase the solubility of the guest molecule in three patterns when graphed with cyclodextrin concentration and drug solubility. AL profiles indicate a linear increase in drug solubility as the concentration of cyclodextrin is increased. AP systems have a curve that deviates in a positive direction from the linearity indicating that the cyclodextrin is proportionally more effective at higher concentrations. AN relationships have a negative deviation from the linearity indicating that at higher concentrations cyclodextrin's effectiveness decreases (Brewster, Loftsson, 2007).

Complex Dissociation

Once a product of drug-cyclodextrin is obtained, the complex must dissociate to release free drug upon reaching the site of action. When drug is encapsulated within the cyclodextrin there are factors that cause the complex to dissociate and release free drug. The drug and cyclodextrin interactions are non-covalent interactions that are in dynamic equilibrium, constantly associating and dissociating. There are two important factors concerning the complex's non-covalent interactions; firstly the complexation strength, defined by K, and secondly the complex lifetime. In a 1:1 complex ratio of drug to cyclodextrin the complexation strength, the K constant, is defined by the equation: $K = kf / kt = [DCyD] / [Df] [CyDf]$ where kf and kt are the forward and reverse rate constants, [DCyD] is the complex concentration and [Df] and [CyDf] are free drug and free cyclodextrin respectively.

Dilution is a major factor assumed to play a role in the dissociation of the complex. 1000-fold dilution of a drug with a low binding constant to cyclodextrin decreased the complexation percentage from 98% to 5.7%. The fraction of complexed drug decreased from 99.5% to 47.5% in the presence of 1000-fold dilution of a drug with a high binding constant to cyclodextrin.

Therapeutically, the complexed drug is diluted either in the plasma or in the aqueous extracellular fluid. A 1mL

drug-cyclodextrin complex administered via intravenous injection is diluted in the plasma by a factor of 1:3,500, in the extracellular fluid the factor is 1:21,000. Dilution alone is not sufficient in orally administered drugs because the complex resides in the gastrointestinal tract before reaching the site of action.

Drug binding to plasma protein is another factor responsible for complex dissociation. If a drug has the ability to bind to plasma protein the complex would dissociate further to release more free drug according to Le Chatelier's principle. Drugs with a high binding constant to cyclodextrin that have the ability to bind moderately to proteins, in 1000-fold dilution, are further dissociated and only 31.9% complexed drug remains. If the drug binds more tightly to proteins the factor of complexed drug decreases to 8.9%.

Furthermore, if a drug is lipophilic and can be taken up by tissue more drug would be released from the complex. The cyclodextrin would remain in the aqueous solution and drug would be taken up by tissue. This mechanism is especially useful in drug administered at a site that has insignificant dilution (Stella et. al. 1999).

The dissociation process is driven mainly by an increase of water molecules in the surrounding environment and happens relatively fast. In a dilute environment there is a concentration gradient which shifts the equilibrium to the left, dissociating the cyclodextrin and the drug, thereby releasing free drug to be absorbed. In the body's dynamic environment, it is not likely that the drug will encounter another cyclodextrin with which to form a complex and in this way it remains free in the solution (Chaudhary, Patel, 2013).

Complexed Drug Pharmacokinetics

The effect of cyclodextrins on the pharmacokinetics of complexed drug must be considered before cyclodextrin complexation can be applied. Kurkov et. al. studied the effect of complexation on the pharmacokinetics of close to 200 drugs. The results concluded that the hydroxypropyl-β-cyclodextrin does not have a significant effect on the pharmacokinetics of the drugs. However, although not altered, some drug is lost due to the complexation. The hydrophilic cyclodextrins are excreted unchanged by the kidneys and any drug still encapsulated is excreted too. This is especially true when the drug has a high binding constant to cyclodextrin (Kurkov et. al. 2012).

Toxicological Considerations

Orally administered cyclodextrins are not significantly absorbed in their intact form from the gastrointestinal tract. The Cyclodextrins range from 1000 Da to over 2000 Da in molecular weight and are hydrophilic with significant hydrogen bonding donors and acceptors, therefore they cannot be absorbed in their original form. Natural α-cyclodextrin and β-cyclodextrin cannot be hydrolyzed by human salivary and pancreatic amylases and in general oral administration is not associated with significant adverse effects and is well tolerated. After IV injection α-cyclodextrin is mainly excreted unchanged in the urine. β-cyclodextrin is nephrotoxic when administered parenterally but non-toxic in the topical, buccal, rectal or oral form and can be found in numerous marketed drugs. The metabolism of γ-cyclodextrin is similar to that of starch and linear dextrins and only small amounts are absorbed intact. After iv injection the γ-cyclodextrin is mainly excreted unchanged in the urine (Brewster, Loftsson, 2007).

Ibuprofen-Cyclodextrin

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) used to treat mild to moderate pain and fever. Ibuprofen works by inhibiting cyclooxygenase, an enzyme that converts arachidonic acid to cyclic endoperoxides. Cyclic endoperoxides are precursors to prostaglandins responsible for inflammation (PubChem, 2004).

Ibuprofen is a poorly water soluble drug. Although it contains a carboxylic acid functional group which can hydrogen bond with water and be solvated, the alkyl groups and the benzene ring are non-polar and make the drug poorly soluble. Ibuprofen is complexed with cyclodextrin derivatives to increase its solubility (Hussein et. al. 2007).

Khaled Hussein, et al. conducted a study on the ibuprofen-β-cyclodextrin complex (Hussein et. al. 2007). A solid complex of ibuprofen, MW 206.3 g/mol, and β-cyclodextrin, MW 1,135.0 g/mol, was prepared. Using controlled Particle Deposition (CPD) with supercritical carbon dioxide the ibuprofen-β-cyclodextrin complex was formed. The CPD method dissolves the desired solution in supercritical carbon dioxide which then enters the pores of the carrier at extremely high pressure and precipitates following a rapid pressure drop.

The "n-hexane wash" method determined whether the complex was formed. β-cyclodextrin and its complexes are insoluble in n-hexane but the free drug is soluble. The supernatant liquid that was insoluble was separated, dried and then analyzed by high-performance liquid chromatography (HPLC). HPLC determined the product yield as well as the content of ibuprofen in the complex. The inclusion yield of the complex was determined using the following equation:

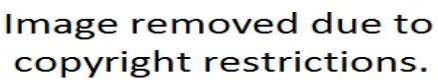
Percent of included ibuprofen= [complexed amount of ibuprofen/free amount + complexed amount] x 100 49.83±3.65 inclusion yield % was calculated.

Infrared spectroscopy of pure ibuprofen showed all the characteristic peaks including the carbonyl peak at 1,706 cm-1. The complexed drug's infrared spectroscopy had a very week carbonyl peak indicating that the drug molecule was encapsulated inside the β-cyclodextrin. Morphological changes were also observed. Pure ibuprofen is rough needle-shaped crystals and β-cyclodextrin is parallelogram shaped. The shape of the complexed particles appeared smaller in size and different than pure drug or pure β-cyclodextrin. This observation adds to the evidence of the ibuprofen-β-cyclodextrin complex formation.

The complex was tested for drug release using a flow system with a dissolution vessel at pH 5 and 37°C. A sample of pure ibuprofen and a sample of complexed ibuprofen, each containing 3mg ibuprofen, were added to the vessel. Samples were taken from the dissolution fluid at set intervals and were studied spectrophotometrically. Kw, the dissolution coefficient, was calculated. Kw corresponds to the time when 63.2% of the drug is dissolved.

The dissolution of pure ibuprofen in vitro at pH 5 showed poor dissolution after 15 minutes and after 75 minutes. The complexed drug had a significantly higher dissolution rate and amount, due to the presence of the β-cyclodextrin. The dissolution rate of the complexed product was 0.086±0.002 min^{-1}, the rate of pure ibuprofen was excluded from analysis since it did not reach 63.2% within 72 minutes. The dissolved amount of drug after 15 minutes was determined to be 71.9±3.62% for the complexed prepared using the CPD method, and 22.0±3.56% for pure drug. After 72 minutes, 93.5±2.89% of drug dissolved from the ibuprofen-β-cyclodextrin complex and from pure ibuprofen sample only 59.5±4.86% of drug dissolved. The complexed drug had a significant increase in aqueous solubility.

Imatinib-Cyclodextrin



Imatinib, known as Gleevec, is an oral tablet or a capsule used for the treatment of Philadelphia chromosome positive chronic myeloid leukemia (CML) or gastrointestinal stromal tumors. Imatinib works by inhibiting protein tyrosine kinase, the growth factor receptor that causes unregulated cell growth of cancer cells (PubChem, 2005).

Aqueous solubility of imatinib is charge dependent and varies depending on the pH. The active compound, imatinib, can be mono, di, tri and tetra protonated. The more protonated the compound the greater the overall charge of the compound. Increased charge increases the aqueous solubility. At a pH below 5.5 the formation of different protonated species of imatinib results in a charged drug that is water soluble. However, the free base form of imatinib is neutral, uncharged, and therefore poorly soluble. To increase the aqueous solubility of the free base form of imatinib, the drug was complexed with β-cyclodextrin and with randomly methylated β-cyclodextrin (RAMEB). Szabolcs Beni, et al. performed a study determining the effectiveness of cyclodextrin and cyclodextrin derivatives in increasing the solubility of the neutral drug form (Beni et. al. 2007).

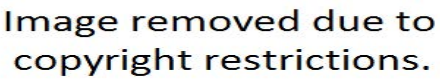
In a solution of Na2CO3 at a pH of 10.5, 2 mg of imatinib was added to a 1 ml solution of varying concentrations of β-cyclodextrin, 0-13 mM. The mixture was shaken for two days at 25.0 ± 0.5°C and a complexed product of 1:1 stoichiometry was formed.

The aqueous solubility of the free base form of imatinib was enhanced by complexing it with β-cyclodextrin. At a pH of 10 the linear AL phase solubility diagram indicated the improvement in the solubility of the drug. With increased cyclodextrin concentration, the solubility of the drug increased linearly, complexation increased the drug solubility tenfold.

Titration of pure imatinib with KOH precipitated above a pH of 6.5. Titration of 1mM imatinib in the presence of fourfold β-cyclodextrin or randomly methylated β-cyclodextrin (RAMEB) did not precipitate due to the complex's effect on the solubility. The most stable form of the imatinib-cyclodextrin complex formed with the neutral form of imatinib. This is because the neutral imatinib is most hydrophobic and interacts most with the hydrophobic interior of the cyclodextrin. The affinity of imatinib towards cyclodextrin decreased as the charge on the drug increased.

^{1}H NMR chemical shift titration method quantified the non-covalent molecular interactions. At a titration of pH 3.5 the predominant imatinib species was the deprotonated form. The mono and tri protonated forms were present in smaller quantities. Interactions between the imatinib forms and the β-cyclodextrin was evident. The data collected suggests that the benzamide fragment of imatinib is involved in the inclusion complex.

2D ROESY NMR spectra was recorded at the same pH to verify the data. The evidence suggested that the imatinib inclusion proceeds through the narrower part of the cyclodextrin host. For steric reasons the inclusion proceeds in an unusual way, through the narrower part of the cyclodextrin. The result

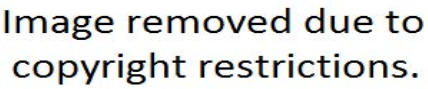


of the study concluded that a tenfold increase in the imatinib solubility was achieved with 12mM β-cyclodextrin complexation. The increased aqueous solubility of imatinib greatly increases the effectiveness of the anti-cancer drug.

Praziquantel-Cyclodextrin

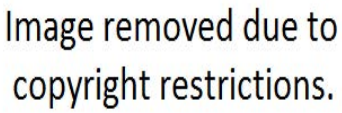
Praziquantel (PZQ) is a Class II drug, according to the BCS, used for the treatment of schistosomiasis. The drug works by causing severe spasm and paralysis to the worms' muscles and the worms are then destroyed in the intestines or passed in the stool. It is classified as a Class II drug due to its high membrane permeability but low aqueous solubility. The low aqueous solubility is due to its lipophilic character (PubChem, 2005).

As a Class II drug, a high dose of praziquantel is necessary in order for it to be effective. Improving the aqueous solubility of praziquantel can potentially result in the classification of the drug as a BCS-Class I compound. To increase the aqueous solubility



of praziquantel the drug was complexed with β-cyclodextrin and hydroxypropyl-β-cyclodextrin. A study was conducted to determine whether this Class II drug can be complexed with cyclodextrin and behave like a Class I drug (Maragos et. al. 2009).

The praziquantel-cyclodextrin complexes were formed via the kneading method. 0.080 grams of praziquantel was mixed with cyclodextrin in varying ratios; 1:1, 1:2, and 1:4 molar ratios. The complexed praziquantel-β-cyclodextrin reached equilibration within 12 hours and the praziquantel-hydroxypropyl-β-cyclodextrin complex reached equilibration after a 24 hour period. Heating the samples at 70°C for an hour prior to the experiment accelerated the reaction and also increased the formation of the praziquantel-cyclodextrin complex. The concentration of praziquantel in the binary system remained constant while it was in a shaking bath at 25°C for 72 hours.



The aqueous solubility of the lipophilic drug had a 4-5-fold increase upon complexation with cyclodextrin. Calculations of the praziquantel solubility in 0.01 M cyclodextrin versus the solubility of praziquantel without cyclodextrin calculated a solubility enhancement factor of 4.5. Preheating the samples at 70°C for one hour prior to the experiment increased the solubility enhancement factor; a factor of 5.5 was calculated for the praziquantel-β-cyclodextrin complex and 6.0 for the praziquantel-hydroxypropyl-β-cyclodextrin complex. Preheating the sample before complexation enhanced the drug complexation by increasing the dissolved amount of praziquantel that interacted with the cyclodextrin to form inclusion complexes.

The content of praziquantel in the product was determined spectrometrically. The isoquinoline ring of the praziquantel inserted in the cyclodextrin cavity. The phase solubility diagrams were linear. The praziquantel complexation was stronger with β-cyclodextrin than with the hydroxypropyl-β-cyclodextrin derivative as revealed by the complexation constants, K values,

obtained from the diagram. Furthermore, the complexation enhancement as a result of preheating the sample was more significant in the hydroxypropyl-β-cyclodextrin complex as reflected in the K values.

Praziquantel complexation with different molar ratios of cyclodextrin was studied. The praziquantel complex with β-cyclodextrin had the highest drug concentration in a 1:2 praziquantel-cyclodextrin ratio. The optimum praziquantel-hydroxypropyl-β-cyclodextrin complex molar ratio was 1:4. The different behaviors of the cyclodextrin derivatives are a result of hydrogen bond formation between the drug and cyclodextrin.

After complexation with β-cyclodextrin and hydroxypropyl-β-cyclodextrin the drug was reevaluated in terms of the BCS. The solubility/dose ratio, in relation to the dose and intestinal content, was calculated using solubility values of praziquantel in the presence and absence of β-cyclodextrin and hydroxypropyl-β-cyclodextrin. At a low dose of 150 mg the praziquantel complexed with β-cyclodextrin or hydroxypropyl-β-cyclodextrin had a higher solubility/dose ratio than pure drug. The solubility was increased while the dose decreased. The low dose form of praziquantel, 150 mg, thus classified as a BCS-Class I drug.

Camptothecin-Cyclodextrin

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Camptothecin is an antineoplastic agent that displays broad spectrum activity in the treatment of various cancers. Camptothecin is used against human lung cancer, prostate, breast, colon, stomach, and ovarian carcinomas and in the treatment of melanoma, lymphomas, and sarcomas. An alkaloid isolated from the bark of the Chinese tree Camptotheca acuminata, camptothecin works by inhibiting the enzyme topoisomerase I. Inhibition of topoisomerase I prevents DNA relegation leading to DNA damage and apoptosis (PubChem, 2005). Camptothecin is practically insoluble in aqueous solution at a pH above 7 the drug is converted to a water soluble but less active carboxylate form. Jichao Kang, et al. performed a study complexing camptothecin with cyclodextrin in attempt to increase the solubility of the pharmaceutically important drug (Kang et. al. 2002).

The solubility of the drug complexed with cyclodextrin was determined by placing 5 mg of camptothecin in 1.0 ml of a 0.02 M HCl solution containing different amounts of cyclodextrin. Analysis of the results gave a linear graph, the solubility of camptothecin increased linearly with the increasing concentrations of various cyclodextrins. Camptothecin in the β-cyclodextrin complex had the highest increase in solubility, increasing by about six-fold. α-cyclodextrin increased the camptothecin solubility three-fold and γ-cyclodextrin increased the drug's solubility five-fold. Of the modified cyclodextrin, RDM-β-CD exhibited the greatest increase in camptothecin's solubility. At 25% weight / volume concentration of RDM-β-CD, the solubility of camptothecin increased by a factor of 170. The methyl groups significantly increase the solubilizing effect of this cyclodextrin derivative by disrupting the intramolecular hydrogen bonding and enlarging the cyclodextrin cavity.

Conclusion

Drug solubility is an important factor in determining the efficacy of a drug. The low aqueous solubility of many pharmaceutical developments is an area of constant research. This paper studied the effect of cyclodextrins on the solubility of poorly soluble drugs. Cyclodextrins and their derivatives, by forming guest-host complexes, encapsulate an insoluble drug and increase its aqueous solubility. The cyclodextrin carries the drug through the aqueous solution and the complex dissociates upon reaching the site of action. The cyclodextrin drug carrier transports the insoluble drugs without altering the drug or causing significant harm to the patient.

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Consequences of Untreated Obstructive Sleep Apnea

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Abstract

Over the past few years, awareness of the prevalence of obstructive sleep apnea has significantly increased. Indeed, sleep apnea may be more common now with the increasing incidence of obesity and the growing number of elderly individuals in our population. How serious is this condition, and what are the potential long-term effects and consequences of obstructive sleep apnea if left untreated? An overlap of many serious conditions with obstructive sleep apnea has been noticed. These conditions include hypertension, heart disease and failure, stroke, diabetes, neurological diseases, depression, and a number of other serious health concerns. The purpose of this paper is to understand what the effects of untreated obstructive sleep apnea might be, and to determine if is reasonable to suggest that sleep apnea is the cause of, or at least a significant contributing factor to, the conditions it is associated with. Research shows that sleep apnea itself does inflict enormous trauma on the body. This trauma is mainly in the forms of hypoxia which leads to oxidative stress, vascular damage, and hyperstimulation of the sympathetic nervous system which can lead to high blood pressure and subsequent heart disease and failure, and by causing repeated episodes of unnatural intrathoracic pressure which can lead to a number serious health concerns and even sudden death. These findings demonstrate that sleep apnea should not be viewed as an incidental condition alongside other serious health problems nor mainly as a side effect of them. It should rather be seen as a contributing factor, if not the primary cause, of many consequential health issues.

Introduction

Untreated obstructive sleep apnea has been strongly linked to many serious diseases and early mortality. It can be the underlying cause of certain diseases, and it can cause rapid progression or early onset of others. Perhaps even more alarming, is that many incidences of sudden death, especially heart attacks, have been attributed to untreated obstructive sleep apnea. The basic understanding of its severity is that untreated obstructive sleep apnea inflicts significant and multi-faceted trauma on the body. This trauma is mainly due to nightly deprivation of oxygen in the blood, sleep deprivation, constant hyper stimulation of the sympathetic nervous system, and enormous strain on the heart. It is noteworthy that there are also secondary physical, mental, and emotional health concerns associated with untreated sleep apnea. In a random sample of several hundred individuals presenting to a sleep clinic with symptoms typical of sleep apnea, it was observed that the vast majority of the patients subsequently diagnosed with obstructive sleep apnea had at least one other serious health issue. The percentage of people with additional health problems tended to be much higher among those 40 years of age and older- i.e. in the individuals who had gone through many more years of trauma to their bodies before seeking treatment (personal observation, Chang, et.al. 2013).

It is also especially critical to explore the effects of untreated sleep apnea because it effects many more people than was previously thought (up to 24% of adult men) (Peppard, et.al. 2000) and because there has been a steady rise in the diagnosis of sleep apnea with the increased incidence of obesity. In addition to the obesity factor, it is now known that the general aging process is a major risk factor in the development of sleep apnea. Consequently, it may be prudent to study the diagnostic methods and treatment options for sleep apnea for those involved in geriatric care (Adegunsoye, Ramachandran, 2012). Several studies indicate that post-menopausal women are at increased risk for developing obstructive sleep apnea because the female sex hormones which help dilate the airway decrease after menopause (Chang, et.al. 2013). This paper will explore the question of how untreated sleep apnea effects the body and the implications of these effects.

Methods

Relevant material was scoured mainly from the web, from conversations and correspondence with a veteran pulmonologist and sleep specialist, and from personal experience observing and interviewing patients and studying polysomnography data at a sleep clinic over a period of about two months. Only materials from authoritative sources were used. An effort was made to gather information on the interaction between sleep apnea and numerous medical conditions thus broadening the scope of the paper. This was done in order get as full a picture as possible on how sleep apnea may affect the body. Material on the effectiveness of treatment of sleep apnea was also considered pertinent to understanding the mechanisms of this condition and are included in the discussion accordingly.

