UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

NEONATAL INTRAVENOUS INJECTION OF CRISPR/CAS9 LIPOSOMAL COMPLEX HAS NO INCIDENCE OF OFF-TARGETS IN MICE

EDUARDA PERES COUTO

Porto Alegre, setembro de 2022

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

Eduarda Peres Couto

NEONATAL INTRAVENOUS INJECTION OF CRISPR/CAS9 LIPOSOMAL COMPLEX HAS NO INCIDENCE OF OFF-TARGETS IN MICE

Trabalho de Conclusão de Curso apresentado ao Curso de Farmácia da Universidade Federal do Rio Grande do Sul como requisito à obtenção do título de grau de Farmacêutica.

Orientador: Profª. Drª. Roselena Silvestri Schuh

Porto Alegre, setembro de 2022

AGRADECIMENTOS

Gostaria de agradecer primeiramente à minha orientadora, professora Roselena Schuh, que sempre me apoiou e esteve presente quando precisei, dando suporte tanto em questões de faculdade, quanto pessoais. Agradeço também aos meus pais e à minha irmã, que desde o início me incentivaram e torceram por minhas conquistas. Meus amigos foram igualmente fundamentais, tanto os antigos, quanto os que fiz durante a faculdade, já que me acompanharam e tornaram essa jornada muito mais leve. Por fim, agradeço à UFRGS, à Faculdade de Farmácia e aos profissionais que nela atuam, pois me possibilitaram ter uma educação pública gratuita e de qualidade, me proporcionando experiências e aprendizados que levarei para sempre junto comigo.

APRESENTAÇÃO

Este trabalho foi escrito em formato de artigo científico para ser submetido ao periódico **Journal of Inherited Errors of Metabolism and Screening**, cujas normas para submissão estão contidas em Anexo.

NEONATAL INTRAVENOUS INJECTION OF CRISPR/CAS9 LIPOSOMAL COMPLEX HAS NO INCIDENCE OF OFF-TARGETS IN MICE

Eduarda Peres Couto[2], Édina Poletto[1,3], Bruna Brazeiro Brum[2], Bruna Medeiros-Neves[1]; Nathalya Tesch Brazil[1]; Flávia Nathiely Silveira Fachel[1], Giselle Renata Martins[1,2], Ana Helena da Rosa Paz[1,2], Helder Ferreira Teixeira[1], Ursula Matte[,2,3], Roberto Giugliani[2,3], Guilherme Baldo[2,3], Roselena Silvestri Schuh[1,2,#]

[1]Programa de Pós-Graduação em Ciências Farmacêuticas da UFRGS, Faculdade de Farmácia, Porto Alegre, RS, Brazil.

[2]Grupo de Pesquisa Células, Tecidos e Genes, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.

[3]Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS, Instituto de Biociências, Porto Alegre, RS, Brazil.

Corresponding author: Roselena S. Schuh

E-mail: roselena.schuh@ufrgs.br

Telephone: +55 51 33085278

ABSTRACT

Gene therapy has been increasingly researched for the treatment of diseases and the CRISPR/Cas9 system is considered a highly advanced and specific tool. However, it is necessary to reduce the chances of off-target cleavage, in order to reduce the harmful effects of mutations and chromosomal rearrangements, which may be a side effect of gene editing. Consequently, it is imperative to highlight the need to identify potential off-target cleavage sites that can cause mutagenesis and consequent tumor induction. In this sense, we analyzed off-target site prediction after hydrodynamic injection of liposomes as vectors of the CRISPR/Cas9 system in newborn mice. The overall characterization of formulations showed complexes of about 133 nm, with positive zeta potential of +43 mV. The biodistribution of complexes after hydrodynamic injection was markedly detected in the liver, lungs, and heart, which became the main target tissues of this study. Off-target experimental analysis based on the potential sites obtained from *in silico* predictions showed 0% of indels in the liver and lungs. We conclude that the set of experiments showed the potential of the chosen gRNA sequence to perform a safe gene editing in the murine *ROSA26* locus.

