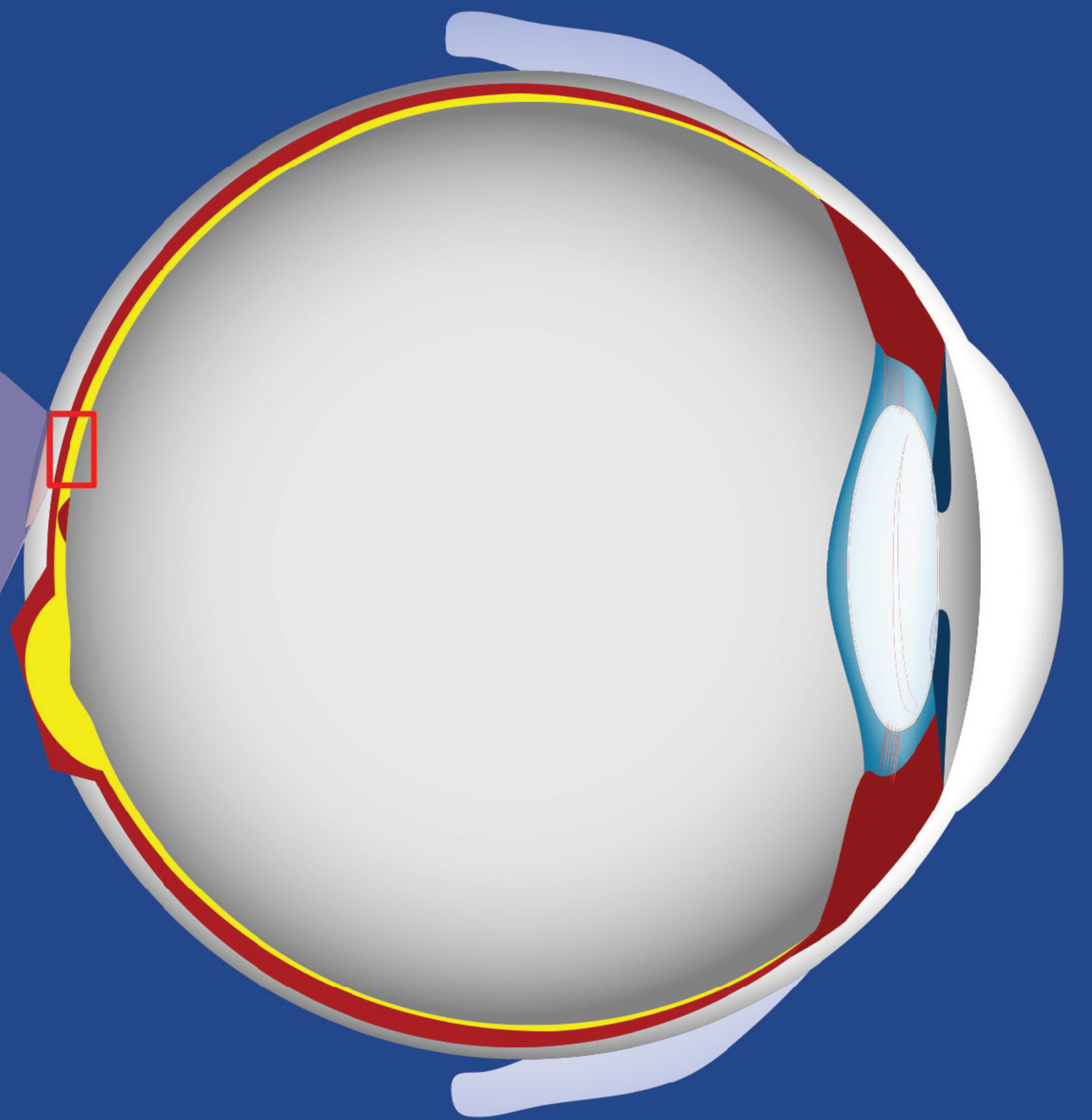
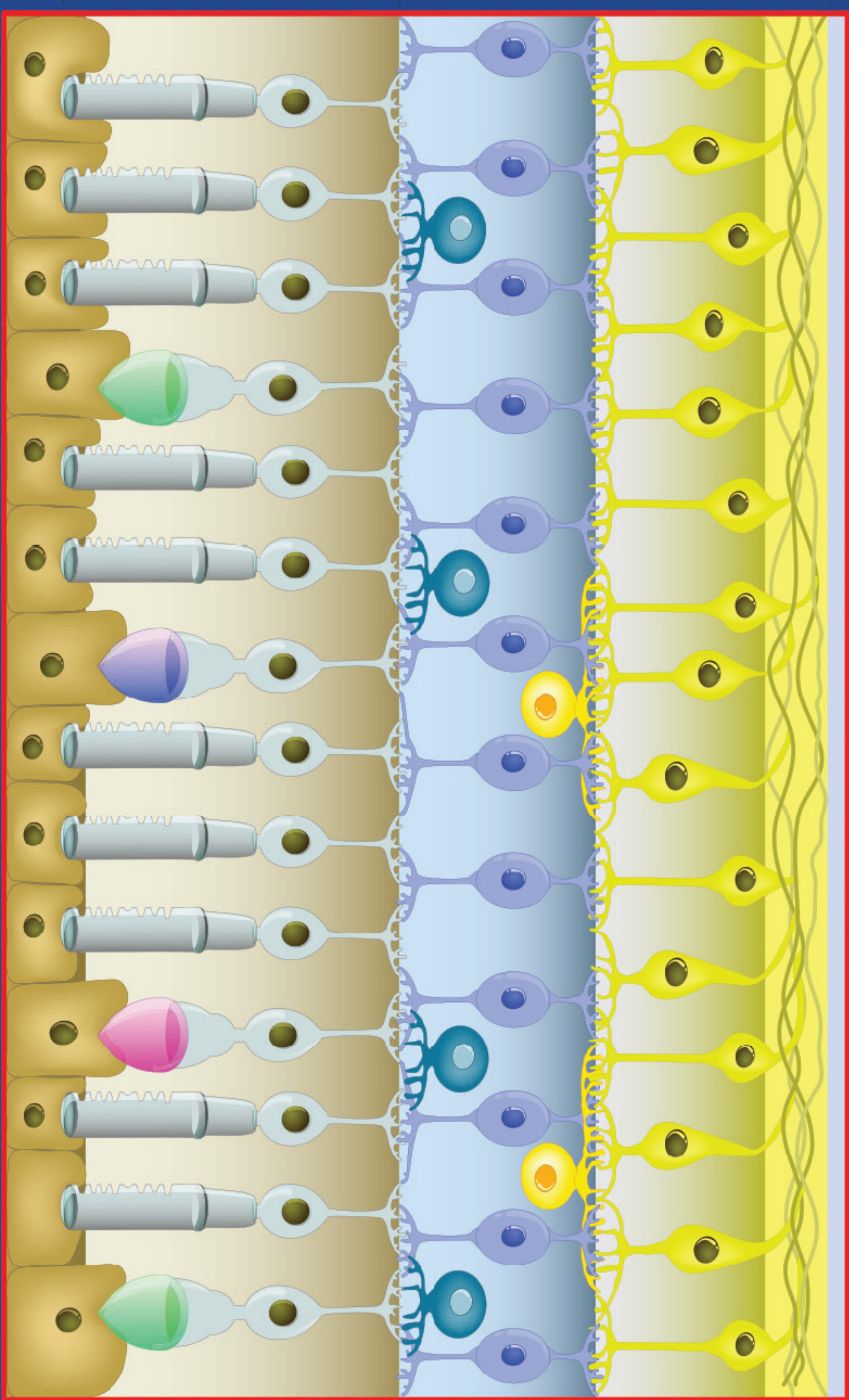


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LANDER COLLEGE OF ARTS & SCIENCES
A DIVISION OF TOURO COLLEGE IN FLATBUSH

Where Knowledge and Values Meet



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LANDER COLLEGE OF ARTS & SCIENCES
A DIVISION OF TOURO COLLEGE IN FLATBUSH

Where Knowledge and Values Meet

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The cover illustration, created by Professor Antony O'Hara of the Digital Multimedia Design, pertains to the two articles about Retinitis Pigmentosa and Macular Degeneration by Rachel Stern and Michael Radparvar respectively.

Do Humans Possess the Capability to Regenerate?

Chasha Wuensch

A Chasha Wuensch graduated in May 2018 with a Bachelor of Science degree in Biology and will be attending pharmacy school.

Abstract

Urodele amphibians, including newts and salamanders, are amongst the most commonly studied research models for regeneration. The ability to regenerate, however, is not limited to amphibians, and the regenerative process has been observed in mammals as well. This paper discusses methods by which amphibians and mammals regenerate to lend insights into human regenerative mechanisms and regenerative potential. A focus is placed on the urodele and murine digit tip models, both of which share critical regenerative stages including wound healing, histolysis, and blastema formation. Formation of the blastema proved to be a crucial process necessary for regeneration, and is responsible for dedifferentiation and pattern formation. Additionally, the necessity of nerves, macrophages, and upregulation of several genes are discussed. The use of cellular therapy and development of extracellular templates shows promising opportunities in the fields of regenerative medicine and tissue engineering for the stimulation of endogenous repair.

Introduction

Organ deterioration can be caused by disease, damage, or aging. The shortage of viable organs for transplantation has grown severe; an average of twenty people die each day waiting for an organ. (www.unos.org) Additionally, there are nearly 2 million people with amputations in the United States alone, with limb loss caused by various reasons including vascular disease, trauma, and cancer. (www.amputee-coalition.org) If a human organ or limb could be stimulated to regenerate it would radically change the quality of life and prognosis of many people.

There are two major classes of regeneration. Regular replenishment of a specific cell type or various types of cells within a tissue is defined as homeostatic regeneration. The mammalian epidermis is continuously undergoing homeostatic regeneration by the shedding and renewal of the epidermal cells. However, when complex tissue is replaced due to a wound this is referred to as reparative regeneration. Differentiation between these two types of regeneration is necessary; it expresses that regeneration requires coordination between multiple cell types, and that single cell level regeneration does not mean regeneration for complex tissue. For example, if an injury causes the loss of the epidermis and the underlying dermis, the skin will replace the lost tissue, but hair follicles derived from the epidermis fail to regenerate. (Simkin, Seifert, 2017) Using other models of reparative regeneration as a guideline, the mechanism by which humans may regenerate can be better understood.

Methods

This study was completed through the use of databases such as Touro College Library and Google Scholar. The National Institute of Health and NCBI were also used to find peer reviewed articles related to both amphibian and mammalian regeneration and regenerative mechanisms.

Urodele Limb Regeneration

Amphibians of the order Urodela, which include salamanders and newts, are the only tetrapod vertebrates capable of repeatedly undergoing regeneration of entire limbs. (Stocum, 2017) The mechanism by which they regenerate has been studied for over a century, with original studies focused on the histology and morphology of a regenerating limb, and more recent work

on manipulating the regenerative process. The mechanisms by which they regenerate have been divided into several phases:

Wound Healing

Within a few hours after sustaining an injury, an enzyme catalyzed blood clot forms, sealing the open wound. Cells from the basal layer of the epidermis detach from the basement membrane and begin to travel towards the injury site. The basal cells relocate in thin sheets which begin to proliferate only once the wound is fully covered, forming a protective layer called the wound epidermis. The wound epidermis (WE) insulates the wound, protecting it from further external damage as well as providing a regenerative microenvironment. WE can grow up to 15 cell layers thick, ultimately forming the apical epithelial cap (AEC). (Campbell, Crews, 2008)

The breakdown of the extracellular matrix of the stump tissue through matrix metalloproteinases (MMP) and other hydrolytic enzymes is called histolysis. Degradation of the extracellular matrix by MMP is directly correlated with the rapid production and release of cells from the limb tissue. The significance of MMP's role was noted when an amputated limb was treated with the matrix metalloproteinase inhibitor GM6001 and the blastema failed to form. In addition to ECM remodeling, MMPs ensure that the basement membrane does not reform. This allows the wound epidermis and the tissues below to be in direct contact, which is necessary for the successful establishment of apical epithelial cap. Matrix metalloproteinases are released up until the developing limb bud is about medium sized, slowing only once inhibitory molecules such as TIMP1 are upregulated. (Vinarsky et.al. 2005)

Blastema

Two to five days post-amputation, cells from the internal tissues adjacent to the limb stump undergo dedifferentiation, forming a heterogeneous mixture of multipotent stem cells and limited progenitor cells. (Wallace et al., 1974) This accumulation of cells is called the blastema, one of the most fundamental stages in the regenerative response. Dedifferentiation is a nuclear reprogramming which prevents transcription of differentiation genes, and reverts cells into an embryonic-like state with greater developmental potential. While cells dedifferentiate during

blastema formation, they retain a form of cell memory, and are predominately restricted to the original cell phenotype. For example, cartilage and muscle tissue originating from the blastema are generally derived from cells with chondrocyte and myocyte lineage, respectively. Dermal cells were the exception, since they possess the ability to form other tissues in addition to the expected dermal tissue. (Tweedel, 2010) The cells are also known to possess a positional memory, specifically found in fibroblast derived blastema cells. Cells adjacent to neighboring cells with disparate positional identities are detected and then stimulated to regenerate the missing cells specific to the amputated structure. (McCusker et. al., 2015)

Macrophage count is significantly higher during blastema formation. Enriched numbers of macrophages reduce inflammation and clear cellular debris while stimulating angiogenesis, fibroblast migration, and production of MMPs. Amputated limbs depleted of macrophages during blastema formation led to scarring of the limb stump. On the other hand, depletion of macrophages after the blastema already entered the growth phase only caused regenerative delay. Dermal scar tissue separates the WE from the tissues below, so while the wound can close, the AEC is unable to form, thereby preventing the regenerative process from occurring. Macrophages are therefore essential in the early stages of blastema formation and influence the final outcome of a regenerating limb. (Godwin et. al., 2013)

The gene activity present in a regenerating limb is only partially known. Several genes upregulated during blastema formation include *Msx1*, *Msx2*, *Nrad*, *Rfrng*, and *Notch*. These genes have been linked to myogenesis inhibition, reduction of muscle regulatory proteins, muscle dedifferentiation, and stem cell self-renewal. Two of these genes, *Msx1* and *Msx2*, are specifically known to be expressed in the apical mesenchyme of all tetrapod vertebrates. Following amputation or a simple wound, *Msx2* is expressed in the damaged epidermis and the surrounding tissue, while *Msx1* is limited to the blastemal cells. Studies show that *MSX* genes affect the early regenerative stages, and in the absence of these genes tissue growth and differentiation is reduced. (Stocum, 2017)

Patterning

There are currently two major models for the study of pattern formation of a developing limb. The polar coordinate model is a one which presents a view on tissue regrowth and how patterning is induced. Cells are thought to have different tissue regrowth patterns based on their positional information or their particular anterior- posterior and proximal- distal sequence. This model also predicts that supernumeraries will only be induced when an ipsilateral graft is performed at angles of 180°. (Maden, Turner, 1978)

Cells with a specific differentiated phenotype may not be equivalent in terms of positional identity. This fact was proven

through several studies where skin taken from the anterior portion of the same limb was grafted in strips, and the homotopic skin grafting led to regeneration. Heterotopic grafting, however, resulted in supernumerary body parts or regeneration failure. (Holder, 1981) Studies involving marked grafts provide direct evidence for the contribution of different tissues to limb tissue regrowth. Cells were proposed to possess the property of intercalation, in which growth is specific to their position. When two cells which are typically non-adjacent come in contact due to causes such as an injury or grafting, the cells will begin to divide. Division begins to replace all intermediary cells, and will terminate once all positional disparities between the two cells are resolved. When there is more than one possible route, intercalation will occur via the shortest path. (Bryant, Gardiner, 1987) This model was tested by placing two typically non-adjacent cells within a limb in contact with each other. Both cells acted via the intercalation response predicted by the polar coordinate model, proving these cells do indeed possess positional information. After the initial cells began pattern formation, the surrounding cells react secondarily to these cells, not necessarily through intercalation. (Holder, 1981) While cells of the regenerating limb were proven to possess positional information, the specific tissue containing this information was unknown. It was known, however, that the blastemal cells are derived from the tissues adjacent to the wound epidermis. Experiments involved with repositioning of the epidermis relative to other tissues of the limb did not have any effect on formation of the limb, nor lead to supernumerary structures. Similar studies done on nerve sheaths and skeletal muscle had the same results, leading to the conclusion that the epidermis, nerve sheaths, and skeletal muscle are not directly related to limb patterning. However, when even small grafts of skin was reoriented in the stump it led to supernumerary structures. Two of the tissue layers of the skin are the dermis and the epidermis. Since the epidermis was proven not to possess positional information, the dermis was determined to be the tissue layer containing the cells responsible for patterning in limbs. The dermis consists of mainly pigment cells and fibroblasts, and since tissue regrowth can occur in limbs without pigmented cells, fibroblasts were determined to be the cells possessing the positional information. It is thought that these fibroblasts form the patterned template for the limb, and the endothelium, myoblasts, nerves, and other cells use this information secondarily to complete limb regeneration. (Bryant, Gardiner, 1987) The polar coordinate model helps explain the way cells interact before and during regeneration, as well as how a specific pattern in a regenerating limb is induced. However, later experiments could not be explained fully with the polar coordinate model. For example, a supernumerary limb developed after an ipsilateral 90° rotation. (Maden, Turner, 1978)

The second model, the Boundary model, states that patterning in secondary embryonic fields is triggered by the boundaries

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of cells of different determination. This model proposes testable predictions of the handedness, or the specific asymmetry in relation to the central axes, of supernumeraries with ipsilateral rotation. One prediction states that a supernumerary growing out of the anterior region of the host will possess the handedness of the host, while a supernumerary growing from the posterior side will have the opposite of the host. However, when the dorsoventral polarity of a supernumerary changes, (with normal anterior-posterior polarity) the posterior region will also have the handedness of the host.

A comprehensive study on supernumerary limbs found that the predictions proposed by the boundary model were accurate, with one exception. A single supernumerary growing from the posterior region of a host (possessing non-changing DV polarity) developed host handedness, instead of the opposite handedness that was predicted. The opposite handedness proposed only occurs when a supernumerary is one of a pair. (Meinhardt, 1983) The PCM and Boundary models together give an encompassing understanding of pattern formation occurs in regenerating limbs.

Retinoic Acid

Retinoic acid, also known as Tretinoin, is a lipophilic molecule possessing the capability to regenerate tissues and entire limbs of urodele amphibians. Retinoic acid (RA) is derived from Vitamin A, and is synthesized in the cell through several enzymes. Once formed, it functions to influence gene expression. RA binds to one of the two classes of ligand activated nuclear transcription factors, either the retinoid X receptors (RXR's) or the retinoic acid receptors (RARs). These receptors act as heterodimers on upstream DNA sequences of RA responsive- genes.

The effect of retinoic acid on regeneration varies based on concentration. In several clinical studies, transection done through the radius and ulna of axolotls caused the radius, ulna, carpals, and digits to regenerate. (Fig. 1, A) However, when a low dosage of retinoids was applied to the amputation, an entire radius and ulna regrew in tandem with the original. (Fig. 1, B) Raising the dosage further led to regeneration of an extra elbow joint (Fig. 1, C), and with a higher concentration still, the limb was completely regenerated. (Fig. 1, D, E) (Maden, Hind, 2003)

Additional regeneration effects were found in a study done on the common frog, *Rana Temporaria*. When treated with high concentrations of retinoids, not only did the complete foot regenerate. (Fig. 1, F, G) but sets of limbs-including the bony pelvis, were regenerated as well. (Fig. 1, H) The first studies done show how retinoids, including retinoic acid, reduplicated the proximodistal axis. Since the pairs of limbs were mirror images of each other, it is clear the dorsoventral and anteroposterior axes had been reduplicated as well. In addition to regenerating limbs from preexisting cells, RA can alter the course of lineage specific cells, and form different structures entirely. For example,

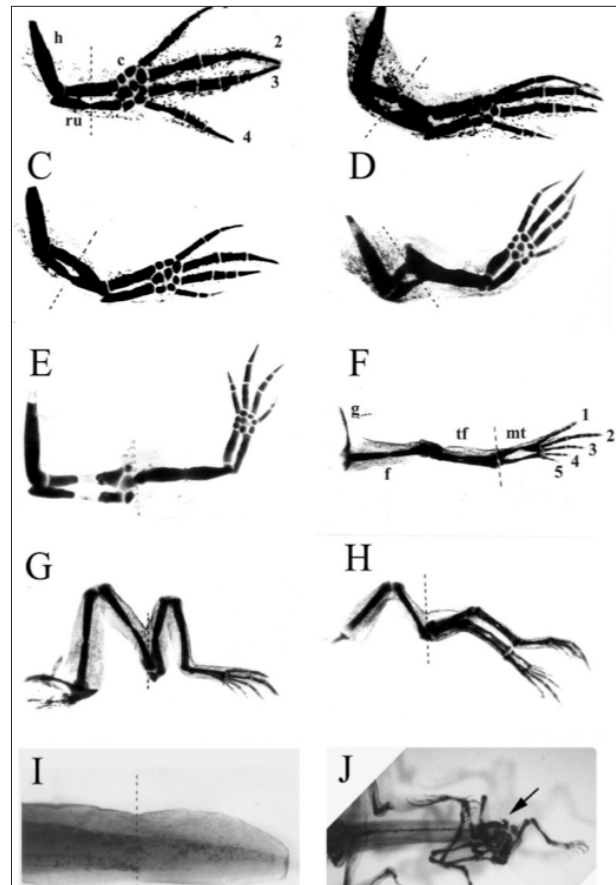


Figure 1 Treatment of an amputated limb with a high retinoid concentration regenerated the complete limb, and in some cases even pairs of limbs, from the amputation plane. (Maden, Hind, 2003)

the tail of a tadpole from the species *Uperdon systoma* and *Rana temporaria* can regenerate completely after amputation. (Fig. 1, I) However, treatment with RA led to the development of multiple hindlimbs, instead of the expected tail. (Fig. 1, J) In addition to retinoic acid's direct relationship with regeneration in amphibian limbs, it has a strong effect on mammalian regeneration, which will be discussed later in this text.

Mouse Digit Tip Regeneration: Mammalian Model

Mice have the capability to regrow epidermis, nail, bone, and other structures of the digit. (Simkin, et. al., 2013) The process by which regeneration occurs for the murine digit is similar to the human response, and has become a primary resource for the study of human regenerative potential.

The response in mice is dependent on the region of amputation. If a wound occurs at the outermost tip of the third phalanx bone (P3) the wound will heal in a regenerative manner. However, if an amputation take place at the proximal region of the P3 bone, or the second phalanx bone (P2), the wound will develop into a scar. These two distinct areas serves as a

guideline for understanding the different processes of scar formation and epimorphic regeneration.

Wound Healing

The immediate response to an injury at the digit tip is similar for both the P2 and P3 bone regions. In the initial stage, inflammation, a blood clot is formed and the blood vessels dilate, allowing inflammatory cells, neutrophils, and other necessary molecules to pass to the wound site. Wound closing time ranges from a few hours to over a week, with fetal mice recovering the fastest. This wound healing process is distinctly longer than the wound healing process occurring for amphibians. In the second stage, histolysis, the divergence between the regenerative-competent and regenerative incompetent region becomes apparent. In the regenerative region, the breakdown of tissue from histolysis causes the bone to completely detach about one week post amputation. The detached bone will fall off due to the accumulation of epidermal cells beneath it, thereby effectively sealing the wound. Amputations at the P2 and the proximal P3 regions undergo histolysis as well, but to a lesser extent and generally the digit remains attached. Unlike the P3 bone, in the regenerative-incompetent region collagen deposits between the tissue layers, inhibiting communication and ultimately leading to the development of a scar. (Quijano et al., 2016)

The developmental genes *Msx1* and *Msx2* are found in regenerating digits and are believed to regulate the expression of bone morphogenetic proteins (BMPs). These proteins are necessary for cartilage and bone repair and have been identified as powerful proliferative molecules. Amputated digits treated with the BMP-inhibitor noggin did not only lose their ability to proliferate, but were unable to regenerate as well. (Manjong et al., 2003) Several of these proteins including *Bmp7*, *Bmp2*, *Bmp4*, and their receptors, *Bmpr1a* and *Bmpr1b*, are expressed in specific regions of the phalanx and communicate via signaling. The regenerative failure seen at the proximal region of the P3 bone has been linked to an absence of BMP signaling. The bone stump of a control digit amputated at the proximal level elongated slightly but ultimately failed to regenerate. However, treating amputations at the proximal P3 region with one BMP bead led to a regenerative response. Specifically, signaling by BMP7 stimulated the greatest elongation of the terminal phalangeal, and caused an overall skeletal elongation of 58%. Two weeks post injury a regenerated digit was integrated within the stump bone. While the bone formed is histologically distinct from the stump bone, the terminal phalanx is still successfully restored.

A small hole connecting the marrow region with the dermis is formed at the ventrolateral area of the P3 bone when there is a severance at the more proximal region of the bone. However, digits regenerated from implanted BMP beads do not completely seal this hole. This can serve as a marker that amputation did occur at the proximal level and not from discrepancies of

severance level. Introduction of one BMP bead caused entire tissues to regenerate at a typically non-regenerative region. This data indicates that the cells necessary for a regenerative response are present, it is the microenvironment which is responsible for regenerative failure. (Yu et al., 2010)

Neural Requirement and the AEC

Nerves play a key role in the regenerative process, and often rely on neurotrophic factors for their maintenance. Neurotrophic factors are biomolecules which encourage neural survival and growth, and have been found necessary for both immature and fully developed nerves. One of these biomolecules, the Nerve Growth Factor (NGF), discovered in the early 1950s, has proved particularly important in regeneration of the peripheral nervous system. Several experiments found that NGF-treated nerves were able to regrow damaged nerve cells that would have otherwise been lost. (Rich et al., 1989) Once a blastema has reached the medium bud stage it no longer requires nerves for differentiation or morphogenesis, but remains nerve dependent for proliferation. Nerves are also involved with transcription; denervation led to a decrease in RNA production by over 73%. (Stocum, 2017)

The AEC plays an equally important role in regeneration. Scar tissue forms on an amputated limb of a urodele when the AEC is taken out multiple times. When the apical epithelial cap was removed from the distal portion of newt's forelimbs, both DNA synthetic activity and overall mitotic index decreased, suggesting the AEC plays a mitogenic role in the regenerating wound. (Globus, 1980)

Nerves and the apical epithelial cap both play fundamental roles in regeneration, suggesting some relationship between the two. One hypothesis is that the nerves and AEC play separate yet synergistic roles in the cell cycle. This idea was formed from the study of labeled blastema cells within an amputation. If either the nerves or wound epidermis were removed, the cells were apprehended in the second growth phase of the cell cycle, ultimately undergoing apoptosis or removal through macrophages. However, if the AEC was re-innervated, mitosis in the blastema increased as much as 10-fold. (Mescher et al., 2000) This mitotic increase only occurred if both nerves and wound epidermis were present. It is thought that the blastema cells are stimulated by a wound to enter the cell cycle, and nerves send mitogenic signals to the undifferentiated cells maintained by the AEC. (Stocum, 2017) Furthermore, the AEC is believed to contain all factors for the cell cycle, yet depends on nerves to express them. It was found that any neural requirements for regeneration are only acquired during late regenerative stages, and with aneurogenic limbs of urodeles, in which the neural tube was extracted during embryogenesis, there is no neural dependency developed at all. (Brookes, 1987)

A final relationship between nerves and the AEC is that they

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both possess the same mitogen, neuregulin 1 (NRG1). Studies show that transcripts of neuregulin 1 are expressed by over half of the blastema mesenchyme cells and by the basal cells of the AEC, while immunostaining techniques revealed the presence of neuregulin 1 in both peripheral limb nerves and dorsal root ganglia. Amputated innervated limbs treated with mubritinib, an inhibitor which prevents signaling by neuregulin 1, were unable to form the blastema. When 16-day innervated blastemas were treated with mubritinib it resulted in the formation of miniature regenerates, identical to the regenerates observed when denervation of a blastema is postponed until a stable quantity of cells have formed. The nerves present were enough to stimulate a partial regenerative response. However, the blastema is also stimulated when NRG1-soaked beads were placed 7 days post-amputation under the wound epithelium of denervated limbs. Implanting beads every four days starting from about 3 to 5 weeks post-amputation allowed some regeneration, however not as much as limbs which were innervated. (Stocum, 2017) These studies indicate the blastema cells produce neuregulin 1 independently of nerves, but in a quantity not great enough to initiate mitosis. Sensory and motor neurons act by inducing both the basal cells and the blastema cells of the apical epithelial cap to increase production of neuregulin 1 to levels required for mitosis. This finding also explains the previously mentioned 10-fold increase of blastema cell production when the AEC is re-innervated.

Blastema Formation

The blastema formed during mouse digit tip regeneration has similarities to the blastema formed in the regenerating urodele. Beginning from 12 hours post amputation for fetal mice to 12 days post amputation in the adult model, cells begin to aggregate at the outer portion of the amputation stump. The cells of the blastema stem from neighboring tissue, are avascular, and proliferate at a significantly faster rate than cells in nonregenerating tissue. Studies show that the cells of the blastema are lineage restricted. It was found that mesodermal cells will form tissues of the dermis, blood vessels, tendons, and the mesenchyme of the digit, but will not participate in the formation of ectodermal cells such as the epidermis, sweat glands, and nail plate. Likewise, ectodermal cells were found to contribute solely to the formation of ectodermal tissue. (Quijano et. al., 2016)

Similar to the urodele model, retinoic acid has regenerative effects on mammals as well. The most advanced clinical study to date was the use of retinoic acid to induce alveolar regeneration in rats and mice. RALDH1, an enzyme responsible for the synthesis of RA, is present in a specific spatial arrangement from postnatal day 1 (PND1) to PND4 in a manner correlating with the patterning of alveolar proliferation. This enzyme is expressed in alveolar parenchyma from PND4, where there is a sharp increase of cell proliferation. When an inhibitor was used

to prevent RA production, air spaces began to develop, the diameter of the alveoli increased, and their overall formation was delayed. RA receptors (RARs) and its isoforms were present in initial alveolar formation, with specific isoforms (RAR γ) acting as stimulants for alveolar development and others (RAR β) serving as negative regulators. Based on these findings it was hypothesized that RA is needed for the primary stages of alveolar development, and can stimulate a regenerative response. A study on the lungs of adult rats with induced alveolar loss proved this indeed was the case. Treatment of these lungs with retinoic acid restored all normal lung parameters within 12 days. A similar test on mice treated with RA from PND30 to PN42 and then grown to PN90 showed complete regeneration of alveolar tissue. From this data, it was deduced that RA reactivates the gene pathways used during embryonic alveolar formation. (Maden, Hind, 2003)

Tissue Remodeling

During the tissue regrowth process, the new tissues of the regenerate are formed. The deposition of soft connective tissue also takes place during the scar forming process and therefore cannot be used as a definitive indicator for the beginning of regeneration. Regeneration of bone, however, is unique to the regenerative process and serves as an indication that regeneration has begun.

Differentiation of regenerated bone begins at the area closest to the body and progresses outwards until the digit tip. As the digit tip lengthens, new bone is added through direct ossification, forming woven or fibrous bone. The regenerated bone is porous, less dense, and therefore weaker than cortical bone.

Furthermore, the woven bone will have a significantly larger volume than the original cortical bone, while still retaining a constant length. Eventually, the pores of the regenerated bone will shrink in size, and the density will increase, yet the regenerate will still remain histologically distinct. (Simkin et. al. 2015)

While the microarchitecture of the original and regenerated digit may differ, many qualities of the original is retained. For example, in several studies the dorsal bend of the nail, the area the ventral fat was situated, and the tapering formation of the digit were restored.

Human Regeneration

Regular cell turnover and renewal occurs in many organs and tissues of the human body, including the cells of the intestinal epithelium, stomach, and epidermis. (Miguel et. al. 2017) While complete regeneration of complex tissue is unusual, reports of distal phalanx regeneration have been documented for both children and adults. In one early case, an infected adult fingertip was amputated and then treated with frequent dressing changes. Three months later, X-ray imaging showed the fingertip had completely regenerated. (Dolan et. al. 2018) Since then there has been

numerous clinical reports on fingertip regeneration, all sealed with tight bandaging or direct suturing, which is thought to create a microenvironment conducive to a regenerative response.

Tissue Engineering and Regenerative Medicine

The fields of tissue engineering and regenerative medicine are dedicated to finding methods to restore both the structure and functions of damaged organs and limbs. (Shieh, Cheng, 2015) There are two central strategies used to obtain this goal. The first is the use of cellular therapies, such as iPSCs and MSCs. For example, a recent study made the use of bone marrow-derived mesenchymal stem cells (MSC-CM) in attempt to regenerate bone. MSC-CM contains cytokines such as IGF-1, transforming growth factor (TGF- β 1), and vascular endothelial growth factor, and was found to increase the development of new blood vessels and cell proliferation and mobilization. In a clinical study done to test the effectiveness and safety of these stem cells, a cavity in the mouth of patients requiring sinus floor elevation surgery or guided bone regeneration surgery was filled with MSC-CM. Six months post-surgery, new bone was present for all the participants.

The typical method for repairing extensive bone damage is through a bone graft. Autogenous bone grafting is osteoinductive, osteoconductive, and osteogenic and is therefore considered the highest standard available when grafting bone. However, the procedure requires retrieving bone directly from the patient, and is painful, expensive, and associated with higher rates of morbidity. (Katagiri et al. 2016) MSC-M is currently undergoing more comprehensive clinical studies and may prove to be a better alternative.

A second a major goal is to transition damaged tissue into a blastema in attempt to promote differentiation and regeneration. Some tissues, including the epidermis, heart, and lung, do not require formation of a blastema to regenerate. However, their microenvironment controls the cellular response to a wound and can therefore also initiate differentiation. One method used to help regulate this microenvironment and facilitate regeneration is the creation of biologic and synthetic scaffolds. One such scaffold, called the ECM scaffold, is formed by decellularization of an organ or tissue, thereby creating a meshwork of proteins in tissue-specific structure. The scaffold's composition will vary based on its source, since the ECM scaffold is composed of the molecules released by the resident stem cells of an individual organ. This biologic scaffold serves as a biochemical signaling platform and a template for the regenerating tissue, and provides both a macro and microenvironment which promotes functional tissue repair. The environment and the processes that occur in the presence of an ECM scaffold are similar to that of a normally developing tissue but are typically absent in the default repair process of an injured tissue. Work is currently being done to regenerate the pancreas, kidneys,

and the muscles of the heart, with the greatest amount of success seen in regeneration of the bladder, skin, airway, and bone. (O'Brien, 2011) Studies on the effectiveness of engineered tissues have been promising; however, some of the testing done for scaffolds efficacy were not standardized processes. (Moreno et. al., 2016) and there is a need for a greater amount of clinical research with more consistent testing methods.

Conclusion

The challenge of stimulating regeneration is to create an environment which permits a regenerative rather than a fibrotic response to a wound. The fact that BMP signaling induced regeneration of the proximal P3 bone in mice shows this endeavor is already a possibility. The regenerative pathways of the urodele limb and murine digit tip serve as a solid basis for achieving this goal in humans. While their regenerative pathways were not identical, both culminate in the formation of the blastema and to complete tissue renewal. A focus in the fields of regenerative medicine and tissue engineering is to create a microenvironment that parallels the blastema, with the purpose of creating integrated multi-tissues for organ and limb formation. The use of synthetic and biologic scaffolds, as well as manipulation of stem cells already made significant steps in generating endogenous repair. Human regeneration once seemed an impossible goal, but current advancements and the regenerative success seen in human fingertips gives hope for regeneration of complete organs and limbs in the future.

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Dentistry: Are Stem Cells the Future?

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Abstract

Stem cell research is currently advancing in every area of medicine. New information about regenerating stem cells is being uncovered on a daily basis. An area of stem cells that has not been focused on until recently is the use of dental stem cells. The objective of this paper is to elucidate the most current research about dental stem cells. Much of what is discussed in this paper has not been implemented yet, and is still in clinical trials. Dental stem cells are important because they could be an alternative way of treating caries, performing root canals, and other traumas to teeth instead of the non-biomaterial currently used. Present research involving dental stem cells includes analyzing the differences between the five dental stem cells, testing out optimal in vitro conditions of dental stem cell proliferation, and implementing lasers in regeneration.

Introduction

For many people, the word dentist brings up memories of fear and pain. Although dentist visits may be traumatic for some people, they are quite necessary. However, there may be a way to help patients with dental caries and trauma to avoid this unpleasantness. By expanding modern technology through ongoing research, regeneration of teeth and neighboring tissue without drills, tools, and cement may very well be the next advancement in dentistry.

The term 'stem cell' was first coined by Haeckel, a German biologist, in 1868. In 1908, Alexander Maksimov, a Russian histologist, suggested the existence of hematopoietic stem cells. Dental stem cells were not acknowledged until 1932, when Feldman, a stomatologist, was able to prove that dental pulp stem cells did grow under certain biological conditions. After this discovery, dentists were able to implement tooth therapy for dental pulp regeneration by using a dentine filling which stimulated pulp. In 2000, Gronthos et al. discovered and isolated the first dental pulp stem cells (DPSCs) (Bansal, Jain, 2015).

