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Review

Advances in gene therapy for hemophilia

Olivia A Robles-Rodríguez[1], José J Pérez-Trujillo[1], Arnulfo Villanueva-Olivo[1], Laura Villarreal-Martínez[2], Luis J Marfil-Rivera[2], Humberto Rodríguez-Rocha[1], Aracely García-García[1], Odila Saucedo-Cárdenas[1,3], María J Loera-Arias[1]* and Roberto Montes de Oca-Luna[1]*

[1]*Departamento de Histología, Facultad de Medicina, Universidad Autónoma de Nuevo León, C.P. 64460 Monterrey, Mexico*

[2]*Servicio de Hematología, Hospital Universitario "Dr. José Eleuterio González González", Universidad Autónoma de Nuevo León, C.P. 64460 Monterrey, Mexico*

[3]*Departamento de Genética Molecular, Centro de Investigación Biomédica del Noreste (CIBIN), Instituto Mexicano del Seguro Social (IMSS), C.P. 64720 Monterrey, Mexico*

**Corresponding authors (Emails, loera.arias@gmail.com; rrrmontes@yahoo.com)*

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Hemophilia is a hereditary disorder that can be life-threatening in individuals who have severe spontaneous bleeding resulting from minor trauma or surgery. Although replacement therapy of the missing exogenous factor has improved patients' quality of life, it has not been possible to establish a long-term treatment. Due to the severity of the disease and the need for repetitive doses throughout the patient's life, replacement therapy has become a high-cost treatment option; therefore, the development of self-sustainable long-term therapies is critical. Hemophilia is a good candidate for gene therapy because it is a monogenic disease that can be counteracted by expression of the missing factor. In this article, we review some of the most relevant advances in gene therapy for this illness.

Keywords. Coagulation; gene editing; gene therapy; hemophilia

1. Introduction

Hemophilia is a hereditary hemorrhagic disorder characterized by spontaneous and prolonged bleeding after surgery or trauma. It is a chromosome x-linked recessive disease that manifests in men, while women are generally carriers with a 50% probability of transmitting the mutated allele to their offspring (Shrestha *et al.* 2016).

The disease is classified into two principal types, hemophilia A and B (HA and HB, respectively). These two types are caused by mutations in genes that codify the coagulation factors VIII and IX (FVIII and FIX, respectively) (Melchiorre *et al.* 2016). Of these forms, hemophilia A is more frequent and is present in the population in a proportion of 1:5000 newborn boys, while hemophilia B is only present in a proportion of 1:30,000 (Franchini and Mannucci 2012). Disease severity is classified depending on the activity of these factors in the organism as mild (5%–30% activity); moderate (2%–5% activity); and severe (<1% activity) (Park *et al.* 2015).

2. A brief history of hemophilia treatment

There is currently no cure for hemophilia; therefore, intravenously administered factor replacement therapies are the predominant form of treatment. Factor obtention methods have varied over time with

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improvements in the amounts and purities of the factors.

In the 1950s and the beginning of the 1960s, hemophilia was only treated with blood or plasma transfusions. These transfusions were a partially effective treatment with the exception of cases of severe hemorrhage because in those cases, the speed and amounts of factor VIII or IX transfusion were not enough for systemic compensation (Franchini and Mannucci 2012). However, it was not until 1964 at which time it was discovered that the fraction that precipitates in cryopreserved plasma has large amounts of factor VIII. This finding formed the basis of program development to care for hemophilia A patients in the United States (Mannucci 2008). Conventional treatment of hemophilia started in 1970 with the availability of plasma-derived clotting factor concentrates obtained from thousands of donors. Even though contamination by hepatitis viruses B and C were detected in these concentrates, patients infected post-transfusion only developed a moderate non-progressive hepatitis, and it was concluded that the concentrate's benefits outweighed the infection risks. This perception changed at the beginning of the 1980s at which time it was found that between 60% and 70% of the patients with severe hemophilia were infected with the *Human immunodeficiency virus* (HIV) (Mannucci and Tuddenham 2001).

In 1983, factor VIII was purified from the blood for the first time with antibodies against factor VIII adhered to a column (Rotblat *et al.* 1983). After obtaining a purified product, factor VIII gene sequence was reported in 1984 (Gitschier *et al.* 1984), which made possible the production of a recombinant factor. However, hemophilia treatment with purified recombinant factor VIII did not start until 1993. Since this date, it became the conventional treatment method for this condition (Graw *et al.* 2005).

