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Review Article

GENE THERAPY FOR HAEMOPHILIA

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Abstract: *The blood disorder, Hemophilia, has its roots embedded deep into the history of genetic disorders. The European royal family is one of the most prominent families to be affected by this disease thus, dubbing it 'the royal disease'. The types of Hemophilia are divided into two based on the type of coagulation factor mutation found in the patient. For treating haemophilia, gene therapy is done by using different vectors such as lentiviral and retroviral vectors but due to production of limited expression different adeno associated virus (AAV) strains are used. Some engineerly modified vectors are currently used to get best possible results. The clinical trials proves the efficacy of these vectors so through their obtained statistical consideration, patient experience and population study once can design vaccines and drugs for haemophilia patients but also due to pre-existing Nabs and pre-existing HCV or HBV infection, the general application of AAV gene therapy is currently limited. The possibility of gene editing for the repair of the mutation is on the horizon.*

Introduction

Haemophilia is a genetic bleeding disorder that has a long reigning history. References to blood related conditions similar to haemophilia date back to the Babylonian period where instances of children dying due to excessive bleeding after circumcision were recorded. Other similar instances have been recorded throughout history where mostly male children were affected by the effects of this disorder. It was not until 1803 that the disorder got its contemporary description given by Dr. John Otto, who described it as a bleeding disorder that can be inherited in which only male members of the family are affected through transmission from unaffected females (Otto *et al*., 2012). The word 'Haemophilia 'comes from the description given by the German physician, Johann Schölein and his student, Friedrich Hopff, who gave the description relating to fatal bleeding, referring to the disease as 'Hämophilie'. Haemophilia literally means 'affinity to blood', however, Schölein proffered to use the term 'haemorraphilia 'which means 'affinity to bleed 'and would serve as a better description of the disorder. This disease was famously dubbed as 'the disease of the kings 'or 'the royal disease 'as several members of the European royal family were known to be affected by it, including Queen Victoria's son Leopald who inherited it through his mother. As the disease started to become better known, its association with coagulation began to come to surface although the true cause was not revealed until quite recently (Schramm, 2014).

The current definition of haemophilia has significantly evolved from Otto's primitive description; however, the core understanding remains the same. Haemophilia is an inheritable bleeding disorder which is prevalent in males. Molecular studies of the disease have unveiled the underlying causes which contribute to the occurrence of haemophilia; the most pivotal being the mutations in the coagulation factor genes in human plasma. Haemophilia is thus divided into two types - Haemophilia A, demonstrating mutations in coagulation factor gene VIII and Haemophilia B with mutations in coagulation factor IX gene. Both these factors remain inactive until there is a haemostatic challenge and the coagulation cascade begins. Factor VIII is a cofactor with no enzyme activity required while factor IX is a serine protease and most definitely requires factor VIII as a cofactor. Once activated, factors VIII and IX for what is known as the tenase complex which in turn activates factor X. The stages of the cascade that follow result in the deposition of fibrin and the formation of the blood clot. The deficiency of either factor VIII or factor IX can impact the activation of factor X which will ultimately result in affecting the subsequent steps of the coagulation cascade, compromising fibrin deposits and resulting in the inability to form sufficient blood clots (Bowen, 2002; Puente *et al*., 2003).

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The genes for factor VIII is located on the X chromosome, near the end of the longer arm at the location Xq28 while for factor IX the gene is also located on the long arm of X chromosome, closer to the centromere area Xq27. They are recessive X-linked genes and are rarely present in females although the occurrence is possible if both the inherited genes are of a particular factor are mutated. Mutations that are found in factor VIIIx and IX can be point mutations, deletions, insertions and inversions of the gene. The most commonly found mutations for haemophilia are point mutations, observed in 90% of the mutations, with deletions being observed at second place, seen in 5-10% of the patients. Insertions and inversions are much rarer among haemophilia patients. These types of mutations can be further divided in their own aspect to reveal further trends that are prevalent in patients of haemophilia through molecular and genetic studies over the years (Belvini *et al*., 2005; Bowen, 2002).

Haemophilia as a bleeding disorder has existed for a considerable period of time. The disease has been understood and interpreted in several different ways by different civilizations throughout time. The oldest known treatment for haemophilia was whole blood transfusion in the year 1840. Meanwhile, during the Russian monarchy by Rasputin, hypnosis was a method introduced as a treatment for haemophilia. The true cause behind haemophilia was revealed in 1946 as plasma separation was made more available and the discovery of the deficiency of a particular plasma protein in haemophilic plasma was made (Aledort, 2007). These proteins, as is now known, are the coagulation factors that are responsible for coagulating the blood and halts excessive bleeding (Bowen, 2002). As the understanding of this genetic condition evolves with time, so do its methods of treatment. The medical world has moved on from primitive methods of treatment and is looking towards gene therapy as an answer for this complex genetic condition. This method looks more closely at the root of the problem with the mutations occurring in the coagulation factor genes and hopes to target them to find a cure. Thus, the future of haemophilia treatment through gene therapy looks to be quite promising (Nathwani *et al.*, 2004).

