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The effectiveness of sickle cell anemia treatment using CRISPR

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Abstract

The rapid advancement of medical biotechnology plays a crucial role in healthcare practices, particularly in genetic engineering for sickle cell anemia. Currently, there is no definitive cure for sickle cell anemia, but in recent years, gene therapy has been extensively researched as an alternative solution. Gene therapy trials on sickle cell anemia patients have been conducted using CRISPR technology, and its effectiveness is still under investigation. The principle involves manipulating the abnormal hemoglobin gene in erythrocytes, which causes sickle cell disease, by extracting stem cells from the patient's bone marrow and replacing the faulty gene with a normal one using CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats). The critical components required are Cas9, an endonuclease restriction enzyme, and gRNA, which guides Cas9 to the target gene for manipulation. Once the target gene is successfully cut by Cas9, DNA polymerase naturally initiates a gene repair mechanism through the HDR (Homologue-directed repair) pathway, a process of repairing damaged DNA sequences in CRISPR. Based on tests conducted on 44 patients, 28 patients showed promising results, with their bone marrow producing functional, normal erythrocytes with significant increase in total hemoglobin (stabilizing at ~12 g/dL) and HbF (stabilizing at ~4 g/dL) within 24 months. These findings suggest that Casgevy, as a CRISPR-based therapeutic intervention, provides a durable hemoglobin modulation effect, which may mitigate clinical complications associated with sickle cell disease

Keywords: Anemia; CRISPR; Hemoglobin; Stem Cells; Sickle Cell

INTRODUCTION

The development of biotechnology, particularly in the field of biomolecular science, began with scientists' discovery of the double helix structure of DNA strands and has continued to evolve. The modernization of medical biotechnology plays a crucial role in healthcare practices, especially for patients with genetic disorders. Genetic engineering, in simple terms, is a method for modifying the DNA sequence isolated from specific cells to be manipulated using various approaches. These include identifying, altering nucleotide sequences, and transferring the synthesized genetic material to the desired recipient target. (1)Gene therapy is one significant product or discovery of genetic engineering that has been highly beneficial in treating various genetic diseases.

CRISPR gene therapy is a method that researchers are extensively studying for its potential applications. This technique uses guide RNA that binds specifically to the target gene and works in conjunction with the Cas9 protein, which acts as a restriction endonuclease enzyme to cut the target DNA. CRISPR is commonly used to identify and treat genetic diseases, including sickle cell disease. The effectiveness of CRISPR exceeded 50% in tests conducted on 28 out of 44 patients with sickle cell anemia. Due to the success of these trials in 2020, the FDA (Food and Drug Administration) approved and regulated CRISPR as a legal treatment

method for sickle cell anemia, which can be used for patients aged 12 and older (2).

Anemia Sickle Cell

Sickle cell anemia is a type of hemoglobinopathy caused by an autosomal recessive genetic disorder (3). The disease was first documented in 1910 by James B. Herrick, who observed abnormally elongated, crescent-shaped red blood cells in the blood smear of a patient (4). The abnormal sickle shape of the blood cells is due to a genetic defect present from birth caused by a mutation in the gene (5). The sickle cell gene is expressed if an individual inherits the gene from either one parent who is a carrier or both parents with the disease. Sickle cell anemia can be detected through a standard blood test, although signs of sickle cells may not be evident in early childhood and may only appear later in adulthood. Therefore, routine screening is recommended for pregnant women and newborns to ensure early detection and management (6).

Epidemiology of Sickle Cell

Sickle cell anemia is commonly found in regions such as the Americas and Africa but is rare in Asia. According to data from the Global Burden of Disease (GBD) in 2021, approximately 7.74 million people worldwide are affected by sickle cell disease, with 3.9 million being women and 3.84 million men (7). The highest prevalence is concentrated in tropical regions such as Africa, the Middle East, parts of South Asia, and among populations in the Americas and Europe with African ancestry (7).