KEYWORDS: liposome, tumor, ROSA26, gene editing, mutation

RESUMO

A edição gênica tem sido cada vez mais pesquisada para o tratamento de doenças, sendo o sistema CRISPR/Cas9 considerado uma ferramenta de clivagem de DNA altamente avançada e específica. No entanto, é necessário reduzir as chances de ocorrerem clivagens fora do alvo pretendido, denominados eventos *off-target*, a fim de evitar os efeitos nocivos de mutações e rearranjos cromossômicos, que podem ser uma consequência dessa edição. Dessa forma, é importante enfatizar a necessidade de identificar potenciais sítios de eventos *off-target* que possam causar mutagênese e consequente indução tumoral. Para isso, realizamos a injeção hidrodinâmica de complexos lipossomais de CRISPR/Cas9 em camundongos recém-nascidos. A caracterização geral das formulações apresentou complexos com cerca de 133 nm, e potencial zeta positivo de +43 mV. A biodistribuição dos complexos após a injeção hidrodinâmica ocorreu principalmente no fígado, pulmões e coração, que se tornaram os principais tecidos alvos deste estudo. A análise experimental com base nos potenciais sítios obtidos através de previsões *in silico* mostrou 0% de indels no fígado e nos pulmões. Concluímos que o conjunto de experimentos demonstrou que a sequência de gRNA escolhida tem potencial para realizar uma edição gênica segura no locus murino *ROSA26*.

PALAVRAS-CHAVE: edição gênica, lipossoma, mutação, ROSA26, tumor

INTRODUCTION

Gene therapy has been increasingly researched for the treatment of diseases considered incurable, both preventive and therapeutic. The ability to transfer genetic material inserted by a vector (plasmid, viral or nanostructured) generated through recombinant DNA is a promising alternative in researching genetic diseases. Several non-clinical and clinical protocols have being studied over the years, focusing on several diseases, such as numerous types of cancer, inborn errors of metabolism, neurodegenerative disorders, and congenital pathologies[1]. With the increasing development of genetic engineering, cloning techniques and the use of plasmids as a "pro-drug" employing genes capable of suppressing or promoting the production of certain proteins, the use of gene therapy for the treatment of diseases has become more palpable [2].

In this context, several systems that promote the integration of genetic material into the genome are being researched. The most innovative has been shown to be genomic editing using the CRISPR/Cas system (Clustered Regularly Interspaced Short Palindromic Repeats). This system is composed of a guide RNA and a nuclease, and together this complex cleaves sequences of interest [3], which can be used for various applications in the genetic field. This Cas9 protein-associated (CRISPR/Cas9) genome editing approach demonstrates great potential for inserting or deleting genes at specific genomic locations. The

CRISPR/Cas9 system is a highly advanced and specific tool for gene therapy. However, it is necessary to reduce the chances of off-target cleavage, in order to reduce the harmful effects of mutations and chromosomal rearrangements. Studies with CRISPR/Cas9 also demonstrated the ability of off-target activity due to hybridization and it was revealed that the modified DNA may contain insertions or deletions, as well as base incompatibility, thus resulting in cleavages and mutagenesis in the genome [4,5]. These studies highlight the need to identify potential off-target cleavage sites that can cause mutagenesis and consequent tumor induction. Therefore, it is essential to check these off-target sites in order to ensure the safety of these treatments.

However, although gene therapy proposes a range of promising treatments, its application depends on the successful internalization of DNA molecules directly into target tissue cells, and it faces several limitations, such as the intracellular stability of nucleic acids and interaction with plasma proteins [6]. The introduction of genetically modified DNA is performed using viral and non-viral vectors [7]. Viral vectors are derived from different classes of viruses and undergo modifications to eliminate their pathogenicity and insert the gene of interest. Despite having high transduction capacity, they have low therapeutic safety, in view of the possibility of reverting to their initial form and binding to the genome in an unwanted location, causing insertional mutagenesis. On the other

hand, non-viral vectors present biocompatible particles in their structure, where the DNA plasmid that contains the therapeutic gene of interest is encapsulated or linked to a positive residual charge on the surface or core of the nanostructure. These structured carriers have versatile properties such as biodegradability, biocompatibility, nontoxicity, reduced immunogenicity, and low cost of production [8,9]. In this sense, liposomes are nanostructured carriers composed of a bilayer of phospholipids containing a water core. DNA can be adsorbed at the vesicle interface or inside the structure when associated with cationic lipids, and thus can be protected against degradation and can be carried inside the cells complexed to this transporter [8].

