Project On

A review on the emergence of adeno-associated virus (AAV) for the management of Rett syndrome with improved safety and Efficacy.

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Faculty of Allied Health Sciences,

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APPROVAL

The Department of Pharmacy, Faculty of Allied Health Sciences at Daffodil International University has approved the project entitled "A review on the emergence of adeno-associated virus (AAV) for the treatment of Rett syndrome with improved safety and efficacy" as satisfactory for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy. The style and contents of the project have also been approved.

BOARD OF EXAMINERS

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DECLARATION

I declare that I am conducting this thesis study under the guidance of **Mr. Galib Muhammad Abrar Ishtiaque**, Lecturer, Department of Pharmacy, Faculty of Allied Health Sciences at Daffodil International University, in impartial compliance with the requirements for the Bachelor of Pharmacy degree. The present project is entirely my original work, and I affirm that neither the project nor any part of it has been submitted elsewhere for a Bachelor award or any other degree.

Supervised by

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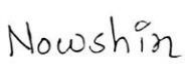

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ABSTRACT

Rett Syndrome (RTT) is a rare neuronal disorder caused by alteration of MECP2 gene. There is currently no cure for RTT, but gene therapy with adeno-associated virus (AAV) vectors holds great promise as a potential treatment option. AAV vectors have shown excellent safety and efficacy profiles in preclinical studies and clinical trials for a variety of diseases. However, systemic delivery of AAV vectors to achieve widespread gene transfer to the brain is challenging due to the limited ability of AAV to cross the bloodbrain barrier and the potential for peripheral toxicity. In this context, the development of AAV vectors with improved brain penetrance and sub-toxic levels of transgene expression is critical. Several modifications to AAV vectors have been tested in preclinical studies for RTT, including the use of alternative promoters, 3' UTRs, and capsids, resulting in improved control of MeCP2 expression and reduced peripheral toxicity. Further studies are needed to determine the optimal AAV vector design and delivery strategy for RTT gene therapy. Nonetheless, AAV gene therapy holds great promise for the treatment of RTT, and ongoing research in this field may pave the way for a much-needed cure for this debilitating disorder.

Keywords: Rett syndrome, MeCP2 gene, Mutation, Gene therapy, Adeno-associated virus.

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Introduction

1.1. RETT Syndrome and It's History:

Two little girls with significant ailment were observed recurrently rubbing their hands while sitting in the knees of their mothers in an Austria medical clinic waiting area. This surprising occurrence influenced Dr. Andreas Rett to carry out additional investigation and look for additional individual's activity and behavior. After one year of 1965, Dr. Rett discovered same cases in twenty two individuals and discovered a identical illness that has since been named after him, Rett syndrome (Pearl & Percy, 1988).

After seventeen years later, it was published in an English literature by which it was introduced with the medical science. That time a Swedish neurologist Dr. Bengt Hagberg with his fellow mate published thirty five cases about this diseases (Hagberg et al., 1983). It was such a situation same as to the previously founded in the German literature by Rett. Alongside in 1980 there were a lot of advanced research done about the process by which methyl group added into DNA molecule. By which, the first relation was build up between this process and genetic changes in gene transcription and translation process.

This diseases originate by inactivating alteration of transcription regulator methyl-CpG binding protein 2 (MECP2) and is a neurodevelopmental disorder with X-linked inheritance (Chahil & Bollu, 2018; Mnatzakanian et al., 2004; Van den Veyver & Zoghbi, 2002)

Occurring in one in ten thousands females, Rett syndrome (RTT) is a rare disorder (Haas, 1988; Rett, 1966), where normal development of neurology and physiology is occurred after but symptoms becoming evident at the age of six to eighteen months (Hagberg, 2002). The progression of indications occurs in different phages including inactiveness, fast reverting and loss of mobility. During the inactiveness stage, walking, running or other movement activity, delicate activity are lately developed, and leading to a delayed diagnosis. The fast reverting stage is noticed by the loss of learned skills like pronunciation, movement of hand and also motor activity, breathing abnormalities, autistic-like features. The stationary period is noticed by an increase in development and intelligence problems, seizures but may see improvement in communication skills. Finally, at the last phage many types of physical disability are shown and some patients also need to depend on other or need support for their normal activity. Regardless of the stage of the disease, some patients with Rett syndrome (RTT) also experience various health issues. These include GI related problems (Motil et al., 2012), complication in heart and lung (Kumar et al., 2017), BMD or bone mass (Shapiro et al., 2010), osteopenia (Haas et al., 1997), occlusal neurosis (Alpoz et al., 1999), dyslipidemia (Justice et al., 2013; Segatto et al., 2014), acute cholecystitis, scoliosis (Leonard et al., 2014), urinary tract problems (Ward et al., 2016), and insomnia (Shahbazian, Young, et al., 2002). Furthermore, patients with this disease are at a higher risk of sudden death, infection in respiratory, less stabile cardiac condition, and failure in respiratory system (Laurvick et al., 2006; Leonard et al., 2014).