The WHO has now recognized sickle cell disease as a global health issue. The global mortality rate (Figure 1) in countries with a history of sickle cell disease is 11 times higher than for other diseases, with 374,000 cases reported, predominantly in East Asia and sub-Saharan Africa. The risk of death due to sickle cell disease is especially high among children. In 2021, 515,000 babies were born with sickle cell disease, with a mortality rate of around 15.3% per 100,000 live births globally for children under the age of five (8).

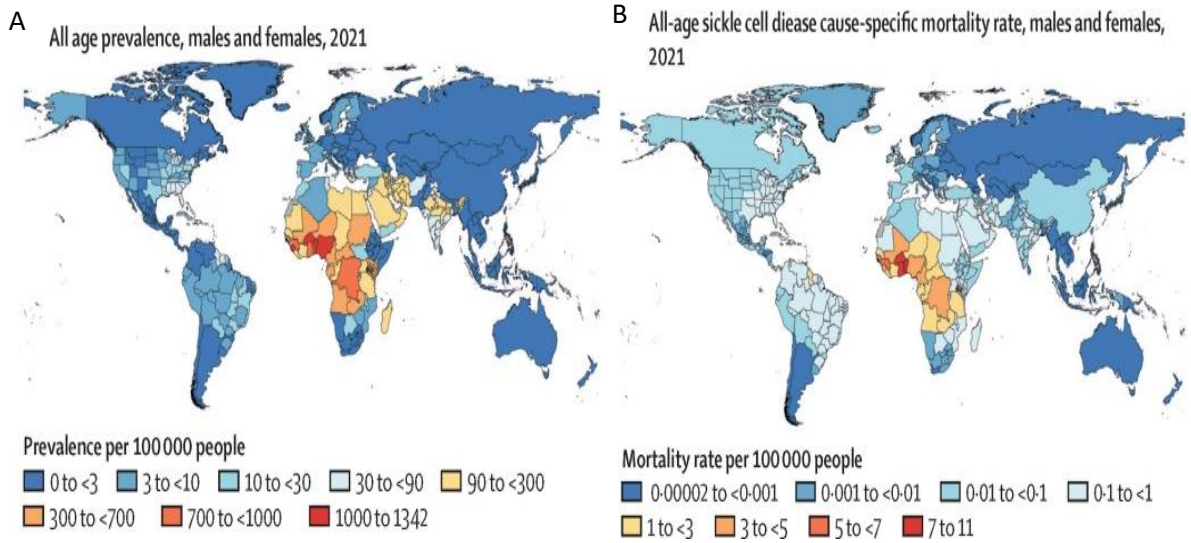

Figure 1. Map of sickle cell distribution per 100,000 population in 2021; A. Prevalence rate, B. Mortality rate (7).

Diagnosis and Clinical Presentation of Sickle Cell Anemia

Anemia is a condition in which a person experiences blood loss, iron deficiency, or a decrease in hemoglobin levels below the normal range (>14 g/dL for men and >12 g/dL for women) (Chaparro dan Suchdev, 2019). The diagnosis of anemia is usually based on hemoglobin concentration and hematocrit tests, which compare the levels of red blood cells, white blood cells, and platelets (10). In contrast, diagnosing sickle cell anemia requires additional testing, such as hemoglobin electrophoresis, to analyze the presence of hemoglobin S in the blood (11).

People with anemia often experience symptoms such as fatigue, dizziness, shortness of breath, pale skin, chest pain, and heart palpitations (Chaparro dan Suchdev, 2019). However, sickle cell anemia presents with more severe symptoms. The most common symptom of sickle cell anemia is vaso-occlusion, blockage of blood vessels. Vaso-occlusion is characterized by intense pain that can occur suddenly. This pain is episodic and can occur in different body areas, depending on where the blockage occurs. Additionally, sickle-shaped red blood cells can cause ischemia, a condition where blood flow to vital tissues or organs, such as the brain, is restricted, potentially leading to stroke and death (5).