Stem cells are undifferentiated cells that can continuously regenerate and differentiate into mature cells. Further research may prove stem cells can be an alternative method of regeneration and repair in lieu of traditional medical procedures (Park, et al., 2016). There are two types of stem cells both based upon the stage of growth in which they are isolated. The first type of stem cells is embryonic stem cells (ESCs). These stem cells are totipotent, capable of giving rise to an embryo or any cell type. They are derived from the embryonic blastocyst. Embryonic stem cells would be the preferred stem cells for regeneration because they have the greatest differentiation ability, but there are ethical concerns about destroying the blastocyst embryo to isolate the stem cells. Therefore, ESC's are no longer being used to regenerate damaged tissues. The second type of stem cells are adult stem cells, such as bone marrow stem cells. These adult stem cells do not pose ethical questions because they are taken from postnatal, matured tissue. However, they do have limited differentiation potential compared with embryonic stem cells (Sunil, 2016).

A big step in stem cell research was the discovery of induced pluripotent stem cells (iPSCs). Induced pluripotent stem cells are made by "recreating" cells by putting new genes that resemble those genes of ESCs into iPSCs. Induced pluripotent stem cells are useful because scientists have reprogrammed them from their original somatic cell-like state into an embryonic-like

state. The use of iPSCs eliminates the limitations that adult stem cells have, such as lifespan and differentiation, without causing any ethical problems. Also, while cells from other parts of the body may cause the body to reject adult stem cells, iPSCs are not rejected because of their embryonic state. Induced pluripotent cells look the same, have the same differentiation potential, and have the same formation as the cells they are programmed to become. Induced pluripotent cells can react with and become neuron, pancreas, cardiac myocytes, and renal lineage cells (Yun-Jong, et al., 2016).

Methods

This document was written by researching peer reviewed scholarly articles and medical journals to assess the newest research and methods used in tooth regeneration. The research question was analyzed and reviewed from many different perspectives. Proquest, Ebsco, and Medline databases were accessed through Touro College Library online and Pubmed. Only research articles published within the last five years were referenced.

Discussion

Studies in the differentiation of dental epithelial cells conclude that iPSCs can differentiate into oral-related cells for generative purposes. Induced pluripotent stem cell technology needs suitable cells for the implementation of genes. Dental pulp cells have been shown to be able to help with the reprogramming of iPSCs. An advantage that dental pulp stem cells have over other cells used in reprogramming is their efficiency, multipotency, and accessibility (Yun-Jong, et al., 2016). During reprogramming, iPSCs are able to form colonies faster than mesenchymal stem cells and bone marrow stem cells (Sunil, 2016). However, iPSCs have recently been connected with tumor formation while adult stem cells have not been, so they may not be as beneficial in regeneration as originally proposed. Nonetheless, no conclusions can be drawn yet as these matters are still undergoing investigation in clinical trials (Takebe, et al., 2017).

Bone marrow is the most common tissue to retrieve adult stem cells from. However, the number of stem cells in bone marrow decreases with age, requiring an alternative place from which to recruit them (Newaskar, 2013). The use of dental stem cells may be the solution in response to the limitations bone marrow stem cells possess. Because dental stem cells are easily accessible and are often removed at some point in one's life,

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they make a good alternative to bone marrow stem cells. These teeth contain dental pulp that have stem cells which have similar characteristics to bone marrow stem cells but the amount of cells do not decrease with age (Sunil, 2016).

Dental-tissue-derived stem cells are used because they can differentiate into other odontogenic cells. Since their discovery, dental stem cells have shown the ability to multi-differentiate into specifically osteogenic, odontogenic, adipogenic, and neurogenic cells. Although bone marrow cells do have the same differentiation ability, differences have been noted between the dental cells and bone marrow multipotent stem cells. It was found that dental stem cells appear to be more prone to odontogenic development, while stem cells derived from bone marrow are more prone to osteogenic development, making dental stem cells a more viable option for repair of dental trauma (Yun-Jong, et al., 2016).

Recently, researchers have been encouraging people to save dental stem cells from extracted teeth, and store them in a cell bank in case a need for regeneration does arise (Newaskar, 2013). When it comes to storing these cells in the US however, there are rules and regulations regarding isolation and storage. Dental pulp stem cells are separated into two categories. The first category produces products that are “minimally manipulated.” This means that the cells are used in a homologous manner in clinical trials. The second category of DPSC, produces products that are used in a non-homologous way, significantly altering the biological functions of the cell by using certain enzymes. The FDA does not approve the use of many enzymes to extract cells. Therefore, cells are isolated using the explant culture, which is a technique that cultures cells from tissues or organs which will eventually migrate to the top of the dish which will then be isolated and stored (Ducret et al., 2015).

Human stem cells are removed from either deciduous teeth or third molars. The tooth germ is put in sterile physiological saline and is placed in the lab within two hours of extraction. The success of the stem cells implantation is based upon time and temperature, so everything must be done accurately. The tooth is cleaned by washing with buffered saline (Dulbecco's Phosphate Buffered Saline without Ca^{++} and Mg^{++}). Povidine iodine and PBSA are used to disinfect the tooth. Once the tooth is disinfected, the pulp tissue is removed with forceps or a dental excavator. The tissue is digested and the cells that are distinct are isolated and filtered. The filtration allows for single cell suspensions (Bansal, 2015). The cells are placed onto growth plates, and tissue pieces are placed onto plates containing more growth medium. The stem cells are then incubated in a humidified atmosphere with carbon dioxide. These cells are left to incubate for ten to fifteen days. Cells are dissociated with trypsin, an enzyme that breaks down proteins so that the cells can stick to walls in which they are being held, and are incubated with antibodies such as CD34, CD35, CD73, CD90, CD105, CD133, CD166 for a total of 45 minutes. After 45 minutes, the

cells are washed and placed with more conjugated antibodies (Newaskar, 2013).

The stem cells from this medium can be preserved by cryopreservation which cools the cells or tissues at subzero temperatures. This causes all biological activity to stop, but pulp death does not occur because that process is stopped as well due to the subzero temperature conditions (Newaskar, 2013). The best cells for cryopreservation are cells harvested near the end of log phase growth (Bansal, Jain, 2015). Magnetic freezing is another method, which allows the cell to remain intact by forcing the temperature to drop below freezing point so that the cell does not have time to freeze and expand and therefore remains intact. Through this process, dental stem cells can be isolated and preserved until they are needed (Newaskar, 2013).

This cell banking process used is time consuming and costly. The cultures used also have the ability to get contaminated by microorganisms in the lab because they are left out for a while. It has been suggested to shorten cell processing before the cryopreservation step to avoid the time wasted and contamination that may occur. Taking dental pulp and cryopreserving it without culture would be the quickest way to go about this process. However, the percent recovery of cells would be very low and only a small amount of healthy cells would be left in the tissue. This would occur because without sitting in culture, the cryopreservation agent wouldn't infiltrate to the center of the tissue. Instead, letting the cells sit in culture (along with the cryopreservation agent) for 5 days allows the agent to infiltrate into the middle of the culture and around 90% of cells would be recovered. These cells would also be able to be easily isolated because they would migrate toward the edges even after five days (Takebe, et al., 2017).

Tooth-derived stem cells are named according to the part of the tooth from which they are isolated (Park, et al., 2016). There are five known types of tooth-derived stem cells: dental pulp stem cells (DPSC), periodontal ligament stem cells (PDLSC), stem cells from apical papilla (SCAPs), dental follicle progenitor cells (DFPC) and stem cells from human exfoliated deciduous teeth (SHEDS). These stem cells come from the tooth but are slightly different depending on which place in the tooth they originate (Benavides, et al., 2018).

Dental mesenchyme is also known as 'ectomesenchyme' because of its early interaction with neural crest. Dental tissues are specialized and are more restricted in their differentiation and do not undergo remodeling the same way that bone marrow tissue does.

Dental pulp cells can easily differentiate into odontoblasts. Odontoblasts are cells that have neural crest origin. Dental pulp cells can be transported in vitro to help regenerate other cells in the mouth. As reported for bone marrow mesenchymal stem cells (BMMSC) the DPSC also have different morphology and different rates of growth. Even within the same colony, different

cell growth and size can be observed. If DPSCs are placed on dentin, some of them will convert into odontoblasts. DPSCs, however, are not limited to oral-related issues. In vitro, they can multi-differentiate into adipose-like and neurogenic-like cells. They also have the potential to differentiate into osteogenic, myogenic, and chondrogenic cells. Their multipotency and rate at which they divide is greater than that of BMSCs. This makes them a better candidate for mineralized tissue regeneration (Yun-Jong, et al., 2016).

The second type of dental stem cells, PDLSCs, are found in the periodontal ligament. The periodontal ligament is located between the tooth and alveolar bone (Lymperi, 2013). These cells have the ability to differentiate into osteoblasts, cementoblasts, adipocytes, and chondrocytes. Because of their location, they can be used for periodontal ligament and cementum tissue inside the mouth (Park, et al., 2016).

After PDLSC's are isolated, they display spindle shapes and have microtubule cytoskeleton. PDLSCs were analyzed in depth and were found to have 3235 proteins. The majority of proteins were mainly part of the cellular metabolism network and showed high proliferation ability (Taraslia, 2018).

The third type of cells, SCAPs, are located in the upper dental papilla. They are usually isolated from third molars and the apex of the tooth (Lymperi, 2013). They are also able to be isolated while the tooth is developing, when the tooth still has immature roots. These cells could produce dentin in vivo. The SCAPs survive pulp necrosis (pulp death) because they are close to the periapical tissue vasculature. This means that after an endodontic disinfection, SCAPs can still give rise to primary odontoblasts which would help in the completion of root formation (Bansal, Jain, 2015).

The fourth type of dental stem cell, DFPCs, are located in the dental follicle surrounding tooth germ in early tooth formation. In vitro, these cells have a fibroblast and plastic-like appearance. In comparison to DPSC, DFPC had higher pluripotent reprogramming factors and developmental factors. This allows DFPC to have greater regeneration of pulp than DPSC. However, more research still needs to be conducted to help assess which dental stem cells are the best for oral regeneration (Karamzadeh, et al., 2017).

The fifth type of dental stem cell, SHEDs, come from exfoliated teeth. It has a faster and higher growth rate and can differentiate into BMSCs and DPSCs (Park, et al., 2016). They can also differentiate into a greater variety of cell types than DPSCs (Gabiec, et al., 2017). SHEDs have the same morphology as PDLSCs, but are larger in size. There are 2032 proteins that were found in SHEDs, with the majority functioning during cell adhesion, motility, regulation of localization and migration (Taraslia, 2018). SHEDs can be good candidates for regeneration purposes and specifically for the enhancement of orofacial bone regeneration in which these characteristics are greatly required (Bansal, et al., 2015).

The table below (Table 1) summarizes the different abilities of each dental cell in vivo and in vitro, along with bone marrow mesenchymal stem cells (BMMSC) for comparative measure.

The main differences in DPSC and BMMSC are in their proliferative ability and how they develop in vitro. Both are limited to dividing into other stem cells according to where they were in vivo. For example, the neurogenicity of dental stem cells is better than that of BMMSC because they come from the neural crest, while BMMSC does not (Yun-Jong, 2015).

	In Vivo -Multipotentiality	In Vitro
DPSC	Oseto/dentigonic Adipogenic Chondrogenic Myogenic Neurogenic (Kim B, et al., 2012)	Dentin pulp like complex Odontoblast-like cells Bone-like tissue (Lymperi, et al., 2013)
SHED	Dentinogenic Adipogenic Chondrogenic Neurogenic (Kim B, et al., 2012) Osteo-induction, and uniquely the ability to recruit bone forming cells and tissue formation (Gabiec, et al., 2017)	Dentin pulp like tissue Odontoblast like cells No dentin pulp like formation Bone formation (Lymperi, et al., 2013)
SCAP	Dentinogenic Adipogenic Chondrogenic Myogenic Neurogenic (Kim B, et al., 2012)	Dentin pulp like complex Odontoblast-like cells (Lymperi, et al., 2013)
PDLSC	Osteo/cementogenic Adipogenic Chondrogenic Neurogenic (Kim B, et al., 2012)	Differentiate into cells that have the properties of cementoblasts, osteocytes, and fibroblast (Gabiec, et al., 2017)
DFPC	Cementogenic Odontogenic Adipogenic Chondrogenic Myogenic Neurogenic (Yun-Jong, 2015)	Adipogenic Odontogenic (Lymperi, 2013) Cementum matrix formation and osteoblast formation. (Gabiec, et al., 2017)
BMMSC	Odontogenic Osteogenic Adipogenic Myogenic Neurogenic (Yun-Jong, 2015)	BMMSCs have both ectopic and orthotopic tissue formation. Bone and bone marrow like, cartilage, muscle, and neuronal cell tissue formation (Yun-Jong, 2015)

Table 1

The above chart is of vital importance because in many research centers, biological flipper teeth as well as implementing of a new tooth are trying to be successfully formed in vitro. So far the experiments have been able to be carried out in animals, but the formation of a tooth still needs much improvement (Gabiec, et al., 2017).

Clinical Trials:

The five dental stem cells have been discovered relatively recently, and now further researchers can study alternative ways

to use them. All of the following studies are currently in clinical trials and have not yet been implemented in dental clinics and private practices.

A study was done last year to determine if dentin repair could become biologically more enhanced if the formation of natural dentin could be stimulated by mobilizing the resident stem cells that are in the pulp of the tooth. The study was done *in vitro* and *in vivo* with the molars of mice.

When one loses a tooth due to dental caries or other trauma, the most commonly used treatment is placing cement in the empty space. The cement which is usually made up of calcium or silicon, never fully disintegrates. Due to this lack of regeneration, the normal tooth mineral level will never be like it once was. In general, when the soft dentin-like tissue gets exposed, a natural repair process starts to take place. Mesenchymal stem cells that are already there move and differentiate into new odontoblast-like cells that create new dentin, called tertiary dentin. When the dentin repairs itself, it creates a new, thin layer of dentin which is then able to protect the pulp from the surroundings. The problem is that this only works for small injuries. Large injuries cannot be fixed with reparative dentin (Neves, et al., 2017).

This restoration process takes place by the Wnt/B-catenin pathway. Wnt/B – cat (a signaling molecule) is an early response for stimulating cellular repair in all tissues. Axin-2, a repressor protein, inhibits Wnt/B pathway. Glycogen synthase kinase 3 (GSK – 3), a component of the Wnt/B transduction pathway, phosphorylates B- catenin and axin, in the absence of the Wnt ligand/receptor binding. This leads to degradation and death of the protein (Neves, et al., 2017). Therefore when the tooth is still developing, and B- catenin is not working, the development of the tooth stops (Han, et al., 2014). In the presence of the ligands, GSK does not work. Because it does not function, the B- catenin does not get phosphorylated, but enters the nucleus, where it interacts with Lef/ Tcf transcription factors. One of these transcription factors is Runx2, which helps reform the tissue into odontoblastic formation (Han, et al., 2014). These transcription factors are able to regulate expression of target genes, including Axin -2. Studies done showed that, following tooth damage, the number of receptors on Axin 2, and therefore Wnt/B signaling, is increased. It is theorized that if a Wnt signaling agonist were added to the stem cells, it may help the formation of reparative dentin. In this way, dentine can be restored following removal of a cavity (Neves, et al., 2017).

Recently, a study was done with the use of Tideglusib, a small molecule that inhibits GSK3 and upregulates Wnt activity, which is currently in clinical trials for the treatment of neurological disorders. This could help with Alzheimer's disease by upregulating Wnt activity (Boutajangout, Wisniewski, 2014). Other small molecules that inhibit GSK3 were also used in experimentation and upregulated WNT activity as well. In this study, three GSK3 inhibitors – BIO, CHIR9902, and Tideglusib were used to arouse

tertiary dentin formation after inducing with pulp exposure (Neves, et al., 2017).

Mouse dental cells were incubated with the three inhibitors. The results showed that BIO induction of Axin 2 was much greater than CHIR99021 and Tideglusib levels. To protect the tooth, a glass piece was placed on top of the sponge in the enamel part because enamel does not regrow from these drugs, only the dentin part does. As part of this experiment, a sponge was soaked with these three inhibitors and placed into holes in mice molars. Sponges were left in the teeth (they were able to dissolve) and the teeth were analyzed at 4 and 6 weeks. Treated teeth and the controls were compared.

It was discovered that mineralization did indeed, increase with all three agonists. When controls were analyzed, there was no obvious mineralization detected. Compared to controls containing collagen sponge and MTA, more reparative dentin was formed with GSK-3 inhibitors and the newly formed odontoblast-like cells expressed high levels of axin -2.

This method can be a very effective way of replacing fillings on teeth. A great way of implanting this procedure would be by using biomaterial, such as Kolspon (collagen sponge), which is soaked in the GSK-3 inhibitors. Wnt agonists elevate Wnt activity which is an immediate response to tooth damage. If Wnt is upregulated with a sponge, mineralization happens much faster and completely finishes the tissue formation (Neves, et al., 2017).

Another ongoing study is testing for the optimal conditions *in vitro* for stem cell formation. The past few years have brought about much new insight into hypoxia-based response. Hypoxia is a deficiency in the amount of oxygen in a biotic environment. Oxygen has been known for quite a few years to have a variety of effects on adult stem cells. Because of this knowledge, new regenerative ideas can be implemented to help enhance the use of stem cells *in vitro*, especially with the targeting of cellular oxygen sensors (Janji, et al., 2017).

When stem cells are used *in vitro*, their differentiation fate is determined by transcription factors and cell cycle regulators such as Oct-4, Sox2, c-Myc, and downstream signaling pathways (Zhou, et al., 2014). It has been shown, although not yet implemented, that low oxygen levels can activate Oct-4 to help maintain stem cell properties (Ratajczak, et al., 2016). Also, ongoing research has shown that hypoxia may induce the expression of hypoxia-inducible factor (HIF-1). HIF-1 regulates the expression of target genes that affect cell proliferation, differentiation, apoptosis, and embryonic development (Zhou, et al., 2014). Through HIF-1, angiogenin is able to work (Ratajczak, et al., 2016).

Based on the effects of hypoxia transcription factors in other stem cells, an experiment was done to try and ascertain if hypoxia stimulates production of angiogenin in the pulp (Janji, et al., 2017). Angiogenin (ANG), a growth factor contained in tissues, causes angiogenesis, the generation of new blood vessels. (Ratajczak, et al., 2016). Also known as ribonuclease 5, angiogenin

is a secreted protein that regulates cell proliferation and differentiation in other cell models during angiogenesis. Angiogenin binds to endothelial and smooth muscle cells. This causes cell migration, invasion, proliferation of the endothelial cells. They can also cause tube like structures to form. Because of these abilities, angiogenin can be used in regenerative techniques. In vivo, small amounts of angiogenin are found relatively early on during hard and soft tissue regeneration. There are several pro-angiogenic factors, including vascular endothelial growth factor, fibroblast growth factor, and endothelial growth factor, which stimulate the growth of angiogenin. The transcription factor HIF-1 helps induce angiogenin as well (Janji, et al., 2017).

Angiogenin is found in other mesenchymal stem cells, such as those derived from the umbilical cord. This has been shown to help with the function and growth of the tissue which could help with infertility due to its ability to create blood vessels (Zhang, et al., 2017).

The results obtained are relevant to preconditioning approaches for cell therapy and tissue engineering. The goal is to improve the pro-angiogenic capacity of transplanted cells (Janji, et al., 2017). In this experiment, human dental pulp cells (DPC) were extracted from third molars. Three different cultures were set up. First, a monolayer culture of DPC was collected and incubated overnight. Then, a spheroid culture of DPC was collected and lastly, a tooth slice organ culture was used. All these samples were rendered hypoxic. The degree of hypoxia used was based on previous studies done with stem cells and hypoxia (Janji, et al., 2017).

Angiogenin was increased in the monolayer culture of DPC when rendered with hypoxia. When angiogenin was first expressed in normoxic conditions, the increase of angiogenin did not reach significant levels at the mRNA level. However, at the protein level, angiogenin was increased after the hypoxia was added compared to the normoxic control. Inhibitor studies were also done with echinomycin, which inhibits HIF-1 function. Echinomycin is a peptide antibiotic that blocks the binding of HIF-1 α (subunit of HIF-1). This study was done to help figure out the role of HIF-1 mechanism. mRNA production of angiogenin was reduced in the presence of hypoxia and echinomycin. Protein production after hypoxia was implemented was also reduced in the presence of echinomycin. It was deduced from this data that under hypoxic conditions, HIF-1 activity is needed for the angiogenin to be increased on monolayer cultures of DPC.

In order to make the sample more like the dental pulp matrix, in addition to the monolayer used, a spherical culture model (3D model) was also used. At the mRNA level, no significant increase of angiogenin occurred. The same was found at the protein level. It was concluded that the effects of hypoxia and the hypoxia mimetic agents that were used did not have the same effect on 3D cultures as it did in the monolayer culture. Cells from the 3D model in different environments are possibly less

sensitive to hypoxia than those of the monolayer. This would be due to the possibility that the cells which are deep within the 3D sphere have already reached low levels of oxygen. The third culture of the tooth sliced organ culture model was then tested. Angiogenin was produced in the dental pulp before and even more so after hypoxia was added.

Overall, when comparing the response at the mRNA levels compared to that of the protein level especially in the tooth slice model, angiogenin did in fact increase in response to hypoxia. The kinetics of angiogenin has yet to be determined because the amount of time left for the effect of hypoxia in other stem cells to take place was not consistent with the time frame of the oral stem cells (Janji, et al., 2017).

The reason why this is a promising technique in the future of regenerative endodontics is targeting cellular oxygen sensors with hypoxia did in fact increase pro-angiogenic activity. In conclusion, this current research is the first of its kind to apply hypoxia and hypoxia mimetic agents in creating a greater scope of angiogenin in dental pulp cells. This specific experiment concluded that although successful, the results depend on the in vitro model and the HIF-1 α activity. Knowing how to modulate angiogenin in dental pulp stem cells is a stepping stone for future studies which will research the role of angiogenin in pulp regeneration. This study will also be very important for understanding cell therapy and tissue engineering in regenerative endodontics (Hong, et al., 2018).

Table 2 contains a list of just some of the factors contained in dental stem cells that help with angiogenesis stimulation (Ratajczak, et al., 2016). Although not yet tested with hypoxia, if any of these are somehow stimulated, angiogenesis may very well be increased.

The use of laser for stem cell regeneration is also being researched. Studies previously conducted have shown that lasers can help in the differentiation of stem cells. The lasers used in these studies were high powered lasers and the effects of low power laser (LPL) were never fully studied in dental stem cells. LPL had previously been used for therapy to help osteoarthritis, inflammation, and soft tissue injuries. When DPSCs were placed in vitro, the light from LPL (810 nm light- infrared light) did not enhance the growth of DPSC. However, when LPL was used to treat a culture for 21 days under optimal conditions, dentinogenesis did occur.

The success of LPL depends on both the biological conditions and the way in which it is applied. There are two ways to apply the laser. The first method uses the laser in a continuous motion and the second way is applying it in pulsed waves. Pulsed waves were shown to be more efficient due to the time in between pulses that allowed the light to penetrate.

Non-ionizing LPL does not affect the stem cells directly but affects the growth factors that regenerate the teeth. The light from the laser hits water, gets excited, absorbs the energy, and

Factor	Function	Population
Angiogenin (ANG)	Endothelial proliferation and migration activation of smooth muscle cells indirect degradation of basement membrane.	DPSCs, SCAPs, FSCs
Angiotensin -1 (ANGPT1)	Endothelial survival, migration, and matrix adhesion. endothelial sprouting and vessel stabilization	DPSCs, SCAPs, and FSCs.
Angiotensin-2 (ANGPT2)	Endothelial proliferation, migration, and sprouting in the presence of VEGF	PDLSC
Basic fibroblast growth factor (bFGF)	Endothelial proliferation, migration, and differentiation. induction of proteolytic enzyme release	DPSC, SCAP, SHED, and PDLSCs
Colony Stimulating factor (CSF)	Endothelial proliferation, migration, and differentiation. induction of proteolytic enzyme release	DPSCs
CXC chemokines, for example, interleukin-8 (IL-8)	Endothelial survival, proliferation, migration, and tube formation. Induction of MMP production	DPSCs
Hepatocyte growth factor (HGF)	Endothelial proliferation, migration, and tube formation, proliferation of vascular smooth muscle cells, stimulation of VEGF and PIGF	SCAP, SHED
Insulin-like growth factor-1 (IGF-1)	Endothelial proliferation, migration and tube formation. stimulation of VEGF and plasminogen activator production downregulation of endothelial apoptosis	PDLSC
Platelet-derived growth factor (PDGF)	Endothelial proliferation, migration, and differentiation. stimulation of VEGF expression proliferation of vascular smooth muscle cells and pericytes vessel stabilization	DPSC

Table 2

transfers energy to dissolved oxygen. This results in the formation of a mild amount of radical oxygen species (ROS). ROS then activate TGF β -1 which cause dentinogenic differentiation. The low levels of ROS are around when the cell is still a stem cell but when ROS is present in mild amounts it helps with differentiation, proliferation, and migration of the stem cells (Hong, et al., 2018).

All the methods mentioned in this paper have not yet been

used aside from trials in the laboratory. Last year, a study was done to assess the awareness of dental pulp cells to those whom it matters most, the dental professionals. Around one hundred eighty-nine dental professionals were involved in this study and over 90% of them had heard of dental stem cells. Only 81% were aware of the use of dental stem cells through the internet or journals. Some, however, were aware due to their undergraduate training. (This would probably be referring to the younger generation). Not everyone knew about banking dental stem cells, and the prospective uses that dentists may be able to apply to improve the restoration of teeth. Those who knew about dental stem cells thought that the main obstacles with implementing dental stem cells were the costs as well as lack of full knowledge about the procedures (most are still in clinical trials). Further awareness can be created by introducing seminars and lectures discussing this topic (Chitroda, et al., 2017).

Conclusions

Dental stem cells are definitely the future in dentistry. There are so many experiments being done that may very well change the future in how a yearly trip to the dentist may look like. Researchers are optimistic about tooth regeneration and regeneration of other traumas in the mouth using stem cells. However, before regeneration using dental stem cells becomes reality, trials need to be conducted on humans. As of 2018, many trials have only successfully been experimented on animals (Benavides, et al., 2018). Although there may still be a long way to go, dental stem cells are becoming more and more of a reality and may very well be our children's only association with cavities.

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Capsaicin and Analgesia

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Abstract

Capsaicin is the active compound responsible for the pungency of hot chilli. Research has discovered its ability to desensitize peripheral nociceptive fibers which is useful in treating chronic pain disorders, specifically neuropathic pain syndromes. Capsaicin treatment comes in a variety of mediums including patches and creams and has been clinically proven to bring relief to patients with disorders such as post herpetic neuralgia, chronic regional pain syndrome and HIV related neuralgia. Exciting new forms of treatment are also in development and promise breakthroughs in the near future in this relatively young field of capsaicin-based analgesia.

Introduction

Chronic pain syndromes are debilitating, robbing many of their quality of life and productivity while putting them at additional risk of mental health disorders. Many do not find relief despite extensive treatments. This has prompted research into finding a compound that will present a viable alternative to traditional analgesics, especially opiates which carry an additional risk of addiction. Capsaicin, the compound responsible for the pungency of the capsicum family and an example of a vanilloid, has been used as an analgesic agent for thousands of years. Recent research has proven just how well suited it is as a treatment for a subset of chronic pain disorders especially neuropathic pain.

This paper will look at the mechanisms of nociception that are targeted by this treatment, the properties of capsaicin and its receptor, TRPV1, that make it so effective. Its clinical applications and limitations will also be examined to give a glimpse of what research has discovered and where it is taking us.

Mechanisms of Subcutaneous Primary Nociception

Nociception is the sensation of noxious stimuli perceived by nociceptive receptors which are usually found in the skin (Caterina & Julius, 2001), although they can also be found viscerally. It is the first step toward the sensation of pain which is the result of a complicated multilayered process including central gating controls and peripheral activity (Hunt, 2009). Nociception itself is a complex process with aspects that take place in both the central (CNS) and peripheral nervous systems (PNS).

There are two general categories of subcutaneous nociceptive primary afferent neurons, myelinated A-fibers and unmyelinated C-fibers. Not all A-fibers are sensitive to nociceptive stimuli, A β fibers are large rapidly conducting fibers responsible for touch and proprioception, and only A δ fibers which are smaller and slower conductors are involved in nociception (Khalid & Tubbs, 2017). Lightly-myelinated A δ fibers are associated with acute pain or first pain which is characterized by lancinating, stabbing, or pricking pain that is felt immediately after exposure a noxious stimulus. Unmyelinated C-fibers are very slow conductors and are responsible for second pain which is characterized by the more global sensations of throbbing, burning or cramping pain (Hunt, 2009; Dubin & Patapoutian, 2010).

Noxious stimuli are usually categorized as either heat (H), cold (C), or mechanical (M). Primary nociceptive afferent fibers are further classified according to the type of stimuli that they respond to, for example C-MH, A-MH. The most common C fibers are polymodal, meaning they respond to multiple types of stimulation such

as C-MC, C-MH and C-MHC. There is also a subgroup of C-fibers known as silent C-MiHi that only become sensitive to noxious mechanical or thermal stimuli after the area has become sensitized by inflammation. Most A-fibers are heat- or mechano- sensitive (A-H, A-M, A-HM) (Dubin & Patapoutian, 2010).

Nociceptors have a variety of voltage gated channels that encode the intensity of the stimulus (Dubin & Patapoutian, 2010). They also release neuropeptides that transmit input further up the neural axis. For example, when C-fibers are triggered they release glutamate and substance P (Hanpaa & Treede, 2012).

When the skin is injured it undergoes physiological changes that protect it from further damage and promote healing, known as inflammation. There are two subgroups of C-fibers. One of them expresses the trkA receptor which binds to nerve growth factor (NGF) and synthesizes neuropeptides such as CGRP and substance P. When they are triggered they contribute to inflammation and healing at the site of the injury (Hunt, 2009). Symptoms of the inflammatory state include flare or reddening of the immediate area and increased sensitivity to both innocuous and noxious stimuli, both in the immediate and surrounding area. The increased sensitivity is due to a reduced threshold of excitation in the C-fibers (Dubin & Patapoutian, 2010) and increased receptor expression and excitation (Lebovitz, et al., 2012).

There is a second subgroup of C-fibers that do not have a role in inflammation, instead they display the Ret receptor that binds to GDNF and express PAP that breaks down ATP to produce adenosine that, in turn, binds to the A1 receptor and reduces nociception. In fact, intrathecal application of GDNF has been shown to reduce neuropathic pain symptoms (Hunt, 2009).

C-fibers that are constantly in a state of excitation, such as those in a chronic pain state, will release a constant flow of glutamate known as a glutamate barrage. This barrage may result in physiological changes to the dorsal root ganglion and higher pain areas of the brain that increases their sensitivity and excitability which is known as central sensitization. Descending controls may also have an impact on determining the sensitization of dorsal root neurons (Hunt, 2009). Central sensitization may also impact the sensitivity of A-fiber neurons, which don't usually become sensitized, causing even innocuous stimuli to register as painful (Baron, 2006). This may be a result of synapses that they form with neurons that have been sensitized by C-fiber barrage (Hunt, 2009).

Peripheral sensitization may also occur post-injury as a result of physiological changes which may contribute to the development of neuropathic pain syndromes (Gillon, Baron, & Jensen, 2015)

The role that C-fibers and A δ - fibers play in the process of nociception is definitely an important one which makes them a worthwhile target in the field of analgesia. These afferent neurons share a receptor TRPV1 which is expressed on all C-fibers and a subset of A δ fibers (Hunt, 2009; (Lebovitz, et al., 2012), this means that if it can be shut off or blocked then peripheral nociceptive input can be greatly minimized or even neutralized.

Targeting TRPV1 is especially prized because it allows a two-pronged strategy against pain. Not only does targeting it, target the beginning of the pain pathway (Trevisani & Szallasi, 2011; Wagner, Roth-Daniek, Sell, England, & Kern, 2012), but it also blocks the glutamate barrage which allows the body to break the pain cycle common in chronic pain patients.

TRPV1

In 1990, the study of a more potent capsaicin analog, resiniferatoxin (RTX), led to the discovery of a new receptor known as TRPV1. Its name comes from the amino acid sequence that designates its membership in a family of receptors first identified in fruit flies. In 1969 a mutation was identified on a visual receptor of a fruit fly, instead of a sustained depolarizing after exposure to light, these mutated receptors showed only transient depolarizing response to light, giving the family of receptors the name transient receptor potential or TRP. The second part of its name, V1, comes from the amino acid sequence that was identified that marked it as a vanilloid receptor (Caterina & Julius, 2001). The amino acid strain was first designated VR1 but when it was joined to the receptor family it was abbreviated to TRPV1.

The discovery of TRPV1 led to the discovery of a subset of thermal-sensitive TRP-type receptors which are also called thermoTRPs (Trevisani & Szallasi, 2011). Other members of this family include TRPV2, TRPV3 and TRPV4. TRPV1 is highly expressed on normal nociceptive C-fibers and A δ -fibers and after inflammation show increased upregulation and sensitivity (Lebovitz, et al., 2012). It is part of the nociceptive pathway and is activated by a variety of stimuli including protons, noxious heat, phorbol esters, fatty acids as well as vanilloids like capsaicin and RTX. The receptor only experiences maximum activation when activated by multiple types of stimuli (Vyklícky, et al., 2008), which is consistent with the polymodal nature of the C-fibers that are involved in nociception.

TRPV1 has been proved to be involved in many forms of pathophysiology including inflammatory bowel syndrome (IBS), osteoarthritis, rheumatoid arthritis, post herpetic neuralgia (PHN) and cystitis amongst others (Andreev, et al., 2013). This makes understanding this receptor necessary for the treatment of many different disorders.

There are two ways that TRPV1 can be targeted pharmacologically, either it can be desensitized by vanilloids or blocked by antagonists. There are two types of desensitization, acute desensitization and tachyphylaxis, which is a reduction in response

to noxious stimuli as a result of repeated application (Vyklícky, et al., 2008). There is also a difference between high dosage and low dosage desensitization such as between the short-term refractory period that takes place after any application of a vanilloid and longer lasting defunctionalization that takes place after the administration of a high dosage application (Finch & Drummond, 2015) (Trevisani & Szallasi, 2011).

Multiple biomechanisms have been discovered by researchers to have a role in the process of sensitization and desensitization of the TRPV1 receptor including the phosphorylation and dephosphorylation of the receptor (Trevisani & Szallasi, 2011), although the process isn't fully understood. The dramatic influx of Ca²⁺ ions in the primary afferent neurons that takes place during vanilloid applications have also proved to be a part of the long-term desensitization mechanism (Vyklícky, et al., 2008). Vanilloid induced messenger plasticity, which refers to changes in the expression of neuropeptides on the receptor such as a decrease of the proinflammatory neuropeptide SP and the increase of analgesic peptide galanin, and is also part of the long-term desensitization or 'defunctionalization' associated with the application of high dosage capsaicin.

Antagonists that block the TRPV1 receptor are a popular avenue of research, but many promising leads often lead to disaster. These compounds often induce spontaneous hypothermia in humans making them impractical for use. However, there have been promising breakthroughs by a group in Russia with a compound that only partially blocked the receptor which shows that partial blockage of the receptor may be a better outcome than a full blockage (Andreev, et al., 2013). This is backed up by research that has shown that partial binding is enough to activate the TRPV1 receptor and that there are multiple levels of activation based on the state of binding of the receptor (Hui, Liu, & Qin, 2003).

There used to be a theory that the main function of TRPV1 is thermoregulation. This was based on its role in thermal sensitivity and the hypothermic effect of capsaicin and TRPV1 antagonists, but this was later mostly debunked (Trevisani & Szallasi, 2011). Other studies have also shown that the gene deletion of TRPV1 in mice still allowed for a level of noxious thermal sensitization (Dubin & Patapoutian, 2010).

TRPV1 is the therapeutic focus of a common analgesic and other drugs on the market. Paracetamol or acetaminophen is thought to interact with TRPV1 as part of its analgesic mechanism. Also drugs such as Taramidol which is used to treat fibromyalgia and nefopam, a benzoxazocine analgesic, also interact with TRPV1. There are also compounds in Eastern medicine that may target TRPV1 (Trevisani & Szallasi, 2011).

Capsaicin

Capsaicin, or 8-methyl-N-vanillyl-noneamid, is the compound responsible for the pungency of hot chili peppers (Factor, et al.,

2011) which are of the genus *capsicum* (Finch & Drummond, 2015). Any ingestion of hot chili will cause a painful burning sensation. However, perhaps ironically, capsaicin has been proved to have extensive analgesic qualities. It is also a potential neurotoxin and large doses can cause permanent nerve damage (Trevisani & Szallasi, 2011).

