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Review

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Comparing CRISPR-Cas9 Gene Therapy Delivery Systems: Implications and Optimization for Hyperargininemia

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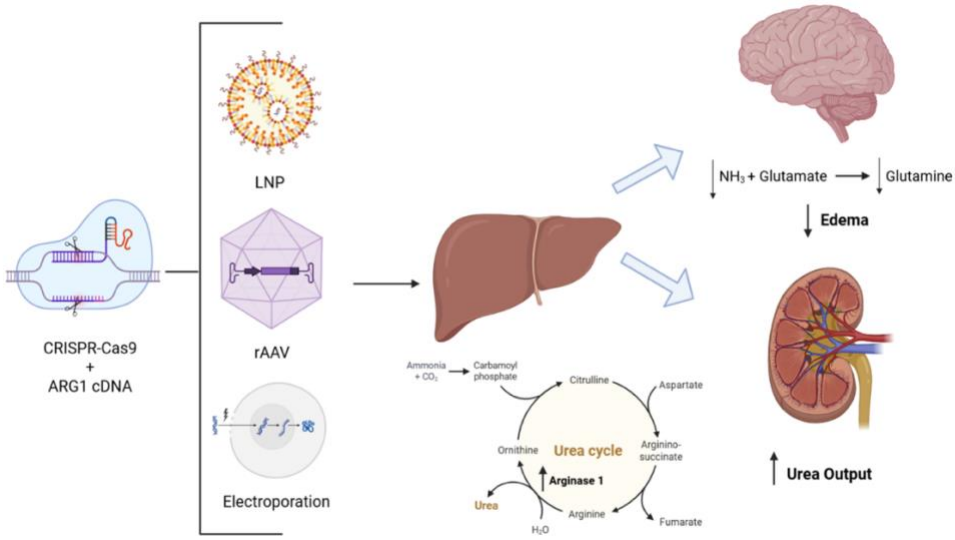
Graphical Abstract[†]



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Abstract

Hyperargininemia is a rare autosomal recessive metabolic disorder caused by a deficiency in the urea cycle enzyme arginase 1, resulting in severe systemic and neurological complications. Given the shortcomings of conventional treatment paradigms, there is an increasing demand for gene therapies, such as CRISPR-Cas9, that address hyperargininemia at its genetic root and reverse its diverse manifestations. Homology-directed repair offers high specificity and durable genetic correction. The strategic delivery of CRISPR components, including homology arm-flanked, wild-type ARG1 cDNA, Cas9 endonucleases, and appropriate sgRNAs, is necessary to enable homology-directed repair in arginase 1-deficient hepatocytes. This review summarizes the methodology, strengths, and weaknesses of three prominent CRISPR delivery methods: lipid nanoparticles, recombinant adeno-associated viruses, and electroporation. We also highlight ongoing efforts to optimize these delivery methods and analyze the relevance of existing preclinical CRISPR-Cas9 studies to hyperargininemia. Among these delivery systems, recombinant adeno-associated viruses currently show the most promise for clinical translation, although further enhancements in safety and scalability remain necessary.

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Rationale, Purpose, and Limitations

Hyperargininemia due to arginase deficiency is a condition that remains challenging to address with conventional treatments, necessitating durable correction at its genetic root. Although CRISPR-Cas9 gene therapy has recently emerged to meet this need, gaps in the literature regarding the implications of delivery system selection remain. The objective of this narrative review is to compare lipid nanoparticles, recombinant adeno-associated viruses, and electroporation in terms of their potential for therapeutic translation. Considerations regarding delivery systems and avenues for further research and optimization are also elucidated to ensure a critical, forward-looking assessment. There are key limitations to this review, namely its reliance on studies detailing delivery approaches for other hepatic genetic diseases, given the relative lack of preclinical and clinical literature on CRISPR-Cas9 therapy for hyperargininemia specifically. Although delivery methods used to correct similar hepatic genetic diseases were discussed in reference to their potential implications for hyperargininemia, the ability to draw definitive, translatable conclusions may be limited.