Currently the most commonly used viral vectors for gene therapy are recombinant adeno-associated viral (AAV) vectors (Eichler et al., 2017). Over the other types of vectors AAV vector offer more advantages due to their ability to avoid causing any known pathology. Additionally, it's contagiosity rate of cells and tissues are almost twenty five nm which is very high, which facilitates pervasion into tissues, many natural groups of species with distinctive surface structure and also many modified groups that encode coat proteins of virus, are not duplicating and have a less DNA insert into the host. Moreover, AAV vectors have relatively low immunogenicity (Hastie & Samulski, 2015).

Recombinant AAV vectors, the most commonly used viral vectors for gene therapy, can be classified into two kind of: single-stranded vectors (ssAAV) and self-complementary vectors (scAAV). While ssAAV vectors can carry larger DNA fragments of up to 4.6 kilobases, scAAV vectors have a smaller capacity of up to 2.4 kilobases but are effective to express the foreign gene or DNA (Hastie & Samulski, 2015). However, in case of all adeno associated virus vectors, it is important to note that the size limit must include not only the coding sequence but also the regulatory elements and ITR required (Gholizadeh et al., 2014; Lykken et al., 2018).

Recombinant AAV vectors are stuffed into virus particles and then purified utilizing iodixanol-based ion exchange chromatography, HPLC and density gradient centrifugation. The viral particles enter the cell after they link to the receptor protein of AAV and begin to make RNA copy and make protein using the genetic information of the foreign DNA. It is important to select the appropriate AAV serotype for the therapy, as each serotype has different tissue and cellular preferences due to their specific receptor proteins (Hocquemiller et al., 2016; Lykken et al., 2018).

Recombinant AAV vectors with a serotype 9 capsid called AAV9 vectors are particularly promising due to their high transduction efficiency in the central nervous system (CNS) following systemic administration (Foust et al., 2009a). AAV9 vectors are especially attractive for RTT gene therapy as they can efficiently infect neurons and mediate long-term transgene expression, even crossing the bloodbrain barrier (Arruda et al., 2005; Herzog et al., 1997; Jiang et al., 2006). Furthermore, AAV9-derived variants, such as PHP.B and PHP.eB, have also demonstrated the ability to cross the blood-brain barrier and transduce neurons, making them suitable for gene replacement therapy in mouse models of CNS disorders (Gray, Matagne, et al., 2011; Saraiva et al., 2016).

1.2. Diagnostic cues of Rett syndrome:

Rett syndrome (RTT) is a continuous intellectual disabilities by which mainly girls are affected at age of six to eight months and manifests in stages (fig 1) with not all symptoms being prominent initially (Amano et al., 2000). The diagnosis of classical Rett syndrome requires the presence of certain obligatory manifestations, including (i) normal appearance during infancy, (ii) slow-down of head growth between

three months to four years, (iii) loss of lustful hand skills between 9 months and 2.5 years, communicative dysfunction, psychic dysfunction, and mumbling, (iv) purposeless, repetitive movements of hand such as clapping/tapping at the age of one to three, and (v) posture dyspraxia with jerky truncal "ataxia" between 2-4 years (Hagberg, 2002). An important norm to diagnosis this diseases are mislaying of previously learned skills or purposeful uses finger or hand, speaking problem, mumbling, tend to be alone from society usually occurring between the ages of 1-2 years.

Additionally, there are supportive clinical manifestations of Rett syndrome, including aordinary hand movements. These consist of nearly constant and revolved hand movement like wringing, twisting, and clapping, which are characteristic of this disorder. Most of the girls have serotypic hand movements, although some have simultaneous rubbing movements of thumb and index fingers against each other. Every patients builds her own monotonously revolved hand stratagem.