Definition of Terms

Sleep apnea: Sleep apnea is defined as a cessation of breathing of ten seconds or more while asleep (Tsara, et.al. 2009). There are two main types of sleep apnea: Obstructive Sleep Apnea, and Central Sleep Apnea.

Obstructive Sleep Apnea (OSA): OSA is an anatomical condition where, while asleep, the muscles in the throat relax to an extent that the airway collapses. Specifically, there may be several different muscles responsible for this. Sleep apnea is more common in people who have a narrow airway. A narrow airway can be genetic, it can be caused by inflammation, or it can be due to obesity. (Obese individuals often have excess submucosal adipose tissue which can build up around the airway thereby causing the airway to compress.) Defects in, or damage to, the face or neck in areas which affect the airway may also be a risk factor for OSA. OSA can affect adults or children, but is more common in adults (Tsara, et.al. 2009). OSA can be a factor in determining the cause of Sudden Infantile Death Syndrome (SIDS), and a family history of OSA may be considered a risk factor in SIDS (Thach, 2008).

Central Sleep Apnea (CSA): CSA is a condition where the apneustic center in the brain stem fails to send nerve impulses necessary for breathing. CSA can occur without an obstructed airway. However, OSA and CSA are often clinically related.

When a patient who has CSA and OSA is treated just for the OSA, the CSA part of the sleep apnea is often minimized or even eliminated (Costanzo, et.al. 2015). It is possible that the disordered breathing caused by the OSA may contribute to the apneustic center malfunction- either directly or by a secondary mechanism. CSA can also be accompanied by irregular breathing patterns. For example, the Cheyne-Stokes respiration is a common symptom of primary CSA (Tsara, et.al. 2009, Costanzo, et.al. 2015). (Cheyne-Stokes respiration is a breathing pattern where there are rapid or deep breaths followed by a decrease and secession of breathing. This is usually in a cycle which can last anywhere from 30 seconds to two minutes.) CSA can also be secondary to illness or trauma, and it can be caused by certain medications and substances. It is noteworthy that if a newborn has sleep apnea, it is often CSA (Tsara, et.al. 2009) and may be a factor in determining the cause of SIDS (Thach, 2008).

Hypopnea: A hypopnea is a partial closure of the airway which is often characterized by loud snoring. (The snoring is caused by the fluttering of the air passage walls as they come in close proximity while breathing.) This partial closing is classified as a hypopnea when the obstruction is severe enough to cause oxygen desaturation in the blood.

Polysomnography: A polysomnography is a sleep study. During a sleep study, the individual is attached to electrodes and other machinery which collect information about the person's sleep. This information will include how long the patient spent in each phase of sleep, whether or not the patient has OSA or CSA, oxygen saturation or desaturation in the blood, breathing patterns, and many other items of relevance. A polysomnography can be used to diagnose sleep apnea.

Apnea-Hypopnea Index (AHI): An AHI is a score of how many events of apnea and hypopnea occur per hour. For example, if six times in the course of an hour, a patient stops breathing for ten seconds or more and/or has an oxygen desaturation, then the patient will have an AHI of 6 (or an event on average of every ten minutes).

Sleep Disordered Breathing: Sleep disordered breathing is a general term used to refer to any abnormal breathing issues or abnormal breathing patterns which can occur when one is asleep.

Continuous Positive Airway Pressure Machine (CPAP Machine): A CPAP Machine is a medical device which blows air through the nose to the back of the airway to keep it open while a person is asleep. It is currently the most effective standard treatment for sleep apnea. The machine can be adjusted to apply more or less pressure as needed, and modern machines are often self-adjusting. Other machines are available to treat more complicated cases, but they too incorporate positive pressure to treat the OSA component of any sleep disordered breathing. There are surgeries which can be performed to help eliminate OSA, but they are only about 50% effective. Some dental devices have been developed to treat OSA, but they too are often insufficient as effective treatment (Peker, et.al. 2002).

Discussion

The most obvious area of concern with sleep apnea is the fact that when breathing stops, oxygen doesn't enter the lungs. This, in turn, leads to oxygen desaturation in the blood. This hypoventilation has been linked to a number of conditions such as systemic hypertension (Peppard, et.al. 2000), pulmonary hypertension (average pressure in the pulmonary arteries greater than 25mmHg (resting)) (Adegunsoye, Ramachandran, 2012), polycythemia (high red blood cell count), heart problems, behavioral issues, emotional issues (Sheu, et.al. 2015), and birth defects (Tsara, et.al. 2009, Adegunsoye, Ramachandran, 2012). It has also been reported that early treatment is effective in eliminating these negative effects, and it is therefore of paramount importance to recognize, diagnose, and treat sleep apnea syndrome at the earliest possible opportunity (Adegunsoye, Ramachandran, 2012).

Systemic hypertension can be due to numerous factors involving OSA. One factor is that the hypoxia may lead to a pressor effect as an attempt by the body to get more oxygen (Peppard, et.al. 2000, Tkacova, et.al. 2014). Another contributing factor to hypertension is fluid and sodium retention due to sympathetic nervous system activation of the renin angiotensin aldosterone system effecting the kidneys (Bradley, Floras, 2003, Nishizaka, et.al. 2004). Many patients with untreated OSA have swelling in their legs (personal observation). It is noteworthy that administration of oxygen does not prevent the hypertensive effect of untreated OSA (Leung, Bradley, 2001).

The increased risk for developing hypertension due to untreated OSA is present even in individuals diagnosed with mild sleep apnea (AHI of approx. 5) (Peppard, et.al. 2000). The risk for developing hypertension is, however, directly related to the severity of the sleep apnea. Several studies show that individuals with more severe sleep apnea were more likely to develop hypertension than those with milder sleep apnea (Peppard, et.al. 2000, Peker, et.al. 2002, Nieto, et.al. 2000). The likelihood of developing hypertension is present regardless of gender, race, or ethnicity, and it is usually manifest in individuals middle age or older (Nieto, et.al. 2000). Severity of sleep apnea is based on AHI and on ODI (oxygen desaturation index) (Tkacova, et.al. 2014). If treatment for OSA is initiated before the onset of hypertension, then chronic hypertension as well as other cardiovascular disease may be avoided. Additionally, treatment for the OSA has a much greater impact on lowering blood pressure than does weight loss (Nieto, et.al. 2000, Tkacova, et.al. 2014, Lavie, et.al. 2000). Even in the event that hypertension has already set in, studies show that it can be minimized significantly after approximately 9 weeks of treatment (Milleron, et.al. 2004). The association between sleep apnea and hypertension is so strong that

sleep apnea should be seriously considered in the diagnosis and treatment of high blood pressure (Lavie, et.al. 2000).

Many studies have demonstrated that endothelial cells are severely damaged and impaired by hypoxic conditions, and the extent of endothelial cell damage has also been associated with the AHI index (Lui, et.al. 2013). The damage may occur in the form of apoptosis of endothelial cells, in the form of cell cycle arrest, and in a number of other ways which play a role in many of the adverse effects of untreated sleep apnea (Iida, et.al. 2002). Endothelial cell damage and dysfunction is connected to inflammation, atherosclerosis, renal damage, impaired vasodilation ability and nitric oxide production, and many other severe health issues (Bruno, et.al. 2013). Additionally, oxidative stress seems to be one of the main causes for endothelial cell damage. The extent of oxidative stress is likewise directly correlated to the severity of the sleep apnea (Del ben, et.al. 2012). It is extremely important to realize that the individual with untreated OSA is at increased risk of arterial stiffness, which can lead to heart disease and other severe health issues even if they experience very minimal symptoms (Kohler, et.al. 2008).

Hypoxia is known to cause an increase in reactive oxygen species. The mechanism for this phenomenon is not clear, but some studies suggest that this is due to the lack of a strong electron acceptor in the absence of oxygen. When there are no electron acceptors, the electrons have no place to go and therefore remain unbound as superoxide radicals or as other reactive oxygen species (ROS) (Kondoh, et.al. 2013). Compounding this problem is the reperfusion following the apneic episode which also causes an increase in ROS. Reperfusion may cause an increase in ROS for two reasons. One is that cell function has been impaired due to the lack of oxygen and is altogether not running efficiently enough to rid itself of the ROS. Second is that the sudden influx of oxygen overloads the cell which cannot process it fast enough. Studies show that hypoxia and subsequent reperfusion can cause damage to the epithelial lining of blood vessels. This damage can cause and/or contribute to atherosclerosis (Sawatari, et.al. 2016).

Aside from the ROS caused by reperfusion, there are other ROS released by a number of granular leukocytes in response to hypoxemia. The increased presence of ROS is a general danger to the body and has been linked to cancer and other illnesses. This is especially a concern for cancers which originate in endothelial cells because they are most directly affected by ROS (Kondoh, et.al. 2013). This is especially the case when the body experiences this kind of oxidative stress night after night for years on end (Adegunsoye, Ramachandran, 2012, Milleron, et.al. 2004). Proper treatment of OSA solves the problem of oxidative stress and can help reverse some or all of the damage caused by it (Del ben, et.al. 2012, Bayram, et.al. 2009).

Pulmonary hypertension in particular, is one cause for right sided heart failure and has an especially grim prognosis if left untreated. Pulmonary hypertension can be caused by a number of conditions, but its link to OSA and general sleep disordered breathing is strong enough that the American College of Chest Physicians recommends that patients presenting with pulmonary hypertension be evaluated for sleep disordered breathing. The pathophysiology of pulmonary hypertension in sleep disordered breathing is that the hypoxia caused by the breathing disorder in turn causes hypertrophy of the pulmonary arteries. This hypertrophy decreases perfusion of blood in the lungs. The situation is even more disastrous for individuals with emphysema or fibrosis of the lung. The heart tries to compensate for the decrease in perfusion by working harder, and this attempted compensation puts enormous strain on the right ventricle. This strain is unsustainable and leads to right sided heart failure and a subsequent general dyspnea even at rest. If left untreated, death typically occurs within three years of diagnosis (Adegunsoye, Ramachandran, 2012). The mechanism of how hypoxia causes the initial hypertrophy of the pulmonary arteries needs elucidation, but it seems to be related to endothelial cell dysfunction. (It is also noteworthy that obese individuals are more likely to develop pulmonary hypertension with OSA (Adegunsoye, Ramachandran, 2012).)

Aside from hypertrophy of the pulmonary arteries which can cause pulmonary hypertension, there is the direct effect of hypoxia in the form of pulmonary vasoconstriction. The reason for vasoconstriction during an hypoxic event is due to an attempt by the body to keep ventilation and perfusion in sync. In the absence of oxygen, vasoconstriction will lessen perfusion. This, however, will also cause an increase in pulmonary artery pressure. This pressure is primarily a concern while the individual is sleeping. However, about 20% of patients with elevated pulmonary artery pressure while sleeping will eventually develop pulmonary hypertension even while awake. This may be because the increased blood pressure can directly cause damage to blood vessels and result in the hardening and stenosis of those damaged vessels.

Another area of concern in untreated OSA is the effect of prolonged sympathetic nervous system stimulation. When a person with OSA has an event, the body responds to the obstructed breathing by activating the fight or flight response. This occurs for each event- all night, every night. One effect of this stimulation is vasoconstriction and an increased heart rate- both of which raise blood pressure. Eventually, this can lead to chronic systemic and pulmonary hypertension (Adegunsoye, Ramachandran, 2012).

Chronic systemic hypertension is known to negatively affect cardiovascular health and increase mortality. It has also been reported that hypertension in individuals with untreated OSA is particularly difficult to control, and that in attempting to control the high blood pressure, beta blockers are the most effective, suggesting that a major contributing factor to the high blood

pressure is sympathetic stimulation (Leung, Bradley, 2001).

Even in the absence of chronic hypertension development, there is, concern of vascular endothelial dysfunction. Over-stimulation of the sympathetic nervous system can, in of itself, lead to vascular endothelial dysfunction. The pathophysiology of the damage is, similar to what is found in patients with chronic hypertension: Vasoconstriction creates more resistance in the blood vessels. Resistance, especially if prolonged, can cause damage to the vasculature and lead to hardening of the blood vessels (Adegunsoye, Ramachandran, 2012). The damage can be quite significant because the effects of sympathetic system over-stimulation linger into the wake hours as well (Tkacova, et.al. 2014).

The constant presence of catecholamine levels also puts the untreated OSA patient at greater risk for thromboembolism because high levels of catecholamines tends to lead to platelet aggregation. This and other phenomena which can predispose an individual to thromboembolism can be reversed with effective treatment. The reversal of which suggests that the hypercoagulability in individuals with OSA is indeed caused by the OSA (Adegunsoye, Ramachandran, 2012).

There is a secondary problem associated with untreated OSA of the sleep deprived individual. The way the body clears the obstructed breathing is by jolting the person out of deep sleep long enough to return muscle tone to the airway and open it back up. This arousal from deep sleep is called a Respiratory Effort- Related Arousal or RERA. A RERA can be present even in the absence of a clinically defined apnea- for example if the breathing stops for less than 10 seconds, or if there is no significant oxygen desaturation during an event (Tsara, et.al. 2009). There are serious health risks associated with sleep deprivation as well as physiological effects and secondary risks. Psychological effects could include depression, apathy, and irritably. Secondary risks could include general fatigue or falling asleep while driving (Adegunsoye, Ramachandran, 2012).

Sleep deprivation and hypoxia are both known causes of inflammation. Inflammation is an independent factor in endothelial cell dysfunction and has also been linked to certain cancers (Lui, et.al. 2013). Several factors which cause inflammation are found in high levels in individuals with untreated OSA. Proper treatment is effective in stopping the inflammatory response (Adegunsoye, Ramachandran, 2012, Shamsuzzaman, et.al. 2003).

Another factor associated with OSA which causes sleep deprivation is nocturia. Nocturia is a condition where an individual's sleep is interrupted multiple times in order to empty the bladder. The pathophysiology of this condition is that attempting to breathe with an obstructed airway causes an enormous amount of intrathoracic pressure which results in a much larger than usual volume of blood to enter the heart. In some individuals, this overfilling causes the release of atrial nauturetic peptide (ANP). ANP in turn causes the kidneys to excrete more water. The reason this system is activated is because the overfilling of blood in the heart mimics the mechanics of increased blood volume. Increased blood volume increases blood pressure. As a result, the body, thinking that it has too much blood, will want to get rid of the excess volume by way of diuresis (Umlauf, et.al. 2004). Patients with OSA report that their nocturia subsides once they comply with effective treatment (Personal communication with patients).

In addition to the mimicking of increased blood volume which causes the release ANP, ANP excretion is also increased due to acidosis. The lack of oxygen caused by OSA can certainly lead to at least a temporary acidosis, and this may further contribute to nocturia. ANP also directly inhibits the excretion of vasopressin. Vasopressin normally plays a role in the decrease in urine production while asleep. If vasopressin is inhibited, then urine production will not decrease and the individual may experience nocturia. In one study of carefully selected individuals diagnosed with OSA, blood levels of ANP, as well as urine levels of ANP and increased urine volume were directly correlated with untreated OSA. It is also noteworthy that an elevated level of blood ANP is related to mortality rates in patients with heart problems, and a correlation between heart disease and untreated OSA has been strongly established (Umlauf, et.al. 2004).

Nocturia is often associated with aging. This association is due to the general aging process, and in men due to prostate hypertrophy, and in women due to decreased bladder volume. Aging is also a risk factor for OSA, and as such, its another possible cause of the increase in nocturia with age in both men and women. The connection between untreated OSA and nocturia is one way to explain why, in many individuals, nocturia is not resolved with the treatment for benign prostate hypertrophy. It has also been documented that the incidence of sleep apnea doubles in women post menopause- probably as a result of low estrogen levels. This correlates well with the increase in nocturia in women post menopause. Attributing nocturia in post-menopausal women to untreated OSA may be more accurate than attributing it to a simple decrease in bladder volume. The effects of over elimination of fluids and how this may affect proper hydration and general fluid management in the body also needs to be considered when evaluating the impact of nocturia (Umlauf, et.al. 2004).

Many individuals with OSA are obese. Obesity is a risk factor in OSA, but OSA can, in of itself, also cause weight gain. Weight gain can be attributed to lack of physical activity. People with OSA often experience chronic fatigue, and this can possibly cause OSA patients to limit their physical activity. However, in addition to this secondary cause for weight gain, OSA can be directly responsible for altered metabolic factors which lead to obesity. Leptin levels are often found to be at abnormally high levels in obese individuals. Patients with untreated OSA often have even higher levels of leptin than obese individuals.

The current understanding is that leptin is found at high levels because of resistance to their appetite suppressing activity. Patients with untreated OSA have sometimes been found to have high levels of leptin and gain weight in the year prior to diagnosis, and these patients tend to return to normal leptin levels and lose the weight once proper treatment is undertaken. It is also noteworthy that a high leptin level is considered a risk factor for cardiovascular disease (Adegunsoye, Ramachandran, 2012, Shamsuzzaman, et.al. 2003, Milleron, et.al. 2004).

Another metabolic factor linked to OSA is impaired glucose tolerance. Insulin resistance has been directly correlated to OSA, and several studies demonstrate that individuals with OSA are several times more likely to develop diabetes mellitus than those without OSA (Adegunsoye, Ramachandran, 2012, Gottlieb, et.al. 2010, Bayram, et.al. 2009).

One of the most extensive areas of study concerning untreated OSA is the effect it has on the heart. In fact, about 50% of patients with heart failure have OSA, and medication for heart failure seems to have little, if no effect on alleviating OSA (Bradley, Floras, 2003). In addition to the effect of pulmonary hypertension on the heart (discussed above), there are additional ways in which untreated OSA can cause cardiac health problems. When the body tries to breath while the airway is obstructed, an enormous amount of negative pressure is generated in the thoracic cavity. This negative pressure pulls large amounts of blood from the superior and inferior vena cava into the right atrium of the heart, thus effectively increasimg afterload (Shekerdemian, Bohn, 1999). The negative intrathoracic pressure increases venous return and distends the right ventricle which effectively impairs left ventricular filling (Bradley, Floras, 2003). Additionally, the negative pressure may prevent the left ventricle from relaxing and filling properly (Shamsuzzaman, et.al. 2003). These mechanisms effectively decrease cardiac output (Adegunsoye, Ramachandran, 2012, Mooe, et.al. 2001, Gottlieb, et.al. 2010). Individuals with OSA can thus have high blood pressure due to vasoconstriction from sympathetic nervous system stimulation but decreased cardiac output due to impaired left ventricular filling. Unnatural configuration of the heart and aortic stretching are also caused by this large negative pressure. This stress on the heart can cause either rapid or reduced heart rate, and even complete sudden heart failure. The longer the apneic episode the greater the risk of cardiac malfunction (Adegunsoye, Ramachandran, 2012).

Aside from rhythmic and filling problems for the heart, there is also a risk for myocardial hypertrophy which can cause irreversible hypertension. The hypertrophy is caused by the constant strain on the heart muscle- just as constant strain on any muscle leads to muscle growth. If the strain is primarily right sided, then the hypertrophy will affect the right side of the heart. If the strain is primarily left sided then it will affect the left side of the heart. If the strain is on both sides then the entire heart can be effected. Hypertension caused by OSA can last beyond the actual apnea episodes, and even in the absence of myocardial hypertrophy, this hypertension can last for many hours after awakening (Peppard, et.al. 2000, Peker, et.al. 2002). However, treatment for OSA can often reverse systemic and pulmonary hypertension if myocardial hypertrophy has not yet set in. This is one on the many reasons why it's important to properly treat OSA at the earliest opportunity (Adegunsoye, Ramachandran, 2012, Jean-louis et.al. 2010).

In addition to myocardial hypertrophy, there is concern that untreated OSA may cause ion channel remodeling in the heart. This condition can lead to sudden cardiac death (Chahal, Somers, 2016). Gene expression effecting potassium ion channels was studied by examining mRNA associated with potassium ion channels. It was discovered that there was significant altering of several potassium pump genes which may result in an extended repolarization period. Potassium pumps are needed to pump positively charged potassium ions across the myocardial cell membranes so that a new muscle contraction can begin. If they don't pump ions efficiently, then this period of repolarization will take longer. One potential problem with this is that there is a danger of ventricular extrasystole (premature ventricular contraction). This kind of arrhythmia can cause sudden cardiac death. The mechanism for how exactly the OSA causes the altered gene expression has not been fully elucidated, but it seems to be multifactorial, including stressors such has hypoxia, sympathetic stimulation, and increased intrathoracic pressure. Individuals who undergo treatment for OSA can reverse the pathologic alterations to the potassium ion channels and regain proper gene expression for potassium pumps that work correctly (Chahal, Somers, 2016, Bradley, Floras, 2003).