Introduction

Hyperargininemia is a rare autosomal recessive metabolic disorder caused by a deficiency in arginase 1, the urea cycle enzyme that catalyzes the hydrolysis of arginine into ornithine and urea [1]. Hyperargininemia is typically characterized by episodic hyperammonemia, the buildup of ammonia in the bloodstream that leads to severe physiological and neurological effects, including cerebral edema, spasticity, and impaired cognition, with an estimated prevalence of 1 in 1,000,000 live births [1,2]. Astrocytes convert excess ammonia into glutamine via glutamine synthetase, increasing glutamine concentrations in the brain and driving osmotic imbalances that result in astrocyte swelling and enhanced GABA-mediated inhibition of brain signaling [3, 4]. Although clinically asymptomatic at birth, hyperargininemia typically presents with seizures, spasticity, neurodevelopmental delays, and intellectual disabilities, typically beginning at age 2-4 [5,6]. These severe and debilitating effects can persist into adulthood and progressively worsen if left untreated, underscoring the urgent need for novel therapeutic interventions.

Advancements in genetic engineering, specifically the development of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 gene editing, offer a promising avenue for permanently treating a myriad of diseases [7]. CRISPR-Cas9 employs a single guide RNA (sgRNA) that directs Cas9 to catalyze the formation of sequence-specific double-strand breaks in DNA. Subsequently, the sequence of interest is incorporated via homology-directed repair (HDR) or non-homologous end joining (NHEJ). Although HDR enables accurate correction of disease-causing mutations, its efficiency is lower in non-dividing cells than the more error-prone NHEJ pathway [8]. In the context of hyperargininemia, this is an important translational barrier as adult hepatocytes are typically quiescent [9]. Therefore, delivery systems must not only achieve effective hepatocyte transduction but also maximize HDR template integration. The optimization of donor template design (i.e., homology arms) or transient knockout of NHEJ components is a promising preclinical approach but carries risks of genomic instability and oncogenic transformation [10]. Notably, novel approaches, such as base and prime editing, which do not depend on HDR, may offer promising alternatives, but their efficacy will nevertheless depend on efficient delivery to hepatocytes.

Unlike conventional treatments for hyperargininemia, such as liver transplantation and dietary adjustments, CRISPR-Cas9 may offer durable genetic correction that can comprehensively address metabolic and neurological aspects of the disease [11,12]. Few studies have specifically explored CRISPR-Cas9 for hyperargininemia; however, preclinical trials targeting other urea cycle enzyme deficiencies indicate that CRISPR-Cas9 has potential for this therapeutic application [13,14]. Furthermore, the feasibility of CRISPR-Cas9 was bolstered by *in vitro* studies demonstrating the restoration of arginase 1 expression and ureagenesis in human codon-optimized induced pluripotent stem cells derived from affected individuals [15].

Successful gene therapy for hyperargininemia requires the efficient delivery of CRISPR-Cas9 components to hepatocytes, the primary site of arginase 1 expression. In this review, we critically evaluate lipid nanoparticles, rAAVs, and electroporation as candidate delivery systems, highlighting their translational potential, limitations, and opportunities for optimization. Given the rarity of hyperargininemia and limited disease-specific preclinical studies, much of the current understanding is derived from related urea cycle disorders and hepatic genetic models [16]. Although these data provide a necessary foundation, their translational relevance must be interpreted critically, as disease-specific biology may alter delivery outcomes. Therefore, targeted preclinical research on hyperargininemia is essential to refine delivery strategies, establish safety and efficacy benchmarks, and accelerate clinical translation.