Intense communication by eye is an eminent trait in most of patients, where they stare intensely to obtain eye contact or express their wishes. This behavior typically appears after the regression period and becomes further strengthened in schoolgirls and adolescents. Some RS females have their own "eye pointing" sign rather than communication by speech or language. For rehabilitation programs, inventive specialists have developed practical eye communication techniques.

Another peculiar feature of RS is disorganized breathing disturbances, obstructive apnea can be seen for thirty to forty seconds. Recently episodic hyperventilation in RS has been investigated (Julu et al., 2001; Kerr & Julu, 1999).

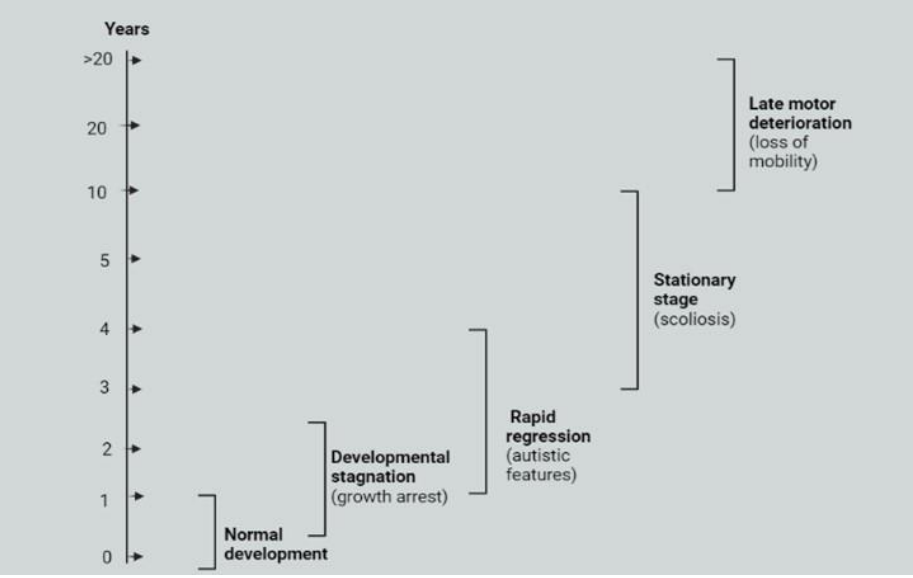

Figure 1: Clinical stages of Rett syndrome.

1.3. Genetic aspects for Rett syndrome:

The mecp2 gene, located on xq28, is responsible for 96% of typical rett cases and 74% of typical rett cases (Ehrhart et al., 2018). This gene found in all human cell and is plentiful among neuron and oligodendrocytes in the brain. The protein is expressed at low levels prenatally and increases during neuronal maturation and synaptogenesis, suggesting its role in neuronal activity and plasticity (Cohen et al., 2003; Jung et al., 2003; Samaco et al., 2004). Although the exact mechanism by which loss of MECP2 causes the clinical features of RTT remains unclear, it is believed that abnormal cortical glutamatergic synaptic responses and excitatory connectivity result in an excess of inhibition and deficits in neuronal plasticity. Most MECP2 mutations occur de novo, with over 98% of cases being sporadic and less than 2% being inherited from one parent (Burd et al., 1991). Inherited mutations leading to familial cases of RTT typically originate from healthy or mildly affected mothers or from gonadal mosaicism (Matijevic et al., 2009). Thus, most patients have a heterozygous state for the MECP2 mutation, with one copy of the gene being normal and the other being mutated (Kyle et al., 2018; Neul et al., 2008).The majority of de novo mutations, which cause RTT, have been found almost solely in the paternal gamete (Trappe et al., 2001). In 800 mutation cases, almost all parts of the MECP2 gene have been found to have: point mutations, insertions, duplications, small or large deletions (Ehrhart et al., 2018). The majority of these causative mutations have been identified in eight single nucleotide polymorphism hotspots as missense and nonsense mutations, namely T158m, R255x, R168x, R306c, R294x, R270x, and R133c which are responsible for almost 74% of all mutations, whereas, R168x being the most frequent one C-terminal deletions responsible for 9%, and large deletions account for another 6% (Kyle et al., 2018; Neul et al., 2008; Percy et al., 2010; Trappe et al., 2001).