The risk of sudden cardiac death in patients with untreated OSA is not limited to extrasystole due to ion channel malfunction. In fact, there are quite a few additional mechanisms by which sudden cardiac death may occur. For example, during REM sleep, there is a general increase in sympathetic stimulation. (This is in contrast to other stages of sleep where the sympathetic nervous system is depressed.) The transition from non-REM to REM thus creates a situation which increases oxygen demand. Experiencing an apnea, with the simultaneously occurring hypoxia, is thus of greater consequence at this point in the sleep cycle. This ensuing trauma to the heart can cause arrhythmias (Chahal, Somers, 2016).

Sudden cardiac death is of even greater concern in the morning hours because that is typically when the longest REM period takes place and because the apneas at this point tend to last for longer periods of time. In addition to the risk of arrhythmias being increased during this longer REM period, there is great risk of myocardial infarction or stroke at this point as well (Mooe, et.al. 2001). In general, blood pressure rises at different points in the sleep cycle. Individuals with

untreated OSA often experience an even greater increase in blood pressure- probably due mainly to the overstimulation of the sympathetic nervous system. The risk for MI or stroke is always increased with an increase in blood pressure, and as such, these individuals will be in the most danger during REM sleep. The longer REM and the prolonged apneas associated with it have claimed many lives (Michael Katzoff MD, personal communication May 2017).

Stimulation of the sympathetic nervous system can also result in tachycardia which further puts a large demand for oxygen on the heart at a time when the heart cannot get oxygen due the apnea. This situation can be even more life-threatening in patients with coronary artery disease (CAD) (Milleron, et.al. 2004). Additionally, a myocardial infarction or stroke is often caused by a plaque rupture which is made more likely by the increase in blood pressure and also because of the intrathoracic pressure caused by the obstructed airway (Chahal, Somers, 2016). In fact, 43-91% of patients who experience a stroke also have sleep apnea (Leung, Bradley, 2001, Shamsuzzaman, et.al. 2003). It is noteworthy that the rise in intrathoracic pressure has also been known to cause the rupture of an aortic aneurism which is typically fatal (Chahal, Somers, 2016).

In patients with pre-existing coronary artery disease (CAD) there is at least a 60-70% increase in sudden cardiac death or myocardial infarction when the individual also has untreated OSA. In addition to heart related problems there is an increase in cerebral vascular complications such as stroke. The cerebral vascular complications may result from the increase in the prothrombotic effect connected with untreated OSA as well as from other factors effecting hemodynamics (Mooe, et.al. 2001, Peker, et.al. 2002). Fortunately, proper treatment for OSA has been shown to decrease the number of new cardiac issues or at least to dramatically slow the rate of coronary artery disease progression (Milleron, et.al. 2004, Peker, et.al. 2002, Jean-louis et.al. 2010). It is likely that treatment would be similarly beneficial to cerebral vascular health (Gottlieb, et.al. 2010).

In general, the sympathetic system stimulation, which often lingers throughout the day in untreated OSA individuals, is known to increase platelet aggregation. This aggregation is detrimental to patients with CAD (Milleron, et.al. 2004). Additionally, prolonged platelet aggregation as well as increased levels of fibrinogen, and general inflammation in individuals with untreated OSA, may be a risk factor in developing a deep vein thrombosis on top of the risk these factors pose to CAD (Milleron, et.al. 2004, Bayram, et.al. 2009).

It is noteworthy that many patients with nocturnal angina experience relief when undergoing proper treatment for OSA. This suggests that the cause of pain, which is normally due to a lack of oxygen, is caused by the OSA and is effectively treated with standard CPAP therapy (Peker, et.al. 2002).

Another area of recent study is the possible connection between untreated OSA and dementia. One study found that the correlation between sleep apnea and dementia may be affected by factors such as age, gender, and duration of the condition. The mechanism for the connection between untreated OSA and dementia are not yet conclusive, but the connection between hypoxia, which is a side effect of untreated OSA, and neurological malfunction has been documented a number of times. For instance, rats which were exposed to hypoxic conditions experienced a greater incidence of apoptosis in their hippocampus, and advanced brain imaging showed reduced grey matter in parts of the brain responsible for executive functions and memory. Damage to these areas in humans may be a factor in the cause of dementia (Svatikova, et.al. 2003, Ju, et.al. 2013).

Another disturbing finding is that rodents placed in hypoxic conditions developed large amounts of cerebral amyloid plaque. Similar results have been found in humans with levels of serum amyloid A being two and a half times higher in patients with untreated OSA (Svatikova, et.al. 2003, Ju, et.al. 2013). Cerebral amyloid plaque is one of the hallmark findings in individuals with Alzheimer's disease. It was also found that there was significant phosphorylation of the tau proteins. Tau proteins are abundant in neural tissue and function in support of microtubules. When they are over-phosphorylated, they can malfunction and cause the structure of the cells in which they are found to become pathologically altered. Additionally, microtubules function as a sort of road or transport pathway for various particles within the cell. The intracellular functions which rely on these transport pathways may stop working properly if the tau proteins become over phosphorylated. Quite tellingly, another common finding in Alzheimer's disease is tau protein phosphorylation. In lab mice, cerebral amyloid plaque buildup and tau phosphorylation has been connected to decreased memory function (Daulatzai, 2015). It is also possible that the general damage to the vasculature throughout the body due to hypoxia can be a factor in dementia because the vasculature in the brain is negatively affected (Chang, et.al. 2013).

It has been shown that treatment of OSA in the early stages of Alzheimer's disease can aid in dramatically slowing its progression. One study demonstrated that with treatment of OSA with CPAP machine, memory and mental processing speed improved in any stage of dementia. Treatment also helped reduce or eliminate neuroinflammation. Additionally, the cardiovascular improvements experienced with CPAP treatment have had positive impact on patients with neurological illnesses. Conversely, some research indicates that the onset of mental decline may begin as much as one decade earlier in individuals with untreated OSA (Emamian, et.al. 2016).

Another connection between sleep apnea and brain function loss in humans is the presence of a common gene known as APOE4 (apolipoprotein epsilon 4) which has been shown to play a role in obstructive sleep apnea as well as being connected

to the development of dementia. The association could be a shared genetic fate, but it could also suggest that the gene is tied mainly to the development of OSA which, if left untreated, could lead to the damage which can cause dementia. Further studies would be necessary to determine the exact nature of the correlation. The long-term effects of sleep deprivation caused by sleep apnea should be considered when determining the long-term effects of untreated OSA on brain function (Chang, et.al. 2013).

A recent study has implicated untreated OSA as one possible cause of Parkinson's disease. The mechanisms seem most likely to include the general oxidative stress caused by hypoxia and reperfusion and the general inflammation characteristic of the body's response to untreated sleep apnea. There is even evidence to support the possibility that the blood brain barrier may be interrupted as a result of cerebral vascular damage which may allow molecules harmful to brain tissue to cross the barrier and reach cells involved in Parkinson's disease and other neurological pathologies (Sheu, et.al. 2015).

Conclusion

Extensive research reveals that untreated obstructive sleep apnea is extremely traumatic and damaging to the body. It has been clearly linked to physical diseases in the heart, brain, endocrine system, and vasculature, and it has been implicated as a cause for psychological and emotion distress, as well as secondary hazards. Mortality rates are consistently higher and earlier in individuals with untreated OSA and as such, individuals presenting to their health care providers with significant health issues associated with OSA should be tested for it. It is extremely important for health care providers to realize the severity of OSA and to understand that it can be present even in patients who experience few of the symptoms commonly indicative of OSA. Even patients with a relatively low AHI of about 4 or 5 are at risk because the trauma to the body is consistent and repeated every time the person sleeps, and this trauma has a tendency to have a cumulative effect on the body. Early diagnosis is key because the negative effects of OSA can be eliminated with early treatment. Treatment should be adamantly encouraged even in individuals in whom permanent damage has already occurred because treatment can reverse some of the damage and certainly help stabilize the patient and prevent the progression of further health issues. Unfortunately, the symptoms of sleep apnea are not always connected immediately to the condition and are often written off as symptoms of other medical or phycological issues. It is therefore prudent of heath care practitioners to be mindful of sleep apnea and diagnose it as early as possible.

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The Relationship between Periodontitis and Cardiovascular Disease

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Abstract

The relationship between periodontal disease and cardiovascular disease has been the subject of much research in recent years. The aim of this study is to review and analyze the relevant literature regarding this relationship, with an emphasis on determining a presence of periodontal bacteria from the periodontal pocket in atheromatous plaques, and to explore the biological role of inflammatory mechanisms that may link periodontitis and cardiovascular disease. Although there seems to be conflicting reports, the overall consensus confirms the presence of periodontal bacteria, such as Porphyromonas gingivalis, in atheromatous plaques. Additionally, the presence of systemic markers of cardiovascular disease in patients with periodontitis, such as acute phase proteins, proinflammatory cytokines, and markers of procoagulant state, has been confirmed. These confirmations could indicate a role for periodontal pathogenic bacteria in atherosclerosis disease process. Additionally, these findings give rise to possible mechanisms linking the two diseases.

Introduction

As early as the early 1900's, oral sepsis and tooth extractions were suggested as causes of cardiac infection. However, due to the lack of compelling scientific evidence, the idea of oral disease as a cause of systemic illness was basically disregarded. That focus was revived in the early 1990's after reports of the connection between periodontal disease (PD) and cardiovascular disease (CVD). Although many subsequent studies have postulated positive associations between PD and CVD, others have maintained that no such correlation exists. Additionally, several potential mechanisms have been proposed describing how PD could cause systemic inflammation, initiate or exacerbate atherogenesis, and possibly lead to cardiovascular catastrophes such as myocardial infarction (MI) or stroke. The purpose of this article is to review the relevant articles, predominant theories, and proposed mechanisms relating periodontitis and atherosclerotic vascular disease. Specifically, does the inflammatory PD contribute causally to heart disease and stroke, or are these two conditions coincidentally associated?

Methods

The information gathered in this paper has been collected from numerous sources including databases such as GoogleScholar, Touro Library, and PUBMED. The information was read, analyzed and compared to determine each study's validity and authority.

Results and Discussion:

Defining PD and CVD

CVD, or more specifically atherosclerotic vascular disease, affects the heart and the blood vessels. Coronary heart disease (CHD)- also referred to as coronary atherosclerosis, or simply heart disease- is a subset of CVD and is characterized by dysfunction of the arteries supplying blood to the muscle tissue of the heart, depriving it of sufficient amounts of blood. Atherosclerosis, or the hardening and narrowing of the arteries, silently and slowly blocks arteries, putting blood flow at risk. CHD includes blockage of blood vessels (thrombosis) and can eventually cause acute myocardial infarction (heart attack). CVD is the number one cause of death in the United States and other industrialized countries, and is among the major causes of mortality worldwide. Traditionally, CVD has been attributed to risk factors such as treated and untreated systolic blood pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, and body-mass index. However, these factors only occur in about half of all patients that experience heart attacks. For example, 40% of CHD deaths occur in patients who have cholesterol levels that are lower than the population average. This has led researchers to investigate additional causes of CHD, including bacterial induced inflammation (Taylor, et. al. 2009).

Periodontitis is a bacteria-induced, chronic inflammation of the gingival tissue in response to dental plaque accumulation. PD is always preceded by gingivitis, an earlier and less severe form of gum disease, though not all cases of gingivitis will progress to PD. Untreated PD leads to deepening of the gingival sulcus, which evolves into a periodontal pocket, destruction of the connective tissue and bone supporting the teeth, including gingival tissue, periodontal ligament, and alveolar bone. Clinical criteria for PD include bleeding on probing, pocket depth and degree of tooth attachment loss.

Evidence of Correlation Between CVD and PD

Many studies have been conducted assessing the possible connection between CVD and PD. The results have varied, and at times proposed conflicting views. The fact that oral pathogens can come to lesions far from the oral cavity has been proven by Lockhart, et. al.. The authors showed how poor oral hygiene or dental disease are risk factors for developing bacteremia after a simple tooth brushing or after a single tooth extraction. One hundred and ninety-four patients participated in the study. The authors assayed blood samples before, during and after the toothbrushing or single tooth extraction to identify Infective Endocarditis (IE)- associated bacteria. The authors found that oral hygiene and gingival disease indices were strongly correlated with IE-related bacteremia after toothbrushing. Patients with a mean plaque and calculus score of 2 or more had an increased risk of 3.78 and 4.43-times for developing bacteremia. Thus, we see that it is easy for bacteria in the oral cavity to enter that blood stream. This, as well as other similar studies, present a strong basis for the idea that oral pathogens can make their way to regions far from the oral cavity (Lockhart, et. al. 2009). Additionally, many studies show that bacteremia is present after oral examination and probing Loos, 2006).

Siddeshappa, et. al. (2016) conducted a study to determine whether nonsurgical periodontal therapy can impact various hematological parameters in patients with periodontitis. Traditionally, the total number of white blood cells (leukocytes) and erythrocyte sedimentation rates in peripheral blood has been used as diagnostic measures to determine whether or not a patient suffers from an infection or an inflammatory disease. Leukocytes become more numerous through the innate immune system response to periodontal bacteremia. The suggestion has been made that higher numbers of leukocytes makes the blood more viscous. Additionally, cells may adhere to the endothelial lining of the blood vessels causing decrease of blood flow. Furthermore, periodontitis has been associated with an increase in plasma fibrinogen and platelet activation which increase the risk of atherosclerosis and CVD. The aim of the study was to investigate the effect of nonsurgical periodontal therapy on total leukocyte count (TLC), differential leukocyte count (neutrophils, lymphocytes, eosinophils, basophils, and monocytes), erythrocyte sedimentation rate (ESR), and the total platelet count on patients with periodontitis. Thirty patients were selected for the study. The results of this experiment were that a decrease in clinical parameters of PD (ex. plaque index, gingival index, etc.) was accompanied by a statistically significant change in TLC, platelet count and ESR. The authors concluded that there is a decrease in hematological parameters after nonsurgical periodontal therapy, which may also reduce the risk of atherosclerosis (Siddeshappa, et. al. 2016).

In a similar vein, animals with experimentally induced periodontitis had more extensive accumulation of lipids in the aorta than did those without t. There was a positive correlation between the severity of PD and the extent of lipid deposition (Jain, et. al. 2003)., A study of 711 subjects, suggestaaed that tooth loss is a marker of past periodontal disease, and is related to subclinical atherosclerosis. Among those with 0 to 9 missing teeth, 46% had carotid artery plaque, and among those with ≥10 missing teeth, carotid artery plaque prevalence was about 60% (Desvarieux, et al. 2003).

However, such correlations are vague and broad. The above studies merely show increased atherosclerotic suseptability. This can be due to an overall systemic increase of inflammatory organisms. A more direct demonstration of correlation can be had by discovery of specific periodontal oral cavity bacteria in the atherosclerotic lesion. Such findings would show that inflammatory PD contributes causally to heart disease and stroke, and that these two conditions are not merely coincidentally associated. Research in this area is more questionable, and not as consistent in its results.

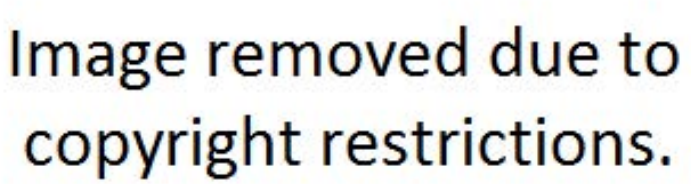
In a study conducted in 2004, 52 patients were studied who were scheduled for carotid endarderectomy to ascertain the presence of periodontal bacteria DNA, including that of Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia, and Tannerella forsythensis, in carotid atheromatous plaque. In subgingival plaque samples of 19 test patients, T. forsythensis, F. Nucleatum, P. intermedia, P. gingivalis and A. actinomycetemcomitans were found. However, no periodontal DNA was detected by PCR in any of the carotid samples. The authors concluded that the presence of periodontal DNA in atheromatous plaques could not be confirmed by this study, and thus no correlation could be established between species associated with periodontal disease and putative bacteria contributing to atheromatous plaques (Cairo, et. al. 2004). This study brings into question the causality relationship of periodontitis and gingival disease to CVD.

Other studies have produced results indicating a small number of matching pathogens. A study was conducted to isolate and identify bacteria from the periodontal pockets of different patients and to compare them with the microorganisms detected in atheromatous plaques obtained from the same patients. Clinical isolates were obtained from 12 patients with periodontal wounds and atheromatous plaques. These samples were then used to identify periodontal bacteria using polymerase chain reaction (PCR) assays. The results were as follows. From the 12 patients studied, 9 presented different periodontopathic species. In two patients Actinobacillus actinomycetemcomitans, a gram-negative coccobacillus, was present in the periodontal pockets and the respective atheromatous plaques. The authors were unable to explain the mysterious absence of P. intermedia and P. gingivalis growth in atheroma samples, even though these bacteria are usually present in periodontal samples. The authors concluded that the presence of A. actinomycetemcomitans in both the atheromatous plaque and the periodontal pockets of the same patients could indicate a role for periodontal pathogenic bacteria in the atherosclerosis disease process (Padilla, et. al. 2006). Similarly another investigation was aimed at testing the validity of the translocation hypothesi and its role in promoting plaque development. The authors used 16s cloning and sequencing, to determine the microbial diversity of the subgingival environment and atheroma plaques of patients concomitantly suffering from periodontitis and obstructive coronary artery atherosclerosis (OCAA). Subgingival biofilm and coronary balloons used in percutaneous transluminal coronary angioplasty were collected from 18 subjects presenting with generalized moderate to severe periodontitis and OCAA. DNA was extracted and the gene 16S was amplified, cloned and sequenced. The authors results were similar to those obtained by Padilla, et al.. They observed significant differences in microbial diversity between the two environments. While subgingival samples mostly contained the phylum Firmicutes, in coronary balloons, Proteobacteria was predominant. Additionally, the most commonly detected genera in coronary balloons were Acinetobacter, Alloprevotella, Pseudomonas, Enterobacter, Sphingomonas and Moraxella, while in subgingival samples Porphyromonas, Filifactor,

Veillonella, Aggregatibacter and Treponema were found. Despite such diversity, 17 identical phylotypes were found in atheroma and subgingival samples, indicating possible bacterial translocation between periodontal pockets and coronary arteries (Serra e Silva Filho, et. al. 2014). Again, these findings are seemingly small pieces of data regarding the overwhelming question of a periodontal-atheroscleric connection. Nevertheless, several studies do indicate a strong correlation with respect to Porphyromonas gingivalis (P gingivalis). Porphyromonas gingivalis has been strongly associated with adult periodontitis. This bacterium is a Gram-negative, nonmotile, obligate anaerobe that can invade and infect epithelium, endothelium, and vascular smooth muscle cells. Another group used ApolipoprotienE knockout (apoE–/–) mice which were orally administered the periodontal disease pathogen Porphyromonas gingivalis. The P. gingivalis was detected in the blood and aortic tissue of the mice. The mice challenged with P. gingivalis presented with an increase in atherosclerotic plaque, as well as expression of the innate immune response markers Toll-like receptors (TLR)-2 and TLR-4 in the aortic tissue (Gibson, 2004).

In order to determine whether recurrent intravenous injections of P. gingivalis, mimicking periodontitis-associated bacteremia, promotes coronary artery and aortic atherosclerosis the researchers chose pigs for this experiment since they develop coronary lesions that closely simulate human disease. Showed Thirty-six pigs were sensitized with 109 killed P gingivalis subcutaneously. Four weeks later all sensitized pigs in the group to be challenged started intravenous injections three times a week for 5 months with 106 to 107 units of P gingivalis while controls received saline. Pigs were euthanized 2 weeks after the last injection, and coronary arteries and aortas were analyzed. The results were significant. The authors found that the pigs that received intravenous P gingivalis challenges demonstrated a >100-fold increase in anti–P gingivalis antibody levels. This was in conjunction with the result that pigs challenged with recurrent P gingivalis bacteremia develop significantly larger coronary and aortic atherosclerotic lesions than those that were not challenged. The average coronary and aortic intimal areas were between≈3 times and ≈5.9 times larger than controls, a significant difference. These lesions were predominantly composed of smooth muscle cells. In addition, hypercholesterolemic pigs likewise challenged with recurrent P gingivalis bacteremia develop larger coronary and aortic atherosclerotic lesions, both having an intimal area that was ≈2.2 times larger than controls. Brodala, N et al. summarized their results in Table 1 and Table 2.