Lipid Nanoparticle-Mediated Delivery

Lipid nanoparticles (LNPs) are composed of ionizable lipids, polyethylene glycol (PEG)-modified lipids, helper lipids, phospholipids, and cholesterol. In ethanol, these components self-assemble into a spherical nanoparticle that encapsulates Cas9, sgRNA, and donor DNA templates [17]. The specificity of LNPs for hepatocytes is mediated by apolipoprotein E (ApoE), a glycoprotein that targets and binds to LNPs in the bloodstream and facilitates a high-affinity interaction with LDL receptors, many of which cluster on hepatocytes [18-20]. LNPs have become an attractive platform for therapeutic delivery in urea cycle enzyme deficiencies due to their potential to mediate liver-specific delivery of CRISPR-Cas9 and other therapeutic contents. LNPs first bind to LDL receptors to deliver the arginase 1 gene (ARG1) to hepatocytes, which are then endocytosed and delivered to an acidic endosomal lumen. Ionizable lipids accumulate positive charges within the endosome, destabilizing electrostatic interactions with nucleic acids and promoting LNP-endosomal membrane fusion [21]. Lipid components in the core and along the LNP surface redistribute, a process assumed to further trigger LNP escape from the endosome into the cytosol [22]. At this point, the wild-type ARG1 HDR template enters the nucleus for integration and transcription, whereas, if delivered as mRNA, Cas9 is translated by cytosolic ribosomes into a functional endonuclease [23].

Methodological Insights

Before translating LNPs to the clinic, ionizable lipids that maximize transfection efficiency and arginase 1 deficiency correction must be identified. In a study delivering ARG1 mRNA and firefly luciferase mRNA to mouse hepatocytes *in vivo*, two lipids were used; one significantly upregulated arginase 1 protein expression and resulted in a 40-fold increase in luciferase expression compared with the other [11]. This indicates that lipid composition can be optimized to improve ARG1 expression and that the optimal proportions of lipid subtypes comprising LNPs must be determined to ensure maximal CRISPR-Cas9 delivery to hepatocytes. These optimization protocols have been applied to LNP-based delivery systems in other clinical contexts. The lipid composition used in the Pfizer-BioNTech and Moderna COVID-19 vaccines (50:10:38.5:1.5 ratio of ionizable lipid: phospholipid: cholesterol: PEGylated lipid) has become a reference for liver-targeted formulations, including those used in the RNA-based therapeutic Patisiran [24,25]. A similar approach has been applied in CRISPR-mediated *in vivo* genome editing of the ANGPTL3 gene, where LNPs enabled stable, long-term therapeutic efficacy for over 100 days following a single-dose administration in mice [26]. Given the universality of hepatic targeting mechanisms for LNPs, these previous studies offer potentially generalizable insights for optimizing LNP composition via lipid selection and formulation tuning to maximize delivery of the CRISPR-Cas9 component for related liver diseases, such as hyperargininemia.

Clinical Takeaways

LNPs offer several clinical advantages over other gene therapy delivery vectors, including high scalability, efficient manufacturing, and cost-effective production [27]. A large cargo capacity enables effective encapsulation and transport of all CRISPR components [28]. However, mild immunogenicity, relatively low pharmacological stability, and editing duration of LNPs remain barriers to clinical translation [29]. Transfection efficiency is reduced because hepatic clearance via Kupffer cell uptake, immune-mediated opsonization, and other interactions with plasma proteins shorten

the LNP's circulation half-life [30]. PEGylated lipids may attenuate these effects by enabling LNPs to resist opsonization, thereby reducing their immunogenicity and prolonging their circulation [31,32].

Additionally, as nonviral vectors, LNPs may trigger inflammatory responses, necessitating careful assessment of long-term safety, efficacy, and potential off-target effects before clinical application [30,33,34]. Although LNP-mediated CRISPR delivery has proven effective in preclinical models of other liver diseases, hyperargininemia-specific studies are lacking [35]. Hence, the translation potential of LNPs in this context remains mostly predictive, underscoring the need for rigorous testing in human-relevant models, such as patient-derived hepatocytes or humanized mouse livers, to better estimate LNP uptake and genome-editing outcomes.

Recombinant Adeno-Associated Virus-Mediated Delivery

Recombinant adeno-associated viruses (rAAVs) are gene therapy delivery vehicles preferred for their low cytotoxicity and capacity for long-term cDNA expression, reducing the need for frequent, repeated administrations [36]. Following injection, rAAVs facilitate cellular transduction of therapeutic cargo by entering the nucleus via an episome, rendering the homology-arm-flanked ARG1 cDNA construct available for HDR [37].