A significant achievement in hemophilia treatment was the creation of a humanized bispecific antibody (emicizumab; ACE910), which binds to FIX and FX and mimics the function of FVIII. In the first emicizumab clinical trial, there was a reduction in patient's bleeding; however, several doses of the antibody were necessary. Continued use of this antibody is expensive, and the generation of antibodies against the reactant has not yet been evaluated (Miao 2016).

3. Strategies to prolong the life of the recombinant factor

Commercial available recombinant FVIII concentrates have a short half-life of 6 to 24 h with an average of 12 h in adults and less in children (Tiede 2015). Optimal prophylaxis with Factor VIII requires 3–4 weekly intravenous infusions. Generally, in children, FVIII has a shorter half-life making it necessary to administer more frequent doses (Nolan *et al.* 2016).

Studies aimed at improving the half-life of factor VIII in blood have been recently carried out. To extend recombinant factor VIII- half-life, its structure has been modified to make it less labile in the body. One of these modifications is the fusion to the Fc domain of IgG1. The binding of the fusion protein to the neonatal Fc receptor prevents its degradation and facilitates its recycling, resulting in a prolonged half-life. This protein fusion has twice the half-life in murine and canine hemophilia models (Dumont *et al.* 2012).

Another strategy for prolonging the half-life of proteins of therapeutic interest is the adhesion of polyethylene glycol (PEG) molecules to the protein's cysteine residues. Also, directed mutagenesis has made cysteine residue addition possible with the aim of favoring PEG molecule adhesion and half-life extension. Importantly, these proteins have less immunogenicity (Mei *et al.* 2010).

4. Neutralizing antibodies against factor VIII

The most severe problem in hemophilia treatment is the development of neutralizing antibodies that inhibit the recombinant factor VIII. Early prophylaxis with the recombinant factor reduces bleeding in joints and other hemorrhaging and reduces chronic damage (Tiede 2015). This treatment is preferred to prevent complications, especially in children with severe hemophilia. Notably, only 1% of children who receive prophylactic treatment develop inhibitory antibodies, while 38% of the children who do not receive prophylaxis develop these antibodies. After antibody development, replacement therapy with the recombinant factor VIII loses its effectivity due to activity neutralization (Klukowska *et al.* 2015).

Alternatively, immune tolerance induction (ITI) is the only proven strategy for antibody removal, and it has been successful in >70% of patients with hemophilia A. This therapy consists of high doses of intravenous recombinant FVIII administered at regular intervals over long-term periods in hemophilia A patients who have inhibitory antibodies (Mizoguchi *et al.* 2016).

In some studies, recombinant FVIII was more immunogenic than the plasma-derived FVIII. However, comparison between studies is limited due to several factors, including heterogeneity, a small number of

patients, and the use of recombinant factors produced in different ways (Gouw *et al.* 2013).

5. Gene therapy using viral vectors

As mentioned earlier, treatment with recombinant FVIII is effective but has some disadvantages, such as a reduced half-life and long-term generation of inhibitory antibodies. Hence, better strategies are required to overcome these disadvantages. In the last decade, gene therapy has emerged as a powerful tool in targeting and correcting disease-specific and dependent gene mutations (figure 1).

Gene therapy is a strategy for delivering a non-defective copy of a gene to the patients and appears to be adequate for hemophilia treatment since a minimal increase and maintenance of systemic levels of the factor can convert severe hemophilia into a moderate form of the disease (Pang *et al.* 2016). Furthermore, due to its monogenic nature, hemophilia is one of the primary targets for gene therapy. Different vectors, both viral and non-viral, have been used for gene delivery, with viral vectors being the most frequently used due to their ability to infect multiple cells and express the gene consistently. Besides, viruses are genetically modified to make them safer and more efficient, with adeno-associated, retroviral, and lentiviral vectors being the most studied.