Additional significant information was reported by the authors. We mentioned previously the lack of evidence showing periodontal bacteria within the atheromatous legions. Previous studies only showed a correlation between the two diseases. Brodala, et al. discovered the following. P gingivalis ribosomal DNA was detected in the carotids (5 of 5) and the aortas (4 of 5) from the 5 P gingivalis-treated pigs on the low-fat diet and the aortas of all 7 P gingivalis–challenged pigs on the high-fat diet. However, none of the aortas or carotids from saline-challenged control pigs, whether sensitized or not, had amplifiable P gingivalis ribosomal DNA. The authors themselves note two limitations in their study. Firstly, the pig model used does not entirely and accurately replicate the bacteremia of oral origin from inflamed human periodontal tissues. Nonetheless, as seen previously in rodents, the P gingivalis–challenged pigs



developed an anti–P gingivalisantibody response as a consequence of P gingivalis challenges in association with increased atherosclerosis. Second, the number of pigs was not sufficient to exclude an effect of gender in this study. Data that was not available at the time of this study suggests that human males have a greater prevalence of asymptomatic carotid disease than females with comparable severity of periodontitis. This study does not account for such differences (Brodala, et al. 2005).

Other systemic markers also present evidence that periodontitis can lead to a systemic inflammatory response. Loos, (2006) outlines several systemic effects of periodontitis. He writes that in severe periodontitis, characterized by extensive plaque buildup, bacteremia occurs frequently, leading to leakage of lipopolysaccharides, a major component of periodontopathogens. Additionally, Loos was involved in several studies which investigated the presence of systemic markers of cardiovascular disease in patients with periodontitis. The author took peripheral blood from patients and analyzed the presence and levels of white blood cells, red blood cells and thrombocytes. He also analyzed the presence of important marker molecules such as acute phase proteins, proinflammatory cytokines, and markers of procoagulant state. The suggestion is that increased numbers of white blood cells may make the blood more viscous and increase blood rheology. Additionally, white blood cells tend to stick to the inside of blood vessels, particularly where there is inflammation. This may contribute to reduced blood flow which in turn increases the risk of microthrombus formation. The author writes that he found that the highest number of white blood cells were present in patients with severe periodontitis, the lowest numbers were found in control groups, and subjects with moderate periodontitis presented levels in between. Thus, the presence of elevated WBC's in the blood stream due to periodontitis is evident. We can then postulate that such inflammatory markers can exacerbate an atherosclerotic lesion.

The presence of other systemic markers of inflammation in periodontitis is also evident. One of the important markers of CVD is proinflammatory cytokines. In particular, interleukin-6 (IL-6) is produced by monocytes, neutrophils, endothelial cells, and B cells, and stimulates the hepatocytes to produce acute phase reactants. The author found that in general, patients with severe periodontitis had much higher levels of IL-6 than the controls. In many controls the levels of IL-6 could not be detected at all. Additionally, when patients with aggressive periodontitis were treated, IL-6 levels decreased, highlighting the correlation between periodontitis and IL-6 levels.

C-reactive protein (CRP) is another systemic marker of periodontitis. CRP is one of several acute phase proteins produced in the liver. The liver produces these acute phase proteins in response to infection or an inflammatory process, mainly due to elevated IL-6. CRP acts as a substance that binds to foreign microorganisms or cells, making them more susceptible to phagocytosis, also known as an opsonization. What is especially important is that CRP is associated with CVD. The relative risk of CVD is increased 90% when CRP levels are equal to or more than 2 mg. Thus, a possible mechanism would also be associated with these findings (Loos, 2006).

Evidence of the Role of Inflammation in CVD

Until recently, most believed that atherosclerosis was an arterial collection of cholesterol, complicated by smooth muscle cell accumulation. According to that theory, endothelial denuding injury led to platelet aggregation and release of platelet factors which would trigger the proliferation of smooth muscle cells within the arterial wall. It is now widely accepted that a major component of CVD in general, and atherosclerosis in particular, involves numerous components of the adaptive and innate immune systems leading to an inflammatory response within atheromatous lesion. A brief synopsis of these findings follows.

The innate immune system plays a major role in the inflammatory response in atherosclerosis. Considerable evidence supports the notion of early involvement of the monocyte/macrophage, the most prominent cellular component of the innate immune system, in atherosclerosis. Observations in human arterial samplings have identified monocyte recruitment as an early event in atherogenesis. Additionally, recent examinations of monocyte recruitment in mouse atherosclerotic lesions have shown that monocyte entry into the atherosclerotic lesion continues throughout lesion maturation. High levels of proinflammatory monocytes in mice are recognized by the high levels of a marker known as Ly6C, or Gr-1, and may correspond to a human monocyte subset marked by the presence of P-selectin glycoprotein ligand (PSGL) These proinflammatory monocytes express high levels of proinflammatory cytokines and other macrophage mediators. Recent studies have also highlighted the possible participation of mast cells, which exhibit numerous functions implicated in atherogenesis. For example, mast cells are known to release certain serine proteinases, histamine and leukotrienes, and heparin, a cofactor in atherogenesis. An additional area of evidence links thrombosis with inflammation. Thrombin may induce the expression of proinflammatory cytokines (Libby Peter, 2009).

The adaptive immune system also contributes to the inflammatory response in atherosclerosis. T lymphocytes populate atherosclerotic plaques and can be stimulated by certain heat shock proteins, components of plasma lipoproteins and other molecules. These T cells, upon further stimulation, produce cytokines and trigger inflammation. Additionally, biomarkers, such as highly sensitive C-reactive protein (CRP), apolipoprotein B (ApoB), and lipoprotein (a) amongst others, have been identified in conjunction with atherosclerosis predictability (Libby Peter, 2009) (Wong , 2012).

Proposed Mechanisms Linking Periodontitis to Cardiovascular Disease

Based on this review regarding systemic effects of periodontitis and inflammatory roles in CVD, we can begin to hypothesize the different mechanisms that link the two diseases. The authors suggest that the host response to bacteremia may vary between patients with periodontitis due to individual variation in inflammatory pathway. It is also possible that inherited genetic variations could enhance these mechanisms.

The authors maintain that a number of inflammatory mediators and markers are present in higher concentrations in the systemic circulation of periodontal patients than in those with healthy oral cavities. There are theoretically two pathways by which this could occur. There are ample data indicating that inflammatory cytokines and other mediators are produced in the periodontal lesion (Siddeshappa, et al. 2016)(Loos, 2006). It has been hypothesized that these mediators could enter into the circulation from the oral cavity. If this occurs, and the mediators achieve adequate concentrations, they would then impact tissues and organs distant from the oral cavity. In particular, these mediators from the periodontium could affect other organs, such as the liver, to initiate an acute-phase response that would impact other organs. The result is that the mediators themselves cause inflammation, as well as stimulate other organs to produce a pro-inflammatory response. This would lead to inflammatory changes in the endothelium such as up-regulation of adhesion molecules and promotion of cytokine production, which directly causes initiation and/or acceleration of atheroma development. It should be noted that there is not strong evidence supporting this mechanism for inflammatory cytokines and other mediators accessing the circulation (Teles, Wang, 2010).

In addition, it has been shown patients with periodontitis have frequent bacteremic incidents and that detectable concentrations of lipopolysaccharides are frequently found in the circulation. Thus, bacteria, or their pro-inflammatory components, may stimulate systemic inflammatory responses as well as local inflammatory responses in atheromatous lesions. This would follow their association with, or modification of serum lipids, engagement of receptors on inflammatory cells and endothelium, invasion of endothelial cells, or seeding of atheromatous lesions with bacteria or bacterial components. Bacteria or their products could then promote inflammatory changes that would contribute to the development or propagation of atheromatous lesions.

There are a number of antibodies that can also effect an inflammatory response. Some of these anti-bodies have been found to react as models of "molecular mimicry," which is defined as the theoretical possibility that sequence similarities between foreign and self-peptides are sufficient to result in the cross-activation of autoreactive cells. This is the case in which antibodies that have arisen due to infection by oral bacteria in the oral cavity make thier way into the systemic blood, engage with the host antigens and regulate their operation. These are cross-reactive antibodies. It is possible that sometimes these antibodies promote cardiovascular disease by exacerbating the endothelial reaction to their presence which causes further endothelial inflammation, or increasing the absorption of lipids into phagocytes.

Several studies indicate that serum concentrations of potentially inflammatory lipids, including LDLs, triglycerides, and very low-density lipoproteins (vLDLs) are elevated in periodontitis patients. These lipid subforms are thought to enter the blood vessel wall more easily, may be more susceptible to modification and therefore more likely to be incorporated in to the atherosclerotic lesion. This would accelerate and promote the development and maturation of the lesions (Schenkein, Loos, 2013).

It is thought that patients who present with periodontal disease are at risk for some or all of the activities outlined above. These mechansims may separately, or in partnership with each other, be implicated in arterial disease in periodontal patients. If these mechanisms work together, their cumulative effects could impact greatly on the severity of the cardiac and arterial disease in the periodontal patient.

Conclusion

In conclusion, to date the research does not absolutely prove that periodontitis causes cardiovascular disease. However, as seen above there is strong evidence supporting this idea, and many possible and logical mechanisms have been delineated through which periodontitis with all of its ramifications could effect cardiovascular disease. More recently, patients being admitted for surgeries with no seeming connection to periodontitis, for example hip replacement surgery, have been required to receive authorization from their oral care providers that they do not have any periodontal diseases. This is due to the mounting evidence, over the last few years, that there is indeed a correlation between PD and the rest of the body. Oral care providers should be wary of these problems and should advise their patients accordingly of the importance of oral care, not only with regard to the oral cavity.

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Should Genetic Testing be Recommended for Long QT Syndrome Patients and Their Relatives?

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Abstract

The Long QT Syndrome (LQTS) is a familial, potentially fatal cardiac arrhythmia. Traditionally, it has been diagnosed by ECG. Molecular studies have provided evidence that LQTS can be caused by a range of underlying molecular abnormalities. Genetic research has proven that different forms of LQTS have different genotypic bases. Therefore, it has become possible to diagnose the specific type of disease genetically. This study examines the advancements made in the past thirty years in understanding LQTS and research regarding the use of genetic testing, in order to determine the benefits of genetic testing for this disease. A survey of original studies which produced the information is presented here, and provides the reader with an understanding of the mechanics of the disease and how they differ in the several genetic variants. Research shows that the benefit of genetic testing must be weighed against the personal implications in may have for a particular patient and his or her family.

Introduction

In the year 1856, the German scientist Freidrich Ludwig Meissner described what may have been the first recorded case of the Long QT syndrome (LQTS). He recorded a case in which a deaf female student died suddenly during a moment of emotional stress. There had been two other children in her family who had died previously under similar circumstances. Since a diagnosis of LQTS requires an electrocardiogram, and at that time the ECG had not yet been invented, (Rivera-Ruiz, 1927) it is not known for certain that these cases were an example of LQTS (Vincent, 2002). Over a hundred years later, a study was performed on a family of 10 children. Four of the children had profound deafness and would often faint during moments of emotional or physical stress (Jervell, Lange-Neilson, 1957). Later, a similar familial tendency to faint during stress was observed in case studies (Ward, 1964). A prolonged QT interval was observed in the ECGs of subjects. At that time, two familial heart diseases known collectively as Long QT Syndrome were fully discovered and defined.

The advent of molecular and genetic science has revolutionized the medical world's approach to this disease, as it has done for that of many others. Molecular studies have divided the cases of LQTS, which seemed in the past like one basic condition, into larger categories differentiated based upon molecular mechanism. Genetic differences were discovered between these types in the lab, and since genetic testing became available to the public in 2004, 15 categories including over 1500 different mutations have been found to be the underlying cause of a prolongation of the QT interval. This study focuses on the three categories which make up the overwhelming majority of cases. In some cases, certain genetic origins and molecular mechanisms have been linked to certain phenotypic characteristics, and some believe that genetic diagnosis may aid in recommendations and treatment for LQTS patients. This study aims to give a full description of the physiological and molecular mechanisms of LQTS based upon the latest research, and analyze the contribution of genetic research to the understanding and treatment of the disease. Most importantly, the primary objective of this study is to answer the question: Should genetic testing be universally recommended to LQTS patients and their relatives?

Methods

The Touro library system was an invaluable source of recent, high-quality academic papers. In addition, the National Institutes of Health and NCBI provided excellent references to PubMed articles which provided useful information. The author sought out independent studies and other primary sources to provide up-to-date reports of developments in the molecular and genetic understanding of the disease. Studies regarding recent attempts at gene-specific treatments and current academic medical surveys are considered in determining when genetic testing is indicated.

Discussion

Physiology of LQTS

The QT interval indicates the length of time from the beginning of the depolarization of the ventricles to the end of their repolarization. A prolonged QT interval will mean that the ventricles are taking too long to repolarize. This usually does not interfere with cardiac function, but occasionally can cause life-threatening ventricular fibrillation. The major mechanism of ventricular fibrillation which is brought about by a prolonged QT interval is the result of Early Afterdepolarizations (EADs). The plateau curve caused by the delay in repolarization can cause some of the heart's cells to begin depolarizing while others are in the process of repolarizing. This can cause heterogeneous activity in the heart tissue, especially the ventricles, with some regions contracting and others relaxing. The result is a quivering or fibrillating heart, which cannot produce sufficient blood output and quickly leads to syncope and death (January, et. al. 2000).

Clinical Manifestation

Normally, depolarization and excitation of the cells of the heart are caused by a quick flow inwards of positively charged ions. These are usually Sodium and Calcium ions. However, the repolarization of the cells is not the result of those same ions flowing back out; it is an outward current of positively charged Potassium ions which causes repolarization. As the Potassium outflow increases, it overtakes the Sodium inflow, and the cell begins to repolarize. In all forms of LQTS, the symptoms are caused by a surplus of positive charges remaining within the cell. When some channels malfunction, the extra positive charge is caused

by reduced outward Potassium current. When other channels malfunction, the extra positive charge is caused by increased or unchecked inward Sodium current. In either case early afterdepolarizations can result, meaning that depolarization may still be going on after the effects of repolarization have subsided. This can cause what is called a U wave (Roden, et. al. 1996). This U wave, however, is not a homogenous contraction of the heart. Some of the cells will show this depolarization, and others will not. If this EAD is still going on when the next beat occurs, it can cause the heart to contract in an uneven way (Antzelevitch, Sicouri, 1994). This leads to a characteristic ECG graph known as Torsades de Pointes. This is French for "Twisting of the Points," and is named for the peculiar way the wave form seems to twist around the isoelectric line (El-Sherif, et. al. 1996).

Torsades de Pointes is not able to sustain a sufficient stroke volume of blood to support brain function. After some time with this abnormal ECG morphology, the patient will faint. Most of the time, the heart will overcome the Torsades and regain a normal heartbeat. At other times, the heart will not be able to "snap out of it" and the rhythm will deteriorate to ventricular fibrillation, without providing any meaningful output of blood. Unfortunately, this situation leads to death within minutes.

A patient who presents with Torsades is considered a medical emergency due to the risk of ventricular fibrillation which may result. However, the situation rights itself in the vast majority of cases. Therefore, DC shock defibrillation is not used as a primary course of action since it can cause recurrent arrhythmias. One of the major aspects of treatment is preventing the patient from returning to a state of Torsades; whatever caused it the first time might cause it again. Therefore, it is urgent to remove or mitigate the effects of any drugs which may have caused the Torsades. It is also advisable to take measures to suppress EADs, among other things. Magnesium is effective at suppressing EADs (Viskin, 1999).

Since the danger resulting from a prolonged QT interval depends not only on the actual length of the interval but also the way it interacts with the surrounding segments of the ECG wave, it is important to have a method with which to accurately measure the clinical significance of any particular QT prolongation. To this end, the concept of QTc was developed, in which the actual length of the QT interval is mathematically corrected to take heart rate into account (Bazett, 1920). Since that time, many other methods have been proposed, and the medical community is currently in debate over which is most useful and accurate.

Molecular Mechanisms

To understand the cellular and molecular mechanics of Long QT Syndrome, we must understand the process of the action potential itself. At rest, nerve cells have a higher level of positive charge outside the cell than they do inside. When a nerve cell is stimulated, the chemical or electrical impulse it receives activates voltage- or ligand-gated Sodium channels. This creates an influx of positive current (depolarization). After a certain amount of positive flow is admitted to the cell, voltage-gated Potassium channels are activated, creating an outflow of Potassium ions and returning the cell to its resting state with more positive charge on the outside. Many different types of channels are involved in these processes. Cardiac muscle cells are unusual in the sense that they act to carry an action potential as efficiently as nervous tissue can, and therefore depend upon a similar set of ion channels (Sanguinetti, Jurkiewicz, 1990). We will delve deeper into this process shortly.

Some years ago, a groundbreaking study uncovered information about the process of repolarization in cardiac tissue. The goal was to understand electrophysiological differences between different areas of the heart. Microelectrodes were used to measure delays in repolarization found in certain canine heart cells, known as M cells. These cells, which had been previously found to show markedly longer repolarization times, are found in the deep sub-pericardial and mid-myocardial regions. The researchers measured the durations of their action potentials to have a mean of 358 ms, as opposed to 282 ms or 287 ms found in other regions of cardiac muscle. Upon further examination of the M cells, it was shown that they show differences in IK when compared to other cells. IK means Potassium ion current, which is the current responsible for the repolarization that ends every action potential. There are two distinct currents found in cardiac cells, the IKr, or rapid-activating current, and the IKs, or slow-activating current. These currents are mediated by two separate transmembrane voltage-gated protein channels. The M cells have smaller IKs (Slow-acting Potassium current) than the other cells, while IKr (Rapid-acting Potassium current) is generally the same. (Of course, the mechanism for the rate of an action potential has many factors, and depends upon the inward rectifier (IK1), Calcium ion inward current, Sodium-Potassium pump inward current, Sodium-Calcium inward current, and others.) (Sanguinetti, Jurkiewicz, 1990). The IKs in the M cells was measured using a selective IKr blocker, E-4031. The Potassium current was measured in the cells with E-4031 and in the cells without E-4031. The difference between the two measurements was the IK due to IKr, and the common portion was the IK due to IKs. It was found that the IKr in different cell types was essentially the same, and reduced IKs current was responsible for the delayed repolarization in the M cells. It was discovered in this study that IKr differs from IKs in its kinetics of activation, more negative threshold for activation, and different protein structure suggested by its selective blockage by methane sulfonamide class III agents like E-4031. However, studies of M cells demonstrated that delayed IKs can play a principle role in delayed repolarization. The researchers predicted that the M cells may be found to be the culprits of diseases such as LQTS (Liu, Antzelevitch, 1995).

Later the same year, it was shown that the plateau portion of repolarization is accomplished principally by the IKs protein. It was also discovered that in the heart in general, blocking either IKr or IKs can prolong duration of the action potential by the same degree. This means that either protein can be a target for antiarrhythmic drugs for tachycardia. By the same token, either protein could also be the culprit of an arrhythmia with extended action potentials, such as LQTS (Zeng, et. al. 1995.) As we will see soon, genetic studies have confirmed that LQTS can result from mutations in the genes which code for either of the proteins, and others as well.

Genetic Variation

It has been known for many years that not all cases of LQTS have the same symptoms. For example, some cases of LQTS are dangerous specifically under situations of emotional stress. Other cases are set off especially by exercise, whereas some cases seem to cause syncope specifically during swimming. It was established that in these cases it was not the stress or exercise of swimming which caused the ill effects, but rather the actual immersion of the face in cold water (Mayumi, et.al. 1995).

Genetic research has since shown that mutations in several genes, coding for several proteins, can cause LQTS. Different types of the disease have been linked to different genes, and over 1500 different novel mutations have thus far been discovered on 15 different genes which code for 15 different proteins. One such gene is the KCNQ1 gene. This gene on chromosome 11 codes for the alpha subunit of the voltage-gated Potassium channel. It has six transmembrane domains. Even though there is ion pore function for this protein alone, some of the functioning of the channel requires it to be co-assembled with the beta subunit. This subunit is called KCNE1. The two subunits together account for the cardiac IKs. Mutations in different segments of this protein channel cause varying degrees and kinds of diseases. The S4 domain contains a voltage sensor, and loss-of function mutations in this area can cause LQTS. In addition, there is a pore helix selectivity filter segment which can, when its shape is altered, severely affect the functioning of the IKs current. In this segment, certain mutations cause more loss of function than others. For example, an I313K mutation in the selectivity filter segment can cause all of the symptoms of LQTS, including syncope and risk of death, whereas an I313M mutation will prolong the QT interval, but the patient will be otherwise asymptomatic. All KCNQ1 and KCNE1 mutations are included in the category LQT1 (Ikrar, et. al. 2008).

