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Cover Picture: The cover picture was created by Professor Antony O'Hara of the Digital Multimedia Design Department, pertains to the article Xenotransplants: The Risks and Benefits by Sima Langner.

Xenotransplants: The Risks and Benefits

Sima Langner

Sima Langner graduated with a Bachelor of Science degree in Biology in May 2022.

Abstract

This literature review discusses how xenotransplants from animals, especially pigs, can serve as viable alternatives to allotransplants. There are many ways of ensuring the health of the host, including testing the donor pig for cytomegalovirus, Epstein Barr virus, simian agent 8, and other viruses linked to transmitted infections. The Galα-1,3-Gal epitope and N-glycolylneuraminic acid are both recognized by human antibodies and induce rejection. To remedy this, double knockout pigs have been developed that lack both. The human complement system serves as another potential pitfall for xenotransplants that can be neutralized by introducing human complement regulatory proteins into transgenic animals pre-transplant. Another potential risk of pig xenotransplants are porcine endogenous retroviruses. These retroviruses can potentially adapt to infect human tissue and further infect those that come in contact with the host. Extensive research has been done on porcine endogenous retroviruses to determine the extent of this possibility. In separate studies, xenografts have displayed great medical advantages for patients diagnosed with type I diabetes and Parkinson's disease.

Introduction

Today, allotransplants, specifically organ, cell, or tissue donations between a genetically non-identical donor and recipient of the same species, have a better chance than ever of being successful. With the rapid improvements in the medical field, patients who receive allotransplants see health improvements and life expectancy increases. However, with allotransplants, there are organ shortages, an issue that cannot be remedied by science. Hundreds of patients languish on waiting lists with failing organs in desperate need of an organ transplant to stay alive, yet the only resource for most allotransplants are deceased donors. The number of those in need of an organ vastly outweighs the number of available organs. Xenotransplants, transplanting organs or tissues between two different species, can be an alternative to allotransplants with the major benefit of having an endless supply of organs available (Cooper et al., 2018). Another major benefit of xenotransplantation is that it allows for modification of the donated organs by way of genetic engineering. This can reduce the number of treatments required by recipients to prevent organ rejection after matching them to the available organs.

The best animals to breed for human organ donations are pigs. The downside of using non-human organs is the risk of infections and issues that a xenograft can bring to the host (Fishman & Patience, 2004). This paper will discuss the steps and procedures that can be performed during the process of xenotransplantation to better match the host and determine if the benefits of using animal organs in humans outweigh the risks.

Methods

The Touro College Online Library Database and Google Scholar were used to access original papers that have studied and discussed different aspects of xenotransplantation. The sources were reviewed to understand the risks and benefits of xenotransplants and to determine if xenotransplants can be a reliable alternative to allotransplants.

Screening Donors for Xenotransplantation

A common concern of allotransplantation is the transmission of infections from the donor organ to the recipient. This concern applies to xenotransplantation as well. Xenozoonosis is the transmission of animal infections to a human host. A study was done using thirty-one adult male baboons, all from the same colony. These baboons were evaluated for antibodies for microbial agents that could potentially cause an infection. This study was undertaken to determine the number of baboons in the population that have specific microbial agents that would pose a risk to the host post-xenotransplantation. From each of the thirty-one baboons, paired serum samples were taken and sent to two different laboratories for herpesvirus and retrovirus testing. After carful testing of the blood sera, 30 out of the 31 animals were positive for cytomegalovirus (CMV), Epstein-Barr virus (EBV), and simian agent 8 (SA8). There were no antibodies detected for simian immunodeficiency virus (SIV), but eight animals had antibodies for simian T lymphotropic virus type 1 (STLV-1), and one of the animals was positive for foamy virus. Ten animals had antibodies to T gondii, one had antibodies for hepatitis B, and three for hepatitis A. None of the animals were positive for lymphocytic choriomeningitis virus, simian hemorrhagic fever virus, Marburg virus or monkey pox virus (Michaels et al., 1994).

The target viruses were chosen for one of two reasons, either they had been linked to donor transmitted infections after allotransplantations, the concern that some endemic primate viruses are possibly lethal to humans, and the accessibility of techniques to differentiate species specific viruses so that the transmission of infections from primates to humans can be documented. Herpesviruses such as CMV and EBV are viruses that are known to transmit infections from the donor and infect the host after allotransplantations. Antibodies to herpesviruses were found in the entire population of adult male baboons (Michaels et al., 1994). Retroviruses can also be transmitted by organ transplantation. STLV-1, equivalent to human T lymphotropic virus type 1 (HTLV-1) is found

in 40% of the studied baboon population. Foamy virus is unusual in humans but very common in nonhuman primate species (Simonds et al., 1992). The presence of foamy virus in a recipient can be used to document transmission of a virus between species through xenotransplantation. T gondii has been transmitted after solid organ allotransplantations and would likely pose the same risk with xenotransplantation. To decrease the risk of xenotransplant transmitted infections the donor animal will need to be tested for these viruses and picked appropriately (Michaels et al., 1994).

The probability of infections being transmitted via organ donation is unclear. For an infection to be transmitted, it must be pathogenic for both the donor and recipient species, as are many bacteria and parasites. There are species-specific animal pathogens that closely resemble their human counterparts and can infect human cells. It is hypothetically possible that host cells, which are naturally resistant, may become susceptible to animal pathogens as a result of the immunosuppression induced in the host to favor the transplantation. There is also the possibility that some of the animal pathogens will maintain species specificity but will undergo local reactivation in the host, causing disease or the transplant to fail (Michaels et al., 1994).

Hepatitis B virus is believed to be species specific and unable to infect baboons. To evaluate the susceptibility of hepatitis B virus in baboons, four baboons were inoculated with hepatitis B virus. Of the four, only two baboons were treated with immunosuppressive therapy. All the animals remained healthy for the duration of the six-month study. Weekly PCR for hepatitis B virus as well as biopsies of the liver all came back negative. This experiment cemented the idea that baboons are not susceptible to hepatitis B virus. A further study was done using two human patients diagnosed with hepatitis B end stage liver disease who underwent a baboon liver xenotransplant. One of the transplant patients survived seventy days, the other twenty-five. Liver biopsies and autopsy material of the transplanted livers showed no evidence of hepatitis B virus and staining for hepatitis B antigens were negative. This further supports the evidence that baboons are resistant to hepatitis B virus (Michaels et al., 1994).

Causes of Rejection and Lowering the Risk

The biggest obstacle to any successful organ or tissue transplant, especially xenotransplants, is organ rejection. Antibody-mediated rejection can prevent xenotransplants from being successful. The surface of pig cells has a Galα-1,3-Gal epitope that is recognized by human antibodies and causes hyperacute rejection. Pigs without the gal epitope, called α1,3Galactosyltransferase knockout (GGTA1-KO) pigs, have been developed with the help somatic cell nuclear transfers and cloning (Arn et al., 2004). However, the Galα-1,3-Gal epitope is not the only factor causing rejection. When kidneys from GGTA1-knockout pigs were transplanted into immunosuppressed baboons, most of the baboons still died from severe acute humoral xenograft rejection. During the rejection phase, induced antibodies to non-Gal antigens were significantly elevated. This can indicate that there are antibodies to non-Gal antigens that develop and further complicate xenotransplants. Many experiments have studied the Gal specific antibodies and discovered that these antibodies cause hyperacute rejection after a few hours and start an acute humoral xenograft rejection that begins anywhere from a few days to a few weeks post transplantation (Chen et al., 2005).

In a controlled experiment using α1,3Galactosyltransferase knockout pigs as donors, six kidneys were transplanted into baboons. Endothelial cells and lymphocytes were both extracted from the GGTA1- knockout donor pigs and tested to be sure that they were indeed Gal-negative. Half of the baboons were treated with a multiagent regimen that combined thymoglobulin (ATG) with different steroids daily, while the other half were given one single dose of thymoglobulin and underwent monotherapy with tacrolimus, an immunosuppressant usually used along with other medications to prevent transplant rejection. Pre-transplantation all the recipient baboons had low levels of non-Gal-specific immunoglobulin G and M (IgG and IgM). Sera was collected from the recipients pre-transplantation and when compared to the GGTA1 knockout pig donors' lymphocytes, there were similar levels of complement-dependent cytotoxicity. None of the GGTA1 knockout pig kidneys in this experiment went through hyperacute rejection as they all survived a minimum of eight days. The baboon that survived the longest was among the three that were treated with the daily multiagent regimen and lived for 16 days post-transplant before developing renal failure. This baboon was one of four to develop renal failure resulting from critical acute humoral xenograft rejection. During the rejection there was increased serum creatinine, decreased urine production, and an increase of non-Gal-specific antibodies. The four baboons to develop acute humoral xenograft rejection had increased non-Gal IgG antibodies by 7 to 26-fold and the sera collected showed strong complement-dependent cytotoxicity. This experiment demonstrated that using α1,3Galactosyltransferase knockout pigs will eliminate the risk of hyper-acute rejection in immunosuppressed baboons but acute humoral xenograft rejection is still an inevitable outcome due to induced antibodies to non-Gal antigens (Chen et al., 2005).

N-glycolylneuraminic acid (Neu5Ge) has been identified as another xenotransplant interfering antigen. Neu5Ge is not synthesized in humans but is present in pigs and other mammals. The enzyme responsible for Neu5Ge is cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), and there is a genetic mutation in humans that causes the absence of this enzyme and the creation of Neu5Ge. If both GGTA1 and Neu5Ge can be removed from the animal donor, xenoantigenicity of animal organs in humans would be greatly reduced. Zinc-finger nucleases is a new development in the production of genetic knockout pigs and has made the process of creating a double knockout pig more efficient. Using zinc-finger nucleases it is possible to create a porcine lacking both GGTA1 and Neu5Ge (Lutz et al., 2013).

To create the modified potential xenotransplant donors, porcine adult liver-derived cells were isolated and used as the starting material for the genetic modifications. Zinc-finger nucleases encoded with DNA were directed to porcine cytidine monophosphate-N-acetylneuraminic acid hydroxylase and transferred into the liver-derived cells. Then, the liver-derived cells lacking CMAH were treated with zinc-finger nucleases directed to porcine α1,3Galactosyltransferas. Somatic cell nuclear transfer of the CMAH and GGTA1 deficient cells led to a successful gestation (Lutz et al., 2013). Somatic cell nuclear transfer is a technique that transfers the nucleus of a somatic cell into an oocyte that had its chromosomes removed. The somatic nucleus controls the development of the embryo (Wilmut et al., 2015). Five fetuses were harvested and underwent a DNA sequence analysis to verify the homozygous disruption of the GGTA1 and CMAH genes. Both genes were disrupted successfully. The CMAH gene was turned off by four base-pair insertions that caused a frameshift mutation. GGTA1 was modified with a combination of a point mutation and three base-pair deletions. Flow cytometry of the double-knockout fetuses red blood cells and adult human red blood cells confirmed that the absence of functioning GGTA1 and CMAH genes eradicated the Gal and Neu5Ge antigens. Fibroblasts were cultivated from one of the fetuses and somatic cell nuclear transfer was used for a second pregnancy. This pregnancy yielded another four double-knockout piglets. The same flow cytometry tests were repeated to confirm that the second generation of pigs displayed the same modifications (Lutz et al., 2013).

Once a viable pig lacking GGTA1 and CMAH was produced, peripheral blood mononuclear cells were extracted to analyze the levels of remaining antigens. Sera was extracted from 10 arbitrary healthy human volunteers and processed in a flow cytometric antibody-binding analysis. The human sera had less IgM and IgG bound to the double-knockout cells than the cells from the single GGTA1 knockout pigs. The use of zinc-finger nucleases to create the double knockout pigs showed that it is possible to greatly lower the antigenicity of pig cells by removing both GGTA1 and CMAH. Using Zinc-finger nucleases, double gene homozygous knockout pig was created in only seven months. The discovery that zinc-finger nucleases can knock out two genes simultaneously in vitro will greatly accelerate the research and experimentation of preparing pigs for xenotransplants. Researchers should be able to further improve the pig as a donor for xenotransplants (Lutz et al., 2013).

A specific part of the immune system that is relevant to organ transplants and organ rejection is the human complement system. The complement system is behind self-non-self-recognition and destruction. The activation of the complement system leads to the creation of the C3/C5 convertases that go on to cleave C5. By cleaving C5 the convertases initiate the formation of membrane attack complexes (MAC). Once there are membrane attack complexes, C5b6 is formed, along with semi stable C56-7, and C5b-8, a C9 convertase. Host cells will express membrane bound proteins known as regulators of complement activation (RCA) to protect themselves from the products of membrane attack complexes. Two regulators of complement activation are decay accelerating factor (DAF) and membrane cofactor proteins (MCP). Decay accelerating factor will accelerate decay dissociation of C3 and C5 convertases, and membrane cofactor proteins will bind to and cleave the complement activation products. Two other proteins, 65-kDa homologous restriction factor and CD59 (20-kDa), control the terminal complement pathway on host cell membranes. CD59 is a glycoprotein found on the cell's surface that prevents C5b-8 from creating C9 (Zhou et al., 2019).

An essential feature of membrane bound complementary regulatory proteins, especially regarding xenotransplants, is their species selective inhibitory activity. An experiment was conducted to understand the defending role of human CD59 and membrane cofactor proteins when in the presence of non-human cells. Full copies of CD59 and membrane cofactor protein CDNAs were obtained from human embryos and transfected into NIH/3T3 (mouse fibroblast cell line) cells to determine whether they would properly work on heterogenous cells and maintain their ability to prevent human complement. The transfected NIH/3T3 cells were incubated with human, guinea pig and rabbit serum. The cells were destroyed by the guinea pig and rabbit complement but were not affected by human complement. This meant that

the CD59 and MCP that were transfected retained their species specificity. According to these results, hyperacute rejection can be regulated by introducing human complement regulatory protein in transgenic animals. This experiment proved that complement regulatory proteins are species specific and the animal equivalent of human complement regulatory protein will not operate effectively against human complement (Huang et al., 2001).

The cDNA of CD59 and membrane cofactor protein were also transfected into endothelial cells from pigs that contained the human decay accelerating factor to study a presumed synergistic action. The endothelial cells that expressed both DAF and MCP proteins survived at a greater rate than those that only expressed one protein (Huang et al., 2001). DAF, MCP, and CD59 all function differently at precise stages of complement activation. DAF inhibits the assembly of C3 and C5 convertases and hastens the decay of C3 convertases. MCP participates in the cleavage of C3b and C4b, and CD59 rivals C9, hindering the formation of membrane attack complexes (Zhou et al., 2019). The majority of human cells express two or more of these regulators, which have the ability to act additively or synergistically. In this experiment the endothelial cells that expressed two proteins had a greater chance of surviving a complement cytolysis. According to these results, the expression of human membrane cofactor protein, decay accelerating factor, or CD59 on a transplanted organ should protect the xenograft from the human host complement system and provide a solution to complement mediated hyperacute transplant rejection (Huang et al., 2001). If the xenogeneic organ would be introduced to human regulators of complement activation, the transplanted organ should effectively be protected from lysis and complete organ rejection (Seya et al., 1999).

Porcine Endogenous Retroviruses

Another point of concern with xenotransplantation is the risk of porcine endogenous retroviruses (PERVs). Multiple copies of porcine endogenous retroviruses are included in all pig genomes. There are two subgroups of porcine endogenous retroviruses, named PERV-A and PERV-B. These viruses can infect human cells in vitro and each has a unique cellular receptor that differs from other porcine endogenous retroviruses. PERV-A is more likely to put humans at risk, as all human-tropic PERV isolates that have been taken from primary pig cells have been PERV-A. In addition, PERV-A is present at a much higher rate on porcine DNA than PERV-B. The viruses that closely resemble PERV are linked to hematopoietic cell malignancies (Patience et al., 1997). If porcine endogenous retroviruses were to be transmitted, they would pose a great risk to the recipient. If the initial infection was to occur under levels of high immunosuppression, it could present the virus with time to adapt to infection of human tissue. Understanding the biology of porcine endogenous retroviruses and identifying the molecules used as receptors to infect the human cells is crucial to preventing PERV from infecting human xenotransplant recipients (Ericsson et al., 2003).

After complex experimentation, two functional human receptors for porcine endogenous retrovirus A were identified. The receptors for PERV-A are known as multiple independent multitransmembrane receptors and have been associated with other gammaretroviruses including baboon endogenous virus, certain strains of murine leukemia virus, and feline leukemia virus. Although the receptor for porcine endogenous retroviruses has been identified, without knowing the physiological role of PERV-A its pathogenic consequences cannot be predicted. To study the pathogenic consequences of viral infections of humans, appropriate animals can be used as models. Baboons have been found to express a functional receptor for porcine endogenous retrovirus A, although expressing a receptor does not directly correlate to cells tolerating the virus. Animal models of porcine endogenous retrovirus infection will be useful in researching different factors including potential immunosuppressants that can affect susceptibility to infection in vivo and the likely pathogenic effects of the infection (Ericsson et al., 2003). Even more important, with the use of animal models, the risk of porcine endogenous retroviruses being transmitted between animals can be explored in a controlled environment. This experimentation between animals will also advance the understanding as to whether xenotransplant recipients can further transmit porcine endogenous retroviruses to those they are in contact with and the population at large (van der Laan, et al., 2000).

Studies have been conducted to perceive how big of a risk PERV presents to xenotransplant receptors. As porcine aortic endothelial cells are the main cells encountering the host's leucocytes and tissues after xenotransplantation, an experiment was conducted to determine whether these cells can release PERV particles, and if xenografts from different breeds of pigs differ in their risk of transplanting endogenous retroviruses. A variety of porcine cells were acquired, and RNA was prepared from primary endothelial cells, lung, hepatocytes, and skin cells from minipigs, Yucatan micropigs, and German land breed pigs. PERV was detected in all the samples. PK15 and human endothelial cells served as control and experimental groups. PERV was found to infect the human cells but the transfer of infectious endogenous virus from

porcine to human cells lines in vitro is not equivalent to the situation in vivo. To ascertain whether PERV will infect human cells in vivo, further experiments have been conducted with human subjects (Martin et al., 1998).

In a study of human patients to determine if porcine xenografts will lead to PERV infection, ten diabetic patients who received porcine fetal islets from non-transgenic Swedish cross bred pigs were monitored. PCR and reverse transcriptase assays tested for any evidence of PERV infection. All patients' serum samples that were collected between 3 days and 7 years post xenotransplantation were negative for PERV RNA. All patients had increased titers of anti-porcine antibodies within week of the xenotransplantation. Five patients displayed rapid clearance of porcine cells which may be responsible for the failure of PERV to establish infection; however, four patients showed excretion of porcine C-peptides for up to 450 days post transplantation. There were also findings of porcine mitochondrial sequences in these patients' serum for six months to one year after their transplantation. These results show that in some patients there was extended xenograft survival along with the negative porcine endogenous retrovirus results. Although this experiment did not have any positive PERV results, it did not rule out the possibility of a PERV infection resulting from a xenotransplant. At the same time the results are encouraging to the future of xenotransplantation. The risk of a xenotransplant recipient developing a PERV infection is likely due to many factors including the source animal, xenotransplant technique, and characteristics of the recipient. While this study bodes well for future xenotransplant recipients, it does not provide a definite method of preventing a PERV infection in transplant recipients (Heneine et al., 1998).

Xenotransplants and Diseases

Type I diabetics experience difficulties achieving the tight metabolic control they need to maintain good health. Less than ideal carbohydrate metabolism also hastens the onset of diabetic complications. To restore glycemic control in those with type I diabetes, whole organ pancreas transplantations have consistently been used to obtain normoglycemia. A more recently developed method of maintaining carbohydrate control is the allotransplantation of isolated islets of Langerhans along with immunosuppressants (Shapiro et al., 2000). Due to the shortages of available allotransplants, the xenotransplantation of porcine islets has been considered as a solution. An experiment involving 12 type I adolescentpatients who had been diabetic for a range of four to ten years were chosen to be the transplant acceptors. If the patient achieved total insulin independence after receiving the first transplant, they received no further treatment. If there was a minimal response to the first transplant, a second one was completed six months after the first. Four patients were recipients of a third transplantation three years after the original procedure. The animals used for this experiment were male seven-to-ten-day old piglets. The piglets were bred in a pathogen free environment and sera from the piglets was analyzed for pathogens (Valdés-González et al., 2005).

Half of the patients who received the porcine islets significantly lowered their need for exogenous insulin (injected insulin). Two patients even attained transient insulin independence. The control group of 11 age matched patients who did not receive a transplant saw zero reduction for the need of exogenous insulin. The experimental group, who achieved reduced exogenous insulin, had stable glycosylated hemoglobin and blood glucose levels throughout the entire study. When the sera of the patients were tested, glucose stimulated immunoreactive porcine insulin was detected in three patients, and of the biopsies done, four patients had insulin producing cells. The patients were monitored for four years post-transplant and were seen to have insulin and glucagon secreting cells three to four years after the first transplant. This experiment showed that islet xenotransplantation can serve as a reliable treatment to help those with type I diabetes. None of the patients displayed endocrinological or immunological complications and all were able to reduce the amount of required insulin to varying degrees (Valdés-González et al., 2005).

Cellular therapy is used to replace neurons as a way of treating diseases of the adult central nervous system. Fetal neurons have been proven to be prime cells for cellular therapy. They can survive and function properly when transplanted into the adult brain. Cellular therapy with fetal neurons is used on patients suffering from Parkinson's disease and Huntington's disease (Fink et al., 2000). Patients with advanced Parkinson's disease who have received transplanted fetal human dopaminergic neurons reacted positively to the transplantation and have shown significant improvement in their motor symptoms, such as improved gait as well as reduced dyskinesia (Freeman et al., 1995). In one study, a patient with advanced Parkinson's disease had improved motor function after receiving bilateral grafts of fetal ventral mesencephalic tissue (Kordower et al., 1995). When using animal models of Huntington's disease to study transplanted embryonic cells the cells were seen to have integrated into the basal ganglia circuitry and aided in repairing partial motor and cognitive deficiencies (Kendall et al., 1998).

Human fetal neurons have displayed impressive results when transplanted into patients with Parkinson's disease and have the potential to improve symptoms of Huntington's disease. The difficulty of using human fetal neurons is the extremely limited available supply, as well as the ethical concerns that they raise. Pigs are a practical alternative to fetal neurons due to their large brain size. Experimental trials were done using human patients and fetal porcine ventral mesencephalon and striatal tissue. The subjects were 12 patients with confirmed Parkinson's disease and eleven patients with Huntington's disease. The Parkinson's disease patients received fetal mesencephalic cell suspensions that were prepared by dissection of the ventral mesencephalic of porcine fetuses on embryonic days 25 to 28. For the transplants intended for the Huntington's disease patients, the lateral ganglionic eminence region of the striatal anlage, the origin of many neuronal elements of the mature striatum, was dissected from the porcine fetuses on embryonic days 35 to 38. All the patients were discharged from the hospital within 72 hours of the procedure. No edema or mass effect was seen on MRIs performed post operation, six months after surgery, and 12 months after surgery. The patients were tested for the presence of porcine endogenous retrovirus nucleic acid sequences, and none were detected (Fink et al., 2000).