In this sense, previous studies of our research group evaluated the hydrodynamic administration of liposomal vectors complexed to plasmids of the CRISPR/Cas9 system and a donor plasmid of the complete cDNA of *IDUA* gene for the treatment of MPS I mice (mucopolysaccharidosis type I), in order to correct the defect in the gene of the alpha-L-iduronidase enzyme (IDUA), responsible for the catabolism of the glycosaminoglycans dermatan and heparan sulfate [10]. Experimental treatment using the complexes demonstrated increased gene expression and enzymatic activity in the previous work, reaching about 5% of normal mice IDUA activity serum and tissue levels [10–12].

Finally, in view of the positive results achieved in previous studies and based on the need to investigate this promising approach, the safety of these complexes were assessed *in vivo* to evaluate the potential to produce off-target effects, awaiting that this treatment can, in the near future, be used as therapy in patients affected by MPS I.

MATERIALS AND METHODS

Vectors

The same PrecisionX CRISPR/Cas9 SmartNuclease™ system (System Biosciences, USA) was used for *in vivo* genomic editing experiments, except that the target sequence for cleavage by the Cas9, 5'ggattctcccaggcccaggg3', was selected at the *ROSA26* locus of the mouse genome and was inserted into the vector [13].

For homologous recombination, a vector containing the *Idua* cDNA that was customized by the company System Biosciences (USA) was used. The construct contains the mouse *Idua* cDNA sequence regulated by an EF promoter and two homologous regions (approximately 1 Kb each) to the *ROSA26* locus of mice, in the region that Cas9 recognizes and cleaves. It also contains a hygromycin resistance gene for future experiments involving clone selection.

Preparation of formulations and complexes

Liposomal formulation was prepared by microfluidization and the liposomal complexes (LC) and fluorescent liposomal complexes were prepared by adsorption of DNA onto blank liposomes at +4 /-1 charge ratio, as previously described [10]. The charge ratio is due to the presence of the cationic lipid DOTAP, which confers a positive charge to the formulation, while the presence of nucleic acids and the pegylated phospholipid DSPE-PEG confer the negative charge.

Physicochemical characterization of liposomes and complexes

The droplet size and polydispersity index (PDI) were determined by photon correlation spectroscopy at 25°C after appropriate dilution of samples in water. The ζ-potential was determined by electrophoretic mobility at 25°C after appropriate dilution with 1mM NaCl solution. The measurements were performed using a Zetasizer Nano-ZS90® (Malvern Instruments, England, GB) equipment.

In vivo **assay**

Animals

Newborn C57BL/6 mice (2-3 days old) (n=6) were used for the experiments. The treatments consisted in one hydrodynamic injection (10% of body weight) of LC complexes (called only “Treated” group) in the superficial

temporal vein of newborn mice. Mice were weekly weighted and blood was collected for determination of serum Idua activity monthly after treatment. After 21 months, mice were anesthetized with isoflurane prior to cervical dislocation and perfusioned through the portal vein with a solution containing at least 20mL of 0.9% NaCl for 3 min to eliminate blood. One untreated control group was used (n=6).

Biodistribution of fluorescent complexes after newborn injection

One experimental group of newborn mice (n=4) received one single injection of fluorescent liposomal complexes in the superficial temporal vein, as described above. One minute after treatment, mice were euthanized by decapitation by guillotine with a sharp blade. Blood was collected in EDTA. Brain, lung, heart, liver, spleen, and kidney tissues were removed and mounted on a metal sample holder using Tissue-tek O.C.T™ (Sakura Fine Technical, JPN). Then, the block was frozen at -80°C, and cut in 30 µm thick slices with a cryostat (Leica CM 1850, JPN). The slices were mounted on a microscope slide and analyzed under a fluorescence microscope (Olympus BX51TF, JPN). The images were taken at 200x magnification.