Pathophysiology of Sickle Cell Hemoglobin

Erythrocytes, or red blood cells, are functional cellular components produced through hematopoiesis in the bone marrow. These cells are round, biconcave, and lack a nucleus, with a cytoskeleton made of a protein network and a phospholipid bilayer membrane serving as an outer protective layer (Barbalato, 2022; Wahyudi, 2020) The main component of erythrocytes is hemoglobin (Figure 2). Hemoglobin comprises heme, which includes iron (Fe) ions and protoporphyrin, an organic compound essential for heme synthesis, and globin, which consists of alpha and beta amino acid chains. Hemoglobin is structured as a tetramer—four protein subunits, each containing two alpha (α1 and α2) and two beta (β1 and β2) subunits, all of which are of equal size and covalently bonded. Each subunit is made up of 7-8 helices, labeled sequentially from A to H, with the heme-binding pocket formed between helices E and F (Ahmed et al., 2020). This structure allows hemoglobin to bind four oxygen molecules, as heme in each hemoglobin subunit serves as an oxygen-binding site (15).

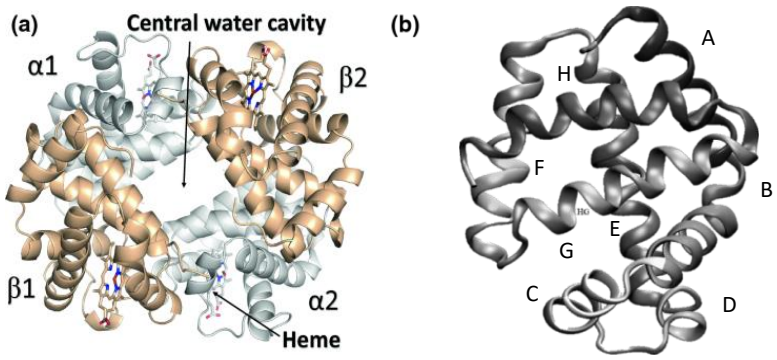

Figure 2. The structure of normal hemoglobin; A. Hemoglobin tetramer shape, B. Hemoglobin helical arrangement (14).

Various variants of hemoglobin cause sickle cell disease, including sickle cell heterozygotes caused by hemoglobin C (HbC), hemoglobin D (HbD), and hemoglobin E (HbE). Sickle cell anemia, however, is associated with sickle cell homozygosity, which results from hemoglobin S (HbS) (Ahmed dan Ibrahim, 2021). HbS forms due to a mutation in the sixth amino acid sequence of the β-globin chain on the short arm of chromosome 11 at locus 11p15.4 (Figure 3) (17). Specifically, this mutation involves a single base change from adenine (A) in the GAG codon (glutamic acid), which is hydrophilic, to thymine (T) in the GTG codon (valine), which is hydrophobic. When deoxygenation or a decrease in oxygen levels occurs in the blood, this amino acid change in HbS leads to its tendency to bind with other HbS molecules, a process known as polymerization (18). This rapid polymerization forms long fibers that damage the erythrocyte membrane and cytoskeleton, causing the cells to become rigid and sickle-shaped, leading to premature hemolysis (19). The hemolysis of erythrocytes results in a reduced erythrocyte lifespan—from the normal 120 days to just 10-20 days (20).

Deoxygenation or decreased oxygen levels within erythrocytes can lead to increased blood viscosity and a reduced affinity for oxygen. Excessively high blood viscosity, or thickening, can result in blockages within blood vessels. As the rate of erythrocyte lysis increases, The presence of nitric oxide enhances the adhesive properties of erythrocytes, causing them to stick to blood vessel walls. This adhesion leads to the narrowing of blood vessels, known as vasoconstriction. Consequently, the oxygen that hemoglobin would typically bind and circulate throughout the body becomes obstructed, leading to a deficiency in blood oxygen levels (22).