One year after the implantation, the total Unified Parkinson's Disease Rating Scale for the Parkinson's disease patients improved by an average of 19% in the off state. Improvements were demonstrated by 3 months post-surgery, and three patients improved over 30%. The Huntington's disease patients did not show such positive results. The entire group's mean total functional capacity score did not change over the year post surgery and the mean motor sub score of the Unified Huntington's Disease Rating Scale worsened by 36%. The results of this study displayed strong positive results for the use of fetal porcine ventral mesencephalon and striatal tissue in place of the equivalent human fetal tissue in Parkinson's disease patients. Of 12 patients suffering from Parkinson's disease, all showed varying degrees of improvement, and none had any detrimental results from the porcine tissue in the year of observation. While the results for the Parkinson's disease patients were supportive of their transplants, the results for the Huntington's disease patients were not. The motor skills of these patients worsened, indicating progression of the disease. It is difficult to say exactly how the transplanted lateral ganglionic eminence effected the Huntington's disease patients when the progression of the disease is also considered. It is possible the fetal porcine cells slowed down the advancing symptoms, but further testing must be done with larger groups of patients to determine whether or not this is true. This experiment demonstrated that porcine fetal neurons are well tolerated in a human host and can be a viable cell source for healthy neural tissue in place of human fetal neural cells (Fink et al., 2000).

Conclusion

According to the current research, the benefits of xenotransplants outweigh the risks. The health of the host can be protected by testing the donor pig for cytomegalovirus, Epstein Barr virus, simian agent 8, and other viruses linked to transmitted infections. To avoid rejection induced by Galα-1,3-Gal epitope and N-glycolylneuraminic acid, double knockout pigs have been successfully developed. To stop the human complement system from destroying the transplanted organ, human complement regulatory proteins can be introduced to transgenic animals pre-transplant. Porcine endogenous retroviruses are another risk that comes with using xenotransplants from these pigs. These retroviruses can potentially adapt to infect human tissue and travel to others in the host's environment. More experiments must be done regarding porcine endogenous retroviruses to fully understand the risk they pose the host and others. When xenografts have been used in human patients, they have shown highly effective results. In different experiments, patients diagnosed with type 1 diabetes and Parkinson's disease received xenotransplants and responded well to the procedure. The research on xenotransplants has evolved considerably and continues to grow. The benefits xenotransplants can bring to the world of medicine are too great to disregard because of their risk potential.

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Gene Therapy for Cancer Treatment

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Abstract

Cancer is a genetic disease in which cells grow uncontrollably and can spread throughout the body. Cancer cells have distinguishing traits which facilitate their unlimited growth; these traits can be used in the development of gene therapy cancer treatment, which includes: CRISPR, a gene-editing tool that can precisely modify human DNA; Kymriah, which induces T cells to kill cancer cells; Gendicine and zinc metallochaperones, which utilize p53, a protein vital for the destruction of cancer cells. Scientists continue to improve gene therapy treatments, making them available to more patients and decreasing the toxicity of treatments.

Introduction

Cancer is a genetic disease in which cells grow uncontrollably and can spread throughout the body. Human cells grow and multiply through a process called cell division, in which new cells take the place of old cells, and, normally, the body rids itself of cells that have damaged DNA. When genes, which control cell division, are damaged, abnormal or damaged cells may develop. Errors that occur as cells divide, damage to DNA caused by toxic substances such as tobacco smoke and UV rays, and inherited mutations are genetic changes that can cause cancer. Damaged cells can form lumps of tissue and tumors, which can be benign or cancerous.

Cancer cells have distinguishing traits which facilitate their unlimited growth. Malignant tumors invade healthy tissue and can spread to other areas of the body, metastasize and form additional tumors; metastatic cancer is classified according to its origin. Cancer cells use a variety of tactics that differentiate them from normal cells and allow them to take control; they ignore signals telling them to stop growing or die by apoptosis; they communicate with blood vessels to grow toward tumors to help them survive; they hide from the immune system to prevent their destruction and can trick the immune system into protecting tumors; they can also change their chromosomes and rely on different nutrients. In the development and treatment of cancer, the differences between cancer cells and normal cells are used to target the abnormal cells. (National Cancer Institute, 2021) For best results, a variety of cancer treatments are used in combination; however, many treatments currently available have debilitating side effects. Gene therapy, an experimental treatment for various diseases, specifically focuses on cancer and is currently undergoing clinical trials in the United States. Scientists believe that, with additional research and advances, gene therapy will replace more invasive cancer treatments.

Methods

Data was compiled using the National Library of Medicine, PubMed databases, and Science.org.

Discussion

CRISPR

A gene-editing tool, CRISPR, which can precisely modify human DNA, was developed. CRISPR, inspired by nature, imitates the mechanisms of microbes. In nature, microbes capture small segments of intruder DNA and store them. If the same invader attacks again, those segments of DNA help an enzyme, Cas, locate and destroy the invader's DNA. CRISPR, using a cutting enzyme, Cas 9, uses virus RNA as a guide which mirrors the DNA of the gene being edited. When the RNA matches with the target gene, Cas 9 cuts the DNA. Scientists have been testing CRISPR's effect on editing immune cells to improve recognition and then attack cancer cells, and they have also used CRISPR to detect specific targets, such as DNA, from cancer-causing viruses and RNA from cancer cells. Researchers can use hundreds of guide RNAs to edit and evaluate hundreds of genes at a time and can pick out genes that will be effective drug targets. CRISPR is a valuable tool for future cancer treatment.

While solutions are being developed, technical limitations, such as off-target editing, negative immune response to the gene-carrying virus, and fear of alterations in the DNA of the germ cells have slowed CRISPRs entrance into the market. At times, CRISPR cuts untargeted DNA and off-target editing, and scientists are worried that off-target edits could turn cells cancerous. Therefore, to improve the accuracy of Cas 9 and the guide RNA, researchers use a virus that can only infect one organ; while this makes CRISPR safer, it also limits the number of viruses that can be used. Nanocapsules have also been used to deliver CRISPR components to specific cells. Another concern with gene editing is that CRISPR may accidentally edit sperm or egg cells, and the changes will become hereditary. Therefore, to prevent changes to germ cells, the cells are edited outside the body only. Additionally, the immune system can attack gene-carrying viruses. In 2001, a patient died when his immune system attacked a gene-carrying virus, and scientists fear that CRISPR edited viruses may be attacked. However, scientists have developed a better understanding of viruses and choose those that appear to be safer, and they continue running clinical trials to test this improved knowledge.

The first CRISPR trial in the United States to test the safety and efficacy of CRISPR edited T cells that help destroy cancer was performed in 2019. The collection of preclinical data began in 2016, and it was over two years before the federal government approved phase 1 of the trial. (Penn Medicine News, 2020) Scientists performed four edits on the T cells: the first two helped the T cell survive; the third reduced toxicity; and the fourth enhanced

the cell's tracking mechanism by adding a gene to locate tumors. Usually, edited T cells survive for approximately a week, but the first two modifications allowed the cells to last for at least nine months. A lentivirus was used to insert the tracking mechanism, which informs the edited T cells to target the NY-ESO-1 antigen. Patients had to have a molecule called HLA-A*02:01 to be approved for the trial; this molecule activates the CRISPR edited T cells, which, unlike CAR T cells, are not active on their own. Two patients with refractory, advanced myeloma and one with metastatic sarcoma met the trial requirements. Results indicated that the treatment was safe, and side effects were likely due to prior doses of chemotherapy. No evidence of an immune reaction to the CRISPR-edited cells was reported. However, only ten percent of the T cells used for the therapy had the four intended genetic edits. Some off-target modifications were found, none of which appeared to have turned cancerous. While no adverse reactions were evident, the CRISPR treatment had little effect on the tumors. The tumors of two of the patients in the trial stopped growing for a short period of time but then resumed growth. The tumors of the third patient were not affected at all. (Stadtmauerer et al., 2020). The long-term effects of this therapy still need to be monitored. To fully assess the efficacy of CRISPR therapy experience with more patients, using advanced editing techniques and monitoring the results for a longer period of time was needed. In March of 2022, using CRISPR- based technologies, Intellia Therapeutics, Inc., a clinical-stage genome editing company, announced that T cell therapy, NTLA-5001, designed to target Wilms' Tumor, WT1, antigen, found in acute myeloid leukemia, was administered to the first patient. The therapy was developed using Intellia's advanced lipid nanoparticle cell engineering platform, which was designed to improve edited cell performance (Intellia Therapeutics, Inc., 2022).

Acute myeloid leukemia and some other forms of cancer are distinguishable by the increased level of Wilms' Tumor 1 antigen, which encodes a transcription factor that displays an important part in cell growth and differentiation. (Haruo, 2010). NTLA-5001, designed for acute myeloid patients with HLA-A*02:01 allele, promotes T cell survival and accurately targets intracellular tumor antigens. To improve T cell receptor-based therapy, T cell receptors (TCRs) specific for shared oncogenic antigens are still needed, and the redirection of T cell specificity, while promoting T cell survival, requires modification. Using fifteen healthy donors, nineteen specific TCRs for Wilms' tumor antigen one were isolated. The TCRs recognized various peptides which restricted common human leukocyte antigen alleles and exhibited a wide range of effective avidities. The researchers then selected five high-avidity HLA-A*02:01- restricted TCRs, and primary acute myeloid leukemia blasts processed the TCRs. Using CRISPR-Cas9 gene-editing tools, the researchers combined TCR-targeted integration into the TCR alpha constant locus with TCR beta constant knockout to prevent TCR alpha beta mispairing and to increase the TCR activity and expression. The engineered lymphocytes were placed into memory stem T cells. A distinct WT137-45 specific TCR exhibited antigen-specific responses and successfully destroyed acute lymphoblastic leukemia blasts and glioblastoma cells in vitro and in vivo. No off-tumor toxicity was present. Researchers are now engineering T cells to express the effective receptor, which is being clinically developed for acute myeloid leukemia immunotherapy and shows potential in the treatment of other WT1 expressing tumors (Ruggiero et al., 2022).

The FDA approved Phase 1/2a study to assess the safety and efficacy of a single dose of NTLA-5001 in adults with acute myeloid leukemia. First, patients will receive standard cancer therapy and then a single dose of NTLA-5001. If proven safe, the researchers will then administer additional doses of NTLA-5001 to a group of patients with a mild number of tumors and one group with a larger amount. Another phase will expand to include up to fifty-four patients. After the first two phases are complete and the dosages are determined for each group, the trial will expand to include more patients and further assess the safety of the treatment. Based on the results of the preclinical trial, Intellia believes the therapy will lead to a safer, more efficacious cancer treatment. (Intellia Therapeutics, Inc., 2022)

Kymriah

Kymriah, a cell-based gene therapy for patients up to age twenty-five with B cell acute lymphoblastic leukemia, is used for refractory, in relapse after transplant, or in second or later relapse, and for adult patients with relapsed or refractory diffuse large B-cell lymphoma after two or more lines of systemic therapy. The active substance in Kymriah is tisagenlecleucel; the drug involves reprogramming the patient's T cells to identify and eradicate CD19 expressing cells, which is done by adding a replication-defective, self-inactivating lentivirus vector containing an anti-CD19 CAR transgene. When CAR binds with CD19-expressing cells, the transgene transmits a signal to induce T cell expansion, activation, and target cell elimination. Monocytes, natural killer cells, and B cells may also be added to the drug. The number of T cells added is patient-specific. Based on its high success rate in driving the cancers into complete remission with minimal

residual disease, on August 30, 2017, tisagenlecleucel became the first gene-modified cell therapy to gain FDA approval. However, the therapy is not without side effects. The most frequently reported reaction to the drug was cytokine release syndrome (Clinical Cancer Research, 2019). Cytokine release syndrome is caused by a large, rapid release of cytokines into the blood from immune cells affected by the immunotherapy" and is characterized by fever and organ failure (National Cancer Institute). The cytokine release syndrome that patients experienced was reversible in most cases and managed with supportive care and anti-cytokine therapy. Half of those who suffered from cytokine release syndrome were admitted to an intensive care unit and, on average, remained there for one week. Two patients, out of the 63 in the trial, died within 30 days of infusion. One patient died with cytokine release syndrome and progressive leukemia, and the second with resolving cytokine release syndrome, abdominal compartment syndrome, coagulopathy, renal failure, and intracranial hemorrhage. Neurological side effects were also present, a majority of which were resolved (The Oncologist, 2020). Since approval, the treatment has not been expanded to a wider age range since its safety has not been established (Clinical Cancer Research, 2019). The use of the treatment is limited because potential benefits do not always outweigh the risks.

Research on the long-term efficacy of Kymriah recently highlighted two patients who continue to display a CAR T cell response ten years post-treatment. Both patients had been diagnosed with chronic lymphocytic leukemia, and when their cancers no longer responded to standard therapy, they became the first participants in the clinical trial of Kymriah. The patients went into remission that year, and in a recent analysis, researchers observed that the modified CAR T cells had a highly activated CD4+ T cell population, which have become dominant in both patients. The data implies two phases of the response to CAR T-cell therapy: one phase is dominated by killer T cells, and the long-term phase is dominated by CD4+ T cells. The CD4+ T cells, which became the majority of T cells and increased their dominance with time, continued to display mechanisms that destroy tumors and continued growth, which demonstrates the efficacy of CAR T cells. This CD4+ T cell dominance led researchers to believe that CD4+ T cells may be foremost in distinguishing T-helper from T cytotoxic cells. (Penn Medicine News, 2022).

P53

Research has shown that mutant P53 is a leading cause of cancer. Professor David Lane discovered P53 and dubbed it the "guardian of the genome" as it prevents mutations from passing down to daughter cells. The wild-type p53 protein is activated by cellular stress, such as hypoxia, DNA damage, and oncogenic stress, and mediates cell-cycle arrest and DNA repair or induces apoptosis depending on the degree of cellular stress (Zhu et al., 2020). The Cancer Genome Atlas program analyzed Tp53 mutations in 10,225 patients with 32 different forms of cancer to study the effects of the mutation (Kandoth et al., 2013). The mutation was present in 3,786 patients, and the mutation frequency varied with cancer type, ovarian and uterine cancer showing ninety percent incidence. In contrast, other cancer types had less than five percent incidence. Analysis of the Tp53 mutation found that it causes instability of chromosomes, which included increased oncogenes and deletion of tumor suppressor genes (Donehower et al., 2019). Mutant Tp53 was found in over half of human cancers, leaving the body unprotected against tumors.

Another study supported the efficacy of p53 restoration. They injected drugs that restore mutant p53 in mice with lymphomas and sarcomas. The results indicate that the restored mutant p53 led to shrinkage of lymphomas and sarcomas without damaging healthy cells (Ventura et al., 2020). The data also implies that drugs that restore mutant p53 in humans can shrink tumors as well.

Mutations in Tp53 cause the mutant gene to survive and take over. Mutant p53 acquires gain of function activities which leads to its dominance. Mutant p53 can interact with many transcription factors, which alters transcription, cell cycle, apoptosis, and cancer cells metabolism. Mutated p53 genes differ from most mutations because they produce a single amino acid substitution in the mutant protein. In addition, mutant p53 alters the cellular metabolism of glucose, lipid, and nucleotides, which correlates with the Warburg effect, that rapidly dividing tumor cells rely mainly on glycolysis to meet their high energy demand. The changes can lead to metastasis and chemotherapy and radiation resistance. Researchers found that mutant p53 ignores anti-growth signals and is responsible for the unlimited replication of tumor cells. Epithelial to mesenchymal transition is a critical factor in metastasis, allowing cells to gain the ability to migrate and invade. Chemotherapy and radiation are used to treat metastatic cancer; however, since mutant p53 activates MDR1, a gene that helps resist these therapies is activated by p53. Restoration of the wild-type p53 could end the activity of MDR1 by reducing its phosphorylation and thus increase the efficacy of chemotherapy and radiation. Professor David Lane predicted that many more drugs to target p53 would be developed in the future (Lane, 2010).

Gendicine

Gendicine, a gene therapy that utilizes p53 to treat head and neck cancer, was developed by Shenzhen Sibiono GeneTech Co. Ltd. and approved by the China Food and Drug Administration (CFDA) in 2003. Gendicine is an adenovirus vector, most commonly used for gene therapy due to its high gene-transfer efficiency, large gene-carrying capacity, selective gene delivery, mild cytotoxicity, potential therapeutic immunogenicity, ease of construction and manipulation, and cost-effective manufacture, that delivers wild type human p53 to tumor cells. Ad-p53 can boost the immune system to fight cancer cells by activating cytokine genes, tumor antigen genes, and co-stimulatory molecule genes. While other Ad-p53 drugs have undergone preclinical and clinical trials, Gendicine is the only one to have obtained approval but only in China.

The CFDA approved Gendicine because it increases the efficacy of chemotherapy and radiation when used in combination, and, since then, it has also been proven successful in the treatment of other cancers. The delivery of Gendicine is minimally invasive and injected intratumorally, intracavity, or intravascularly. Thirteen published studies have shown that standard therapies combined with Gendicine yield significantly longer survival rates. Gendicine, as a primary treatment, can also be used for ovarian cancer, malignant pleural effusions, or peritoneal ascites. The major adverse effect of Gendicine is fever within 24 hours of administration, which occurred in fifty to sixty percent of patients but was easily resolved. It is thought that the feve that many patients develop after treatment may have a positive effect; it alludes to the possibility that rAd-p53 can induce an immune response that kills tumor cells (Bioxue et al., 2016). Immune responses to adenoviral vectors have been studied extensively. Most patients have been exposed to adenovirus and have antibodies to neutralize the virus; nonetheless, the clinical efficacy of Ad5-based therapies appears effective and does not elicit an adverse immune response. In clinical trials, the anti-tumor effect of Gendicine was not at all inhibited by pre-existing antibodies. The anti-vector neutralizing antibody levels increased in patients after receiving Gendicine with no negative outcome. Over the course of twelve years, thirty Chinese clinical studies have been published, and approximately 30,000 patients have been given Gendicine, which provides strong evidence of its safety. Nonetheless, researchers continue to work on improving the safety and efficacy of Ad-vectors (Zhang et al.,2018).

Domestically, the FDA is more cautious than the CFDA when approving new drugs. Ad-p53, Gendicine in China, was originally developed by Introgen Therapeutics and Gendux under the name Advexin in the United States (Zhang et al., 2018). Advexin was developed for the treatment of head and neck cancer and Li-Fraumeni syndrome, a rare autosomal dominant mutation that increases the risk of developing cancer (Chin-Hang Kong, 2009). The Phase III trial conducted by Introgen compared the efficacy of Advexin with methotrexate, a chemotherapy drug. Patients, with p53 profiles that were positive for Advexin efficacy, had increased survival rates. Patients, with p53 profiles negative for Advexin efficacy, had increased survival following treatment with methotrexate. Outcomes showed that both Advexin and methotrexate increased survival depending upon p53 positive and negative profiles. Biomarker analysis indicates that Advexin suppresses tumors in negative p53 profiles, but the FDA refused to approve Advexin, despite having accepted the data of the biomarker. However, the refusal may be due to Advexin's inability to improve survival compared to standard therapy. The FDA then suspended the trial for Advexin in the United States since Introgen's Biologics License Application for the therapy was incomplete. The FDA has authorized the use of Advexin on a compassionate basis for patients with Li- Fraumeni syndrome under authorized protocol. Introgen intends to appeal the decision against Advexin (Chin-Hang Kong, 2009).

Shortly after launching Gendicine, the Chinese State Food and Drug Administration (SFDA) approved type 5 Ad derivative of E1B-55 kDa molecule for head and neck cancer treatment which was also originally developed in the United States under the name ONYX-015. The Chinese obtained exclusive license of ONYX-015 in the world and labeled it Oncorine. The United States suspended a phase III study of ONYX-015 to treat head and neck cancer. China's trials of Oncorine to treat head and neck cancer had similar results to that of ONYX-015 in the United States. Ad vectors are injected intratumorally and are well tolerated by patients; however, efficacy for some advanced cancers would increase if treatment were delivered intravenously. On target specificity of tumors is an area that still needs additional investigation. Modification of the viral coat proteins will reduce toxicity and improve on-target specificity Trials using Ad-p53 in combination with standard therapy, done in both the United States and China, show similar results that the Ad-p53 or Ad derivative of E1b-55 kDa are safe and can be beneficial for patients who have not responded to standard therapies (Ma et al., 2009).

Despite the many trials that have been conducted in the United States and other Western countries, Ad-p53 products have not received government approval and cannot be marketed. One reason for the delay is the inadequate information from Chinese studies. The Chinese

reports only summarize case reports and do not include the long-term effects of the therapy or an adequate control group. The Chinese obtained very different outcomes in their trials with Ad vectors than trials done in Western countries. The significantly better outcomes seen in the Chinese studies may be due to the fact that they included early-stage patients, who should have been treated with standard therapies and instead treated with a combination of Ad-p53 and standard therapies. Unfortunately, additional errors were found in the Chinese trials (Bioxue et al., 2016). None of the studies that the researchers analyzed focused on the P53 mutation in patients with Malignant Pleural effusion, so the effect of the P53 mutation on the response to Gendicine treatment was not assessed. However, previous studies have shown that Ad-p53 can inhibit the growth of human lung adenocarcinoma cell line containing the mutant and wild-type p53 genes. Other drawbacks of the data are the small sample size, the studies were performed in China, and different responses to treatment may occur in patients from other countries (Bioxue et al., 2016). Outcomes of the United States and other Western studies should not be compared with the studies done in China.

Much of the clinical data in China has not been well reported at international conferences, and, until recently, researchers from other countries have been unaware of cancer-related gene therapy progress in China (Ma et al., 2008). However, cancer patients from all over the world travel to China to receive treatment. The results are reported in Chinese domestic journals, but access to the information is unavailable to non-Chinese medical scientists. On the other hand, the available clinical data from Chinese studies provide helpful leads for future clinical studies. The growing number of patients who receive gene therapy treatment in China increases the need for phase IV studies in the United States. If Chinese studies meet international standards, they will be able to contribute to gene therapy research. However, the director of China's State Food and Drug Administration was found guilty of bribery to approve new medicines, which has led to further concern in international medical and pharmacological societies. China's gene therapy companies claim they were not involved in the fraudulent activity; however, the gene therapy approval process in China must be better monitored. (Ma et al., 2008). Despite the drawbacks, the evidence present suggests that Gendicine is an effective and safe treatment that improves the quality of life compared with standard therapies alone (Bioxue et al., 2016).