1.4. Function of MECP2 gene:

It is suggested that mecp2 has an effective role in early after birth development and less during embryonic stages. MeCP2 encodes a protein known as Mecp2, which has a crucial role in regulating gene expression by modifying neuclesome - the DNA and protein complex that packages DNA into chromosomes. MeCP2 belongs to the methyl-CpG binding protein family (Hendrich & Bird, 1998) and comprises of three domains, namely the methyl binding domain (mbd), transcriptional repression domain (trd), and a c-terminal domain, along with two nuclear localization signals (NLS). The MBD binds distinctively to methylated CpG dinucleotides (Klose et al., 2005). Unmethylated four-way DNA junctions also binded by MBD with similar affinity (Galvao & Thomas, 2005), interact with high decree chromatin. The downstream TRD contributes in the mechanism of precise control of gene expression by hiring transcriptional regulators and CRCs. MeCP2 relies on the nucleosomal core and exposed DNA to connect to them. This section contains conserved metamorphic poly-proline sequences that interact with group II WW domain splicing factors to create linkages (Buschdorf & Strätling, 2004). Though its function in

MeCP2 is critical, the C-terminal region is little understood. This is clear from deletions of this section that result in RTT and from a mouse model lacking the C-terminus that displays a number of RTT symptoms.(Shahbazian, Young, et al., 2002).

During human development, the processes of neuronal maturation and synaptogenesis take place as early as embryonic weeks 12 and 20, gradually (Marsh et al., 2008). The absence of MeCP2 expression within this time frame could be the cause of reduced brain size and neuronal function in individuals with RTT. Disordering in MeCP2 function may impede proper maturity of neuron and formation of synapses and leading to unusual growth of central nervous system (Armstrong, 2002).

In rodent development, MeCP2 expression is first observed in the spinal cord and brainstem around day E12. During embryonic neurogenesis, mRNA for MeCP2 is found in subcortical regions (Jung et al., 2003; Shahbazian, Antalffy, et al., 2002). Reaction against immune response in MeCP2 is detected in the thalamus, caudate putamen, cerebellum, hypothalamus, and hippocampus beginning at days E14–16. MeCP2 expression in the cerebral cortex starts at embryonic day 14 and is initially limited to deeper cortical layers before eventually spreading to more superficial layers by E18. MeCP2 protein levels increase throughout cellular differentiation. That's why in neuron MeCP2 express high after birth till become adult (Shahbazian, Antalffy, et al., 2002).

1.5. MECP2 gene Alteration:

The first disorder to be linked with defects in a protein involved with the addition of methyl group with DNA and packed chromatin, is Rett syndrome. Numerous studies, totaling more than a dozen, have been published to date, which have identified mutations in the MECP2 gene in individuals with Rett syndrome. The data below provides a summary of the results from these studies (Amano et al., 2000; Amir et al., 1999; Bienvenu et al., 2000; Cheadle et al., 2000; De Bona et al., 2000; Hampson et al., 2000; Huppke et al., 2000; Lam et al., 2000; Obata et al., 2000; Wan et al., 1999; Xiang et al., 2000).

Point mutations (Table 1)

Several types of mutations, including missense or protein truncating mutations, mainly involving C→T transitions, have been identified in more than 70 cases related to the MECP2 gene. However, despite extensive efforts, alteration wasn't found in that gene around twenty to twenty five patients with this diseases. This could be because the methodology used is not capable of detecting large deletions or intragenic inversions. Although some studies utilizing FISH and Southern blotting have attempted to detect such mutations, they have not been successful thus far (Fan et al., 1999).

The extremely large 3'UTR region had not been thoroughly investigated. While the majority of detected mutations were de novo, eight were relatively frequent mutations that responsible for approximately 60% of cases in female with this diseases (fig 2). All eight of these mutations were c→t transitions. In addition,

small deletions, primarily in the C terminal region, responsible for 10% of mutations. This was thought to be due to the presence of a series of ccacc repeats located at the 3' end of exon 4. Within the region between the MBD and TRD, there had been 41 reported mutations where, 38 of these mutations were the result of a single mutation R167x (501c>T), which was the most commonly detected mutation in Rett syndrome. Rest of three mutations were consist of two missense and one nonsense mutation. Again, seventeen several alteration was found in MBD, 11 had found, while 3 hotspots exist: R116w (316c>T) for 14 times, R132c (396c>T) for 11 times, and T157m (473C>T) for 22 times. But the mutations different in the TRD region, where nonsense mutations leading to truncated proteins tend to be more prevalent. However, missense mutations become more common again. There were 3 hotspots for curtail mutations:
R254x (762c>T) for 24 times, R271x (807c>T) for 23 times, and R293x (881c>T) for 23 times.