Vectors used for gene therapy

Gene therapy is currently most effective way of treating haemophilia. Gene therapy is the introduction of the functional genes to the target cells by the help of viruses as carriers (Nathwani *et al*., 2017). In case of haemophilia if the therapeutic genes able to produce clotting factor (FVIII or FIX) to threshold level 1% to normal, which has been considered as sufficient result for gene therapy. Gene therapy usually done through two ways, i) by introducing genes for clotting factor into the stem cells using vectors, ii) And alternative way of introducing genes into non-dividing cells of tissues and other liver cells. These genetic modifications are done by in-vivo and ex-vivo therapies. In-vivo therapy, it is the modifications of target cells and ex-vivo therapy are first isolation of target cells and then modification (Chuah *et al*., 2013).

In haemophilia gene therapy many types of vectors are used based on the variations and they vary in their transgene factor or configuration whether it's self-complementary or single stranded, also vary with their effecting promotors, enhancers, sequence optimization, and content of pyrimidine, purine and dinucleotide motifs. Variations in their configurations could alter transduction and expression potency (Gater *et al*., 2011).

Retroviral and lentiviral

First type of vectors is Retroviral and lentiviral vectors. γ-retroviral vectors are used to introduce only when cells are in dividing phase. Various clinical trials of gene therapy are done successfully using these vectors in mice, dogs and rabbits for treating both haemophilia A and B. Since it is effective only for dividing cells these vectors show transient expressions in humans for the treatment of haemophilia A. Lentiviral integrate into the target cells even in more dividing cells and resulted in continuous expressions of FIX in hepatocytes in liver. If these expressions are exposed to antigen presenting cells (APS) in liver this would increase the chance of producing immune responses against FVIII or FIX (VandenDriessche *et al.*, 1999). But using specific hepatocytes promoters would lower these chances and there is still possibility that cytotoxic T-cells eliminates these FVIII and FIX expressions To reduce these expressions hematopoietic specific target sequence of the micro RNA (miRNA) are incorporated into the vector. miRNA target sequences are complementary to the hematopoietic target sequence due to this APCS does not able to express antigen presenting T-cell response which results in prolonged expression of factors FVIII and FIX in hepatocytes (Matrai *et al*., 2011). Nathwani *et al*., (2004) performed experiments on immunocompetent mice by introducing copies of a microRNA target sequence into vector and this results in expression of these miRNA in targeted cells which allow them to mark specific transgene vectors and caused the destruction of transgene vectors in cells (Brown *et al*, 2006). This selective suppression strategy would help in down regulations of transgene expression in HPSC. These type of engineered vectors shows more strong and stable expression and decreased immunogenicity in mice.

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Both these vectors are also used in ex-vivo in hematopoietic stem cells (HSCS). By gene therapy HSCS produce their cells such as erythrocytes, megakaryocytes and platelets that results in the production of clotting factor in blood so these cells are targeting to treat haemophilia. Evan and Morgan performed experiments to treat haemophilia A in mice by taking bone marrow cells and transducer them with FVIII containing γ-retroviral as vectors and experiment results in the prolonged correction of haemophilia (Matrai *et al*., 2010).

Adeno-Associated vectors

Adeno-Associated vectors (AAV) being the most used vectors for treating haemophilia among all of them because of their nonpathogenic and defective replication characteristics. Enhancement in transgene expression levels were done by using liver-specific promoters and portal vein administration. Recombinant AAV vectors are modified genetically by removing coding sequence of AAV and adding gene of interest with suitable promoter. So this rAAV contains only transgene sequence and proteins guiding them to target cells. rAAV were first used in blood disease transfer trials, since then many AAV serotypes are known to be used and are differentiated by their tissue specific reaction (Gao *et al*., 2002). Current blood disease clinical trials hold the utilization of present serotypes AAV5, AAV6, AAV8 and bioengineered AAV-rh10, AAV-Spark100, AAV-Spark200 AAV serotypes.