Zinc Metallochaperones

Another p53-based gene therapy, zinc metallochaperones, has impacted the ability to repair mutant p53. Zinc metallochaperones regenerate the p53 mutation that occurs in the DNA-binding domain, thus allowing the p53 gene to transcribe RNA, preventing a loss of function by impairing the binding of zinc to the p53 protein. For proper folding, the p53 protein requires binding to a single zinc ion. Initially, scientists attempted to bind or modify the mutated p53 gene to make it function in the cell's environment; however, no functional compounds were found. A class of drugs was discovered, zinc metallochaperones, which changes the environment to accommodate the mutated protein rather than the protein itself (Blanden, et al., 2015).. Zinc metallochaperones reactivate p53 by restoring the wild-type structure by reestablishing zinc-binding, which changes the conformation of wild-type p53. The zinc metallochaperones also reactivate p53 through post-translational modifications brought on by cellular reactive oxygen species-ROS, which causes apoptosis of the cancer cell via p53 They bind zinc and other divalent metal ions, which are strong enough to remove serum albumin but weak enough to donate zinc to mutant p53. Further research led scientists to discover that the homeostatic mechanisms to maintain intracellular zinc levels are induced by zinc metallochaperones. Therefore, when zinc levels are stable, the homeostatic mechanisms can deactivate the zinc metallochaperones (Kogan & Carpizo, 2018). Research of the pharmacodynamics of zinc metallochaperones has led scientists to recognize that the ON/OFF switch mechanism of zinc metallochaperones allows for the brief reactivation of the p53 mutant and enables on-target efficacy, and avoids off-target toxicities. The alternate route, targeting the environment rather than the protein, has created a new method of drug development.

For the treatment of tumorigenic p53 mutations, researchers analyzed 20 of the most common mutations and found that eighty percent impair zinc affinity, thermodynamic stability, or both. Blandon et al. explain that for treatment, mutations will be classified into three groups that will be useful when categorizing patients. Synthetic zinc metallochaperones repair both mutations that decrease zinc affinity and mutations that destabilize DNA binding domains without impairing zinc binding. Zinc metallochaperones can repair mutations that are associated with new cancer cases in over 120,500 patients each year in the United States. (Blanden et al., 2020)

Preclinical studies of cancer models show that a zinc metallochaperone therapy, ZMC1, improved survival and inhibited tumor growth, specifically for the zinc-deficient allele. In the BRCA1-deficient breast cancer model, scientists discovered that ZMC1 and the PARP inhibitor Olaparib, an enzyme that helps repair DNA damage in

cells, combine to increase efficacy. Olaparib has been approved for the treatment of advanced BRCA1/2 mutant ovarian and breast cancers. Some tumors have developed resistance to Olaparib but are still affected by ZMC1 treatment. Researchers are testing combinations of ZMC1 and other chemotherapy drugs. (American Association for Cancer Research, 2020)

Scientists thought ZMC1 would work well with chemotherapy and radiation; however, their hypothesis was proven incorrect. The reason for the lack of cooperation was found in the reactive oxygen species activity, ROS, which negates the signal on p53 that is generated with chemotherapy and radiation. The signaling events that chemotherapy and radiation would normally induce to activate p53 are already being stimulated by ZMC1. ROS enables ZMC1 to act on its own but inhibits it from working together with chemotherapy and radiation. Although ZMC1 does not operate together with chemotherapy and radiation, it does work well with other targeted agents, and other zinc-binding agents work well with chemotherapy and radiation by inducing p53 signals on mutant p53. (Zaman et al.,2019).

Conclusion

Research has shown that gene therapy, including CRISPR, Kymriah, Gendicine, and zinc metallochaperones, can be effective in treating cancer. While scientists have developed an array of genetic treatments to attack cancer cells, many gene therapy treatments have not been brought to market for fear that the long-term effects could interfere with the human genome. Hopefully, with additional data and safety improvements, all patients will be able to benefit from gene therapy treatments in the near future.

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An Analysis of Different Treatment Options for Type 1 Diabetes Mellitus

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Abstract

Type 1 Diabetes Mellitus is a highly dangerous autoimmune disease. Type 1 diabetes is most commonly seen in children and young adults as pancreatic Beta (B) cell destruction is highest at this age. Patients with Type 1 Diabetes are required to take insulin injections to compensate for their lack of insulin, but many patients still have episodes of hyperglycemia. This paper analyzes and compares the effectiveness of different treatment options. The standard approach is to prescribe insulin injections, but this analysis finds insulin injections in conjunction with oral medications such as Metformin and Sodium-glucose co-transporter (SGLT) inhibitors has a positive effect on patient health. Metformin in addition to insulin therapy decreased metabolic syndrome. SGLT inhibitors decreased blood glucose levels in patients and have an increased efficacy to prevent hyperglycemia. If a patient were unable to take oral medications, rapid acting insulin aspart decreases blood glucose levels effectively compared to human insulin. Additionally, long acting insulin proved more efficient in reducing blood glucose levels than intermediate acting insulin. Extensive research is being conducted by different pharmaceutical companies for a potential cure. The first clinical trial using stem cells has shown positive results for the patients as they could now go days without taking insulin. More research is needed; however, insulin therapies can be adjusted to each patient to provide the most beneficial results, and insulin therapy alone no longer is needed to be the first and only choice as the other options appear to provide beneficial results.

Introduction

Diabetes Mellitus is a chronic health condition that occurs when blood glucose is too high. There are various subclasses of Diabetes Mellitus. The chronic conditions are Type 1, an autoimmune disease, and Type 2, in which insulin receptors don't recognize insulin. Potential reversible conditions are Gestational Diabetes which happens during pregnancy and Prediabetes.

Type 1 diabetes is a T cell autoimmune disease, characterized by the destruction of pancreatic Beta cells. Consequently, the body does not produce enough insulin, leading to hyperglycemia, an increase in levels of blood glucose. Hyperglycemia dramatically increases the risk of various cardiovascular diseases such as atherosclerosis, angina and high blood pressure. Type 1 Diabetes is usually present in infants and children because the rate of β cell destruction tends to be more aggressive at this age. (Kelly et al., 2003). There is no known way to prevent Type 1 Diabetes. As many as 37.3 million people have Diabetes and in adults Type 1 accounts for 5-10% of all cases. The rate of new cases within youth increased by 1.9% per year in the United States between 2002 and 2015 (Center for Disease Control and Prevention, 2020).

Autoimmune diseases have a complex genetic basis. For Type 1 diabetics, two gene regions of importance have been identified that are associated with the disease's genetic component: The Human Leukocyte Antigen Locus (HLA) and the insulin gene. The HLA region is the major genetic determinant of disease risk, accounting for 42% of the familial inheritance of Type 1 Diabetes. The insulin gene region contributes a further 10% of genetic susceptibility (Kelly et al., 2003).

While there is no known way to prevent Type 1 Diabetes, there are different treatment options that exist or are in the process of being developed. This paper will analyze different treatment options available for Type 1 diabetes and seek to determine which is the most effective option.

Methods

This comprehensive review of treatment options for Type 1 Diabetes Mellitus was based on the critical analysis of data collected from PubMed and other databases accessed through Touro College and University System's library including ProQuest and EBSCO. Among the keywords and phrases used to retrieve data included "Type 1 Diabetes treatment options," "Therapy for Type 1 Diabetes," "Type 1 Diabetes age correlation" and, "stem cells and Type 1 Diabetes."

Genetic Component of Type 1 Diabetes

The first diabetes susceptibility genes to be identified were the human leucocyte antigen (HLA) genes, located on chromosome 6p21 within the major histocompatibility complex, as well as the insulin gene region on chromosome 11p. A study in which genome screens were conducted confirmed that the IDDM1 locus (the HLA gene region) is the major genetic determinant of disease risk. It accounts for 42% of the familial inheritance of type 1 Diabetes. The IDDM2 locus (the insulin gene region) contributes a further 10% of genetic susceptibility (Davies et al., 1994).

HLA class 1 is expressed in all cells, class 2 expression is restricted to B lymphocytes, dendritic cells, macrophages and activated T lymphocytes. Class 1 and 2 express cell surface glycoproteins which are involved in the presentation of antigens to T cells. Cytotoxic T cells (CD8+) recognize antigen in the context of class I, whereas helper T cells (CD4+) recognize antigen in the context of the class II molecules. The class 2 HLA-DR, HLA-DQ, and molecules are involved in the activation of helper T cells.

The risk of these molecules for type 1 diabetes is most likely related to their role in antigen presentation and the activation of a helper T cell mediated immune response. This function is determined by the binding clefts of the molecules. In a study using x-ray crystallography, HLA molecules associated with type 1 diabetes share similar chemical

properties in their binding antigen domain. The characteristics of protective HLA molecules were different from the predisposing molecule structure. (Cucca et al., 2001).

The structural differences between the predisposing and protective surface molecules is seen in the peptide selectivity and binding affinity of the antigen binding cleft pockets 1,4 and 9.

P1 in the predisposing diabetes molecules contains a glycine residue at position 86 of the β chain which prefers to bind large aromatic side chains. The valine residue encoded at position β86 in the protective molecule prefers small hydrophobic residues.

P4 is important for the binding selectivity of the HLA DR molecule. In predisposing diabetes molecules, the alanine residue at β74 is selective for acidic residues, but for the protective molecules it cannot bind to acidic residues (Cucca et al., 2001).

P9 in the protection molecules carry an aspartate residue at position β57, whereas predisposing molecules carry an uncharged amino acid residue at this position. This alters the shape of P9 and alters the preference of the molecule for particular anchor residues in the bound peptide (Kelly et al., 2003) In summary, the structural differences between the predisposing and protective HLA molecules may result in differences in their ability to bind to diabetic antigens.

Treatments Options

Insulin Therapy

Type 1 Diabetics are insulin deficient and thus require an insulin supplement. The goal of exogenous insulin therapy is to mimic normal endogenous insulin secretion of the pancreas. Type 1 diabetics use insulin injections as their supplement as opposed to oral insulin because the acidity in the stomach would break it down (Gradel et al., 2018). Therefore, insulin needs to be injected subcutaneously to be released in the body. There are four different types of insulin injections. Rapid-acting begins to work within a few minutes and lasts a couple of hours. Regular or short-acting, which takes about 30 minutes to work fully and lasts 3 to 6 hours. Intermediate-acting, which takes 2 to 4 hours to work fully and lasts up to 18 hours. Finally, Long-acting, which can work for an entire day (Center for Disease Control and prevention, 2021).

Insulin Aspart vs Human Insulin

A study showed the difference in glycemic control of insulin aspart, an analogue of human insulin, and human insulin. The study included 423 basal-bolus treated patients with Type 1 diabetes, who take long acting and rapid acting insulin together. The researchers gave the patients an algorithm-driven dose optimization over 3 months. Glycated hemoglobin levels (HbA1c) were significantly lower in insulin aspart treated patients compared to the human insulin treated subjects by 0.17 with a P value less than 0.05. Additionally, blood glucose profiles showed lower levels with insulin aspart after breakfast, with a mean of 8.4 vs 10.1 mmol/l ($P<0.0001$), and dinner, 8.2 vs 9.3 mmol/l; ($P<0.01$), compared with human insulin. (Tamas et al., 2001). This study indicates the higher absorption rate of the insulin analogue and is why many patients have switched over for some time now. There may be other factors that lead to a higher absorption.

Short Acting vs Rapid Acting

A systemic review was done to determine the efficacy of taking rapid acting insulin aspart compared with short acting human insulin. In 13 randomized controlled trials it was shown that insulin aspart resulted in a significant decrease of .11% in glycated hemoglobin levels compared with regular human insulin. But there was an increased risk of a hypoglycemic episode with insulin aspart as was shown in six of the randomized controlled trials (Rys et al., 2011). This large review demonstrates the positive effect of taking rapid acting insulin compared with regular insulin. But, hypoglycemia is a risk factor and one must be careful in how they use rapid acting insulin to prevent hypoglycemia episodes.

Rapid Acting vs. Different Timings

Rapid acting insulin is usually taken before a meal when there will be a rise in glucose level. One study compared and analyzed the impact of three pre-meal timings of rapid-acting insulin on postprandial glucose excursions in type 1 diabetes. Ten subjects were used in the study and all were treated with insulin aspart. The subjects were randomly assigned to administer the insulin either 30, 15, or 0 minutes before the meal using a cross over design, each patient received different treatments during the different time periods.

Their glucose levels were measured before and after the meals. The time spent in euglycemia, normal concentration of blood glucose 3.5-10 mmol/l, was highest when the insulin was given 15 minutes before the meal. Another finding from the study indicates that when insulin is administered 15 min before, the patients' blood glucose declines slightly before mealtime. This finding shows that administering the insulin must be only when the patient's pre-prandial glucose levels are greater than 5.0 mmol/l (Luijf et al., 2010). The small sample size as well the resulting decrease in blood glucose suggests a need for further investigation.

Intermediate Acting vs Long Acting

A meta-analysis of twenty-six randomized control studies and 6,776 patients was done to show the effect of using intermediate acting vs long acting insulin on HbA1c levels. The results showed that compared to Neutral Protamine Hagedorn, (NPH) an intermediate acting insulin, Glargine and Detemir, long acting insulin, significantly reduced HbA1c levels in the body (Tricco et al., 2014).

A sub-group analysis of hemoglobin A1c was done using 12 randomized controlled trials which included 4,002 patients who had poorly controlled hemoglobin A1c (>8%). When given once daily, Glargine significantly improved blood glucose levels compared to NPH, with a mean difference of –0.65%, –0.96% to –0.35% and Detemir once daily –0.41%, –0.74% to –0.08% for the three different trials.

The analysis also sought to compare the cost effectiveness of taking long acting insulin compared to intermediate acting. Fourteen studies were found on the cost effectiveness: five of which compared Detemir to NPH. The results reported that Detemir costs less and is more effective. Another eight studies compared Glargine to NPH. The former was less costly and more effective in two of these analyses, while six studies found that Glargine was costlier and more effective than NPH (Tricco et al., 2014). Intermediate acting is taken twice daily compared to long acting which is daily. This meta-analysis demonstrates the effective use of long acting insulin and thus creates a possible opportunity for diabetic patients to feel comfortable taking less injections each day.

Attempts were made to try to create a more practical and easier therapy for patients. A study was done to try and show the effects of mixing rapid acting insulin analogues with insulin Glargine in children. The study was done using 55 children whose mean age was 13.4 years old. The children mixed the two types of insulin in the same syringe and were injected by their medical care provider. A group of 55 similar children served as the control. Data was collected 6 months prior and post the mixing of insulin began. HbA1c values were collected after the six months and were 8.54% vs 8.61% with a P value of 1.00 indicating there is no statistical significance in the difference. (Fiallo-Scharer et al., 2005). These findings suggest that there is no issue with mixing the two insulins in one syringe. Additionally, the results are especially encouraging for those patients who wish to minimize the number of total daily injections because of needle fear or forgetting injections.

Oral Medications in Conjunction with Insulin Therapy: Metformin

Metformin is an oral anti-hyperglycemic medication and it is commonly used to treat type 2 diabetes. This drug increases both hepatic and peripheral insulin sensitivity by inhibiting the amount of glucose the liver produces and by increasing glucose uptake in cells. People with type 2 Diabetes can control their blood glucose level by using metformin and insulin therapy. One study shows the effect of including metformin as an add-on therapy to insulin in overweight adolescents with type 1 Diabetes Mellitus. The results show that at the end of the experiment there was no improvement in glycemic control (Libman et al., 2015).

A different study was done to investigate the effects of metformin on type 1 diabetics. Twenty-nine patients with type 1 Diabetes included metformin as an adjunct to their insulin therapy for 12 months. Their glycated hemoglobin levels (HbA1c) were quite high while only using insulin therapy. They were compared to a placebo control group whose weight; blood pressure and other factors did not differ from the experimental group. The results of the experiment show that there was an increase in insulin dosage by 0.11 IU/kg/d in the control group, whereas in the test group dosage decreased by 0.03 IU/kg/d. Metabolic syndrome prevalence in the control group was 44.8% compared to 41.4%, in the test group. The resulting p value was higher than 0.05 indicating that there was no statistically significant difference in their metabolic syndrome prevalence. But, after treatment with metformin, metabolic syndrome was decreased in the metformin-insulin group by about 8.9% after treatment compared to the insulin alone group which decreased by 2.5% ($p = 0.028$). However, HbA1c did not differ between the groups ($p > 0.05$) (Beysel et al., 2018). These results show a positive effect on the inclusion of metformin in treatment as it reduced metabolic syndrome which is a factor that greatly influences a diabetics probability of developing cardiovascular diseases. But, blood glucose levels were not reduced indicating a need for better adjunct therapy for patients whose blood sugar remains very high.

Sodium-glucose cotransporter (SGLT) inhibitors:

Sodium-glucose cotransporter (SGLT) inhibitors, a new class of oral hypoglycemic agents, lowers serum blood glucose levels. There are two classes of the inhibitors SGLT2 and SGLT1.

SGLT-1 is responsible for glucose absorption in the small intestine, and for the reabsorption of nearly 10% of the filtered glucose load in the renal proximal tubule. SGLT-2 is primarily expressed in the renal proximal tubule and is responsible for the reabsorption of 90% of the filtered glucose load. Most patients with type 1 diabetes do not have adequate glycemic control with just insulin therapy. The currently available SGLT-2 inhibitors, canagliflozin, dapagliflozin, and empagliflozin, have similar

characteristics and have similar effects on glycemic control. However, sotagliflozin acts on both sodium–glucose cotransporters 1 and 2.

In a study done to observe the efficacy of canagliflozin, the goal was to reach a decrease in HbA1c of more than .4%. Three hundred and fifty-one patients were included in the 18-week study. At the end of the study, 36.9% of patients with insulin and canagliflozin 100 mg, 41.4% with insulin and canagliflozin 300 mg, and 14.5% with insulin and placebo reached a reduction in blood glucose level greater than .4%. (Henry et al., 2015).

Another study was done to test the efficacy of empagliflozin as an adjunct to insulin treatment. A total of 75 patients with high HbA1c levels were randomized to receive once-daily empagliflozin 2.5 mg, 10mg, 25mg, or placebo for 4 weeks. The goal of the study was to increase urinary glucose excretion (UGE). The results show that there was an increase in UGE and a statistically significant ($p < 0.05$) decrease in HbA1c of 0.49% was noted for the empagliflozin group after 28 days in comparison with the placebo group (Pieber et al., 2015).

Compared with the previous two studies, a much larger randomized, placebo-controlled study was done to evaluate the safety and efficacy of sotagliflozin in combination with insulin therapy. The study was conducted at 133 sites in 19 countries and included 1,402 patients with type 1 diabetes. Eligible patients were randomly assigned, in a 1:1 ratio, to receive either sotagliflozin (400 mg per day) or placebo for 24 weeks. The patients that received the medication took it before the first meal of the day. The primary goal of the study was to reach a glycated hemoglobin level (HbA1c) lower than 7.0% at week 24, without episodes of severe hypoglycemia or diabetic ketoacidosis. The secondary goal of the study was a possible reduction from the baseline to week 21, body weight and blood pressure. A significantly larger proportion of patients in the sotagliflozin group than in the placebo group 200 of 699 patients [28.6%] vs. 107 of 703 [15.2%], ($P<0.001$) reached the primary endpoint goal of a blood glucose level less than 7 percent. There was a greater reduction in the glycated hemoglobin level from baseline in the sotagliflozin group than in the placebo group, the difference was −0.46 percentage points ($P < 0.001$) (Garg et al., 2017).

A glycated hemoglobin level lower than 7.0% was achieved with no weight gain in 171 patients in the sotagliflozin group and 51 patients in the placebo group. The reduction in body weight was significantly greater in the sotagliflozin group than in the placebo group with a difference of -2.98 kg ($P<0.001$). Among patients with a systolic blood pressure of 130 mm Hg or higher at the start of the experiment, reduction in blood pressure from start to week 16 was significantly greater in the sotagliflozin group than in the placebo group with a difference of, -3.5 mm Hg. The rate of diabetic ketoacidosis was higher in the sotagliflozin group than in the placebo group (3.0% [21 patients] and 0.6% [4], respectively) (Garg et al., 2017). It appears from this study that patients using sotagliflozin in adjunct with insulin showed increased improvement reducing their blood glucose level and overall health and there is greater benefit to using this medication compared to other SGLT-2 inhibitors. Yet, there is still a chance of ketoacidosis and further research might need to be done to perfect the drug.

Stem Cells

A curative treatment for Type 1 diabetes mellitus involves pancreas transplantation, but due to the incidence of transplant rejection and complications associated with immunosuppression, alternatives are being explored. One such alterative is the use of stem cells. This treatment revolves around the idea of pluripotency, the ability of a stem cell to differentiate into multiple lines of cells. There are four different types of stem cells: human Embryoinc stem cells (hESC), induced pluripotent stem cells (iPSCs), and adult stem cells that are being tested to generate functional islet cells. There is another class of cells that is being testing called progenitor cells which are descendants of stem cells that then further differentiate to create specialized cell type that belong to the same tissue or organ. Stem cell differentiation can be manipulated by controlling the cells environment when placed in a medium.

Human embryonic stem cells compared to Induced Pluripotent stem cells.

Induced pluripotent stem cells are created by essentially reverse engineering of an already differentiated cell. This method can be done using the CRISPR-Cas9 system. HESC are made when inner mass cells are taken from the fertilized blastocyst egg.

A study showed the efficacy of hESC compared to pancreatic progenitor iPSC that were made from pancreatic progenitors. Using key markers at the mRNA and protein level they were able to assess beta cell development of the stem cells i.e. MAFA and G6PC2. This study was done in seven stages, each with a new cell line. In the thirteenth day of the stage (S) 7, hEBSC cells injected into the mouse models showed transcript levels of key markers, INS, MAFA and G6PC2 that were indistinguishable from human islet preparations. S7 also included an iPSC line that produced key markers for beta cells, although not as efficiently as with the hESC line used. The embryo-developed S7 reversed diabetes in mice approximately four times faster than iPSCs from pancreatic progenitors.

The iPSC line also produced less insulin producing cells compared to the hESC. Although the data showed that hESC-derived S7 cells did not rapidly secrete insulin in response to high glucose, a statistically significant ($P < 0.0001$) accumulation of human C-peptide, a peptide that is measured to tell the difference between insulin the body produces and insulin that is injected into the body, was seen in incubation of the cells in vitro (Rezania et al., 2014). This study showed the reversal of diabetes with the S7 cells when injected in the mouse models.

In another study, researchers successfully generated billions of functioning beta cells using iPSC and hESC together. In vitro the cells exhibited glucose sensitive insulin secretion just as human pancreatic B cells. When injected into mice, the cells from both hEBSC and iPSC cell lines secreted insulin directly into the bloodstream and showed increased human insulin secretion. (Pagliuca et al., 2014)

Vertex Pharmaceuticals initiated a 17-person phase I clinical trial that would be the first test in people of islet cells derived from stem cells, VX-880 is an investigational allogeneic stem cell-derived, fully differentiated, insulin-producing islet cell therapy manufactured using proprietary technology (Nature, 2021).