Off-target analysis

Part of the animals' tissues was flash frozen immediately after collection and stored at -20ºC. Liver and lung samples were selected for initial analysis, as they were the organs with the highest percentage of editing and enzyme production in the previous studies[10]. Total DNA from these tissues was isolated using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's recommendations. Possible sites of off-target action of the gRNA GGATTCTCCCAGGCCCAGGG(NGG) were identified using the COSMID online tool (https://crispr.bme.gatech.edu/) [14]. Five possible sites were identified on chromosomes 2, 5, 11, 17, and X. To analyze these regions, primers were designed so that each region was amplified by PCR and produced an amplicon of approximately 700 bp. The primers used were:

Chr2: F-tcaactgtttgagccagctcaagg and R-ggctttgcctggctaacagattac;

Chr5: F-acggcaaaggtagcaggcag and R-agcacgcccactacagggtt;

Chr11: F-gtagataaggagctcaggtagcc and R-ctgccccagatgtagtctgaac;

Chr17: F-gaagtgtatggctgccatgtgc and R-gtggagtttggatggccttcg;

ChrX F-gcctggagcctcaagaaatgtc and R-cgtctctggagatgccttcatag.

The regions of interest were amplified using the enzyme Phusion Green High-Fidelity DNA Polymerase (2 U/µL) (ThermoFisher Scientific) as recommended by the manufacturer. The amplification reactions were performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems) with an initial

denaturation step at 98ºC for 3 min, followed by 35 cycles of 98ºC for 10 sec, 64ºC for 10 sec and 72ºC for 20 sec and finished with a step at 72ºC for 10 min. Amplicons were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's instructions. The sequencing of the samples was performed at the Molecular and Protein Analysis Unit (Centro de Pesquisa Experimental, HCPA, BR) using the ABI 3500 Genetic Analyzer equipment with 50 cm capillaries and POP7 polymer (Applied Biosystems, USA). PCR products were labeled using 5.0 pmol of forward primers from each region and 1 μL of BigDye Terminator v3.1 Cycle Sequencing reagent Kit (Applied Biosystems, USA) in a final volume of 10 μL. The labeling reactions were performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems) with an initial denaturation step at 96ºC for 1 min followed by 35 cycles of 96ºC for 15 sec, 50ºC for 15 sec and 60ºC for 4 min. After labeling, the samples were purified by precipitation with BigDye XTerminator Purification Kit (Applied Biosystems) and electroinjected into the genetic analyzer. The resulting chromatograms were analyzed using the ICE Analysis software (Synthego, USA), which compares the sequences of samples submitted to the editing protocol with that of a control, and estimates the percentage of indels generated at the site of possible cleavage. The higher the percentage of indels, the greater the CRISPR/Cas9 system activity at the site.

Ethics

All experiments were approved by the ethics committee of our institution (Research Ethics Committee - Hospital de Clínicas de Porto Alegre #20160482). Animal procedures were carried out in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health[15], monitored by our veterinarian and designed to minimize animal suffering. Possible gender effects were analyzed in all tests, and no significant differences were found between males and females if not specified.

Statistics

Results were presented as mean ± standard deviation of at least three independent experiments. Group differences were analyzed by Student's T test or One-Way ANOVA, with Tukey as *post hoc*, using the PASW Statistics 18 software (v 18.0; SPSS, IBM, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Physicochemical characterization of complexes

The results of physicochemical characterization of complexes are summarized in Table 1. LC exhibited droplet size of approximately 133 nm , while

PDI was below 0.15, indicating monodisperse formulations. In addition, there was no difference inter days when comparing droplet diameter, although PDI results increased with storage time. ζ-potential was around +43 mV.

Table 1. Physicochemical characterization of liposomal complexes.

Formulation 0/7/30 days	Mean diameter (nm)	PDI	ζ-potential (mV)
LC	133.0 ± 3.29	0.101 ± 0.01	+41.50 ± 0.95
	139.0 ± 3.05	0.132 ± 0.02*	+43.80 ± 1.41
	138.9 ± 1.01	0.143 ± 0.01*	+46.07 ± 0.55

PDI.: polydispersity index; LC: CRISPR/Cas9 and *Idua* donor plasmids liposomal complex. *Significant difference ($p < 0.05$).

Formulation	Days	Mean diameter (nm)	PDI	ζ-potential (mV)
LC	0	133.0 ± 3.29	0.101 ± 0.01	+41.50 ± 0.95
	7	139.0 ± 3.05	0.132 ± 0.02*	+43.80 ± 1.41
	30	138.9 ± 1.01	0.143 ± 0.01*	+46.07 ± 0.55

PDI.: polydispersity index; LC: CRISPR/Cas9 and *Idua* donor plasmids liposomal complex. *Significant difference ($p < 0.05$).