LQT2 is a result of a loss of function in the HERG channel. HERG stands for Human Ether a go go related gene, so named for the fact that a mutation in its homolog in fruit flies causes them to "dance" when exposed to ether. More than 80 mutations were known in this gene in 2000, and many more are known today. HERG is a gene located on chromosome 7 that codes for the pore-forming subunit for the IKr channel. There are several mechanisms by which HERG mutations cause reduced IKr. One of them is that a certain degree of codominance exists in heterozygous individuals with certain mutations. The protein which comprises the HERG channel is actually a tetrameric structure formed by the co-assembly of four copies of the HERG gene product. Depending on how the subunits combine, the mutant gene products might be excluded from the ion pores, causing a 50% reduction in pore function. On the other hand, the mutants might be included in some of the complete proteins, causing more than 50% to be defective. Other mutations in the HERG gene are truly dominant, and produce channels which activate at voltages which are more negative than usual. One mutation, N629D, was found to make the pore channel nonselective and allow ions other than K+ to pass through. Interestingly, many mutations in the HERG protein affect not the actual functioning of the ion pore, but its "trafficking," i.e. its ability to be properly packaged and transported to the plasma membrane. In these cases, the mutant proteins are marked as abnormal by the cell's quality control systems and are kept in the endoplasmic reticulum and degraded. In these scenarios, applying E-4031 to affected cells was shown to allow trafficking of the proteins to the plasma membrane, and the drug could later be washed off. Applying this method in vivo is still an area of study for these particular mutant forms. This is a form of gene-specific therapy for LQT2. Another form may be increasing serum K+ concentration, since HERG current is highly sensitive to extracellular K+ concentration. (January, et. al. 2000)

More work was done recently on attacking the gene-specific therapy problem from the trafficking angle. It has been shown that certain mutations cause misfolding of the protein, and this is what prevents it from leaving the endoplasmic reticulum. This was discovered by causing chemical unfolding of several different mutant forms and measuring the difference between the stabilities of each mutant's folding. It was found that there was a direct correlation between the conformational stability of the protein and its degree of trafficking deficiency (Harley, et. al. 2012).

LQT3 is has a very different molecular basis from that of both LQT1 and LQT2. The first two forms of Long QT syndrome are caused by malfunction of Potassium channels. These channels function to create an outflow of positive current during repolarization of the heart's muscle cells. These mutations are examples of what is known as "loss of function" mutations. In other words, the protein coded for by the gene does not work as well with the mutation as it does in the wild type. LQT3, however, is a "gain of function" mutation. It causes the Sodium channel pores to become more effective than in the wild type. This means that the depolarization process lasts longer, thereby slowing repolarization and extending the QT interval (Mazzanti, et. al. 2016).

Gene-Specific Recommendations and Therapies

Phenotypic differences have been found between LQT1 and LQT2. Heterogeneity has been noticed among LQTS patients regarding the effect that exercise has in precipitating Torsades de Pointes and ventricular fibrillation. It was discovered that patients matched for age and gender showed differing QT adaptation patterns during exercise. Patients with LQT1 showed a steady QTc prolongation, even at higher heart rates. Since each heart beat occurs at a faster rate, and the QT prolongation remains the same, it is more likely that the repolarization process will interfere with the following QRS complex, bringing about Torsades de Pointes and ventricular fibrillation, causing syncope or death. In LQT2, however, the QT interval prolongation is actually reduced as the heart rate increases, meaning that the QT interval occupies the same proportion of the total ECG sequence that it does at lower heart rates, such as during rest. Therefore there is no greater risk of syncope in LQT2 during exercise than at any other time. This is consistent with the molecular and genotypic understanding of the differences between these two disease types. Exercise creates an increase in sympathetic activity, i.e. the heart is being driven not only by its internal pacemaker, but also by hormonal and systemic nervous impulses and stimulants, such as epinephrine and other adrenergic agents. In this situation, the predominant current for the increase in the rate of repolarization has been proven to be the IKs current, precisely that one which is affected by LQT1. Therefore, it is understandable that LQT1 patients will be more susceptible to syncope brought on by exercise-related stress. These differences are generalizations; actual recommendations to patients still must be based upon observation and testing under different types of stress (Sy, et. al. 2010).

In addition, many symptoms have been found to be associated with different forms of LQTS. As stated, LQT1 is associated with syncope brought on by exercise, with swimming being a particular stimulus in certain cases. LQT2 is associated with cardiac events connected with mental stress such as fear or anger. LQT2 patients also faint when they hear sudden noises such as alarm clocks and phones. LQT3 seems to be associated with attacks which come while a person is resting or sleeping. Knowledge of the genetic basis of the disease can thus be helpful in advising the patient which activities to avoid. However, even though statistically there is a significant association of these properties with these forms of the disease, a genetic diagnosis is by no means conclusive of a specific expression. Among the 1500 variations discovered thus far, most do not exactly fit the general trend. Therefore, although genetic testing can be helpful in planning an avoidance or treatment plan, clinical manifestation is the ultimate deciding factor (Mangset, Hoffman, 2014).

Several gene-specific therapies have been proposed for LQTS. Beta Blockers are effective for LQT1 and LQT2, whereas they can have negative effects in the case of LQT3. Potassium supplements also are suggested for LQT2, since an elevated blood potassium level has been shown to enhance the activity of the HERG channels. Mexiletine, a methane sulfonamide Sodium channel blocker, may be effective for LQT3, as described before. Although some of these have shown promise, comprehensive and clear proof of their efficacy and safety has not yet been established (Viskin, 1999.) Recent studies have shown Mexiletine to be effective at reducing the QTc of more than two thirds of patients with LQT3. These studies had certain limitations, however, and were not sufficient basis for widespread use of this gene-specific therapy (Mazzanti, et. al. 2016).

Implications of Genetic Testing

Since 2004, genetic testing has been made available on symptomatic people to discover what the genetic source of their syncopal episodes may be. However, in many cases there is a dilemma for the patient. Most cases of LQTS do not require genetic testing for diagnoses, and if pathology is suspected in a newborn based on heredity, risk of serious effects can usually be effectively determined by ECG. In addition, even if the genetic testing comes up clear, preventive measures will still be taken for anyone whose QTc is over the limit of .45 ms. Therefore, the primary justification for genetic testing is not identification, but rather the possibility that gene-specific therapy will be available. As explained, most gene-specific therapies which require a genotypic diagnosis are still not available to the public. Those therapies and recommendations which are available can be safely used without genetic testing. This creates a dilemma for many patients and their relatives. On the one hand, there is the patient's right to know. A clear, empirical knowledge of the source of their disease helps some patients adjust and cope psychologically. On the other hand, who would want to know for certain that they carry a gene which may or may not have deleterious effects on their children? Since there are effective screening methods, and this knowledge can be a significant source of fear and anxiety as well as causing moral dilemmas, many people are uncomfortable going for testing. Sensitivity to this discomfort has been termed by some as "the patient's right not to know." This consideration is rarely addressed by health care providers, and the National Society of Genetic Counselors is making an effort to educate providers about the psychological impact of genetic testing (Mangsett, Hoffman, 2014).

A longitudinal study was done to observe the long-term effects of congenital LQTS. Individuals from 328 families with multiple cases of LQTS were observed over the course of several years. The probands had usually fainted at some time during their childhood years, with an episode of fainting or death occurring in 50% of them by age 12. The majority were women, showing that the effects of the gene are more serious in females. The yearly rate of fainting spells till age 50 was 5.0% per year for those enrolled in the study, and the rate of death was .9%. This

was higher not only than the rates in unaffected family members, but also higher than the rates in affected family members. This shows that LQTS genes can have a variable level of expression even among affected people (Moss, et. al. 1996). Since the disease can have varying levels of effect even among those who are stricken by it, it may be true that testing of all family members can help in discovering mildly affected people.

More recently, however, a survey was taken to determine the effects of genetic abnormalities on people who were otherwise clinically normal in terms of ECG and symptoms. Interestingly, no ill effects were found to be associated with genetic defects alone, even in situations where those same genetic defects produced significant disease in other patients. Some suggest that this might be affected by the fact that genetically positive patients tend to take care to avoid activities which bring on syncopal episodes. This information was not part of the study however, and it is therefore unclear whether clinically normal but genetically abnormal individuals need to take any action at all to protect themselves from syncope or death (Lampert, 2015).

One other argument has been made in favor of genetic testing of family members of affected individuals. Some drugs which are administered by psychiatrists to relieve psychological symptoms either function directly through changing the electrophysiological workings of the heart, or have side effects which can affect or alter the action potential of the heart cells. It is suspected that an increased rate of sudden death among psychiatric patients may be caused by underlying QT interval abnormalities which may, when augmented by the effects of the drugs, come to have deadly effects upon the heart's rhythm (Sayako, et.al. 2014.) However, the effects were only observed in a small percentage of the psychiatric population.

Certain specific mutations have been identified as conclusively pathogenic. Individuals who carry these variants are advised to take beta blockers as a prophylactic measure. Although these are official recommendations due to an abundance of caution, there is still no evidence that these mutations pose a significant risk in unaffected people, and the testing of relatives is not universally required (Vincent, 2001).

Conclusions

The Long QT Syndrome is a great example of how a little information can be a dangerous thing. This is an area in which there has been a recent surge in pure information: exact mutation types, statistics, symptoms, etc. However, the tying together and understanding of this information is lagging far behind its production, and that which exists is ambivalent and confusing. Since people rely on their healthcare providers not only for information, but also for confidence and reassurance, it can be difficult for the provider to respond appropriately to questions about this disease. One patient commented that he gets different recommendations from the same doctor on different occasions. In these situations the doctor must try to convey confidence to the patient while honestly admitting that he or she does not know the full significance of genetic results, and possibly nobody in the world knows (Mangsett, Hoffman, 2014).

Genetic testing is definitely a powerful tool in the understanding of LQTS. However, both methods of interpreting the genetic data and the treatments that might be tailored to the specific types have not yet been developed in a way which provides institutionally recognized necessary medical benefit. In addition, in most cases, only minor benefits have been shown to come from testing unaffected relatives for identification purposes. This makes it difficult to argue that all patients and relatives should universally submit to genetic testing, since research has observed emotional and ethical difficulties which can arise from the testing itself. However, a specific genetic diagnosis can influence the recommendations and, to some degree, the treatment that a patient will be provided with. In addition, since treatment is recommended for some variants even in the absence of ECG symptoms, relatives of individuals with those variants have more reason to submit for genetic testing. A physician and genetic counselor should therefore discuss the benefits as well as the pitfalls of testing with the patient or relative, and the patient or relative should decide whether or not to submit to testing. In the coming years, the approval of developing gene-specific treatments may make genetic testing more important for affected individuals.

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The Correlation Between Stress and the Development of Dissociative Identity Disorder

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Abstract

Stress is a huge part of life. Our body responds to stress in different ways and most of the times the body overcomes the stress. There are a few incidents when the body is not capable of dealing with the stress and the toll it takes on the brain is undeniable. One result of intolerable stress is Dissociative Identity Disorder (DID) in which biopsychology comes to life, as we see how the body is affected by psychology, and vice versa. The mediators that help the body adapt to stressors become detrimental when a person faces trauma or chronic stress. Glucocorticoids, cortisol, and glutamate are all involved in either helping the body endure stress or causing plasticity in parts of the brain that are essential to being mentally healthy. This paper delves into the nature of DID, and how stress creates changes in the hippocampus and amygdala, the two parts of the limbic system that are smaller in patients with DID..

Introduction

In 1957 the world was exposed to what was then known as Multiple Personality Disorder (MPD) through the storyline of the movie, Three Faces of Eve. A classic example of MPD, a woman named Eve White was having symptoms of headaches and blackouts while being unaware of her disorder. One of the three identities, known as Black, is the reason her husband abandons her and is also why she tries to kill her own daughter. Black knows about White, but White does not know about Black. Through therapy the third identity emerges, known as Jane. Jane knows about all three identities and eventually all three identities merge (Lehman, 2014). This story of the Three Faces of Eve is a glimpse into the world of MPD. As time progressed new research has been developed and continues to develop regarding MPD, currently referred to as Dissociative Identity Disorder (DID), broadening the subject in all areas.

DID is a psychophysiological disorder that affects about 1-3% of the world's population (Vermetten et.al.,2006). DID diagnosis requires a minimum of two identities residing in one body. Each of these identities have their own biology, memories, perceptions and preferences. Common symptoms of DID include hallucinations and amnesic periods. An average of 8-13 identities develop and Daniel Goleman (1923) states that it is possible for one to be living with up to 60 different identities within oneself. The different identity types include the childlike personality type, the protector, and the persecutor. A misconception that the host personality is in fact the patient's original identity since it is the identity or alter ego will commonly seek treatment. The split in identities originates from the childhood of the patient. People with DID have a history of either sexual or physical abuse typically between the ages 3-8. Coherent with the common identity types, the childlike personality is the age in which the abuse took place. The persecutor is the identity that mirrors the abuser. The protector is the identity that feels responsible to protect the rest of the personalities from any abuse or trauma (Goleman, 1985). Each identity can be categorized as either an emotional personality or a normal personality. The emotional personalities are the ones that react and respond to the trauma they experienced. The normal personalities are the ones that deal with normal and daily functions (Nijenhuis, Steele, 2010) . DID is often misdiagnosed as other personality disorders due to the complexity of the various identities. An identity in itself may have a personality disorder such as Obsessive Compulsive Disorder and be treated for that. From an outsider's perspective what might seem like a suicide attempt may actually be one identity trying to kill another and thus must be dealt with appropriately. Additionally, DID is very similar to Post Traumatic Stress Disorder (PTSD) except that in a patient with DID the trauma happens in early childhood and in PTSD it occurs in adulthood (Gillig, 2009). In addition, 80-100% of patients diagnosed with DID are diagnosed with PTSD as well (Vermetten et.al., 2006).

There are cases in which one alter ego can be deathly allergic to bee stings, or may need glasses to see, while another alter ego in the same body may not have any reaction to bee stings or may not need glasses to see. There is a case study involving a 33-year-old female diagnosed with DID who suffered from blindness after a car accident. She was confirmed to be visually impaired after going through tests such as ocular fundus inspection, Humphrey refractometry, and intraocular tension measurement, which are all ways to test for vision or lack thereof. She even failed to show signs of involuntary refluxes such as eye watering and winking. After years of treating the patient with therapy for DID, one of the identities became visually active and soon many of the identities followed and retrieved vision. Because only some identities were blind, just like any identities can fluctuate, the patient was fluctuating between sight and blindness within seconds. Proof of complete blindness in one identity and complete sight in another was seen by visual evoked potential (VEP) recording system. In a blind identity the pattern evoked potentials showed a disorganized blind pattern. When the VEP was run on a sighted identity, there were organized patterns cohesive to a visually active person. The VEP results for the blind identity shows lack of vision, yet insufficient for complete blindness. The blindness that was relayed by the tests can be explained as psychogenic blindness that was activated by a temporary loss of vision caused by the accident. The psychogenic blindness is proof that in DID psychology takes a toll on the biology of a person (Strasburger, Waldvogel, (2015).

Dissociative identity disorder creates questions within the realms of sense of self; how can it be that two completely different identities are being controlled by one brain? Experiments using Positron Emission Tomography (PET) were performed to see how the brain creates these two different senses of self. To help explain this phenomena Damasio and coworkers created a theory that there is a core self, constantly synchronized

with the body and controlled by the body's biology, and the autobiographical self that are memories and is influenced by environmental factors. According to this theory only the autobiographical self is capable of change, yet the core self remains intact because it comes from the same biological makeup. Since the autobiographical manner of processing memory can be disrupted, the neutral personality state processes trauma in a non-autobiographical way, causing different senses of self in the brain. A study consisting of 11 females with DID in four different conditions was done. These four conditions included a neutral memory exposed to both the neutral and traumatic personality state, as well as a traumatic memory exposed to both a neutral and traumatic personality state. There were decreased amounts of regional cerebral blood flow (rCBF) perfusions when a traumatic memory was exposed to the traumatic personality state. The rCBF perfusions were unchanged when a traumatic memory was exposed to the neutral personality state, and when a neutral memory was exposed to both traumatic and neutral personality state. The areas that showed a decrease in rCBF perfusion correlated with the areas associated with the autobiographical senses-of-self. Decreased perfusion in the visual association area and the middle occipital gyrus causes the lack of integration of information and blocks emotional processing; this is the defense system manifested by patients with DID. Another symptom of DID is depersonalization, which is caused by decreased perfusion in parietal and occipital areas of the brain. Additionally, there is decreased perfusion in the medial prefrontal cortex that plays a big part in a person's sense of self. The only part of the brain that shows increased perfusion of rCBF is the parietal operculum regulating emotions in response to pain. This comes forth as emotional dissociation in the traumatic personality state of a DID patient (Reinders et.al., 2003).

Testing the autonomic nervous system through heart rate and skin conductance is an additional test that proves the authenticity of the various alter egos. Nine patients diagnosed with DID were brought in each day for testing. Each patient had three identities involved in the study: the host personality and two other identities. As the researchers hypothesized, each identity showed differing results of heart rate and skin conductance when exposed to specific stimuli. Since the autonomic nervous system is completely voluntary it is safe to assume that the identities are genuinely distinct (Putman et.al., 1990).

Amnesia and Memory Transfer

According to the DMV, DID should be a possible diagnosis when there is an "inability to recall important personal information that is too extensive to be explained by ordinary forgetfulness." Amnesia is the most dominant symptom in a patient with DID and according to the Journal of Abnormal Psychology is said to be a psychological disorder as a response to stress or trauma. Furthermore, it's questionable whether inter-identity memory transfers exist. A study was conducted proving that DID patients only show memory transfer of implicit memories/words, but when there was an emotional or explicit word the patient regressed into an amnesic state. When these memories are restored it's possible to develop dissociative memories in which at times the trauma is remembered and at times the patient has amnesia.

The amnesic periods are only found between different identities and not within one identity. Therefore, in order to get to that amnesic state the identity fluctuates. Accordingly, the psychological perspective to DID is believed to be that DID patients are capable of switching identities in order to forget, and to avoid re-experiencing the trauma (Elzinga et.al., 2003).

Trauma During Childhood

Vermetten et. al. (2006) describe DID as a childhood-onset posttraumatic developmental disorder. The post traumatic model of DID states that when a child goes through the traumatic experience of physical or sexual abuse it is natural that the child will dissociate, mentally compartmentalize, and have amnesic periods as a response. This coping mechanism removes unbearable memories that the child cannot deal with. As the child grows up, each painful experience gets compartmentalized and is expressed as a different identity (Piper, Mersky, 2004).
Studies show that childhood trauma is imperative to the development of DID. Traumatic is a relative word and is not referring to a one-time beating. The trauma endured is unusually sadistic abuse and it happens about twice a week for 50 weeks out of the year for 10 years. Statistically the type of abuse that will cause DID is 60-75% physical abuse, and 68-83% sexual abuse (Piper et.al., 2004).

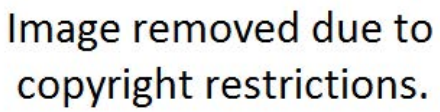
DID specifically only develops when the trauma occurs in childhood because children are not yet equipped with the resources to react. Furthermore, children are more prone to peritraumatic dissociation, and do not personalize the trauma that they are experiencing. DID can be classified as a developmental disorder due to the child not being able to handle dealing with the trauma and it takes a toll in later years. When a normal person deals with stress their mental health is improving. However, when the stress is overbearing it reduces the mental health of a person (Goleman, 1985). Stress in early development biologically embeds itself in a person and has long lasting effects. When a child experiences abuse versus being cared for it will affect his social experiences in later life (Mcewen et.al., 2015).

Hippocampus and Amygdala

Stress related trauma plays a role psychologically and biologically in patients with DID.

Changes in the limbic system have been linked to stress factors (Gulyaeva, 2014). Parts of the limbic system such as the hippocampus and amygdala are two major parts of the brain that are involved in the formation of DID. Patients with DID

have a significantly reduced sized hippocampus and amygdala. The hippocampus and amygdala are located in the medial temporal lobe (Figure 1) and although they have different functions they ultimately influence each other (Phelps, 2004).



The hippocampus is the part of the brain that is involved in episodic memory and the primary memory system of the brain. Learning and stress regulation are also part of hippocampal function. The amygdala controls fear conditioning and processes emotion. Although the hippocampus and amygdala reside adjacent to each other, studies introducing a blue box that gives a shock proved that the hippocampus and amygdala have independent functions. The study was performed by introducing a blue box that gives a shock.. People with focal lesions on the amygdala understood that the blue box causes a shock but failed to show any sign of fear. Focal lesions to the hippocampus caused one to fear the shock but fail to register that the shock comes from the blue box (Phelps, 2004).

The hippocampus and amygdala are dependent on each other when it comes to emotional memories. The amygdaloid complex consists of the subunits of the amygdala that are difficult to distinguish in MRIs. The amygdaloid complex is divided by function, receives, and relays information involved in fear conditioning and response. The forebrain receives input from the amygdala to stimulate attention, and the brainstem stimulates fear. The hypothalamus receives input from the amygdala for hormone release during stress that eventually affects the hippocampus. Through animal studies researchers were able to see that stress hormones were attacking androgenic receptors on the basolateral amygdala that were affecting hormones in the hippocampus. Further research revealed through fMRI scans that activity of the hippocampus causes less activity of the amygdala and vice versa(Phelps, 2004). When studies were done removing the temporal lobe (which includes both hippocampus and amygdala), symptoms normally seen in patients with DID, such as amnesia and hypermetamorphosis were present. (Cristinzio et.al., 2007).