One retroviral vector, based on retrovirus C, has been modified to express FVIII with a deleted B domain to reduce the size of the transgene and simplify cloning in the vector. Also, to improve its safety, it was modified to avoid producing viral particles. Before being tested in humans, it was evaluated in rat, rabbit, and dog hemophilia A models, where FVIII was efficiently produced (Greengard and Jolly 1999; Roehl *et al.* 2000). Afterward, the vector was tested in a clinical trial phase in patients with severe hemophilia A (table 1). It was the first trial applying gene therapy to treat these patients. Of the 14 patients who received the vector, nine patients had a 1% increase in FVIII 5 days after administration. In some subjects, the retrovirus persisted in peripheral blood mononuclear cells 1 year after administration. Also, competent replication trials of the retrovirus were negative, making this vector safer but inefficient (Powell *et al.* 2003).

On the other hand, lentiviral vectors have the ability to transduce both dividing and quiescent cells, which is an advantage over retroviruses. Therefore, lentivirus may be able to increase the efficiency of transduction in stem cells. Lentiviral vectors have been administered intraosseously by transducing hematopoietic stem cells with FVIII *in vivo*. These transduced cells conserve their differentiation potential into any blood lineage,

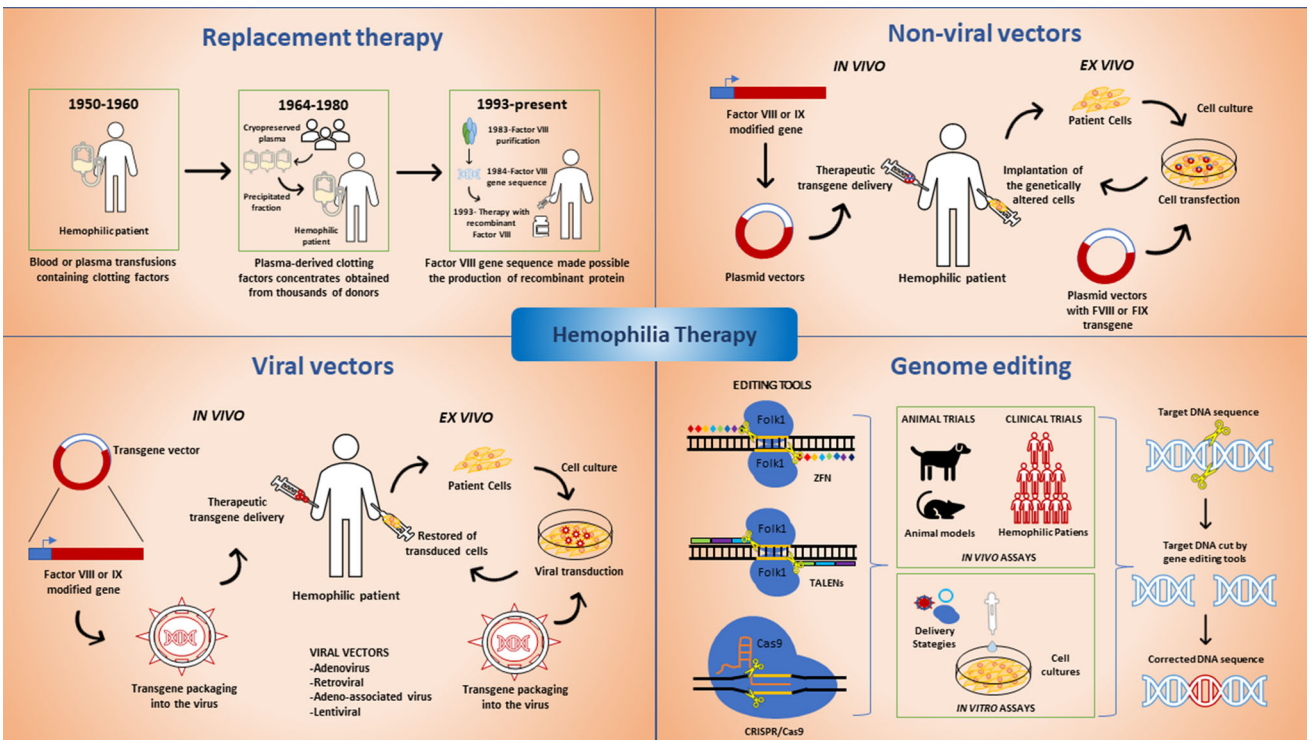

Figure 1. Therapy strategies for Hemophilia.