On October 18, 2021 the company announced positive Day 90 data for the first patient from the clinical trial of VX-880. Prior to treatment with VX-880, the patient's insulin dose was 34 units per day. Fasting and stimulated C-peptide levels were undetectable, which indicated that the patient was not making their own insulin. The patient received half the target dose of VX-880 through a hepatic portal vein infusion with a combination of immunosuppressive agents. At Day 90, fasting C-peptide was 280 pmol/L, reflecting restored basal insulin production and increased to a peak of 560 pmol/L. This shows restored glucose-responsive insulin production. Also, at Day 90, HbA1c improved from 8.6% at baseline to 7.2%, and daily insulin dose decreased from 34 units per day prior to treatment with VX-880 to an average dose of 2.9 units per day over a 7-day period at the Day 90 visit, reflecting a 91% decrease in daily exogenous insulin use. (Vertex Pharmaceuticals Incorporated, 2021)

The patient mentioned above is Brian Shelton, a 64-year-old male who has been living with Type 1 diabetes for over 50 years. On June 29, he got an infusion of VX-880 together with an immunosuppressant to prevent his body from attacking the newly engineered cells. Mr. Shelton said in an interview that the suppressants "cause him no side effects" and he finds them far less onerous or risky than constantly monitoring his blood sugar and taking insulin (Kolata, 2021). In an additional interview he explained that he can go days now without having to take insulin and the treatment has given him a new-found freedom on life (Vollmayer, 2021).

Conclusion

Treatment options for Type 1 Diabetes Mellitus are limited and require more research. Many doctors believe that since Type 1 diabetes is an autoimmune disease, the best course of action is to provide insulin injections to compensate for the lacking insulin. While as a first line of treatment this may be necessary to prevent hyperglycemia and diabetic ketoacidosis, it has been reported that most patients are not stable even with insulin. Other options including Sodium-glucose cotransporter (SGLT) inhibitors, Sotagliflozin, canagliflozin, and empagliflozin, have shown statistically significant benefits in reducing blood sugar levels when taken in conjunction with insulin therapy. Metformin has shown to benefit the patient metabolic syndrome. Stem cell research has had its first successful clinical trial and proves to have very effective outcomes. This suggests that Type 1 diabetic patients have a potential cure on the horizon. However, more research is needed to evaluate the effectiveness and efficiency of all currently available possibilities. https://pubmed.ncbi.nlm.nih.gov/25103565/

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What is the Best Method to Cure HIV?

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Abstract

Human Immunodeficiency Virus (HIV) is a sexually transmitted retrovirus impacting millions of people worldwide. Strides have been made to cure and/or prevent various genetic mutations via gene editing. Scientists hope to rid of HIV on a genomic level which will also prevent Acquired Immunodeficiency Syndrome (AIDS) - a biproduct of HIV. HIV utilizes a mechanism that counteracts the innate, adaptive, and intrinsic immune systems. Because the virus's replication takes place in the lymphatic organs, the infection traverses the entire bloodstream. Due to the complexity of HIV and AIDS, their constant evolution, and their effect on a genomic level, no cures are currently available, but some treatments are recommended. Antiviral drugs, Nucleoside Reverse Transcriptase Inhibitors, fusion inhibitors and CCR5 antagonists are mainly used. Studies show that while CD4 receptors are integral to human survival the CCR5 receptors are not. This paper compares and contrasts the TALEN, PiggyBac, CRISPR, and Sleeping Beauty gene editing methods in order to find the best technique to eradicate HIV. Sleeping Beauty and PiggyBac use transposable elements as non-viral gene delivery with slight variations. TALEN is another class of gene editing method which uses endonuclease proteins as their editing tools. After comparing many papers on Google scholar and Touro library, the most efficient method is called CRISPR-Cas9. This method is the most precise and versatile yet simple. CRISPR-Cas9 uses one piece of guide RNA and an enzyme called Cas9 which is the scissors. Delivery of CRISPR-Cas9 into cell is achieved by utilizing an integrative lentiviral vector. Overhanging RNA called scaffold RNA is then inserted into the genome. Experimentations show the accuracy and efficiency of CRISPR-Cas9 in conferring HIV-1 resistance in Vivo. Based on the aforementioned studies, properties of the CRISPR- Cas9 system include versatility, availability, simplicity, easy manipulation and efficacy. CRISPR- Cas9 should therefore be utilized for HIV-1 protection.

Introduction

Human Immunodeficiency Virus (HIV) is a sexually transmitted retrovirus currently impacting an estimated 39 million people worldwide (UNAIDS, 2006). Originating in Africa in the year 1959, HIV has high morbidity and high mortality rates, contributing to the death of more than 25 million people. Although sexually transmitted by nature, a third of infections are attributed to the use of contaminated needles amongst drug users. Recently, advancements in the understanding of molecular components opened doors undreamed of before in research. Great strides have been made to cure and/or prevent various genetic mutations via gene editing. Researchers are using numerous engineering methods (i.e. TALEN, PiggyBac, CRISPR, and Sleeping Beauty) to edit and alter the human genome. Using these new methods, scientists hope to get rid of HIV on a genomic level which will also prevent Acquired Immunodeficiency Syndrome (AIDS) - a biproduct of HIV. This paper will compare and contrast the various gene editing methods in order to find the best technique to eradicate HIV.

Methods

Comparison of peer reviewed literature using Google scholar and Touro Library.

Discussion and Background

HIV is multifaceted and multifarious; constantly evolving and impacting its hosts. Typically, eight to ten years post-infection, HIV can develop into Acquired Immunodeficiency Syndrome (AIDS) which almost entirely suppresses and compromises the immune system. Following a suspected HIV exposure, it is important to use direct virus detection tests, as opposed to antibody and antigen tests. Although costly, early detection is critical for the prevention and further spread of HIV. (Price, et. Al. 2004, Sharghi, et. al. 2005)

HIV Subtypes

HIV is well-defined as a retrovirus. However, due to its versatility, it is constantly evolving. Currently, the main groups of HIV viruses are called HIV I and HIV I,; with HIV I being the more transmittable and thereby contributing to about ninety-five percent of all infections. HIV I is made up of three subvariants (M, N, O) and the M subgroup, the most prevalent HIV I, is further split into eight branches (A, B, C, D, F, G, H, J.) (Keele, et. al. 2006, Korber, et. Al. 2005, Thomson and Majeraet,. 2005) Subtype C is the most prevalent; accounting for over 47% of the M subgroup.

Initial Attack

HIV utilizes a mechanism that counteracts the innate, adaptive, and intrinsic immune systems. (Mahalingam, et. al. 2002 Bieniasz, 2004) The virus is virtually undetectable to the human immune system mainly due to its diminutive size of 10 KB and its delayed damage technique. (Barre-Sinotissi, 1996, Emerman and Malim 1998, Balabanian et. al. 2004, Cicala et. al. 2002) Part of the makeup of the virus consists of a glycoprotein (GP) GP120 on its outer envelope and a transmembrane glycoprotein GP41. HIV is an enveloped virus with a fatty bilayer. (Ray and Doms, 2006) Both GP120 and GP41 form the spike protein on the virus's outermost surface, and which, respectively, facilitate viral detection and entry into the cell. Initially, cluster of differentiation 4 (CD4) receptors which are

found on the outside of the host's cell membrane, and are vital for the production of CD4 proteins, are the main binding sites for GP120. (Ray, 2006) Once bound, normal cellular activities associated with CD4 become blocked. After the onset of the virus binding, the chemokines and co-receptors, which are responsible for the control of cells' immune behavior ranging from chemotaxis and cell adhesion to mediator release, are also bound and blocked via irreversible configurational changes. Receptors such as C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) are detrimental to the viruses' success. (Eckert and Kim, 2001)

Post Attack

Post entry into the cell's cytoplasm, the virus's genome is irreversibly transcribed into the host's genome via reverse transcriptase using the host's transcriptase enzyme. (Coffinet. al., 1997) Reverse transcriptase is not equipped with a proofreading mechanism and is therefore prone to error. HIV encodes for three structural genes, GAG, POL, and ENV. (Coffin, et. al. 1997) These genes respectively code for matrix polyprotein used for viral assembly and infection; reverse transcriptase and other crucial enzymes for genomic integration; and envelope proteins such as GP 120 and GP41 that enable entry into the cell. Once transcribed, the host's immune system is compromised. CD 4+ cells which are helper T cells (Th cells) are destroyed. The virus uses the host's replication mechanisms to create copies of itself and thereby compromises the host's immune system.

Impact

HIV is prone to travel quickly throughout the body and affects every cell making HIV a greater risk and more difficult to contain; also allowing for HIV to transform into AIDS which affects the entire immune system. The virus's replication takes place in the lymphatic organs which leads to a primary amplification. In the lymph system, the infected T lymphocytes traverse into the bloodstream. Secondary amplification now occurs mainly in the gastrointestinal tract, spleen, and bone marrow. Because a large number of cells are affected after the secondary infection, a hallmark sign of severe infection is a gradual but pronounced depletion of activated, as well as, naïve and memory CD 4+ T lymphocytes located in the gut-associated lymphoid tissue (Unlike destroyed CD 4+ T-helper cells in the peripheral blood which usually return to normal with antiviral treatments.) AIDS is the final stage of this disease. (Mehandru et. al. 2004, Douek, et. al. 2003)

Current Treatments

Due to the complexity of HIV and AIDS, their constant evolution, and their effect on a genomic level, no cures are currently available, but some treatments are recommended. Some antiviral drugs are recommended by doctors to lower the virus's spread. (Lalezari, et. al. 2012), as well as Nucleoside Reverse Transcriptase Inhibitors (NRTIs) which slow down the virus's spread by blocking reverse transcriptase. (Eriqe, 2022) Also, there are fusion inhibitors that block HIV from entering the CD4 T lymphocyte of the immune system; (Wolstein, et. al. 2002) as well as CCR5 antagonists which block CCR5 coreceptors on the surface of certain immune cells which limits the ability of HIV to enter the cells. (Lazzarin, et. al. 2003)

New Developments

With advancements in technology and science, new techniques are emerging. One of the newest developments in research is genetic modification in which genes can be edited, altered, deleted, and added to the human genome. Various methods with slight variations are currently in existence. Some methods attempt to create a CCR5 Delta 32 mutation that consists of a 32 base-pair deletion which in turn introduces a premature stop to replication. Others use lentiviral vectors delivered siRNAs as a strategy to protect cells from HIV-1 infection. (Maslennikova, et. al. 2021)

Sleeping Beauty (Transposon)

One class of gene editing procedures uses transposable elements as non-viral gene delivery vehicles to alter portions of the genome. One such method is called Sleeping Beauty. In this method, Sleeping Beauty, uses a cut and paste procedure ultimately transferring DNA from one molecule to the next. (Furushima, et. al. 2012) Some studies have been conducted using Sleeping Beauty transposons to mediate gene transfer of anti- CCR5 and CXCR4 coreceptor siRNAs. siRNAs are small interfering RNAs that disturb the expression of specific genes that have a complementary nucleotide sequences by degrading the mRNA post transcription, thereby preventing translation. PCR simplification and exposure to HIV determined that the lack of these coreceptors is ideal for successfully conferring HIV resistance. However, the studies were conducted in a lab using cultured polypotent cells. In order to determine validity, clinical trials must be conducted using hematopoietic stem cells in vivo.

PiggyBac (Transposon)

Another method of treatment is called PiggyBac - also a DNA transposon. The translocation of genes occurs via

excision from the donor site and insertion into the target site by insertional mutagenesis and transgenesis. (Jung, et. al. 2006) This method promises less potential harm via reintegration. Transposons need to be excised once they complete their job. This makes it less likely to cause genomic damage during mutagenesis, in which multiple transposition events occur in a single genome. PiggyBac restores the site of the insertion to its pre-transposon state with extreme precision (the changes notwithstanding.).

TALEN (Endonuclease Protein)

Another class of gene editing methods uses proteins as their editing tools. One such method is called TALEN. TALENs are chimeric proteins (formed from parts of various animals) that contain two functional domains. (Gaj, et. al. 2013) One is a DNA recognition Transcription Activator Like Effector (TALE.) TALE is comprised of a right and left side which recognizes specific genes and binds them. (Gaj, et. al. 2013) The other is a nuclease domain (N) which dimerizes with another nucleus domain across from it and creates a cut. TALEN is designed to introduce a double-strand break within the nucleus by binding to specific genes, as long as the transcription activators like effectors are lined up correctly. The break creates a mutation in the genome, knocking out genes, which is then healed using cellular repair systems. (Gaj, et. al. 2013) A number of studies were conducted using TALEN to knock out the genes coding for CCR5 coreceptor. Although it is very difficult for TALEN to be transported due to its size and highly repetitive sequences. Great success has been recorded regarding CCR5 deletion and HIV protection. However, the problem lies in the fact that TALEN sequences are prone to rearrangements following their introduction into human cells by HIV. This renders TALEN a method which still requires some perfecting.

CRISPR-Cas9 (Endonuclease Protein)

The most efficient and highly utilized method is called CRISPR-Cas9. CRISPR's unique technology enables geneticists to edit the genome by either removing or adding sections of the DNA sequence in every cell. (Holt, et. al. 2010, DiGiusto, et. al. 2016, Moreno-Mateos, 2015, Upadhyayand Sharma.. 2014, Perez, et. al. 2008) This method is the most precise and versatile yet simple method for genetic manipulation. Additionally, CRISPR-Cas9 is readily available and inexpensive when compared to other methods. Individuals can order their guide RNAs from websites such as http://chopchop.cbu.uib.no/ and https://www.benchling.com/crispr. CRISPR-Cas9 uses two molecules, which are injected into the blood by the millions, to introduce mutations into the DNA of every cell. One molecule is a piece of RNA called guide RNA which consists of a small piece of predetermined sequence (about 20 BP long.) (Holt, et. al. 2010, DiGiusto, et. al. 2016, Moreno-Mateos, 2015, Upadhyay, et. al. 2014, Perez, et. al. 2008) The second is an enzyme called Cas9 which in essence is the molecular scissors that creates the double-strand break at a specified location in the genome. Delivery of CRISPR-Cas9 into cell lines is readily achieved by utilizing an integrative lentiviral vector (which are a family of viruses that can enter cells.). Post insertion, Cas9 is guided by the RNA, using complementary base pairs, to the correct location, and at that point, Cas9 creates the break. Overhanging RNA called scaffold RNA is then inserted into the genome. The cell recognizes the damage in the DNA and repairs itself with minimal damage done (Holt, et. al. 2010, DiGiusto, et. al. 2016, Moreno-Mateos, 2015, Upadhyay, et. al. 2014, Perez, et. al. 2008) Using guide RNA allows scientists to bind specifically to target sequences with extreme accuracy without affecting other regions in the genome. (Mahalingam, et. al. 2002). In one particular study, researchers achieved an average of 42% (±2.1%, n = 3) cleavage efficiency of CCR5 gene from stem cells conferring HIV-1 Resistance in Vivo. (Xu, et. al. 2017)

CCR5 Receptors

Studies show that while CD4 receptors are integral to human survival the CCR5 receptors are not. (Berger, et. al. 1999) The body can manage without the CCR5 receptors. CRISPR-Cas9 can be used to knock out the genes coding for the CCR5 receptor which will prevent HIV from binding to the cells' periphery. Using the CRISPR-Cas9 method can help solve the HIV and AIDS pandemic while avoiding the complications that are attached to traditional treatments. Genetic manipulations are the key to eradicating the virus.

Natural Immunity

The effects of knocking out CCR5 receptors aren't just theories. Studies show that many people are born (mainly in Europe) with a gene defect in the region that codes for the CCR5 receptor. (Lopalco, 2010) Those people not only live a completely healthy life but also are entirely immune to contracting HIV and therefore are safe from AIDS. By using genetic modifications scientists will be able to bypass any issues vaccines may face when HIV mutates.

Conclusion

Based on the aforementioned studies, properties of the CRISPR- Cas9 system include availability, versatility and easy manipulation and should be important points when considering utilizing CRISPR-Cas9 for HIV-1 protection.

Cas9 nuclease can cleave the CCR5 genomic locus around the predicted cleavage site with high efficacy and specificity- as directed by CRISPR. It is important to highlight that importing the components of the CRISPR-Cas9 system into cells using lentiviral vectors, is extremely simple and effective. Although all of the gene therapies have similar results, in which the positively transduced cells with disrupted CCR5 expression are resultantly conferred with HIV-1 resistance, the aforementioned reasons of reliability and simplicity are enough to conclude that CRISPR-Cas9 will be the most effective in conferring HIV-1 resistance.

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Cerebral Organoids as Models for Neurological Disorders

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Abstract

Despite the devastating effects of neurological disorders on millions of people each year, for decades, brain research remained stagnant in the face of scientific advancement in other areas. Ethical concerns, debilitating costs, and a lack of suitable models created an unfriendly environment for the study of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (AD), amyotrophic lateral sclerosis (ALS), autism spectrum disorder (ASD), and gliomas. Recent developments in the field of stem cells, including Yamanaka's discovery of the four transcription factors necessary to induce pluripotency, and the subsequent culturing of induced pluripotent stem cell models known as organoids, opened new opportunities for brain-centered studies. Today, cerebral organoids, mini in vitro 3d models of the human brain, are used to determine disease pathogenesis and potential treatments for neurological disorders. This review explores the achievements, challenges, and possibilities of cerebral organoids as disease models.

Introduction

Scientists have long viewed the brain as an unsolved mystery. Its microscopic neurons and intricate pathways make it difficult to observe and understand. A major obstacle in this field of research is the lack of suitable cerebral models. Obtaining functional human brain tissue is a sensitive procedure (Devine et al., 2011; Gabriel, Gopalakrishnan, 2017). Furthermore, the human brain is more complex than many other species', so animal models serve as poor substitutes for live human subjects (Lancaster et al., 2013; Plummer et al., 2019). These challenges limit the availability and effectiveness of treatment for neurodegenerative diseases. Alzheimer's disease (AD) is the leading cause of dementia worldwide, affecting over 40 million people as of 2019 (Park et al., 2021), while Parkinson's disease remains the most widespread neurodegenerative movement disorder (Devine et al., 2011). Other common neural afflictions, such as autism spectrum disorder (ASD) and amyotrophic lateral sclerosis (ALS), also have no cure (Marchetto et al., 2010; Seminary et al., 2018), while glial tumors, responsible for many brain cancers, are among the most fatal human tumors (Linkous et al., 2019; Goranci-Buzhala et al., 2020). Despite these statistics, until recently, brain research was limited and very costly (Plummer et al., 2019). A finding in 2006 changed the status quo with Japanese researcher Shinya Yamanaka's groundbreaking discovery of induced pluripotent stem cells (iPSCs).

Pluripotent stem cells, or cells from which all cells in the body can be derived, are important to science because of their ability to differentiate into many specialized cell lines. Particularly in the field of neuroscience, pluripotent stem cells are intriguing because they have the potential to serve as in vitro models of the ever-elusive brain. Until the 21st century, however, pluripotent stem cells remained out of reach, primarily because they were difficult to obtain and raised ethical questions due to their embryonic origins. With the Nobel prize-winning breakthrough in 2006, in which Yamanaka identified the four transcription factors necessary to generate pluripotent stem cells from adult somatic cells (namely Sox2, Oct4, c-Myc, and Klf4), the entire research industry changed (Takahashi, Yamanaka, 2006). Scientists now use iPSCs to culture mini-models known as organoids, which can then be employed to study disease pathogenesis and drug efficacy. In 2012, a method for generating brain organoids using Yamanaka's four factors was outlined. The process involved inducing pluripotency in human embryonic stem cells (hESCs), with careful introduction of many additional transcription factors to guide the development of these cells into 3d cultures of neuroepithelial tissue (Eiraku, Sasai, 2012). While this method is still in use, further experimentation honed the process to reduce culture time and generate differentiated brain regions, creating what are now known as cerebral organoids. This review aims to determine whether cerebral organoids can serve as effective in vitro models for treatment of neurological disorders.

Methods

Data for this paper was collected using the Touro College Library and PubMed databases. Keywords included, but were not limited to, "cerebral organoids," "brain organoids," "induced pluripotent stem cells," and "stem cell models."

Cerebral Organoids

Cerebral organoids are in vitro 3d models of the brain derived from human iPSCs. The first documented cerebral organoids were produced by Lancaster and Knoblich, researchers who are also credited with coining the term. To produce the organoids, the researchers used embryoid bodies, cells derived from human iPSCs, to generate neuroectoderm, the precursor to the central nervous system (Lancaster et al., 2013). For the purpose of creating cerebral organoids, neuroectoderm can also be generated from hESCs, as was done in earlier studies. However, recent studies have refrained from using hESCs; this is due to ethical concerns rather than ineffectiveness. Both methods have proven equally capable of serving as in vitro brain models (Linkous et al., 2019; Marchetto et al., 2010). In the case of Lancaster and Knoblich, the neuroectoderm was generated from human iPSCs and then cultured in Matrigel, a matrix that serves as a scaffold for tissue development. Most importantly, the Matrigel drops

were placed in a spinning bioreactor for better absorption of nutrients, a novel technique that formed larger, more consistent, and more stable models than those that were kept stationary. At 8-10 days, the neural cells differentiated into brain cells with specialized functions; at 15-20 days, a fluid-filled cavity formed inside the neuroepithelium; within a month, the neuroectoderm differentiated into clearly defined brain regions, containing a cerebral cortex, choroid plexus, and meninges, among other identifying brain regions (Lancaster et al., 2013). As in earlier models, the cerebral organoids contained both neurons and glial cells, as well as the axons, myelin, and synapses that characterize the human brain and enable cross-communication between cells (Linkous et al., 2019). Once prepared, cerebral organoids are useful for studying the pathogenesis and treatment of neurological diseases. In addition to these critical functions, which will be discussed shortly, cerebral organoids offer the pivotal opportunity to observe brain development in real-time, a role that cannot be filled by fully-developed animal or human brain models. Previously, brain development was mapped out by in utero imaging such as ultrasound, a method that, although successful, has its limitations. With the advent of cerebral organoids, scientists can use reverse transcription polymerase chain reaction (RT-PCR) and staining techniques to track neural development. They observed the differentiation of early neural tissue into three regions, with the forebrain expanding faster than the hindbrain, according to forebrain and hindbrain markers. However, care must be taken when using cerebral organoids to model brain development; researchers found that the early hippocampus and ventral forebrain were not structured identically to those formed in vivo (Lancaster et al., 2013).

Disease Modeling

Cerebral organoids can be induced to exhibit disease pathogenesis by culturing them from iPSCs derived from affected patients. The first recorded incidence of cerebral organoid disease modeling was concurrent with the first instance of cerebral organoid culture. The researchers used the organoids to model microcephaly, a neurological disorder caused by mutations in the CDK5RAP2 gene, resulting in a drastic reduction in brain size. They converted a patient's skin fibroblasts into iPSCs using Yamanaka's four transcription factors, then used the iPSCs to culture cerebral organoids. The organoids displayed markers of microcephaly such as smaller embryoid bodies, indicating an accurate disease model (Lancaster et al., 2013). Another way to generate cerebral organoid disease models is to introduce patient-derived stem cells into a fully-formed cerebral organoid culture. This method was used to create cerebral organoid glioma (GLICO) models. Glioma stem cells, the parent cells for tumor formation, were obtained from cancer patients; they were then co-cultured with individual, healthy cerebral organoids. The cells proliferated in the organoids, imitating the process of tumor formation in vivo (Linkous et al., 2019). This latter method has the added benefit of enabling observation of disease pathology.