Vector biodistribution after intravenous injection

We observed that our complexes go primarily to the lung and liver, as seen in fluorescence analysis (Figure 1). The number of transgene copies in these organs, along with kidney, liver, and spleen made us perform off-target analysis in these organs, as well as on any abnormal-appearing regions.

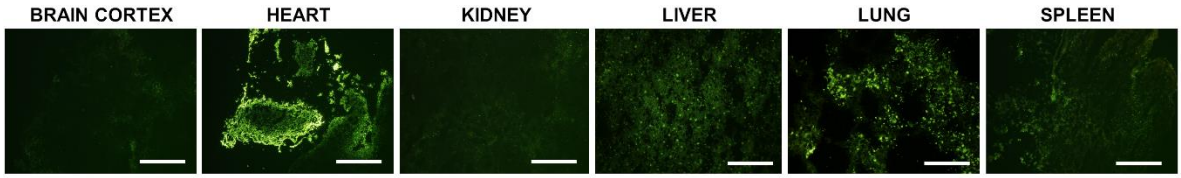

Figure 1. Biodistribution of fluorescent liposomal complexes after injection. The biodistribution of 'fluorescent LC' complexes in newborn MPS I mice after a single injection in the superficial temporal vein was analyzed under a fluorescence microscope. Images were acquired in fluorescence (Ex/Em = 596/619 nm) at 200x (scale bars 100 μm) magnification. Untreated MPS I mice had no detectable fluorescence in any of the analyzed organs.

Off-target analysis

Five possible off-target sites were analyzed in lung and liver samples from treated animals. These tissues were chosen because they showed the highest

biodistribution [10] and the highest percentage of editing (about 3%) compared to the others and, therefore, would present a greater chance of occurrence of off-target mutations.

Table 2. Potential off-target sites obtained with COSMID tool (https://crispr.bme.gatech.edu/).

Result	Query type	Mismatch	Ends with RG	Chr Position	Strand	Cut site	Score
GGATTCTCCCAGGCCCAGGGCGG -- hit GGATTCTCCCAGGCCCAGGGNGG -- query	No indel	0	Yes	Chr6:113076075-113076097	+	113076091	0
TGATTCTCCCAGGCCCAGGGAAG -- hit GGATTCTCCCAGGCCCAGGGNGG -- query	No indel	2	Yes	Chr5:113445565-113445587	-	113445571	20,12
GGCTTCTCCCAGGCCCAGGATGG -- hit GGATTCTCCCAGGCCCAGGGNGG -- query	No indel	2	Yes	ChrX:166717699-166717721	-	166717705	6,15
GGAATCTCCCAGGCTCAGGGAGG -- hit GGATTCTCCCAGGCCCAGGGNGG -- query	No indel	2	Yes	Chr2:69238534-69238556	-	69238540	2,07
GGAGTCTCCCAGGCCTAGGGTGG -- hit GGATTCTCCCAGGCCCAGGGNGG -- query	No indel	2	Yes	Chr11:73171299-73171321	+	73171315	2,47
GATTCTCCCAGGCCCAGGGCGG -- hit GATTCTCCCAGGCCCAGGGNGG -- query	Del 19, or Del 20	0	Yes	Chr6:113076076-113076097	+	113076091	0,63
GATTCTCCCAGGCCCAGGGAAG -- hit GATTCTCCCAGGCCCAGGGNGG -- query	Del 19, or Del 20	1	Yes	Chr5:113445565-113445586	-	113445571	20,63
GATTCTCCCAGGCCCAGGGCGG -- hit GGTTCTCCCAGGCCCAGGGNGG -- query	Del 18	1	Yes	Chr6:113076076-113076097	+	113076091	0,79