Amygdala and Hippocampal Size Difference

The difference in hippocampal volume between a patient with DID and a healthy person is significant. The hippocampus in a patient with DID is 19.2% smaller and the amygdala is 31.6% smaller than that of an average person. In addition to stress causing smaller hippocampal volumes in patients with DID, other disorders such as PTSD that are associated with abuse in childhood show smaller hippocampal volumes, which can be seen on MRI readings. On the contrary, people who experienced childhood abuse and did not develop PTSD or DID, showed a normal sized hippocampus. There is no proven decrease in the size and volume of the amygdala in PTSD patients with no childhood trauma. In contrast, a PTSD patient with record of childhood abuse revealed a 15% decrease in amygdala size (Vermetten et.al., 2006).

Methods

To research the different mechanisms and causes that stress has on DID, search engines such as Touro College Library, Proquest, and Google Scholar were used. Keywords such as "dissociative identity disorder", "glucocorticoids", and "hippocampus and amygdala" were used to retrieve appropriate information. Multiple articles and medical journals were found presenting the different perspectives to answer the question above.

Discussion

Not much is known regarding the neurobiology of DID, but the way that stress affects parts of the brain associated with DID is discussed. The parts of the brain affected may be the underlying cause of the symptoms of DID.

The homeostasis of the body is disrupted as a result of stress (Krugers et.al., 2010). Allostasis is the body adapting to stressors through mediators. Mediators can be steroid hormones such as glucocorticoids. Through glucocorticoids the body is able to adjust and adapt to the stress that it is handling. These effects of glucocorticoids can either be helpful for the person's survival or detrimental to the brain. The brain can be altered through the expansion or retraction of the dendrites or change in synaptic density. Receptors in the brain are important factors in brain alterations (Mcewen et.al., 2015),

Glucocorticoids

Glucocorticoids are adrenal steroid hormones that are produced in the zona fasciculata of the adrenal cortex in response to stress. One major class of glucocorticoids is cortisol.
Glucocorticoid or cortisol secretion from acute stress is crucial for the survival of a person. Stored energy is retrieved to

enable the body to react appropriately to stressors. The harmful effect of glucocorticoids appear in the hippocampus and can take a toll on a person's memory, specifically explicit memory (Sapolsky, 2003).

HPA Axis

A major gland involved in stress regulation is the adrenal gland. Located superior to the kidney, the adrenal gland either reacts to stress in a fight or flight response or supplies energy for the body's response via the HPA axis. The adrenal gland is made up of the adrenal cortex and the adrenal medulla. The stimulation of stress causes the adrenal medulla to secrete epinephrine (adrenaline) and norepinephrine (noradrenaline) so the body can respond in a fight or flight manner. When the stress is continuous the adrenal cortex secretes glucocorticoids or cortisol which mostly bind to proteins. The cortisol that does not bind to proteins exists as free cortisol and supplies the body with the energy it needs to react to the stress. Cortisol acts in different reactions to produce glucose through gluconeogenesis, fatty acids from triglycerides, and amino acids from proteins to create new energy or to repair cells that were damaged by the stress (Hannibal, Bishop 2014).

Hypothalamic pituitary adrenal axis (HPA axis) is a negative feedback mechanism that helps regulate glucocorticoid production in times of stress (figure 2). The reaction begins in the hypothalamus, the link between the nervous and endocrine system. The paraventricular nucleus in the hypothalamus responds to a relative amount of stress and causes the release of corticotropin releasing hormone (CRH). The CRH thus stimulates the anterior pituitary to release adrenocorticotropic hormone

(ACTH) that travels through the blood to the adrenal cortex. Glucocorticoids are released and supply energy to the body. When there is an excess of glucocorticoids the hypothalamus and anterior pituitary receive signals to stop the production of CRH and ACTH in order to inhibit the overproduction of glucocorticoids (Zhu et.al., 2014). However, this negative feedback mechanism may be disrupted. Only a small amount of stress is required to activate the HPA axis, but when there is too much stress it is possible for the HPA axis to become hyperactive.

HPA Axis Hyperactivity

Hyperactivity of the HPA axis can be caused by many factors, including excess glucocorticoids. Excess glucocorticoids in the brain can be attributed to receptor changes. Two different glucocorticoid receptors in the brain are: glucocorticoid receptors (GR) and mineralocorticoid receptors(MR). GR and MR are found on both the hippocampus and the hypothalamus. MR are more likely to attract glucocorticoids even when glucocorticoid levels are low. GR only receive glucocorticoids when there is an excess amount of glucocorticoids from stress (Krugers et.al., 2010). If there is a decrease in GR in the hippocampus it can help cause the hyperactivity of the HPA axis (Zhu et.al., 2014).

A study using metyrapone was conducted on rats to determine the correlation between glucocorticoids and HPA axis hyperactivity that causes depressive behaviors. Enzyme 11-b-hydroxylase, found in the adrenal cortex, is responsible to convert

deoxycorticosterone to glucocorticoids. Metyrapone, is used to block the function of 11-b-hydroxylase therefore terminating the process which would result in hyperactivity and depressive behaviors (Zhu et.al., 2014).

MR-NNOS Pathway-Hyperactivity of HPA Axis

An additional pathway that decreases the activity of GR in the hippocampus is the MR-nNOS pathway. Glucocorticoids react with neuronal nitric oxide synthesis enzyme (nNos), causing the overproduction of nitric oxide (NO) in the hippocampus. The GR in the hippocampus, which is crucial for the HPA axis to maintain homeostasis, interacts with the NO causing the negative feedback mechanism to be disrupted. The relatively decreased amount of GR in the hippocampus explains why excess glucocorticoid exposure would generate hyperactivity of the HPA axis.

The reason that the hypothalamus is not involved with the hyperactivity of the HPA axis is because of the low amount of MR in the hypothalamus. Thus, the MR-nNOS-NO pathway is non-existent in the hypothalamus and the hypothalamus is only capable of activating the negative feedback of the HPA axis (Zhu et.al., 2014).

Different Pathways of Glucocorticoids

There are two effects that glucocorticoids can have: genomic or nongenomic. A non-genomic effect is a transcription independent mechanism that can interact with a cell membrane specifically or nonspecifically. The genomic effect works through interaction with Glucocorticoid Response Element directly or indirectly. Direct interaction is through receptors themselves and indirect interaction is through other transcription factors. Direct interaction of glucocorticoids is responsible for glutamate secretion. Indirect interaction of glucocorticoids uses endocannabinoids to control glutamate and GABA (Mcewen et.al., 2015).

NMDA and AMPA Receptors

One type of glutamate receptor is N-methyl-d-aspartic acid (NMDA) receptor found on the hippocampus. NMDAR is activated by glutamate with glycine and when activated permits charged ions to travel through the cell membrane (Mcewen et.al., 2015). Studies show that ketamine, an NMDA antagonist, causes the same symptoms seen in DID: amnesia, out of body experiences, and time standing still (Vermetten et.al., 2006).

Alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor(AMPAR) or quisqualate receptor is a transmembrane receptor. AMPAR only requires glutamate to activate the ion channel. Recent research show that activated glucocorticoids regulate AMPAR and effect learning and memory in the hippocampus. Learning and memory are disrupted by the synaptic connectivity in the hippocampus. Plasticity of the brain is in the control of the pre- and postsynaptic sites, and can be controlled by either limiting the neurotransmitters or the neurotransmitter receptors at the pre and postsynaptic sites, respectively. Further research proves that AMPAR also causes changes in synaptic function and plasticity of the brain. Miniature excitatory postsynaptic currents (mEPSC) are used to measure the strength of a synapse. When the nongenomic effect (fast) on AMPARs was measured the increase in the mEPSC of the hippocampus and amygdala were similar to those experienced upon glucocorticoid exposure. The genomic effect (slower) of AMPARs is detrimental to the function of neurons (Krugers et.al., 2010).

Brain Plasticity

Brain plasticity is caused by the conjunction of glucocorticoids and amino acids.

Dendrites are altered in the way of expansion or retraction, and increase or decrease of dendritic synapses. For example, CRH, released in the hippocampus, down regulates thin spines. Parts of brain affected by hormones and intra and extracellular mediators are the hippocampus, amygdala and prefrontal cortex (Mcewen et.al., 2015).

Studies show that chronic stress leads to permanent alterations to the brain. Regardless if the chronic stress subsides, the brain creates a new way to handle stress and hippocampal changes remain. Additionally, experiments with rats prove gene alterations due to stress, including delayed alterations. The delay in gene response comes from epigenetic regulators. Even though the dendrites can regrow they are different in that the dendrites grow more proximal to the cell body (Mcewen et.al., 2016).

The neuroendocrine model was postulated regarding the effects that stress has on the hippocampus and the role of the amygdala in hippocampal plasticity. It was proven that hippocampal plasticity can be achieved through limiting input from the amygdala.

A part of the hippocampus, the dentate gyrus, is known for its constant rearrangement and its reproduction of its cells to replace lost cells. Reproduction of the new cells is inhibited by the amount of adrenal hormones in the brain through a mechanism involving NMDA receptors. Stress exposure results in glucocorticoid production and the release of glutamate from the hippocampus which blocks the reproduction of neurons in the dentate gyrus. Only chronic stress has effect on proliferation in the dentate gyrus, because small amounts of stress were seen to have no effect on neurogenesis in the dentate gyrus.

In acute stress the new cells that are produced take part in stress response. When there is an excess amount of glucocorticoids caused by chronic stress the dendrites in the hippocampus retract and do not partake in stress response (Gulyaeva, 2014).

Hippocampal and Amygdalar Role in Memory

Both the hippocampus and the amygdala contain GR and MR. As a result of stress, the role of the amygdala in fear conditioning is altered. According to Sapolsky (2016) when the hippocampus is affected by stress the amygdala will start to process the memories. For example, amygdala long term potentiation will substitute the hippocampal long-term processing. Studies show that when hippocampal dendritic processes go through atrophy it causes the extension of the amygdalar processes'. The mechanism as to why this happens is currently unknown. Despite the fact that the hippocampus is irrelevant in memory processing, the hippocampus is said to process explicit memories. The reason is that the hippocampus and the amygdala work together to process the memories. Since the hippocampus cannot process the memories normally and neutral it acts like the amygdala and processes the memories inaccurately, causing flashbulb-like memories. The mechanism is controlled by the sympathetic nervous system and is activated by stress. Stress causes catecholamines to be released by the adrenal medulla and stimulates

the vagus nerve. Thus, the vagus nerve stimulates the nucleus of the tractus solitarius (NTS) to have an effect on the amygdala. The amygdala begins to play a part in memory processing from the glucocorticoids in the hippocampus, amygdala and the NTS (Phelps, 2004).

Animal studies show that by exposing glucocorticoids to the hippocampus it causes a decrease of pyramidal neurons and dendritic branching that are used to integrate memories (Nijenhuis, Steele, 2010).

Genetic Factors

Genetics are very influential in the development of any disorder. People that are born with a smaller hippocampal or amygdalar volumes have been seen to be more prone to developing DID (Vermetten et.al., 2006). When one is born with an overabundance of glucocorticoid receptors, there is an increased probability to develop mood disorders and are more responsive to antidepressant drugs. The opposite effect is on those who are born with less GR than an average person (Mcewen et.al., 2016).

It is more common for a male to develop DID than a female. The hippocampus in a male naturally becomes reduced 1-1.5% per year while the amygdala is unchanged. Female resistance to this reduction is attributed to the production of estrogen which somehow acts as a prevention (Vermetten et.al., 2006).

Conclusion

It is clear how much damage stress can do in terms of brain plasticity. Through mechanisms of how stress affects the brain it can be understood how DID can possibly develop. Treatment regarding DID is not proven to be 100% efficient or effective, but it may improve and make the disorder livable. Therapists dealing with DID patients will try to bring forth all their identities and help the patient deal with the trauma they experienced that sparked this disorder. As seen in the Three Faces of Eve, therapists may attempt for years to merge all the personalities together in order to diminish symptoms of dissociation. Cognitive behavior therapists introduce the patient to various coping mechanisms as an alternative to switching identities. One form of treatment that is not effective is to diminish personality states that are not positive or that may be detrimental to the environment. Since those identities exist to protect the patient from abuse it should be incorporated appropriately into their comprehensive personality (Gillig, 2009). Antidepressants may be another form of treatment for DID because in rodents neurogenesis of hippocampus was induced by them (Vermetten et.al., 2006). Recent studies show that the drug paroxetine may be useful in treating DID. Paroxetine has proven to regenerate hippocampal growth by 4.6% and to limit the symptoms of DID and PTSD . The correlation between DID and PTSD has shown to be very similar in neurobiology and causation of the disorders (Nijenhuis et.al., 2010). So many factors are involved in the development of DID, thus making the disorder very rare. The treatments noted are just a step in the direction to help understand the disorder and to further the subjects of research on DID. Even though it is understood how stress causes plasticity to the hippocampus and amygdala creating symptoms involved in DID, there is still so much about DID that is unknown to the world and is a mystery till today.

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Is Gene Therapy a Viable Option for Cancer Treatment?

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Abstract

The use of gene therapy as a medical treatment option was first introduced to the world in 1990, when a four-year-old girl became its first patient. Since then gene therapy has met great success but also severe drawback. Incidences with severely negative outcomes on patients gave gene therapy a bad name and many began skeptical towards its use, but the constant work and progress on the safety and effectiveness of gene therapy is making it a more viable route of treatment. This paper focuses on gene therapy as a form of cancer treatment. Viral insertion of the modified genetic material is the most effective method of insertion, targeting a large number of cells, although physical insertion may be safer and more economical. The mechanism by which many gene therapies work is suicide genes, genes that cause the cell they enter to lyse. The paper goes on to discuss the H-19 locus on the genome, which plays a significant role in cancer development and conversely, treatment. BC-819 is a plasmid that is synthetically produced to treat cancer based on targeting the H19 locus. Research and test models of the BC-819 show promising results for many cancer patients.

Introduction

Cancer is the second leading cause of death worldwide, taking the lives of over 8.2 million people every year. The standard cancer treatments, such as chemotherapy and radiation therapy, are often inadequate and debilitating, destroying healthy fast-growing cells in the process of treatment. Over the past decade, gene therapy has become a more prevalent option for treating cancers. Gene therapy avoids targeting healthy cells, selecting only cancerous cells for treatment. There are three approaches to gene therapy immunotherapy: stimulating a patient's immune system to recognize and attack cancer cells, oncolytic virotherapy, which generally uses viruses to infect and kill cancer cells, and gene transfer, which is the insertion of genetic material into the cancerous cells. This paper aims to consider the viability of gene transfer therapy. Gene transfer therapy is an exciting new technology that is shifting the paradigm of cancer treatment. It involves inserting a foreign gene directly into the cancerous cells or surrounding tissue's genome (Cross, Brumester 2006). With all the strides and progress made in gene transfer there are still problems that need to be rectified. In early safety test cases, gene transfer scared many by causing the death of a patient (Raper, et. al. 2003). Also, in some cases, gene transfer methods have promoted leukemia in their attempt to cure the patient of his disease (Thomas, et. al. 2003). Additionally, there is still plenty of research yet to be done in this area due to its relative newness. This paper will assess how much of an option gene therapy is for cancer patients, taking into account its numerous benefits and sometimes severe drawbacks.

Methods

Research literature for this paper was obtained through the Touro College Online library. Searches done on the Touro College Online library led the student to Proquest and Pubmed, where majority of the articles were obtained. Articles found on other scholarly sites were also used. The articles discussed experimental studies done and the thorough analysis of these articles allowed for the assessment of gene therapy's practicability. Review articles also assisted in composing the formal analysis.

Discussion

The standard treatment method for cancer today is chemotherapy. Chemotherapy can cause an array of both short term and long term side effects. Short term side effects are side effects that are present during the time of treatment and are often reversible, while long term side effects cause more severe and permanent damage. Short term side effects include hair loss, nausea, and vomiting, which can sometimes hinder patient compliance. Long term side effects such as arthritis, appendicitis, and thyroid damage have less of a probability of occurring, but do occur in some patients (Ramirez, et. al. 2009). The above mentioned are general side effects, but each patients' individual circumstance can pose other possible risks. If patients could be assured that chemotherapy would remove the cancer in totality, undergoing chemotherapy would be more tolerable, yet in many cases the chemotherapy fails to rid the body from the cancer and therefore, is often not a preferable option.

In this paper, gene therapy will be analyzed to assess whether it is a better option of treatment for cancer patients or whether it is yet another treatment method that provides partial results and causes severe side effects. How efficient gene therapy is in treating cancer, what side effects it includes and what the severity of the side effects are all questions that need to be addressed.

Insertion Approaches

There are two approaches to gene insertion; it can be done by means of either a viral or a non-viral vector (Amer, 2014). A viral insertion uses a virus as a vector to harbor the drug. A non-viral vector, which generally uses naked DNA or toxic material for the cell as a vector, can be inserted either chemically or physically. Physical insertion can be done by a gene gun or ultra sound. Another physical approach is that of electroporation, which uses high voltages of electricity to disrupt the cell's membrane, allowing the drug to enter the cell (Baranyi, et. al. 2013).

Viral Insertion

Viruses have long been a choice for a vector because of some important properties they contain. Viruses are small pathogenic particles that contain either DNA or RNA encoded in a protein coat. Some viruses also contain a lipid bilayer surrounding the protein coat. The mechanism in which a virus infects a cell is by implanting itself on the host cell's membrane and inserting its viral DNA into the host cell. The host cell then transcribes and translates the viral DNA, which codes for the creation and

assembly of more viral particles. These new viruses cause the cell to burst and proceed to infect more cells. The mechanism by which the virus operates is useful, for genetic material that will lead to cancer cell death or degeneration can be placed in a virus vector, which is essentially the outer protein coat of the virus deprived of its viral genome, and then infect the target tissue area. Before using it as a vector, the virus has to be rendered non replicative so it no longer behaves as a pathogen. Viral vectors are advantageous since they can be produced in high concentrations and have minimal side effects (Amer, 2003).

The most popularly used viral vector is the adenovirus. The adenovirus is favored since it can be made in high titers and can infect both dividing and non-dividing cells. When using a viral vector such as the adenovirus, steps must be taken to ensure that the virus will not reproduce in the host's body like it naturally would. Prevention of the adenovirus replication can be achieved by removing early regions of the adenovirus vector's genome (Pulkkanen, et. al. 2005).

Comparing Adenovirus to Retrovirus

A comparison of the efficiency of the adenovirus and the retrovirus as vectors for gene transfer was done. Ten patients with malignant glioma, a spreading brain tumor, were treated with a beta galactosidase gene via retrovirus and adenovirus vectors. This was done by inserting a catheter into the tumor and injecting the patient with retroviruses and adenoviruses for three consecutive days. This was followed by resection of the tumor one to two days later. X-gal staining was then used to highlight the beta galactosidase gene and to evaluate its efficacy in gene transfer. Findings showed that beta galactosidase was well tolerated with both vectors. In all but two patients, no systemic or tissue complications were apparent. The gene transfer was successful, with an efficacy between <.01- 4% for the retrovirus and an efficacy <.01-11% for the adenovirus. The adenovirus was thus more efficient then the retrovirus as a gene transfer vector (Puumalainen, et. al. 1998).

Malignant Glioma Adenoviral Gene Therapy

Although adenoviral vectors have some of the highest efficacies of gene transfer amongst other viral vectors, they still fall short of producing significant effects on the treatment of tumors. A study of the treatments of malignant glioma was conducted with the aim of evaluating the safety of the adenoviral vector as well as determining the maximum possible dose that would be tolerated. Fourteen patients with relapsed malignant glioma were treated with adenoviral vector containing the Herpes simplex virus thymidine kinase (HSV-tk) and its promoter (IG.Ad.MLPI.TK), and were then treated with ganciclovir, an antiviral drug. Prior to this the retrovirus had been used as a vector for HSV-tk gene therapy treatment. However, the adenovirus was used in this study because of its advantages of high titer production and efficacy of gene transfer.

The patients underwent as much debulking of the tumor as was considered safe. The wound bed was then infiltrated with around 50 evenly spaced injections of the HSV-tk gene. The patients were treated with different dose levels and then monitored for any adverse events. The patients reported either adverse events or serious adverse events. From surgery until completion of the ganciclovir treatment, 27 adverse events and 5 serious adverse events were reported. However none of the adverse events or serious adverse events were from the adenoviral vector.

After surgery, the patients were kept in strict isolation in the ICU, and viral cultures were taken until there were two consecutive days of negative culture results. None the cultures taken were found to be positive, indicating that the viral vector did not shed during its administration and did not pose a hazard to the environment.