For thousands of years capsaicin extracts were used as part of native medicine as a primitive analgesic. Aztec physicians may have used it to treat tooth ache and sources show that RTX, which is derived from the latex of the palm *Euphoria Resinifera*, may have been used in Ancient Rome to treat the arthritis of the Emperor Augustus. In Northern India a native plant containing capsaicin called bhut jolokia has been used by the people who live there to treat arthritis (Finch & Drummond, 2015). Modern pharmacy, however, was late to discover its qualities and did not recognize its potential until the year 1878 (Trevisani & Szallasi, 2011).

The Hungarian chemist Endre Högyes is credited with the discovery of the effect of capsaicin on sensory fibers but his find remained lost until the turbulent years and scientific innovation of World War Two. Another Hungarian chemist, Nicholas (Miklós) Jancsó further developed the use of capsaicin by developing its potential as a promising analgesic. In the 1970's capsaicin research exploded, with the number of papers on record jumping exponentially. Capsaicin continues to be of a topic of interest for the general scientific community and continues to be extensively researched until today (Trevisani & Szallasi, 2011).

Originally the analgesic properties of capsaicin were thought to come from its counterirritant and warming properties (Hanpaa & Treede, 2012) but now we know that there are many mechanisms involved (Vyklícky, et al., 2008). It is a TRPV1 agonist (Factor, et al., 2011) and it is the complex interaction between TRPV1 and capsaicin that is the crux of its analgesic abilities.

Initial exposure to capsaicin activates the TRPV1 ligand gated channels on the primary afferent nociceptive neurons which cause depolarization, the initiation of an action potential and transmission of a pain signal to the spinal cord (Backonja, et al., 2008). This is followed by a lasting refractory period known as desensitization (Factor, et al., 2011) and if the dose is high enough it generates a longer lasting local desensitization which is also known as defunctionalization (Finch & Drummond, 2015).

In high dose applications when TRPV1 is activated, high levels of calcium enter the cell overwhelming the mitochondria and causing apoptosis. The nociceptive nerve fibers in the area where the capsaicin is administered begin to die which prevents the initiation of new nociceptive input, causing a defunctionalization of the nociceptive ability in that area. After the application of a high dose capsaicin patch for one week there was an 80% decrease in the density of epidermal nerve fibers (ENFs). However, if the dosage is not too high the damage is reversible, which was corroborated by the study. Partial regeneration was

present twelve weeks after the treatment and complete regeneration at twenty-four weeks (Wallace & Pappagallo, 2011). This is consistent with the pain relief that patients who use high dosage capsaicin treatment experience, often pain relief lasts for up to three months per application (Finch & Drummond, 2015).

This is one of the reasons that capsaicin is so well suited to treating chronic pain, especially neuropathic pain. Long lasting, comprehensive relief without daily drug use is very attractive for chronic pain sufferers. Also, the ability of capsaicin to temporarily destroy nociceptive nerve endings provides a way to cut off nociceptive input, the glutamate barrage to the spinal cord. This allows the spinal cord and higher pain areas to minimize or perhaps extinguish the effects of central sensitization or 'wind-up,' which is the hyperactivity of neurons in the spinal cord and brain, due to neuroplasticity that makes them extra sensitive to pain input. Central sensitization is characteristic of neuropathic pain and extremely hard to treat. Doctors who have been using the high dose capsaicin patch have reported anecdotally a reduction in the symptoms of windup in their patients (Wagner, Roth-Daniek, Sell, England, & Kern, 2012).

Medications

There are many forms of capsaicin based pharmacological treatments. Initially, low dose, topical creams were the form of choice, but studies found them to have a disappointing effect. In a comprehensive study they were found, at best, to have a moderate effect and many subjects reported poor or no effect (Trevisani & Szallasi, 2011). The creams come in two different strengths 0.075% under the name Zostrix® (Finch & Drummond, 2015) and 0.025% with lidocaine under the name Axain® (Trevisani & Szallasi, 2011).

The most popular form of capsaicin-based treatment is the Quetenza® 8% patch. The dosage of the patch is significantly higher than the creams which allows the treatment to enable long-term desensitization within the treatment area. The patch promises relief for up to three months (Wallace & Pappagallo, 2011) which is consistent with capsaicin mediated nerve fiber death. This means that the patch only needs to be reapplied every three months, which greatly increases patient compliance.

The potential for capsaicin to cause irreversible nerve damage may make physicians wary of using the high potency patch. However, a study shows that the recommended dosage of up to four patches per application (Wallace & Pappagallo, 2011) is within estimated dietary consumption of cultures that consume a diet heavy in capsaicin such as Mexico, Thailand and India (Hanpaa & Treede, 2012).

The patch has shown to be effective for a variety of syndromes and can be cut to size so that it can be applied almost anywhere on the body. Capsaicin's unique mechanism of analgesia means that it has no known drug interactions which allows it to be incorporated into a treatment plan that includes other analgesics too (Wallace & Pappagallo, 2011).

Other forms of capsaicin include an injectable form that can be used to treat osteoarthritis, tendonitis and some forms of neuropathic pain. There is also an intranasal form that has been clinically proven to be effective in the treatment of migraines (Trevisani & Szallasi, 2011).

Clinical Applications

Capsaicin treatment has been adapted for the treatment of many disorders. The most obvious of these are pain related including PHN, stump pain, headaches, trigeminal neuralgia and osteoarthritis amongst others (Wagner, Roth-Daniek, Sell, England, & Kern, 2012). Interestingly, capsaicin has also been used for the treatment of urinary incontinence as well (Trevisani & Szallasi, 2011).

The treatment of PHN by capsaicin has been the focus of many studies into the efficacy of capsaicin-based analgesia. PHN is one of the most common forms of neuropathic pain after chronic back pain and painful diabetic neuropathy (Wallace & Pappagallo, 2011). PHN is a chronic pain disorder that develops the varicella zoster (shingles) virus comes out of dormancy. The treatment options of PHN are limited and often poorly tolerated which makes the disorder very hard to treat (Backonja, et al., 2008). However, multiple studies that have investigated the outcome of using a capsaicin patch have seen favorable results with minimal side effects and marked relief experienced by the patient (Backonja, et al., 2008; Wallace & Pappagallo, 2011). Some patients with HIV associated neuropathy have also reported relief from capsaicin patch treatments (Capsaicin dermal patch: a guide to its use in non-diabetic peripheral neuropathic pain, 2011).

A tertiary hospital studied the results of their hospital-wide adoption of capsaicin patches to treat non-diabetic patients with a variety of neuropathic pains. These included PHN, CRPS-I, knee arthroplasty related pain, painful scarring, femoral cutaneous neuropathy, HIV related neuropathy and neuroma. They found a 23% reduction of pain in a population of twenty patients that had been treated with the patches. They also noted that six of the patients had not found relief with any other treatment that they had tried so far. They also found that patients that had a higher basal quality of life experienced a better response to the drug (Gimenez-Mila, et al., 2014).

A novel way that capsaicin is being used is to facilitate rehabilitation for knee arthroplasty patients. Often pain following knee arthroplasty surgery can impede recovery and rehabilitation, leading to prolonged hospital stays and a more painful, prolonged recovery. Capsaicin has been introduced as a novel way to dull the pain through temporary desensitization of the local area for the duration of the initial recovery. This allows the patient to proceed with exercises and activities that will improve their function and help them get back on their feet quicker. It was expected that patients may report increased post-operative pain because the capsaicin had been applied, but pain scores were found to be well within range (Hartrick, Pestan, Carlson, & Hartrick, 2011).

Trials of capsaicin being used as 'molecular scalpels' for the treatment of cancer-related neuropathic pain are showing promising signs (Trevisani & Szallasi, 2011). The principle behind this method of treatment is that physicians can use the neurotoxic ability of capsaicin to target very specific cells to treat chronic, intractable pain that cancer patients experience. The treatment carries obvious risks which is probably why the clinical applications that are being pursued right now are very limited, but the potential of this technique is very exciting.

Drawbacks

Although capsaicin is an exciting new frontier in analgesia it has some risks and limitations that must be addressed. Exposure of the mucosa of the throat to capsaicin can cause coughing and bronchoconstriction. However, bronchoconstriction from such a scenario is very rare and even coughing had a very low incidence rate during the clinical trials (Wallace & Pappagallo, 2011).

Capsaicin can only be applied to a local area. In high doses that would be needed to give systemic relief it can cause a drop in body temperature and may be cardiotoxic (Lebovitz, et al., 2012). The hypothermic effect of capsaicin is posited to be a result of the defunctionalization of the TRPV1 receptor. This warps the thermosensitivity of the preoptic heat sensors and fools the animal into thinking that they are warm. This is reflected in the animal's loss of ability to regulate their own body temperature (Trevisani & Szallasi, 2011).

Researchers have also found a correlation between a diet high in spicy food and an elevated risk of cancer. However, further research needs to be done to see if this is applicable to all forms of capsaicin exposure or just ingestion of capsaicin. Capsaicin is poorly metabolized by the skin (Wallace & Pappagallo, 2011) so topical application may not carry the same risk of ingestion.

The patch system, despite having many advantages, has the drawback of needing a minimal infrastructure and attending medical personnel to be able to incorporate them into a treatment plan (Gimenez-Mila, et al., 2014). Also, they often require the administration of a local topical anesthetic such as Lidocaine before application due to the sensation of painful burning that is experienced during the hour long application time (Wagner, Roth-Daniek, Sell, England, & Kern, 2012).

The disadvantage of the creams is that they require multiple, daily applications which greatly decreases patient compliance. It may also induce a painful burning sensation that will discourage its application and hasn't even been proven to be an effective analgesic because of its low dosage (Backonja, et al., 2008).

Conclusions

The ingenuity that is being employed to find pathology and techniques suited to capsaicin treatment is astounding and there is no doubt that the scientific community will continue to come up with new ways to use the unique capabilities of capsaicin

to treat other pathologies. However, there is a need for the development of a standardized schedule of treatment for using capsaicin in a clinical setting (Factor, et al., 2011).

A better understanding of the biology of neuropathic pain would also be an advantage for the development of capsaicin-based pharmacology. If the mechanisms of pain disorders were better understood, treatments could be targeted to directly counteract them in a technique similar to the molecular scalpel being used for cancer-related neuropathic pain.

Perhaps capsaicin could also be adapted for the treatment of phantom limb pain (PLP). The source of PLP is a combination of sensitization and inflammation that takes place in both the central and peripheral nervous systems. Perhaps the desensitizing ability of capsaicin could be effective in this scenario by reducing peripheral sensitization and central nervous system input.

Capsaicin is a novel pharmacological agent that promises an alternative to traditional analgesics and opioids in the treatment of neuropathic pain and other pain syndromes. Research into capsaicin is fairly recent but clinical data has yielded favorable results and new research seems hopeful of making new breakthroughs in techniques and applications of capsaicin in medicine.

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Does In-Vitro Fertilization Increase the Risk for Birth Defects?

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Abstract

Since 1978 when the first “petri dish” baby was born, In-Vitro Fertilization (IVF) has been used as a tool to give couples struggling with infertility the opportunity to have children. Using this method of Assisted Reproductive Technology (ART), the woman is given medication to stimulate her ovaries for the maturation of multiple eggs, which are then retrieved via needle aspiration, fertilized in a petri dish, and inserted in the uterus with the hopes of achieving a successful pregnancy. Many times IVF is completed with another technique known as Intracytoplasmic Sperm Injection (ICSI), where the sperm is injected straight into the egg increasing the chance of fertilization. In 2002, researchers discovered a clear association between an increased risk for birth defects with the use of In-Vitro Fertilization and Intracytoplasmic Sperm Injection. Since then, multiple studies were conducted to determine whether it is instigated by a mechanism of IVF itself, or influenced by the infertility problems of the couple (Hansen et al., 2002). There are various reasonable explanations for the findings of the surfeit occurrence of birth defects in pregnancies with use of IVF or ICSI. Firstly, the advanced age of couples undergoing infertility treatments could be the basis for the underlying increase in birth defects. Maternal factors such as obesity, metabolic disease, and chronic health issues are independent factors proven to increase the risk for birth defects as well (Davies MJ et al., 2012). Factors associated with the treatment such as, freezing and thawing of embryos, exposure of oocytes or embryos to a culture medium, or ICSI gamete manipulation, could also contribute. Additional explanations point to the medications given to induce follicular numbers, or to speed the maturation of follicles into oocytes (Hansen et al., 2002). This paper reviews numerous studies that have been done to resolve the question at hand.

Introduction Infertility

Intervention with medical assistance is required for one in ten couples who have difficulty conceiving naturally. Conception is a complex process that requires many factors to be working in synchrony to develop into a successful pregnancy. The problem can be attributed to female or male components. After one year without conceiving, couples are referred to a specialist to determine the root of the infertility. When the woman is over the age of 35, they are advised to seek medical advice after six months without conceiving. The most common causes of female infertility are irregular ovulation, obstructed fallopian tubes or, congenital anomalies of the uterus. Male infertility is most often caused by azoospermia, when no sperm is produced, or oligospermia, too few sperm. When faced with any of these factors, Assisted Reproductive Technology may be required.

In-Vitro Fertilization Process

The first step in the process of IVF is to produce ovarian hyperstimulation by giving medications to stimulate egg production. Injections of gonadotropins are given daily until multiple follicles develop into optimal size. Next, Human Chorionic Gonadotropin (HCG) is administered to speed the maturation of the follicles into oocytes. The eggs are then retrieved via needle aspiration using transvaginal ultrasonography and stored in a special culture medium until insemination. In a case with normal sperm, 50,000 to 100,000 motile sperm/ml are transferred into the petri dish with the mature eggs. When quality of the sperm is abnormal, such as poor motility or morphology, intracytoplasmic sperm injection (ICSI) is used to fertilize the egg. During ICSI, an embryologist uses a micro needle to inject one single sperm directly into the egg cytoplasm. After insemination, the embryo or embryos are placed in a catheter and injected into the endometrium in

hopes of implanting onto the uterine wall (Wdowiak et al. 2016). Cultured embryos often have difficulty exiting from the zona pellucida membrane. Artificial disruption using laser perforation may be required to aid in the process and increase the chance of implantation (Zakharchenko et al., 2015).

Methods

The research discussed in this paper was compiled from various published articles taken from Touro’s database, including, Proquest Science, EBSCO, PubMed, and Google Scholar. All articles are original scientific papers that were analyzed and explored to obtain accurate data.

Discussion IVF & Birth Defects

Researchers wondered whether the process of Assisted Reproductive Technologies, specifically IVF, can affect the risk of congenital birth defects, or developmental delays. Investigators used data from The Reproductive Technology Register in Western Australia, to analyze pregnancies between the years 1993 to 1997, that were a product of IVF or ICSI. They surveyed all pregnancies that reached 20 weeks and were terminated because of fetal abnormalities. The Western Australian Birth Defects Registry listed all of the birth defects in pregnancies in Western Australia, from natural pregnancies and pregnancies that required the use of ART. With the data from these two institutions, researchers examined all pregnancies that were terminated, stillborn, or live births during these years, to determine whether birth defects were more common in infants conceived with use of IVF or ICSI. The presence of major birth defects diagnosed until the age of one year was examined.

There were a total of 168 out of 4000 infants conceived naturally, 75 out of 837 conceived via IVF, and 26 out of 301 from

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ICSI, that were diagnosed with birth defects before the age of one year. The authors reported that the risk of birth defects was twofold in pregnancies that required Assisted Reproductive Technology. The results remained significant when only singleton births and full term births were taken into consideration. Other studies attribute the increase in risk of birth defects to the multiple births and preterm births that often are associated with use of ART. This research restricted the analysis to singleton and full term births so the results would not be explained by these factors. The overall risks remained the same when factors such as, maternal age and parity, sex of the infant, and association of siblings with birth defects, were taken into consideration. Furthermore, when analyzing the prevalence of birth defects in terminated pregnancies, the results remained similar to the original analyses. The prevalence of birth defects in terminated pregnancies was 4.5% in natural conception, 9.4% with use of IVF, and 8.6% in the ICSI category (Hansen et al., 2002).

After use of IVF the most commonly seen birth defects were cardiovascular, musculoskeletal, urogenital, gastrointestinal abnormalities and cerebral palsy. Also seen was an increased risk for multiple birth defects in singleton births specifically in the areas of cardiovascular defects, musculoskeletal, urogenital and cerebral palsy. The risk of respiratory disorders increased in cases of multiple births brought about by IVF. No association of increased risk in trisomy's, such as Down's, Edward's and Turner's was found (Davies et al., 2012).

Parental Factors

Factors related to maternal and paternal age and health were examined to find reliable information on the association of birth defects and parental issues. A study was done examining the occurrence of birth defects diagnosed before age five and including pregnancies that were terminated at any gestational age. The risks of birth defects were compared in pregnancies that were spontaneous versus assisted conception. Additional data was analyzed in women who reported infertility but eventually became pregnant without the use of ART. With this information it can be determined whether infertility is the major factor in the cause of birth defects or if the technology is to blame. A total of 308,974 births was used for analysis. The risk of birth defects increased significantly in pregnancies of women who experienced a history of infertility but were not treated with ART, versus spontaneous pregnancies without history of infertility. This indicates that the association of birth defects may be attributed to the patients' medical factors rather than the IVF. On the other hand, some patients use treatments such as clomiphene citrate which is given outside the clinic and data on who used this medication is not available for analyses. Clomiphene citrate is used for women experiencing anovulatory infertility to stimulate an increase in estrogen, a hormone that supports the growth of oocytes. This treatment could be the sole reason for increase

in birth defects without use of IVF treatments, and may explain why women with a history of infertility had children with birth defects more commonly (Davies et al., 2012). Another study was done examining the mental health of children born to infertile women who conceived spontaneously. The study indicated that there was a significantly higher increase in mental disorders such as schizophrenia, mood disorders, and psychological development compared to children born to women without fertility problems (Svahn et al., 2015).

Another study was done to examine the degree at which maternal health issues were related to the increase in birth defects after IVF and ICSI. The South Australian Birth Defects Register contained all of the data on maternal medical conditions following various fertility treatments. The maternal factors that were considered were maternal age at delivery, parity, BMI, smoking habits, hypertension, diabetes, asthma, epilepsy, and gestational conditions such as, pre-eclampsia, impaired glucose tolerance, gestational diabetes, anemia, and UTI occurrences. With regard to health conditions, only smoking and obesity were linked to an increase in the risk for birth defects over the general population risk. With regard to ICSI, parity, anemia, and UTI occurrence, were also associated with an increase in birth defects. Additionally, the report indicated that the women with the greatest risk for an increase in birth defects with use of IVF were aged 29 and younger compared to fertile women of their age. The study suggests that the uterine environment of younger women is more likely to be able to sustain an embryo with birth defects or, the implantation success in older women is limited which helps to prevent developmentally compromised embryos from implanting. Women aged 40 and older were at a lesser risk for an increase in birth defects compared to fertile women of their age. A possible explanation may be the influence of the gonadotropins that may protect oocyte development (Davies MJ et al., 2012).

Paternal factors also add to the increased risk for birth defects with use of ART. According to one study, children born to fathers over the age of 40 are at an increased risk for childhood fatality due to congenital abnormalities and malignancies. When paternal age increased to over 45, the childhood fatality rate increased from 1.24% to 1.65% compared to fathers aged 30 to 34 (Urhoj et al., 2014). When the sperm of infertile men was analyzed under light microscopy, it was noticed that there was an excess of DNA damaged sperm. DNA damage limits the ability of apoptosis to occur. Typically, apoptosis removes the damaged sperm but with IVF, fertilization can take place with damaged sperm that would have been excluded in natural conception. This increases the risk for miscarriage and birth defects (Lewis & Kumar, 2015). Additionally, men with reproductive issues often require the use of ICSI because of the poor motility and morphology of their sperm. ICSI can be problematic when abnormal sperm is inadvertently injected, or the injection itself

could damage the chromosome spindle formation in the oocyte (Winston & Handyside, 1993).

Multiple studies imply that the increase in birth defects or mental disorders with use of IVF is likely associated with parental factors. One study pointed to alternative treatments that may be used in addition to IVF. Overall, parental issues play a large role in the increase in birth defects with use of ART, although there are other factors that are likely associated as well.

Cryopreservation

Cryopreservation involves preservation of an embryo or oocyte in liquid nitrogen after cooling the sample in a cryoprotectant to -30 degrees Celsius (Winston & Handyside, 1993). The two methods for cryopreservation are slow freezing and vitrification. Currently, vitrification is the preferred method as it instantly solidifies the samples preventing the formation of ice crystals causing less damage to the oocytes or embryos. Slow freezing involves the use of a less toxic concentration of cryoprotectants however, the limited ability to prevent ice crystals is thought to increase the risk for abnormalities.

There are severe problems involved in oocyte cryopreservation which is a common practice used for women approaching advanced maternal age. First, oocytes are temperature sensitive due to the high content of cytoplasmic liquid and, the arrangement of the chromosomes in the sensitive spindle formation. Disruptions during the freezing and thawing process lead to a threefold increase in aneuploidy when spindle formation is disturbed. The risk of polyploidy also increases, as frozen oocytes are more vulnerable to polyspermic fertilization when the cortical granules are unevenly distributed (Jiminez-Trigos et al., 2012). Caution must be taken with these results because most studies are done on mice and the effect of chromosomal segregation is different between species. Additionally, cryopreservation of oocytes reduces successful fertilization by inducing changes in the zona pellucida specifically, zona hardening, and, producing changes on the sperm receptors of the zona (Winston & Handyside, 1993). Data on the results of cryopreserved oocytes is limited as the number of births resulting from this technique is small. Further results on the health status of children born afterwards is not yet available, however reports do not indicate an increase in birth defects or developmental delays (Schattman, 2015). Some reports reveal that oocytes that developed in vitro have a decreased live births and pregnancy rates.

By analyzing the effect of vitrification on the oocyte, it can be determined if this technique causes adverse effects to oocyte functionality. The mitochondrial function of vitrified oocytes was observed by analyzing the condition of ATP synthase in the oocytes. ATP synthase in Metaphase II stage was analyzed by looking at the ratio of FAD to NAD(P)H. If the ratio is abnormally elevated, it insinuates a decrease in the ATP synthase function of the oocytes which results in abnormal spindle formation.

Additionally, the stage at which the oocytes are vitrified effects the outcomes. Vitrification can be performed at the Metaphase II stage which contains sensitive spindle formation, or the germinal vesicle stage, before the spindle is formed. The germinal vesicle stage has to go through maturation after thawing which may also effect the value of the oocyte. Live births have been seen from vitrification at both of these stages and it is not yet known which has a better success rate (Gao et al., 2017). Ultimately, the ideal method for freezing oocytes is not yet known, and it has been shown to increase the risk for aneuploidy or polyploidy, and decrease risk for fertilization. However, cryopreservation of oocytes is an opportunity for women who wish to preserve eggs for future use because of medical or age related issues. It is currently the only option to preserve fertility for women undergoing treatments such as radiation or chemotherapy which may affect their ability to conceive.

The transfer of frozen embryos has been used more frequently in the past few decades as it has shown several advantages. Multiple studies show that using frozen-embryo transfers increases the rate of live births compared to fresh-embryo transfers. Most reports point to the non-stimulated menstrual cycle that is associated with frozen transfers. When fresh-embryos are transferred, the artificial hormone induced menstrual cycle adversely effects the endometrial receptivity leading to implantation failure (Aflatoonian et al., 2016). Studies have been done to determine differences noted between fresh-embryo and frozen-embryo transfers. A study of, 1508 infertile women diagnosed with Polycystic Ovarian Syndrome and were undergoing their first IVF treatment, were randomized in two groups. They received either fresh-embryo or frozen-embryo transfers. The women were all between the ages 20 to 34 and were in a healthy weight range. All patients went through the standard IVF procedure and subsequently a transfer of up to 2, day-3 embryos. The frequency of live births was considerably higher after the frozen-embryo transfers. The rates showed a 49.3% versus 42% difference. It was proposed that when frozen-embryos are transferred, it allows time for the reproductive system to recover from the induced ovulation by hormones. The exposed endometrial lining has time to shed, increasing the chance that the embryo will implant. The results may not be pertinent to the overall population undergoing IVF since these subjects were PCOS patients. Another study was done to assess whether infertile women without Polycystic Ovarian Syndrome who were undergoing IVF showed the same results. The same method was used and in this case, the transfer of frozen embryos did not increase the chances for a higher live birth rate. Reasons for the different results may be due to the method of cryopreservation or the timing of the freezing (Vuong et al., 2018). In both studies no increase in the risk for congenital malformations was noticed (Zi-Jiang et al., 2016). Other studies note, that when comparing the risk of birth defects in the fresh-embryo and

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frozen-embryo cycles, there was a significant increase in the risk of birth defects for the former. Plausible justifications may be related to the cryopreservation that would eliminate the developmentally compromised embryos, because they would be less likely to survive the freezing and thawing process. Additional explanations that would eliminate the inferior embryos would be the separation of the embryo from hormonal stimulation drugs (Davies et al., 2012).

Various researchers have attempted to observe the effects of the cryopreservation process and the health status of the resulting children. Most studies were done involving results from single-center analyses. A multi-center study between the period of 1995 to 2013 was done to analyze the miscarriage rates, birth weight, pregnancy complications and congenital malformations in IVF centers worldwide. It was determined that the suboptimal results were more likely caused by parental factors such as advanced age, concurrent metabolic, genetic and epigenetic alterations, which affects the health of gametes (Keshishian, 2014). After much research into the matter, the consensus appears to be that there is an increase in live birth rates after the use of frozen-embryo transfers although the reason is still unknown. It is most likely from the natural exclusion of unfit embryos during the freezing process, or the separation of the embryo from hormonal drugs giving the uterus time to readjust for the implantation process. The presence of birth defects does not seem to be affected by the freezing process and is more likely associated with parental issues. This interpretation is consistent with the majority of studies done to analyze the effects of embryo cryopreservation.

Although cryopreservation of embryos is associated with an increase of pregnancy rates and a decrease in preterm labor, it is important to determine that there are no long term unforeseen consequences with regard to this technique. National registries in Denmark were used to examine the intellectual abilities of children aged 15 to 16 born from cryopreserved embryos. A standardized test was given to all children ages 15 to 16 and overall test scores were evaluated. No apparent difference in the test scores between children conceived via IVF, fresh or frozen, and natural conception, was noticed. The study was limited as they did not observe the children that did not enter the school system due to intellectual disabilities (Spangmose et al., 2019)

Culture Medium

Fertilization normally takes place in a protected well-controlled setting in the oviduct. During IVF, the male and female gametes are brought together in a medium that is intended to provide conditions for the sperm to go through the process of capacitation and produce acrosome reactions, in order for sperm penetration, fertilization, and cleavage to occur (Bavister, 1981). Studies done on mouse embryos show that different conditions in the culture can lead to changes in gene expression. Good quality human day 4 cryopreserved embryos were donated to

observe the effect of culture media on transcription. The embryos were randomly selected to be cultured in one of two media (HTF or G5) and one of two oxygen levels. The two media used were Human Tubal Fluid mixed with glucose and phosphate, and the second, a more enriched version of the medium containing additives such as additional amino acids (G5). The two HTF cultures and two G5 cultures contained either 5% or 20% oxygen levels. The embryos were also classified for maternal age and developmental stage at the time of transfer. After culturing, 89 embryos were transferred to PCR and further analyzed. Microarray data analysis was used to observe any correlation between differentially expressed genes (DEG's), the upregulation or downregulation of genes, and whether they were caused by different culture conditions, or whether biological factors such as maternal age and developmental stage played a larger role. The number of differentially expressed genes was significantly higher with regard to biological factors when comparing it with the differentially expressed genes from different culture conditions. Notwithstanding the conviction that maternal age only effects oocytes, this data indicates that maternal age and developmental stage has a much greater effect on transcription. Although the correlation between transcription errors and culture conditions was minimal, it was noted that human embryos developed best in the G5 5% oxygen level culture medium. G5 medium caused an upregulation in genes involved in regulation of phosphorylation and mitosis, and the lower oxygen level led to an upregulation of genes involved in cell morphogenesis which aids in embryo development (Mantikou et al., 2016).

Intravaginal culture (IVC), also known as INVO, is an ART technique where oocytes and early embryos are cultured in a gas permeable air-free plastic device placed in the vaginal cavity for incubation. The advantages are that INVO mimics the environment of the uterus and excludes use of a culture medium to sustain the fertilization and early growth of the embryo. IVF involves the use of a culture medium which influences embryo quality with changes in oxygen and carbon dioxide concentrations, and, PH or temperature changes. If any of these factors become unproportional, embryo fragmentation can occur. Oxygen in the uterine cavity is less than 5%. The INVO device is able to offer an environment similar in that way. The almost anaerobic oxygen concentration level guarantees a safe atmosphere for the energetic metabolism required for successful gamete viability, fertilization and embryo development. Carbon dioxide is another important factor involved in embryo development as it directly effects PH levels (Lucena et al., 2012). The INVO device is absorbent to gas in the uterus and allows for a balanced equilibrium of CO₂ to uphold the PH level of 7.2 to 7.4 (Garcia-Ferreya et al., 2015). On the other hand, in traditional IVF, the concentration of carbon dioxide can be altered when the large gas-filled incubators are opened. This effects the PH equilibrium and temperature of the culture medium, decreasing

embryo quality. A study was done to assess the outcomes of INVO and compare the pregnancy results to common existing IVF techniques (Lucena et al., 2012). Embryos were cultured in the INVO device and then transferred to the uterus on day 3 or day 5 after fertilization. The implantation rates, pregnancy rates, and miscarriage rates were considered. The results showed that the INVO-ICSI procedure had similar outcomes to the traditional IVF-ICSI procedure. Pregnancies were evaluated 14 days after embryo transfer by measuring hCG-beta subunits in the blood, and ultrasounds were utilized to reveal gestational sacs, and heartbeats 21 to 28 days after the transfer. INVO-ICSI was concluded to be alternative option for couples struggling with infertility, with advantages in cost effectiveness, and psychological benefits, allowing a more direct involvement in fertilization which was shown to reduce stress levels in patients (Garcia-Ferreira et al., 2015).

Another study was done to assess the effect of culture conditions on Imprinting Disorders. Syndromes, such as, Angelman Syndrome, Beckwith- Wiedmann Syndrome, and Silver-Russel Syndrome, were found to be more prevalent after IVF. These syndromes are referred to as genomic imprinting disorders and are an epigenetic phenomenon that restricts the expression of one parental allele leading to activation of only one copy of a chromosome. Imprinting genes are controlled by the Imprinting Control Region (ICR) where abnormal cytosine methylation occurs on various genes causing them to deactivate. A study was done that observed the methylation levels of ART produced human embryos at day 3 of cleavage and the blastocyst stage. The specific ICRs analyzed were KCNQ1OT1, SNRPN, and H19, which are the ICR regions where most maternal methylation occurs in the syndromes studied. In the Beckwith-Wiedmann Syndrome children conceived with ART, there were 90% observed methylation errors at KCNQ1OT1 as compared with 50% in the general population of cases with Beckwith-Wiedmann Syndrome. SNRPN methylations occurred in 46% of the Angelman Syndrome ART children versus 5% in the general population. The ART children with Silver-Russel Syndrome were observed to have 92% of the H19 hypomethylations while only 40% of the general population had these defects (White et al., 2015). Some researchers ponder whether the prolonged exposure to a culture medium plays a role in development of these imprinting methylation errors (Davies et al., 2012). Day 3 embryos as well as blastocyst embryos were studied to determine if time spent in the media culture relates to an increase in methylation errors. It was noted that 76% of the day 3 embryos, and 50% of the blastocysts, exhibited defects in imprinting methylations (White et al., 2015). This report demonstrated that the extent of time spent in the culture does not pose a greater risk for the imprinting errors to occur. Multiple studies show that media culture does not significantly affect the outcome of IVF, and therefore does not play a role in the increased risk for birth defects.

The absence of natural reproductive fluids in early embryonic development has shown to influence the outcomes of gene expression in the embryo. Downregulation of embryonic nutritional elements and upregulation of apoptosis factors in the oviduct, have been seen with the absence of seminal fluid (Bromfeild et al., 2014). In mice studies, seminal fluid has been shown to influence fetal development by inducing immune responses from the female reproductive tract. The production of T-regulatory cells which act as a protection to the embryo is activated by seminal fluid. T-regulatory cells are programmed to recognize the male antigens present and to suppress inflammation that would normally occur with an unidentified antigen. This aids in the adaptation of the uterus required for implantation and placental development. Mice studies have shown that with the absence of seminal fluid, fetal development, phenotype, and metabolic function are altered (Schjenken & Robertson, 2015). Follicular fluids have also been shown to influence embryo development. A study was done to detect methylation levels in pig blastocysts to show differences between fluids present in IVF and natural embryos. Whole-genome DNA sequencing was performed for methylation analysis. The addition of reproductive fluids into the culture for IVF produced embryos of higher quality that were similar to embryos produced in vivo. The methylation patterns were closer to the methylation of naturally produced embryos. Additionally, fewer irregularities in genes involved in imprinting, development, and reprogramming, were noticed in the IVF culture that included reproductive fluids (Canovas et al., 2017).

Globally it has been proven through multiple studies that the culture medium does not play a role in the increased risk for birth defects. Reports on the amount of time spent in the culture and the culture conditions were analyzed and no increase in congenital anomalies was seen. There is a slight association between improved gene expression and addition of natural reproductive fluids to the culture.

Alternative Treatments

For couples who are having difficulty with conception, especially at advanced maternal age, the ideal approach is uncertain. Infertile couples have a 2 to 12% chance per year of becoming pregnant spontaneously. Alternative treatments include but are not limited to intrauterine insemination and ovulation induction. These treatments are less costly than IVF however the success rates are well below those of IVF. During intrauterine insemination, semen is condensed in a laboratory and then injected past the cervix into the uterus (Van Vorhis & Bradley, 2007). Ovulation induction involves use of Clomiphene Citrate to stimulate follicles and hence egg growth. Clomiphene citrate works to increase estrogen levels which then signals the anterior pituitary gland to release Luteinizing Hormone which stimulates ovulation. Ovulation induction is most often used in women with Polycystic Ovarian Syndrome which is the most

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common cause of anovulatory infertility. Multiple studies found links between use of ovulation induction medications and an increase in birth defects such as neural tube and cardiac defects. This was reportedly due to the fact that these medications are given to PCOS patients who often struggle with obesity (Balen & Rutherford, 2007). One study suggests an association between use of clomiphene citrate and craniosynostosis (Reeffhuis et al., 2003). This is likely due to the multiple births that are expected with ovulation induction. Multiples are at increased risk for mortality and long term consequences such as congenital anomalies and developmental delays (Corchia et al., 1996).

The use of prefertilization and preimplantation genetic diagnoses combined with IVF significantly increases the rates of successful pregnancies. With advanced maternal age, egg quality is reduced resulting from abnormalities in the spindle formation of the chromosomes. Eggs enter prophase I in the first meiotic division during the fetal period, and remain in that phase until ovulation. When meiosis resumes many years later, the spindle formation can be disturbed, leading to a failure to fertilize, abnormal embryo development, and fetal loss (Van Vorhis & Bradley, 2007). Genetic diagnosis of oocytes, also known as polar body analyses, is useful to differentiate between mutations in genetic material of the oocyte. Each polar body contains complementary genetic material to the oocyte. This can reveal any genetic alterations of the oocyte which would then be excluded from use in IVF. The procedure incorporates, laser microdissection of the zona pellucida with a UV-A laser, and extraction of the polar body with a micropipette (Clement-Sengewald et al., 2000). After genetically normal oocytes are recognized by polar body analyses, they are fertilized and cultured until either day 3 embryos or blastocyst stage when they can then be transferred into the uterus. Polar body analyses are only useful to detect problems with maternal genes. Therefore, another technique known as preimplantation genetic diagnosis is commonly used to select embryos for transfer using genetic analysis that is performed on cells biopsied from embryos. The procedure normally does not affect embryo development because the cells at this stage are totipotent. The practice allows for a choice of embryos that are unaffected by the heritable condition or chromosomal mutations prior to pregnancy and thus avoiding termination of pregnancy (SenGupta & Delhanty, 2012).

Conclusion

Multiple studies show a clear association between the use of IVF and an increase in birth defects. The research discussed above was done to determine whether certain techniques involved in IVF are related to the risk. In multiple studies, cryopreservation of oocytes and embryos was not seen to increase the risk for birth defects or intellectual disabilities. It was noted however, to increase the live birth rate. The various culture conditions were examined and they were noted to have slightly

different effects on transcription but no overall effect on the risk for birth defects. Many reports are limited due to the ethical and practical difficulties that are involved while studying topics such as human embryonic development. Birth defects are rare and thus the observational results were done on small sample sizes. Larger studies are required to give a definite answer to the underlying cause of the association. A number of studies indicated that the infertility problems of the couples undergoing IVF are a likely answer for the increase in congenital anomalies. ICSI has also been thought to increase birth defects because of the increase in chromosomal abnormalities in men with low sperm counts. There is an association between IVF and Imprinting Disorders, however the underlying cause is not known. The generally older age of patients undergoing IVF adds to the plausible complications such as implantation failure, miscarriage, congenital anomalies, and fetal death. Some of these problems can be addressed with Preimplantation Genetic Screening (PGS) which screens for embryos with chromosomal abnormalities or Preimplantation Genetic Diagnosis (PGD) to search for a specific genetic disease. Overall, the comprehensive data related to children born from IVF shows that majority are not born with birth defects. The risk is greatly reduced when IVF is used together with PGD and PGS. Couples considering IVF should discuss genetic testing options with their doctor to determine if this service is appropriate for their situation.