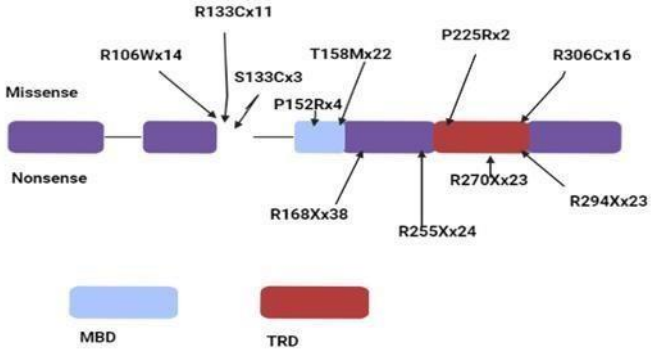

Figure 2: Hotspot of alteration of MECP2 gene in RETT syndrome in girls.

All three of these mutations result in the loss of a highly conserved arginine codon and all were caused by a C>T transition. The MECP2 gene had a very high mutability of CpG dinucleotides, which could explain why arginine codons were targeted for mutation. However, it was not yet clear whether the absence of reported mutations at position 268 indicates selectivity in arginine codon or whether mutations of R268 were lethal. In the TRD, the most frequent missense mutation is R306C (916C>T), which had been observed 16 times and also results in the loss of an arginine codon caused by a C>T transition. The same arginine codon through R306H (917G>A) had lost and reported for two times. To summarize, a total of 208 point mutations have been reported, with 33 being distinct. Out of these substitutions, 173 (83%) are C>T, and 142 (68%) affect an arginine codon (table 2). This leads wrong amino acid in protein chain or curtail protein chain. It's worth noting that only 33 out of 486 amino acids, or 7%, are arginine in the normal protein (Bienvenu et al., 2000).

	Point mutation in MBD	Point mutation in TBD
Total different	17	13
Missense	16	8
Nonsense	1	5
Total number	71	96
Missense	69	26
Nonsense	2	70
Loss of cytosine	65/71 (92%)	93/96 (97%)
C>T	48/71 (68%)	86/96 (90%)

Table 1: Mutations of the MECP2 gene in girls with Rett syndrome

Region of the gene	Different mutations	Total mutations
MBD	4/17 (24%)	27/71 (38%)
MBD-TRD	3/4	40/41 (98%)
TRD	5/12 (42%)	85/96 (89%)
Total	12/33 (36%)	142/208 (68%)

Table 2: The loss of specific protein genoms in the MECP2 gene

1.6. Current treatment practice for RETT syndrome:

A coordinated multidisciplinary approach to medical care and management is the preferred option in Rett syndrome as it is a multi-systemic disease. Intensive early intervention, as applied to other neurodevelopmental disorders, is recommended shortly after the diagnosis at an early age (Warren et al., 2011). Symptomatic medical management is the current standard for Rett syndrome, as there are no specific treatments available. Along with traditional drug treatments (such as antiepileptic drugs for seizures and selective serotonin reuptake inhibitors for anxiety), preventive approaches such as nutritional management (emphasizing caloric intake and vitamin D levels), prevention of gastrointestinal and orthopaedic complications, and personalized rehabilitation therapies are becoming increasingly recognized as standards of care (Ward et al., 2016).

First and only one medicine for RTT syndrome:

On March 10, 2023, the FDA approved Daybue (trofinetide) as the first oral solution containing a synthetic analog of the amino terminal tripeptide of IGF-1. Studies have shown that treatment with IGF1 can improve disease symptoms (Bienvenu et al., 2000; Burd et al., 1991; Laurvick et al., 2006; Tropea et al., 2009). Trofinetide is thought to be effective in treating rett by correcting abnormal neuronal and glial function as a result of its anti-inflammatory and trophic characteristics. It can also inhibit abnormal increase in the number of astrocyte, stop aberrant microglial activation, normal protein production in synap, shape of dendron and signal of neuron, among other things. Trofinetide can also increase the antioxidant response in people with rett (Derecki et al., 2012; Feng et al., 2014; Ross et al., 2016). In a phase two study conducted on adolescent or adult, Daybue showed effective, safe, tolerable at a dose of seventy milligram per kg administered for two times in day for twenty eight days (Neul et al., 2008).