The adenovirus was thus considered to be a safe vector internally and externally. However, in regard to the results of the patients' tumor responses, the adenovirus does not appear as promising. Unfortunately, none of the tumors responded to the successful gene transfer. Overall the patients did not fare well. The median survival time was four months, with four patients surviving for over a year. The median survival time attained in this study with the injection of the adenoviral vector was no better than the survival time in respective studies of malignant glioma with no gene therapy treatment. According to the study, even the survival of patients with the favorable prognosis was most likely due to the nature of the tumor and not the gene transfer.

It is clear that the adenoviral vector used in this study is a safe method of choice but not significantly effective in diminishing the tumor growth. It is not definitive from this study if the adenoviral gene transfer had even any effect on the tumor. (Smitt, et. al. 2003).

In contrast to viral vectors, non-viral vectors are generally more economical and easier to produce in large quantities. They also have limited immunogenicity which allows for re-dosing. There is no concern of a gene recombination causing the virus to become competent and pose a danger to the body (Amer, et. al. 2014).

Physical Insertion

Physical insertion involves injecting naked DNA or liposomes directly into the target cell through a breach in the membrane made by rapid needle or jet injections, particle impact, electric pulse, laser radiation, or ultrasound. A novel method of physical insertion is the Jet injection, which was first introduced in 1947 as an alternate to needle injections. Jet injection uses a pressurized gas, like carbon dioxide, to drive an ultrafine high speed stream of DNA into the target tissue in the form of plasmids. Comparisons done between jet and needle injections demonstrated that gene expression was fifty times greater when

done by jet injection than it was done by the standard needle injection.

A phase I study was conducted to determine the safety and feasibility of jet injection on patients with skin metastases from melanoma and breast cancer. Seventeen patients received five jet injections of B-galactosidase, a LacZ- expressing DNA plasmid, into a single cutaneous lesion. To monitor the clearance of the plasmid in the blood stream, real time quantitative PCR of blood samples was done throughout the study. After two to six days, the lesions were resected and a series of tests were performed to determine the efficiency of the plasmid uptake, as well as the transcription of DNA to mRNA and translation to a protein.

All the patients responded well to the jet injections. Four weeks after jet injection, all the patients were alive and none showed any adverse effects from the jet injection. Within forty-eight hours any small bleeding and jet penetration at the injection site disappeared. Additionally, the LacZ plasmids were successfully taken up by all the tumors, with variation in amounts detected in each tumor (Wolfgang, et. al. 2008).

Because this was a phase I study, research was taken to determine the safety of the jet injection and did not cover the efficacy of jet injection in reducing cancerous growth. The LacZ gene did not have any association to reduction of cancerous growth, but rather served as a marker to determine if jet injection was a viable method of gene transfer. Research on humans using jet-injection-based gene transfer as antitumor therapy is limited and quite recent. There have been studies done on mice, though, with encouraging results.

A study was conducted on mice containing human colon carcinoma to test the effectiveness of gene transfer jet injection in its ability to inhibit tumor growth. The mice were injected four times with a suicide gene, Escherichia coli cytosine deaminase, and then after forty-eight hours treated with 5-flurocytocine, an antifungal drug. Tumor volumes were monitored, and starting on day five, there was a significant decrease in the size of the tumors treated with the jet injected suicide gene in comparison to the control groups' tumors. Additionally, protein and mRNA levels revealed that the suicide gene was sufficiently expressed (Walther, et. al. 2005).

Although this study was not conducted on humans, it still has significant findings, chiefly that non-viral jet injection of suicide genes is an alternate method to injection via viral vectors, with comparable therapeutic response. Though there are studies, as mentioned above, of successful adenoviral vector gene transfers, the adenoviral vector does have limitations that the jet injection does not. When using the adenovirus as a vector, there is always the concern that it may have a pathogenic effect on the patient, or that the patient's immune system will respond to the viral proteins and inhibit the vector in completing its task. Jet injection looks promising for cancer treatment, but it is only useful for subcutaneous cancerous growths, like that of melanoma and breast cancer, since it cannot penetrate very deep.

Suicide Genes

Once the genetic material is successfully transferred into the host cancer cells and incorporated into the nuclear genetic DNA, there are a few methods by which it represses tumor growth. A key method being the injection of suicide genes, which are genes that cause apoptosis, or cellular death, when expressed. These genes are usually transcribed by various promoters found within the host cell. The H19 RNA gene is an example of one such promoter. The H19 gene locus was the first imprinted non-coding RNA identified. Recently, extensive study has been done on the role of the H19 gene and tumorigenesis. It is found that there is an abnormal expression of the H19 RNA gene in many cancerous cells, causing cancer cell proliferation, genetic instability, vascular angiogenesis, and tumor metastases. In a number of studies, blocking the H19 gene led to tumor regression and necrosis (Amer, et. al. 2014).

H19 Locus and tumorigenesis

The H19 RNA gene is greatly expressed in fetal organs but is rapidly turned off at birth. In tumor cells, the H19 gene becomes highly expressed or shows an abnormal expression pattern when compared to normal non-cancer cells. In cancer cells, the H19 gene expression can be activated by a combination of various transcriptional modulators and regulators that have malfunctioned. The interplay of the H19 gene locus and the modulators in tumorigenesis is highly complex, involving many regulatory factors that rely on many other regulatory factors (Matouk, et. al. 2013).

Hypoxia and H19

One such approach to the H19 gene's upregulation in cancerous cells is through hypoxia. Hypoxia is the loss of oxygen to areas of cancerous growth, and is considered a major trigger for metastasis, tumor angiogenesis, and chemotherapy resistance. Hypoxia is also considered to increase H19 expression in tumor cells. A study was done to investigate the relation between hypoxia and H19 upregulation in tumor cells. Carcinoma and Hepatocellular carcinoma cell lines were placed in an Aneropack rectangular jar and supplemented with Gaspak to create a hypoxic environment. Some cells where left with normal oxygen conditions as a control. The cells were then monitored by a hypoxic indicator. After twenty-four hours, the cells were examined and RNA from each cell was extracted and amplified through PCR. Viewed on the gel, the cells under anaerobic conditions expressed the H19 RNA significantly more than the cells under normal oxygen conditions.

In a similar study, mice were injected with Hep3B, cells containing hepatitis B, which caused the proliferation of Hepatocellular carcinoma on their dorsal side. A group of those mice were then injected with siRNA, a H19 gene knockout. The results showed that the mice that were treated with the siRNA showed

a significant retardation of tumor growth of 82%. Thus, from this study it is clear that H19 plays a large role in tumor growth, and is activated by hypoxia, which is common in cancerous growths (Matouk, et. al. 2007, Matouk, et. al. 2005).

c-Myc and H19

Another factor that induces H19 transcription is the c-Myc transcription factor. C-Myc is a transcription factor that, together with its obligate partner, protein Max, another transcription factor, binds to E-boxes, which are enhancer sequences on the DNA that initiate transcription. C-Myc then promotes gene transcription by initiating chromatin remodeling on the DNA or RNA polymerase clearance. To assess the role of c-Myc in tumor cells with increased H19 expression, a study was designed in which c-Myc was inserted into breast and glioblastoma cell lines. Cells inserted with c-Myc showed a seven-fold to ten-fold increase of H-19 expression based on Real-time PCR readings. Breast and lung cancer cell lines were also used to determine the correlation between elevated levels of c-Myc and H19 and tumor growth. The cell lines containing elevated levels of H19 and c-Myc were treated with siRNA to knock down H19 expression. The cells with knockdown H19 exhibited significant retardation of tumorigenesis. Thus, c-Myc was established as another factor that induces H19 upregulation and thereby increases tumor growth (Barsyte, et. al. 2006).

E2F1 and H19

Another basis for increased H19 expression in cancer cells is the E2F1 regulatory factor. E2F1 belongs to the E2F protein family that regulates DNA by binding to promoters. E2F1 binds to the H19 promoter and initiates its transcription. E2F regulation is based on the stages of the cell cycle. E2F1 is considered a key factor in the transition from G1 to S in the cell cycle, as it promotes the transcription of genes whose protein products are necessary for the progression of the cell cycle and for imitating DNA duplication. Thus, increased E2F1 expression when it is not the appropriate cell stage or time for cell replication can lead to cancerous growths.

A study conducted assayed the correlation between increased E2F1 and H19 gene expression in the S phase, as well as E2F1's impact on tumor growth. First, epithelial breast cells were transfected with a luciferase reporter gene, a selectable marker gene that, when expressed, causes the cells to emit a bioluminescence. Half of the cells were transfected with a luciferase reporter gene carrying the wild type H19 gene and the other half with a mutated promoter site of an H19 gene, so E2F1 binding is inhibited. The breast cells were then serum starved, and after twenty-four hours, some cells were placed in a fresh medium to stimulate their entry into cell cycle. After a set time, the cells were then compared using FACS analysis, a fluorescent strength intensity test, because the luciferase reporter that was used contains bioluminescence. Results showed that serum-starved cells had very little H19 expression, while cells in S phase had remarkably increased levels of H19. The cells transfected with the mutated promoter site had low H19 expression compared to the wild type cells that had overall increased H19 expression, especially at S phase (Fig. 1).

The correlation between H19 and E2F1 binding to its promoter is demonstrated when the comparison between the wild type and mutated transfected cells are noted. The cells with wild type H19 promoter sites showed a higher concentration of luciferase activities, since E2F1 was able to bind to the promoter site and activate transcription, while the cells with the mutated promoter sites exhibited a lower concentration of luciferase activities since E2F1 was not able to bind to the mutated promoter site. Additionally, H19's and E2F1's definite role in G1/S phase is observed by the fact that the greatest percentage of cells were recorded during the S phase of the cell cycle.

The second step of the study was to examine the roles of H19 and E2F1 in cancer proliferation. Breast cancer cell lines were obtained and the levels of E2F1 mRNa and H19 RNA were calculated. Normal breast cells were used as a control. The data showed that the expression of E2F1 and H19 were generally corresponding. In the healthy breast cells there were low levels of both E2F1 and H19 expression, while most of the cancer cells showed notable activation of E2F1 and H19 gene. In one line of cancer cells however, the E2F1 expression was high but the H19 expression was comparatively low. This discrepancy was attributed to heterogeneity of breast tumors. In general, there is a correlation between E2F1 and H19 upregulated gene expression in cancerous tissue (Berteaux, et. al. 2005).

Based on the studies discussed above, increased H19 expression is regulated by a number of regulatory factors, such as c-Myc and E2F1. Their upregulation is also triggered by environmental stress conditions such as hypoxia and S phase induced cells after serum starvation. However, cells under normal conditions do not demonstrate significant H19 expression (Ayesh, et. al. 2002). These findings reinforce the evidence that H19 is upregulated in many cancer cells, for hypoxia and serum starvation are considered normal stages in tumor growth. Thus, the tumor's growth causes its further proliferation. As it outgrows its blood supply, some portions of the tumor lack sufficient oxygen and reside in hypoxic microenvironments, which in turn triggers the increased expression of H19, further promoting cancerous growth.

Some of the explanations of H19 gene upregulation in tumor cells have been presented, and the therapeutic methods involving the H19 locus will now be discussed.

BC-819 Gene Therapy

In the past couple of years, BC-819, a plasmid involving a suicide gene and the H19 promoter, has been developed and has successfully improved treatment of a number of cancers. BC-19 is a double-stranded DNA vector that contains Diphtheria toxin A sequence, which isused to destroy the cancer cell, and an H19 promoter sequence. It is mixed with Polyethylenimine transfectant (PEI), which allows for easier entry of the plasmid into the rapidly dividing tumor cells. In some cases, PEI is not used and BC-819 is injected intratumorally or by hepatic artery infusion (Matouk, et. al. 2013).

Once BC-819 is in the cancer cell, the H19 promoter is activated and transcribed continuing with the Diphtheria toxin A sequence, which causes cell death by disrupting protein synthesis. BC-819 can actively select tumor cells to destroy, since only tumor cells have increased levels of H19 transcriptional factors. BC-819 will enter healthy cells as well, but since they lack the H19 regulatory factors, they will not transcribe the plasmid and the cells will not be destroyed. BC-819 is an ingenious development that acts as a 'search and destroy' unit by only killing cells that contain H19 transcriptional factors thereby triggering their own demise (BioCanCell, 2017). BC-819 has been semi-successful at treating bladder, pancreatic and ovarian cancer patient (Fig. 2)s.

Bladder Cancer

In the United States, bladder cancer is the fourth most common cancer in men, with an estimated 74,000 annual incidents. Around 70% of bladder cancer patients suffer recurrence within five years. A chief goal of battling bladder cancer is in preventing its recurrence. For decades, the standard care option was the BCG vaccine but the vaccine included drawbacks such as recurrence, resistance to the treatment, and negative side effects (Matouk, et. al. 2013).

A phase 2b study was conducted testing the efficacy of BC-819 treatment of bladder cancer. Patients who had confirmed recurrent bladder cancer and for whom BCG and chemotherapy had failed were recruited and BC-819 with PEI was administered to them. First, they were given six weekly treatments of BC-819, and at week nine, safety and efficiency of transfer were assessed. In cases of no toxicity or recurrence therapy was discontinued in patients and follow up maintenance therapy was given for the duration of the year. From the first cohort, nine out of eighteen patients had complete resolution of the target lesion within eight to ten weeks. Overall, 63% of patients had recurrence-free tumors for the first three months after treatment and 48% had tumors for a year after treatment. Additionally, the patients tolerated the treatment well with only mild to moderate adverse effects.

Reports of Phase III trials have not yet been published but trials are in progress as of the year 2016.

Pancreatic Cancer

The eighth leading cancer cause of death in the United States is pancreatic cancer, with a poor prognosis of five year survival. The standard treatment for pancreatic cancer is gemcitabine, a chemotherapy drug. However, gemcitabine has limited effect because of its poor intracellular metabolism. Other methods have been tried in combination with gemcitabine in the hope of a more effective treatment, but none proved worthwhile. Recently, BC-819 and gemcitabine were tested together on pancreatic patients in a phase 2b study that showed more promising results. Patients received four week treatments of gemcitabine

and were then administered with BC-819 through endoscopic ultrasound. Continued treatment with gemcitabine and BC-819 was done for as long as the cancerous growth did not progress. After three months, nine out of eleven patients showed encouraging results. Two had partial recovery and seven reached a point of stable disease. There were several adverse events mostly relating to liver function, but it was concluded that the adverse events were not related to the BC-819 , but were rather due to the advanced stage of the cancer that all the patients had (Matouk, et.a. 2013).

Ovarian Cancer

For women, ovarian cancer has a high mortality rate and is the fifth leading cause of cancer related death in the United States. Ovarian cancer patients generally have a poor prognosis, because the initial detection of the cancer is usually after it has reached an advanced stage. The typical course of treatment includes surgical removal of the tumor followed by chemotherapy. Unfortunately, most patients with the advanced stage tumor experience relapse after treatment. In the hopes of finding a better alternative treatment course, a phase ½ study testing the efficacy of BC-819 plasmid was conducted on fourteen ovarian cancer patients. All fourteen women had been pretreated with extensive chemotherapy. Different doses of BC-819 were administered to different groups of the patients. The first cohort of patients were treated for three weeks with BC-819, rested for a week, and were then treated for six more consecutive weeks. This was followed by a four week rest period. The second and third cohort were treated with increased dosages for three weeks, with four weeks of rest and then an attempt at repeat treatment when possible. Of the fourteen subjects, only 3 completed the study, while the rest withdrew prematurely due to overall clinical deterioration. There were fifteen reported severe adverse events, yet none were from the drug. There were, however, five adverse events that were possibly related to BC-819 administration. The best outcome of the treatment was a stable disease, with insufficient shrinkage or growth to qualify as either a partial response or progressive disease. The patients in the study all had advanced tumor growth, but the findings suggest that with less advanced stage ovarian cancer, BC-819 treatment would yield a partial response (Lavie, et. al. 2017).

BC-819 shows great promise for cancer patients. Although not every patient treated in the studies mentioned above had a positive outcome from the treatment, no one's medical state was worsened. The study of the BC-819 treatment is still in progress. The studies mentioned above are phase one or two studies, which means they are being done to determine the maximum tolerable dose of the drug, its safety, and efficiency. A phase three trial is generally the final test performed before the drug can be open to the public. A phase three trial is presently ongoing for bladder cancer, and a phase one/two has been completed for ovarian and pancreatic cancer.

Based on the various studies presented, gene therapy appears to be a viable option for cancer treatment. Although in each study there were some patients who did not fare well with this form of treatment, as an overall option, gene therapy looks like a promising alternative for cancer patients for whom standard treatment is insufficient.

Gene Therapy and Leukemia

However, there have been studies that have shown that in its attempt to rid the patient of his illness, gene therapy can actually promote one of the deadliest cancers, leukemia.

In 2002, a group of infants with severe combined immunodeficiency (SCID) were treated with gene therapy, but four out of the nine patients developed leukemia within the next five years. This alarmed many patients and researchers, and was a major setback in the advancement of gene therapy.

SCID is caused by a genetic mutation, making a patient with it lack the IL-2 receptor γ (IL2RG) gene. To restore the absent gene, the infants in the study were treated with a therapeutic gene via a retroviral vector. However, the retroviral vector works by inserting its genome near a transcription start site in the host genome, allowing the virus' long terminal repeats, which are repeated identical sequences of DNA that enable insertion into the host genome, to unintentionally turn on transcription of other nearby sites. In these infants with SCID, the LMO2 oncogene site was found near the insertion site and was turned on, promoting leukemic growth (Hacein-Bay-Abina, et.al. 2008).

The events of this gene therapy treatment were unfortunate, and did remove some of the enthusiasm for gene therapy at the time. However, the fact that this occurred on SCID patients and not on cancer patients must be taken note of, thus gene therapy might not be the right choice for SCID patient, but that does not rule out cancer patients. Cancer patients may not have an oncogene site near the insertion site for their therapeutic gene, which completely removes the possibility of inducing leukemia. In the research done, no reports have been made demonstrating that gene therapy for cancer patients further induced new cancerous growths.

Conclusion

Although gene therapy as a treatment option for cancer has had some setbacks and inconclusive results, it still provides a large source of hope for cancer patients. The paradigm of treating cancer is slowly shifting due to the ongoing progress of gene therapy. Based on the studies presented above, overall gene therapy, whether administered through a viral vector or a non-viral vector, was successful in treating a portion of the

patients. Additionally, even in the studies done in which small or no substantial recovery was obtained, there were no considerable adverse effects on the patients treated with gene therapy. This greatly contrasts standard treatments like chemotherapy that cause an array of adverse effects on the patient without necessarily providing complete removal of the cancer. Thus, even though gene therapy may not provide a complete cure against cancer, it is a promising alternative to standard cancer treatment. With the constant hard work and progress of medical researchers and physicians that is presently taking place, it is anticipative to say that gene therapy will provide great relief to many cancer patients in the coming years.

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Luteinizing Hormone and Alzheimer's Disease: Impact and Possibilities of Treatment

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Abstract

Alzheimer's disease is a common neurodegenerative disease that is the 6th leading cause of death in the United States. An estimated 5 million Americans are afflicted nationwide and the cost to the economy is valued at 259 billion dollars. Additionally, unlike other major causes of death in the United States, there is no treatment for Alzheimer's Disease. Alzheimer's is a progressive disease and it is strongly tied to aging. In most cases symptoms make their first appearance in the late 60's and gradually worsen, eventually leading to a loss of cognitive function and death. The two outstanding changes in the brain associated with Alzheimer's disease are neurofibrillary tangles and beta amyloid plaque. The presence of these is used to diagnose Alzheimer's disease after death. Certain hormonal changes that are associated with age, such as a rise in luteinizing hormone levels, are strong contenders for the age-related causes of Alzheimer's disease. Elevated gonadotropin levels have been shown in studies to correlate with amyloid beta accumulation in human and animal brains. The precise mechanism of action and the causation are not yet fully understood. Nevertheless, some studies have shown that lowering levels of Gonadotropin-releasing hormone (which releases luteinizing hormone) through the treatment with Leuprolide acetate, a gonadotropin releasing hormone agonist, have led to lowered risk of mortality by Alzheimer's disease in both mice and humans. This paper will discuss the association between elevated luteinizing hormone levels and Alzheimer's disease as well as the possibility of a Gonadotropin-releasing-hormone blocking based treatment for Alzheimer's.