Past five years experimentation has proved that rAAV vectors shows promising results for treating haemophilia into a therapeutic reality. AAV-mediated gene therapy for haemophilia B was 1st targeted at muscles that doesn't form FVIII or FIX. These muscle cells which are capable of post-translationally modification of macromolecule to supply a practical transgene product. However protein cleavage and glycosylation modifications don't seem to be as effective as in hepatocytes. Herzog *et al*., transferred genes into the muscle directed cell in haemophilia dogs which results in stable FIX expression. These dogs also had a mis-sense mutation within the FIX gene sequence, so gene transfer with constant vector in dogs carrying associate FIX null mutation results in the formation of high-titer inhibitors. This shows that the mutations that occurs within the FIX gene sequence control the formation of inhibitors (Hargoz *et al*., 1999)

The liver is also a possible way of partial and complete phenotypic correction of haemophilia using AAV mediated gene therapy (Arruda *et al*., 2010). Transducing hepatocytes ends up in T-cells production, inducing immune tolerance to the FIX antigen (Mingozzi *et al*., 2003). Stronger FIX transgene factor are made to reduce the dose of vectors. They is done by the use is codon-optimised FIX, stronger promoter/enhancer elements, or self-complementary, double-stranded AAV vectors (scAAVs) (MacCarty *et al*., 2003). These engineered scAAV vector beats one of the transition limiting step in AAV, This reduction of dose can also achieved by using of other AAV serotypes, such as. AAV8, which will reduce the T-cell activation, increase transduction of hepatocyte and improve vector transport (Vandenberghe *et al*., 2006). In mice, gene trials using lentiviral vector and AAV8 or AAV9. AAV vectors are more efficient and also decrease the chances of inflammation in liver-directed gene therapy (Cantore *et al*., 2007).

Exceeding studies of haemophilia has shown that the vector particles will evade phosphorylation and ubiquitination once their surface exposed residues of tyrosine are mutated (Zhong *et al*., 2008). This will ends in preventing the degradation of proteasome, resulting in a 10 times higher FIX expression level. Since FVIII transgene factor is larger than the FIX factor, and also the AAV vector have packaging limitations, it is absolutely not unattainable for AAV vector to treat classical haemophilia. To get better of this limitation, a BDD kind of FVIII along with small promoters are being used (Jiang *et al*., 2006). With higher vector doses, prolonged FVIII expression can be achieved when gene therapy of AAV vector-mediated factor transfer of genes are performed in mice and dogs, establishing a proof-of-concept that haemophilia A factor medical aid with AAV is probably possible.

Clinical studies

Basic issue of clinical program is GT products that are similar biological products. Their advance trials shouldn't estimate only safety but Also bioactivity preliminary efficiency it is produced through (Guidance of industry, 2020)

Efficacy endpoint

Approval was not based on factor activity level. If we look scientific basis they have ability to use factor activity level to support traditional approval. When use methodology of conventional factors for plasma that is treated for patients of GT products and compassion that estimate levels show control of recombinant and derived plasma counterparts. Other factors are lack of molecular characterization protein that translates differently in vivo to recombinant that concentrate produce products in vitro. When we use genetic engineering increase the coagulation activity of protein that proved sponsor seeking which based on factors level of activity that give evidence and correlates factor levels with related clinical outcome the production of valid evidence between activity and clinical help for example evidence of multiple GT products can be generalized.

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Design study

For clinical study sponsor that consider the pre and post-administration directions. Patient enrolled that are not required for dose judgement that replace the prophylactic therapy reduce bleeding events before 6 to 12 months. This method provides stability of dose efficacy assessment which is followed by GT products administration while keeping under 6 months observing subject to collect data (Guidance of industry, 1998).

Statistical consideration

To help BLA for customary endorsement, we suggest a non- inadequacy clinical preliminary plan with ABR as the essential viability endpoint, utilizing inside subject correlation. We suggest that you build up a NI for looking the ABR GT investigational item to the current prophylaxis treatment inside correlation preliminary. Theory tests are certainty stretch to oblige the matched idea of ABRs when GT for similar subject. Descriptive measurements, including graphical shows like histograms and scatter plots of ABRs can add important data to treat the effects. They include subject examination configuration gives additional benefits for the treatment impacts for investigational items that control factors likewise impact draining results. Data gives statistical or clinical contemplations of non- mediocrity prelaminar describe in FDA direction (in-vitro companion 2014)

Patient expérience

Patient experience gives significant extra data to the clinical advantages of GT items. Include item advancement Sponsor urge FDA gather patient information experience and submit information in BLA. We plan to assess the advantage hazard profile of the investigational item with regards treatment scene time of our review an advertising uses (Non inferiority clinical 2016)

Population study

Product of antibodies is used for blocking coagulation factor gene to target its therapeutic potential. Therefore choose sponsor to eliminate patients for pre-existing GT product antibodies. Sponsor should consider strongly development of companion diagnostic to find antibodies of GT product. In vitro helper needed to diagnose appropriately patient select to study the submission of marketing of submission of BLA for GT product and companion diagnostic that coordinate to support marketing authorization. Development of clinical include studies of pre-existing antibodies for the safety and efficacy products. Both children and adults of haemophilia act as pediatric medicine that is critical for drug development. Investigation of clinical are involve greater than minimal risk for the chance of benefit of individual subjects which is generalizable yield for knowledge of disorders. Establishment of applicable adequate made to obtain permission of parents or assent of child.