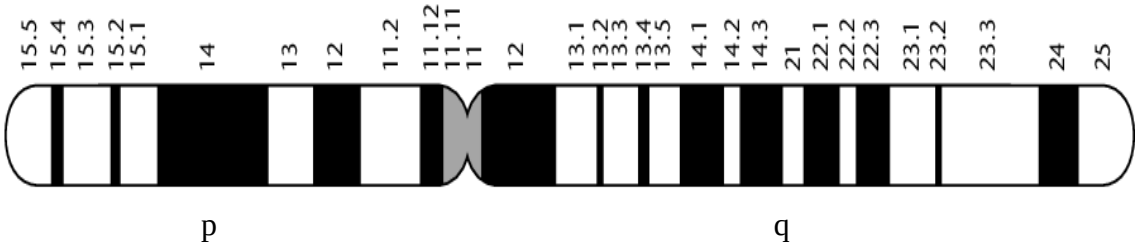

Figure 3. Gene mutation point on the eleventh chromosome arm p15.4 (21)

CRISPR

Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) is one of the simplest, most versatile, precise, and accessible gene editing methods for geneticists and researchers to rewrite the genetic code of an organism (24). The application of CRISPR in humans requires two main components: guide RNA (gRNA) and the Cas9 protein (Figure 5). Together, these components target the specific gene to be manipulated. The guide RNA is derived from CRISPR RNA (crRNA) and trans-activating RNA (transcrRNA) (25). crRNA contains the complementary gene sequence of the target DNA and binds to it using a homology approach between the target DNA and Cas9, while transcrRNA functions in the maturation and activation of crRNA (26). The Cas9 enzyme, commonly used to target human DNA, belongs to the class 2 type 2 nucleases and acts as a molecular "scissors" to cut the target DNA.

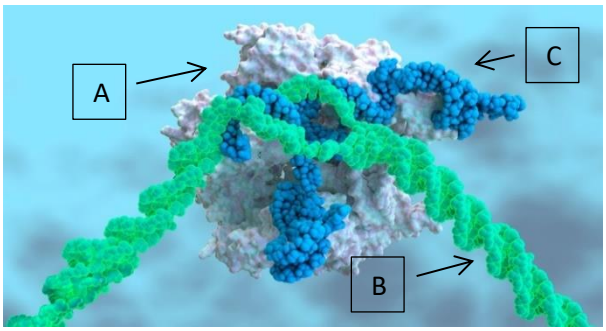

Figure 4. Components of the CRISPR system; A. Cas9 enzyme, B. target DNA, C. gRNA (guide RNA) (27)

The CRISPR-Cas9 system is highly universal and effective for manipulating human genetic codes. Depending on the patient's condition, it typically requires only one treatment over approximately six months. Unlike other gene editing techniques, CRISPR can be performed naturally without advanced protein engineering, making it a relatively cost-effective option (28). One of the first successful implementations of CRISPR was in 2019 in the United Kingdom, where a woman with sickle cell disease underwent CRISPR gene therapy and successfully returned to normal health without serious symptoms (29). Since then, 75 other patients with sickle cell disease have also achieved successful outcomes using CRISPR therapy (30)However, CRISPR gene therapy is not yet considered safe for patients under 12 or over 64, as further testing is still required.

CRISPR Procedure Overview

CRISPR-Cas9 has revolutionized the field of genetic engineering, largely due to its high success rate. Its ability to enhance or inhibit gene expression both in vitro and in vivo is a key factor in this success. Before gene editing can begin, the Cas9 protein and guide RNA (gRNA, 9-19kb) are transfected into bacterial plasmid vectors to create a stable cell line. This transfected cell line helps minimize cell death during CRISPR (31). CRISPR-Cas9 uses the Cas9 enzyme to cut a specific gene on the DNA strand, creating double-strand breaks (DSBs) (32). Naturally, the body's repair system attempts to fix these broken genes by rejoining them. There are several pathways for gene repair, but the primary ones used in gene editing are non-homologous end joining (NHEJ) and homology-directed repair (HDR).