Gene therapy-AAV for RTT syndrome:

The use of vectors in the treatment of neurodevelopmental disorders presents a significant opportunity. A modified virus containing the correct version of the MECP2 gene can be used to target the central nervous system (CNS) with minimally invasive delivery, while specifically targeting the appropriate cell type(s) in the target tissue(s) for lifelong treatment following a single low dose. However, the complexity of the CNS poses many obstacles to achieving the ideal adeno-associated virus (AAV) gene therapy, including the blood-brain barrier (BBB), invasiveness of delivery, and adequate viral spread from the delivery site (A Kotterman & Schaffer, 2015; Castle et al., 2016; Mastakov et al., 2002; Ojala et al., 2015; Rosenberg et al., 2014).

In the early studies of CNS, first-generation of adeno associated virus namely AAV2 and secondgeneration namely AAV5 or AAV8 vectors were used (Aschauer et al., 2013; Burger et al., 2004; Taymans et al., 2007; Watakabe et al., 2015). But nowadays, a third-generation vector, AAV9, has been found to distribute widely in the brain and spinal cord, targeting both neurons and astrocytes (Cearley & Wolfe, 2006). AAV9 can cross the BBB following intravenous injection, making it a minimally invasive treatment option (Cearley et al., 2008; Foust et al., 2009b; Gray et al., 2013). Additionally, AAV9 distribution is superior to other serotypes when injected intra-cranially or intrathecally (Cearley et al., 2008; Cearley & Wolfe, 2006; Foust et al., 2009b), allowing for overall lower dosing. AAV9's ability to undergo axonal transport has been suggested as one reason for its broad distribution. AAV9 has demonstrated promising results in rodent research models and non-human primates, making it the gold standard for AAV-mediated gene therapy in the CNS and in treating RTT syndrome (Girod et al., 1999; Rabinowitz et al., 1999).

AAV has a single-stranded (ss) DNA genome of 4.7 kb, which can be modified by replacing up to 4.4 kb in foreign DNA of human. Self-complementary (sc) AAV vectors are able to infects host cells 10100

times higher, but they have a packaging capacity of only 2.2 kb, which makes it difficult to deliver larger gene (McCarty et al., 2001). In neonatal Mecp2-null mice, injecting AAV9-MECP2 under the promoter of chicken β-actin resulted in varying transduction efficiencies across cerebrum, ranging from seven to forty two percent cell are infected, along with hypothalamus exhibiting the maximum of infection and striatum showing the lowest (Gadalla et al., 2013a). In male mice this low infection efficiency increased the length of life time almost sixteen weeks and enhance the loss movement activity, but it had no impact on lung disorders. In comparison, when AAV9 which are selfcomplementary along with MeCP2 gene was administered systemically using a shortened Mecp2 promoter, the brain's transduction efficiency was very low at 2-4%. Nevertheless, mice lived for fifteen weeks, indicating that even modest MECP2 reexpression in the brain and/or non-CNS tissues could improve lifespan. AAV9 mainly targeted the liver and spleen, with some cells receiving ten copies of the vector, causing liver damage. Thus, future studies should aim to enhance the selectivity of gene therapy with AAV9 prior to proceeding the clinical trials. In a follow-up investigation, scAAV9MECP2 was delivered systemically using a shortened Mecp2 promoter. The transduction efficiencies in the brain were diverse, with the encephalon having ten percent, while the cerebral and medulla oblongata had twenty five percent (Garg et al., 2013). While this resulted in some enhancements of traits, it failed to alleviate the dyspnoea. To reduce liver expression, a secondgeneration AAV9 vector was created with a modified 3′UTR and a panel of miRNA binding sites (Gadalla et al., 2017). The lifetime of Mecp2 mutant mice was increased when the vector is administered by injection into the posterior cerebellomedullary cistern, but behavioral improvements were not observed unless the vector was used in high dosages. But, administering the vector directly into the ventricular space of brain of newborn Mecp2-null mice led to improved Rett-like phenotypes, increase longevity, and high brain transduction efficiency. The significance of endogenous regulatory components in the gene expression cassette is emphasized by these findings. (Gadalla et al., 2017).