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CAR T-cell Therapy for Acute Lymphoblastic Leukemia

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Abstract

Despite all the available therapies, Acute Lymphoblastic Leukemia (ALL) remains extremely difficult to eradicate. Current available therapies, which include chemotherapy, radiation, and stem cell transplants, tend to be more successful in treating children than adults. While adults are more likely than children to relapse after treatment, the most common cause of treatment failure in children is also relapse. Improved outcomes for all ALL patients may depend upon new immunotherapies, specifically CAR T-cell therapy. CAR T-cell therapy extracts a patient's own T-cells and modifies them with a CD19 antigen. This modification allows the new T-cells to recognize and kill cancer cells that contain the antigen on their surfaces, like leukemia cells do. Although CAR T-cell therapy may cause toxicities such as Cytokine Release Syndrome (CRS), they are mostly short term and reversible. Trials indicate that almost all patients who undergo CAR T-cell therapy will enter complete remission. Though a large percentage of those patients will experience a relapse, relapse rates of CAR T-cell therapy are lower than other treatments. By reviewing the available research literature regarding CAR T-cell therapy, this paper examines the effectiveness of this therapy in different patient populations and demonstrates that CAR T-cell therapy significantly improves event-free survival rates in ALL patients.

Acronyms Used:

ALL - Acute Lymphoblastic Leukemia
ACT - Adoptive Cell Transfer
CAR - Chimeric Antigen Receptors
CRS - Cytokine Release Syndrome
CSF - Cerebrospinal Fluid
Ph - Philadelphia chromosome
TKI - Tyrosine Kinase Inhibitors
CNS – Central Nervous System
WBC – White Blood Count

Introduction

The leading cause of disease related death in U.S. pediatric patients is cancer, most commonly Acute Lymphoblastic Leukemia (ALL) (Tumaini, et. al., 2013). For many years, cancer treatments were limited to radiation therapy, chemotherapy, or surgery. More recently, newer types of treatments called immunotherapies, have become available. Cancer immunotherapies are therapies that enlist and strengthen the power of a patient's immune system to fight and attack the cancer. Of the immunotherapies discovered, adoptive cell transfer (ACT), in which a patient's own immune cells are collected and used to treat their cancer, has achieved the most successful outcomes. The most effective ACT therapy is CAR T-cell therapy (Brentjens, et. al., 2013). In 2017, CAR T-cell therapy was approved by the FDA as a treatment for ALL and for adults with advanced forms of lymphoma. However, while it may seem that CAR T-cell therapy is poised to revolutionize cancer therapy, some of the optimism surrounding it is tempered by concerns about its safety and potentially severe toxicities (Lim, June, 2017), calling into question if CAR T-cell therapy is an improved treatment for ALL and relapsed ALL.

Methods

The research used in this paper was located and compiled from papers obtained through Touro College's access to online publications. Google Scholar and Blood Journal were used for additional references. The articles were critically selected,

compared, and analyzed to evaluate if CAR T-cell therapy is an effective treatment for ALL.

CAR T-cell Therapy

CAR T-cell therapy is relatively straight forward. T-cells, which play a critical role in orchestrating an immune response, are responsible for killing cells that are infected by pathogens. In order to retrieve these T-cells, blood is drawn from a patient and the T-cells are separated from the rest of the blood. The T-cells then undergo genetic modification via the insertion of genes that encode for tumor specific chimeric antigen receptors (CARs). These receptors allow the T-cells to recognize and subsequently attach to a specific antigen. The antigen CD19 was chosen because it is universally expressed on all ALL tumor cells and not on pluripotent hematopoietic stem cells (Tumaini, et. al., 2013). Once the T-cells have been successfully engineered to express the CD19 antigen, they are expanded in a lab to form hundreds of millions of cells. This process has been refined and advanced over the years and can now quickly create large quantities of T-cells that have genetically engineered receptors on their surface to treat both pediatric and adult ALL patients. The CAR T-cells are then infused back into the patient's body where they continue to multiply, recognize, and kill the cancer cells containing the antigen on their surfaces (Tumaini, et. al., 2013). This step is considered to be an in vivo expansion, which requires the new host to support these engineered T-cells. Therefore, the administration of the T-cells is preceded by a lymphodepleting regimen, as lymphopenia (the condition of having an abnormally low level of lymphocytes in the blood) generates changes that support T-cell expansion and survival (Klebanoff, et. al., 2005). As a result, most adoptive cell therapy protocols incorporate some version of lymphotoxic therapies prior to cell transfer.

Like most cancer treatments, CAR T-cell therapy has serious side effects. The most common side effect that patients usually experience is cytokine release syndrome (CRS). Notably, CRS has been seen in patients treated with other immunotherapies and is therefore not limited to CAR T-cell therapy (Lee, et. al., 2014; Teachey et al., 2013). CRS is caused by the cytokines

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released from the T-cells as CAR T-cells rapidly expand within the patient's body. It can also be caused by other immune cells, such as macrophages that might produce cytokines in response to the cytokines produced by the infused CAR T cells. CRS patients have high levels of IL-6 (the cytokine secreted by the T-cells) and is characterized by systemic symptoms that usually begin with a fever (Giavridis, et. al., 2018). The onset of the fever can range from a few hours after the treatment to more than a week after CAR T-cell infusion (Brudno, Kochenderfer, 2016). The fever is followed by nausea, chills, headaches, muscle pain, and difficulty breathing (Maude et al., 2014).

CRS can lead to many different related toxicities that attack organ systems. Cardiovascular toxicities most commonly cause tachycardia, although more severe cases of CRS have prompted hypotension, arrhythmia (irregular heartbeat), and decreased cardiac ejection fraction. CRS can lead to pulmonary edema and hypoxia, the deficiency in the amount of oxygen reaching the tissues that may require mechanical ventilation. It can also lead to reduced renal perfusion, the volume of blood delivered to the kidneys per unit time, which can cause a kidney injury. However, CAR T-cell related renal injuries are mostly reversible. The same goes for other laboratory abnormalities that CRS causes, such as elevated levels of bilirubin and/or serum transaminases. Patients also commonly become neutropenic and lymphopenic when undergoing and following CAR T-cell therapy because they are severely immunocompromised and are not protected against opportunistic infections, such as salmonella, bacteremia, and urinary tract infections. Viral infections such as influenza, respiratory syncytial virus, and herpes zoster virus, have also been known to affect patients following CAR T-cell infusion (Brudno, Kochenderfer, 2016). Unfortunately, in such a setting, fevers, tachycardia, hypotension, and other regular symptoms associated with CRS can be difficult to differentiate from sepsis, which is a life-threatening infection. In an early trial, a patient with chronic lymphocytic leukemia who received chemotherapy prior to his CAR T-cell treatment died with fever, hypotension, and renal failure four days after the administration of the CAR T-cells. It was discovered later that there were elevated serum levels of inflammatory cytokines before CAR T-cell infusion, suggesting that the patient had a prior infection that caused his death (Brentjens, Curran, 2012).

CAR T-cell therapy also has neurological toxicities associated with the treatment. The toxicities can be diverse, as they do not always localize to one specific area of the nervous system. The occurrence of neurologic toxicity is quite variable, with published reports stating that there is a 0% to 50% chance of neurological toxicities developing (Brudno, Kochenderfer, 2016). Neurologic events are not always associated with CRS toxicities, which suggests that in some cases, they might have a different mechanism than many of the other usual toxicities caused by CRS, such as fever and hypotension (Maude, et. al., 2014).

Ironically, CRS is considered to be an “on-target” effect of CAR T-cell therapy, as the presence of the cytokines show that the T-cells are working in the body. Various grading systems for the “CRS-related adverse events” caused by immunotherapies have been proposed. They depend on many things, such as the temperature of the fever, the number of severe signs of toxicities, and cytokine levels in the patient. A category of severe CRS is defined as CRS requiring pharmacologic and medical intervention. In such cases, tocilizumab, an IL-6 receptor antagonist that is used to treat rheumatologic disorders, is used as a first line agent. While not approved for this use by the FDA, it has effectively treated CRS-related toxicities in clinical trials with no life threatening or toxic effects (Maude, et. al., 2014), and is now a widely used off-label for the patients who have received CAR T-cell infusions. (Brudno, Kochenderfer, 2016).

Systemic corticosteroids have also been used for CRS-related toxicities. However, there is some evidence that corticosteroids can possibly inhibit CAR T-cell persistence and anti-malignancy efficacy. For this reason, corticosteroid therapy is only used as a last resort if the tocilizumab does not succeed in ameliorating the CRS. However, because neurologic toxicities may not come along with CRS, these toxicities may differ from that of CRS alone. It is unclear if tocilizumab has any beneficial effect on neurologic toxicities, as severe neurologic toxicities are commonly treated with systemic corticosteroids right away, rather than initially beginning with tocilizumab.

Toxicities caused by CAR T-cells are diverse. Management of these toxicities requires continuous and vigilant monitoring, aggressive supportive treatments, and, in some cases, intensive care. Another harmful side effect of CAR T-cell therapy is that the engineered CAR T-cells with the CD-19 receptor could damage other tissues that express the antigen recognized by it. This mechanism of toxicity can be mostly eliminated by searching for any expression of the targeted antigen on normal tissues in the body prior to the development of the CAR (Lamers, et. al., 2013).

Other Therapies

There are several other therapies used to treat ALL. The goal of these treatments is to remove all traces of the ALL from the patient. The most commonly used treatment for ALL is chemotherapy. The chemo treatment is divided into three phases. The first stage is known as the induction phase and usually takes about a month. The next phase, consolidation, also referred to as intensification, is, as its name suggests, extremely intense as well, and typically lasts for a few months. The last phase, maintenance, or post-consolidation, is less intensive, and lasts for about two years (Pui, et. al., 2008).

The goal of induction, is to remove more than 99% of the initial leukemia cells from the patient and to restore normal haemopoiesis, the production of blood cells and platelets in the bone marrow. Afterwards, if both the blood and bone marrow

show no evidence of persistent leukemia and blood counts have returned to normal, then the patient achieves remission. Studies show that 96 to 99% of children with ALL enter remission after the induction treatment. Adults have a lower remission rate with only 78-93% of adults entering clinical remission (Pui, et. al., 2008). Nevertheless, a remission is not necessarily a cure, and since the first month is such intense treatment, many complications and serious infections can arise. As a result, this phase of treatment requires lengthy hospital stays and frequent visits to the doctor, as these complications can be life-threatening. Recent advances in supportive care have helped lower these complication rates, making them considerably less common than they have previously been. Examples of supportive care are better nursing care, proper nutrition, prescribed antibiotics, and red blood cell or platelet transfusions as needed (Locatelli, et. al., 2012).

There are three major types of drugs that children with standard-risk ALL receive during the first month of treatment. These are chemotherapy drugs L-asparaginase and vincristine, as well as a steroid drug, usually dexamethasone (Goekbuget, et al., 2005). For children in high-risk groups, a fourth drug from the anthracycline class, typically daunorubicin, is added. ALL patients also require chemotherapy via the cerebrospinal fluid (CSF), as there may be leukemia cells that spread to the brain and spinal cord. This treatment is known as intrathecal chemotherapy and given through a lumbar puncture/spinal tap. Patients with high risk leukemia or leukemia in their CSF receive this treatment more frequently than other ALL patients do. In the past, along with intrathecal therapy, patients were also given prophylactic cranial irradiation. Recent studies have found that children who are given more intensive chemotherapy may not need radiation therapy at all. Doctors try to avoid giving radiation to the brain, especially in younger children, because even low doses may result in problems with cognition, growth, and development (Locatelli, et al., 2012).

The next phase, consolidation, is the phase of chemotherapy that further reduces the number of leukemia cells still in the body. Several chemo drugs are used together to prevent the remaining leukemia cells from developing a resistance. Intrathecal therapy is continued at this time, and patients with high-risk leukemia usually receive more intense chemotherapy. During this phase, patients with Philadelphia chromosome-positive ALL may benefit from the addition of other types of cancer therapies, such as targeted cancer drugs or stem cell transplants (Pui, et. al., 2008).

If the leukemia remains in remission after induction and consolidation, the third phase, maintenance therapy, can begin. Most treatments use medication given either as pills or intravenously, and a steroid, usually prednisone or dexamethasone. Depending on the type of ALL and the risk of recurrence, other drugs may be added as needed. In the beginning of the maintenance phase, most treatment plans include one or two repeat intensified treatments like the initial induction. These four-week

intensifications are called re-induction or delayed intensification. Some children at higher risk may receive more intense maintenance chemotherapy and intrathecal therapy. The total length for the three phases of chemotherapy for most ALL treatment plans is two to three years. However, patients with a higher risk of relapse are given several extra months of treatment as an added precaution (Pui, et. al., 2008).

Throughout the entire chemotherapy process, the combination of anti-cancer drugs used often causes serious side effects. This is mainly because the chemotherapy drugs affect healthy body cells as well cancer cells. Many organs, such as the kidneys, liver, testicles, ovaries, and lungs, can be damaged by these drugs. In an effort to reduce the number of side effects, the chemotherapy is given in cycles, and each round of treatment is followed by a rest period, so the body has time to recover. Some of the main side effects of chemotherapy are loss of hair and appetite, vomiting and nausea, constipation, and mouth sores. Chemo drugs also affect the normal cells in bone marrow, which can lower blood cell counts. This leads to an increased risk of infections, due to low white blood cell counts, easy bleeding and bruising from low platelet counts, and constant fatigue and shortness of breath, as patients do not have enough red blood cells (hemoglobin) to carry the oxygen needed in their bodies (Pui, et. al., 2008).

As with most treatments, steps can be taken to reduce the toll these side effects can have on the patients. Drugs, such as Ondansetron (Zofran) can be given to decrease nausea and vomiting. Transfusions or drugs can be administered to raise a platelet or red blood cell count, and antibiotics are given at the earliest sign of a developing infection. Even tumor lysis syndrome can be prevented. This potentially life-threatening side effect of chemo is usually seen in the induction phase of treatment. As the leukemia cells are killed by the chemo drugs, they break open, releasing their contents into the bloodstream. These components can overwhelm the kidneys because they are unable to filter out and remove all these substances from the blood at once, and the excess amounts of certain minerals can affect the heart and nervous system. Administering certain drugs and extra fluids during treatment can help the body eliminate these substances (Goldman, et. al., 2001). Patients need to be carefully monitored while being treated with cancer drugs to reduce the risk of these side effects as much as possible.

High-energy radiation used to kill cancer cells is another therapy used to treat ALL. Most often, external beam radiation therapy is used, in which a machine delivers a beam of radiation to a specific part of the body at a certain angle. Although radiation is not used as the main treatment for ALL, it is used in certain situations, such as preventing or treating leukemia that has spread to the brain, (though lately, radiation has been omitted from treatment plans) spinal fluid, and testicles. Before a bone marrow or peripheral blood stem cell transplant takes place,

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the whole body often needs radiation. Rarely, radiation can help shrink a tumor if it is causing breathing problems by pressing on the trachea, although chemotherapy is often used instead, as it normally achieves the same effect more rapidly (Cherlow, et. al., 1993). The side effects of radiation therapy depend on the location at which the radiation beam was targeted. The treated area can appear sunburned and undergo hair loss. Radiation to the abdomen can sometimes cause nausea, vomiting, or diarrhea. Effects of radiation that targets large parts of the body may include fatigue, shortness of breath, and an increased risk of infection due to lower blood cell counts (Pearce, et. al., 2012).

One of the most serious, but not very common side effects of ALL chemotherapy and radiation therapy is an increased risk of getting another neoplasm, a new and abnormal growth of tissue in some part of the body that can become cancerous later on. Although effective treatments for ALL now result in five-year survival rates above 70%, the treatments used are ironically carcinogenic. Studies show that there is a substantial chance of secondary neoplasms among patients treated for ALL with chemotherapy and radiation. Children five years old and under, as well as patients who received radiation as a form of therapy, are at a higher risk for second tumors arising in their central nervous system, as well as patients who received radiation as a form of therapy. (Neglia, et. al., 1991).

Allogenic hematopoietic stem-cell transplantation, the transplantation of multipotent hematopoietic stem cells, is the most intensive form of treatment for ALL. This stem cell transplantation seems to benefit several types of high risk ALL patients, such as patients with poor initial responses to treatments, patients who have relapsed, and those with Philadelphia chromosome-positive disease (Pui, et. al., 2008). The Philadelphia chromosome (Ph) is the most frequent cytogenetic abnormality in adult ALL. Most patients with Ph+ ALL cannot be treated with chemotherapy alone, as chemotherapy can only induce a complete remission for a few months until most patients experience a relapse. The five-year survival rates for those who have Ph+ ALL and were treated with chemotherapy alone are less than 10%. However, those who underwent allogenic stem cell transplantation during early remission have a 35-65% chance of long-term survival (Lee, et. al., 2005). Bone marrow transplants (BMT) are sometimes needed as well for patients with underlying malignancies or genetic disorders (Slavin, wt. al., 1998).

Targeted cancer drugs are also being used as a treatment for ALL. Cancer cells are cells that undergo changes to their genes. These changes cause the cancer cells to grow faster and work differently from others. Targeted cancer drugs use these differences in cell genes to differentiate them from other normal cells and target the specific gene changes. The main targeted cancer drugs used for ALL are tyrosine kinase inhibitors (TKIs). They block tyrosine kinases, chemicals that cells use to signal to each other. The main side effects of targeted cancer drugs, specifically

TKIs, are fatigue, a sore mouth, rashes or reddening of the skin, and loss of appetite (Pui, et. al., 2008).

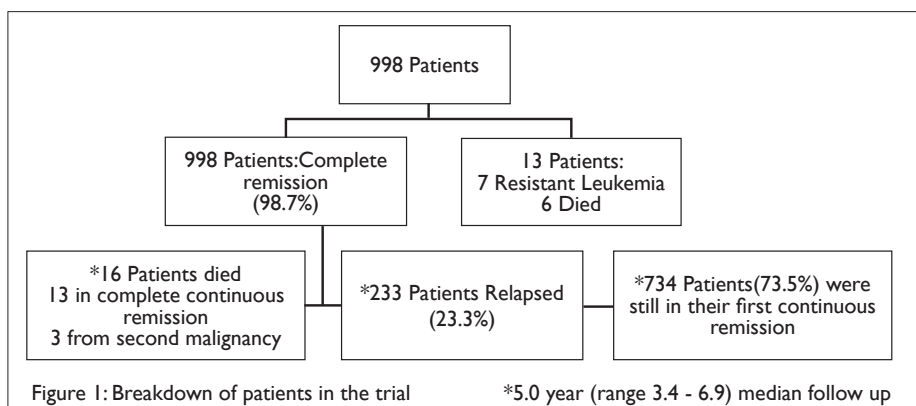
Success Rates

Chemotherapy affects children and adults with ALL differently. Children that undergo chemotherapy treatment have a much greater chance of survival than adults, yet only 80 – 85% of children can be cured. A large chemotherapy trial comprised of children up to eighteen years old was conducted on 1,114 patients. Of those patients, only 998 became protocol patients, and were divided into three groups; a standard risk group, those without central nervous system (CNS) disease, a risk group, those with CNS disease, and an experimental group. They all received induction therapy and some variation of continued chemotherapy, including cranial irradiation as needed. Additionally, an intensive reinduction therapy was added for patients in the standard risk group who had increased risk of failure during the trial. The event-free survival for the 110 patients who did not receive reinduction therapy was almost 30% lower than those with reinduction therapy. In this trial, a complete remission was measured by several parameters: the absence of leukemic cells in the blood and CSF, fewer than 5% lymphoblasts in marrow, and no evidence of localized disease. Relapse was defined as reappearance of lymphoblasts or localized leukemia infiltrates at any site (Reiter, et. al., 1994).

Figure 1 presents results of this trial. Of the 998 patients, 985 (98.7%) entered complete remission. Thirteen patients did not enter remission; seven had resistant leukemia, one died of renal failure, and five died an early toxic death. At the 5.0-year (range 3.4 - 6.9 years) median follow up, 233 patients experienced a relapse (23.3%). Thirteen more patients died while in complete, continuous remission and three patients developed a second malignancy. There were 734 patients (73.5%) still in their first continuous complete remission.

The six-year, event-free survival estimate was for 888 patients. The 110 patients who did not receive reinduction were excluded from this estimate. Of all three branches together, the estimate was $74\% \pm 2\%$. Detailed analysis showed that male patients had higher white blood counts (WBC), were six years old or greater, or had T-ALL, had a better outcome than others. For example, $69\% \pm 5\%$ of T-ALL patients with $WBC \geq 20,000/\mu L$ had event-free survival at six years. This is a comparatively larger number than $58\% \pm 3\%$ for the complementary group of patients with an immunophenotype other than T-cell ALL. These other immunophenotypes had no predictive strength for treatment outcome (Reiter, et. al., 1994).

There was a trial for 525 patients under 19 with a first-time relapse of T-cell or B-cell ALL. The patients were treated with intensified, short course multi drug chemotherapy. A major aim of this study was to improve outcomes through a third intensive chemotherapy course (R3) containing HD cytarabine and



etoposide. Results showed that 440 patients (84%) achieved a second complete remission, 25 patients (5%) died during induction, and 60 patients (11%) did not respond to treatment. Thirty-one patients (6%) died in the second complete remission as a result of treatment-related adverse effects. Of those patients who achieved second complete remission, 246 patients (56%) suffered subsequent relapse, and six patients (1%) experienced a secondary malignant neoplasm. At a median follow-up of twelve years, 153 (29%) of 525 patients remained in complete continuous remission. The study concluded that neither R3 nor adaptation of chemotherapy intensity was capable of improving event-free survival or of overcoming prognostic factors. Therefore, induction regimens need to be improved for high risk patients, and allogeneic stem cell transplant should be recommended in patients achieving second complete remission (Tallen, et. al., 2010).

Adult recovery rates are not as high as children's. ALL accounts for about 15 – 20% of all adult leukemias. Although some of these patients enter complete remission, most of them relapse and die. With chemotherapy alone, those younger than sixty have a 30 - 40% chance of recovery (Mohty, et. al., 2010). Anyone older than sixty has less than 10% chance. In many cases, chemotherapy is not enough and adult patients need other therapies, such as transplants. However, the patients still need to undergo chemotherapy maintenance after the induction therapy and transplant. (Goldstone, et. al., 2008).

A study was performed on 609 adults with relapsed ALL, all of whom were previously treated in the Medical Research Council study, in which the overall survival of newly diagnosed patients was 38% at 5 years. By contrast, in this chemotherapy study, the overall survival at 5 years after relapse was 7%. Factors predicting a good outcome after salvage therapy were young age (overall survival of 12% in patients younger than 20 years vs overall survival of 3% in patients older than 50 years) and short duration of first remission. Treatment received in the first complete remission did not influence the outcome after relapse. This study concluded that adults who have an ALL relapse cannot be rescued using currently available therapies, even if stem

cell transplantation is available. Prevention of recurrence would be the best strategy for long-term survival from this disease (Fielding, et. al., 2007).

The most common cause of ALL treatment failure is relapse, as approximately 15 – 20% of children experience relapse. With intensive chemotherapy and transplantations, 30-50% of all children with relapsed ALL can be cured. However, because relapsed ALL is

difficult to treat, most relapsed children still die, despite the aggressive therapies (Locatelli, et. al., 2012). CAR T-cell therapy may be able to overcome the limitations of conventional therapies and induce remission in patients with relapsed and refractory ALL (Maude, et. al., 2014). In one trial, thirty patients were selected. Twenty-five patients were aged from five to twenty-two, and the other five patients between twenty-six and sixty. Of these patients, twenty-six had B-cell ALL in a first to fourth relapse, three had primary refractory B-cell ALL, and one patient had relapsed T-cell ALL. Eighteen patients had experienced their relapsed ALL after allogeneic stem-cell transplantation. All the patients in this study experienced CRS. Eight patients developed severe CRS, and all required respiratory support and vasopressor support for hypotension. However, all neurotoxicity was reversible and there was no lasting damage from the CRS (Maude, et. al., 2014).

One month after the infusion, twenty-seven patients (90%) obtained complete remission. Of these patients, nineteen remained in remission. Fifteen patients received no further therapy, and five withdrew to receive other treatments. At six months, the event-free survival rate was 67%, and the overall survival rate was 78%. This is a much better rate than the <25% complete remission rates of the recently approved drugs (nelarabine, liposomal-encapsulated vincristine, and clofarabine) for ALL. This study showed an encouraging sustained remission of up to two years (Maude, et. al., 2014).

In another trial, 53 pretreated adults received CAR T-cell therapy. A total of thirty-six patients (68%) received CAR T-cell therapy as a third or later salvage treatment, twelve had primary refractory disease, nineteen had undergone allogeneic hematopoietic stem-cell transplantation previously, and thirteen had received the drug blinatumomab previously. A total of sixteen patients had Philadelphia chromosome positive ALL, and ten of the sixteen patients had disease that was refractory to the drug ponatinib. After infusion, 26% of the patients had severe CRS. Complete remission was defined as less than 5% bone marrow blasts, the absence of circulating blasts, and no extramedullary sites of disease regardless of cell-count recovery. Relapsed

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disease was defined as the reappearance of blasts in blood or bone marrow or in an extramedullary site after a complete remission. One patient died from multiorgan failure and severe CRS on day five, and complete remission was observed in 83% of the patients. At a median follow-up of twenty-nine months (range one to sixty-five), the median event-free survival was 6.1 months, and the overall survival was 12.9 months. Patients with a low disease burden (<5% bone marrow blasts) before treatment had distinctly enhanced remission duration and survival, with a median event-free survival of 10.6 months and a median overall survival of 20.1 months. Patients with a higher burden of disease ($\geq 5\%$ bone marrow blasts or extramedullary disease) had a greater incidence of the cytokine release syndrome and neurotoxic events and shorter long-term survival than did patients with a low disease burden. This is a better outcome than the three to nine months of median overall survival seen from chemotherapy (Park, et. al., 2018).

Of the 44 patients who had a complete remission after the infusion of CAR T-cells, 26 did not undergo further therapy, including nine who were alive and seventeen who had a relapse or died. One patient received alternative treatment for minimal residual disease–positive disease, and seventeen patients progressed to transplantation. The median time from the CAR T-cell infusion to transplantation was 74 days (range, 44 to 312). Of the seventeen patients who underwent allogeneic transplantation after the CAR T-cell infusion, five patients were alive and had a complete remission, six had a relapse, and six died from transplant-related toxic effects. This study showed that CAR T-cell therapy had favorable long-term remission rates in a population of patients with low disease burden, who had significantly longer event-free survival and overall survival with markedly lower incidences of toxic effects than did those with a high disease burden (Park, et. al., 2018).

In another trial, 20 patients (aged 1-30 years, including eight patients who underwent allogeneic stem-cell transplantation) with relapsed or refractory ALL were infused with CAR T-cells. CRS was recorded in 16 patients, and all toxicities associated with the therapy were reversible. Complete remission was observed in 70% of the patients. Many of the patients in the trial underwent further stem cell transplantation therapy, which led to the conclusion that CAR T-cell therapy is an effective bridge to stem cell transplantation for patients with chemo refractory B-ALL. Because most patients who entered remission eventually underwent stem cell transplantation, this study was not able to assess the durability of response to the CAR T-cells, yet it was associated with a favorable long-term survival. Additionally, this study showed that CAR T-cells mediate a complete remission in refractory ALL that is substantially higher than the 8-20% reported with clofarabine, a drug that was approved in 2004 for refractory pediatric ALL (Lee, et. al., 2014).

Conclusion

CAR T-cell therapy is currently being used as a treatment for patients who have already been treated with other therapies and relapsed. This makes the effectiveness of the therapy difficult to gauge, as CAR T-cell therapy trial outcomes cannot be compared to first time treatment data of other therapies such as chemotherapy. Nevertheless, the trials have shown that the toxicities from CAR T-cell therapy are manageable and are no longer a big concern. The trials also show that although it does not have perfect results, CAR T-cell therapy is far more successful in treating relapsed ALL than other therapies have been, as the complete remission rates and longer survival rates are higher for relapsed ALL than any other treatment. Some studies indicated that many patients may have needed additional stem cell transplants and other therapies after undergoing CAR T-cell therapy, so it cannot always be used as a lone therapy. However, some patients did achieve event-free remission from only CAR T-cell therapy. There were no attempts in proving that CAR T-cell therapy should not be done, making this therapy a great treatment option for ALL, especially relapsed ALL.

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The Effects of RF-EMF on the Child Brain

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Abstract

It has long been debated whether or not cell phones have a deleterious effect on the brain. Recent studies indicate that the electro-magnetic field emitted by cell phones called RF-EMF is linked to cancer. Guidelines created to limit the exposure have not been changed since 1981 and do not consider children. The mechanism thought to cause cancer is reactive oxygen species (ROS), which cause the creation of micronuclei. RF-EMF poses a greater threat to children than adults. This is due to the major anatomical differences between the head of a child and an adult. The skull of a child is much thinner than that of an adult. Additionally, the marrow in the skull of a child is much more vulnerable to RF-EMF. Another difference is the presence of myelin in the brain of a child. Until the age of two production of myelin sheath occurs at a frenzied pace. After age two production slows but continues into adulthood. The uncompleted myelin sheaths, as well as the unprotected axons, can be easily damaged by RF-EMF. This can lead to axonal degeneration and decreased action potential speeds. Another difference is the presence of neural stem cells. Neural stem cells differentiate from neuroepithelial tissue. These cells then commit to oligodendrocytes or astrocytes and undergo cell division to form immature glial cells. Research shows that children contain a substantial amount of these stem cells, whereas adults do not. RF-EMF inhibits cell division resulting in a decreased number of immature glial cells. Because of these anatomical differences, parents should be wary of the amount of "screen time" they provide their children. The guidelines of acceptable SAR should also be changed to take the risks to children into account.

Key Abbreviations

ROS- Reactive Oxygen Species.

RF-EMF- Radio Frequency Electro Magnetic Fields.

ELF-EMF- Extremely Low Frequency Electro Magnetic Fields

SAR- Specific Absorption Rate

SAM- Standard Anthropometric Mannequin

GSM- Global Systems for Mobile communication

MWF- Myelin associated Water Frequency

Introduction

It has been estimated that as of the end of 2017 there have been more cell-phone subscriptions than humans (International Telecommunication Union 2016). Cell phones use radio-frequency waves to carry information from one phone to another via base towers. As of May 2011, the IARC officially recognized RF-EMF as a Group 2B human carcinogen (International Agency for Research on Cancer 2011). This means that RF-EMF is now classified as a "possible human carcinogen". There are those who believe that it should be moved up to the "known carcinogen" category due to the studies done on rats that show a positive correlation between cell-phones, and cancer (Belpomme et. al 2018). With the arrival of 5G networks and the ever-increasing dependency on cell-phones the potential risks of these networks must be determined. One area of study that has not been as defined is the potentially greater hazards of RF-EMF to children than adults.

It is thought that there are two mechanisms in EMF that cause cancer. The first is thermal radiation and the second non-thermal. Thermal radiation is due to the friction caused by polar molecules as they move along with the electromagnetic field. This effectively heats up the brain the same way a microwave heats up food. This can cause denaturing of DNA. Non-thermal radiation is the emitting of a particle that denatures DNA or splits ROS (reactive oxygen species) creating free radicals that are detrimental to human health. It is now being discovered that even ELF-EMF, such as those that provide electricity, can

cause cancer when one is exposed long-term. This is also seen in RF-EMF, as studies show that one who uses a cell phone for ≥ 10 years will double his chances of getting acoustic neuroma (Khurana et. al 2009)

Another factor that must be considered is the intensity of the radio-wave. The average GSM (Global Systems for Mobile communication) phone operates on a wavelength of 800 MHz to 1900 MHz. This is broadcasted at different strengths depending on the signal strength and how hard the phone has to work to connect to the closest base station. There are guidelines in place to limit the amount of radiation emitted by a phone. This is limited to 1.6 W/Kg in a 1-gram cube of tissue. It is estimated that up to 80% of the radiation emitted by the phone is absorbed by the head when one is talking on the phone normally. The aim of this paper is to explore the potential effects RF-EMF waves emitted by a cell-phone have on a child's brain.

Methods

To complete this study, online scholarly databases were searched for relevant articles. Databases included Google Scholar, as well as ProQuest, EBSCO, and EMF-PORTAL. Key words included "head," "child," as well as "cell phones" among many others. While most of the material found is available to the public, many of the articles needed special access that was provided by Touro College for the use of this paper.

Background

In 1982 the IEEE (Institute of Electrical and Electronic Engineers) was commissioned to find a level of intensity of RF-EMF exposure that could be considered safe. It is worthwhile to keep in mind that the IEEE is an organization dedicated to "advancing technology for the benefit of humanity" according to the mission stated on their webpage. They are also not a group whose main focus is health sciences. Their studies resulted in a near unanimous conclusion of 1.6 W/kg to be safe for non-occupational use (IEEE, 1982), however no rationale was provided for

this number. These guidelines have remained unchanged since a remodeling of the guidelines in 1991 to a two-tier system based on a “controlled environment,” i.e. workers in a profession who are aware of the potential hazards of the job, and an “uncontrolled environment,” i.e. the general population.

Cell phone compliance with these guidelines are tested through SAM (Standard Anthropometric Mannequin), a model head with dimensions based off of the 90th percentile of U.S. military recruits in the year 1989. The corresponding body of the head would be a six foot, two inches, 220 lb. male. The model is filled with dielectric material of similar composition to that of brain tissue. A phone is then positioned next to the head and the model is subjected to a phone call. A probe is inserted between the mannequin’s head and the cell phone, which measures the radiation levels emitted and calculates it as W/kg based off of 1-gram of tissue. This is referred to as the psSAR (peak spatial Specific Absorption Rate), or the SAR of a cell phone. It must be noted that this method can only measure the effects of thermal radiation.

There is another way of calculating SAR called the FDTD (Finite Difference Time Domain) method. This method is a computational program in which a magnetic field is arranged as a three-dimensional grid. The permeability, permittivity, and conductivity are then entered into the program for the material contained in each specific cube. Through this the program can compute based off of all the materials in the cubes how much radiation was absorbed by each specific tissue.

There have been multiple experiments done to prove the hazards of RF-EMF. One of the more popular studies exposed rats to a GSM-like frequency and monitored long-term and short-term effects. The most recent study exposed rats to RF-EMF to monitor long-term effects and found a positive correlation between these waves and cancer (National Toxicology Program 2018). One of the disadvantages of this study was the inability to expose rats in the same manner that a human is exposed. Additionally, the SAR that was used for this experiment was higher than that of normal cell phone use.

Another study that has gained popularity in recent years is the computer simulation of an RF-EMF. This involves studying the characteristics of the electromagnetic field and the specific features of the tissues being exposed. The researchers will then create virtual models and simulate exposure to an RF-EMF. The shortcoming of this type of study is that one can only monitor the effects of thermal radiation. The FDA uses a four-member family for all their simulations consisting of male and female adults and children. Both the SEMCAD program, which monitors EMF, and the virtual family are available to the public.

Potential Effects

These studies, as well as most other recent experiments conducted by experts in the field, yield results that indicate that

RF-EMF’s are potentially hazardous to one’s health. The question remains as to whether RF-EMF causes cancer. Most recent studies find a positive correlation between RF-EMF and malignant glial cell tumors (Falcioni et al 2018). This study is unique as the researchers projected a lower SAR over a longer period of time. This suggests that long-term exposure may in fact be the cause for concern and not the SAR. If this is the case, it would render today’s safety standards useless, as they are based on SAR. Another study found that there was an increased risk for glioblastoma and acoustic neuroma in heavy cell phone users (Hardell et al. 2013). The tumors observed were usually ipsilateral to the preferred side of cell phone use. These results are extremely important. Acoustic neuroma is a benign tumor on the vestibulocochlear nerve, just inside the inner ear. This is the closest organ to which one holds the cell phone while talking on the phone. While acoustic neuroma is not malignant, it can cause deleterious effects by putting pressure on the brain stem. The mechanisms by which RF-EMF potentially causes cancer are unclear. It is highly unlikely that RF-EMFs have enough energy in them to significantly heat up brain tissue to the point that would cause denaturing of DNA. It is highly debated whether they have enough energy to break a strand of DNA. However, even if there is not enough energy to denature or break strands of DNA, there would be enough energy to create ROS which can lead to genetic damage in the long term.