In some cases, disease pathogenesis can be induced in healthy stem cells. This can be especially useful where affected patients are not accessible as subjects, or to provide isogenic stem cell models, which allow diseased organoids to be compared to healthy controls. This was the case in a study of AD, which obtained stem cells expressing the parental apolipoprotein E3 (APOE3) gene. CRISPR/Cas9 technology was used to induce the cells to express apolipoprotein E4 (APOE4), a gene commonly associated with increased risk for AD. Using the two cell lines to derive cerebral organoids, the researchers were able to compare the effects of both gene variants on neural development, an added benefit over using a single diseased cell line (Lin et al., 2018). A similar technique was employed to derive stem cell models with markers of Parkinson's disease (PD). Healthy embryonic stem cell lines, induced to express the SNCA-A53T mutation associated with PD, were used to generate dopaminergic neurons as disease models. Some cells were deliberately not induced and were set aside as isogenic controls. For the same reason, patient-derived iPSCs carrying the SNCA-A53T mutation were used to generate dopaminergic neurons; the mutation was corrected in some cells to provide isogenic controls (Ryan et al., 2013). It must be noted, however, that only dopaminergic neurons, and not cerebral organoids, were generated in this case. Thus, although they expressed the [a]-synuclein and Lewy body pathology characteristic of PD, it is unclear whether these stem cells serve as effective models for the disease. In addition to providing healthy and diseased isogenic models, cerebral organoids confer the added advantage of allowing scientists to choose which genes or characteristics to express. One study on autism spectrum disorder (ASD) examined 53 iPSC lines from 25 affected patients, each carrying different genetic variants of the disease. The stem cells were differentiated into glutamatergic neurons, which were used to compare neural activity in the various mutations (Deneault et al., 2019). However, because the iPSCs were not cultured into GABAergic neurons, which have an inhibitory effect on the brain, or into the many other cells that make up cerebral organoids, the results may have been skewed. Nevertheless, the approach could

be used to compare the effect of different mutations of a disease. With regard to characteristics, an in-depth study of AD generated 1300 organoids with different key markers of the disease. Their iPSCs included Pittsburg compound B (PiB) positive and negative cell lines, a second strong indicator of AD, in addition to iPSCs derived from APOE3 and APOE4 cell lines (Park et al., 2021). They were then able to generate organoids with any combination of these markers, among others. This diversity enables scientists to model a disease under a variety of circumstances, creating the potential for drug tailoring based on which hallmarks of AD a patient possesses.

Treatment of Disease

It is important to determine whether cerebral organoids can serve as models to test potential treatments for disease. To determine the efficacy of ionizing radiation in treating glioma, scientists compared the effects of radiation on their 3d GLICO disease models to the effects of radiation on simpler, 2d glioma models. They observed that radiation had reduced ability to inhibit tumor growth when compared with the drastic apoptotic effects of radiation on 2d glioma models. The poor response observed in organoids concurs with the real-life observation that radiation is usually ineffective in treating glioma patients (Linkous et al., 2019). Thus, the outcomes indicate that 2d glioma models are unreliable because they give artificially inflated results, whereas cerebral organoids serve as better models because of their greater accuracy. Although this doesn't irrefutably prove that cerebral organoids are ideal models for treatment of disease, it does indicate that they perform better than the 2d models formerly used.

When it comes to testing treatments for disease, cerebral organoids can also be used to rule out ineffective treatments, saving years of wasted effort. In their study of microcephaly, scientists used RNA interference (RNAi), a process that uses RNA to selectively suppress gene expression, to inhibit destructive CDK5RAP2 activity in microcephalic organoids. Accordingly, they observed that the number of neurons increased in vitro, confirming that CDK5RAP2 is the gene behind microcephaly. This conclusion suggests that selective RNAi of CDK5RAP2 might be a possible treatment option for patients with microcephaly. The researchers also made another important conclusion; when they introduced healthy CDK5RAP2 into the diseased culture, larger neural tissue was observed. The latter method, however, proved toxic to cells, eliminating it as an effective treatment option (Lancaster et al., 2013). Likewise, in a study of Rett Syndrome, a neurodevelopmental disorder classified as an ASD, iPSC-derived neurons were used to test treatments for the disease. The neurons, while not complete organoids, possessed MeCP2 mutations, a major disease marker, as well as the decrease in synapses and soma size present in Rett Syndrome. Treatment of the cells with IFG-1, an insulin-like growth-factor often studied in relation to Rett Syndrome, had a positive effect on synapses but stimulated excitatory neurons to abnormal levels. While this does not preclude IFG-1 as a potential candidate for treatment of the disease, it does indicate that care must be taken when the therapy is used (Marchetto et al., 2010). Thus, although stem cells models, and not cerebral organoids, were used in this case, the study highlights the capacity of organoids to identify or eliminate treatment options.

Drug Testing

Cerebral organoids provide the opportunity for accurate, personalized medicine through the use of brain models derived from iPSCs of an affected patient. One study on anticancer therapy used glioma tissues derived from Johns Hopkins surgical patients to test the efficacy of temozolomide (TMZ) and doxorubicin in treating glioblastoma. The success rates, at approximately 30% reduction and 80% dose-dependent reduction of cultured tumor cells, respectively, indicate that patient-derived iPSCs can serve as effective models for drug testing (Plummer et al., 2019). Whether the indicated dosages would be ideal for said patients is unknown, as the study did not examine the effect of these drugs on the patients. This could potentially be an area for future study. Another study compared the effects of TMZ and bis-chloroethylnitrosourea (BCNU), another anticancer therapy, on both GLICO and 2d models. They found that while the drugs exhibited drastic dose-dependent decrease of tumor cells in 2d models, with 80% and 90% success rates, respectively, the drugs were only moderately effective in reducing tumor cells in GLICO models, with respective 24-43% and 5% decreases (Linkous et al., 2019). The limited effectiveness observed in GLICO models compares to the weak response to these drugs evident in live subjects. Once again, this confirms the theory that cerebral organoids are superior to 2d cultures as glioma models, because their behavior more closely resembles that observed in actual glioma patients. Additionally, the similar outcomes obtained by both studies in regard to the efficacy of TMZ in treating gliomas (a 30% vs 24-43% reduction in tumor cells) indicates the reliability of cerebral organoids as models for drug testing.

Although the amount of research available on drug testing using cerebral organoids is limited due to the relative novelty of the process, multiple drug screenings performed using alternate stem cell models indicate the

strong potential of cerebral organoids for drug testing. For example, a drug screening of motor neurons cultured from patient-derived iPSCs identified ropinirole, a drug used to treat PD, as a candidate for treatment of sporadic amyotrophic lateral sclerosis (ALS), a disorder involving loss of movement. To evaluate drug efficacy, a new screening system designed to screen for multiple phenotypic changes was introduced. Disease models were treated either with DMSO, a solvent, designating them as controls, or with a drug dissolved in DMSO. They were then evaluated for multiple markers of ALS, including changes in neurite length, number of formed stress granules, and leakage of FUS protein aggregates and LDH. Subsequent increases and/or decreases in these markers were used to indicate the success of a particular drug (Fujimori et al., 2018). Although this technique was only tried using motor neurons, and not cerebral organoids, the system could be applied to other diseases.

Similarly, a comprehensive screening of over 1,000 drugs on iPSC-derived AD cortical neurons indicated that a drug cocktail of bromocriptine, cromolyn, and topiramate could lower the count of toxic β-amyloid plaques. Three different solvents, used as positive and negative controls, helped evaluate the efficacy of the drugs and narrow down the drug pool to 27 possibilities. A widely accepted fingerprinting method was then utilized to determine the best combination, taking factors such as efficacy and toxicity into account (Kondo et al., 2017). It is unknown whether this cocktail demonstrated improvement in actual trials, but it's possible that the methodology used in this study could be applied to screen cerebral organoids.

Challenges of Cerebral Organoids

One challenge to using brain organoids for drug testing is finding a technique for visualizing markers of disease in the organoids. To overcome this challenge, researchers developed a new screening platform called microTMA, a spheroid tissue microarray that enables the viewing of multiple organoid cross-sections on a single slide, for a comprehensive 3d image. Using this platform, the developers successfully tracked the effect of anticancer drugs on glioblastoma brain sphere models. This screening system performs similarly to the older Polaris/Inform system, a screening technique that is beyond the scope of this paper, but reduces the image acquisition time by more than 95% compared to the original screening technique (Plummer et al., 2019). For disease-specific screening, a more customized approach may be necessary. For instance, when developing in vitro models of AD, one study used two forms of positron emission tomography (PET), PiB-PET and tau-PET, to screen cerebral organoids for β-amyloid plaques and phosphorylated tau protein, two pathological hallmarks of AD. They consistently applied these screening techniques throughout the study to monitor drug efficacy (Park et al., 2021).

Critics of cerebral organoid research point out that organoids vary in size from sample to sample, which may lead to inconsistent results. However, this argument can be made irrelevant by using identical samples. For instance, researchers of AD put their organoids through intensive quality control to determine that only organoids uniform in shape and size were used (Plummer et al., 2019). Nevertheless, it should be noted that this method requires the discarding of many organoids that fail to meet physical requirements. Another solution, employed on stem cell models of ALS, is to use a bulk culture system in which multiple models are derived from many stem cell lines. Rather than disposing of heterogenous models, a clustering system was developed to separate the models based on different disease pathologies. The models were then used to study differing forms of ALS, suggesting that there is an advantage to diversity (Fujimori et al., 2018). However, there are times when uniformity is needed. A novel protocol, adapted from the original method proposed by Lancaster et al., gives researchers more control over organoid development, avoiding the problem of heterogeneity altogether. In this process, neurons are differentiated directly from iPSCs, avoiding the formation of embryoid bodies that spontaneously form unwanted germ cell layers. This method, successfully used to model microcephaly, results in brain organoids with fewer variations and defects (Gabriel, Gopalakrishnan, 2017). Perhaps further research using this protocol could contribute to uniformity of cerebral organoids.

Areas for Future Research

There remains the question of whether drug absorption in brain spheres is comparable to drug absorption in vivo. In actuality, the human brain is isolated from circulation by a blood-brain barrier (BBB) that selectively allows only 5% of drugs across its borders in significant enough dosages to have a pronounced effect (Ribecco-Lutkiewicz et al., 2018). To avoid the pitfall of testing drugs that are not permeable to the BBB, researchers of AD tested only FDA-approved drugs known or suspected to have BBB permeability (Park et al., 2021). While a solution, this is a severe limitation of the organoids because it confines the study to available treatments. Towards that end, several attempts have been made to generate an in vitro model of the BBB using iPSCs to generate a 2d monolayer of epithelial cells. These models have been shown to effectively evaluate drugs for BBB-permeability (Ribecco-Lutkiewicz

et al., 2018). However, to this author's knowledge, no study to date has cultured an in vitro model of brain organoids in conjunction with an epithelial BBB. The introduction of a BBB might provide more therapeutic options.

Brain organoids with a BBB may provide a more complete model for drug testing, but still fail to accurately model drug penetration due to their lack of a circulatory system. Some propose that this factor is negligible due to previous studies indicating high drug penetration in organoids (Plummer et al., 2019). However, it is possible that drug screening platforms using current in vitro models are misleading. The developers of cerebral organoids note that cell death occurs in the core of the brain tissues after several months in vitro, most likely because of their lack of a blood supply, which restricts their oxygen and nutrient intake. Also of note, these missing factors probably explain why the organoids stop growing once they reach a certain size (Lancaster et al., 2013). Perhaps further research could lead to the development of a cerebral organoid containing a capillary system, which would better simulate the real-life internal environment.

Moreover, some cerebral organoids developed to model AD did not contain microglial cells due to the unique circumstance regarding their origins (Park et al., 2021). This is a major downside because microglial cells function in an immune capacity in the central nervous system. In addition, microglia express certain genes that are associated with an increased risk for late-onset AD, making them an important component in any in vitro AD model (Abud et al., 2017). In a study that did incorporate microglia-like cells into their cerebral organoids, the cells expressing APOE4 experienced reduced β-amyloid uptake and impaired response time when cultured in organoids, indicating that microglia may contribute to the β-amyloid buildup that is so detrimental to AD (Lin et al., 2018). Thus, introducing microglial cells into cerebral organoids might generate different responses to drug testing. Although actual microglia are difficult to obtain, owing to their complex lineage which traces back to yolk sac erythromyeloid progenitors that migrate into the neural tube during development, iPSC-derived microglial cells make an acceptable substitute. In fact, microglia-like cells generated from iPSCs are shown to mimic the activity of human fetal and adult microglia (Abud et al., 2017).

There is one area where cerebral organoid research is critically lacking. Although PD is the second most common neurodegenerative disease, stem cell research on the topic is severely lacking (Zambon et al., 2019). Several studies have generated midbrain dopaminergic neurons with markers of PD, but these are missing the complete cerebral environment necessary for accurate drug testing (Ryan et al., 2013; Laperle et al., 2020; Devine et al., 2011; Fernandez-Santiago et al., 2015; Zambon et al., 2019). Others have developed full cerebral organoids, but with a focus on external factors, such as response to viral infection, rather than treatment options (Schultz et al., 2021). Despite the capacity of cerebral organoids for identifying potential drug candidates, little research has been done in this realm with regard to PD. In fact, to this author's knowledge, no published study to date has produced cerebral organoid models of PD with the express intention of performing a drug screening. It is likely that future cerebral organoid PD models, with an emphasis on treatment, could lead to a breakthrough in this disease.

Conclusion

Since their entrance into the scientific industry in 2013, cerebral organoids have changed researchers' approach to the study of neurological disorders. Derived from the iPSCs of affected patients, or from the stem cells of healthy subjects, cerebral organoids exhibit optimal growth when cultured in Matrigel placed in a spinning bioreactor. Perhaps avoiding the step of embryoid bodies could further enhance the process by reducing inconsistencies. Once formed, cerebral organoids can serve as in vitro models, enabling the observation of brain development and/or disease progression. If healthy organoids were produced, disease pathology can be introduced via affected stem cells, offering the opportunity to study isogenic disease and control groups. In addition to RT-PCR and staining, advanced screening techniques, such as the MicroTMA platform or other disease-specific imaging technology, may be necessary for visualizing disease pathology in the organoids. Overall, cerebral organoids perform better than their earlier, 2d counterparts, indicating success in that their results compare to phenomena seen in human subjects. Thus, they appear to serve as effective models for the investigation of potential treatments, and can likely be used to rule out ineffective therapies and explore new treatment options. Furthermore, cerebral organoids show satisfactory progress in the area of drug screening. Although more studies are necessary to make a definitive conclusion, similar trials carried out on alternate stem cell models suggest that cerebral organoids could be used to identify drug candidates. Cerebral organoids could be improved by the introduction of a blood-brain barrier and a capillary system, which would portray drug permeability and penetration more accurately. Moreover, the addition of microglia-like cells, which were omitted in some organoids, seems to be a critical aspect of some disease models, especially of AD. Further research centered on treatment, particularly in regard

to PD, could possibly lead to breakthroughs in this field. Consequently, while there is a long way to go, the signs indicate that cerebral organoids can successfully serve as in vitro models for the analysis and treatment of neurological disorders, potentially opening new doors in medicine.

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How is H. pylori Implicated in the Etiology of Cancer?

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Abstract

Gastric cancer is a major public health concern due to the many deaths it is associated with. H. pylori is present in almost all gastric cancer patients; thus, it is fundamental to understand how H. pylori is implicated in the etiology of gastric cancer. The research in this paper is primarily based on studies acquired from Touro College Online Libraries. The bacteria have the means to survive the harsh environment of the stomach by creating a safe microenvironment and propelling themselves toward safer territory. If the bacteria attach to host cells, H. pylori injects cytotoxin-associated protein A (CagA) and vacuolating cytotoxin A (VacA) into their host, inducing inflammation and disrupting cell functions. H. pylori continues to survive at the surface epithelia through various functions and causes damage to epithelial cells. Following gastritis, ulceration or gastric cancer may develop. Mechanisms of the proliferation of cells are abundant but stem from the destruction of cells due to inflammation. The prognosis of gastric cancer is poor due to the advanced stage of cancer at the time of detection. Surgical removal of the tumor, although sometimes employed, is not always recommended or successful. Since a large majority of individuals are infected with non-pathogenic strains of H. pylori, treatment isn't always suggested. Moreover, some research suggests that there are benefits to being infected with H. pylori.

Introduction

It was long believed that duodenal and gastric ulcers were caused by stress and lifestyle. In the 1980s, Warren and Marshall (reported in Huang 2016) the association of a spiral-shaped, Gram-negative bacterium with gastric and duodenal ulcer biopsies. They published these findings but were not believed by many because of the belief that bacteria cannot live in the stomach due to its harsh conditions of a low pH. Failure to induce animals with H. pylori prevented animal experimentation. Dr. Marshall infected himself with the bacteria and developed pain and inflammation. Endoscopy revealed the same spiral shape bacteria (Huang, 2016). This supported his and Warren's hypothesis. Antibiotic treatment trials prevented the relapse of ulcers. They were awarded the Nobel Prize in 2005 for their link of Helicobacter pylori to gastritis and peptic ulcer disease ("The Nobel Prize in Physiology or Medicine 2005"). H. pylori infects about half of the world's population, although it is not equally distributed in each geographical location. It is not fully understood how it is contracted but is assumed to come from contaminated drinking water or through the fecal-oral route. Infection generally occurs during childhood; if not treated with antibiotics, it can last a lifetime (Perkins et al., 2013).

Gastric cancer is one of the leading causes of death worldwide. Although a large percentage of individuals in every population are infected with H. pylori, many are asymptomatic (Min Ho Lee et al., 2019). Less than one percent of those infected with H. pylori will develop gastric cancer; however, those with gastric cancer present with H. pylori in most cases (Min Ho Lee et al., 2019; Xie et al., 2008). Many studies show that H. pylori infection is associated with a host of stomach problems, including gastric adenocarcinoma, peptic ulcers, gastric inflammation, and chronic gastritis (Min Ho Lee et al., 2019). Because of this, the WHO has classified H. pylori as a class I carcinogen (Min Ho Lee et al., 2019).

This paper is intended to be a critical review of research projects that have been conducted to better define the mechanisms by which H. pylori leads to gastric cancer. A better understanding of the connection would provide specific methods of prevention as well as treatments to target the progression of the bacterial infection. Just as Dr. Warren and Marshall successfully proved that H. pylori is linked to gastritis and peptic ulcer disease, perhaps it can be determined how H. pylori is implicated in the etiology of gastric cancer.

Methods

Data for this paper was compiled from the databases of Touro College Online Libraries, primarily ProQuest, and was supplemented by additional websites. Keywords used include H. pylori, gastric cancer, CagA, and gastroesophageal reflux disease (GERD).

Discussion

Mechanisms of Infection

H. pylori is a bacterium only found in the digestive tract (Fung, 2018). Although it lives in the stomach, H. pylori is a neutrophile with optimal growth conditions at a slightly acidic pH (Liao, 2020). Since the pH in the stomach is about two, the bacteria have two ways of surviving the harsh environment. First, H. pylori contains the protein urease, which breaks down urea into ammonia and carbon dioxide. The urease breath test contains carbon thirteen marked urea, which gets converted to carbon thirteen marked carbon dioxide in H. pylori positive patients. Ammonia allows the bacteria to neutralize the acid, creating a microenvironment with a higher pH. Second, by use of their flagella, the bacteria swim away from the low pH of the stomach to the mucosa layer, which has a higher pH. (Huang, 2016). The TlpB protein detects low levels of pH (Liao, 2020). Once in the mucus covering of the epithelial lining, the bacteria can circulate freely in their microenvironment. Some strains of the bacteria are equipped with fimbriae, allowing them to attach to the surface of the epithelial cells that make up the glands that secrete the mucus. The bacteria then divide, creating

microcolonies at the junctional complexes (Huang, 2016). This is where the problem starts.

Virulence factors of H. pylori

H. pylori can live in the mucus layer without causing symptoms. This is the large subset of those infected by H. pylori but not affected by it. They present with moderate gastritis and mild changes in acid secretion (Fung, 2018). Microcolonies that are attached to the surface of the epithelial cells have been found to cause more prominent inflammation, leading to complications (Dorer et al., 2010). It has been reported that the most well-known virulence factors of H. pylori are the cytotoxin-associated protein A (CagA) and vacuolating cytotoxin A (VacA) proteins present in pathogenic strains of H. pylori (Saha et al., 2010). These bacterial proteins are secreted into the host after contact is made via type IV and type V secretion systems (T4SS, T5SS). Once injected, CagA distorts epithelial junctions and cell morphology, promotes inflammation, and, more importantly, takes control over signaling pathways. Both CagA and VacA migrate to the periplasm of the bacterial cell through Sec-related proteins, which operate with ATP.

VacA generates vacuole production and eventually leads to apoptosis. It does this by changing the mitochondrial membrane potential in the host cell and causing the mitochondria to release cytochrome c, which starts the breakdown of cells (Min Ho Lee et al., 2019). VacA also prevents the actin filaments from adhering to the parietal cells at the lumen (Saha et al., 2010). The bacteria remain attached to the surface of the epithelial cells and inject these proteins. This causes chronic inflammation in the gastric mucosa and the presence of many pro-inflammatory cytokines such as nuclear factor-kB (NF-kB) and interleukin 8 (IL8). NF-kB activates the specific genes that control inflammation in the gastric mucosal layer, one of which is IL8 (Min Ho Lee et al., 2019). IL8 is associated with tumor growth and secondary tumors. Additionally, IL8 plays a role in angiogenesis, the formation of new blood vessels (Kang et al., 2013). Studies have shown that those with increased levels of IL8 in their blood are at an increased risk for gastric cancer (Epplein et al., 2013). IL8 inhibits the H, K ATPase on parietal cells from functioning, raising the pH (Saha et al., 2010).

Essentially, by releasing CagA and VacA, H. pylori increases inflammation. Thus, it is important to understand how some of the strains of H. pylori adhere to the surface.

Survival of H. pylori at the surface epithelia

Anemia is a less common symptom of H. pylori. H. pylori thrives in iron-deficient hosts. CagA acquires iron from the host cell by altering cell activity. Catalase, one of the proteins of H. pylori, breaks down hydrogen peroxide, which is produced by inflammation, into water and hydrogen gas, which are less toxic substances. This is thought to aid in the survival of H. pylori (Fung, 2018).

Some strains of the bacteria make their way through the pits and down into the base of the gastric glands, where they colonize epithelial cells. Deep in the glands are stem and epithelial precursor cells. It is here where the bacteria affect proliferation, in addition to inflammation and hyperplasia leading to gastric cancer (Fung, 2018).