In a recent study, it was discovered that intracranial injection of MECP2 protein containing only the domain that bind with methyl and the domain interact with NCOR increased traits and longevity in neonatal mice (Tillotson et al., 2017). According to these results, MECP2's main function is to link DNA to the complex that contains NCOR. So, if this type of MECP2 protein is used in gene therapy, Mecp2 expression can be more precisely controlled by adding extra regulatory sequences to scAAV9 vectors. But to maximize the therapeutic utility of gene therapy in patients, more researches should take into account including regulatory components of vector and managing the timing of treatment. Again, as studies in mice may not be able to provide accurate data on dosages, determining the right dosage for patients presents an additional challenge for gene therapy. As a result, gene therapy treatments for neurological illnesses are being tested in alternative animal models like dogs and non-human primates. Non-human primates are a useful model for researching gene therapy for neurological illnesses because they resemble humans in terms of physiology, behavioral traits, network among neuron (Gopinath et al., 2015). But using

larger animal models for research is more expensive also necessitates a larger research time. However, it is noteworthy that MECP2-deficient primate models have been made and may be conducted to accelerate RTT gene therapy treatment (Chen et al., 2017; Liu et al., 2014). The necessity of measure of dosage was recently brought to light by the finding that systemic delivery of high-dose AAV9 caused significant hepatic and brain damage in 3 non-human primates (Hinderer et al., 2018).

Intendent of my studies

RETT syndrome is a rare neurological disorder due to genetical changes mostly in the girls and rarely in boys. Still now there is no cureness of this disease. Gene therapy by AAV may be the best way in the treatment RTT syndrome though it is on trial till now.

My study of this paper will focus on,

- The history, clinical features of RTT syndrome.
- Genetic basis and the mutation of the responsible gene.
- Gene therapy trials on mouse model along with the dose and safety issues.

Methodology

By reviewing approximately 27 papers the study this paper has made. The papers were collected through google scholar, PubMed, Sciencedirect. For the figure Biorender was used.

All the information gathered here were assorted and checked to be accurate. Papers used for this study were collected from 1997 to 2023.

Result and Discussion

4.1. Dose Expand with AAV by Systemic Route Lead Narrow Therapeutic Window

In this paper, the correlation between vector dose and therapeutic benefits is being reviewed, where a polypeptide protein derived from c-myc with human methyl CpG binding protein 2 in complementery DNA delivered by scAAV2/9 vector under the control of a 229-bp in murine region Mecp2 endogenous core promoter (MeP229) (Gadalla et al., 2013b; Gray, Foti, et al., 2011), which will be named as "firstgeneration vector".

Juvenile male $Mecp2^{-/y}$ and wild-type (WT) mice were injected with either vehicle or the firstgeneration vector at the age of four to five weeks in the tail vein, at doses of $1*10^{11}$ (low dose), $1*10^{12}$ (moderate dose), or $1*10^{13}$ (high dose) viral genomes (vg) per mouse (vg/kg).

As observed in prior to researches on this knockout animal (Guy et al., 2001, 2007; Shahbazian, Antalffy, et al., 2002) $Mecp2^{-/y}$ mice treated with vehicle alone displayed signs of RTT-like symptoms (Guy et al., 2007) from 4 to 5 weeks of age, with increasing severity leading to death or censoring of all mice by 20 weeks of age. $Mecp2^{-/y}$ mice treated with the low dose of the first-generation vector showed no significant difference in survival (median survival = 9.36 weeks versus 11.64 weeks, respectively; $p = 0.2$, MantelCox test) or severity scores compared to the vehicle-treated $Mecp2^{-/y}$ mice. The treated $Mecp2^{-/y}$ animals had a noticeably greater average body weight than the untreated $Mecp2^{-/y}$ mice after eleven weeks, which is the norm survival duration for untreated $Mecp2^{-/y}$ mice ($p < 0.05$). On the other hand, compared to the untreated mice, the group of $Mecp2^{-/y}$ mice received a medium dose (1*1012) showed a substantial increase in longevity and body weight. In contrast to the control group's average survival rate of 11.64 weeks ($p = 0.001$, Mantel-Cox test), this group's average survival rate was 27.3 weeks. Additionally, this group's average body weight at 11 weeks was noticeably higher ($p < 0.05$). However, at this dosage, the severity of the RTT-like phenotype was comparable to that of the control group. Acute toxicity and death occurred in the highest dose group within ten to fifteen days of injection. It is important to note that the vehicle-treated wild mice were different from the $Mecp2^{-/y}$ animals in all parameters.