Another adverse effect that RF-EMFs can have is on memory. It has been reported that RF-EMF impair memory, cognitive function, and learning. Experiments show that rats that have been exposed to RF-EMFs for four weeks have performed poorly on inhibitory avoidance tests. The suggested mechanism for learning and memory impairment is that RF-EMF stimulates the opioidergic system in the amygdala, hippocampus and other areas crucial for memory consolidation. This may in turn impair the release of NO, which plays a role in memory consolidation (Ahmadi et al. 2018). Despite this, many researchers have not found RF-EMF to impair memory, learning or cognitive function (Klose et al. 2014).

RF-EMF has been blamed for many other conditions that are not associated with the brain, such as hypofertilization and cancers of the eyes and glands. These are extremely broad fields and are beyond the scope of this paper. Researchers are of the opinion that the mechanism that causes all these ailments is long-term exposure to RF-EMF. However, RF-EMFs do have the energy to potentially create ROS. ROS can be created by transfer of energy from the EM wave to the oxygen molecule or the transfer of a free electron. This has been shown to cause the creation of micronuclei (Kesari et al 2014). A micronucleus contains a chromosome or part of a chromosome that is not included in the daughter nucleus after the nuclear envelope forms around the chromosomes during mitosis. This part of DNA that has been left out of the nucleus is enclosed in its own nuclear

envelope and is attached to the nucleus. Formations of micronuclei have also been associated with DNA double-strand breaks that lead to the incorrect copying of the chromosomes and formation of the nuclear envelope around them. Micronuclei have been used as markers for researchers and healthcare providers to reveal DNA damage and potential cancerous cells. If these micronuclei are a direct consequence of the EMF, then we can see a definite link between RF-EMF and DNA damage. However, even if the mechanism of the formation these micronuclei is not DNA damage, research has shown that the chromosomes inside the micronucleus have reduced functioning (Hatch et. al 2013). Eventually, due to the close proximity of the micronucleus to the nucleus, the contents of the micronucleus will be released into the nucleus. This often results in incorporation of non-functional DNA into functional DNA which can cause the cell to turn cancerous. Thus, even if the mechanism for micronuclei formation is not DNA damage, it is almost inevitable that the formation of micronuclei will lead to DNA damage.

Each of these effects is highly debated amongst researchers. It is not clear whether the cause is the cell-phone or perhaps some other environmental stressor. However, there is overwhelming evidence that cell-phones have a negative effect on the brain of a child.

**Discussion:
Bone Thickness and Density**

The head of a child differs anatomically in multiple ways from the head of an adult. These anatomical differences cause the brain to be more vulnerable to cell-phone radiation. One example is the thickness of the skull. The skull vault is comprised of two flat compact bones with a diploe or spongy bone sandwiched in between. The average skull of an adult is between 6 to 8 mm thick depending on the location on the skull. A study conducted measured the thickness of the cranial vaults of children (birth-18) using CT scans. It was found that skull thickness increases as a child grows older (Smith et. al 2012). Not only does the thickness of the skull develop over time, but so does the cranial capacity. This is to accommodate for the ever growing and developing brain of the child. Additionally, the skull of an adult is completely ossified. The skull of a fetus, however, is made of cartilage and it slowly ossifies until birth. Even after birth there are areas that are not ossified called fontanel. One of these fontanel or soft spots do not ossify and close until six years after birth. One advantage of the incomplete ossification is to assist the baby in descending through the birth canal. Skull thickness increases throughout development due to the remodeling of osseous tissue by osteoclasts and osteoblasts. This is particularly

interesting since remodeling of bone is usually due to stress on the bones. Subsequently, the bones restructure themselves to create new lines of stress. Yet the cranium undergoes little to no stress from the weight of the body as many of the other bones do. Remodeling of osseous tissue in the cranium has been shown to continue until age 18. The thickness of the skull of a child under the age of 1 has been shown to be between 3 and 4 mm (Table 1). The skull of a 20-year-old, on the other hand, has an average thickness of 6 to 8 mm depending on the location on the skull (Delye et. al 2004). This is a significant increase in the protection provided to the brain. Additionally, the bones have been shown to increase in density as the child gets older. The density of the skull of a child under the age of 1 is between 750 and 850 mg/cm³ (Table 2), whereas the density of a 20-year-old is around 1000 mg/cm³.

The added thickness and density of the skull during the development of the cranium is likely due to an increased compact bone formation and decreasing amount of spongy bone tissue. Thus, when a child is exposed to RF-EMF the compact tissue which is the protective covering is not as thick. This not only exposes the brain to RF-EMF, but it also exposes the vulnerable red bone marrow contained in the diploe in between the trabeculae to these waves. This could account for the correlation of leukemia and RF-EMF observed in children in Rome that were within a 2 km radius of base towers that communicate via RF-EMF (Michelozzi et. al 2002). Other studies found the same results in different communities, although the biological mechanism is still unclear. Yet, perhaps since the compact bone

Age	Mean Thickness (mm)	Standard Deviation (mm)	Age	Mean Density (mg/cm ³)	Standard Deviation (mg/cm ³)
0-6 (months)	3.4	0.3	0-6 (months)	777.1	35.9
7-12 (months)	4.1	0.5	7-12 (months)	822.5	39.9
5	5.2	0.7	5	935.5	65.9
10	5.9	0.8	10	973	33.1
15	6.2	1	15	999.2	59.4
20	7.3	0.9	20	1013	60.6

Table 1 and 2 (modified from Delye et. al 2004))

is not fully developed, it leaves the marrow exposed. These waves could then potentially damage the marrow cells, creating cancerous white blood cells that interfere with the production of red blood cells. The lack of red blood cells carrying oxygen in the blood results in the devastating symptoms of leukemia. Another potential hazard of RF-EMF that can come about due to lack of bone protection is damage to the brain itself. The less radiation that the skull absorbs results in more radiation hitting sensitive brain tissue.

Myelin

A major anatomical difference between the brain of children and adults is the amount of myelin surrounding a neuron. Myelin is a fatty substance which surrounds neurons in the CNS and PNS. Its functions are to insulate neurons to allow action potentials to propagate quickly, as well as to provide protection for the neuron. Among other components, myelin is mainly comprised of sphingomyelin, long hydrocarbon chains, and sphingolipids. It is produced by Schwann cells in the CNS and oligodendrocytes in the PNS. Demyelination has been associated with many diseases such as MS. It is also associated with many psychiatric disorders such as developmental disorders and schizophrenia. Demyelination leaves neurons vulnerable to toxins. It also increases the internal resistance of the membrane, which leads to the decreased velocity of an action potential and less of a chance of this potential propagating into a post synaptic potential (PSP). When demyelination proceeds past a given value, axon degeneration occurs.

Production of myelin begins in the brain at the fourth month of gestation and continues rapidly until the age of two. This rapid myelination relates to the rapid development of cognitive skills, showing the importance of myelin in the brain development. The production then slows; however, myelin is continually being synthesized throughout adulthood. One way to be able to quantify the amount of myelin in the brain is to differentiate between white and gray matter. While myelin is contained in the gray matter, it is most heavily concentrated in white matter. MRI imaging is used to create an MWF (Myelin associated Water Fraction). MWF is a scale that measures the movement and volume of water molecules that are trapped between the lipid bilayer of the myelin sheath. Calculating the water content can provide a quantifiable measure

of the amount of myelin structure above these water molecules. Units are given in mean VFM, or mean myelin water fraction on a scale of zero through twenty-five on a Gompertz curve. Studies using MWF show that the fastest myelination occurs in the first two years after birth (Dean III et. al 2015). MRI images from this same study show a clear progression of myelination throughout the first five years of life (Figure 3).

This study also established a direct association between cognition scores and myelination. It is not by coincidence that the most rapid development in cognition, learning and motor skills occurs during the fastest rate of myelination.

RF-EMF can be a risk for demyelination in children whose myelin development has not been completed. Even adults whose myelin sheaths are fully developed are at risk of demyelination due to RF-EMF. The risk exists because formation of free radicals by RF-EMF can cause lipid peroxidation. This can then cause the myelin to be oxidized, resulting in the formation of a free radical chain. The demyelination results in an exposed axon that risks further damage by RF-EMF, which could potentially degenerate the axon. Demyelination can also result in slower action potentials and the decreased likelihood of PSP propagation in the post-synaptic membrane. This may be the mechanism for the lower cognition scores observed by (Dean III et. al 2015) in children with lower VFM. Lower myelination levels can also result in the plaques of dead neurons associated with MS as demyelinated neurons die. Children whose myelin is still not fully developed in certain areas in the brain are at an increased risk of myelin and axonal degeneration. The more RF-EMF that is absorbed by the brain in a child, the more exposed the neurons can become as demyelination progresses.

Neural Stem Cells

When a child is born, most of the neurons needed are already differentiated from their stem cells. Development of the brain consists of axonal growth as well as formation of new synapses. Another area of development is the differentiation of glial stem

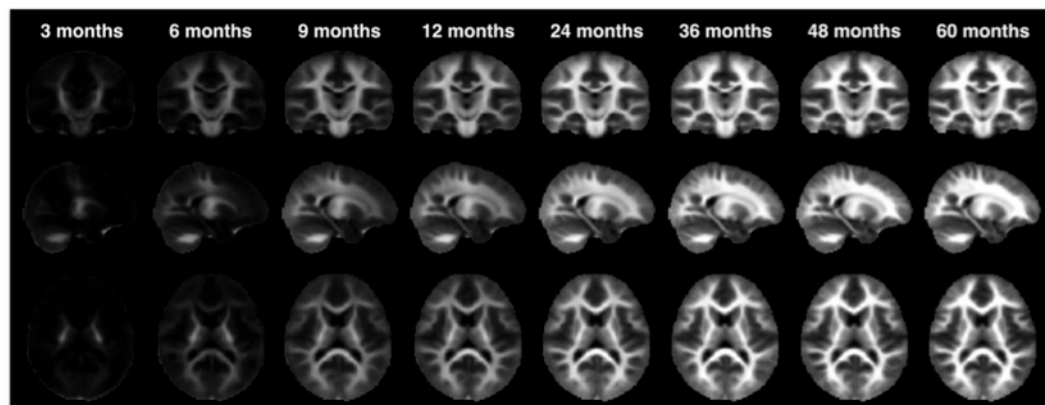


Figure 3

cells into immature glial cells. Studies show that the number of mature oligodendrocytes and astrocytes significantly increase in the first three years after birth (Kjær et al. 2017). In this study, researchers found that while there was an insignificant increase in neurons in the first three years of life, there was almost a threefold increase in the number of glial cells from birth until age three. Oligodendrocytes added an average of 6 million new cells per month, which is about two new oligodendrocytes per second. After the age of three, addition of new oligodendrocytes declined, adding only another 10 million from the age 3 until adulthood, whereas addition of new glial cells is negligible.

All neural cells including neurons and glial cells are originally derived from neuroepithelial cells of the neural tube. The neuroepithelial cells that will eventually become glial cells differentiate into precursors of oligodendrocytes and astrocytes, and then migrate toward the neurons. These precursors then differentiate into immature oligodendrocytes or astrocytes. The differentiation of the neuroepithelial cells into oligodendrocyte precursor cells is accomplished on three distinct waves of proliferation. The waves are initiated by sonic hedgehog signaling (SHH), a signal that controls differentiation of cells in an embryo. These signals cause the formation and migration of precursors from neuroepithelial tissue in both the CNS and PNS in the early days of gestation. Less than 3% of the precursor cells do not differentiate and remain as precursor cells in the adult brain. Once the precursor cells have reached their destination, they differentiate into immature glial cells. The precursor cells will then express certain sequences of transcription factor codes that will determine whether this cell will be an astrocyte or an oligodendrocyte. If the cell undergoes genetic commitment to oligodendrocytes, it will develop a PDGFRα receptor that will be sensitive to platelet derived growth factor (PDGF), a key component in the differentiation of a precursor into an oligodendrocyte. Upon stimulation from PDGF the cell will then undergo chromatin condensation and will form heterochromatin. This will cause silencing of certain genes and the activation of specific sequences of transcription factor, which results in the formation of an immature oligodendrocyte (Goldman, Kuypers 2015).

Exposure of glial cells to RF-EMF results in a decreased conversion of precursor cells to immature glial cells. A recent study found that long-term exposure to RF-EMF resulted in a dramatic decrease of proliferation (Eghlidospour et al 2017). One must keep in mind that in this particular study a higher SAR (2.287 W/kg) was used than that of a cell phone. Despite this, the RF-EMF was not found to induce apoptosis of these cells which had been previously suggested. However, the results did show an inability of precursor cells to undergo proliferation into immature oligodendrocytes, which resulted in a lower number of available oligodendrocytes to perform myelination of the neurons. The brain of a child contains many more of these precursor cells than an adult brain does. The development of a neuron, including

its axonal growth and formation of new synapses, depend on the myelination by the oligodendrocytes. If oligodendrocytes are withheld from differentiation, a lower number of mature cells available for neuron myelination can result. The lack of myelination can affect action potential speeds and cause axonal degeneration. Additionally, the inability to differentiate not only results in less immature oligodendrocyte cells, it will also result in less immature astrocytes. This is because astrocytes and oligodendrocytes share the same precursor cell.

Conclusions

Both biologically and socially, the early years of development are extremely sensitive to environmental factors of a child. The SAR guidelines have still not been changed to accommodate the developing brain of a child. There has been little effort to monitor how children get phones or how long they use them. As technology roots itself deeply in our lives and society depends increasingly on its conveniences, there must be an understanding of the potential effects that they can have on children. While the effects of exposure continue to be debated amongst researchers, it is clear that the guidelines are not sufficient when it comes to children. The current methods of testing SAR do not take into account many variables that change from children to adults. Additionally, the guidelines have not been changed since 1981 despite the upgrade to the “known carcinogen” category. The difference between the brain of an adult and that of a child, as well as the importance of the development of the brain, should warrant a difference in guidelines.

One may question the relevance of these studies, as they all discuss the brain and the normal method of talking on the phone. In current society talking on the phone has been replaced by texting and using phones for virtual reality gaming. Therefore, one may question whether the studies done are relevant to modern society. Yet studies show that while the head absorbs 80% of the radiation while one is talking on the phone, the head absorbs 50% of the radiation during gaming (Fernandez 2018). This is still a considerable amount. Especially with the current culture of letting children use cell-phones to stream movies and play games online. The effects of cell-phones, whether socially or biologically, clearly affect children. While the purpose of this paper is not to suggest that society abandon cell-phones entirely, there should be some sort of regulation for giving a cell-phone to a child, the same way there are rules regarding giving a minor tobacco or alcohol.

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Triggers of Spermatogenesis

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Abstract

Of the 7% of men affected with infertility, about 54% suffer from pre-testicular and/or testicular factor induced azoospermia/oligospermia. This agenesis of spermatozoa has been the subject of much andrology research over the past 50 years, with a particular focus in the triggers of spermatogenesis. While much of their work is limited to murine populations, researchers have put a lot of emphasis on the spermatogonial stem cell (SSC) niche as the source of the trigger(s). By following physiological patterns exhibited in the seminiferous epithelium, researchers have been able to detect distinct morphological stages that correlate with spermatogonial germ-line action. Different niche cells appear to release different concentrations of active compounds, androgens, and receptors during different stages. Specifically, in the steps leading up to and during SSC differentiation, Sertoli cells and germ line cells release retinoic acid and retinoic acid receptors. Retinoic acid appears to trigger SSCs in vitro as well. Testosterone, released by Leydig cells and potentially testicular macrophages, appears to play an essential role in a spermiation-SSC differentiation axis, as well as a role in GDNF production by peritubular myoid cells, both required for proper maintenance of SSC populations and commitment to meiosis. With new and promising research being done on the whole of the SSC niche, as opposed to just Sertoli cells, scientists are closer than ever to uncovering the secrets of male fertility.

Introduction

The mechanisms of spermatogenesis, the process by which male gametes are produced in the testes, are held within the constant and timed divisions of spermatogonial stem cells (SSCs). In a healthy human adult male, to maintain adequate virility, about 1000 spermatozoa are produced per second (Johnson et al., 1980). About 7% of men suffer from infertility (Lotti & Maggi, 2015), whether idiopathic or organic. In as many as 90% of these cases, azoospermia, a lack of sperm in the ejaculate, or oligospermia, a low sperm count (below 15 million sperm cells per cc of semen), is typically observed (Sabra & Al-Harbi, 2014). Some of this data can be attributed to post-testicular factors, including anatomical blockages like tumors and congenital absence of the vas deferens; but much of this data, perhaps 60%, is due to pre-testicular and testicular issues. For example, a lack of anterior pituitary hormones such as Luteinizing hormone and Follicle stimulating hormone (FSH), deficiencies in SSC count or serum testosterone could all be factors (Bernie, et al. 2013). All of these factors appear to be of the utmost importance for the steps of spermatogenesis to be carried out properly, or to be carried out at all. Perhaps the most essential of these steps in the journey from SSC to spermatozoon is the first. What triggers commit undifferentiated spermatogonia to meiosis? Understanding the triggers of spermatogenesis can ultimately lead to uncovering the secrets of both male fertility and infertility. This review attempts to collect and analyze the relevant research on this subject.

Methods

Data was collected using ProQuest and PubMed databases through Touro College's online library. Among the key-phrases used were "triggers of spermatogenesis", "spermatogonium", "the cycle of seminiferous epithelium", and "testosterone and spermatogenesis."

The Spermatogonial Stem Cell

The testes are the male gonads, where both androgens and sperm are produced. Sperm is more specifically produced in the

seminiferous tubules, which are tightly coiled throughout the testes. Sperm cells are developed within the walls of the tubules and, by the process of spermiation, are released into the lumen prior to their passage to the epididymis, where they are stored until released into semen. In humans, this timed and coordinated process, from type A pale spermatogonia to spermiation, takes 64 days (Heller & Clermont, 1963). In order to maintain virility, mature sperm production must be continuous, tightly coordinated, and staggered over the 64 days. Otherwise, instead of being released continuously, sperm would be released into the lumina of multiple tubules in a pulsatile fashion, resulting in staggered fertility (Griswold, 2016). All sperm cells stem from SSCs. They are the cell that gives rise to the rest of the germ-line.

Another necessary characteristic of spermatogonia is the ability to replenish SSC populations as they are continuously committed to meiosis. In this manner, an adequate sperm count is maintained. At the beginning of the germ-line in human testes, SSCs can be identified as two types: type A dark and type A pale. The exact functions of these cells, or whether they are actually different, are not completely agreed upon (Di Persio et al., 2017). However, most of the literature subscribes to the following theory. Pale cells transition into type B spermatogonia, which are committed to meiosis, as well as mitotically dividing to maintain the proper number of SSCs. Dark cells remain on reserve in case of damage to the testicle or SSC population. According to Clermont, dark cells can be considered the true, most undifferentiated, SSCs in the spermatogonial germ-line, while pale cells are best described as the actively dividing and self-replenishing progenitors of B cells (Clermont, 1969; Amann, 2008; Dym et al., 2009). Type A pale spermatogonia are the cells which are regularly triggered by some mechanism to enter the process of spermatogenesis (Gilbert, 2000).

Many labs regularly use murine SSCs for their studies. It is important to also be familiarized with the nuances of murine SSCs and how they differ from humans. In mice, type A spermatogonia (known in mice as type A single spermatogonia) only exhibit qualities found in human type A pale cells. Dark cells do not

exist in murine SSC populations. Before committing to meiosis, type A single cells divide into chains, or syncytia, of 2 (known as Apaired), 4, 8, or 16 (all three known as Aaligned). After this, the type A cells transition into A1, A2, A3, A4, B spermatogonia, and primary spermatocytes, in that order, before initiating meiotic division. Without a type A dark cell reserve, murine SSC populations run the risk of dwindling if commitment to meiosis is not properly regulated. This extensive germ-line, compared to the human model, is thought to be necessary for the proper maintenance of SSC populations. What hasn't been observed is by what mechanism these type A spermatogonia are triggered to commit to spermatogenesis.

There have been many reports that both human and mouse SSCs can be converted to an embryonic stem cell-like state without the addition of new genes (Shambloott et al., 1998; Donovan & de Miguel, 2003; Shim et al., 2008). The limit of the induced pluripotency of these cells appears to be indefinite (Mahapatra & Gallicano, 2014). This quality has led many researchers to look solely extrinsically from SSCs for the source of their differentiation to spermatids. This conclusion doesn't appear to be correct. Although the existence of an extrinsic signal seems likely, as the divisions of SSCs appear to be carefully timed and regulated by an outside mechanism, we cannot ignore the fact that intrinsic factors unique to SSCs, such as certain receptors, are required as well (Lin et al., 2008).

In recent years, the dogma of where to look for influences of SSC commitment to meiosis has shifted from just the seminiferous compartment toward the whole testicular environment of the stem cells, otherwise known as the SSC niche (Potter & DeFalco, 2017). This niche is made up of cells found in both the seminiferous compartment, such as Sertoli cells, as well as the interstitial compartment, such as Leydig cells and peritubular myoid cells.

The SSC Niche Sertoli Cells, the Cycle of Seminiferous Epithelium, and Retinoic Acid

The most adjacent cells to SSCs, Sertoli cells (once referred to as nurse cells) are essential in assisting the successful maturation of spermatids from SSCs. They do so through direct contact with germ-line cells and by creating the specific environment needed for proper sperm development. These triangular cells are bound by tight junctions, and are seen extending from the basement membrane to the lumen of a tubule, with SSCs, developing luminally directly between them. These branching cells are also responsible for forming the blood-testis barrier through tight junctions, which prevents unwanted substances from entering the seminiferous tubules, as well as regulating which tubular substances can enter the bloodstream (Anatomy and Physiology). This makes them an obvious candidate for influencing SSC differentiation.

A murine spermatogonial transplant study, designed to correct genetic male infertility in mice, observed an interesting relationship between Sertoli cells and SSCs. Whether host, foreign, or rat stem cells were injected into the lumen of a mouse seminiferous tubule, Sertoli cells were observed transporting them to the basal layer, a movement that requires the breaking and making of tight junctions between adjacent Sertoli cells (Brinster & Avarbock, 1994; Russel & Brinster, 1996). It can be inferred that Sertoli cells have a specific mechanism for recognizing SSCs, perhaps through cadherins (Griswold, 1998). It follows that the relationship between Sertoli cells and SSCs may be more essential than previously thought.

The number and size of murine Sertoli cells is potentially regulated by FSH released by the pituitary during the pubertal stage (Steinberger & Jakubowiak, 1993; Meachem et al., 1996). These conclusions continued to be confirmed by later studies performed with prepubertal murine Sertoli cells but proved difficult to be repeated with cells from older mice (Griswold, 2018b). The studies of the effects of FSH on murine Sertoli cells in culture also exhibited a positive correlation between the exposure of FSH and the production of Androgen Binding Protein (ABP) by Sertoli cells. There was evidence that spermatogenic cells took up ABP, and that overexposure to ABP led to an increased number of SSCs in vitro and in vivo (Gerard et al., 1994; Jeyaraj et al., 2002). A homologue to ABP was isolated in human Sertoli cells (Hammond, 2011). Further studies on this chemical may be able to confirm an undeniable link between FSH production, the number of Sertoli cells, and the number of SSCs in vivo. A reasonable conclusion is that the number of Sertoli cells has a high correlation to the number of SSCs in vivo.

"This process [spermatogenesis] has been simplified morphologically by recognizing cellular associations or 'stages' and 'phases' of spermatogenesis, which progress through precisely

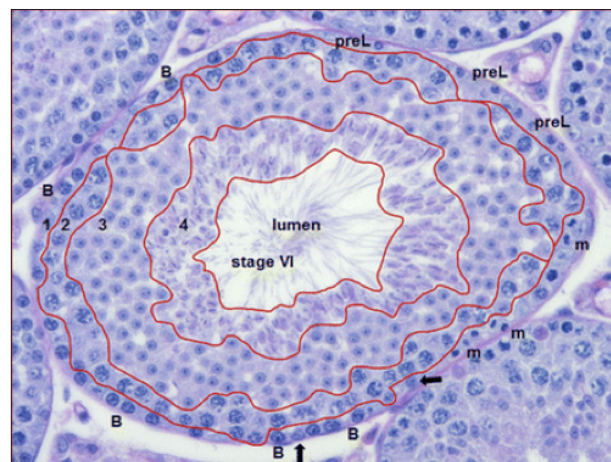


Figure 1: A cross section of a seminiferous tubule highlighting the cellular associations between cells in the epithelial layers, maturing luminally (de Rooij, 2017).

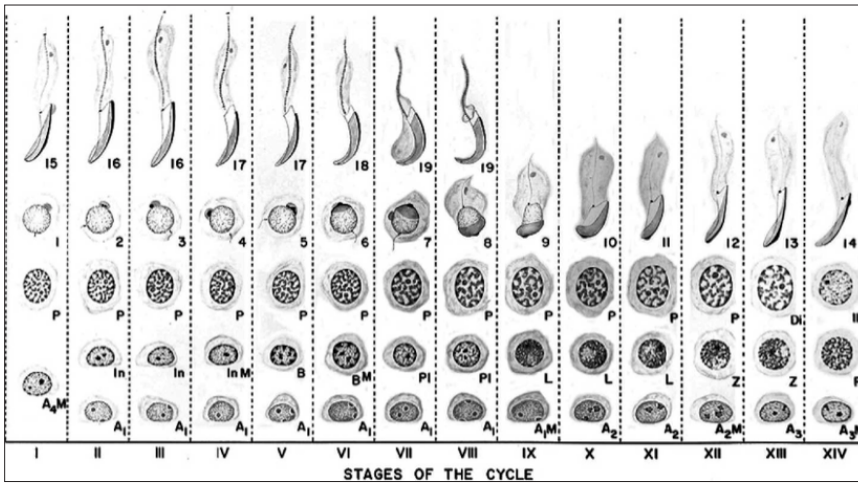


Figure 2: A representation of the linear associations between certain germ cell types at each stage of a cycle (Amann, 2008)

timed and highly organized cycles. These cycles of spermatogenesis are essential for continuous sperm production, which is dependent upon numerous factors, both intrinsic (Sertoli and germ cells) and extrinsic (androgens, retinoic acids), as well as being species-specific.” (Hess & de Franca, 2007) Observations of Sertoli cells during different stages of this cycle of the seminiferous epithelium has given insight to possible triggers of the all-important first step of spermatogenesis.

Simply, the cycle of the seminiferous epithelium is the asynchronous cycle through which SSCs, located basally, eventually develop into spermatids and are released into the lumen (Figure 1). An SSC must go through four complete cycles to become an elongated spermatozoon. After every cycle (cycles begin at stage IX and end at stage VIII), a germ cell graduates into a more luminal layer of epithelium; at stage IX after the fourth cycle, the spermatid is released into the lumen. Every cycle has fourteen stages. Each stage in a cycle is characterized by the linear association (luminally to basally) between certain germ cell types (Figure 2), as well changes in neighboring Sertoli cells.

Understanding stages VII-IX is essential for understanding what triggers differentiation in SSCs. Stage VII seems to be the start of some obvious morphological changes in type A1/type A pale spermatogonia. At stage IX, type A pale spermatogonia appear to be officially committed to meiosis, by differentiating into the next cells in the germline. Neighboring Sertoli cells appear to go through many changes with the cycle, with notable changes occurring at stages VII-IX as well (Parvinen, 1982; Parvinen, 1993; Johnston et al., 2008).

Morphologically, Sertoli cells experience dramatic changes with every stage of the cycle. Perhaps the two stage ranges that expressed the most dramatic changes were stages VII-IX and stages XIII-III. Stages VII-IX appeared to show an increase in retinoic acid receptors, retinoic acid itself, and ABP. Stages XIII-III showed an increase in FSH and androgen receptors (Linder et

al., 1991). These findings “fit the known functional modes of the cycle with spermiation, onset of spermatogonial differentiation and meiosis, and tight junction dynamics in mode A [stages VII-IX], and spermatogonial expansion in mode B [stages XIII-III].” (Griswold, 2018b)

It has been known since 1925 that vitamin A, more specifically retinol (a precursor of retinoic acid), is needed for spermatogenesis. Sertoli cells were also found expressing retinol uptake and retinoic acid production (Hogarth & Griswold, 2010). Many vitamin A depletion studies have found dramatic cessations of sperm production, while reintroduction of vitamin A led to an

almost equally dramatic, synchronous, start of spermatogenesis throughout the whole length of every tubule (Morales & Griswold, 1987). The vitamin A depletion would usually last for weeks and would often leave rats and mice in very unhealthy states, so unequal function of SSCs at reintroduction was expected. The true magnitude of the need for retinoic acid for spermatogenesis may need a different model to be properly observed.

Since stages VII-IX, the period in which Sertoli cells have been observed producing retinoic acid and its receptors, directly coincide with the commitment of SSCs to meiosis, a connection between retinoic acid levels and type A pale spermatogonial differentiation was proposed.

Subsequent experiments found that both drug-induced and diet-induced vitamin A depletion led to an accumulation of type A spermatogonia in mice, with no progression into later cells in the spermatogonial germline. WIN 18,446, a drug previously used in studies aimed at developing a male birth control pill, was found to directly interfere with the aldehyde dehydrogenase that converted retinal to retinoic acid. When mice, who were given this drug for 9 days, were finally injected with retinoic acid, expression of Str8 mRNA and proteins (which were previously known to be stimulated by retinoic acid), was observed in both Sertoli cells and SSCs. After 24 hours, differentiation in these cells was observed (Hogarth et al., 2013). The drug-induced studies are more valid than the diet induced studies, because total body effects of vitamin A depletion was avoided by the short periods the drug was given in.

In short, these studies laid incredibly strong evidence that Sertoli cells, a source of retinoic acid, triggered the commitment of undifferentiated spermatogonia to meiosis. An obvious limitation to these studies is the fact that all of the test subjects were murine. Human studies of retinoic acid with equal specificity have yet to be conducted.

Leydig Cells, Testicular Macrophages, and Testosterone

Adult Leydig cells, located in the interstitial space between seminiferous tubules, are responsible for the production of testosterone. Luteinizing hormone binds to receptors on the Leydig cell. This activates LDL cholesterol displacement from the cell membrane and its conversion to pregnenolone in the mitochondria. Pregnenolone is then transported to the smooth endoplasmic reticulum to be converted to testosterone through a series of enzyme mediated reactions (Mechanisms in Medicine., 2011).

Interstitial and peritubular testicular macrophages protect SSCs and other testicular structures from both foreign and auto-immune attacks (Mossadegh-Keller et al, 2017). Other mechanisms of these macrophages are not completely known or understood, but some researchers speculate that they might indirectly influence SSC differentiation by producing small amounts of testosterone. More plausible is the hypothesis that interstitial testicular macrophages interact with Leydig cells to affect the production of Leydig cell steroidal agents, like testosterone (Trpimir, 2014).

The average serum testosterone for men over 30 years of age is 350-750 ng/dL (Davis, 2018). In the testicle, the concentration of testosterone can be up to a factor 125 times that amount; this is due to the local production of testosterone (Comhaire & Vermeulen, 1976). A study on rats found that spermatogenesis decreased significantly when testicular testosterone concentrations dropped below 70mM, five times average serum concentrations (Zirkin et al., 1989). Due to an unusually high concentration of testosterone in the testes required for proper spermatogenesis, some point to testosterone as a major extrinsic signal in SSC differentiation.

Effectors of testosterone can be identified by the presence of androgen receptors on their cell surfaces. In the testicle, almost all cell types express androgen receptors and thus respond to testosterone. Knocking out (KO) this receptor in isolated cell types shows how testosterone acts in these cells. Surprisingly, androgen receptors have not been found on germ cells, indicating that testosterone must have an indirect effect on SSC differentiation, if any effect at all. "Germ cells from testicular feminized mice that lacked testosterone receptors were transplanted into the seminiferous tubules of azoospermic mice expressing functional androgen receptors. When recipient testes were analyzed between 110 and 200 days following transplantation, multiple colonies of qualitatively normal donor-derived spermatogenesis were seen in each recipient testis." (Sorkitis et al., 2003) Studies on androgen receptor KO Leydig cells seem to show spermatogenesis arresting at spermiation, but the protocols with which these experiments were carried out are questionable (Xu et al., 2007, Smith & Walker, 2014). The agent used to knock out androgen receptors was anti-Mullerian hormone. Later attempts

at mimicking these methods found that not all Leydig cells were androgen receptor free, while some Sertoli cells were affected.

In three independent studies, androgen receptor KO Sertoli cells exhibited normal growth and proliferation in all subjects, showing that testosterone isn't required for those functions (De Gendt et al., 2004; Chang et al., 2004; Stanton et al., 2012). However, analysis of the seminiferous epithelium found that the blood-testis barrier under-expressed necessary cadherins, resulting in a loss of integrity in its structure.

Further analysis found that spermatogenesis slowed at the formation of elongated spermatids and arrested completely at spermiation, suggesting that testosterone's interaction with Sertoli cells is required for these actions. Additionally, the differentiation of spermatogonia also ceased shortly after spermiation did; this suggests that the mechanism of spermiation is involved directly with the proper maintenance of the cycle of seminiferous epithelium.

From the data presented, it seems likely that testosterone is at least indirectly involved in the differentiation of SSCs. Although it's not fully understood, other studies suggest that testosterone is essentially involved in a regulatory axis of spermatogenesis.

Peritubular Myoid Cells and GDNF

Peritubular myoid cells (PMCs) make up the smooth muscle-like layer that surrounds the seminiferous tubules. Some main functions of these contractile cells are to provide support as well as some peristaltic movement throughout the tubule. A few studies have suggested that PMCs may indirectly affect SSC differentiation.

Glial cell line-derived neurotrophic factor (GDNF), thought to affect the maintenance of SSC populations and SSC renewal, is present in both murine and human Sertoli cells (Fouchécourt et al., 2006). When GDNF levels are depleted, SSC populations dwindle while SSC differentiation is agitated. When GDNF levels are elevated, SSC populations are much higher than normal, while differentiation is arrested completely (Meng et al., 2000).

It has been suggested that FSH stimulates the production of GDNF by Sertoli cells, as observed by one study. Another study attempting to recreate the previous study's data was unsuccessful (Crépieux, 2001). But what was generally agreed on was that, by some unknown mechanism, Sertoli cell production of GDNF was essential for maintaining SSC populations and differentiation.

An androgen receptor KO study on peritubular myoid cells incidentally found that SSC populations decreased significantly. Further analysis found that GDNF levels were severely lowered, suggesting that testosterone-stimulated peritubular myoid cells synthesized GDNF. This was later confirmed by a study conducted in 2014 (Figure 3). The results of which bring up questions about which cell plays a more significant part in SSC population maintenance. Why original stains missed GDNF concentrations

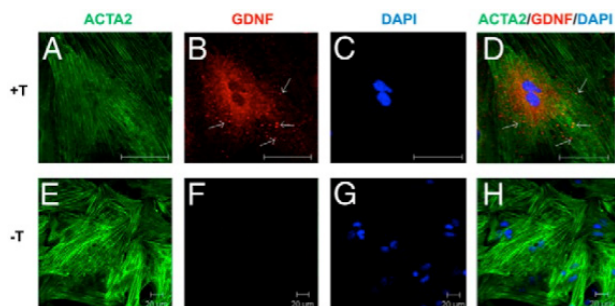


Figure 3: Stains of peritubular myoid cells and SSCs in culture with and without testosterone (Chen et al, 2014). A-D are grown with Testosterone. E-H are grown without. The molecule denoted at the top of each column is stained for

in peritubular myoid cells is probably due to the fact that these cells are very thin and don't stain as well as Sertoli cells.

Germ-Line Cells

Spermiation, the process of mature spermatids being released into the lumen prior to their passage to the epididymis, takes place at the apical surface of the seminiferous epithelium. However, the interruption of this process further impedes the basal processes of SSC differentiation and commitment to meiosis. Interestingly, the process of spermiation directly coincides with this SSC differentiation. This suggests evidence of a regulatory axis between the tightly coordinated processes of spermiation and SSC differentiation.

As spermatids are elongated, in preparation for spermiation, all nonessential organelles and cytoplasm are collected into a section attached to the head of the spermatid. Through a series of Sertoli cell interactions with these elongated cells, and through testosterone stimulation of a Src pathway (see Figure 4), Sertoli cells break and remake tight junctions, mechanically severing this residual body from the elongated spermatid and releasing the sperm into the lumen. At the same time, this pathway allows for conformational changes throughout the entire length of the Sertoli cell, allowing basal germ-line cells to move apically (Yan et al., 2008a; Yan et al., 2008b; Cheng & Mruk, 2010).

Studies on germ-line cells have also found that descendent cells from SSCs exhibit a mechanism of taking up retinol and producing retinoic acid, a mechanism that is very similar to the mechanism of Sertoli cells. This discovery led to the manufacturing of a Sertoli cell specific variant of the drug WIN 18,446, called WIN 14,446. When administered before the first wave of retinoic acid could be initiated in mice, at 2-3 days old, the effects of WIN 14,446 were very similar to WIN 18,446. However, when administered at day 9, after the first wave, there appeared to be no effect (Griswold, 2018c). This suggests that Sertoli cell production of retinoic acid may be essential for starting the cycle of spermatogenesis, while germ-line cells may be the primary source of retinoic acid afterward.