These delivery systems require the design of three distinct plasmids to be effective: the vector plasmid, which carries the wild-type ARG1 expression cassette; the helper plasmid, which contains E4, E2a, and VA genes for viral genome replication; and the packaging plasmid, which provides rep and cap genes for replication and capsid production [36,38]. The extended episomal persistence of these vectors (up to 1 year in the liver) accounts for their widespread use, providing sustained gene expression in hepatocytes [39]. Among the 11 known serotypes of rAAV, serotype 8 shows a notable affinity for hepatocytes and has been shown to transduce 90-95% of the therapeutic content into mouse hepatocytes [40, 41].

Methodological Insights

The current research on rAAV-based therapies for hyperargininemia has shown promising outcomes. In a study by Lee *et al.*, administration of rAAV therapy in ARG1-deficient mice with an exon 4 deletion successfully restored hepatic arginase 1 expression, leading to improved survival, reduced ammonia levels, and mitigated growth retardation [42]. These findings not only define the therapeutic potential of rAAVs for metabolic liver disease but also demonstrate that maximal phenotypic correction may depend on supraphysiologic levels of ARG1 activity, a more demanding criterion than protein re-expression alone. Furthermore, given the relatively low cargo capacity of ~4.7 kb in standard rAAVs, oversized rAAVs have been explored as an optimal strategy for the all-in-one delivery of therapeutic components [43]. This strategy has demonstrated success in carbamoyl phosphate synthetase 1 (CPS1) deficiency, as reported by [44], who observed enhanced CPS1 expression, reduced ammonia levels, and improved survival using an oversized rAAV vector. Nitzahn *et al.* achieved similar outcomes with a split-rAAV system, where the CPS1 transgene and its regulatory cassette were packaged into 2 separate vectors [44,45]. Given that ARG1 cDNA, homology arms, sgRNA, and Cas9 may exceed rAAV capacity, particularly without a smaller Cas9 variant, oversized and split-rAAVs warrant consideration for CRISPR-mediated hyperargininemia therapy [46]. The success of these approaches in CPS1 deficiency, a related urea cycle disorder, underscores the need to explore analogous approaches for the treatment of arginase 1 deficiency.

Clinical Takeaways

One principal advantage of rAAV-based approaches is episomal persistence in the host cell's nucleus and the potential for long-term, stable gene expression [37,47]. Unlike retroviruses, rAAVs do not typically integrate into the host genome at high levels, reducing the risk of off-target effects from random insertions. This makes rAAVs particularly suitable for the treatment of pediatric metabolic disorders, where permanent hepatocyte modification may carry safety concerns. Yet, stable expression is essential for disease control, and integrated vector genomes have the potential to achieve this [47]. rAAVs are preferable for regenerative medical applications for treating diseases resulting from mutations in a single gene, such as Duchenne muscle dystrophy, lipoprotein lipase

deficiency, and sickle cell anemia [48,49]. rAAVs also have relatively long circulation half-lives, with rAAV8 demonstrating a half-life of over 11 h in vivo [50]. This extended circulation time allows sustained gene expression without the need for high-dose administration, mitigating the risk of vector-related toxicity [36]. Notably, rAAV serotype 8 is hepatocyte-specific and therefore a promising candidate for treating arginase 1 deficiency [40,41].

Despite these advantages, rAAV-based approaches face barriers to clinical translation. Immunogenicity remains a key concern, potentially compromising transgene expression in host models [51]. Although the FDA has approved six AAV-based therapies, patients' immune responses to these treatments, particularly at higher doses, are not yet fully understood [52]. Site-directed mutagenesis of rAAV capsids may reduce immunogenicity but is challenging to study due to complex pre-existing rAAV immunity patterns worldwide. This often requires immunosuppressant therapy, a burden further exacerbated by pre-existing anti-Cas9 immunity [53]. Moreover, rAAVs present scalability challenges due to high production and purification costs and low cell culture yields [54-56]. Overcoming such limitations requires further investigation into anti-immunogenic approaches and the development of low-cost manufacturing methods to improve their feasibility for the treatment of hyperargininemia.