4.2. First-Generation Vector Lead Hepatic Toxicity

For further examine, as the toxicity issues observed with high doses of the first-generation vector upon systemic administration, MeCP2 expression levels were assessed in various peripheral tissues. At the end, bio-distribution in several organ of the vector genome was quantitatively analyzed by using qPCR, then it was found, via immunohistochemistry and analysis, that a high percentage of c-myc tagged cell were present in hepatic cell. Although endogenous MeCP2 levels in liver cells are typically lower than those in brain neurons (Ross et al., 2016; Skene et al., 2010), Myc-derived MeCP2 levels in treated WT mice's liver cells (as detected by anti-Myc immunolabeling) were higher than those found in neurons. Approximately twenty times higher than the endogenous observed. In treated $Mecp2^{-/y}$ mice, Mycpositive cells were also found in other peripheral tissues, such as the heart and kidneys (Brown et al., 2016).

4.3. Second-Generation Vector With Reduced Hepatic Toxicity

It has been proven in the past that employing systemic delivery, a higher dose of the AAV vector is necessary to achieve significant brain transduction. However, the first-generation vector caused considerable toxicity when used at large doses, necessitating the creation of a new design. To address this issue, several changes to the expression cassette and capsid were attempted in an effort to reduce cellular expression levels and/or liver tropism. The use of different expression cassettes with a condensed JeT promoter (Tornøe et al., 2002) and a brief synthetic polyadenylation signal was one of these modifications (Levitt et al., 1989). An in vivo search for capsid sequences that had lower liver tropism than AAV9 led to the discovery of the scAAV9.47 capsid included with the original firstgeneration vector (KarumuthilMelethil et al., 2016; Pulicherla et al., 2011). When compared to the first-generation vector, the moderate dose (1*1012 vg per mouse) of these modified vectors administered systemically to four to five week-old $Mecp2^{-/y}$ mice did not noticeably enhance the RTTlike assemble seriousness rating ($p > 0.05$ for all measures, ANOVA, and Mantel-Cox tests). It did, however, result in dramatically increased survival and better body weight. Sadly, these changes led to the emergence of liver disease resembling that seen with the first-generation vector. An endogenous Mecp2 core promoter fragment (MeP229) that had been shown to largely reproduce the endogenous tissue-level pattern of MeCP2 expression was used in the firstgeneration vector (Gray, Foti, et al., 2011).

An extended promoter fragment (MeP426) with additional regulatory elements and a possible silencer element was inserted in the second-generation vector. This was done to more effectively manage the levels of MeCP2 the vector produced in transduced cells. Additionally, a new 3' UTR was developed by combining a piece of the natural MECP2 3' UTR with a chosen set of microRNA binding sites that are involved in the control of Mecp2 (Feng et al., 2014; Visvanathan et al., 2007).

Mecp2$^{-/y}$ mice were administered a medium dose (1*1012 vg per mouse) of the second-generation vector intravenously in order to evaluate its therapeutic efficacy. However, there was no difference in the RTTlike aggregate severity score between the treated and vehicle-treated mice in terms of survival. A study of MeCP2 expression levels in transduced cells revealed that expression was tightly regulated, with fewer cells displaying very high levels of expression, despite the lack of therapeutic benefit seen with systemic delivery of the second-generation vector compared to the first-generation vector. Furthermore, the secondgeneration vector did not cause any noticeable changes to the hepatic architecture or vacuolation, in contrast to the first-generation vector. When compared to mice treated with second-generation vectors, liver samples from first-generation vector-treated mice had a considerably higher density of inflammatory foci (Robinson et al., 2012).

Conclusion

Identifying and addressing barriers to successful translation is crucial for achieving optimal outcomes in human clinical trials of MECP2 gene therapy. The present study highlights challenges associated with systemic delivery, such as low brain transduction efficiency and peripheral overexpression toxicity upon dose escalation, which create a narrow therapeutic window. To achieve successful therapy, widespread brain expression with appropriate control of MeCP2 levels is necessary. Expression cassettes producing sub-toxic levels of MeCP2 could overcome issues of cellular overexpression and enable direct delivery via the cerebrospinal fluid compartment. AAV9 may not be efficient enough for systemic delivery, but combining the safer second-generation cassette with capsids that have improved brain penetrance could pair effective CNS gene transfer with safe levels of peripheral MeCP2 transgene expression and enhance translational promise.

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