Table 1. Recent advances in gene therapy against hemophilia year

Year	Phase	Strategy	References
2001	Phase 1 clinical study	Patients with hemophilia A were injected with fibroblasts that expressed factor VIII	Roth *et al.* (2001)
2003	Phase 1 clinical study	Patients with hemophilia B were injected intramuscularly with an adeno-associated virus that expressed factor IX	Manno *et al.* (2003)
2003	Phase 1 clinical study	Patients with hemophilia A were injected with a retroviral vector carrying a deleted B-domain human factor VIII	Powell *et al.* (2003)
2011	Phase 1 clinical study	Patients with hemophilia B were injected with a self-complementary adeno-associated virus vector expressing a codon optimized factor IX (FIX) transgene (scAAV2/8-LP1-hFIXco)	Nathwani *et al.* (2011)
2014	Phase 1 clinical study	Patients with hemophilia B were injected with a self-complementary adeno-associated virus vector that expresses optimized factor IX (scAAV2/8-LP1-hFIXco)	Nathwani *et al.* (2014)
2017	Phase 2 clinical study	Patients with hemophilia A were injected with a codon-optimized adeno-associated virus serotype 5 (AAV5) vector encoding a B-domain-deleted human factor VIII (AAV5-hFVIII-SQ)	Rangarajan *et al.* (2017)
2017	Phase 2 clinical study	Patients with hemophilia B were injected with a single-stranded adeno-associated viral (AAV) vector consisting of a bioengineered capsid, liver-specific promoter, and factor IX Padua (factor IX-R338L)	George *et al.* (2017)
2018	Phase 2 clinical study	Patients with hemophilia B were injected with an adeno-associated virus-5 (AAV5) with a liver specific promoter driving expression of a codon-optimized wild-type human FIX gene	Miesbach *et al.* (2018)

and they maintain their ability to renew (Miao 2016). Platelets are ideal for FVIII expression since these factors are stored in granules that could protect them from inhibitory antibodies. When hemorrhaging occurs, FVIII can be secreted by the platelets and promote coagulation (Kuether *et al.* 2012). In mice, long-term stable expression of FVIII in platelets was achieved with a single intraosseous dose of the lentiviral vector expressing FVIII under a specific platelet promoter, which partially corrected hemophilia (Wang *et al.* 2015).

Lentiviral vectors were also used to express FVIII in mouse hepatocytes, in which transduction was optimized by adding a glycoprotein envelope from baculovirus GP64. Also, to reduce endoplasmic reticulum stress, FVIII cDNA was modified by including 256 amino acids with 11 potential asparagine-linked oligosaccharide linkages of the B domain, which increased the secretion of FVIII. Mice showed elevated FVIII levels and decreased levels of inhibitory antibodies against FVIII (Staber *et al.* 2017; Miao *et al.* 2004).

In a clinical trial including six patients with severe hemophilia B, patients received different escalation-doses (low, intermediate, and high dose) of the adeno-associated virus expressing FIX (AAV-FIX). FIX expression increased by 2 to 11% of the average plasma values for 6 to 16 months. In the long-term study, patients enrolled in the escalation-doses trial and four additional patients received a high dose of AAV-FIX. All patients showed an increase of 1 to 6% in FIX expression over a median period of 3.2 years, also 4 of the 6 patients in the high dose group showed a transient increase in alanine aminotransferase, suggestive of liver damage, but resolved over a median of 5 days (Nathwani *et al.* 2011; Nathwani *et al.* 2014).

Recently, by using an adeno-associated vector that expresses a hyper-functional variant of the FIX gene (*Padua*), it was possible to increase the factor's expression with only one injection of the vector in hemophilia B patients, which maintained a mean level of 33.7% ± 18.5% of the normal FIX value with drastically reduced bleeding (George *et al.* 2017).

Adeno-associated viruses have for gene delivery; however, the size of the FVIII gene exceeds the packing capacity of adeno-associated vectors (Wu *et al.* 2016). Nevertheless, FVIII delivery has been achieved in patients with hemophilia A using an adeno-associated virus that expresses optimized FVIII with a deleted B domain (AAV5-hFVIII-SQ). Patients in this study, who received a high dose of AAV5-hFVIII-SQ, presented normal FVIII values for a period of 20 to

24 weeks. Furthermore, after gene transfer, episodes of bleeding were reduced from an annual mean of 16 events to one event (Rangarajan *et al.* 2017).