Discussion and Conclusion

Much of the research presented has been done strictly on murine populations, with few parallels found in humans. Almost the entirety of the research on retinoic acid has been done in mice. Future studies must be done in human populations in order to obtain a true understanding of spermatogenic processes in humans. Other topics that require more insight are (1) the concentration of intra-testicular testosterone needed for proper spermatogenesis, (2) the specific mechanisms of the regulatory axis between spermiation and SSC differentiation, particularly if the conformation of the Sertoli cell sets off differentiation, and (3) how the cycle of seminiferous epithelium in murine populations differs from humans.

Additionally, a topic that goes hand-in-hand with triggers of spermatogenesis is the asynchronous nature of the cycle of seminiferous epithelium. Throughout the length of the tubule, sperm are released into the lumen constantly, in an uninterrupted fashion. This is due to the fact that the stage (of the cycle) at one portion of the tubule is not the same as the stage being carried out in adjacent portions. All portions of every tubule are releasing sperm into the lumen at a different time. In retinoic acid depletion studies, after spermatogenesis was completely halted, injections of retinol stimulated an almost synchronous restarting of the cycle in every part of every tubule. After about six months, the cycles had returned to their asynchronous nature. Obviously, this is a very confusing phenomenon. When asked directly about it, Dr. Michael Griswold, who is currently researching this issue states that "the key to the cycle is the conversion of A spermatogonia to A1 spermatogonia. Any variance in the timing of this conversion would ultimately lead to asynchrony." (Griswold, 2018a) Perhaps the carefully coordinated cycle of seminiferous epithelium isn't as carefully coordinated as researchers once believed. This evolutionary phenomenon proves to be incredibly important in the maintenance of mammalian fertility.

The ideas presented here is the relevant research done on the topic of triggers of spermatogenesis. Obviously, the research is heavily focused on the Sertoli cell. A reason for this is the fact that the field is mainly contributed to by those that can be called "Sertolists". The Sertoli cell, because of its adjacency to germ cells, has been the obvious focus of these researchers. An example of dangers of this bias is the peritubular myoid cell and the late discovery of its extremely important role in GDNF production. Yes, peritubular myoid cells are small and hard to stain, but many published micrographs didn't even include the interstitial space. Perhaps the original researchers weren't expecting the interstitial space to be involved.

In February 2018, researchers successfully observed human induced pluripotent stem cells (hiPSCs) transforming into actively differentiating SSCs in vivo in mice (Fang et al., 2018). When these results were attempted to be recreated in vitro with just hiPSCs and Sertoli cells, there was no change. The entirety of the

niche must be present for proper functioning. As more research is done on the rest of the SSC niche, the field is beginning to experience a rise in extra-Sertoli cell understanding. And perhaps, with this understanding, the mysteries of non-obstructive azoospermia and oligospermia will be uncovered.

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Degeneration of Rods and Cones in Retinitis Pigmentosa

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Abstract

Retinitis Pigmentosa, most commonly characterized by night blindness and loss of peripheral vision, is a rare genetically inherited group of diseases affecting the retina of the eye. It is estimated that 1 in 4000 people in the USA are affected by some form of the disease. Retinitis Pigmentosa (RP), is caused by a mutation or change in one or more of 55 genes. There are many causes to this disease as RP presents with many different symptoms and biological effects on the eye. These are then grouped together because they share a common result, deterioration of vision. Presently, there is no cure for the disease. Most of the present therapies are aimed at preserving visual function and preventing or slowing further cell death. Restoring vision is particularly difficult because human photoreceptors are not produced and do not divide after birth and therefore their loss is irreversible. Experimentation in gene and stem cell transplantation to retard the advance of the disease, as well as drug therapies and surgeries to diminish the effects of the disease are ongoing and have found some limited success.

List of Acronyms:

RP: Retinitis Pigmentosa

RGCs: Retinal ganglion cells

NGF: Nerve growth factor

IPS: Induced pluripotent stem

DHA: Docosahexaenoic acid

Introduction

The eyeball consists of three main layers and is one of the most complex organs in the body (fig.1). The outermost layer, the fibrous tunic, is made up of the sclera and the cornea. Then the middle layer, the vascular layer, consists of the ciliary body, iris and choroid. The innermost layer is the nervous tunic which consists of the retina. The retina contains two types of photo-

receptor cells, the rods and cones. Near the beginning stage it is hard to detect the disease, especially if there is no familial history, as most daily functions are not affected. The slow regressive pattern of deterioration that defines RP presents in the onset of the disease with night blindness and the reduction of peripheral vision. The lack of peripheral vision in the day becomes apparent when incidents like not seeing pedestrians walking, not noticing cars approaching from the side while driving, or missing handshakes when greeting people occur. As the disease advances patients become sensitive to light. This often leads to reading difficulties and difficulty perceiving pale colors particularly in the blue and yellow tones.

In the last stages as the cones begin to be more affected the vision field continues to narrow, and the light sensitivity continues to grow. Often patients are still able to read a little, usually with a magnifying glass, until the central visual field vanishes. Usually patients can still perceive light.

Methods

Articles and studies researched in this paper were obtained through the EBSCO and ProQuest databases with access provided by the Touro College Library. Images and diagrams that are used throughout the paper were obtained from the research articles cited.

How is RP Inherited?

RP is a progressive inherited disease that occurs in one of three ways: autosomal recessive inheritance, autosomal dominant inheritance, or X-linked inheritance. Autosomal recessive inheritance happens when both the mother and father are carriers of the recessive gene mutation giving them a 1 in 4 chances of having a child with the disorder. The parents typically do not show any signs or symptoms of the disease. Autosomal dominant inheritance happens when one parent has a dominant gene mutation giving them a 1 in 2 chance of having a child with the disorder. In this form of the disease there is often a parent and other members of the family with the disease.

X-linked inheritance is when the mutated gene is on one of the X-chromosomes. It can be passed on to sons who inherit only one X-chromosome and cannot offset the gene with their Y-chromosome. Sons from mothers that are X-linked carriers

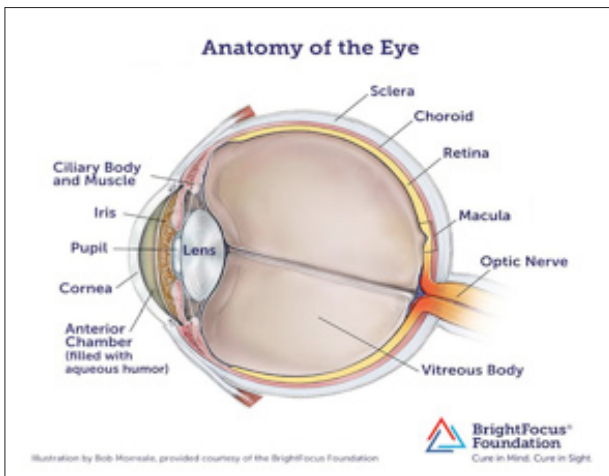


Fig 1

receptor cells, the rods and cones. The rods and cones absorb and convert light into electrical signals that are then sent to the brain. The rod and cone cells are present throughout the retina with the exception of the fovea, the center of the retina, where only cone cells are present. Rod cells are accountable for peripheral and night vision whereas cone cells are accountable for more direct visual functions like reading, driving and facial recognition. The rods allow us to see in dim light and the cones allow us to see color and details.

RP can manifest itself as early as the first years of life,

have a 1 in 2 chances of having the disease. Fathers with X-linked RP cannot pass the disease on to their sons. Girls inheriting only 1 affected X-chromosome have a 1 in 2 chance of being carriers. Girls usually need both X-chromosomes affected to get the disease. There are a small minority of woman who get the disease with only one X-chromosome affected. X-linked inheritance is by and large thought to be the most severe form of RP and can begin in early childhood (Melamud, 2006).

In a situation where only one person in the family is affected, the disease is referred to as simplex. These cases could be the result of small families where there are few possible relatives to be affected, or it could be the result of a new gene mutation.

RP is primarily a monogenic inherited disease. This form of inheritance refers to a single gene controlling a genetic trait. Digenic inheritance refers to a disease that is inherited through the mutation of two interacting genes. There are many diseases caused by single gene mutations but so far very few have been found which are caused by mutations in at least one copy of two genes interacting. Digenic inheritance is a rare form of the genetically complex RP.

How are the Rods and Cones Damaged?

RP is caused by the loss of use of the rods and cones. It is typified by the initial loss of rod function followed by the death of the cones. It is the genetic mutation that causes the initial rod death, but the subsequent cone death is thought to be caused by its dependence on the rods. It is the loss of the cones that causes the severe blindness associated with the disease.

The rhodopsin genes' function is to encode a protein called rhodopsin in the rod cells. Rhodopsin absorbs the light that is then converted into electrical signals in the rods. The most common cause of autosomal dominant RP is a sequence variation in the rhodopsin gene (Schuster, 2005).

Mutations in the rhodopsin genes obstruct their ability to function properly and are the cause of over 25%, of autosomal-dominant RP cases. The alteration at a specific point accounts for the difference in the disease expression within families. The majority of the mutations affect the folding, and stability of the rhodopsin molecule (Terry, et. al. 2017). In other forms of the disease the mutations produce a protein that is toxic to the cell, or the mutation doesn't allow the cell to function properly. As a result, the rods followed by the cones cease to function and slow progressive vision loss occurs (Jensen, 2016).

Discussion

While there is a great deal of research on the genetic sources of the disease, there is still much to be discovered and studied to extend our knowledge and treat RP effectively. For example, most of the current research focuses on the etiology of RP. However, because of the many differing genetic mutations that cause RP there is very little research that can be done on the

disease as a whole, but it is rather approached in a very specific type by type manner. Since most of the accepted approaches target specific versions of the disease, they are only beneficial to particular groups of patients.

In the past few years there has been a vast amount of scientific research into treatments for RP. These include surgical, pharmacological and non-invasive treatments.

Recent studies have shown that as the rods degenerate, the cones are deprived of glucose and eventually die. The photoreceptors are dependent on glucose in order to function properly. One of the symptoms of RP is the breakdown of this process.

Muller cells, which are non-neural cells of the retina, serve to protect the retina by providing a conduit to transport glucose to the photoreceptors, remove debris and provide electrical and mechanical support of the neural retina. The Muller cells are among the first to show signs of metabolic changes in the retinal degeneration of RP and are among the last cells left in the degenerate retina. As RP progresses, the Muller cells follow a chaotic metabolic path. Unlike the precise cohesive response Muller cells exhibit in healthy retinas, they appear to react to the degenerating photoreceptors in an unfamiliar non-homogeneous manner. The remodeling of the retinal cells caused by cell stress and death is an unavoidable consequence of the progression of the disease (Pfeiffer, et. al. 2016).

As the photoreceptors degenerate, the surviving rods and cones reorganize (fig. 2). This is followed by the adjustment of inner retinal components such as the neurons, the glial cells and the blood vessels. This process of remodeling cannot be explained as an attempt to produce new cells as retinal cells cannot be reproduced or regrown. It is rather seen as a reaction to one or more possible negative inputs that causes the remodeling and the eventual cell death. The course of the photoreceptor cell death is dependent of the underlying genetic mutation, the type of RP. The path of the inner retinal layer degeneration is

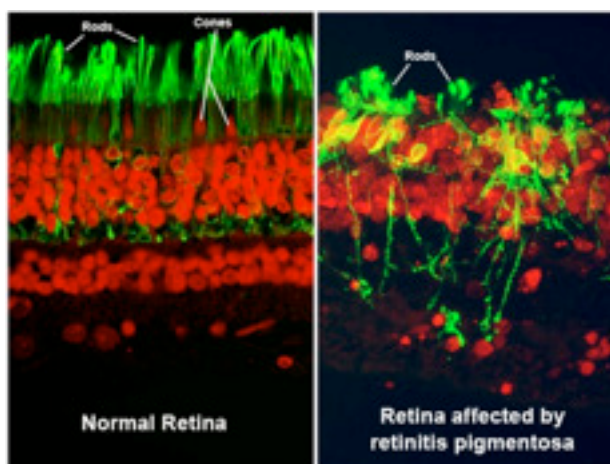


Fig. 2 Normal retina vs. reorganization of the retina in a patient with RP (Raw.com)

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similar in spite of the differing underlying genetic mutation. This is significant in the search for a cure or a reversal of at least part of the progressive path of the disease.

The complex biological process of retinal remodeling is an active field of retinal investigation, as it shows promise for therapeutic treatments of the disease (Terray, et. al. 2017).

Scientists have tried a number of ways to surgically reverse this process. One of the methods is that they have attempted to bypass the ineffective rods by surgically replacing them and restoring the transportation of glucose to the cones. The cones remain latent for a period of time before they die and if the glucose supply can be replenished before that time they can be regenerated. In a study of rod transplantation, the rods from healthy mice were transplanted into RP affected mice that were totally lacking rods but still had some functioning cones. These mice were compared to a control group of RP mice to measure the normal degeneration for the same period of time. Two weeks after surgery the number of functioning cones were compared between the two groups of mice. Those mice that received transplanted healthy rods showed 40% greater cone survival than untreated mice. Another surgical method that has shown potential as a treatment for RP is the placement of glucose directly under the retina so as to regenerate and reactivate the cones (Mohand-Said, 2000).

The objective of the rod transplantation surgery is to help the survival of the cones as they gradually deteriorate over time and ultimately to prevent blindness from occurring. This is different than other surgical treatments being tried, such as cataract surgery. In cataract surgery the lens of the eye is removed and replaced because the lens has become cloudy and thus the person's vision is cloudy. For RP patients with progressively limiting vision the start of cataracts can advance the loss of vision significantly. Therefore, cataract surgery has been found to be beneficial in some cases of RP. The difference between the rod replacement surgery attempts and cataract surgery is their goals. While they both are trying to restore as much sight as possible, the rod replacement surgery is trying stop the progression of the disease by replacing the genetically affected rods. By contrast the cataract surgery is treating only the symptoms and not the underlying cause (Dikopf, et. al. 2013).

There are also a vast number of non-surgical research studies being done. One of the first symptoms of RP is night blindness and difficulties with dark adaptation. Researchers have investigated the use of antipsychotic drugs on RP given that the side effect of certain specific drugs of this type is increased light sensitivity. The increase in light sensitivity produced by these drugs was compared to their known adverse side effects to determine if they could be a useful treatment in RP.

Most antipsychotic drugs act as dopamine antagonists. They block the dopamine receptors. In order to reach the photoreceptors, incoming light must first pass through all the cell layers

of the retina. The first layer is composed of the ganglion cells. The ganglion cells are a type of neuron in the inner surface of the retina that transmit information to the brain. Between the photoreceptor and the ganglion cells there are bipolar cells that transmit signals from the photoreceptor to the ganglion cell. The ganglion cell receptive fields are subdivided into two parts, center and surround. They operate in an ON center OFF surround or an OFF center ON surround manner. In the ON center/OFF surround a small ray of light in the center increases the cells response. An Off center On surround has the opposite effect where a small ray of light in the center inhibits the cells response.

The antipsychotic drugs haloperidol and clozapine were tested to see whether they could similarly alter the light responses of the retinal ganglion cells (RGCs) in the rat retinas.

The drugs have the effect of transforming abnormal long latent ON-center RGCs into Off-center cells. It is believed that these RGCs might have been Off-center cells early in the onset of the disease. Antipsychotic drugs are divided into two categories, typical and atypical. The difference between these two categories is the side effects they produce. Atypical antipsychotic drugs like clozapine are considered second generation antipsychotic drugs that have fewer negative side effects than the first-generation drugs like haloperidol. The responses of the retinas of the rats were recorded before and after the application of the various drugs. Both haloperidol and clozapine increased light sensitivity of the affected rat retinas. For those retinas that exhibited an abnormally long latency ON response to the onset of a small spot of light, both haloperidol and clozapine brought about a change and a reduction in the long latency ON response. Both these drugs act as an antagonist to receptors in the rats. The haloperidol acts as a D2 receptor antagonist while the clozapine acts on the D2 receptor as well as a 5-HT2A (serotonin) receptor antagonist. On the whole, the results imply that antipsychotic drugs may be useful in temporarily improving vision in patients with RP (Jensen, 2016).

Other pharmacological treatments such as the supplementation of different vitamins, DHA (docosahexaenoic acid) a type of omega 3 fatty acid, oral valproic acid and topical brimonidine tartrate NGF drops, are all being explored in the hope they can help of protect and slow the progression of the disease.

Vitamin A is a powerful antioxidant that has been found to be essential for good vision, eye health and the ability to help protect the eyes from night blindness and age-related degeneration. Supplementation of vitamin A over a period of 4 to 6 years showed a significantly slower rate of visual decline. The study included different genetic types of RP as well as children in the early stages of the disease. These results contrasted the results of the supplementation of vitamin E. Patients receiving vitamin E showed a greater loss of retinal function than placebo groups.

In a 4-year study of DHA supplementation vs. placebo the loss rate of cone and rod function was not appreciably different.

Another study comparing the effects of taking DHA with vitamin A or taking vitamin with a placebo showed that the addition of the DHA did not alter the results of the effects of the vitamin A on RP patients. The rate of decline of the progression of the disease remained the same (Sacchetti, 2015).

Nerve Growth Factor

NGF, nerve growth factor, is important in the regulation, growth and survival of the neurons. It has been found to promote the recovery of neuronal damage in several animals. NGF drops were administered in a 10-day trial to see the short-term effect of the drops on patients with advanced stages of RP. The possible adverse effect of the drops was monitored closely. The study showed neither adverse effects nor significant improvement in RP patients.

Stem Cell

Stem cell transplantation is a potential treatment that is showing great promise. Allogeneic stem cell transplantation, which are stem cells taken from matching donors, or human embryos, have the potential to be effective and safe. However, there is the ongoing controversial debate on the morality of using embryonic tissue in medical experiments or treatments. This issue coupled with the greater risk of rejection and need for immunosuppressant therapy when using allogeneic stem cells has encouraged scientists to investigate the use of autologous stem cells transplantations, which are treatments using stem cells derived from the patients themselves (Bassuk, et. al. 2016).

IPS: Induced Pluripotent Stem Cells

IPS cells, induced pluripotent stem cells are autologous stem cells that come from the person's own skin or blood cells. These cells are then reprogrammed to enable them to develop into any type of human cell.

The use of IPS cells as a treatment for RP has shown some promise in restoring some of the lost vision. Because of the many different genetic causes of RP there is hope of developing specific IPS cells to target precisely the various forms of the disease independently. The goal of this treatment is to edit and correct the patient's pathogenic mutation in very specific type by type RP cases. X-linked RP is a very aggressive form of the disease. IPS cells have been used in studies to try to correct the specific X-linked form of the disease and have been found to correct 13% of the gene mutation that causes the X-linked RP. Researchers are optimistic with their success of the gene editing treatment of X-linked RP thus far. It establishes that this treatment should be continued to be studied and used as part of autologous transplantation of other photoreceptor degeneration cases.

There are still other challenges with this treatment due to complications with incorrect placement, the development of

tumors and disturbance of other types of cell which need to be resolved in order to further the use of this stem cells research (Bassuk, et.al. 2016).

In contrast to the pharmaceutical approach to the early onset of light sensitivity and vision loss, the nonpharmacological interventions are based on strategies of light protection. The use of specialized electronic glasses to protect the eye from contact with light has been employed. In more advanced stages of RP there is the Argus II which combines both glasses with surgery. The Argus II involves the implantation of an artificial device that must connect to a functioning optic nerve. Electrodes are then implanted into the eye and connected to the glasses that can convert images into electrical pulses that the brain perceives as patterns of light.

Conclusion

There is promising research for treating the symptoms of RP. There is also promising research into fixing the underlying genetic mutation that is associated with RP, with the use of stem cell therapy. Continued research is needed before the stem cell therapy can be used as a widespread treatment. It is the goal of researchers to treat patients earlier on in the progression of the disease, before complete visual function is lost.

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The Effects of Aging on Skeletal Muscle ATP Production

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Abstract

The study of the aging process and its prevention is an incredibly popular field; the natural course of the human body is to age and theories abound on how to avoid it. Age affects every system and pathway of the body and alters many of the bodily functions. This paper, using articles from Touro's library database examines the different areas in which aging negatively affects ATP production. The multifaceted process associated with the production of ATP in the human body was analyzed. Several features such as the mitochondria, physical activity, and hormones are linked to ATP production and the relationship was further explored. The findings reveal that a dysfunction or lack of these factors, a feature of aging, is the initiator of the decrease in ATP production that is attributed to the natural aging process. Together, a lack of mitochondrial function, physical inactivity, reduced growth hormone levels, and increased insulin sensitivity levels contribute to the decline in ATP production that is concomitant with the progression of age. The diminishing presence of ATP then stimulates further dysfunction or lack of these elements, which in turn drives the aging process even further.

Introduction

Aging is a phenomenon that all creatures go through. It is believed to be a degenerative process caused by accumulated damage that leads to cellular dysfunction, tissue failure and death. The physiological changes which lead to senescence in humans may involve spine curving (kyphosis), reduced fertility, loss of hearing and eyesight, frailty, cognitive decline and many other symptoms (Trifunovic et. al., 2004). All these changes in the body are triggered by a variety of molecular and biochemical modifications. One of the major pathways that is altered as people age is the cellular respiration pathway which produces ATP, the body's main source of energy.

Methods

The research discussed in this paper was collected from various different databases. The majority of the articles were compiled from databases such as PubMed and ProQuest, with access to restricted articles gained by using Touro's library system. Some articles were also found through google scholar.

An Overview of Cellular Respiration

Cellular respiration is a set of metabolic reactions to convert biochemical energy from nutrients into adenosine triphosphate (ATP). ATP is the primary energy carrier in living organisms. When the cell requires energy, it breaks down ATP through hydrolysis; a high energy bond is broken where a phosphoryl group is removed and energy is released. In humans, aerobic cell respiration has three parts. Glycolysis is the breakdown of glucose into two pyruvate molecules where two molecules of ATP and two molecules of NADH are produced without necessitating oxygen. The next step is known as the Krebs cycle, the citric acid cycle or the tricarboxylic acid cycle. The carbons in the pyruvate molecules are pulled apart and the energy stored in those covalent bonds is released. This step produces two molecules of ATP, six NADH molecules and two FADH₂ molecules. The third and final step is the electron transport chain and oxidative phosphorylation. Here, oxygen is required and about thirty-four molecules of ATP are produced. The second two steps of cellular respiration, which are the main

energy-producing steps, occur in the mitochondria, unlike glycolysis which takes place in the cytoplasm (Ferne et. al., 2004).

Oxidative phosphorylation is the metabolic pathway in which cells use enzymes to oxidize nutrients. Electrons are fed from reduced substrates such as NADH, FADH₂, fatty acids or glycerol phosphates into the electron transport chain located in the inner membrane of the mitochondria. Big membrane-bound enzymes such as the dehydrogenases of β -oxidation, glycerol phosphate dehydrogenase, NADH-Q oxidoreductase (Complex I) or succinate-Q oxidoreductase (Complex II) pass electrons down the gradient of redox potential to ubiquinone (Q) which is a mobile lipid-soluble carrier. The electrons then get passed down from ubiquinone to the final acceptor, oxygen, through Q-cytochrome c oxidoreductase (Complex III), cytochrome c (a second, water-soluble, mobile carrier) and cytochrome c oxidase (Complex IV). As the electrons move from less electronegative to more electronegative molecules, they lose energy, and with that energy, Complex I, Complex III and Complex IV pump protons against their electrochemical gradient from the matrix to the intermembrane space. A protonmotive force, consisting mostly an electrical gradient (membrane potential) and a small chemical gradient, (pH difference) is set up due to the pumping of protons. This protonmotive force then pushes the protons back into the matrix through the ion channel ATP synthase which harvests the energy and creates ATP (Saraste, 1999).

Being that the main organelle involved in cellular respiration is the mitochondrion, dysfunction of this organelle must be the driving force behind the lack of energy in cells, resulting in cell death. This implies that dysfunctional mitochondria must be of central focus in aging. The decline of mitochondrial function with age has been recognized for some time and has been linked to a decreased number of mtDNA and mitochondrial protein levels in human heart, brain and skeletal muscle cells. Additionally, aged muscle suffers from functionally impaired respiratory chain units, inhibiting respiration associated with ATP production (Zapico et. al., 2016). The mitochondria in elderly tissue has often been found to be larger, fewer in number and contain abnormal cristae, vacuoles and paracrystalline inclusions (Hutter et. al., 2007). When the quadriceps of thirty participants whose

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ages ranged from 78.5 ± 5 years were examined, an inverse relationship between the fatigability level and the level of oxidative phosphorylation (ATP_{max}) was reported. Higher fatigue levels, a condition which is extremely common with advancing age, is caused by a decrease in mitochondrion capacity for oxidative phosphorylation (Santanasto et. al., 2014). In another study, permeabilized muscle fibers from the vastus lateralis were studied from twenty-four young (approximately thirty years) and thirty-one older (approximately sixty-five years) subjects. This study was to determine if there was a change in mitochondrial respiratory capacity and coupled respiration (respiration linked to ATP production) with age and the results showed a decline in both (Porter et. al., 2015). There are many possible reasons for this decline in mitochondrial function but the most accepted theory seems to be the Free Radical Theory of Aging.

Mitochondrial Free Radical Theory of Aging

Oxidative damage is a main cause of cell degeneration. Since the mitochondria are involved in many redox reactions, they are extremely prone to oxidative stress. The source of oxidative damage is the free radicals known as oxidants or reactive oxygen species (ROS) which are a byproduct of redox-reaction and therefore produced during aerobic respiration (Harman, 1956). Normally, a small fraction of the oxygen consumed by the mitochondria is converted into reactive oxygen species, but defects in the respiratory chain lead to greater production of these free radicals (Lee et. al., 2000). Superoxide Dismutase (SOD), an enzyme whose activity is present in subcellular compartments where there is $O_2^{\cdot-}$ formation, was used as a tool to reveal that the mitochondria are a principle source of endogenous oxidants. Some of the SOD activity was recognized in the matrix space and the other half in the inner and outer membranes of the mitochondria (Imray, Fridovich, 1991).

Ground state diatomic oxygen is not too reactive itself because its two unpaired electrons are located in different molecular orbitals and possess parallel spins. As a result, in order for O_2 to simultaneously accept two electrons, they must both possess antiparallel spins relative to the unpaired electrons. Consequentially, O_2 preferentially accepts electrons one at a time from other radicals (such as transition metals in certain valences). In living organisms, therefore, oxygen must participate in coordinated, serial, enzyme-catalyzed one-electron reductions in order to be reduced by two or four electrons. The enzymes that carry out these reactions generally possess active-site radical species such as iron. This method of O_2 reduction generates $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2). When in the company of free transition metals (specifically iron and copper), $O_2^{\cdot-}$ and H_2O_2 together produce the hydroxyl radical ($\cdot OH$) (Wood, 1988). This extremely reactive radical is presumed to be the species responsible for initiating the oxidative destruction of biomolecules (Harman, 1956). When mitochondrial DNA of cells which can no longer

reproduce undergoes oxidative damage it results in mutations and blocks in replication, leading to mitochondrial dysfunction and ultimately a decline in ATP production.

One study demonstrated that older people have significantly higher oxidative damage to DNA and that mtDNA abundance decreases with age. The decrease in mtDNA is attributed to the lessened content of mRNA transcripts that encode mitochondrial protein. The skeletal muscle of the elderly population was found to contain a reduced amount of mitochondrial proteins and oxidative enzymes (enzymes that catalyze redox reactions and increase the rate at which ATP is produced aerobically). Accumulated oxidative damage can be the reason for lessened mtDNA content in aging muscle. DNA oxidation, the process of oxidative damage on Deoxyribonucleic Acid, was observed to increase with age. Also, there is a higher activity of antioxidant enzymes in older rat muscles, indicating that the ROS production rate increases with age; the ROS production rate surpasses the activity of the antioxidants defense enzymes (Short et. al., 2005). The decline in mtDNA content is a contributing factor to dysfunctional mitochondria because it means that there is a lack of template availability for transcription and translation of crucial mitochondrial proteins. The reduction of mitochondrial protein synthesis and oxidative enzyme activity helps explain the reduced ability of mitochondria to perform oxidative phosphorylation in older muscle. This study concluded that there is a decline in mitochondrial ATP production rate (MAPR), and its point of validity comes from the fact that the subjects were well suited for the study. They had a large group of one hundred and forty-six healthy men and women ranging in age from nineteen to eighty-nine. Their physical activity was taken into account and they were subjects who were not regularly doing exercise (less than thirty minutes less than two days a week). In response to the studies which show that physical activity improves muscle mitochondrial biogenesis, the physical activity of the subjects was taken into account. Only men and women who were not regularly exercising, with activity totaling less than thirty minutes for less than two days a week, were chosen. Also, the participants were kept on a standard diet for three days before muscle biopsies from their vastus lateralis were taken for study. A key finding of this study was the decline in the MAPR of skeletal muscle with age due to a combination of reduced mitochondrial content and a functional alteration in the existing mitochondrial population (Short et. al., 2005).

In response to the production of ROS, uncoupling agents such as dinitrophenol (DNP) are produced. These agents are lipid-soluble weak acids that can cross the mitochondrial membrane. Once inside the mitochondria, these proteins eliminate the link between oxidation and phosphorylation. They accomplish this by setting up a catalytic cycle that dissipates the proton-motive force, allowing the continuation of substrate oxidation all the while lacking the driving force for coupled ATP synthesis.

This is a thermogenic process and occurs in many living cells including skeletal muscle cells (Brand, 2000). The first known UCP, UCPI is the one that controls brown fat which is one of the body's ways of regulating thermogenesis. In the brown adipose tissue, the proton leak through UCPI is a mechanism that exploits energy generally used to create ATP to generate heat. This method of thermogenesis regulated by brown fat UCPI is important in numerous physiological conditions such as arousal from hibernation and cold exposure in newborns. The question of the reason for this process arises. What reason is enough to validate a reduction in ATP level and such a high energetic price? To strengthen this question, it is known that thermogenesis is not enough of motivation because this uncoupling process is done by both endotherms and ectotherms. The reasoning behind this seemingly troubling process is the diminution of ROS production, a protection against cellular degeneration (Brand, 2000). When calculated, the respiratory capacity used for phosphorylation and respiratory function linked to ATP production in older adults was lower than in the young (Porter et al., 2015). Not only do the presence of free radicals (which are a result of aging) lower the skeletal muscle ATP production, but the cell's natural response and method to protect itself against oxidative damage, which involves uncoupling proteins, is also a major contributing factor to the reduction of ATP production.

A more indirect but lethal effect oxidants have on the mitochondria is that they disrupt the activity of the lysosomes. Oxidative damage is responsible for depositing lipofuscin in the lysosomes. Lipofuscin is the yellow pigment granules made up of lipid residues of lysosomal digestion, and accumulation of lipofuscin alters the lysosomal autophagic ability. Because the lysosomes take care of recycling unnecessary material and organelles (such as viruses or damaged particles), their failure results in a buildup of unwanted particles in the cell (Konig et al., 2017). Decreased macroautophagic activity from lysosomal leakage was observed in rat cells, and is a central reason for the accumulation of damaged mitochondria in aged cells. This buildup is associated with a decrease in mitochondrial efficiency and an increase in oxidant generation (Roberg, Ollinger, 1998). The decline in autophagic activity as a common characteristic of aging makes for clogged lysosomes due to lipofuscin and mitochondrial deterioration. This process in which mitochondrial ROS encourage lipofuscin generation while the accumulation of lipofuscin disturbs the mitochondrial autophagy is known as the "garbage catastrophe theory of aging" (Hutter et al., 2007).

Therefore, oxidative destruction of molecules involved in aerobic respiration (such as oxidative enzymes, mitochondrial proteins or mtDNA) leads to defective mitochondria. The mitochondria themselves are primary targets of oxidative damage and increase their production of ROS once destroyed. Putting these two processes together results in a disastrous vicious

cycle in which the synthesis of malfunctioning electron transport chain subunits results in oxidative phosphorylation impairment, decreased ATP production and further ROS generation.

Physical Activity

There are many studies which say that the primary contributing factor to reduced mitochondrial function is the lack of physical activity. In one study, thirty-nine volunteers were to complete graded exercise tests to determine their daily physical activity, fitness, and exercise efficiency. The group of volunteers included ten young active people about thirty years old, ten older active people about sixty-seven years old and nineteen older sedentary people. Mitochondrial energetics from biopsies of the vastus lateralis were measured via magnetic resonance spectroscopy and respirometry. The results of this study showed a higher fitness level in the young active compared to the older active and older sedentary. The mitochondrial respiration, maximum mitochondrial capacity, Maximal ATP production/Oxygen consumption (P/O) ratio, and exercise efficiency were similar in the young active and older active and were significantly lower in older sedentary. This study concluded that constant physical activity makes for better mitochondrial capacity (Distefano et al., 2018).

Besides for physical activity having an effect on the mitochondrial efficiency (ATPmax/max O₂ consumption), the reverse is also true. Lessened mitochondrial efficiency seems to have a negative effect on older peoples' physical activity. A study was done to analyze whether or not slower walking speed in older adults is related to reduced mitochondrial capacity and efficiency. A group of thirty-seven older people (ages ranging from seventy to eighty-nine) who were of normal weight (under 286 lb for the men and under 251 lb. for the women) were chosen. They were able to walk unassisted and had no symptoms of cardiovascular or pulmonary disease. They had no pain or stiffness in the legs, hips, knees, feet, or ankles when walking and no difficulty bending or straightening the knees. The participants were told to complete a 400-meter walk within 15 minutes at their normal walking speed. Afterward, muscle fibers biopsies from the vastus lateralis were taken to be observed. A key finding was that muscle mitochondrial efficiency correlated with the participants walking speed. This study concluded that the loss of mitochondrial capacity and efficiency with age may be important contributors to the reduction in mobility and increase in disability. This is due to the relationship between mitochondrial capacity and walking speed (Coen et al., 2012).

The link between physical activity and mitochondrial energetics is that aerobic exercise enhances muscle mitochondrial biogenesis. Resistance training in older people can increase the protein synthesis rate. The content and function of certain proteins in the muscle which are vital to its functioning, depend on the protein synthesis rate (Short et al., 2005). Exercise counteracts decay which happens in aging muscle by heightening

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muscle protein turnover. Because muscle mitochondrial content and function is reduced with age, the resting and maximal oxygen consumption also decline with advancing age (Short et. al., 2004). The VO₂max of an individual is closely associated with mitochondrial ATP production and so a decline in the VO₂max with age means that it is correlated with a decline in ATP production. In muscle samples taken from healthy individuals ranging from eighteen to eighty-seven years, the VO₂max while cycling declined approximately eight percent per decade (Short et. al., 2005). If constant physical activity throughout one's life is what can keep the VO₂max at a decent level, it must be that physical decline, a common occurrence in aging, is in part a contribution to the reduction of ATP production.

Sarcopenia and Physical Decline

Sarcopenia, a natural part of the aging process, is the progressive decline of muscle mass, strength and physical function. The loss rate is believed to accelerate after the age of sixty-five. The deterioration of muscle quantity and quality leads to a decline in strength and power and a slowing of movement, all features of sarcopenia (Baumgartner et. al., 1999). A deterioration in muscle mass is followed by a decline in physical activity which has been negatively linked to mitochondrial function.

Age-related hormonal changes seem to be a possible mechanism for the onset of sarcopenia. Insulin-like growth factor I (IGF-1) is a protein that is encoded by the IGF-1 gene. This hormone plays a fundamental role in childhood growth and continues to have anabolic effects in adulthood. The production of IGF-1 is stimulated by growth hormone. Growth hormone (GH) is made in the anterior pituitary gland, released into the blood stream, and then stimulates the liver to produce IGF-1. IGF-1 is the stimulation for systemic body growth, and has proliferating effects on many cells in the body, especially skeletal muscle (Laron, 2001). After twenty years of age, the secretion of growth hormone decreases by approximately fourteen percent per decade. This decline is followed by a reduction in IGF-1, which is responsible for cell production, energy metabolism, and inhibition of apoptosis (Giannoulis et. al., 2008).

Testosterone, an anabolic steroid which increases muscle protein synthesis, begins to drop in male serum levels in the late thirties and continues to decline throughout adulthood. A group of older men were treated with testosterone in order to increase their testosterone level to that of younger men. The treatment attenuated one symptom of sarcopenia, the loss of muscle mass (Storer et. al., 2016). In females, the positive correlation between plasma estrogen levels and muscle mass suggests that reduced estrogen following menopause may result in reduced muscle mass as well (Van Geel et. al., 2009).

Although there was no change in muscle mass or strength when healthy males were treated with GH alone, the addition

of testosterone to the treatment resulted in a 6.8% increase in muscle strength. There was also an increase in lean body mass (LBM=total body weight minus the fat) which indicates the correlation between lean body mass and strength; a change in LBM results in a change in strength. Along with increase in LBM came an increase in VO₂max. (Blackman et. al., 2002). An effective mechanism to increase the endogenous levels of somatotrophic hormones (such as GH and IGF-1) in the blood is training and exercise. A thirteen-week combined sprint and resistance training in middle-aged men improved the body composition and increased circulating GH. There was also an increase in IGF-1 level following the exercise which shows that training can counteract the natural decline in somatotrophic hormones during aging (Sellami et. al., 2017). A decrease in GH and testosterone, vital anabolic hormones, plays a big role in the loss of muscle mass which is a key element in the development of sarcopenia.