GGATTTCCCAGGCCCAGAGTGG -- hit GGATTTCCCAGGCCCAGGGNGG -- query	Del 15	1	Yes	Chr17:8602794-8602815	+	8602809	5,72
GGATTCTCCCAGGCCCAGGGCG -- hit GGATTCTCCCAGGCCCAGGNGG -- query	Del 1, or Del 2, or Del 3	1	No	Chr6:113076075-113076096	+	113076090	24,51
GGATTCTCCCAGGCCCAGGGCG -- hit GGATTCTCCCAGGCCCAGGGGG -- query	Del PAM 3	1	No	Chr6:113076075-113076096	+	113076090	40,51
GGATTCTCCCAGGCCCAGGGCG -- hit GGATTCTCCCAGGCCCAGGGNG -- query	Del PAM 1, or Del PAM 2	0	No	Chr6:113076075-113076096	+	113076090	20,51
GGGATTCTCCCAGGCCCAGGGCGG -- hit GNGATTCTCCCAGGCCCAGGGNGG -- query	Ins 19	0	Yes	Chr6:113076074-113076097	+	113076091	0,83
GGGATTCTCCCAGGCCCAGGGCGG -- hit GGNATTCTCCCAGGCCCAGGGNGG -- query	Ins 18	0	Yes	Chr6:113076074-113076097	+	113076091	0,85
GGGATTCTCCCAGGCCCAGGGCGG -- hit GGANTTCTCCCAGGCCCAGGGNGG -- query	Ins 17	1	Yes	Chr6:113076074-113076097	+	113076091	1,02
GGATTCTCCCAGGCCCAGGGCGGT -- hit GGATTCTCCCAGGCCCAGGGNNGG -- query	Ins PAM 2, or Ins PAM 3	1	No	Chr6:113076075-113076098	+	113076092	40,7
GGATTCTCCCAGGCCCAGGGCGGT -- hit GGATTCTCCCAGGCCCAGGGNGNG -- query	Ins PAM 1	1	No	Chr6:113076075-113076098	+	113076092	40,7

The on-target site is the first one, Gt(ROSA)26S or target locus. Chr6: 113,076,075–113,076,097. The regions containing the off-target sites were amplified by PCR and sequenced by the Sanger method. The sequences obtained were analyzed by the ICE tool (Synthego, USA), which compares the characteristics of the chromatograms between possibly edited samples and a

control, generating an indels score and the possibility of knockouts (Figure 2); therefore, it is possible to evaluate the activity of cleavage and generation of mutations in the analyzed sequences.

There were no signs of alterations in any sample analyzed for all proposed sites. All samples showed 0% indels, with a sequence similarity score of 0.99-1 (maximum).