The NHEJ pathway is most commonly utilized (Figure 5) because it coincides with the cell cycle's interphase. When DSBs occur, the broken nucleotide pairs are not always cut evenly; instead, some nucleotides may degrade on one side only, creating single-strand overhangs, a process known as resection (33). The incompatible ends of the breaks are then bound by the Ku80 and Ku70 heterodimer proteins at each broken end. With the help of the DNA-PKcs enzyme (DNA protein kinase catalytic subunit) and the Artemis protein acting as an exonuclease, the single-strand overhangs are trimmed, leaving only double-strand DNA segments of equal length. Once the double-stranded DNA is ready, DNA ligase attaches the ends according to the Cas9 cut, forming a complete DNA strand. However, some research suggests that this pathway is prone to errors, which can lead to frameshift mutations and result in gene dysfunction. This occurrence is known as a gene knockout (33, 34).

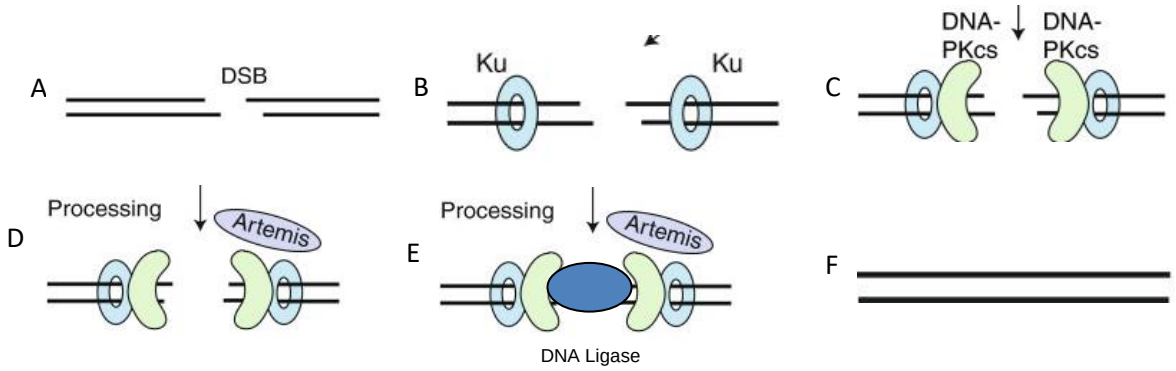

Figure 5. NHEJ pathway repair process; A. DNA resection, B. Ku 70/80 protein attachment to DSBs, C. Recruitment of DNA PKcs, D. Exonuclease by artemis protein, E. DNA repair and reunification by DNA ligase, F. DNA returns to normal (35).

In contrast to the non-homologous end joining (NHEJ) pathway, a homologous DNA template can guide the repair of double-strand breaks (DSBs), directing the Cas9 protein to achieve precise gene editing. This process, known as homology-directed repair (HDR), aims to create a desired gene sequence using a homologous chromosome as a template. This technique results in what is referred to as a gene knock-in (33,36,37). Like NHEJ, HDR involves nucleotide resection at the DNA double-strand break (Figure 6). At the ends of the DSBs, a protein complex called the MRN complex, consisting of MRE11, RAD50, and NBS1, acts as a sensor and begins resecting the DNA at the 5’ ends (38). This resection continues until it reaches a specific breakpoint at the same sequence on both 5’ ends. Once resection is complete, the exposed single-strand DNA is coated with Replication Protein A (RPA), which protects the strand from further degradation by nucleases (39), The binding of RPA to the single-strand DNA forms a nucleofilament (40). The RPA is replaced by recombinase enzymes RAD51 and BRCA1, which locate the homologous DNA and catalyze its invasion into the DSB site (41). Once the homologous DNA is identified, it elongates, and the lower 3’ strand searches for its complementary sequence on the sister chromatid to begin replication, facilitated by DNA polymerase, until it reaches the 5’ end of the lower strand. After replication is completed, the new DNA strand naturally reattaches to the original strand. DNA polymerase also repairs the upper strand by joining the 3’ end to the 5’ end. Once both strands have been restored to their normal structure, DNA ligase binds them together, re-forming the double helix (38,42).