Another possible cause of sarcopenia is the acceleration of apoptotic pathways. Apoptosis is the highly regulated and controlled process of cell death in multicellular organisms. This process can happen via the intrinsic pathway in which the cell kills itself because it senses cell stress, or the extrinsic pathway, where the cell kills itself because of signals from the other cells. Both pathways initiate the activity of caspases, enzymes that destroy proteins. Apoptosis is a homeostatic process which keeps our body balanced between all the new cells arising from stem cells and the billions of cells dying each day. This very beneficial process becomes a problem when it accelerates with age and results in the loss of muscle mass and strength. Although the mechanism for the acceleration of these pathways is unknown, what is known is that there are many contributing factors such as oxidative stress and impaired insulin sensitivity (Marzetti et. al., 2012).

Insulin Resistance

A major finding in old age is insulin resistance. Insulin is a hormone produced by the pancreas and regulates the amount of glucose in the blood. Resistance to this hormone can result in type 2 diabetes. Age associated decline in mitochondrial function seems to be related to insulin resistance. The MAPR was found to be approximately thirty percent less in patients who were insulin resistant compared to those who were not, and that was consistent with the finding of altered mitochondria (Petersen et. al., 2004). The altered mitochondria and reduced MAPR is known to be a contributing factor to insulin resistance. In addition to that, insulin stimulates transcription and translation of mitochondrial proteins and genes thereby increasing MAPR and this effect is lacking in those with type 2 diabetes. So, whether insulin resistance causes a decrease in mitochondrial function or the reverse, both possibilities are plausible for they seem to each have an effect on the other (Short et. al., 2005).

In a study done to determine if insulin enhances the capacity of

mitochondrial ATP production in skeletal muscle, both subjects with and without type 2 diabetes were recruited and muscle biopsies of the vastus lateralis were examined. The muscles were infused with a low dosage of insulin and a high dosage of insulin and were studied after eight hours. Mitochondrial protein complexes, whose syntheses are dependent on coordinated transcriptional regulation of mitochondrial and nuclear genes, must be available for mitochondrial ATP production to occur. In the subjects without diabetes, the high dose insulin infusion resulted in a higher mitochondrial fractional synthesis rate. Additionally, there was an increase in the activities of the oxidative enzymes citrate synthase and cytochrome-c γ -oxidase subunit IV (a part of complex IV). (Citrate synthase is the pace-making enzymes of the first step in the citric acid cycle and increased activity of the citric acid cycle may increase the rate of ATP production.) There was also an increase in the mRNA level from both mitochondrial (NADH dehydrogenase subunit IV) and nuclear (cytochrome c oxidase subunit IV) genes encoding mitochondrial proteins. After eight hours of high dose insulin infusion, the mitochondrial ATP production rate of the individuals with type 2 diabetes did not change. Clearly, insulin infusion increases mitochondrial protein synthesis and the mitochondrial capacity for oxidative phosphorylation in skeletal muscle (Stump et al., 2003). Insulin is not only important for regulating the blood sugar level but it is also heavily involved in ATP production. Resistance to this hormone, resulting from dysfunctional mitochondria, causes a decrease in mitochondrial protein content thus reducing mitochondrial respiration and ATP production.

Conclusion

The free radical theory of aging which gives reason for the malfunctioning mitochondria is itself a result of dysfunctional mitochondria. Increased ROS production, a byproduct of mitochondrial decay disrupts lysosomal activity creating the additional accumulation of damaged mitochondria followed by an increase in oxidants. An additional result of oxidative damage is the decrease in muscle mass, muscle strength and movement. The opposite is also true, physical activity has been shown to increase muscle mass and counteract the production of free radicals (ROS) while physical inactivity has shown to increase ROS levels, increase sarcopenia and decrease insulin sensitivity. Insulin resistance is another result of oxidant accumulation and physical inactivity and causes additional ROS content by disturbing mitochondrial protein synthesis. Essentially, all of these factors are negative feedback loops that influence each other. Studies which failed to see the correlation between these features and decreased ATP production with age, may be because they failed to look at all of these factors as a whole and recognize that when they all come together, create a vicious cycle. Although it is unclear how and where this natural process starts, once it does, there is no way to end it. The decrease in ATP production

in skeletal muscle is an ordinary part of the aging process which results in driving the aging process even further.

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Energy Drinks: Cardiovascular Effects and the Specific Components Responsible

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Abstract

Energy drink usage as a stimulant is on the rise among adolescents and young adults. While these drinks have positive energizing effects, they pose significant health concerns. This paper examines the adverse cardiovascular effects of energy drinks and the components that could possibly be responsible. Analysis was conducted by reviewing and comparing many studies available in this area of research. Studies of energy drinks in general and energy drinks versus drinks containing caffeine alone were analyzed. Additionally, this review investigated studies of the specific ingredients in energy drinks such as caffeine, taurine, guarana, and sugar. This review found that energy drinks cause varying degrees of adverse cardiovascular effects when consumed in specific dosages. While the highest levels of consumption led to arrhythmias, syncope and death, lower levels led to increased QTc interval and increased blood pressure. Longer lasting effects of energy drinks, such as possible energy-drink induced hypertension, were also uncovered. The effects of energy drinks were deemed greater than those caused by caffeine alone. When analyzing energy drinks' ingredients, caffeine, sugar, and guarana were found likely responsible for the adverse effects of energy drinks, while the effect taurine was less convincing. Overall, while energy drinks were found to be safe in small and infrequent amounts, significant public health concerns were related to increased and habitual use.

Introduction

Energy drinks are widely used stimulants that are said to enhance performance, alertness, and concentration. Marketing of these beverages specifically targets adolescents and young adults, many of whom are said to take advantage of these drinks and their stimulant effect. Despite the perceived 'invincibility' connoted by names such as 'Monster' and 'Red Bull,' these pick-me-ups may pose significant health concerns. Substances present in energy drinks such as caffeine, sugar, and taurine have numerous hemodynamic effects. As such, these drinks are suspected of leading to various health threats including adverse cardiovascular events. These range from high blood pressure to cardiac arrhythmias, which, in more extreme cases, can lead to sudden cardiac death. The goal of this review is to evaluate the following question: What are the energy drink-related cardiovascular health concerns, are they valid, whom do they concern, and how do energy drinks lead to these events?

Methods

This review was conducted by typing keywords and phrases such as "energy-drinks," "energy drinks and cardiovascular effects," "the effects of energy drinks," "energy drinks and mechanisms," and the like into database search engines such as EBSCO and ProQuest. The databases were accessed through Touro College, Einstein Medical College, and Johns Hopkins University. Many articles were also retrieved through Google Scholar.

Results and Discussion

How widespread is the usage of these drinks, and who is using them?

Energy drink sales have been steadily increasing since the turn of the century and are not expected to decrease in the near future. In 2006, the industry's retail market value was already \$5.4 billion (Packaged Facts, 2007), a value dwarfed by 2016's \$25 billion market (Packaged Facts, 2017).

Young adults are the heaviest consumers. Whether one gender drinks more heavily than the other is arguable. According

to the NIH, the most common drinkers are men of ages 18-34 (NIH, 2018). Furthermore, in a study of 496 college students, 51% reported using energy drinks more than once a month on average. Of these users, most said they used one energy drink at a time. More concerning though, is that a considerable subgroup admitted to using three or more drinks at a time when mixing energy drinks with alcohol. In contrast to the NIH data, this study found 53% of the users to be female, trumping the 42% who were male by a significant percentage. It should be noted that while the results of this study are comparable to those of similar studies, they should serve solely to paint a general picture, as the 496 participants were all from one college (Malinauskas et al., 2007).

Studies of the rampancy of energy drink usage in adolescents also show worrisome results. The CDC reports that 30-50% of adolescents consume energy drinks (CDC, 2016). Even higher rates were found in two studies done in Canada and the US, in 2012 and 2014, respectively, which found that two thirds of participants used energy drinks occasionally, with others consuming them monthly, or even weekly (Azagba, et al., 2014; Miller, et al., 2018). Therefore, when weighing the damage of energy drink usage, we must bear in mind that the affected population not only includes many adults under 30, but also a great number of adolescents, on whom the damage may have longer-lasting consequences.

The Substances in Energy Drinks

While energy drinks contain various ingredients, their main active ingredient is caffeine. It is well known that the amount of caffeine in energy drinks varies greatly, with most drinks containing between 50 and 360 milligrams. More importantly, many of these drinks fall into the 100 mg – 200 mg range. A formal study that measured caffeine content of 14 energy drinks found values ranging from 67 mg – 240 mg, and an even wider range of 34 mg-330 mg in energy shots (smaller, more concentrated drinks) (Attipoe, et al, 2016). These doses

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are not higher than the amounts of caffeine in many coffees such as those sold by Starbucks and Dunkin Donuts. This, and the fact that there is a decaffeinated '5-hour Energy' drink, suggests that caffeine may not be the only factor causing the effects attributed to energy drinks.

Consequently, this review should also consider the other ingredients and their functions. These substances include glucose, ginseng, high amounts of taurine, B vitamins, green tea extract, guarana (a seed that contains caffeine), green coffee extract, carnitine (an amino acid), glucuronolactone, and inositol. When analyzing the effects of these ingredients, though, caffeine should not be disregarded. The fact that much is known about caffeine's effect on the body and that energy drink manufacturers employ high amounts of caffeine in the making of their drinks implies that caffeine may still be the most important ingredient to study.

Cardiovascular/Hemodynamic Effects of Energy Drinks/Caffeine

Many studies have analyzed the hemodynamic influence of energy drinks. Some studies' results are consistent, while others are contradictory. These differences are often due to differences in dosages of energy drinks. Since caffeine is considered their most active ingredient, this review will group studies by caffeine dosage in the tested drinks when trying to compare studies and their results.

Whereas caffeine is known to have numerous energetic and hemodynamic effects on the body, the FDA advises that doses of less than 400 mg of caffeine a day are generally safe. Still, the administration says that safe dosages can only truly be set on an individual basis, as levels of sensitivity vary throughout the population (FDA, 2018). This is one of the many factors that can account for variation between results of studies, even when similar dosages of caffeine were used.

Energy Drink Dosage as a Factor: Extreme Caffeine as a Danger

A retrospective analysis of 101 patients who arrived at the emergency room having consumed extreme amounts of caffeine showed that many displayed conditions such as tachypnea, tachycardia, and arrhythmia. Seven patients experienced cardiac arrest. Of the 101 patients, most survived after treatment, but three of the seven who developed cardiac arrest died. The patients' estimated caffeine dosages were between 1.2 g to 82.6 g of caffeine with a median ingestion of 7.2 g. Although hard to believe, some of these patients consumed these dosages using energy drinks. Consequently, while this study illustrates the dangers of extreme caffeine usage, the results hardly concern the general public, as a person would have to drink 37 cans of a highly caffeinated energy drink within a few hours to reach the minimum lethal dosage of caffeine in this study, 6 mg. (Kamijo, et al, 2018) More important though, is that this study shows

that caffeine and energy drinks certainly affect blood flow, heart rate, and other vital variables. Whether or not smaller amounts of caffeine can induce the same conditions remains to be determined.

More Moderate Dosages: Case reports

Even in smaller amounts, caffeine, and energy drinks specifically, can have serious pathogenic effects. There have been many case reports describing episodes of energy-drink related cardiovascular incidents, including cardiac arrest, which developed after the patients had consumed much lower dosages than those mentioned in the above study.

In one such case, a 28-year-old, otherwise-healthy motocross racer experienced cardiac arrest after just seven or eight cans (640 mg) of a caffeinated energy drink (Berger, Alford, 2009). In another, a 16-year-old volleyball player experienced repeated episodes of orthostatic tachycardia and rapid changes in blood pressure, diagnosed as reversible postural tachycardia syndrome. This was ultimately attributed to her usage of Red Bull, of which she had started to drink 4-5 cans daily (400 mg at most) (Terlizzi, et al., 2008). Moreover, doctors report cardiovascular events linked to dosages not only less than the previous study, but to doses that are less than FDA-recommended safe consumption. A 23-year-old woman presented with supraventricular tachycardia at 219 bpm after just one GNC speed shot (250 mg of caffeine) and a Mountain Dew (55 mg/12oz) (Nagajothi, et al., 2008). Lastly, and most intriguing, is the case of a 28-year-old basketball player who fell unconscious while playing and later died after drinking only three 250 ml energy drinks, amounting to only 180 mg of caffeine (Avci, et al., 2013). Although case reports cannot stand alone as evidence, that the patients in these cases were otherwise healthy and that doctors ruled out other probable causes, as well as the myriad of similar case reports, give reason to believe that the energy drinks could have caused these negative outcomes. It is important to note that high physical activity after consumption is a characteristic of many of these cases (Avci et al., 2013; Berger, Alford, 2009; Terlizzi, et al., 2008), suggesting that exercise plays a role precipitating these side effects.

More Moderate Dosages: Formal Studies

In order to scientifically test if energy drinks have these cardiovascular and hemodynamic effects on the general public, many researchers have conducted studies which gave specific amounts of energy drink to young, healthy participants, measured vital signs before and after ingestion, and determined the drink's hemodynamic effects either using participants as their own controls or splitting the participants into case and control groups.

Energy drinks containing 320 mg of caffeine seemed to consistently raise QTc intervals as well as systolic blood pressure. Two studies that had healthy adult cohorts consume such

energy drinks found similar results regarding these two parameters (Fletcher, et al., 2017; Kozik, et al., 2018). These results are concerning because prolonged QTc intervals are known to lead to dangerous cardiac arrhythmias, or changes in heart rhythm (Priori, et al., 2003). Furthermore, the second study found QTc to rise to extremely high levels of above 500ms in eight participants (Kozik, et al., 2018). As with the above case studies, these extreme findings developed after physical activity, and QTc normalized once participants rested. This points to the positive relationship between the degree of physical activity and the severity of the effects of energy drink consumption.

While more moderate dosages do not cause electrocardiographic effects in most people, they still have consistent hemodynamic effects. Researchers in at least seven different studies found that energy drinks with caffeine dosages ranging from 77 mg to 215 mg did not increase QTc (Brothers, et al., 2016; Elitok, et al., 2015; Garcia, et al., 2016; Hajsadeghi, et al., 2015; Ragsdale, et al., 2009; Shah, et al., 2016; Steinke, et al., 2009). On the other hand, in at least nine studies where blood pressure changes were measured, results showed that energy drinks containing anywhere between 114-215 mg of caffeine consistently raised systolic blood pressure (Brothers, et al., 2016; Elitok, et al., 2015; Garcia, et al., 2016; Grasser, et al., 2014a; Hajsadeghi, et al., 2015; Marczynski, et al., 2014; Ragsdale, et al., 2009; Shah, et al., 2016; Steinke, et al., 2009). Many of these studies found an increase in diastolic blood pressure and/or heart rate as well (Elitok, et al., 2015; Grasser, et al., 2014a; Hajsadeghi, et al., 2015; Marczynski, et al., 2014; Shah, et al., 2016; Steinke, et al., 2009). Although many of these studies used seemingly small participant groups, the number of necessary participants was statistically calculated beforehand. Moreover, since multiple studies have shown similar results, the size of the studies is less of a concern. In total, the studies show that even moderate amounts of caffeine tend to raise blood pressure. A rise in blood pressure can be induced by many factors, including exercise alone, and this may not be a public health concern if the effect is transient.

Small Dosages: Formal Studies

In terms of the potential detrimental effects mentioned above, energy drinks with a small amount of caffeine do not seem dangerous for healthy people. Studies in which participants were given dosages of up to 80 mg found hemodynamic and electrophysiologic parameters to be for the most part unchanged, with the exception of an increase in heart rate found in one study (Hajsadeghi, et al., 2015; Ragsdale, et al., 2009).

Effects on Patients with Cardiovascular Disease

While it seems that caffeinated energy drinks used in moderation are safe for most healthy people, for the large population of those with cardiovascular abnormalities and diseases, they may not be as safe. Studies of the cardiovascular effects of energy

drinks show that while these drinks may not directly cause more serious hemodynamic or electrophysiologic effects in this group than in healthy subjects (Gray, et al., 2017), these same effects may be safe for healthy people, but may indirectly cause more serious consequences in people with underlying cardiovascular compromise. People with cardiovascular diseases are often predisposed to dangerous arrhythmias, which can lead to syncope and sudden cardiac death (Priori, et al., 2003). Ingesting energy drinks, which affect the sympathetic nervous system, cardiac function, and blood pressure, increases the chances for arrhythmia development. For example, one serious type of arrhythmia-caused myocardial event is known to occur when a ventricular premature beat sparks during the T-wave and leads to circus action in the heart. Therefore, substances like energy drinks, which lengthen the QT interval, increase the possibility of this extra beat landing on the T-wave. Although it would be hard to test these concepts in a formal study, many case studies have reported patients with previous cardiovascular diseases whose arrhythmias developed after energy drink consumption (Dufendach, et al., 2012; Rottlaender, et al., 2012; Rutledge, et al., 2012).

Furthermore, since many cardiovascular diseases go undiagnosed, the dangers of caffeine consumption affect a larger population than just those with diagnosed cardiac diseases. Many case studies describe people whose cardiac syndromes came to light only after an adverse effect, caused by energy drinks, lead to a cardiovascular evaluation (Dufendach, et al., 2012; Rutledge, et al., 2012).

Longer-Lasting Effects

A further concern is that studies indicate that moderate doses in repeated amounts may have a long-term effect. In two studies, participants were instructed to drink the energy drink for seven consecutive days. Hemodynamic measurements were taken on the first and seventh days at baseline and after consumption. In one study, on the first and seventh days, heart rate increased by 7.8% and 11%, systolic blood pressure by 7.9% and 9.6%, and diastolic blood pressure by 7.0% and 7.8%, respectively. While the increases in heart rate and systolic blood pressure were not statistically significant, the increase in diastolic blood pressure was (Steinke, et al., 2009). The second study did not find any statistically significant changes between the days but did notice a trend of increased blood pressure after chronic consumption of the energy drink (Shah, et al., 2016). These data point to the possibility that the effects of the drinks on blood pressure can, in fact, last more than a few hours.

These results are strengthened by those of a differently designed study of 333 adolescents, in which hemodynamic measurements were taken at baseline, during emotional stress, and during recovery without the ingestion of caffeine. Serum caffeine at the time of the study was determined to be nonexistent or trivial. At the start of the lab sessions, the teenagers were asked about

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their habitual caffeine consumption. The study found that total peripheral resistance was significantly higher in those who reported high habitual caffeine consumption (about 258 mg per day) during exercise and at rest. The researchers also noticed a slight trend of increasing systolic and diastolic blood pressure correlated with high, medium, and low habitual consumption groups. This suggests that daily caffeine ingestion and blood pressure are directly proportional (James, et al., 2018). As increased blood pressure is a precursor to many serious cardiovascular complications, the implication that energy drinks cause not only transient, but lasting increases in blood pressure is of concern. It is possible that these long-lasting effects more strongly influence young chronic drinkers than adults, but more extensive studies would have to be conducted to confirm this hypothesis.

Caffeine Consumption Patterns Considered

When analyzing safe amounts of energy drinks, caffeine consumption patterns should not be overlooked. Since caffeine offers consumers a positive feeling, they tend to want to increase consumption. The efficacy of caffeine is in keeping drinkers awake, many habitual users gradually sleeping less and drinking more. Lastly, with habitual consumption, some drinkers develop tolerance to caffeine (Shah, et al., 2016). On the one hand, this may render habitual consumers less susceptible to the sudden negative developments associated with caffeine. On the other hand, this tolerance may lead them to slowly increase their caffeine intake to a level that will affect them negatively. Therefore, it is likely that many energy drink consumers may start drinking at a safe level and later reach dangerous levels of consumption (Malinauskas, et al., 2007). To reinforce this point, most of the energy drink-related case reports were not of suicide attempts, but rather of consumers who had mistakenly increased their dosage to a dangerous level.

What in Energy Drinks is Causing these Effects? Energy Drinks versus Caffeine

While it is clear that energy drinks can have serious cardiovascular effects, the question of which ingredients are causing those effects is still a disputed topic. There have been many studies in which researchers tried to isolate the effects of one ingredient as it relates to the general effects of the drinks.

Even so, it is understood that while energy drinks' other ingredients can have some effects, their caffeine content plays a big role in the health effects mentioned. Proving this, researchers tested the hemodynamic effects of caffeinated energy drinks versus those of decaffeinated or non-caffeinated energy drinks. In both studies reviewed, studies found changes in various blood pressure parameters to be greater after the caffeinated version of the same drink (Kurtz, et al., 2013; Phan, Shah, 2014).

Knowing caffeine to be the most apparent active ingredient, researchers attempted to clarify whether the other substances in energy drinks could possibly strengthen the drinks' effects.

As a first step towards analyzing the other ingredients, many studies attempted to test the consumption of energy drinks versus the consumption of caffeine. In four such studies, energy drinks were found to have a greater effect on the parameters that researchers watched. While not all the studies observed changes in identical parameters, variables such as QTc interval, systolic and diastolic blood pressure, cardiac contractility and stroke volume were found to be more affected by energy drinks than by the caffeinated control drink (Baum, et al., 2001; Doerner, et al., 2014; Fletcher, et al., 2017; Franks, et al., 2012). Three studies similar to the previous ones reported that they did not see any difference between energy drinks and caffeine in these parameters. This could give reason to doubt the previous studies but does not for the following reasons: Two of the studies not only did not see a difference, but also did not observe any cardiovascular effects after either drink, so their results may not be a good means for comparison of the two. (Brothers, et al., 2016; Laizure, et al., 2017) Also, one of the studies used a Guru energy drink that lacked taurine and sugar, two ingredients that are often focused on in evaluation of the drink's effect. (Laizure, et al., 2017) Lastly, the third study only measured parameters for twenty-five minutes following energy drink consumption (Pettit, et al., 2013). This is not a valid analysis as many studies have shown that the effects of energy drinks are apparent one, two, or three hours after drinking and many times cannot be seen during the first hours (Fletcher, et al., 2017; Grasser, et al., 2014a; Elitok, et al., 2015). Moreover, in a graph of this study's data, heart rate elevation after energy drink consumption starts to slightly surpass the caffeine-induced elevation just at the end of the twenty-five-minute interval (Pettit, et al., 2013). Based on the time taken to see results in other studies, it is highly probable that the researchers would have seen a more prominent difference had they measured parameters for longer. Therefore, although the above studies report varying results, it is likely that energy drinks have a greater effect on the cardiovascular system than caffeine itself. This begs the question of which ingredients in energy drinks could be causing these effects.

Energy Drink Ingredients and Their Effects

Energy drinks contain many ingredients that may be active, including taurine, guarana, sugar, and caffeine, among others, as mentioned earlier. Some of these substances have not yet been widely researched, others have been researched but remain highly questionable, and a few seem to have a significant role in the results seen. This review will explore some of the oft-studied ingredients.

Taurine

Taurine is a naturally occurring nonessential amino acid. It has been studied numerous times, but with results that create a very wide spectrum of possible effects. Some studies do show amplified cardiovascular effects when caffeinated drinks containing taurine were compared with non-aurine-containing caffeinated drinks (Baum, et al., 2001; Doerner, et al., 2014). The validity of their conclusions is doubtful, though. One study showed a subtle, yet significant increase in left ventricle contractility, but did not report the ingredients of the energy drink in question other than its taurine and caffeine content. Since it is possible that there were many other role-playing ingredients, this cannot serve as a controlled study of taurine's effects (Doerner, et al., 2014). Another study with a similar deficiency attributed an increase in stroke volume and contractility to taurine but did not account for the glucuronolactone present in the study drink that was not present in the control (Baum, et al., 2001). Glucuronolactone is an organic metabolite often used in energy drinks. Whether or not it has cardiovascular effects is unknown, but the fact that this study disregarded its presence in the drink in question weakens the conclusion that the augmented effects were due to the taurine. Moreover, aside from the ambiguity of these results, many studies of taurine have shown that it does not have detrimental cardiovascular effects, but rather, it has cardioprotective effects. One such study showed that taurine had systolic and diastolic blood pressure-lowering effects and also led to a decrease in plasma catecholamine levels (Fujita, et al., 1987). These results suggest that not only does taurine not intensify caffeine's negative hemodynamic effects, it attenuates them. It should be noted though, that this study tested taurine alone versus a placebo, as opposed to the above studies which investigated taurine in combination with caffeine. Thus, the prospect that the body reacts differently to taurine in these two circumstances cannot be discounted. As such, the discrepancies throughout the research on taurine and the limitations of the studies make it hard to determine whether the compound contributes to energy drinks' negative cardiovascular effects. Studies that are better controlled would have to be done in order to solidify any claims in this area.

Guarana

Guarana, also called zoom or Brazilian cocoa, is a seed originating from the Amazon basin and is present in many energy drinks. Guarana is known to be between 2-7.5% caffeine in composition (Beck, 2005), a concentration higher than any other known plant in the world, which contributes to its cardiovascular effects. It also contains compounds such as theobromine, theophylline, tannins, and saponins. Aside from these compounds, it is important to note that guarana is a fatty seed. Therefore, guarana is not as readily soluble as caffeine, a polar substance, and its components take longer to dissolve into the

blood stream. Although the seed's longer absorption period and strong effects have not been confirmed with certainty, a study that tested the effects of guarana versus a caffeine pill containing the same amounts of caffeine observed an increase in systolic blood pressure after ingestion of guarana that was not seen after the caffeine ingestion. This result was observed at the two-and-a-half-hour mark, pointing to guarana's slow dissolution span (Meyer, Ball, 2004). Thus, although the amount used in the study was greater in concentration than the guarana in energy drinks, it is possible that when guarana is combined with caffeine in energy drinks, its composition and solubility produce greater cardiovascular effects than caffeine alone.

Sugar

The sugar content in one serving of most energy drinks ranges from about 27 g - 37 g in smaller cans to about 57 g in larger drinks. In comparison to the American Heart Association's recommendation for maximum added sugar intake per day, most of these measurements match or exceed the suggested maximum doses of 25 g and 37.5 g, for men and women respectively (AHA, 2018). It stands to reason that if energy drinks contain so much of this risky substance, sugar can play a role in the cardiovascular effects seen.

A small, yet well-designed study tested the participants' reactions to four drinks on four separate days in order to test the effects of sugar, caffeine, and taurine. Subjects consumed Red Bull (containing sugar, caffeine, taurine, and other ingredients), sugar-free Red Bull (identical to the first drink except for the sugar content), water with 120 mg of caffeine, or water itself, after which cardiovascular parameters were measured. Researchers found that all of the caffeinated drinks caused similar increases in systolic and diastolic blood pressure. They noted, however, that although the blood-pressure raising effect of all three beverages seemed to be the same, the mechanisms raising the BP varied between the different drinks. While the two caffeinated, yet sugar-free drinks raised total peripheral resistance, the sugar drink raised many myocardial parameters such as stroke volume, cardiac output, and contractility. These increases were not seen after any other drink. Moreover, the sugar-containing drinks lowered total peripheral resistance. These results suggest that while caffeine alone might have a similar effect on blood pressure when compared to caffeine and sugar combined, the former seems to act through a vascular route, and the latter by a cardiac route (Miles-Chan, et al., 2015).

The above effects seen after the sugar-sweetened, caffeinated Red Bull appear to be caused by an additive effect of the two active substances, as the sugar does not have these effects on its own. In a study of sugared drinks' effects on the cardiovascular system, researchers showed that while fructose raised blood pressure, sucrose and glucose – the two sugars used in energy drinks – did not. These sugars did raise cardiac output and heart

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rate, though (Grasser, et al., 2014b). Therefore, the effects of the Red Bull energy drink – to increase cardiac output, heart rate, and blood pressure – could not have been caused by sugar alone but must have been an additive result achieved by combining the two substances.

This study further suggests that taurine's affects are negligible, as the caffeinated drinks with and without taurine seemed to have the same cardiovascular effects.

Caffeine - Mechanisms

Aside from merely understanding that caffeine is the most active cardiovascular agent in energy drinks, we can also attempt to clarify the possible mechanisms by which it acts. Caffeine, a methylxanthine, is a stimulant known to work by the following three mechanisms: Firstly, caffeine competitively inhibits adenosine receptors. Adenosine is a fundamental component in sleep regulation and plays a role in making a person tired. Secondly, caffeine stimulates catecholamine release, causing raised levels of epinephrine (adrenaline) and norepinephrine (noradrenaline), which stimulate the sympathetic nervous system and increase blood pressure. Lastly, caffeine increases the release of calcium from the sarcoplasmic reticulum, thereby stimulating muscle contractility (Rana, et al., 2010). All of these mechanisms can explain effects attributed to caffeine in energy drinks.

Other Possible Mechanism by which Energy Drinks Adversely Affect the Cardiovascular System Endothelial Dysfunction

Another explanation for energy drinks' adverse effects on the cardiovascular system is that they have been shown to lead to endothelial dysfunction by increasing platelet aggregation and decreasing the reactive hyperemia index (Worthley, et al., 2010). Increased dysfunctional platelet aggregation can lead to thrombosis and cardiac arrest. Also, a reduced reactive hyperemia index indicates vessels that are not able to adapt to normal increases in blood pressure as well as they should be. This wearing-down of the blood vessels makes the system more susceptible to adverse effects because it cannot adapt as well to changes in pressure and flow.

Biochemical Changes in Heart Muscle

Long term energy drink consumption by male Wistar rats followed by heavy exercise has been shown to increase glucose and glycogen concentration in their heart muscle. After feeding these rats energy drinks versus water for thirty days and then having them swim to exhaustion, researchers found increased levels of glucose and glycogen in the heart muscle of the rats exposed to energy drinks. Although the buildup of glycogen seems contradictory to caffeine's usual effect of increasing glucose concentration by activating AMPK (Amp activated protein kinase), it is possible that when the compound is chronically

activated, glycogen synthase is activated allosterically and overcomes AMPK's usual inhibition of glycogen formation. This is of concern due to glycogen's association with incidents caused by the pre-excitation syndrome arrhythmia. Glycogen has been found in increased concentrations in the heart after adverse events caused by pre-excitation syndrome (Munteanu, et al., 2018). This suggests another possible mechanism by which energy drinks precipitate arrhythmias.

Conclusions

There are two public health concerns. The first, is that while arrhythmia-related deaths are rare and most patients with an increased risk of abnormal heart rhythms do not actually have fatal arrhythmias, the introduction of energy drinks will increase the frequency of fatal arrhythmias in this population. The second and more troubling concern is the possibility that energy drinks are causing slight increases in blood pressure in habitual consumers, who are generally young. Increased blood pressure by even small amounts has been convincingly shown to increase the risk for stroke and death due to cardiovascular events. On a public health level, if more and more adolescents and young adults use energy drinks and have increased blood pressure, the rate of hypertension-related deaths will rise in the foreseeable future. The benefits of these drinks must therefore be weighed against the adverse possibilities.

Since many of the currently available studies used small subject groups, the need for larger studies to further clarify results cannot be overlooked. With time, the long-term effects of these relatively new substances can be studied. Population studies comparing those who consumed higher levels of energy drinks versus those who consumed caffeine through coffee and versus those who abstained from both should be conducted.

As with most foods – especially those that contain active substances – energy drinks consumed occasionally and in moderation seem to be reasonably safe for healthy people. Consumers should be careful not to increase consumption to the point that it becomes habitual or reaches the unsafe levels discussed in this study.

Consumers should be warned not to overuse these substances and to avoid using them before strenuous activity, as many of the more serious adverse events mentioned above occurred after increased activity. Government authorities should consider requiring manufacturers to warn buyers about the potential side effects mentioned in this review to prevent the occurrence of serious energy-drink related adverse events.

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Should Subclinical Hypothyroidism Be Treated?

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Abstract

Subclinical Hypothyroidism, SCH, has been detected with increasing frequency in recent years and has brought about major controversies regarding management and treatment [Meier et al., 2001]. The condition is characterized as having a high concentration of thyroid stimulating hormone, yet normal thyroid hormone levels and is often asymptomatic. Scientific articles retrieved from various databases helped determine some of the long term risk factors associated with SCH, including progression to overt hypothyroidism, fatty liver disease, cardiovascular disease, neuropsychiatric complications and reproductive malfunctions. Studies determining the clinical and metabolic effects of L-thyroxine hormone replacement therapy on symptoms and potential risks of SCH on various patient populations were investigated and compared to controls that were not treated or were given a placebo drug. After considering both the arguments promoting treatment and others opposing it, conclusions were drawn regarding the promotion or discouragement of hormonal treatment for patients in different age brackets and stages of life. In general, the greatest benefits of treatment are observed in patients with thyroid-stimulating hormone levels ranging over 10mIU/L, younger patients with slightly elevated thyroid-stimulating hormones who are at risk for cardiovascular disease and for pregnant women with gestational SCH. On the contrary, L-thyroxine treatment therapy is not as crucial for the elderly population or patients with slightly elevated thyroid-stimulating hormone values when there are no apparent symptoms of hypothyroidism being experienced.

Introduction

Describing a condition as 'subclinical' implies that it is asymptomatic, thereby suggesting that a less aggressive treatment plan is needed and possibly rendering the condition unworthy of treatment altogether. However, there is controversy regarding whether the condition is indeed 'subclinical' and if it is necessary or beneficial to treat. Hypothyroidism is an endocrinological disorder in which there is inadequate production of thyroid hormones, triiodothyronine (T3) and thyroxine (T4) by the thyroid gland. These hormones strongly influence energy metabolism, digestion and body heat production. Additionally, they play a key role in cardiac contraction rate, memory, psychological health and sleep. In the case where the gland itself fails to secrete sufficient hormone concentrations, the disease is considered primary. However, if the root of the problem is traced back to insufficient stimulation of a structurally healthy gland due to decreased thyroid-stimulating hormone (TSH) released from the adenohypophysis, then the condition is regarded as secondary or central. Diminished secretion of thyrotropin-releasing hormone (TRH) from the hypothalamus would cause the condition to be considered tertiary hypothyroidism [Hadley & Levine, 2007; Wiersinga, 2010]. Subclinical hypothyroidism (SCH) ranks as a grade of primary hypothyroidism and is characterized biochemically as an elevated serum TSH concentration, with a normal serum-free T4 level, as well as T3 level [Meier et al., 2001]. When a patient's thyroid function is normal and hormone secretion is balanced according to homeostasis, he/she is considered euthyroid. Euthyroid adults should exhibit TSH levels within a range of 0.2-7.6 mIU/L, a T3 concentration of 4.3-12.5 mcg/dL and a free T4 concentration of 0.7-1.7 ng/dL [Robertson, Shilkofsky 2005]. Some experts have suggested that the TSH upper limit should be only 2.5 or 3 mIU/L, while others argue that the serum TSH distribution gradually shifts towards higher values with age and must be adjusted accordingly. Others have proven through their studies that the accepted reference range for serum TSH should be altered as patients age and that maintaining a 4.5 mIU/L upper limit for TSH concentration results in the prevalence of SCH to be significantly overestimated in the

elderly population. Nonetheless, while there is controversy over the appropriate upper limit of normal serum TSH, most laboratories agree that it is 4 to 5 mIU/L [Surks et al., 2007]. Biochemical testing and laboratory test results alone are required in order to diagnose SCH since patients often present with vague symptoms elevated TSH levels. Much research has been done regarding whether to treat SCH in populations of various age brackets and stages. A general consensus has been reached based on the existing and potential risk factors involved and the success rates of levothyroxine thyroid hormone replacement.

Methods

Relevant information was accumulated via original research literature obtained from databases such as Google Scholar, Proquest, Pubmed and Touro College's Online Library. Medical journals, JCEM and JAMA, served as a source of information as well. Books, including a medical book and science textbook, were used to provide background information. All content was critically analyzed and compared to assure validity.

Epidemiology

Based on the results of population-based studies, the prevalence of SCH in adults ranges from 4 to 15 percent [Turnbridge et al., 1977] and affects approximately 10 million people in the United States [Huber et al., 2002]. In one of the best longitudinal studies conducted, Turnbridge et al. (1977) found that 7.5% of women and 2.8% of men of all ages in Whickham, England have TSH levels exceeding 6 mIU/L. Twelve such studies done within different cultures, similar to theirs, were reviewed, and they concluded that primary thyroid-gland malfunction occurs in approximately 5% of any population. Of the 16,533 participants in the United States Third National Health and Examination Survey (NHANES III), 4.3% had SCH [Hollowell et al., 2002]. Of the 25,862 participants included in the Colorado Thyroid Disease Prevalence Study, an elevated TSH level was found in 9.5% of the population [Canaris et al., 2000]. Furthermore, the frequency of SCH is found to be higher in females than males,

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rises with age and is less in blacks than in whites [Kanaya et al., 2002]. It is important to note that since thyroid hormones contain iodine, the inhabitants' thyroid functioning is highly dependent on the iodine reserve of their specific region. When analyzing population-based studies, it's crucial to consider how the degree of iodine repletion or depletion in that geographical environment affects its populations intake and in turn, thyroidal health [Laurberg et al., 1998]. For example, in Africa, lack of dietary iodine is the chief determinant of thyroid pathology, instigating a spectrum of iodine deficiency disorders, including hypothyroidism [Okosieme, 2006].