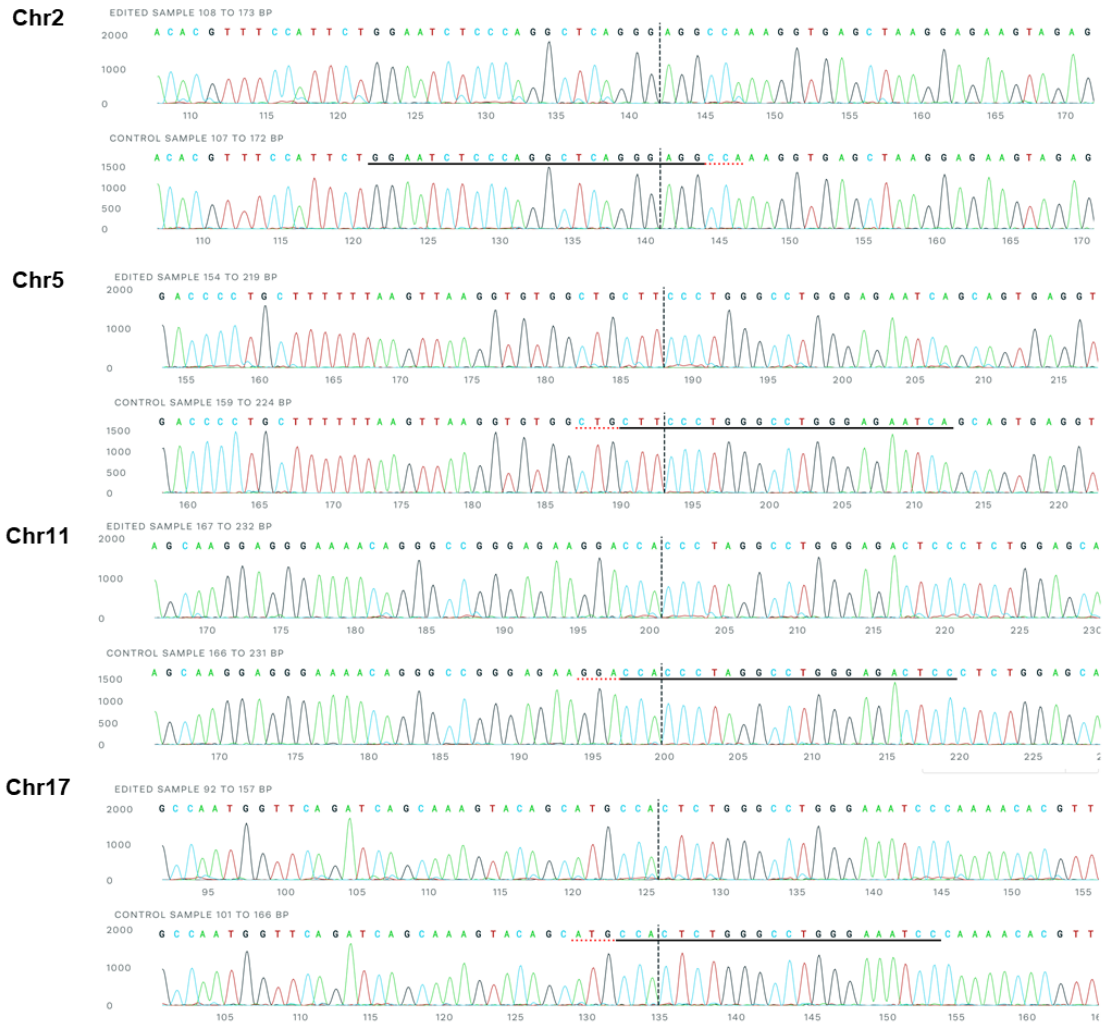

Figure 2. Experimental off-target analysis. Example of chromatograms used in the analysis. Each off-target shows sequence variations in relation to the target, because the target is specific and unique in the genome. The red dotted underline represents what the PAM sequence would be; underlined in black is the gRNA sequence. Vertical dots indicate the location where cleavage would occur if the CRISPR/Cas9 system were active at this site.

DISCUSSION

In this study, we evaluated the safety and the possibility of tumor induction after hydrodynamic administration of the CRISPR/Cas9 system and the *IDUA* gene donor plasmid complexed to liposomal vectors in newborn mice and their potential to produce off-target effects.

Physicochemical properties of the liposomal complexes showed stability of the formulations, since they were small (about 133 nm) and monodisperse droplets (PDI < 0.15), even when associated with DNA. These characteristics were expected and desirable, once they enhance the chances of penetration into the target cells. This may be achieved due to the microfluidization procedure and the presence of pegylated phospholipids, which bring stability to the preparations by avoiding aggregation and leading to small-sized nanostructures [10,16]. The

positive ζ-potential provided by the cationic lipids is also essential to provide stability and to promote interaction with cell membranes[16,17].

The biodistribution of fluorescent labeled complexes, demonstrated high affinity of LC primarily to the lung and liver, corroborating with previous publications of our reasearch group[10]. The efficient delivery to hepatocytes by hydrodynamic injection forces the permeability of the plasma membrane to allow DNA to enter the cells [18]. The lung accumulation of cationic liposomes has also been reported [10,19–21], as its fenestrae capillary bed may entrap the complexes, what might induce effective gene expression in this organ [21].

When CRISPR/Cas system is delivered into a cell, the gRNAs will guide Cas enzyme to locate on a specifically targeted DNA sequence that is complementary to it. Then, Cas nuclease cuts the double strands of DNA and forms a double-strand break (DSB)[22,23]. DSBs can be repaired through several endogenous repair pathways, including the predominant non-homologous end joining (NHEJ) and less-frequently the homology-directed repair (HDR)[24]. While NHEJ directly link two broken DNA molecules together, HDR uses donor DNA template to precisely repair DSBs for gene modification [22,25].

Although an ideal engineered nuclease would have singular genome-wide specificity, many studies demonstrated off-target events when using CRISPR/Cas9 gene editing tools [26,27]. Multiple mismatches between the guide

RNA and its complementary target DNA sequence can be tolerated depending on the quantity, position, and base identity of mismatches, leading to potential off-target events[28]. An off-target event can be defined as a programmable nuclease-induced DNA cleavage at a site anywhere in the genome other than the intended on-target site. Usually, off-target sites are similar in sequence to the desired target sites. However, they may present up to seven mismatches; small indels that cause DNA or RNA bulges; or even a different PAM sequence [29].