Compared to gene knockouts, gene knock-ins are more complex and require precise techniques. This complexity arises because the HDR pathway is less common than NHEJ and only occurs during specific stages of the cell cycle. NHEJ primarily happens during the interphase, specifically in the G1 phase of the cell cycle, which lasts around 11 hours. In contrast, HDR occurs only during the DNA replication phase (S phase) of the cell cycle, which lasts about 8 hours (43). The lower likelihood of HDR occurring results in reduced knock-in efficiency. However, this probability can be increased by inhibiting the NHEJ pathway, thereby redirecting the repair process towards HDR (36,37).

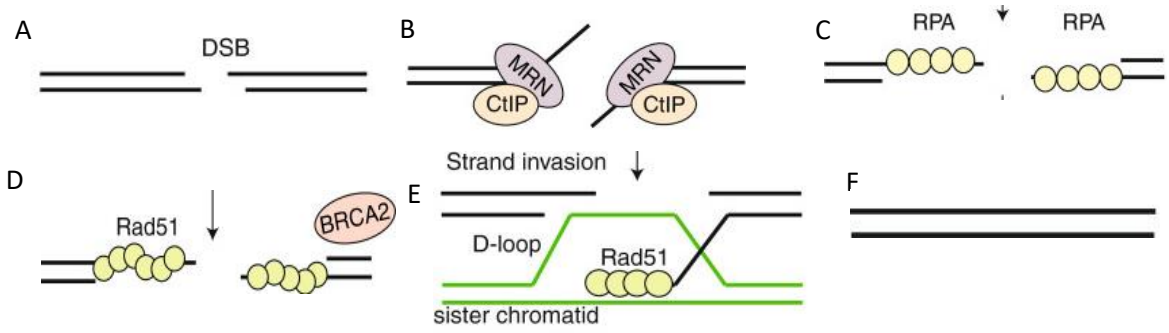

Figure 6. HDR pathway repair process; A. Form a chain of DSBs, B. Both ends of the DSBs are attached to the MRN complex protein, C. The upper single strand will be enveloped by RPA, D. RAD51 and BRCA2 replace the position of RPA acting as a tracer for the presence of homologous DNA E. Homologous chromosomes undergo elongation, and are homologously paired, F. The double strand is reattached with DNA ligase to form a double helix (38).

GENE THERAPY FOR SICKLE CELL ANEMIA

Sickle cell disease has been known for over a century, and its genetic mutation was identified seventy years ago (44). In recent years, gene therapy research, particularly using various approaches like CRISPR, has gained significant attention among scientists studying sickle cell disease. Unlike bone marrow transplants, which carry the risk of rejection due to donor mismatch, or blood transfusion therapy, which aims to reduce blood viscosity and increase the number of functional erythrocytes but requires monthly transfusions (45,46), CRISPR has shown promising advancements, progressing to clinical trials.

In 2020, a major development came from Vertex, a healthcare company that introduced a CRISPR-based gene therapy called Casgevy (47). Casgevy has successfully passed clinical trials and is tailored specifically for individual patients. The therapy was tested on 44 sickle cell patients aged 12 to 64, with 29 patients successfully completing the trial. After two years of regular monitoring and evaluation, 28 of the 29 patients were declared temporarily healthy, experiencing no severe symptoms and able to live normal lives (47,48).

Casgevy therapy does not aim to change the sickle-shaped erythrocytes back into their regular round, biconcave shape. Instead, it focuses on correcting the hematopoiesis process in the red bone marrow to restore the production of normal erythrocytes. The therapy specifically targets hematopoietic stem cells (HSCs) in the red bone marrow. These HSCs are oligopotent stem cells, meaning they can only differentiate into certain types of cells, including erythrocytes (49). In the Casgevy trials, the regeneration of HSCs within the patient's body is utilized to restore damaged erythrocytes right from the early stages of hematopoiesis in the bone marrow (50).