Clinical Findings

While the clinical manifestations of SCH vary, patients sometimes report mild signs and symptoms similar to those experienced due to overt hypothyroidism. However, these symptoms are characterized as neither sensitive nor specific [Meier et al., 2001]. Based on the Colorado Thyroid Disease Prevalence Study involving 2,336 participants with SCH, symptoms reported by patients with SCH included dry skin (28%), poor memory (24%), slow thinking (22%), muscle weakness (22%), fatigue (18%), muscle cramps (17%), cold intolerance (15%), puffy eyes (12%), constipations (8%) and a hoarser voice(7%) [Canaris et al., 2000]. Conflicting data is seen from a community-based cross-sectional study where 1,423 participants, ranging from ages 18-75 years old, had TSH screenings and their symptoms were evaluated. The data collected indicated that subclinical thyroid disease is not associated with lower well-being or impaired health-related quality of life. Therefore, attempts to clinically identify SCH based on reported symptoms are unsuccessful [Bell et al., 2007]. However, significant evidence highlights numerous long-term consequences of SCH, though they do not appear obvious from patients' reports.

Progression to Overt Hypothyroidism

A significant number of patients with SCH eventually develop overt hypothyroidism. In a study done by Huber et al., after a mean observation period of 9.2 years, 23 out of 82 patients (28%) who entered the study with subclinical hypothyroidism developed overt hypothyroidism, as defined by low T4 concentration and elevated TSH (>20 mIU/L). The risk of progression to overt hypothyroidism was seen to correlate to the initial serum TSH level of the patient; the higher the concentration, the greater the risk. Of the 82 women with TSH concentration values ranging from 4-6 mIU/L, the frequency of progression to overt hypothyroidism was 0% after 9.2 years. When only the patients with a serum TSH concentration above 6 mIU/L were evaluated (n = 61), after 10 years, the cumulative incidence of overt hypothyroidism was 55.3% [Huber et al., 2002]. Overt hypothyroidism is particularly common in older patients whose TSH levels exceed 10 mIU/L or those who have circulating thyroid

antibodies [Ayala et al., 2000]. Obviously, overt hypothyroidism poses a greater threat to one's overall health related quality of life as it presents with classical symptoms of fatigue, depression, weight gain, cold intolerance, bradycardia, constipation, dry skin, facial edema and loss of hair [Carle et al., 2014]. More severe dysfunction of the thyroid gland also stimulates a negative feedback mechanism in which more and more TSH is secreted, ultimately causing the gland to swell, referred to as goiter.

Fatty-liver Disease

Since thyroid hormones play a fundamental role in lipid metabolism, mild hypothyroidism may cause hypercholesterolemia and play an essential role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Non-alcoholic fatty liver disease is defined as "extrahepatic accumulation of fat in the absence of excess alcohol consumption" [Liu et al., 2017]. A cross-sectional study reported the prevalence of fatty-liver disease and abnormal liver enzyme levels to increase progressively with rising TSH levels compared to patients with euthyroidism, suggesting associations between the spectrum of SCH and liver malfunction. In one study population, the incidence of fatty-liver disease and abnormal liver enzyme level was 29.9% and 20.1%, respectively, for patients with SCH [Chung et al., 2012]. SCH patients experience higher amounts of liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT), an enzyme released into the bloodstream when the liver is damaged [Liu et al., 2017].

Cardiovascular Disease

Thyroid hormones are known to affect the heart and vasculature. As a result, the impact of SCH on the cardiovascular system has become an important subject of research [Liu et al., 2010]. While it is undisputed that overt hypothyroidism is linked to increased risk for cardiovascular diseases (CVD), conflicting data raises controversy whether SCH is also associated with heart disease. Heterogeneity among individual studies can be explained by the different TSH cutoffs used, varying cardiovascular disease definitions or differences in participants' age, gender, or severity of SCH (as measured by TSH level) [Rodondi et al., 2010]. Clinical hypothyroidism has been known to cause an increase in blood pressure. Since thyroid hormone directly acts on arterial smooth muscle cells of blood vessels, causing vasodilation, when hypothyroidism occurs, a lower hormone level increases the vascular resistance and in turn the level of blood pressure. In a cross-sectional survey the incidence of high blood pressure, hypertension, in the SCH group was significantly higher than the control, the euthyroid group. The conclusions drawn from this study coincide with previous data collected by Luboschitzky et al. in 2002 [cited in Liu et al., 2010].

Furthermore, since SCH has been linked with abnormal lipid metabolism, it is consequently related to increased risk for coronary heart disease. An increased serum total cholesterol

and low density lipoprotein cholesterol (LDL-C) was detected as a feature of SCH in some of the literature, while contrasting research found cholesterol levels to be in normal range. Nonetheless, several studies suggested a connection between SCH and development of atherosclerosis, and more recently published research established SCH as a risk factor for myocardial infarction [Meier et al., 2001; Anderson et al. 2015]. A meta-analysis from 7 studies, including 2,020 patients with SCH, confirmed a substantial trend of increased risk of coronary heart disease by highlighting events like nonfatal myocardial infarction, angina and necessary coronary revascularizations common to patients with elevated serum TSH concentrations, especially of those that were 10 mIU/L or greater. Results from a meta-analysis of patient-level data from 11 prospective cohort studies showed greater risk for coronary heart disease mortality for patients suffering from SCH with serum TSH concentrations above 10mIU/L. Rodoni et al. reported increased systemic vascular resistance, arterial stiffness, altered endothelial function, increased atherosclerosis and altered coagulability to be associated with SCH and the acceleration of development of coronary heart disorder. On the contrary, minimal TSH elevation (4.5-6.9 mIU/L) was not linked to cardiovascular disease or mortality [Rodoni et al., 2010].

Neuropsychiatric Symptoms

While there is evidence that SCH is linked to neuropsychiatric complications, there has been conflicting research as well. One study assessed the history of major depression in subjects with SCH compared to euthyroid subjects. The frequency of depression was substantially higher in those who met the criteria for SCH (56%) than in those who did not (20%), suggesting that SCH may lower the threshold for the occurrence of depression [Haggerty et al., 1993]. Similar results were found in a later study where signs of neuropsychiatric dysfunction like somatization, cognitive impairment disturbances, psychomotor retardation and sleep disorders were seen in 63.5% of a population with mild thyroid failure [Demartini et al., 2010]. However, another study revealed opposite research as patients with SCH scored equally as well as the euthyroid control group on 14 cognitive function tests [Jorder et al., 2006].

Reproductive Malfunctions

Alteration of the thyroid's physiology under the impact of placental human chorionic gonadotropin (hCG) during the first trimester, results in adjustments to TSH levels. During the first trimester, a white woman maintains an upper limit TSH concentration of 2.5 mIU/L and 3.0 mIU/L during her second and third trimester periods [Stagnaro-Green et al., 2011]. SCH during gestation poses fewer risks than overt hypothyroidism would, but still puts women at increased risk for preterm delivery at or before 34 weeks of gestation, placental abruption (3 times more

likely to occur in women with SCH) and/or pregnancy loss compared to euthyroid women. Severe preeclampsia, defined as gestational hypertension is noted as a risk factor for pregnant women with SCH [Casey et al. 2005; Novakovic et al., 2018]. A meta-analysis of 15 cohort studies involving 1,896 pregnant women with SCH arrived at conclusions similar to Novakovic et al. and showed that SCH during gestation causes significant impairment to the intelligence and neurological development of the offspring [Liu et al., 2018].

It's possible to acquire SCH as a result of pregnancy; an estimated 15% of pregnancies in the U.S are affected by gestational SCH [Blatt et al., 2012]. Over the gestational period, increased metabolic needs result in increased production of thyroid hormones by approximately 50% compared to euthyroid, non-pregnant women. Consequently, necessary daily iodine intake levels rise by 50%. More renal blood flow and glomerular filtration results in an increase in iodide clearance from blood plasma. Thus, more iodine is necessary in the diet of a pregnant woman. The stress imposed on the thyroid gland during gestation results in SCH for many women with compromised dietary iodine levels. Clinical studies have proven that iodine deficiency during gestation causes impaired development of the brain and impedes mental function development of the fetus [Farebrother et al., 2015]. Measuring the IQ of children whose mothers had not been treated for SCH during pregnancy revealed mean IQ scores of 7 points lower than controls [Haddow et al., 1999]. Casey et al. (2005) proposed that preterm parturition results in prematurity which contributes to neurodevelopment delay and reduces intelligence quotient.

Effects of Levothyroxine Hormone Replacement

In general, the research showing correlation between T4 replacement and improvement of hypothyroid symptoms is conflicting and is probably due to differences in the populations studied, various TSH cut-off concentrations and age range. Levothyroxine, which is a synthetic form of T4, is usually administered to patients suffering from overt hypothyroidism. Nevertheless, there is much controversy whether patients exhibiting mild thyroid failure with similar symptoms should be prescribed levothyroxine as well [Meier et al., 2001]. Some, but not all, research conducted show how treatment facilitates improvement of hypothyroid symptoms experienced by patients with SCH. Subjects with mild thyroid failure who were treated with L-thyroxine showed significantly greater improvement in overall hypothyroid symptoms than did subjects who were given placebo [Cooper et al., 1984]. Nonetheless, in all the clinical trials reviewed, with ongoing dosage monitoring, L-thyroxine succeeded in lowering previously elevated TSH concentrations to healthy levels.

By decreasing TSH concentration to euthyroid levels, L-Thyroxine prevents the progression to overt hypothyroidism

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[Ayala et al., 2000]. Following T4 treatment, ultrasound verified 80% thyroid volume reduction in patients with goiter [Romaldini et al., 1996]. In one study, L- thyroxine and a placebo drug were randomly administered to 63 female patients over a span of 48 weeks in order to achieve euthyroid TSH levels. In the thyroxine treated group, the mean serum TSH concentration was 12.8 ± 1.4 mIU/liter before and 3.1 ± 0.3 mIU/liter after treatment, whereas TSH levels remained unchanged in the control group.

When lipid concentrations were measured after thyroxine treatment, it was observed that the total cholesterol and low density lipoprotein cholesterol decreased substantially, especially in those with TSH concentrations of 12 mIU/L and above. From the observed improvement, it is approximated that treatment reduces the risk of cardiovascular disease and mortality by 9-31%. In contrast, no change in any variable of thyroid function occurred in the placebo group (Meier et al., 2001). Substantial improvement of cardiac function in treated patients was reflected by a sensitive measure of myocardial contractility [Cooper et al., 1984]. In a study done in Copenhagen, Denmark, the participating cohort included patients over 18 years old with the mean age being 55.2 years. The total of 12,212 subjects were subdivided into 2 groups, one with TSH levels of 5-10 mIU/L and another with TSH levels above 10 mIU/L; some were treated from both groups. In this large cohort study no association between treatment and risk of myocardial infarction or mortality was revealed, besides in younger patients where it may seem that treatment had marginal protective advantage. However, limitations of the study such as no access to patients' blood pressure or serum lipid levels and a relatively short follow-up period of 5 years cause it to have less credence since patients could have suffered from cardiac complication later on in time [Anderesen et al., 2015].

A study involving 415 patients with SCH (defined as TSH ≥ 4.2 mIU/L) administered levothyroxine to measure its effect on non-alcoholic fatty liver disease and abnormal liver enzyme levels. After treatment with LT4, the incidence of fatty liver disease in patients with more pronounced SCH (TSH concentration ≥ 10 mIU/L) reduced from 48.5% to 24.2%, while in patients with milder SCH (TSH concentration 4.2-10 mIU/L), frequency of fatty liver disease and alanine aminotransferase was not significantly altered by LT4 supplementation. However, mild SCH patients with elevation of total cholesterol who received LT4 treatment showed decreases in the incidence of fatty liver disease and serum alanine aminotransferase levels. Those with higher TSH values experienced a decrease of 5.61 IU/L in serum aspartate aminotransferase and a small decrease in serum alanine aminotransferase. Patients with milder SCH showed a reduction in serum alanine aminotransferase from 19.09 IU/L to 17.95 IU/L, yet serum aspartate aminotransferase remained stable throughout the study in the untreated, mild SCH control

group. Observing how these parameters became less prevalent in patients who received treatment, but remained comparably stable in patients who weren't treated, provides concrete evidence of the success LT4 therapy has with prevention of non-alcoholic fatty liver disease [Liu et al., 2017].

In another randomized double-blind, placebo-controlled trial, TSH values decreased by 8.6 mIU/L. Patients treated with with LT4 showed improved psychometric memory score involving their thinking, memory and attention versus untreated control patients [jaeschke et al., 1996]. Unfortunately, in another study, LT4 treatment was not enough to generate complete remission of depressive symptoms, suggesting psychiatric evaluation in patients affected in this way by SCH is needed [Demartini et al., 2010].

Regarding SCH during gestation, miscarriage was significantly less frequent among treated women compared to untreated women in a national U.S. cohort of 5,405 pregnant women. However, the treated group also had increased odds for preterm delivery, gestational diabetes and preeclampsia [Maraka et al., 2017]. Conflicting data claims LT4 decreased the rate of preterm delivery considerably in pregnant women who received treatment compared to their control who did not (SCH defined as TSH ≥ 4 mIU/L; 5.3% incidence for treated women versus 29.4% in control group) [Nazarpour et al., 2017]. Further complicating the matter, another study revealed no difference in the prevalence of preterm delivery, gestational hypertension, miscarriage rates or adverse fetal outcomes between women who received LT4 treatment and those who got the placebo [Casey et al., 2017]. During pregnancy, beginning treatment for compensated maternal iodine status to ensure proper brain development in the fetus is extremely time-sensitive; until the end of the second trimester, iodine treatment protects the neurological system from consequences of iodine deficiency. Since head circumference reflects brain mass, the head circumference of an underdeveloped child will be significantly diminished. Microcephaly, where head circumference is more than 3 standard deviations below the norm, was diagnosed in 27% of children of untreated mothers as opposed to 11% frequency in their treated control [Cao et al., 1994]. Yet, opposing data says that no significant discrepancies are noted when comparing IQ scores and neurodevelopment of 5 year old children of mothers who received LT4 to those who took the placebo drug [Casey et al., 2017]. Additional research trials are needed to determine whether LT4 therapy during gestation prevents adverse outcomes, possibly induces some issues or simply doesn't have any effect at all.

In opposition to treating SCH, various studies, such as the meta-analysis of 12 clinical trials (9 of them with TSH concentration cut-offs of less than 10, 12 and 15 mIU/L), show no changes in hypothyroid symptoms or quality of life in the treated group versus the control, the group who received the placebo

drug [Villar et al., 2007]. While SCH and cognitive dysfunction have been linked, T4 treatment in a population over 65 years of age proved it to be ineffective for aiding cognitive function [Parle et al., 2010]. Similarly, a double-blind, randomized, placebo-controlled, parallel-group trial involving 737 adults (65 years of age+, TSH levels ranging from 4.60-19.99 mIU/L with a mean TSH of 6.40 ± 2.01 mIU/L) concluded that there are no apparent benefits to L-thyroxine treatment in the quality of life in older adults. There was no benefit observed in regard to hypothyroid symptoms, executive cognitive function as measured by the letter-digit coding test or degree of lethargy experienced by the group whom received treatment. However, after 12 months, the mean TSH level was 5.48 ± 2.48 mIU/L in the placebo group, as compared with 3.63 ± 2.11 mIU/L in the levothyroxine group; clearly, in terms of reaching healthy TSH levels, the treatment was successful [Stott et al., 2017].

While research supports that untreated SCH during pregnancy is linked to many adverse obstetric outcomes, many claim that insufficient evidence exists supporting the benefit of LT4 therapy on clinical improvement for women with SCH. Implementing guidelines to treat all pregnant women with SCH would lead to prescription of LT4 for up to 600,000 pregnant women in the U.S every year which would obviously have enormous impact on the cost of healthcare [Maraka et al., 2017]

Strengthening the argument that SCH should not be treated, there is potential danger for a patient to overdose on levothyroxine, causing TSH values to decrease abnormally. Statistical analysis claims that the incidence of this is as frequent as 10-33% as treated SCH patients exhibit unusually low TSH levels. Overtreatment would completely reverse the original condition inducing symptoms of excess thyroid hormone. Many times, this is due to polypharmacy and drug interaction; research done reiterates the importance in monitoring TSH therapy in the older population, because of risk of over-replacement. In their trial with 339 thyroid hormone users, 41% had a low TSH, 16% had a high TSH, and 43% were in the euthyroid range after treatment [Parle et al., 1993; Somwaru et al., 2009]. Additionally, those against treatment resent the lifelong commitment to daily medication in asymptomatic patients, and the cost for both the hormone and for consistent monitoring of its efficacy.

Conclusion

Based on the results of T4 replacement successfully reducing hypothyroid symptoms and potential risks associated with SCH in certain populations, it is recommended that LT4 replacement be given to those patients who present with abnormal thyroid function tests. The most obvious benefit appears to be limited to patients with baseline TSH levels of ≥ 10 mIU/L as they are most likely to experience progression to overt hypothyroidism, atherosclerosis, myocardial infarction and non-alcoholic fatty liver disease. The research regarding pregnant women with SCH

poses no doubt to experts and healthcare providers in their unanimous decision to err on the side of caution and prescribe levothyroxine substitution for both the mother's and fetus's advantage. Adverse obstetric effects and risk factors far outweigh any arguments against treatment. However, treatment of patients with minimally elevated TSH values (between 4.5 and 10 mIU/L; differing depending on individual study TSH cut-off) demonstrated minimal change in health related quality of life and only burdened patients with responsibilities to monitor treatment and cover its cost, suggesting that these patients not be treated. However, based on the increased protection against cardiovascular disease and mortality in treated younger patients (<65 years) with TSH ranging from 7 to 9.9 mIU/L, some recommend treatment. But in the elderly population where LT4 treatment exacerbates the issue of polypharmacy and may instigate harmful drug-interactions, coupled with the fact that there is a lack of treatment trials showing benefit of treatment in this population, only those who present with symptoms suggestive of hypothyroidism should be treated. Of course, long-term monitoring and annual clinical evaluation of TSH concentration should be implemented to help ensure optimal dosage. Whether reference ranges should be adjusted for age, implementing a higher cutoff for starting treatment in older patients, awaits further research. Future research clarifying the reference ranges for thyroid function tests, specifically the upper limit for TSH, will help resolve conflicting data and enable researchers to easily compare data from different clinical trials in order to determine whether or not the condition requires levothyroxine treatment.

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Are There Any Viable Treatments For Age Related Macular Degeneration?

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Abstract

Stem cells seem to offer an alternative venue for treating many cell related diseases, such as age-related macular degeneration (ARMD). ARMD is a progressive neurodegenerative medical condition, which primarily affects the retinal pigmented epithelium (RPE), resulting in degeneration of photoreceptors. Scientists have been successful in implanting stem cells into the eyes of rats. These injected cells sustained visual function and photoreceptor integrity without any cancerous cell formation. There are numerous treatments available to slow down the progression of ARMD. Depending on the type of ARMD, doctors may either recommend leading a healthier lifestyle or that one should undergo surgery. Numerous risk factors can increase one's chances of getting ARMD depending on one's age and race. Bad habits, such as cigarette smoking, can contribute to the progression of ARMD. It is important to get a comprehensive dilated eye exam periodically. Many eye diseases that don't have any warning signs can be detected with such an eye exam.

Introduction

The most versatile cell types in the human body are embryonic stem cells. Stem cells have three unique general properties that distinguish them from other cells in the body. One is that these cells can replicate themselves even after long periods of inactivity. Secondly, stem cells are unspecialized. These cells are not programmed to become any specific tissue. For example, stem cells can't work with other red blood cells to carry oxygen containing molecules through the blood stream. Thirdly, under certain physiologic or experimental conditions, stem cells can become the specialized cells that make up tissues and organs (National Institutes of Health 2016).

When stem cells divide, they can make either more stem cells, or can differentiate into a specialized cell which is tasked with a specific function. For example, these stem cells can turn into motoneurons which are used for motor skill or into cardiomyocytes to help pump blood through the arteries. However, Adult stem cells can only generate cells that are like the tissue in which they are located in (Tuch, 2006). For example, hematopoietic stem cells in the bone marrow form only cells that give rise to the different types of blood cells and cannot make nerve cells for the brain.

Embryonic stem cells are made from oocytes that have been fertilized in vitro and not in a women's body. The process of priming the cells in an in vitro fertilization clinic is known as cell culture. Human embryonic stem cells are primed by placing an embryo into a culture dish that contains a culture medium. With the right physiological conditions, these cells begin to divide and spread over the surface of the dish forming millions of unspecialized stem cells. Although scientists have not agreed upon a battery of tests to ensure they are growing only unspecialized cells, there are some common tests performed to determine the cells fundamental properties. One technique used is by looking for particular cell surface markers, such as transcription factors, that are generally produced by undifferentiated cells.

Stem cells in their culture dish can remain undifferentiated as long as they are under the appropriate conditions. However, cells automatically begin to differentiate once they are allowed to cluster together to form embryoid bodies. These cells can then begin to form tissue specific cells, such as muscle or nerve

cells. Although differentiation is a sign of a healthy culture, this process is generally uncontrolled, and therefore an inefficient way to produce specialized cells. In order to create a more controlled experiment, scientists change around the chemical composition of the culture medium or by inserting specific genes to specialize the cells in an organized fashion.

Human embryonic stem cells have many applications in clinical use and in research. One useful application is in cell-based therapies, situations where stem cells are induced to turn into specialized cells. Through this process, new cells which are needed to repair destroyed tissue can be formed. For example, these specialized cells can replace bone marrow, muscle, and brain cells that are injured or malfunctioning. These cells form an internal repair system by cell mitosis for as long as the organism is alive. Due to their ability to replace damaged cells, stem cells seem to offer an alternative venue for treating many cell related diseases as well. If scientists can reliably find a way to differentiate the cells in an organized manner, then they may be able to cure many diseases in the future (Weiss, Troyer, 2006).

One of the diseases that scientists believe embryonic stem cells can treat is age related macular degeneration (ARMD) (Shroff, 2015). Over 10 million Americans have vision loss related to Macular degeneration. Macular degeneration is the third most common pathological condition leading to vision impairment (Resnikoff, et. al., 2004). ARMD is a progressive neurodegenerative medical condition, which primarily affects the retinal pigmented epithelium (RPE), resulting in degeneration of photoreceptors. This causes loss of vision in the center of the field of vision. The disease is caused by the deterioration of the macula, a region that is located at the center of the retina and is responsible for focusing central vision in the eye. This allows people to ride their bike or recognize a familiar face. Common symptoms include not being able to see in dim light, seeing spots, and distorted vision.

There are two types of ARMD. There is the wet as well as the dry form. Most of the cases of ARMD are the dry form. Dry ARMD is also known as non-neovascular ARMD as well as non-exudative ARMD since there are no fluids that leak into the macula from blood vessels. Although people with this form of ARMD may have fair central vision of at least 20/40, they

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may have other functional limitations, such as fluctuating vision, poor night vision, and pixelated vision. In this form of ARMD, the deterioration by the macula is linked with the appearance of drusen. Drusen are small yellow deposits that form under the macula. These deposits are buildups of shapeless acellular debris from the basement membrane of the RPE. This buildup of drusen causes the macula to thin and dry out, thereby causing the macula to lose its function. This process is known as atrophy. The amount of vision loss that occurs is directly proportional to the amount as well as the location of the drusen. Advanced cases of dry ARMD are also known as geographic atrophy (GA) since large areas of the retina stop functioning. Patients with GA usually see large blank spots in their central vision because their macula has deteriorated. Usually, most people over 50 years old have a drusen buildup in at least one of their eyes. Currently, there is no known cure for this form of the disease.

However, some cases of dry ARMD progress into the wet form. In the wet form, there is an abnormal blood vessel growth under the macula. This type of growth is known as choroidal neovascularization (CNV). Once CNV has occurred in one eye, the patient has a higher risk that the other eye will get CNV as well. These new blood vessels are generally weak and can rupture easily. When they rupture, they leak fluids, such as blood and lipid containing solutions. This causes the macula to bulge, thereby causing a distorted central vision. These conditions can cause severe vision loss in a short amount of time. In this form of ARMD, patients may see spots because of the fluid buildup under the macula. Therefore, periodic eye exams are vital, especially if the patient is at a higher risk of contracting ARMD.

There are three stages in ARMD. There is early, intermediate, and late stage ARMD. During early stage, most people don't realize any of the symptoms. Therefore, it is crucial to have regular eye exams. Early ARMD is diagnosed by the appearance of drusen the size of 63 microns by the macula. In the intermediate ARMD, there may be some vision blur in the central vision and by late ARMD there is a noticeable vision loss in the central vision.

Medical experts aren't exactly sure what causes ARMD but once present, many factors contribute to its progression. A person's age is by far the biggest factor for ARMD. Once one turns 50, they should visit an optometrist for a comprehensive dilated eye exam. Many eye diseases that don't have any warning signs can be detected with such an eye exam. This exam includes a visual acuity test, a visual field test, dilation, and tonometry. Annual exams are recommended once a year once one turns 60. However, if one is African American, then they should get one when they turn 40 since they have a higher risk for glaucoma. Although there aren't any cures presently available, there are numerous treatments for ARMD. Many doctors may prescribe a strict diet and exercise to slow down the progression. However, none of them can cure the disease, except potentially stem cells.

Although stem cells may offer new therapies, their use has

been very controversial. People oppose stem cell research since one is harvesting a fertilized egg. They believe that this is wrong since this is tantamount to killing an unborn child, and therefore one should respect the value of a human life. However, others feel that morally it's our duty to prevent or alleviate a person from suffering.

Methods

Literature for this article was obtained primarily using Touro College's Online library. Other databases, such as PubMed, were used. Additionally, Google Scholar was valuable for finding necessary and relevant articles.

Discussion

The retina of the eye is tasked with converting light into vision. At the center of the retina is the macula. In the retina, there are the rods and cones which process light into nerve impulses. Behind the photoreceptor layer is the retinal pigmented epithelium (RPE). The RPE is tasked with delivering nutrients and removing wastes from the photoreceptor cells. In ARMD, RPE stops functioning, causing the cones and rods to deteriorate. Today, scientists are using stem cell research to understand how diverse cells in the retina interact with one another. This has led to discovering new ways of replacing photoreceptors and the underlying RPE.

Generally, replacing dead cells with stem cells is very challenging since the stem cells would have to establish new connections with the surrounding nerve fibers that ultimately relay the message to the brain. However, the eye is a great target for stem cell research since there are many barriers in the eye, such as tight cell junctions, therefore making it relatively self-contained. This prevents the migration of cells outside of the eye. Furthermore, with the use of an ophthalmoscope it is easy to assess the effectiveness of the treatments. Doctors can also compare the treated eye with the other eye to evaluate the effectiveness of the treatments.

RPE cells are easier to integrate with existing retinal cells since they don't need to connect with nerve fibers. With stem cells research, one can use new RPE cells to replace dead RPE cells. If the stem cells are replaced before the photoreceptors have completely deteriorated, then the new RPE cells may be able to prevent existing photoreceptors from dying, thereby preventing the progression of the disease. Stem cells can also be used to discover new therapies. When damaged RPE cells are stressed, they produce characteristics of ARMD. These damaged cells can then be studied to evaluate the different surface markers present which can help in early intervention and in getting a better diagnosis.

Scientists have come up with different methods to replace the RPE layer. One method is using human embryonic stem cells. These cells are naturally pluripotent when harvested and can renew for long periods if properly maintained in vitro. They can

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transform into the ectoderm, endoderm, and mesoderm. These three primary germ layers can differentiate into all the cells in the body. However, there are numerous obstacles raised with this method. One of the problems is whether it is ethical to harvest embryos since they can turn into a fetus (Narsinh, et. al., 2011). Another challenge that must be overcome is the limited supply of human embryo donors.

Another method researchers have used is induced pluripotent stem cells

(hiPSCs). These are usually epithelial cells that are reprogrammed to behave like embryonic stem cells. They can then be used to grow rods and cones or RPE cells. The landmark discovery of hiPSCs has been hailed as a significant advance in stem cell research. This method circumvents the ethical debate with embryonic stem cells since no human embryo is destroyed ex utero. However, there are numerous problems with this method as well. Extensive research has shown that these cultured cells can turn into benign or malignant tumors (Ho, et. al., 2012). Therefore, these issues preclude clinical use of these cells for now.

Another method which scientists are experimenting with is adult stem cells. This method entails growing RPE specific stem cells from adult stem cells. One source for such cells is from eyes donated to eye banks. These cells are less likely to be rejected if used in implants. However, there usually is a limited amount in each tissue, therefore making it harder to find and purify. Furthermore, these cells can't be stored for any length of time before they turn cancerous (Reya, et. al., 2001).

Delivery Method for Stem Cells

Although there are no FDA approved cures for ARMD, implantation of healthy RPE into the macula may prove to be an effective treatment. The following procedure was used to deliver RPE into rats. After the rat was put under a general anesthetic, a hole was created in the eye using a sharp needle. Then, a blunt needle was inserted into the hole until it reached the RPE layer, into which the stem cells were injected (Westenskow, et. al., 2015).

In one study, animal models, such as rats and mice with macular degeneration, were treated with stem cells that become RPE cells (Lu, et. al., 2009). To simulate real life conditions, the rats were under a 12-hour light /dark cycle. One day prior to their transplantation, all animals in the main experiment were administered cyclosporine to prevent organ rejection. Before the researchers administered the cells, they washed the cells in balance salt solution. Two weeks after the transplantation, the rats received an intraperitoneal injection of dexamethasone. This steroid was injected to treat the resulting inflammation.

These injected cells sustained visual function and photoreceptor integrity without any cancerous cell formation. Visual acuity increased when the models received a dose between 5000-100,000 RPE cells. Following the procedure, the rats were

monitored to check for any adverse reaction. They found that visual function was sustained for at least two months. The cells themselves survived for at least half a year before they started to deteriorate. However, this deterioration in visual acuity can be due to insufficient cyclosporine. Another hypothesis of why the cells deteriorated can be because the transplantation may need to be repeated several times in order to sustain their therapeutic effect.

ARMD Treatments

For early dry ARMD, doctors recommend a diet that is high in antioxidants, such as strawberries and oranges. However, if the patient has an advanced stage of dry ARMD, such as GA, then the doctor may prescribe supplements that increase vital vitamins that support the cell.

Until recently, the only treatment for wet ARMD was laser photocoagulation. This treatment by an ophthalmologist is a minimally invasive procedure which uses a laser to burn and destroy leaking blood vessels. However, this treatment cannot restore vision that has been lost already. One drawback to their use is that most CNV lesions are too big to be treated by laser coagulation. Another is that there is a high chance that the leakage will reoccur over time. Because of these limitations, scientists have been looking for alternative therapies that are safe and effective for a long period of time.

One alternative treatment that is widely used today is Anti-vascular endothelial growth factor (VEGF) therapy. This therapy includes periodic intraocular injections of a chemical called anti-VEGF. Normally, VEGF is beneficial for the circulatory system since it helps promote the growth of new blood vessels. However, having too much by the macula can have deleterious effects on it since they promote the growth of weak new blood vessels. This intravitreal shot of anti VEGF stops the development of new blood vessels by the macula, thereby preventing any further leaks. However, there are numerous side effects with this treatment. Patients have complained about vitreous floaters. This happens since the doctor punctures the vitreous layer of the eye. Another side effect observed was an elevated eye pressure. This can cause glaucoma if left untreated.

Human Implant

Although stem cells have been proposed as a potential treatment in treating ARMD, there are still numerous drawbacks to their use. Safety concerns have been raised since there is a risk that these cells may turn into unwanted cell types which can lead to tumor formation in the eye and potential immune rejection. Although stem cells were discovered in the late 1990s, scientists did not know how to find a safe way of implanting them. The first reported transplanted hESC-derived cells into the macula was in 2012. hESC-derived retinal pigment epithelium cells were transplanted into the subretinal portion of the of the eyes of

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dry ARMD patients (Schwartz, et. al., 2012). Ninety-nine percent of the stem cells differentiated into RPE cells. These differentiated cells integrated into the host forming a homogenous RPE layer. Post-surgery, structural evidence showed that the cells had attached and continued to persist during the study. During the first four months, there were no signs of hyperproliferation, cell rejection, or abnormal growth.

The scientists didn't find any signs of adverse proliferation or any other type of ocular disease. This method proved to be a safer method for treating ARMD than other conventional methods, such as vitreoretinal surgery and immunosuppression. The results of this study provide the first evidence for medium to long term safety as well as graft efficacy. These results suggest that stem cells can be a safe alternative for many medical diseases that require tissue replacement. Therapeutic goals for the future will be to treat the patient in the earlier stages of macular degeneration, thereby preventing the photoreceptors from decaying.

Risk Factor

A study was conducted to evaluate the effects of antioxidants on ARMD. In this clinical experiment, they enrolled 3,640 participants who showed signs of ARMD. These participants had drusen buildup in at least one of their retinas. The size of drusen buildup was between 63-150 microns. Participants were randomly assigned supplements that contained either 500 mg L-ascorbic acid, 400 IU tocopherols, or a placebo. The researchers found that compared to the placebo a high dose of L-ascorbic acid and various tocopherols may delay the progression of ARMD and other forms of vision loss. These supplements showed no adverse effects, proving their safety (Age-Related Eye Disease Study Research Group, 2001).

A study was conducted to assess the risk of smoking in men. This study consisted of 21,157 US male physicians who did not have any known diagnosis of ARMD. Of them, 11% were current smokers, 39% were past smokers, and 50% that never smoked. Over the course of 12 years, they found 268 incidents of ARMD. Current smokers who smoked a pack a day had a 240 percent risk increase compared to nonsmokers. Past smokers had a 30 percent increased risk of contracting ARMD. Current smokers who smoke less than 20 cigarettes had only a modest risk increase of 26 percent (Christen, et. al., 1996). Unlike other risk factors, such as age, smoking is an avoidable risk factor.

Since there aren't any cures that are reliable for ARMD, it is therefore recommended to avoid smoking altogether. However, it could be that the study on smoking isn't 100 percent accurate. It could be that the subjects who didn't smoke had a slower progression since they ate foods rich in omega-3 fatty acid. This acid can be found in many common household products, such as fish and canola oil. In a study conducted with 681 twins, they found that having 2 or more servings of fish reduced the risk of ARMD by 45 percent (Seddon, et. al., 2006).

Although some fruits and vegetables can slow down the progression of ARMD, such as those rich in L-ascorbic acid and tocopherols, other vegetables can be bad for ARMD. One study tried to find out if dietary nitrates can increase your chance of getting ARMD. These nitrates can be found in leafy vegetables, such as lettuce and spinach. In this study, there were 2,856 participants. The scientists took numerous potential cofounders into account such as age, sex, smoking, energy intake, and fish consumption. The participants were monitored for 15 years. The scientists found that those who had a dietary nitrate intake had a 39 percent increase incidence of having ARMD (Gopinath, et. al., 2018). However, this percentage increase cannot be said to apply for all ethnic groups.

A prospective cohort study of 6,176 participants between the ages of 45 to 85 was used to find the prevalence of ARMD between four racial groups. These groups were white, black, Hispanic, and Chinese. The method they used to test for ARMD was by taking a picture of the fundus through the dark-adapted pupils, whereupon they were able to measure the drusen size. What they found is that Hispanics had a 1.8 percent increase in prevalence compared to the black ethnic group. The Chinese had a 2.2 percent increase, and the white had a 3 percent increase compared to the blacks. Differences in age, gender, pupil size, body mass index, smoking, alcohol drinking history, diabetes, and hypertension status did not explain the variability among the 4 racial/ethnic groups (Klein, et. al., 2006).

A person's age is by far the biggest factor for ARMD. In a population-based study, scientists tried to find the prevalence of ARMD in a sample of Hispanic individuals over the age of 50. All 4,774 participants had an initial ophthalmic evaluation to determine a baseline for comparison. Over the course of the study, the scientists monitored drusen size, drusen type, and the area covered by drusen. They found that the prevalence of early and late ARMD increased with age. The prevalence of early ARMD varied with age as follows: people between 50 and 60 had a 20 percent increase, while those aged 80 and older had a 54 percent increase. The prevalence of late ARMD varied with age as well. Those in the age group of 50 to 60 had a .1 percent increase, while those 80 years and older had a 4.3 percent increase (Muñoz, et. al., 2005).

Conclusion

Currently, there is no FDA approved cure for ARMD. However, one promising cure currently being researched is stem cell transplantation. However, there are numerous obstacles that still need to be overcome before they can be safely implanted into patients. Depending on the type of ARMD, doctors may either recommend leading a healthier lifestyle or that one should undergo surgery. As one ages, one is more prone to getting ARMD. Therefore, it is important to get a comprehensive dilated eye exam periodically.

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