Ulceration vs. Gastric Cancer

According to many research studies, those who develop ulcers do not develop gastric cancer. The reason for this "protection" is largely unknown but thought to be associated with specific sites of colonization and levels of hypochlorhydria. Ulcers develop via changes in cell structure and inflammation, leading to apoptosis of epithelial cells. CagA is responsible for inflammation. Chronic inflammation leads to peptic ulcers in ten to fifteen percent of those infected with H. pylori. Persistent inflammation in the antrum (Fig. 1) results in increased acid production in the corpus, and this causes damage to the duodenum, which responds with gastric metaplasia. Some argue that H. pylori can colonize the modified duodenum as opposed to regular duodenal epithelial cells, which H. pylori does not infect. Ulcers form in the corpus and antrum, i.e., gastric ulcers, as well as in the duodenum.

Treatment of ulcers is the same as the treatment of H. pylori, whereas treatment for gastric cancer is not particularly effective since detection most often occurs at advanced stages (Fung, 2018). In light of these findings, H. pylori infection of the antral mucosa is thought to be associated with gastric and peptic ulcers, while infection of the oxyntic mucosa, mucus of the fundus and corpus (Fig. 1), is associated with gastric cancer (Waldum et al., 2015).

Evidence for a Causative Role in Gastric Cancer

Most patients that have gastric cancer are positive for H. pylori. Although rare, some gastric cancer patients do not have H. pylori, and these patients generally have less malignant tumors (Tanaka et al., 2022). H. pylori positive gastric cancer patients treated for the infection were compared to a control group who did not receive antibiotics. The assessment was conducted to take note of additional tumor development. In the group that was treated, fewer patients developed additional tumors. Moreover, no significant side effects were associated with the eradication of H. pylori (Kikuchi et al., 2008).

Proliferation of Cells

Inflammation serves to promote cancer development and progression due to its fertile and pro-growth environment (Epplein et al., 2013). Different theories exist as to how gastric cancer develops from the inflammation caused by H. pylori.

CagA activates NF-kB, although it is arguable how significant CagA is to the activation. NF-kB regulates the expression of the enzyme phospholipase D1 (PLD1), which is upregulated in those with gastric cancer as well as other cancers. PLD1 breaks down phospholipids and is associated with cell growth (Kang et al., 2013).

CagA causes upregulation of cyclo-oxygenase-2 (COX-2), which is an enzyme responsible for the synthesis of prostaglandins (Fig. 2). COX-2 is angiogenic, which provides nutrients to cancerous cells. E-cadherin is responsible for cell adhesion and is produced from epithelial cells. COX-2 frees E-cadherin from the cell membranes, promoting the spread of tumors. When inflammation is present, E-cadherin is liberated, and soluble E-cadherin is found in elevated levels in the blood, a risk factor for cancer. The loss of cell-cell adhesions is thought to aid H. pylori in progressing further into the lamina propria, where it can continue to cause damage. One study suggests that the presence of Cox-2 prevents apoptosis of malignant tissue (Anwar et al., 2012).

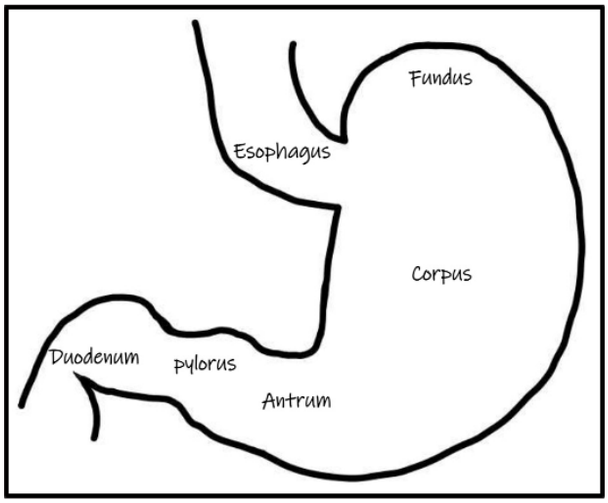

Fig. 1 Diagram depicting regions of the stomach

Once in the host, CagA becomes phosphorylated and causes abnormal epithelial cell proliferation (Saha et al., 2010). Non-atrophic gastritis begins with the presence of an inflammatory response of white blood cells in the gastric mucosa of the corpus (Fig. 1). Gastric glands are destroyed due to chronic inflammation causing atrophic gastritis. Due to the loss of the parietal cells, the pH of the stomach rises, which is called hypochlorhydria, which leads to hypergastrinemia, excess gastrin production (Fig. 2). Hypochlorhydria triggers stem cells and precursor cells to proliferate. During this time, cells may mutate, leading to adenocarcinoma (Fung, 2018). Additionally, it is suggested that since the function of acid in the stomach is to kill ingested organisms when the acidity is lessened, other microbes may be able to survive, leading to cancer (Waldum et al., 2015).

Hypergastrinemia also stimulates the chief cells to produce pepsinogen and the proliferation of enterochromaffin-like (ECL) cells (Fig. 2). Because of this, H. pylori positive patients have high levels of pepsin, the active form of pepsinogen, as well as gastrin, in their blood (Horiuchi et al., 2016; Waldum et al., 2015). A positive pepsinogen test is a risk factor for atrophic gastritis (Horiuchi et al., 2016). The use of netazepide, a gastrin antagonist, was found to reduce atrophy and inflammation in the oxyntic mucosa. ECL cells produce histamine and send it to neighboring parietal cells to help with the secretion of acid. One study demonstrated that loxtidine, a histamine-2 blocker, was found to protect against neoplasia, indicating the possible mutated histamine from ECL cells. Additionally,

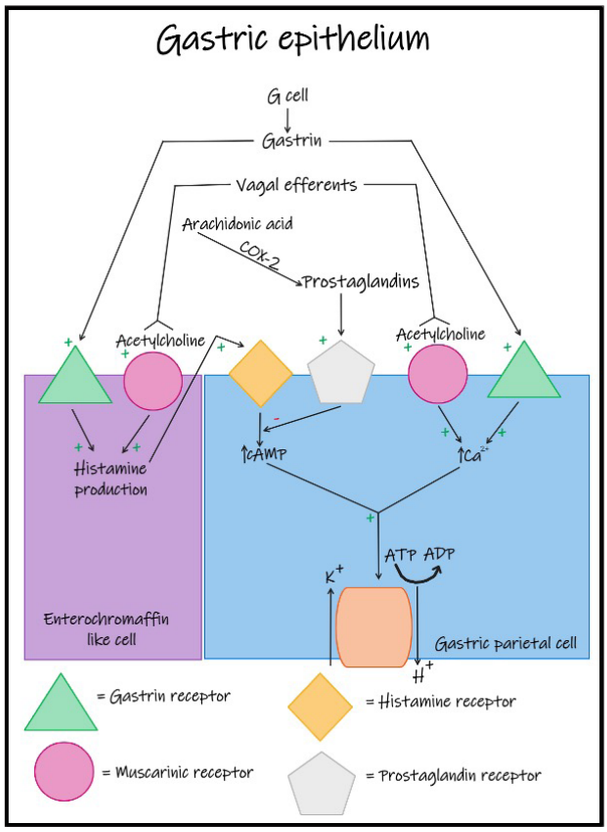

Fig. 2 Diagram depicting gastric epithelial cell processes

if unresolved, chronic hypergastrinemia leads to tumor formation in ECL cells (Waldum et al., 2015).

Treatment of Gastric Cancer

As mentioned previously, the prognosis for gastric cancer is poor due to late-stage detection of cancer (Fung, 2018). If found early, endoscopic resection, surgical removal of the tumor, is recommended. Unfortunately, this treatment isn't one hundred percent effective due to recurrent tumor development shortly after treatment in three to ten percent of patients. Moreover, predictions as to which patients are likely to have recurrent cancer have not been successful, requiring yearly endoscopies to determine whether a tumor has developed (Sato et al., 2019).

Treatment of H. pylori - Benefits and Drawbacks

Since most individuals with H. pylori are asymptomatic and complications are not usually prevalent, treatment is not always necessary. Current medical practice is to treat all those who test positive. Testing is recommended for those with a family history of gastric cancer and those who live in geographical locations with high incidence and prevalence rates of gastric cancer, as well as those with symptoms (Crowe, 2019). Early detection and treatment of gastric cancer are crucial for survival; therefore, prevention is a major factor (Tanaka et al., 2022). However, many individuals infected with H. pylori do not develop complications such as gastric cancer or ulcers. Furthermore, it generally takes more than thirty years for H. pylori infection to progress to gastric cancer (Sato et al., 2019).

Perhaps instead of testing and treating all those in the above-mentioned categories, testing can be confined to specific parameters. ,Although more likely to develop complications, these individuals may still be asymptomatic and not endangered. H. pylori is currently detected via a urease breath test, a stool test, or an endoscopy (Crowe, 2019). It is also suggested that there are benefits to the inhabitance of H. pylori, and eradicating it unnecessarily isn't suggested. Moreover, H. pylori infection is not always easily treated as it is often resistant to antibiotics requiring multiple rounds of treatment. Tests for successful eradication are common practice (Jonaitis et al., 2021). Frequent use of antibiotics for unnecessary reasons is not recommended. Testing for H. pylori should perhaps include blood work in addition to standard detection methods. Testing can include markers of elevated gastrin, CagA, pepsinogen, etc., to determine if the bacterium is causing harm to the one infected.

A cost-effectiveness study that took place in China analyzed the monetary benefits of testing the general population above the age of forty for H. pylori. The researchers compared the cost of testing and treating the individuals positive for H. pylori to the cost of treating those with gastric cancer due to undiagnosed and untreated H. pylori. They concluded that testing their population did not prove to be beneficial monetarily. The study was done on both males and females but suggested that testing males only might be beneficial since gastric cancer is more prevalent in men (Xie et al., 2008).

Another study of individuals with H. pylori focused on those who had close relatives with gastric cancer. The researchers compared those treated with antibiotics and a proton pump inhibitor to those given a placebo. The authors reported that the eradication of H. pylori reduced gastric cancer by fifty-five percent. Additionally, those with successful eradication of H. pylori were at a seventy-three percent lower risk of developing gastric cancer. Those with unsuccessful eradication were at comparable risk to those who took the placebo. Participants in this study were monitored for slightly over nine years post-treatment (Choi Il Ju et al., 2020).

Esophageal Consequences and other Potential Benefits

One school of thought believes that CagA positive strains of H. pylori are actually beneficial for those with GERD in preventing Barrett's esophagus and adenocarcinoma of the esophagus (Mishra, 2013). Significant differences aren't observed with those who have GERD or Barrett's esophagus in regard to the prevalence of H. pylori; however, a study found all those with long segmented Barrett's esophagus lacked CagA positive strains of H. pylori. This suggests that CagA strains lower the acidity in the stomach, which causes less damage to the esophagus when gastric juice leaks through the sphincter. Eradication of H. pylori reduces the pH of the stomach, leading to Barrett's esophagus and adenocarcinoma (Vaezi et al., 2000).

On the other hand, another study showed that patients with H. pylori eradication were not found to have a significant impact on the erosion of the esophagus (Na et al., 2020). However, the study did not mention if the strains of bacteria were CagA positive or not, which can cause discrepancies. If they are negative for CagA, then the strain is less pathogenic, does not alter the acid level in the stomach, and would therefore make sense not to have an impact on symptoms or progression of GERD.

Aside from esophageal benefits, other advantages have been noted. Some research has shown that the presence of H. pylori has a protective effect on asthma as well as decreased diarrhea in children (Mishra, 2013).

Conclusion

H. pylori is a precursor for gastric cancer. The various means through which a malignant tumor develops have been outlined. Knowing the damage H. pylori causes, some of the symptoms of H. pylori, such as gastritis and iron-deficient anemia, can be understood. While prevention of gastric cancer is crucial as many lives are lost each year, eradicating H. pylori from all those who test positive may have consequences. Eradicating H. pylori to prevent complications that seldom occur has to be weighed against the benefits of its presence in preventing other complications.

Owing to the high resistance associated with traditionally-used antibiotics for H. pylori infection, other medications are being researched that target specific pathways that H. pylori optimize. Menadione, a laboratory-manufactured vitamin K (often referred to as K3), has been found to demonstrate antibacterial effects by preventing NF-kB from being activated, reducing inflammation, preventing the expression of VacA, and so forth (Min Ho Lee et al., 2019). Other ways of combating H. pylori may help reduce antibiotic resistance.

Since H. pylori has been around for a long time and is present in most individuals, some argue that the bacterium is part of the normal gastric flora. "H. pylori is just one bug that is isolated from a bacteria-rich stomach environment. And, like many bacteria in our digestive system, it is not only harmless when kept in balance with the other microbes, but may even be beneficial (Mishra, 2013)."

Perhaps research should focus on early detection of gastric cancer and other preventative measures to help minimize mortality from gastric cancer. Focus on methods of treating late-stage gastric cancer will undoubtedly save many lives.

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Physical Therapy in the Treatment and Prevention of Pelvic Floor Dysfunctions in Women

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Abstract

Pelvic floor dysfunctions are a widespread health issue that significantly disrupts the quality of life of those afflicted. This paper aims to present the research that proves how physical therapy, specifically pelvic floor muscle training, is an effective way to treat and prevent pelvic floor dysfunctions in women. Extensive research was done on the topic by exploring numerous scholarly databases and analyzing relevant articles on experimental trials, studies, and their results. After gaining an understanding of pelvic floor dysfunctions, studying the methodology of pelvic floor physical therapy, and examining numerous successful clinical trials, it has been determined that physical therapy is indeed beneficial in treating and preventing certain pelvic floor dysfunctions. Further research must still be conducted to establish the exact efficacy of pelvic floor physical therapy, determine which combination of treatment modalities achieves optimal results, and see if it is effective for various kinds of dysfunctions. However, pelvic floor physical therapy may be the key to restoring the quality of life in women suffering from pelvic floor dysfunctions.

Introduction

Pelvic floor dysfunctions (PFD) are extremely prevalent. Millions of women are affected by this widespread health problem which significantly affects their quality of life and decreases productivity. As many as 46% of women report having at least one form of PFD, and many suffer from more than one dysfunction (Milsom, 2015). PFDs can be described as weakness or damage to the muscles or tissue of the pelvic floor, primarily due to trauma or overuse of the muscles, and hinders their ability to contract or relax correctly. It causes uncomfortable symptoms such as urine or stool leakage, bloating, constipation, pelvic pain, and frequent urination. Besides for being a severe medical condition, PFDs create social difficulties as well. People with PFDs will avoid social interactions and physical activities, fostering embarrassment and negative self-perception, which affects their emotional and psychological health (Dumoulin et. al., 2018). This withdrawal may be further debilitating to a woman's overall well-being because physical activity is crucial to maintaining health and preventing anxiety, depression, high blood pressure, heart disease, obesity, and some cancers (Bø, Sherburn, 2005).

Women's Health Physical Therapy, a relatively new and growing specialty in the physical therapy world, refers to specific training done by a physical therapist to assess and treat diagnoses pertaining to women, especially those related to the pelvic floor muscles (King, 2013). With the emergence of this field, pelvic floor physical therapy became patients' first choice line of treatment due to its low risk and availability. The question then became, is physical therapy an effective way to treat PFDs? Moreover, if it is an effective treatment, can it also be used as a preventative measure for those at risk for PFDs?

Methods

This research was done by critically analyzing peer-reviewed and original articles accessed through Touro's online library database. Literature was taken primarily from the PubMed, ProQuest, and Google Scholar databases. A comprehensive review and analysis were done on each article to determine its relevance to the thesis of the effectiveness of physical therapy in treating and preventing pelvic floor disorders.

Anatomy of the Pelvic Floor

In order to properly understand the connection between weak pelvic floor muscles and their dysfunctions, a brief review of the anatomical structure of the pelvis is required. The bony pelvis, or pelvic girdle, is comprised of two hip bones fused to each other at the pubic symphysis. Each hip bone contains three parts; the ilium, the ischium, and the pubis. The broader, superior section is known as the greater pelvis, and the narrower, inferior section is known as the lesser pelvis. The pelvic inlet separates the greater and lesser pelvis. The pelvic outlet is the inferior opening of the pelvis, and it is closed by the pelvic floor (Herschorn, 2004). The space between the pelvic inlet and the pelvic outlet, which contains the urinary bladder, colon, and reproductive organs, is known as the pelvic cavity, or true pelvis. The female pelvis differs from that of a male because it has a wider diameter and a more circular shape. This wider pelvic outlet predisposes women to pelvic floor weakness (Herschorn, 2004).

The pelvic floor is made up of numerous muscles, classified into superficial and deep muscle layers. The levator ani and coccygeus muscles comprise the deep muscle layer and form the pelvic diaphragm. Two major muscles make up the levator ani: the iliococcygeus and pubococcygeus muscles. These originate from the pubic bone and insert into the coccyx. The puborectalis muscle originates from the pubic symphysis and encircles the rectum. Although it lies between the deep and superficial layers, the puborectalis is generally viewed as part of the levator ani. The pelvic diaphragm supports the pelvic viscera, maintains muscle tone, and can adjust the muscle tone to balance intra-abdominal pressure. The superficial muscle layer contains the ischiocavernosus and bulbocavernosus muscles, thin strips of transverse perineal muscle, and the

sphincter urethrae muscles. This layer comprises the urogenital diaphragm, which lies anteriorly over the pelvic outlet, under the pelvic diaphragm. The superficial muscle layer closes the urogenital hiatus, the gap through which the urethra and vagina pass through. It supports and acts as a sphincter at the distal vagina, and aids in continence (Herschorn, 2004). Both muscle layers of the pelvic floor work together as one functional unit. They are enveloped in a fascia that connects to the pelvic organs' fascia and helps support the organs (Bø and Sherburn, 2005). Sacral nerve roots, the pudendal nerve, and levator ani nerve innervate the muscles of the pelvic floor. These nerves, along with the pelvic floor muscles, help maintain urinary and fecal continence by offering continuous muscle tone to the pelvic floor (Wallace, 2019).

The pelvic floor muscles have numerous vital functions. They regulate bladder and bowel continence by maintaining control of the sphincters, provide support for the pelvic organs by keeping them inside the pelvic cavity, manage sexual function, and aid in lumbar and hip stability (King, 2013). In order to properly carry out these functions and avoid dysfunction, a strong, stable pelvic floor is essential (Herschorn, 2004).

Pathology of PFDs

The bone, muscles, and connective tissue of the pelvic floor together provide support for the pelvic girdle and its organs and aids in urinary, defecatory, and sexual function. In order to function properly, contraction, relaxation, and coordination of the pelvic floor muscles and urinary and anal sphincters are necessary. Therefore, the inability to properly contract or relax the pelvic muscles impairs voiding or defecation and causes pelvic pain and sexual dysfunction (Faubion, et. al. 2012). While pregnancy and childbirth are the leading risk factors for developing PFDs (Milsom, 2015), increasing age, number of births, and BMI are also factors correlated with a greater prevalence of PFDs (Wu, et. al., 2014).

Just like any other group of muscles, the pelvic floor may become overstretched or too tense. Hypotonus or low-tone dysfunctions are caused by a loss of muscle, nerve, ligament, or fascia of the pelvic floor muscles and create weakness and laxity of the muscles. These dysfunctions can occur as a result of traumatic injury, childbearing, gynecologic procedures, obesity, chronic constipation, or hormonal changes. Some examples include stress incontinence, urgency incontinence, and pelvic organ prolapse (Wallace et. al., 2019). Hypertonus or high-tone dysfunctions are due to the pelvic floor muscles becoming too tense. (Fox, 2009) Often, it is a result of repeated voluntary urine holding. Examples include pelvic myofascial pain, dyspareunia, vulvodynia, and sexual dysfunction (Wallace et. al., 2019). Abnormal gait or posture, skeletal disproportion, and excessive sitting may also cause pelvic dysfunctions. The hip, abdomen, pelvis, and spine are an interconnected kinetic chain, each one affecting the others during movement. Any disfunction in one area may cause another area to overcompensate and create further dysfunction. However, the specific cause of a person's PFD is often never identified and may be due to several contributing elements (Faubion et. al., 2012).

The three most common types of PFDs are urinary incontinence, fecal incontinence, and pelvic organ prolapse. Urinary incontinence is involuntary control of the bladder. It ranges in severity and causes random leakage and sudden urges to urinate. Similarly, fecal incontinence is the inability to control bowel movement causing stool leakage. The most common type of incontinence is stress-related, where leakage occurs due to physically straining activities that put pressure on the pelvic floor like coughing, sneezing, sports activities, or sudden changes in position. Urgency incontinence is when one experiences frequent strong urges to urinate and nocturia. A person can also have mixed incontinence, a combination of stress and urgency incontinence (Jundt et. al., 2015). The third widespread PFD is pelvic organ prolapse. This is when the pelvic organs drop from their original positions or press into the vagina due to weakness in the muscles and tissue supporting the organs. These three are all low-tone dysfunctions.

Pelvic Floor Physical Therapy

Pelvic floor physical therapy (PFPT) is a specialized form of physical therapy which aims to reduce symptoms of PFDs and improve the function, strength, and coordination of the pelvic floor muscles. The main goal of PFPT is pelvic floor muscle training (PFMT), an exercise program created to increase the strength, endurance, and relaxation of the pelvic floor muscles (Dumoulin et. al., 2018). To achieve maximum results, physical therapists combine PFMT with other therapeutic modalities such as behavioral education, manual therapy, biofeedback, electrical stimulation, and home-based exercise programs. PFPT is minimally invasive and is used as the first line of treatment for most pelvic floor disorders (Wallace et. al., 2019).

At an initial evaluation, a physical therapist will begin by reviewing the patient's demographics, symptoms, the onset of the condition, medical history, medications, and psychological or social stressors aiming to identify contributing factors to the condition. A thorough orthopedic assessment will be conducted, focusing mainly on the lumbar spine area, abdomen, hips, posture, gait, and lower

extremity strength and range of motion. Both internal and external examinations will be done to evaluate the pelvic floor muscles' strength and coordination and detect sore or tender areas (Fox, 2009).

There is no one standard method used to assess the physiological and functional condition of the pelvic floor. A visual inspection is generally done first, followed by a study of the contraction and relaxation of the patient's pelvic floor muscles. The contraction will yield an inward squeeze and lift of the pelvic floor when done correctly. The contraction is often not performed appropriately, resulting in straining instead of contracting the muscles. Many women require guidance for proper contractions. Digital palpation is a method used to evaluate the contraction, relaxation, areas of pain, tension, tenderness, and tone of the muscles. Women are asked to squeeze around the palpated finger, and the strength of their contraction is rated as either absent, weak, normal, or strong (Faubion et. al., 2012). Other diagnostic assessments are done to evaluate the pelvic floor muscles. Anal or vaginal manometry measures the resting pressure and pressure increase during contractions and gauges muscle tone and contractility using a perineometer or other pressure sensor. Electromyography (EMG) measures the electrical activity in the pelvic floor muscles and its accessory muscles to determine if the nerves innervating the muscles are intact and the precision of each contraction (Pedraza et. al., 2014). Ultrasound and MRI have also recently been used to evaluate the action of pelvic floor muscles during contraction, specifically to measure the actual muscle lift inside the pelvis. More research needs to be done to prove its validity, but ultrasound is a popular clinical diagnostic test due to its economic availability (Bø and Sherburn, 2005).