Even though gene therapy with viral vectors has been the most studied gene therapy method to date, it has some disadvantages, including the immune responses against the vector and its oncogenic capacity (Gabrovsky and Calos 2008). In different non-adult animal models, gene expression decreases over time due to the vector dilution and loss of transduced cell population, which can limit the therapeutic effect, and the use of viral vectors in pediatric patients due to cell division during liver growth (Cunningham *et al.* 2008; Wang *et al.* 2011; Hu *et al.* 2011; Wang *et al.* 2012).

6. Gene therapy using nonviral vectors

Advances in efficiency, specificity, gene expression duration, and safety led to an increased number of non-viral vector products entering clinical trials (Wang *et al.* 2018). One of the non-viral vectors tested for hemophilia treatment is based on the *PiggyBac transposon system*, which transfers the complete cDNA of FVIII. This vector was tested in a murine model of hemophilia A. In this model, expression of the factor in the bloodstream was achieved as long as 300 days after administration, and no antibodies in the blood against FVIII were detected (Matsui *et al.* 2014).

Another promising strategy for the transgene expression is the use of "safe harbors", in which it has been proven that it is possible to insert the gene of interest without altering other gene expressions in the cell's genome. The most frequently used safe harbors are the loci ROSA26, H11, or AVS1, or the locus of ribosomic DNA (hrDNA). In a recent study, a non-viral vector (termed pHrneo) was used to deliver a reconstructed FVIII transgene (hFVIII-BDDAK39) in the hrDNA locus. FVIII transgene site-specific integration and expression was efficiently accomplished in the cell line HL7702 (Liu *et al.* 2007).

Transkaryotic implantation system implies isolation of the patient's somatic cells, transgene insertion in the cell's genome, isolation and propagation of clones stably expressing the gene, and its implantation in the patient. This delivery system was tested in a clinical study of hemophilia A patients, from whom skin fibroblasts were isolated. FVIII transgene was inserted in the fibroblasts genome, cells producing FVIII were cultured and injected into the patients. Four out of six patients showed an increase in FVIII activity with a concurrent reduction in the number of hemorrhages (Roth *et al.* 2001).

7. Genome editing to treat hemophilia

One of the disadvantages of in vivo and ex vivo gene therapy using viral and non-viral vectors for gene delivery is its insertion into any part of the genome, which can cause undesired mutations. Therefore, it is crucial to generate a strategy for site-specific gene integration into the genome. Currently, procedures using site-directed endonucleases performing specific breaks in the genome to integrate complete genes into safe sites of the genome are being conducted.

Correction of specific mutations or the insertion of complete genes is possible using site-oriented endonucleases. For several years, endonucleases, including zinc finger nucleases (ZFN), transcriptional activator-like nucleases (TALEN), and, more recently, the CRISPR/Cas9 system, have been used in gene therapy for hemophilia. These endonucleases break specific sites in DNA and activate intrinsic repair mechanisms in which desired sequences can be inserted or deleted (Park *et al.* 2016).

Different studies have reported successful in vitro correction of two of the main mutations that cause hemophilia A. Since inversion of intron 1 (140 kbp) in the FVIII gene is one of the most frequent mutations that cause hemophilia A, the TALEN system was used to correct it in induced pluripotential stem cells (iPSCs) to fibroblasts, and FVIII expression was reestablished (Park *et al.* 2014). Likewise, after using the TALENS system, in situ correction of the inversion that involves intron 22 was achieved; this inversion causes 45% of the cases of hemophilia A. The inversion was achieved in hemophilia A patient-specific iPSCs (Wu *et al.* 2016).

Afterward, with the CRISPR/Cas9 system, the intergenic inversion involving the intron 22 (600 kbp) of the FVIII gene was corrected in iPSCs induced from cells isolated from the urine of hemophilia A patients. The use of somatic cells isolated from urine is a safe method for obtaining somatic cells from hemophilia patients since this method does not represent a condition-related risk. These cells can be induced by transcription factors to form pluripotent stem cells and can differentiate into cell lines from the three germinal layers. In this study, it was proven that iPSCs corrected with CRISPR/Cas9 expressed the FVIII gene by mRNA analysis. These cells were differentiated into endothelial cells and were transplanted into a murine

hemophilia A model. Mice injected subcutaneously with these cells survived for a longer time than those not receiving the cells at the tail clip challenge, and also showed an increase in the FVIII activity in plasma (Park *et al.* 2015).