Initially, patients who meet the Casgevy therapy criteria must undergo blood transfusions to reduce and maintain their HbS levels at less than 30% of the total hemoglobin for eight weeks before stem cell collection. The patients are then administered drugs such as filgrastim or plerixafor to mobilize the stem cells from the bone marrow over 4-5 days (51). Following

this, apheresis is performed using a device that separates erythrocytes from stem cells. The collected stem cells are gathered according to the required amount, approximately 20 x 10^6 cells per kilogram of body weight per patient, with an additional 2 x 10^6 cells per kilogram collected as "rescue cells" in case of unforeseen complications during therapy. Meanwhile, patients receive blood transfusions to replace the blood lost during the procedure. The apheresis process typically takes place over two consecutive days, with an additional day for collecting rescue cells if clinically feasible. If the required number of cells is not collected, the procedure must be repeated after a minimum interval of 14 days. Once the stem cells are collected, they are sent to a laboratory for genetic modification using CRISPR technology (47).

In vitro gene editing with CRISPR requires several key components: the Cas9 enzyme, guide RNA (gRNA), plasmid vectors, a DNA template sequence, and the patient's hematopoietic stem cells (HSCs). The process begins by targeting the specific DNA within the HSCs that needs to be edited. Researchers design the gRNA to match the problematic gene location on the target DNA. The gRNA is loaded with a gene sequence based on the protospacer of the target gene, allowing the Cas9 enzyme to detect and bind to the gene. Cas9 then acts as an endonuclease, cutting the gene sequence. Multiple cuts can be made at different locations by altering the gene sequences within the gRNA. Once the gene is cut, double-strand breaks (DSBs) occur in both DNA strands. Naturally, proteins attempt to repair these breaks using the cell cycle's repair mechanisms, primarily through the non-homologous end joining (NHEJ) pathway. However, since NHEJ repair carries the risk of mutations due to potential errors, the repair process is redirected to the homology-directed repair (HDR) pathway. This is achieved by introducing a modified DNA template as a blueprint for DNA polymerase to accurately repair the damaged gene. The lower 3' strand seeks a complementary sequence from the sister chromatid of the DNA template to replicate with the help of DNA polymerase until it reaches the 5' end of the lower strand. Once the transcription is complete, the new DNA strand naturally reconnects with the original strand. DNA polymerase is also used to repair the upper strand by joining the 3' and 5' ends. After both strands are restored to their normal structure, DNA ligase binds them together, re-forming the double helix (38,42).

The DNA modification is carried out on all HSCs, which are then cultured to multiply the cells, a process that typically takes six months. Once ready, the modified HSCs are stored in patient-specific vials per dose (at least 3 x 10^6 cells/kg of body weight) and returned to the hospital for transplantation. Before the transplantation, doctors perform myeloablative conditioning in the bone marrow by administering busulfan intravenously to destroy the existing cells, making room for the new stem cells. Patients are also given antihistamines and antipyretics beforehand to prevent allergic and inflammatory reactions (such as pain and fever). This conditioning leaves patients severely anemic and highly susceptible to infections, necessitating hospital care. After 48 hours to 7 days of myeloablative conditioning, depending on the patient's clinical condition, the stem cells must be transplanted into the bone marrow via intravenous infusion. These stem cells then work within the bone marrow to produce fetal hemoglobin (HbF), which will develop into functional blood cells. The administration of

Casgevy demonstrated a significant and sustained increase in total hemoglobin (Hb) and fetal hemoglobin (HbF) levels over a 24-month observation period. These findings suggest that Casgevy, as a CRISPR-based therapeutic intervention, provides a durable hemoglobin modulation effect, which may mitigate clinical complications associated with sickle cell disease. The long-term stability of hemoglobin levels highlights the potential of gene-editing therapies in addressing hematological disorder (47).

CONCLUSION

CRISPR, as a genome-editing technique, offers a promising alternative treatment for curing sickle cell anemia by using the Cas9 endonuclease enzyme and gRNA to correct abnormal genes in hemoglobin. CASGEVY, a gene therapy product based on CRISPR technology, has undergone rigorous evaluation and, as of 2023, has been proven safe for use in patients with sickle cell anemia. It is approved for individuals aged 12 to 64, with a success rate of 63%.

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