The physical therapist must determine the cause of the patient's symptoms and categorize the disorder into either low-tone or high-tone dysfunctions. The low-tone dysfunctions are caused by general muscle weakness in the pelvic floor and can be addressed with strengthening modalities such as Kegels and other strengthening exercises. High-tone dysfunctions are caused by exceedingly strong muscles and are addressed through muscle relaxation training. Biofeedback and electrical stimulation can be beneficial for both kinds of dysfunctions.

After the evaluation, the physical therapist will create a treatment intervention program choosing from numerous therapeutic modalities. The program is individualized for each patient based on the results of their diagnostic evaluation. The primary modality is therapeutic exercises for core strengthening, postural correction, and PFMT. The Kegel exercises are frequently utilized for training, reinforcing, and increasing elasticity of the pelvic floor muscles. Other modalities include manual therapy, electrical stimulation, and biofeedback. Manual therapy is a series of techniques applied to treat tension in the pelvic floor muscles and fascia by releasing internal and external soft tissue. Techniques used are stretching, trigger point therapy, massage, and connective tissue manipulation. Electrical stimulation is a painless and effective treatment for incontinence, urgency, and frequency and strengthens the pelvic floor. An electrode is placed into the vagina and stimulates the muscles to contract in a coordinated manner. Alternatively, electrical stimulation can be used to relax and soothe tight pelvic floor muscles. The effect it produces is similar to the muscular response of the Kegel exercises. Biofeedback is a system where sensors monitor the muscular activity of the pelvic floor, and the results are displayed to the patient while the exercises are performed. This technique offers patients motivational support based on the reinforcement method of the operant conditioning theory. With biofeedback, patients can see which muscles are being used and learn how to isolate the correct muscles (Fox, 2009). Through an improved awareness of correct contractions and relaxation, a patient can relearn how to manipulate his pelvic floor muscles in various positions properly. After the treatment period, patients are given a home exercise program to maintain the skills and incorporate them into their routines.

How does PFMT Work?

Dr. Arnold Kegel, an American gynecologist, was the first person to introduce PFMT exercises to treat and prevent incontinence and pelvic organ prolapse, based on his theory of strengthening the weak pelvic floor muscles (Park and Kang, 2014). Kegel defined pelvic muscle training as tightening and contracting the pelvic floor muscles multiple times a day in order to strengthen them. He claimed that training the pelvic floor muscles yielded a cure rate of 84% for various incontinence types. Kegels' trials were uncontrolled and not randomized, yet multiple controlled and randomized trials have since supported his claim and demonstrated the effectiveness of PFMT (Bø and Sherburn, 2005). Each muscle of the pelvic floor has its own function, yet combined, the pelvic floor muscle can do one voluntary mass contraction, which can be described most accurately as a squeeze and inward lift of the area surrounding the pelvic organs. The contraction stabilizes the muscles, resists downward movement, and closes the urethra (Bø, 2004). The muscle strength training theory is that leakage will stop or decrease by improving the strength, timing, and support of contractions, and pelvic pain will be reduced, giving the patient a

greater quality of life (Bø and Sherburn, 2005).

Physical therapists teach patients how to contract the pelvic floor muscle right before a stressor or physical exertion and to maintain the contraction throughout the stressor. This way, the urethra and bladder are unable to descend, preventing leakage from occurring (Bø, 2004).

Generally, skeletal muscles are strengthened by altering their morphology to have a greater cross-sectional area, enhanced neuromuscular function, and increased muscle tone. The pelvic floor muscles are regular skeletal muscles, so their muscle strength training should be like that of typical skeletal muscles. Furthermore, connective tissue is found inside and surrounding all skeletal muscles, providing strength, stiffness, and support for muscle loading. Exercise and strength training increase connective tissue mass, which also indicates effective muscle training (Bø, 2004).

PFPT in Treating PFDs

Numerous studies demonstrate the efficacy of PFPT in treating PFDs. Dumoulin and colleagues conducted a study that reviewed clinical trials to assess the effectiveness of PFMT on urinary incontinence. This was done by comparing the results of two randomly assigned groups, one that engaged in PFMT and one that did not engage in any treatment or used inactive treatments such as management techniques. Cure or improvement of the condition, quality of life, frequency of leakage, amount of urine loss, and side effects are factors considered in the study. They found that after doing PFMT, women were, on average, five times more likely to report being cured and two times more likely to report cure or improvement from any urinary incontinence. Leakage episodes from the PFMT group were reduced to one less episode in a 24-hour period, and side effects were rare. The PFMT group had an improved quality of life and a higher treatment satisfaction rate compared to the control group, who had a greater likeliness of pursuing additional treatment (Dumoulin et. al., 2018).

Tosun et. al. also wanted to determine whether symptoms of urinary incontinence could be decreased through PFMT and if strengthening the pelvic floor muscle to grade 5 on the Oxford scale can completely abolish incontinence. The oxford grading system is a six-point scale used to measure pelvic muscle strength. It starts at zero, which rates no contraction, and goes up till five, which rates strong. They conducted a randomized and controlled clinical trial of 130 incontinent women divided into a PFMT group and a control group. Urinary incontinence symptoms and their severity were evaluated by a gynecologist utilizing the Incontinent Impact Questionnaire and Urogenital Distress Inventory. Urine loss and frequency were measured by bladder diaries, stop tests, and pad tests. A physical therapist then evaluated pelvic floor muscle strength and function using palpation, perineometer measurements, and ultrasound imaging. These evaluations were done before and after the treatment period. The PFMT program for the experimental group consisted of patient education, instruction on correct pelvic muscle contractions, and exercises individualized towards each patient's strength and tolerance, which were adjusted as they progressed. After a 12-week treatment period, results showed that the PFMT group had significant improvement in symptoms after treatment, whereas the control group had no significant difference. The PFMT group achieved better results in all measured assessments than the control group. According to their bladder diaries, nearly all the patients who reached a grade 5 in muscle strength, were cured of incontinence and nighttime urination (Tosun et. al., 2015). This experiment further proves that PFMT is an effective treatment for urinary incontinence.

Research suggests that women with PFDs have a reduction in the cross-sectional area (CSA) of the levator ani muscle. When skeletal muscles are strengthened, their CSA increases. Therefore, measuring for an increase in the CSA of levator ani muscle would be an effective way to see if PFMT is a successful treatment for PFDs. Bernardes et. al. did this by performing an experiment to test the efficacy of PFMT in increasing the CSA of the levator ani in women diagnosed with pelvic organ prolapse. Women were randomly allocated into three groups. The treatment for the first group consisted of only pelvic floor muscle contractions. The second group did a more comprehensive treatment consisting of pelvic floor muscle contractions, hypopressive exercises, and diaphragmatic breathing. The third group was the control group and received no treatment. The CSA of the levator ani muscle was measured using two-dimensional ultrasonography before and after the three-month PFMT treatment period. It was found that groups one and two had considerable differences in the CSA of the levator ani, while the control group did not. These findings suggest that PFPT significantly increases the CSA of the levator ani in women with pelvic organ prolapse (Bernardes et. al., 2012).

PFPT in Preventing PFDs

Since multiple studies indicate that PFPT is an effective treatment for PFDs, and the risk factors for PFDs are known, researchers sought to determine if PFPT could go as far as preventing PFDs from occurring in people who are at risk. In a systematic review data from six different clinical trials suggests that those who performed PFMT during

pregnancy decreased incontinence during late pregnancy and up to six months postpartum compared to those who engaged in usual care (Woodley et. al., 2017).

A randomized and controlled trial was conducted to determine whether PFMT during pregnancy was an effective prevention for urinary incontinence. Three hundred and one women attending their routine ultrasound scan at 18 weeks gestation, who were pregnant with their first child and continent before pregnancy, received instruction on the anatomy of the pelvic floor and correct pelvic muscle contractions. They were then randomly split into two groups. The experimental group received intense training consisting of PFMT twice daily and a weekly one-hour exercise class given by a physical therapist, which included pelvic floor muscle contractions, breathing exercises, and muscle strength training for the abdomen, back, and thighs. The control group engaged in standard antenatal care. After the treatment period, the PFMT group was 33% less likely to self-report urinary incontinence at 36 weeks gestation and 39% less likely at three months postpartum. These results indicate that supervised, intense PFMT in early pregnancy decreased the risk of self-reported urinary incontinence by approximately one-third in late pregnancy and three months postpartum compared to usual care (Hay-Smith, 2003).

Discussion

While there is a plethora of research on the effects of PFPT on PFDs, many question the accuracy of the research. Many of the trials done on the subject do not have an ideal sample size. Sample size strongly influences the outcomes of a trial, as a huge sample size may overestimate the results, while a small sample size may not reveal results accurately due to low power and higher margins of error. The length of the training periods, type of exercises, frequency, duration, and intensity also impact the results of a trial. Since researchers have different interpretations of 'intense training,' different intensity levels yield different outcomes (Mørkved and Bø, 2013). Therefore, a set, intense exercise program must be established specifying the duration, frequency, and intensity of each exercise so that studies can compare results accurately.

Patient supervision during training is another aspect that must be considered. If a physical therapist is present during training, he can verify that the exercises and contractions are being done correctly. Often, patients are not aware of how to perform a proper pelvic floor contraction and instead contract their gluteus, abdomen, or hip adductor muscles. Improper contractions can cause straining of the pelvic floor, which results in further dysfunction (Bø, 2006). If the contractions are done wrong, the entire study is counterproductive because the patients can be worsening their conditions instead of improving them. When patients are not supervised, there is no way to know if they adequately adhered to protocol, which is a crucial factor in the outcome of a trial. The results will then be ineffective because the true effect of PFMT cannot be evaluated. In future studies, new methods for monitoring patient adherence in a supervised manner must be developed to determine if the results are accurate.

Another significant aspect that questions the accuracy of this research is that diagnosis, results, and adherence are mostly based on self-reports from the patients using general questionnaires, interviews, or diaries. Patients are subjective and can over or underestimate their symptoms, compliance, and participation. Perhaps, better techniques must be utilized in evaluating patients to gather more objective data. Additionally, there is a lack of research studying the timeliness factor in the studies done. How early a person begins muscle training may have a positive effect on treatment and prevention outcomes (Romeikiene and Bartkevičienė, 2021). Also, researchers only analyzed the effects of PFMT for up to six months postpartum. More research is required to see if PFPT is an effective long-term treatment as well.

There is an abundance of clinical trials, systematic reviews, and experiments regarding the prevention and treatment of urinary incontinence. However, there is a lack of adequate research done on the effects of PFPT in the prevention and treatment of pelvic organ prolapse and other kinds of PFDs. Based on the few trials and reviews that do exist, it seems that PFPT is effective, although there is no concrete evidence that it is beneficial for all kinds of PFDs. Therefore, it is still an area of dispute and requires further investigation. One reason for the lack of studies may be because intense physical exertion, especially when the activities are performed incorrectly, can trigger or exacerbate PFDs. In the postpartum period, there is an increase in physical activity due to the return to the lifestyle prior to pregnancy and caring for a baby, which involves lots of exertion. For this reason, trials and reviews are unable to offer strong evidence that PFPT is effective because other factors may be worsening PFD symptoms at the same time. (Romeikiene and Bartkevičienė, 2021).

Conclusion

After studying the causes and detrimental effects of PFDs and exploring the field of PFPT, one can understand and appreciate the positive impact PFPT may have on the lives of suffering women. Research thus far indicates that PFPT is an

effective way to treat and prevent PFDs in women. Additional investigation is still required to determine the exact efficacy of PFPT under more supervised and controlled conditions. There is hope that further research will prove the effectiveness of PFPT, making this preferred treatment method more widespread and accepted in the medical world and giving women the quality of life they desire.

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Can Stem Cell Transplantation Restore Insulin Levels in Diabetics?

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Abstract

A recent public interest in stem cell research has led to new approaches to treat various pathologies, including diabetes. Diabetes mellitus is characterized by elevated blood glucose levels due to the inability of the β cells in the pancreas to produce insulin. Therefore, the standard diabetes treatment is introducing insulin to the bloodstream. With the advancement of stem cell therapy, a new approach to treating diabetes mellitus is being researched. Scientists are working to differentiate pluripotent stem cells into mature insulin-producing pancreatic beta cells. These cells are then transplanted into in vivo models and observed after a glucose challenge for normal blood glucose and elevated C-peptide levels. The subjects in each study are monitored, and the efficacy of each implantation is evaluated. Scientists have engineered novel retrievable encapsulation devices to prevent an autoimmune attack that can be easily removed in the event of tumor growth. It is evident that there is much potential to stem cell therapy and beta-cell encapsulation as an alternate treatment for diabetes.

Keywords

Diabetes Mellitus
Insulin
β cell
Stem Cell Therapy
C-peptide
Encapsulation Device

Can Stem Cell Transplantation Restore Insulin Levels in Diabetics?

Diabetes mellitus is a chronic health condition defined by hyperglycemia resulting from insufficient or dysfunctional insulin. Insulin is a hormone secreted by beta (β) islet cells in the pancreas in response to blood sugar levels in the body. Diabetes is divided into two categories based on the age of onset and causes. Type I diabetes is an autoimmune disease where the body creates cytotoxic T cells that aim to destroy their pancreatic β cells. These T cells recognize their cells based on their β cell antigens, insulin, and the GAD56 antibody. Type II diabetes is often caused by an unhealthy lifestyle and subsequent progressive β cell dysfunction. Standard care for diabetes requires constant monitoring of blood sugar levels and strict adherence to lifelong insulin injections to keep within the normal range of 80-110 mg dl-1. Though people with diabetes can often live almost normal lives, compliance is exhausting, and negligence in diabetic management can lead to severe complications that may induce blindness, kidney and heart dysfunction, peripheral neuropathies, or premature death (Melton, 2011). Unfortunately, the above plan is only a preemptive method, and cannot replace the highly specific work of the pancreatic β cell. Therefore, replication or restoration of functional β cells and autoimmune prevention is a sought treatment method for diabetes mellitus.

Stem Cells

Stem cell therapy is a potential way to form many cell lineages, and therefore an excellent source for cell replacement therapy, especially for diabetes. Mesenchymal stem cells, or stromal cells, are nonhematopoietic, multipotent cells that are self-renewable. They can be isolated from tissue such as the umbilical cord, liver tissue, adipose tissue, and bone marrow. Mesenchymal stem cells have many advantages that enable scientists to study them and use them for experiments. These cells are easily generated to large masses of cell numbers, can maintain their plasticity, are multilineage, and are anti-inflammatory. They also perform potent secretome functions and contain immunomodulatory properties. (Kotikalapudi et al., 2021) Another advantage is that mesenchymal stem cells are less ethically controversial in comparison to other stem cell types (Lu et al., 2020). Therefore, they are well studied and experimented with to combat tissue degeneration immune-based pathologies such as heart disease, osteogenesis, graft vs. host disease, and Crohn's disease.

Methods

Various databases, including Touro College Online Libraries, JSTOR, and PubMed were used to compile information for this paper. A spectrum of peer-reviewed, original articles on the topics of Diabetes Mellitus, stem cell therapy, islet cell transplantations, and ethics were found, and the relevant data was incorporated into this paper.

Discussion

Formation of Beta Cells

To efficiently study diabetes mellitus, stem cells were generated and used to study the disease on a cellular level, to transplant into mice to create simulated components of the disease as preparation for experiments, and most importantly, to create the functional cells to transplant into human and nonhuman subjects. Methods for cell generation vary, but all techniques follow a basic procedure. Biopsies can be obtained from the skin, liver, pancreatic , embryonic, cadaver, and umbilical cord tissue (Maehr et al., 2009). These cells were differentiated into spheroids, 3D clusters that mimic the dimensions and gradients of signals that are found in early-stage embryonic cells (Fattahi et al., 2021). The cells were then infected with

retroviruses that contain transcription factors such as OCT 4, SOX 2, and KLF4. After which, they were tested for pluripotency markers by confirming the presence of alkaline phosphate activity and reactivity to antibodies against pluripotency markers. Once the pluripotent stem cells resembled human embryonic stem cells, they were analyzed for correct differentiation into the endodermal, mesodermal, and ectodermal germ layers. The last step was to direct differentiation into glucose-responsive insulin-producing cells (Maehr et al., 2009).

Preparation of Host for Experimentation

Experimentation included transplanting stem cells into diabetic subjects and then monitoring and recording each success. Prior to experimentation, scientists prepared animal hosts that would undergo the islet cell transplantation. In many studies, researchers replaced the islet cells of immunocompromised mice with dysfunctional beta cells. Other studies involved the creation of a good host by injecting 180 mg/kg of Sigma (streptozotocin) into nonobese diabetic severe combined immunodeficient male mice (Sapir et al., 2005). Alternatively, some studies were performed based on human experimentation. The subjects in these studies were chosen based on their diabetic history, BMI, and a fasting C-peptide level ≥100 pmol/L (Lu et al., 2020).

C-peptide level was recorded to track the success of each transplantation. C-peptide is produced along with insulin and is responsible for the correct folding and linking of alpha and beta sheets in insulin. Since it is produced at the same rate as insulin and is also released by β cells in equal amounts, C-peptide can be used as a marker for insulin production

Efficacy of Multiple Islet Transplantations

In one experiment, umbilical cords were obtained from reportedly healthy women post-delivery. They were then cut, incubated, cultured, cryopreserved, and tested in a quality control lab. Patients received transfusions of these cells every three months along with dexamethasone injections as prevention. Both the experimental group and control group were given intensive insulin therapy and diabetes education. They were then monitored and followed up with blood tests, standard meal tolerance tests at 3, 6, and 12 months and then yearly. The primary efficacy endpoint was clinical remission of the experimental group participants, as noted by a 10% increase from baseline fasting and postprandial C-peptide level, indicating improved β cell function through the one-year follow-up. At that point, 40.7% of participants in the mesenchymal stem cell treated group kept up clinical remission, which is 2.5 times higher than the control group, who maintained 11% clinical remission on a standard insulin regimen. Even better, two treated participants remained insulin-free within the first three months of introduction and only restarted insulin a little over a year later. Another went 3.8 months without introducing insulin, within six months after the second transplantation. Additionally, almost half of the treated group had increased fasting and postprandial C-peptide levels instead of only 1/5th of the control group. (Lu et al., 2020)

A similar study was performed to test the efficacy of pluripotent stem cell-derived islet cell engraftment on 17 human subjects. These subjects were monitored and given mixed-meal tolerance tests at specific intervals, after which their glucose and C-peptide levels were observed. The test's primary efficacy endpoint was detectable levels of circulating C-peptide after increased blood glucose. Subjects who achieved this were classified as responders, whereas nonresponders had undetectable glucose levels. Explants from the responders showed more significant numbers of graft-derived β cells than non-responders. Additionally, 63% of engrafted cells from subjects highlighted insulin expression, and 35.3% of subjects had positive C-peptide as early as six months post-transplant (Shapiro et al., 2021).

The study led by Sapir et al. suggests the use of liver cells as a possible pancreatic progenitor to create functional insulin-producing cells. First, recombinant adenoviruses were constructed. Then adult and fetal liver tissues were isolated, cut into slices, digested by 0.03% collagenase type I, and cultured. The medium was changed daily. The liver cells were then infected with recombinant adenoviruses and placed under 40 rounds of RT-PCR to denature their genes. The cells were also placed under SF (steroidogenic factor) treatment with PDX-1 until there were normalized human β-actin cDNA. Observations proved that only about half of human liver cells were susceptible to recombinant adenovirus infection, and 20-50% of the PDX-1 expressing cells responded to activation of the insulin promoter.

Insulin secretion of the cells was measured, and the liver cells were then challenged with the introduction of increasing concentrations of glucose and tested for C-peptide reactivity. Mice were prepared as mentioned above, and once the mice's blood glucose levels were measured to be ≥300 mg/dl twice consecutively, the adult liver cells were transplanted into them. After fasting 6 hours, the mice received a glucose injection. Blood was drawn from their tails to monitor C-peptide, mouse insulin, and amylase levels. Results indicate that treated adult human liver cell implantation in the mice caused

a gradual, steady, and significant decline in blood sugar levels that lasted throughout the 60 days of the experiment. Conversely, C-peptide levels increased at the same rate as the drop in blood glucose levels. The normal lives that the mice lived in the 60 days of the experiment and their reversal to hyperglycemia and low C-peptide levels following removal of the cells establish the potential for β cells to be replaced by PDX-1 treated hepatocytes in vivo in the future. This approach is another way to circumvent the ethical issues involved in using fetal or embryonic stem cells to achieve this (Shapiro et al., 2021).

In an experiment to test the capacity of β stem cells to function in vivo and to determine the period length of insulin response post-transplantation between stem cells and pancreatic progenitor cells, the cells were placed under the renal capsules of immunocompromised mice. After a two-week surgical recovery period, both groups of mice were injected with glucose, and after 30 minutes, their serum was collected. In contrast to the pancreatic control progenitor transplanted mice that undergo a 3-4 week maturation phase before producing insulin-secreting islets, 73% of the β-cell transplanted mice showed increased human insulin levels in the bloodstream following a glucose challenge. Even more, two out of six control mice died within eight weeks post-transplantation versus zero out of six β-cell transplanted mice. After at least four months of observation, five out of six control mice died compared to one out of six that received β stem cells, proving that the β cell transplanted mice survive better (Pagliuca et al., 2014).

Multiple studies defend the potential of stem cell therapy. Interestingly, an experiment proved that bone marrow stem cells are insufficient when used alone but were successful when implanted along with mesenchymal stem cells (Urbán et al., 2007). Likewise, scientists experimented to improve the insulin function and survival of islet cells by transplanting them along with adipose tissue-derived stem cells. Adipose tissue- derived stem cells possess anti-inflammatory properties and the potential to revascularize, both of which promoted the grafted cells' survival (Ohmura et al., 2010). Another study was performed over a 50-week course to observe and evaluate the sustainability of device encapsulated stem cells when implanted into non-diabetic immunodeficient mice. Thankfully, all the implants differentiated and the hosts exhibited biologically relevant plasma human C-peptide levels beginning at about five weeks post-transplantation and up to 40 weeks post-transplant, with some animals maintaining normal C-peptide levels beyond that time (Robert et al., 2018).

The Problem and Solution:
Immunoprotective Devices
Cell Encapsulation

Despite the significant success in treating diabetes with β cell transplantation, it is hindered by the need for long-lasting immunosuppression. Immunosuppressive drugs increase infectious disease and malignancy and thereby patient morbidity, but they also cause problems with the regulation of β cell regeneration (Lee et al., 2009). There is also an increased risk of teratoma formation when breeding induced pluripotent cell products (El Khatib et al., 2016). To combat this, new engineering solutions such as cellular encapsulation can almost eliminate the issues above and safely protect against teratoma formation and immune rejection. Scientists engineered an optimal device that allowed for adequate exchange of nutrients and oxygen to sustain the encapsulated cells. It also efficiently transported glucose and insulin to execute the beta cell's purpose. Simultaneously, the capsule inhibited the diffusion of immune cells, antibodies, immunoglobulins, and proinflammatory cytokines from invading. Last, the device was biocompatible and could control potential tumorigenic cells.