Viral vectors have been used for the delivery of the CRISPR/Cas9 system. However, one of the disadvantages of using them is their hepatotoxicity. Still, there are studies in which good therapeutic results have been achieved.

The CRISPR/Cas9 system has also been used to induce different mutations that cause hemophilia B. When hemophilia severity associated with each of the mutations in murine models was compared, it was found that a specific mutation (Y371D) causes more severe hemophilia B than other mutations. This murine model was later treated with different adenoviruses, naked DNA expressing the CRISPR/Cas9 system, and factor IX to correct the mutation in hepatocytes. The mice treated with the adenoviruses presented a higher degree of mutation correction but did not show a better therapeutic effect due to the adenovirus-associated hepatic toxicity (Guan *et al.* 2016).

Administration of an adeno-associated virus expressing Cas9 and guide RNA to wild type adult mice achieved the rupture of FIX gene in hepatocytes, which was enough to produce hemophilia B. Afterwards with the same vector, it was possible to insert the FIX cDNA by homology-directed repair (HDR) and the non-homologous end-joining (NHEJ) in one of the introns of the FIX mutated in hepatocytes, reestablishing the production of FIX and correcting hemophilia B (Ohmori *et al.* 2017).

One of the endonucleases most recently used to insert FVIII into safe loci (such as the multi-copy ribosomal DNA locus) is a TALENickase. Insertion of several copies of the FVIII gene was introduced with this endonuclease in iPSCs of patients with hemophilia A, and the FVIII mRNA and protein were detected (Pang *et al.* 2016)

Another safe locus is the AAVS1; it has an open chromosomal region that allows the insertion of an exogenous gene. Human full-length FIX cDNA was inserted into the AAVS1 locus of iPSCs of a Hemophilia B patient using CRISPR-Cas9. Then, these cells were differentiated to hepatocytes secreting FIX and transplanted into the NOD/SCID mice, which had hFIX antigen in plasma, representing an excellent tool for further exploration of in vivo research (Lyu *et al.* 2018).

A promising strategy is the use of the FVIII locus. A universal genome correction to restore FVIII expression was done in iPSCs derived from a patient with hemophilia A by insertion of the B-domain deleted FVIII gene in the FVIII locus. This study is the first demonstration that the FVIII locus is a suitable site for the integration of the normal FVIII gene. The restoration of the FVIII expression and a significant increase in FVIII activity were demonstrated in endothelial cells differentiated from hemophilia A patient-derived gene-corrected iPSCs in vitro. However, it is essential to confirm those results in vivo for future ex vivo clinical assays in patients with hemophilia A (Sung *et al.* 2019).

The last universal genome correction for hemophilia A was done in the H11 locus. This locus is an intergenic sequence; thus, the insertion of a transgene does not induce the gene disruption observed with AAVS1. Patient iPSCs corrected and differentiated to endothelial cells successfully secreted functional FVIII protein in vitro. This type of therapy could provide more efficient long-term therapeutic options in the future for patients with hemophilia and other monogenic diseases (Park *et al.* 2019).

8. Challenges and perspectives

One of the challenges in gene therapy is to ensure the safe delivery of the gene of interest. Therefore, clinical studies that prove that viral vectors are safe and do not have adverse effects that threaten patients' lives continue to be performed. Genome editing has proven to be efficient due to the precision by which the mutations are corrected. However, improvements in the editing system are necessary to avoid undesired alterations that may occur in the genome. For this reason, changes continue to be made in the nucleases so that they splice only the desired site. It is also necessary to demonstrate that the modified, undifferentiated cells are not capable of migrating to other sites of the organism and forming teratomas. Once these safety points are improved, these strategies can be used as treatment alternatives for hemophilia. Therapeutics using CRISPR/Cas9 is feasible; still, preclinical studies will detect and address potential immune and genetic repercussions that may arise in patients.

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