Future prospects of gene therapy

The assortment of AAV serotypes, the range of vector volumes, and the various production processes make products very complex. Nonetheless, the standard thread between these tests is the following: patients under the age of 18 years old enough; patients with less than 30-50 days of exposure to FVIII / FIX; fundamental liver sickness or severe contamination of hepatitis B or C (HBV/HCV); the presence of NAbs in the AAV serotype higher than a cutoff; and earlier history of inhibitors in FVIII or FIX. Gene therapy is not yet intended for use in all people with haemophilia. Quite, in the United States (US), in the same way as other different nations, the high predominance of hepatitis C in patients with haemophilia beyond 35 years old (2018) implies that more than 35% of grown-up patients who may benefit by gene therapy will not be allowed in these liver-guided methods. The ability to treat HCV with new antivirals will remain unclear at this time (Mazepa *et al*., 2016)

The capacity to alleviate existing NAbs in AAV will extend the number of eligible people as approximately 30–70% of patients have NAbs in particular serotypes (Calcedo *et al*., 2011). However, attempts to remove existing NAbs using immunosuppression plasmapheresis, insertion of empty capsid, novel bioengineered capsids, or localized vector infusion have recently been reduced (Corti *et al*., 2014). Finally, patients with more than 30% of HA and patients with 5% HB make inhibitors of the protein, (Miller *et al.,* 2012) which decrease the achievement of hemostasis with protein. Immune tolerance induction (ITI) is the only known treatment strategy for inhibitors, with moderate success (~60%) in patients (Hay *and* DiMichele, 2012). The aim of ITI is to permit the use of protein FVIII/FIX in therapeutic changes as it achieves more hemostasis than other bypassing agents (BPAs) (Miller *et al.,* 2012).While ITI is more expensive than BPA lifelong treatment for hemostasis, it comes with a significant economic burden (~ $ 1 million per 20 kg patient) and demands, as evidenced by approximately 20% of patients withdrawing from International ITI (ITI) study (Crudele *et al.,* 2015). There is growing evidence of HA / HB dogs and mice that AAV-induced genetic therapy in immune models is a strengthening of the immune system and the effectiveness of this nation can bring about positive

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improvements in the health of these patients (Nakade *et al.*, 2014). To overcome these hurdles should broaden the availability of gene therapy for the large number of patients with haemophilia.

Genome editing

The ideal gene treatment would fix the DNA level of the defective gene. With progress in genome editing growing substantially, future uses of innovation are expected. As ethical and security issues cannot be accepted for germline genome editing, genome editing may be conducted at embryonic level. Genome editing components should be provided to somatic cells for the treatment of genetic diseases (Hay *et al.*, 2006).

Genome editing engineered nucleases

These include Zinc-finger nucleases of the first generation (ZFNs), TaL effector nucleases of the second generation (TALENs), and CRISPR-associated protein (Cas) 9, in the third generation. ZFN and TALEN feature a FokI enzyme, which makes the dimmer to cut DNA, while Cas9 protein interfaces and cleavages a DNA site known as RNA guidelines (gRNA). The immunological adaptive mechanism against prokaryote phage infection has been distinguished by CRISPR/Cas9. Only by modifying the gRNA sequence can it produce DSB at arbitrary sites of DNA , and used to modify the genome in various sectors (Nakade *et al.*, 2014).

DSBs Modification Mechanism

The DNA repair mechanism is used for genome editing. There are 2 DNA repair mechanisms: 1)Non-homologous end joining (NHEJ) 2) Homologous-directed repair (HDR). The major DSB repair process is NHEJ, including insertions and mutations at the DSB site, which lead to gene expression interruption. HDR performs in the S/G2 cycle phase, and may be used as a template to repair (or alter) DNA. Due to the exceedingly poor efficacy of DNA alteration via HDR, the knock-in approach has been created for the effective introduction of a target gene into the DSB site (Suzuki *et al.*, 2016). In addition, a technology homology-independent targeted integration was also created to ensure the knock-in direction by integrating the Cas9 sequence at both ends in opposing orientations. This is a significant way to modify the genome for the treatment of genetic diseases (Charlesworth *et al.*, 2019).

Conclusion

Clinical trials of treating haemophilia by gene therapy gave promising results by producing sustainable level of both FVIII and FIX factors by using different types of vectors. Difference in these results maybe occurs due to different vectors and dose quantity. Regardless of all these differences recent clinical advances in treatment allows to consider the future of haemophilia and how this therapy work. Several obstacles are still on the way to gene therapy but by successfully passing all these it may be possible that licensed gene therapy product for haemophilia will be available in market in next few years. So it is important to examine all the possible gene therapies modifications in hope of developing new treatments for treating both haemophilia A and B.

Conflict of Interest

The authors declare absence of conflict of interest.

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