Additionally, due to T cells as the primary immune rejector of grafts, the devices must be capable of preventing their activation. Chang and his associates measured the activation of antigen-specific T cells in response to mice with ovalbumin expressing cells encapsulated in nanoporous immunoprotective devices. They first stained the T cell receptor in the transgenic mice before transferring them into wild-type mice. This was done to assess the capability of nanoporous immunoprotective devices in vivo. The cells were then placed into the mice, and their lymph nodes and spleen were removed and checked for growth of ovalbumin-specific cells. Analysis of the dye loss in the unprotected ovalbumin challenge transplant was indicative of T cells being present. The mice with encapsulated cells showed minimal dye loss. This meant that the encapsulated cell transplants can protect against antigen-specific T cells and indicated proliferation for the T cells within the mice.

Both macro and micro encapsulation devices have been invented and studied. Microcapsules are advantageous in maximizing their surface area to volume ratio but have limited control over pore thickness and size, and retrieval, if necessary, has been proven difficult. Alternatively, macrocapsule technology has been more successful. One reason is its capability to be retrieved easily. This is useful in case of an event such as cell escape, reverse differentiation, or teratoma formation where the cells must be immediately removed (Chang et al., 2017).

The macro encapsulation device is a planar pouch of a bilaminar polytetrafluorethylene membrane system. (Others have been designed from polycaprolactone, an FDA approved synthetic fiber (Chang et al., 2017). It is made of long-lasting durable material, and its semipermeable membrane allows for diffusion of nutrients and insulin. Monodisperse alginate beads encapsulate the cells. The even layer is created by a novel microchannel emulsification device (Bitar et al., 2019). This device's implantation in the subcapsular space of the renal compartment or dorsal subcutaneous space is minimally invasive and can be retrieved if needed. Upon testing the device, a few points were noted. When determining the function of encapsulated fetal β cells, the cells were found to have replicated by 3.6%, proving that not only can β cells proliferate within the capsule, they may actually be promoted. Interestingly, adult human islet cells had contrasting results. The C-peptide levels of encapsulated adult cell transplanted animals were only 5% of those of controls one month after transplantation. The results were the same three months post-transplant as well. Further investigation led to the suggestion of cell death of the encapsulated adult β cells. The researchers explored the factors that may have allowed the fetal islet but not the adult islet cells to survive, suggesting differences in ischemic tissue time, growth factors, cytokines, or blood vessels in older and younger tissue (Lee et al., 2009).

Researchers who used an alginate derivative, triazole-thiomorpholine dioxide, as encapsulation material achieved significantly higher levels of human C-peptide in the mice they were studying compared to those in other treatment groups. They saw that 150 days post-transplant encapsulated β cells restored their glucose to normal levels. Again, at 174 days post-transplant, the stem cells still stained positive for human insulin. Additional positive staining for β cell marker Nkx6.1 is proof that the cells maintained their differentiation throughout the experiment (Vegas et al., 2016).

Even with all its benefits, macrocapsules are impaired by their capacity to be packed densely with cells, consequently causing a hypoxic environment for the cells, hindering their insulin secretion (An et al., 2017). Scientists strive to further understand and solve this issue.

Cell Retrieval Devices

β cell encapsulation itself is a tremendous achievement in avoiding long-term immunosuppression. Likewise, approaches in the case of cell retrieval have also been worked upon. An and his colleagues designed a Ca2+ releasing nanoporous polymer thread coated in alginate hydrogel that can be easily retrieved via a minimally invasive laparoscopic surgery. This can be very useful for retrieving and replacing cells that would otherwise be lost in the peritoneal cavity. A thread reinforced alginate fiber for islets encapsulation, named TRAFFIC, was designed with characteristics that enable easy handling, retrieval, and implantation while also being durable. Inspired by spiders' highly adhesive, nonporous silk, the device is made of a rigid polymer thread with a thickness-controlled alginate hydrogel layer. A Ca 2+ releasing mechanism was incorporated into the thread to maintain stem cell regulation. The string was then folded (to limit surface tension that caused the coating to clump) and twisted to create a stable helical structure that resembles a rope. The thread was first placed in the alginate solution, then placed in a Ca/Ba solution to cross-link further. This created the uniform hydrogel layer, which may have infiltrated the porous surface, adding to the thread's adhesion. Then the thread was placed into another alginate-like solution containing cells for encapsulation.

Once created, the engineers dealt with avoiding fibrosis of the thread when placed in the peritoneal cavity and learned that making the thread thicker to 11 mm would prevent this problem. After confirming the mechanical strength, biocompatibility, and transfer property, TRAFFIC was ready to be tested for its potential usage. The device was transplanted laparoscopically into diabetes-induced mice and dogs. The alginate hydrogel layers seemed to be adequate in protecting against xenografts without immunosuppression. This was tested by transplanting both the device with encapsulated cells and naked, unencapsulated cells into mice. As expected, the un-encapsulated cells were quickly rejected by the host mice within two weeks as compared to the islet cells within TRAFFIC that were protected by their hosts (An et al., 2017).

Researchers also studied other factors that can make stem cell therapy more advantageous such as hemocompatibility and immunomodulatory potential. Davies and her colleagues compared the genotypic and phenotypic profiles of stem cell recipients to the bone marrow-derived mesenchymal stem cell donors. They reported that doing so minimized the risk of immune reactions such as rejection and transmission of donor-derived disease or infection (Davies et al., 2016).

Ethical Ramifications to Stem Cell Treatments

Despite all the beneficial effects islet cell transplantation may have, there still are important ethical and safety concerns to consider. One involves choice of cell tissue origin, as embryonic stem cells involve the destruction of a human embryo. With time, new ways to produce pluripotency in mammalian stem cells have been discovered,

avoiding the traditional embryo destruction method. For example, mesenchymal stem cells hold tremendous potential due to their plasticity, differentiation rate, and many places of origin other than embryonic tissue. This discovery has increased the study as a treatment for diabetes and other diseases, but one must keep in mind its tumorous potential (Volarevic et al., 2018).

Conclusion

Beta cell transplantation achieving insulin independence has come a long way. In 1994, only 12.4% of allographs performed resulted in insulin independence for over a week, and only 8.2% for over a year. However, by the year 2000, one research center reported 14.3% insulin independence (Shapiro et al., 2000). In the past decades, the statistics have exponentially increased, with about half of subjects maintaining insulin independence for extended periods in 2020 and 2021. Technology provided tremendous advancement in stem cell research and aided in overcoming safety challenges in transplantation. Islet transplantation's most significant advantage is its elimination of severe hypoglycemia. Several allograft recipients have maintained insulin independence for over 20 years with minimal to moderate exogenous insulin administration(Shapiro et al., 2021). Therefore, stem cell therapy is a promising approach and will hopefully soon fundamentally alter how people with diabetes are treated today (Chang et al., 2017).

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Crohn's Disease: Risk Factors as Pathways to Treatment

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Abstract

Objective: This paper discusses the research available regarding risk factors of Crohn's disease. Risk factors are analyzed to determine which are the most likely to cause pathogenesis. Discussion includes a definition of the disease, including new ways to diagnose and treat it. Prevalence amongst different populations is reviewed to consider a broad variety of environmental and genetic factors. Risk factors and research of affected populations lead to the study of the microbiome and how that must be further studied to broaden understanding and treatment options.

A Definition Disease and an Overview of Risk Factors and Treatments of Crohn's

Crohn's disease falls under the category of an IBD or Irritable Bowel Disease, which is diagnosed based on intestinal inflammation. "Crohn's disease is a chronic inflammatory disease of the gastrointestinal tract with symptoms evolving in a relapsing and remitting manner" (Torres, et. al. 2017). The journal article defines Crohn's disease as an inflammatory disease of the gastrointestinal tract. Inflammation can occur anywhere along the tract and can affect multiple layers of the tissue (Kalla, et. al. 2014). The inflammation does not always remain exclusively in its original area. Crohn's disease does not have any known cure, but it does have many treatments that can effectively control the condition and heal the inflammation. This period of healing can last a long time and is referred to as remission. A return of symptoms is called a flare-up. Treatments for Crohn's disease include pharmaceutical drugs, surgery, and specialized diets. Risk factors are varied; however, studies have suggested combinations of genetic factors, environmental factors, antibiotic usage, microbial causes, and family background influence. Although there are a multitude of risk factors, research does suggest that microbial dysbiosis may be the most significant.

Methods

Peer-reviewed journal articles accessed through the Touro online library and the ProQuest database were used to gather data for this article. Other research websites and various papers to gain a comprehensive understanding of the subject matter.

Results

A deeper understanding of Crohn's disease is vital to understanding the experiments conducted to determine possible risk factors and treatments. It is also needed to understand the reasoning behind the attempted treatments and their level of success. Crohn's disease is a complex disease because of its spectrum of severity and affected areas. It is an idiopathic autoimmune disorder and is classified through a biopsy of the tissue. Gastrointestinal tissue affected by Crohn's will have a "cobblestone effect", discontinuous inflammation or ulceration (otherwise known as skip lesions), and rectal sparing (Kalla, et. al. 2014). The "cobblestone effect" refers to the affected tissue's fissuring and serpiginous ulcerations (Caio, et. al. 2021). Crohn's disease is classified by three different phenotypes; inflammatory, stenosing, and penetrating (Caio, et. al. 2021). It is also classified using the Montreal classification, which classifies based on the affected area: L1- Ileal, L2- Colonic, L3- Ileocolonic, L4- Upper Gastrointestinal, P- Perianal. Each of these areas presents with different symptoms (Kalla, et. al. 2014).

Studies of the suggested pathogenetic mechanisms of Crohn's Disease reveal that there isno simple explanation. As many as 170 different risk factors have been identified in association with Crohn's disease (Rogler and Hausmann, 2019). Study results tended to be inconclusive, and the general consensus among researchers is that more data must be gathered if we wish to understand this complex disorder.

Epidemiological studies have indicated that there are environmental risk factors for Crohn's disease (Genin, et. al. 2020). Crohn's disease has long been considered a disease in Western countries. The rates of Crohn's disease are much higher in Western countries such as the United Kingdom, France, and the USA. However, westernizing countries have seen a rise in the prevalence of Crohn's disease as well, suggesting a possible connection between a western lifestyle and this disease (Aniwan, et. al. 2017). Western countries experienced an extreme rise in cases during its surge of socioeconomic advancement during the latter half of the 1900's. The swift rise of incidence in South America, Eastern Europe, Asia, and Africa correlates to the socioeconomic advancement that is taking place in those countries now (Ng, et. al. 2017).

An epidemiological study in France traced diagnoses of Crohn's disease from 2007-2014 to find any statistically significant areas with the disease. The study found that there were sixteen spatial clusters (specific localized areas with higher prevalence) in France where the prevalence of Crohn's disease was significantly higher. Northern France contained most of the clusters, and also included a super-cluster in the Northern/Northeastern border that accounts for 35% of the cases in the study (Genin, et. al. 2020). The researchers concluded that the areas of higher prevalence were also more urbanized and underserved compared to other areas of France. This conclusion matched earlier studies in France that found that Northern France had a much greater prevalence of

Crohn's disease and that areas that were economically disadvantaged had a higher rate as well
(Nerich, et. al, 2006).

One obvious risk factor is smoking. It has been strongly correlated with relapses (an increased risk by 65%), and stopping can be as effective as immunomodulatory therapy (Kalla, et. al. 2014). Studies have demonstrated that smoking reduces the effect of treatments while the patients are undergoing them. A group of researchers analyzed eighteen observational studies and five randomized control trials. They found that smokers (defined as having at least five cigarettes a day for five months) treated with biologics were less likely to respond when compared to non- smokers (Lee, et. al. 2021). Biologics are anti-TNF drugs that interfere with inflammatory responses, and are a preferred treatment for Crohn's disease. Lee's study also found that smoking prevented clinical remission, although this was only seen in the observational studies and not in the randomized clinical trials. The difference between Lee's analysis and Kalla's analysis can possibly be explained by Lee's later publishing date (2021) versus Kalla's (2014). The later publishing date gave Lee's study more data to analyze.

Another study that analyzed the effects of smoking on Crohn's disease also found that smoking had no correlation with disease severity. However, the study did found there was a difference in the gut bacteria between smokers and non-smokers. Using the Kruskal-Wallis test, the study found that the Peptostreptococcaceae genus of bacteria was higher in smokers with Crohn's disease, and the Eggerthella lenta genus was higher in non- smokers (Pascal, et. al. 2017). Further studies could analyze the effects of these bacteria on the course of Crohn's disease.
Others have studied obesity as a risk factor for IBD. The study differentiated between Ulcerative Colitis and Crohn's disease. A large randomized group of IBD patients were selected for the study. Test subjects answered lifestyle questions such as smoking and eating habits. Patients' BMI were also collected at the beginning of the study. Although obesity did not emerge as a statistically significant risk factor for all ages, it did emerge as statistically significant when adjusted for the forty- five years and older bracket. However, this finding was unique to Crohn's disease. Ulcerative colitis had no correlation to obesity in the experiment. (Mendall, et. al., 2011).

Genetic factors have been examined as well. As of 2018, more than 170 genetic risk factors were associated with Crohn's disease and at least seventy different chromosomal loci were identified (Kupka, et. al. 2018). Many other inflammatory diseases, such as ankylosing spondylitis, psoriasis, diabetes, and lupus share the same genetic risk factors (Rogler and Hausmann, 2019). In 2001, research groups independently released studies that proved the connection between the NOD2 gene (also known as the CARD15 gene) and Crohn's disease (Cho 2008, Kupka, et. al. 2018; Rogler and Hausmann, 2019). This discovery opened the door for research into genetic factors of Crohn's disease (Rogler and Hausmann, 2019). Further research in the NOD2/CARD15 gene showed that there are multiple mutations that are all associated with Crohn's disease (Kupka, et. al. 2018).

NOD2/CARD 15 has three different mutations identified as risk factors for Crohn's disease. These mutations are classified as R702W, G908R, and 3020insC and account for at least 81% of NOD2/CARD15 mutations in patients with Crohn's disease. An analysis of multiple studies has determined that a heterozygous carrier of the NOD2/CARD15 mutation has a 2-4 higher risk of developing Crohn's disease and a homozygous carrier is seventeen times more likely to develop Crohn's disease (Kupka, et. al. 2018). These studies have been replicated consistently in populations with European ancestry but not in populations of African or Asian descent (Cho, 2008). This gene codes for a protein that is an innate immune receptor for a part of the bacterial cell wall. When the NOD2/CARD15 gene is mutated, the body's ability to recognize the bacterial cell wall is compromised (Rogler and Hausmann, 2019).

Multiple studies confirmed that Crohn's disease runs in families, with Ashkenazi Jews, (ancestry in Central and Eastern Europe) used as a model for Crohn's disease research. Ashkenazi Jewry has the highest percentage worldwide of IBD diagnoses with a rate that can be 2-4.3-fold greater than the rest of its epidemiological population (Mayberry, et.al. 1986). Although Crohn's disease risk factor genes have been identified in Ashkenazi Jewish patients, they do not account for the much greater rate of diagnosed patients (Hui, 2014; Sugimura, et. al. 2003). The three NORD2/CARD15 mutations that have been identified in Ashkenazi Jews have also been identified in 30-40% of all Crohn's disease patients. Therefore, they do not account for the greater prevalence of Crohn's disease in Ashkenazi Jewry. A team of researchers studied the genetics of sixty-four Ashkenazi Jewish families and 147 non-Jewish white families. They identified a new haplotype of the NORD2/CARD 15 gene at the IBD1 locus that was unique to the Ashkenazi Jewish patients. This led to a hypothesis that it is rather this gene that contributes to the higher rate of Crohn's disease in Ashkenazi Jews and gives its Ashkenazi Jewish carriers a predisposition to Crohn's disease (Sugimura, et. al. 2003).

A team of researchers in Israel studied a group of Jewish

Crohn's disease patients with perianal disease. These researchers found that none of their patients with perianal disease had any of the classic Crohn's disease genetic markers. Although the NORD2/CARD15 gene is common in Jewish Crohn's disease patients, the patients in the study did not have this genetic risk factor. After extensive genotyping and phenotyping, the researchers established that there was no known genetic risk factor for perianal Crohn's disease in their study. However, they did find that being a Sephardic Jew, (ancestry from the Iberian Peninsula), was a statistically significant risk factor for developing perianal disease as a Crohn's disease patient (Karban, et. al. 2007). This is interesting because studies on Jewish patients with Crohn's disease have only found being an Ashkenazi Jew as a risk factor, while this study found being a Sephardic Jew as a risk factor as well.

The NOD2/CARD15 discovery opened the door to the next part of Crohn's disease research-the connection between Crohn's disease and intestinal bacteria. Researchers found that IBD was associated with immune dysfunction connected to intestinal flora (Rogler and Hausmann, 2019). Human bodies have large amounts of intestinal flora that live in a commensal environment in the gut. However, some bacterial invaders need to be attacked by the immune system. The immune system's job is to differentiate between the two and only kill the harmful bacteria. There is strong evidence that a major IBD cause is when the immune system cannot differentiate between the good and bad bacteria in the gut. One indication that there is a connection between IBD and intestinal bacteria is that the areas with the highest concentration of bacteria are the areas with the highest prevalence of IBD (Cho, 2008).

Multiple studies have been done to analyze the connection between gut bacteria and Irritable Bowel Disease. A study done by researchers in Spain and Belgium found that Crohn's disease had clear parameters for gut dysbiosis and Ulcerative colitis did not. This study was built on previous studies which found that patients with Crohn's disease had decreased bacteria from the order Clostridiales and increased bacteria from the order Enterobacteriales as compared to healthy patients (Pascal, et. al. 2017). Their study used 669 new patients and 1376 patients that already had their gut bacteria genetically sequenced, creating an unusually large study over four countries: Spain, Belgium, Germany, and the United Kingdom.

Bacteria were studied in the experiment through fecal samples that underwent genetic sequencing for different bacteria. Patients enrolled in the study provided samples at 3-month intervals over one year and a sample if they suffered a flare-up. First degree relatives of the patients were also included and provided samples as well. Results of the study showed that the samples from patients with Crohn's disease showed the highest rate of bacterial instability over time as compared with their healthy relatives and those with Ulcerative colitis. This was in contrast with patients with Ulcerative colitis, whose microbiomes were more stable than their healthy relatives (Pascal, et. al. 2017).

The human microbiome is mostly made up of anaerobic bacteria from the phyla Firmicutes and Bacteriodetes (Oberc and Coombes, 2015). An early study on the connection between the microbiome and Crohn's disease found a decrease in the Clostridium leptum group (Manichanh, et. al. 2006). This group is a butyrate producer, which is a short chain fatty acid (SCFA). SCFA are an energy source for the intestinal epithelium. Butyrate also is known to lower inflammatory mRNA and acidify the intestinal lumen, which scientists consider a possible blocker for pathogens like Salmonella and E. coli (Oberc and Coombes, 2015). One study found that microbes in patients with IBD lack proper pathways for amino acid synthesis and have increased amino acid uptake (Gaboriau-Routhiau, et. al. 2009).

Analysis of the microbiome in patients with Crohn's disease has been complicated by scientists' inability to determine what treatments the Crohn's disease patients had already undergone. One study tried to remedy possible exposure to risk factors and treatments by studying pediatric patients who had not undergone any treatment for Crohn's disease (known as treatment naïve) (Gevers, et. al. 2014). This study also analyzed bacteria in tissue samples instead of stool samples for a more accurate portrayal of the microbiome. It compared confirmed pediatric treatment naïve patients with control pediatric patients that had non-inflammatory abdominal symptoms. Results of the study showed a decrease in the variety of the microbiome in patients with Crohn's disease and changes in the amounts of certain taxa of bacteria. Specifically, taxa Pasteurellaceae (Haemophilus sp.), Veillonellaceae, Neisseriaceae, and Fusobacteriaceae were all new discoveries that positively correlated with Crohn's disease. Fusobacterium, from the family Fusobacteriaceae, had been previously suggested as a biomarker for Crohn's disease (Strauss, et. al. 2011). When these results were compared with stool samples collected from the same patients, many of the bacteria were missing from the stool. These results suggest that Crohn's disease is connected with microbiome dysbiosis, and can be diagnosed through a tissue biopsy of the affected area (Gevers, et. al. 2014)

One hypothesis for a cause of the microbiome dysbiosis is antibiotic usage. Antibiotics have been found to reduce the variety of bacteria in the microbiome and to

shift the microbiome entirely (Jakobsson, et. al. 2010). In the study on pediatric patients with treatment naïve Crohn's disease, a small subset of subjects was on antibiotics during their tissue biopsy. The dysbiosis was more extreme in these patients, with a 10-fold increase in the Fusobacteriaceae in the ileum and the Enterobacteriaceae in the rectum. The Veillonellaceae were decreased in the stool and rectum while the Pasteurellaceae were decreased in the ileum. This led researchers to hypothesize that antibiotics have a strong effect on the microbiome and can possibly be connected to the development of the microbiome dysbiosis associated with Crohn's disease (Gevers, et. al. 2014).

Discussion

Crohn's disease remains an elusive disease. Scientists are still struggling to understand what triggers its development. However, they have found promising leads in understanding its connection to socioeconomic advancement, westernization, genetic factors, and microbiome dysbiosis. Microbiome dysbiosis, in particular, seems to be a particularly intriguing path towards simpler diagnoses and treatments. If we can crack the code of the microbiome in the gut and its connection to Crohn's disease, there's a possibility of understanding what is going wrong in the GI tract in patients with Crohn's disease.

There are still many areas of research waiting to be studied. Crohn's disease is still lumped together with Ulcerative Colitis, although all research points toward there being a big difference between the two. The mind-body connection involved in Crohn's disease has also been severely understudied. There is also a lack of knowledge in the general public about microbiome dysbiosis and its connection to Crohn's disease. It would seem that the microbiome has to be understood to develop accurate diagnosis and treatment. More information about the microbes that live in every person needs to be available to the general public, especially to people living with Crohn's disease.

Conclusion

Through researching and understanding the risk factors of Crohn's disease, one can determine possible triggers that are involved in the development of Crohn's disease. Although research points towards a more complex explanation rather than one simple cause, it also demonstrates that we are closer to understanding Crohn's disease. Microbiome dysbiosis and socioeconomic factors both have strong potential to be the focus of future research on Crohn's disease. However, Crohn's disease research is always evolving, with areas clearly needing cutting edge techniques. Advances in microbiology and genetic sequencing and analyses will continue to support better understanding and treatments, leading to better outcomes for sufferers of this complex disease.

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