Electroporation-Mediated Delivery

CRISPR-Cas9 delivery via electroporation is performed by applying electrical pulses, rendering hepatocytes susceptible to CRISPR sgRNA, HDR donor template, and Cas9 enzyme uptake. *In vivo*, 2-4 electrodes are arranged in an array-like pattern and are typically inserted directly into the liver parenchyma, hepatic artery, or portal vein before stimulation [57,58]. Laparoscopic incisions, typically used for diagnosis and treatment of diseases affecting the abdominal and reproductive systems, may be used for minimally invasive placement of intrahepatic electrodes [59]. Despite these advantages, electroporation-based therapies can cause off-target effects by forming hydrophilic pores in the plasma membrane, thereby enabling controlled molecular exchange [60]. Such electrical pulse-induced stimulation leaves cells vulnerable to cytoskeletal damage, proteasomal degradation, and lipid oxidation, all of which contribute to increased hepatotoxicity and cell death [60].

Methodological Insights

In vitro studies by Sin *et al.* investigated the delivery of sgRNA, the Cas9 enzyme, and a repair template comprising ARG1 exons 7 and 8 via electroporation. They determined that this approach partially rescued ARG1 expression in mouse hepatocyte-like cells, compared to no expression in unrepaired ARG1-deficient control mice. In addition, the mRNA levels of other urea cycle enzymes increased, suggesting upregulation of ureagenesis [61]. Although promising, this partial restoration highlights that electroporation may be less efficient than viral vectors in vivo.

Hepatocyte preconditioning has emerged as a strategy for reducing electroporation-mediated cytotoxicity. In vitro electroporation of donor mouse hepatocytes, followed by reversible portal vein embolization and subsequent transplantation, has demonstrated enhanced safety and control, proving more plausible yet logistically complex than in vivo transfection [62]. Because ROS-induced apoptosis is likely to occur under hypoxic conditions associated with portal vein embolization, antioxidants such as reduced glutathione and superoxide dismutase may also be administered as a preconditioning strategy [63-65].

Rathbone *et al.* employed such an approach, performing retrograde perfusion and isolating mouse hepatocytes before electroporation, resulting in a transfection rate of 89%. When Cas9 was delivered as a ribonucleoprotein rather than mRNA, the target CRISPR-Cas9 editing efficiency increased from 47% to 78% [66]. Antioxidant preconditioning may thus be essential to ensure the long-term survival and repopulation of CRISPR-Cas9 ARG1-transfected hepatocytes in the liver parenchyma following electroporation.

Delivery efficiency may also be enhanced by optimizing the duration and strength of the electroporation pulse. Yao *et al.* determined that electroporation with low voltage (220-400 V) and high capacitance (950 μF) was ideal for *in vitro* delivery of foreign genes to hepatocytes [67]. A separate study found that 8–10 pulses of 20 ms at 250 V/cm were optimal for

transfecting blood-clotting factor VII into hemophilic hepatocytes [68].

Table 1: Comparison of CRISPR-Cas9 Delivery Systems.

Parameter	LNPs	rAAVs	Electroporation
Editing Duration	Transient [29]	Long-term [47]	Transient [66]
Hepatocyte Specificity	High [18,19,20]	High for certain serotypes (AAV 8, AAV 9) [40,41]	Variable *in vivo*, high *in vitro* [62]
Circulation Half-Life	Low [19,32]	High (rAAV 8: 11 h) [48]	N/A
Cargo Capacity	High [28]	Generally low (4.7 kb) [42-44]	Very high [69,70]
Production Complexity	Low [27]	High [54]	Low [71,72]
Scalability	High [27]	Low [55,56]	High [72]
Safety	Nonviral, potentially immunogenic [30,33]	Viral, potentially immunogenic [51,52]	Nonimmunogenic, potentially highly cytotoxic *in vivo*, generally safer *in vitro* [60,62]

Qualitative descriptors summarize trends reported in cited studies. Data are largely extrapolated from other hepatic disease models; relevance to ARG1 deficiency may vary.