Introduction

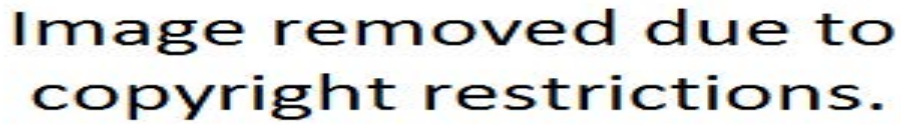
Alzheimer's disease is named after Dr. Alois Alzheimer who first discovered the disease in 1906. Dr. Alzheimer had a patient with severe memory loss, aphasia, and unnatural behavior who died of complications related to her disease. Upon her death, he performed an autopsy and noticed two unusual features in the brain, tangles of fibers and protuberances. We now know these as neurofibrillary tangles and beta amyloid plaques. It is difficult to accurately measure tangles and plaques during the lifetime of a patient with the disease; therefore, Alzheimer's disease is diagnosed based on symptoms. The symptoms that are used to diagnose Alzheimer's disease today are much like those of Dr. Alzheimer's original patient. The common symptoms are memory related issues, trouble with organizational skills, getting lost, confusion, and personality changes. Eventually the deterioration escalates and the patient may hallucinate, become unable to recognize others, and then become unable to talk or eat. Lastly, the parts of the brain that directly control living processes are affected and death occurs. (National Institute on Aging, 2012)

The progression of Alzheimer's disease does not happen quickly, in fact Alzheimer's is a very slowly progressing disease, and there is usually 5-10 years between the first appearance of symptoms and death. This being the case, it is theorized that even before symptoms occur, perhaps years prior, there is a form of "pre-Alzheimer's". This can be defined as asymptomatic changes in the brain which are the true first steps of Alzheimer's disease. The ability to detect these changes would greatly assist in the struggle to find a cure and is currently being researched. (National Institute on Aging, 2012)

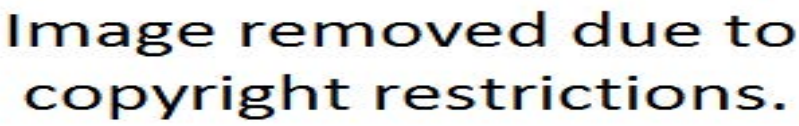
Unfortunately, it is not possible with our current level of technology to reliably detect the changes in the brain that are present in Alzheimer's, even advanced Alzheimer's. Although we have come a long way with neuroimaging methods, they are unable to determine Alzheimer's disease directly. Instead, after the onset of symptoms, brain imaging techniques such as MRIs and PET scans are used to rule out other probable causes of brain dysfunction; such as stroke or other forms of dementia. If these are not indicated, a diagnosis of probable Alzheimer's is given until death and autopsy. Upon autopsy, if there are neurofibrillary tangles and amyloid plaques, the diagnosis is then confirmed (National Institute on Aging, 2015).

Neurofibrillary tangles and beta amyloid plaque are the two main physical phenomena that define Alzheimer's disease, but the mechanism that they use to influence Alzhiemer's disease and cause the deterioration of the brain is not yet understood. Neurofibrillary tangles are also called tau tangles as they are composed and caused by defective tau proteins. Tau proteins are microtubule-associated proteins that are common in the central nervous system. In a healthy person, these tau proteins hold microtubules in place and keep them steady. However in Alzheimer's disease the tau proteins become hyperphosphorylated and they cease to support the microtubules. The microtubules unwind and collapse and the hyperphosphorylated tau builds up. Together they form filamentous clumps which are the neurofibrillary tangles of Alzheimer's disease. It is not certain if the tangles participate in the cause of Alzheimer's disease or are just an effect of it (C. Bancher, 1989). However, the density of the tau tangles correlates with the extent of dementia and they are located in the affected brain areas. A possible mechanism by which the brain degenerated by neurofibrillary tangles is as follows. When disrupted microtubules are abundant, the neuron is weakened and it becomes unable to transmit impulses at a normal rate. The body's immune system detects compromised neurons and triggers apoptosis of the cells (Wang, Xia, Grundke-Iqbal, & Iqbal, 2013).

The more significant Alzheimer's disease marker is beta amyloid plaque. In the healthy brain, tangles and plaques are present in small quantities and pose no problem. It is when the beta amyloid amounts build up that issues arise. It is understood that this build up is the result of a difference between the amount of beta amyloid plaque that is formed and the amount that is removed. Beta amyloid plaque is formed by the breakdown of



amyloid precursor protein (APP), which is present in the synapses of neurons. APP is present all over the brain and is thought to perform a function relating to neuron growth (Thinamaran & Koo, 2008). APP is broken down by several enzymes known as alpha, beta, and gamma secretase. When amyloid precursor protein is broken down by a combination of alpha and gamma secretase it forms a protein called αAPP which may have effects that protect the brain (Krishnaswamy, Verdile, Groth, Kanyenda, & Martins, 2009). However, when beta secretase takes the place of alpha secretase, it forms beta amyloid, which is sticky and insoluble allowing it to form plaques. The process of how beta amyloid plaque is removed from the brain is not fully understood but it could be performed in a variety of ways including protein transport through the blood or destruction by enzymes, such as insulin. Beta amyloid plaque is neurotoxic and it can inhibit synapse formation, disrupt mitochondrial action, contribute to inflammation in the brain, and promote the hyperpolarization of tau proteins which in turn causes neurofibrillary tangles. Beta amyloid plaques may be more important than neurofibrillary tangles and tau proteins in the onset of age related Alzheimer's. Other diseases have tangles but Alzheimer's disease is associated with plaque formation. (Lee, Goedert, & Trojanowski, 2001) Additionally, it is supposed that beta amyloid plaque forms earlier in the progression of Alzheimer's disease and contributes to the neurofibrillary tangles (Villemagne, et al., 2013). This theory, that beta amyloid plaque is the most significant factor in the



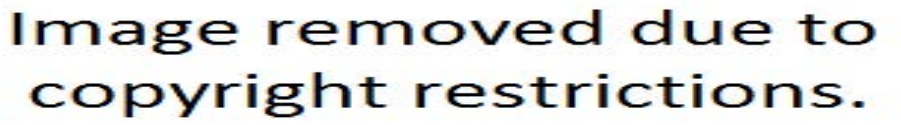
pathogenesis of Alzheimer's is called the "amyloid hypothesis" (Burnham & Thornton, 2015).

There are two forms of Alzheimer's and they have inherently different causes. Early onset Alzheimer's is when Alzheimer's disease occurs to people under 65. Most people with this form of the disease have a mutation in one of three genes, APP, PSEN 1, and PSEN 2. These mutations run in families and create a disposition to suffer from early onset Alzheimer's. (Bird, 2015) This is a genetic disease but it is far less common than late onset Alzheimer's disease and it accounts for about 5 percent of total Alzheimer's cases. In late onset Alzheimer's disease, there is also a genetic component connected with the APOE gene but the main risk factor is age. The older someone is, the more they are at risk of late onset Alzheimer's disease. The reasons why age should increase risk for Alzheimer's disease are not yet understood. Research is being done into the natural changes that occur in the body with age as possible contributors to Alzheimer's disease. The age-related changes to the hypothalamic/pituitary/gonadal feedback loop that regulates sex hormone levels in the blood is a strong candidate for this research.

The sex hormones are produced in the context of a feedback loop with Gonadotropin-releasing hormone in the hypothalamus and the gonadotropins, luteinizing hormone and follicle stimulating hormone which are released by the pituitary gland. In a healthy individual, Gonadotropin-releasing hormone is released from the hypothalamus in surges in response to internal and environmental factors. It travels to the anterior pituitary gland where it stimulates gonadotropic cells to produce luteinizing hormone and follicle stimulating hormone These hormones travel to the gonads, the testicles and the ovaries, and they stimulate them to release testosterone and estrogen respectively. To prevent too much of these hormones from being made, testosterone and estrogen in sufficient quantities provide negative feedback that discourages the further production of GnRH by the hypothalamus. However, with advancing age the process changes. Testosterone levels fall gradually in men, and in women estrogen drops rapidly after menopause. Without the negative feedback of the endpoint, gonadotropin levels tend to rise, although this is not true in all cases. (Jones, 2012)

For many years it was thought that the lowered levels of testosterone and estrogen that come with age were involved in the cause of Alzheimer's. It was assumed that these hormones had neuroprotective properties and their absence allowed Alzheimer's disease to creep in. However, it was found that giving testosterone or estrogen directly did not lower risk for Alzheimer's disease and in fact raised it. (Manly, et al., 2000) Since supplementing with testosterone/estrogen was not useful in preventing or understanding Alzheimer's disease, the next logical step was to look at the heightened levels of gonadotropins that relate to the lower sex hormone levels. However, even in this case distinctions must be made.

Luteinizing hormone and follicle stimulating hormone are both released by the anterior pituitary gland in response to Gonadotropin-releasing hormone. Structurally, LH and FSH are similar. They both are heterodimeric glycoproteins and are composed of an alpha and beta subunit. The alpha subunit of luteinizing hormone and follicle stimulating hormone are identical, however, the beta subunit is different and this is how they connect to different receptors and have a different scope of action. In men, luteinizing hormone is released throughout the day and stimulates Leydig cells to produce testosterone. Follicle stimulating hormone stimulates Sertoli cells which help sustain the



maturing sperm cells. In women, follicle stimulating hormone stimulates growth of the follicle in the ovary which increases production of estradiol and estrogen. When the follicle is mature, there is a critical level of estrogen that is reached and that stimulates a surge in luteinizing hormones, which in turn causes the release of the egg from the follicle. LH and FSH are different in both structure and function and how they interact with amyloid plaque as well. Many studies have shown links specifically between luteinizing hormone and the levels of beta amyloid plaque in the brain and this will be presented and evaluated.

If increased levels of luteinizing hormone contribute to Alzheimer's disease, it would be useful to have an explanation for how it does so. The presence of luteinizing hormone in the brain is not expected as it is not lipid soluble and in theory cannot cross the blood brain barrier. However luteinizing hormone is found in the brain and it has been found that LH can pass through this barrier in small amounts (Lukacs, Hiatt, Lei, & Rao, 1995). It is also a possible that luteinizing hormone is synthesized in the brain itself. LH receptors are found not only in the gonads but also in the brain which would imply that it may be active there as well. It may be that signals which are mediated by luteinizing hormone receptors affect the processing of APP and influence it so it is more likely to be cleaved by beta secretase than alpha secretase. This would result in a higher level of beta amyloid plaque formed relative to αAPP and could trigger a cascade of beta amyloid plaque buildup and ultimately Alzheimer's disease.

Possibility of Treatment

Since elevated levels of luteinizing hormone could be a factor in the pathogenesis of Alzheimer's disease, they are also a target for prevention or reversal of the disease. By lowering levels of luteinizing hormone in the blood, the contribution luteinizing hormone is making to the disease could be negated. A potential medicine that could be effective in this therapy is leuprolide acetate. Leuprolide, also called leuprorelin, is a synthetic hormone that is currently prescribed under the name Lupron to treat prostate cancer, sex hormone imbalance, and even less serious issues such as early onset of puberty (World Health Organization, 2015). Leuprolide acts as an agonist to the Gonadotropin-releasing hormones. It binds to the Gonadotropin-releasing hormone receptors in the pituitary gland and interrupts their stimulation. This results in a downregulation of luteinizing hormone and follicle stimulating hormone. Leuprolide is delivered by injection and it can be self-administered after training. It has mild short-term side effects including dizziness, itching, and headaches. As a long-term treatment, it can weaken bone density (Norsigian, 2005). However, with the research we currently have, the long-term side effects do not seem to be very significant, specifically when weighed against the pathology of Alzheimer's. The effectiveness of Leuprolide as a treatment to lower luteinizing hormone levels, improve cognition, and prevent or even reverse the accumulation of amyloid plaque will be discussed in this paper.

Methods

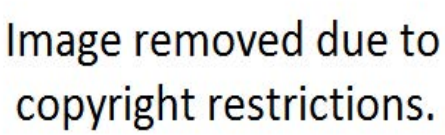
All the information that was used in this article was gathered from online using the Touro Library search and Google Scholar. The search terms that were used were leuprolide acetate, leuprorelin, Alzheimer's disease, beta amyloid plaque, luteinizing hormone, gonadotropins, hormone, gonadotropin releasing hormone. The articles were drawn from accepted and peer reviewed scientific journals and are all available online. Websites that were used were selected from dependable sources, government websites or scientific institutions.

Discussion: Alzheimer's Disease and Heightened Luteinizing Hormone Levels in Humans

In one study, plasma samples were taken from 284 patients seen at a tertiary care center and measured for concentrations of luteinizing hormone and follicle stimulating hormone. The patients were divided into three groups, 134 with probable Alzheimer's disease, 45 with frontal temporal dementia (FTD), and 105 cognitively normal controls. The researchers logged each patient's score on the Mini-Mental State Examination (MMSE) to measure severity of dementia. They recorded length of sickness, the sex and age of the subjects, and, in the case of women, if they were taking an estrogen supplement. It was found that there was no relationship between follicle stimulating hormone levels in men between the controls and the Alzheimer's disease group and although there was one with luteinizing hormone, it was not significant when controlled for age. The important finding was that in the women who were not taking estrogen, a significant difference in luteinizing hormone level was observed compared to the controls after a univariate analysis (Figure 4)

In women who were not taking any form of estrogen, this study found elevated levels of luteinizing hormone and follicle stimulating hormone compared to controls. The luteinizing hormone levels were higher in the Alzheimer's group compared to the frontotemporal dementia group which indicates that they were not just an effect of dementia or brain deterioration. There was no significant difference found for women who were taking estrogen but that is not unexpected, considering that estrogen is part of a negative feedback loop that limits luteinizing hormone production. Within the men, there was no difference found between the FTD group and the Alzheimer's disease group. It is possible that this is due to the small sample size. An important part of this study found that in estrogen free women, there was a connection between elevated levels of gonadotropins and Alzheimer's disease.

In a previous study by the same authors, a difference was



found for men as well. The study tested 40 males who were diagnosed with Alzheimer's disease in a long-term care center and compared them to 29 controls. The study found that the Alzheimer's disease patients were significantly higher in both luteinizing hormone and follicle stimulating hormone levels relative to the control group. (Bowen, Isley, & Atkinson, 2000).

In another study, 585 normal and healthy men between the ages of 70 and 87 years were tested for luteinizing levels to try to find a relationship between luteinizing hormone levels and issues with memory recall. Men were chosen specifically because in previous studies, this correlation had not been demonstrated for men as strongly as in women. The study found that higher levels of luteinizing hormone were related to worse performance on CVLT-II which is a test for immediate recall (Hyde, et al., 2010).

In an even larger study that evaluated the effects of elevated levels of gonadotropins on cognition in elderly women, 649 women without dementia were given cognitive testing and blood samples were taken. Plasma levels of luteinizing hormone, estradiol, follicle stimulating hormone, and beta amyloid were recorded. The study found that elevated levels of luteinizing hormone were associated with worse cognitive performance and depression (Rodrigues, et al., 2008).

In the Australian Imaging Biomarkers and Lifestyle study of aging, more than 1000 people were assembled to conduct a longitudinal study to aid understanding Alzheimer's beginnings and progression. The subjects were given full cognitive evaluation, their blood was analyzed and many of them had their brains imaged by MRI machines and various brain imaging technology. The participants were grouped by cognitive issues. The categories were healthy, subjective memory impairment, mildly impaired, and Alzheimer's patients. It was not the primary focus of the study to test specifically for gonadotropin levels. Nevertheless, the blood that was taken was tested for it amongst other things. Some researcher reviewed the data with an eye on luteinizing hormone and it was found that increased levels of luteinizing hormone in the blood were correlated with beta amyloid presence in the brain as measured by imaging techniques. This was only found in the subjective group, which suggests that the most important link between luteinizing hormone and beta amyloid plaque is in the preliminary stages of Alzheimer's disease (Ellis, et al., 2009).

Animal Trials

In studying disease pathology, and possible treatment options, it is not always possible to use humans as some potential treatments may have negative side effects, it is therefore better to first test their potential with animals. Mice are often used because of their biological similarities to humans. Some researchers have studied ties between luteinizing hormone, beta amyloid plaque, and cognition in transgenic mice that have genes that lead to Alzheimer's disease development. To test for cognition in mice, researchers use a test known as 'spontaneous alternation'. It is a behavioral test which tests spatial learning and memory. In this test, the animal is put in the center of a maze and can move freely. Spatial memory is tested by observing whether the mouse remembers which arms of the maze it has already explored. It should be noted that the test results can also be influenced by factors such as attention or sensory stimuli (Hughes, 2004).

In a study of 21, one month old transgenic Tg2576 mice, the effects of high luteinizing hormone levels in the mice on amyloid beta plaque deposition were tested. The animals were bred to over-express human APP to ensure that there would be beta amyloid plaque buildup. In this breed in general, beta amyloid plaque grows throughout the first 6 months of age and by the time they are 10 months old, the level of beta amyloid plaque is enough to form an accumulation. The mice were randomly split into two groups. One was a control group that received saline solution and the second group received leuprolide. The mice were tested with mazes to examine spontaneous alternation and their blood levels of luteinizing hormone were assayed to evaluate the effects of leuprolide. The brains were sliced on the sagittal plane and preserved to find the amount of beta amyloid deposition. The mice that were treated with leuprolide were found to have significantly lower levels of luteinizing hormone compared to the saline group especially 3 months after the treatment. The leuprolide-treated mice performed better on the maze task than the control group. Additionally, the leuprolide-treated mice had lower levels of beta amyloid plaque and this correlated with the improved cognition (Casadesus, et al., 2006).

Mice which were bred to have an elevated level of amyloid beta precursor proteins had higher levels of luteinizing hormone and faster cognitive decline. The group that was treated with leuprolide, to help lower luteinizing hormone levels, had

less beta amyloid plaque deposition and better cognitive performance than the controls.

In another study done with mice, Tg2576 mice that were bred to express the human amyloid precursor gene were crossed with mice that had the genes for luteinizing hormone receptors removed, so that they couldn't use luteinizing hormone. There were two control groups. One of the control groups were APP expressing mice that were not crossbred. A second control group was mice that had luteinizing hormone receptors and did not express the human APP gene. Finally, a group of normal mice was a third control. The animals were raised for 16 months, sacrificed, and then their brains were sectioned. An imaging software was used to compare the beta amyloid concentrations in the brains of each group. The most important finding in this study was that there was significantly less beta amyloid plaque in the mice which had luteinizing hormone receptor knockout compared to the mice with APP that did have luteinizing hormone receptors. (Lukacs, Hiatt, Lei, & Rao, 1995)

The studies mentioned above all find a correlation between elevated luteinizing hormone levels and Alzheimer's disease or beta amyloid plaque deposition. If luteinizing hormone is a part of the cause of Alzheimer's, it presents exciting treatment possibilities through the lowering of luteinizing hormone levels especially to prevent deterioration in those at risk for Alzheimer's. A promising candidate for a medication that can accomplish this safely is leuprolide.

Leuprolide as a Treatment for Alzheimer's Disease

Leuprolide interferes with GnRH in humans and therefore brings about lower levels of luteinizing hormone. It is not an approved treatment for Alzheimer's disease as of now. However, since leuprolide is an approved treatment for prostate cancer, some studies have observed the effect that leuprolide has had on Alzheimer's disease in the patients using it for other reasons. Clinical trials are currently underway to test this possibility under the code ALADDIN which is an abbreviation for Antigonadotropin-Leuprolide in Alzheimer's Disease Drug Investigation

One study of the impact of leuprolide on Alzheimer's disease in patients with prostate cancer was done in 2010. The study looked at the risk of death by Alzheimer's disease in 6,647 men who were treated for prostate cancer. In the study, 1700 men who were treated with leuprolide were compared to 4,947 controls who were treated using other drugs that do not limit luteinizing hormone production. After 4 years of follow up, 81 of the group in the study had died from Alzheimer's disease. Those who died of Alzheimer's were found to be from the control group rather than from the group of patients who took leuprolide for at least 4 months (D'Amico, Braccioforte, Moran, & Chen, 2010).

The results were that Leuprolide can aid in slower regression and improved cognition in Alzheimer's patients. Voyager Pharmaceuticals is not currently pursuing further research into Lupron and most likely will not do so in the future as it declared bankruptcy in 2012. In an interview for medicalresearch.com, Richard Bowen cited lack of marketability to account for the dearth in leuprolide research. "Unfortunately, there is no intellectual property protection for this treatment making it unlikely that a pharmaceutical company will take the lead." (R. Bowen). It is vitally important to find a cure for Alzheimer's and perhaps the funding will come from a government the government or foundation, and leuprolide research will be continued.

Conclusion

Alzheimer's disease is a common debilitating disease that is one of the main leading causes of death in the United States. One of the main changes detected in the brain of a patient with Alzheimer's disease is beta amyloid plaque. These plaques can only be detected after death, upon autopsy. Studies have shown that a rise in luteinizing hormone is linked to increased beta amyloid plaque and Alzheimer's. Studies have been done to lower Gonadotropin-releasing hormone levels through the treatment of Leuprolide acetate, a Gonadotropin-releasing hormone agonist. This treatment is not being financially backed and is therefore on hold for the time being; however, the possibility of this treatment for Alzheimer's disease is somewhat promising and deserving of more research.

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