These foundational findings underpin current optimization efforts, although electroporation systems have since evolved to microsecond- and nanosecond-pulsing, microfluidic-precision control, and CRISPR-adapted delivery systems. To date, these have not yet been tested in human hepatocytes with ARG1 deficiency. Additional studies will be required to delineate pulse parameters and device configurations that optimize ARG1-targeted CRISPR-Cas9 delivery for clinical translation in hyperargininemia.

Clinical Takeaways

The advantages of electroporation over other delivery methods include high cargo capacity, ability to simultaneously permeate large cell volumes, and simple, cost-effective production, with microcell electroporation being particularly economical [69-72]. However, electroporation-induced editing is transient, necessitating repeated administrations for sustained restoration of genetic expression [66]. Additionally, hepatocyte specificity is variable *in vivo*, which is why *in vitro* electroporation and subsequent transplantation are often preferred to minimize invasiveness and off-target effects [62]. However, the persistent hepatotoxicity associated with pore formation, which has not yet been adequately addressed by preconditioning or pulse optimization, is the most concerning. Unlike LNPs and rAAVs, electroporation is highly invasive *in vivo,* limiting its clinical practicality for chronic conditions such as hyperargininemia. Nevertheless, further research should aim to optimize dosing, preconditioning strategies, and other electroporation protocols to minimize toxicity and enable clinically viable liver-targeted electroporation gene therapies for hyperargininemia (see Table 1).

Discussion

The discovery of CRISPR-Cas9 gene editing offers promising therapeutic options for difficult-to-treat diseases, including urea cycle enzyme disorders such as arginase 1 deficiency. Compared with traditional treatments, such as liver transplantation and dietary restriction, CRISPR-Cas9-mediated HDR of the ARG1 gene has shown greater potential in addressing metabolic and neurological manifestations of the disease [11]. However, the success of this approach hinges on selecting a delivery vehicle

that is efficacious, safe, and capable of long-term therapeutic effects.

Among the discussed approaches, we propose that rAAVs are the most promising for clinical application, offering sustained gene expression and high hepatocyte specificity. Adverse immune responses arising from pre-existing immunity remain a critical hurdle to clinical translation. Although LNPs provide a nonviral, safer alternative, their limitations in pharmacokinetics, endosomal escape efficiency, and transient expression necessitate further optimization before clinical use. Electroporation has high efficiency for ex vivo hepatocyte transfection but is cytotoxic, tissue invasive, and transiently expressed, limiting its clinical applicability, particularly in juvenile patients or those requiring repeated dosing.

The long-term effects of CRISPR-Cas9 remain uncertain as a novel gene-editing technology. Several factors, including variability in symptom severity among patients, patient age, and the presence of pre-existing immunity to Cas9 in certain populations, may affect the effectiveness of gene therapy for hyperargininemia. Therefore, it is essential to optimize delivery systems for individual patients' needs. Future clinical trials of hyperargininemia gene therapy should ideally conduct head-to-head comparisons of these delivery modalities to establish their efficacy *in vivo and* explore personalized approaches tailored to patient-specific factors. Together, these considerations highlight the cumulative findings of the current literature and emphasize actionable pathways to advance hepatic regenerative medicine, enabling the development of effective and safe CRISPR-Cas9 therapies in the clinic.

Conclusions

CRISPR-Cas9 gene therapy has shown preclinical promise, yet delivery remains a key bottleneck for effective clinical translation. This is particularly the case for hyperargininemia, where fine-tuning of liver-directed delivery is paramount to correct arginase deficiency. rAAVs currently demonstrate potential to be the most viable delivery approach due to their high hepatic specificity and ability to mediate persistent gene expression. Despite this, several refinements are necessary to balance safety and efficacy for widespread clinical implementation.

Abbreviations: ARG1: Arginase 1 gene, LNP: Lipid Nanoparticle, ANGPTL3: Angiopoietin-like 3 gene, rAAV: recombinant adeno-associated virus, bFGF: Basic Fibroblast Growth Factor, CPS1: Carbamoyl Phosphate Synthetase.

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