When an off-target cutting event occurs, it can be repaired via the NHEJ pathway, which is intrinsically error-prone, typically resulting in small indels at the site of the break. If it causes a frameshift mutation, there may be loss of gene function due to the production of truncated polypeptides and/or nonsense-mediated mRNA decay[29]. In addition, if an off-target cutting event occurs simultaneously with a second cutting event, it can generate a chromosomal rearrangement, such as an inversion or translocation, or a large deletion between the two break points [30]. Genomic rearrangements could lead to loss of heterozygosity (LOH), which is a serious safety concern. Studies reported that human preimplantation embryos also employ this alternative HDR mechanism, where DSBs are repaired by interallelic gene conversion, utilizing the homologous wildtype allele as a template. As a result, the DSB locus and adjacent area become identical to the template DNA, leading to LOH.

Consequently, it could lead to homozygosity of deleterious alleles and disease in offspring, and may also erase parent-specific epigenetic DNA modifications leading to imprinting abnormalities [31]. Other concerns reported in the literature about the off-target genotoxicity are gene inactivation and indel formation at unintended loci, which may affect cell viability or instead promote tumorigenesis[24].

Considering that the extent of off-target activity is highly dependent on the gRNA [29], it is necessary to identify potential off-target sites and to examine off-target effects experimentally when using CRISPR/Cas systems. There are many different tools which can be chosen to perform *in silico* off-target predictions, although the researcher may choose any of them [30]. In this study, we chose to use COSMID software because it is an easy and reliable prediction tool. We have performed off-target analysis in the lung and liver due to the number of transgene copies in these organs, as seen in fluorescence analysis and previous reports [10]. Comparing the characteristics of the chromatograms of the possibly edited samples and a control, it was verified that they had high similarity score, what demonstrates a great activity of cleavage and that no signs of alterations or indels were found in the analyzed sequences. Other studies in animal models also presented the absence or rare occurrence of off-target events, denoting CRISPR/Cas9 genome editing safety and specificity [32–36]. However, it is

important to consider that off-target mutations may occur at sites beyond those predicted *in silico* [29] therefore, studies involving whole-genome sequencing would be important to acknowledge the safety of this approach.

The probability of occurrence of tumors and activation of oncogenes causing deleterious effects are risks of gene therapy. When the CRISPR system recognizes sequences similar to the target sequence, cleavages that lead to off-target mutations can occur, which can lead to the malfunction of important genes [27]. This off-target potential has already been reported in several studies[37–39]. As the CRISPR system is composed of gRNAs (guide RNAs) that bind to a target genomic locus, mutations may occasionally occur in unwanted genomic loci, and it is important to identify the presence of mutations outside the genomic on-target site. Several studies demonstrate that CRISPR amplification showed increased insertions and/or deletions (indels) in the target DNA, confirmed by NGS (New Generation Sequencing) and DNA cleavage assays[40], reinforcing how imperative is to perform safety experiments before its use in clinical therapy.

CONCLUSION

This study assessed the safety after hydrodynamic administration of the CRISPR/Cas9 system and the IDUA gene donor plasmid complexed to liposomal

vectors in newborn mice. The off-target sites analysis based on the potential sites obtained from in silico predictions showed 0% of indels. This set of results demonstrated that the chosen gRNA sequence has potential to perform a safe gene editing in the murine *ROSA26* locus, and the set of experiments performed bring hope to the use of this tool in clinic studies. In this sense, our future efforts will focus on studies involving whole-genome sequencing to acknowledge the safety of CRISPR/Cas9 gene editing approach.

DECLARATION OF INTEREST

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

ACKNOWLEDGEMENTS

The authors were supported by National Council for Scientific and Technological Development (CNPq) (grant numbers 470888/2014-8 and 141742/2014-3) and FIPE/HCPA (grant number #20160482).

AUTHORS' CONTRIBUTIONS

EPC, EP, BBB, BMN, MTB, FNSF, GRM, AHRP, HFT, UM, RG, GB and RSS participated in Conception and design; Acquisition of data; Critical revision; and Final approval.

REFERENCES

1. Sharma G, Sharma AR, Bhattacharya M, Lee SS, Chakraborty C. CRISPR-Cas9: A Preclinical and Clinical Perspective for the Treatment of Human Diseases. Molecular Therapy. 2021;29(2):571.
2. Gonçalves GAR, Paiva R de MA. Gene therapy: advances, challenges and perspectives. einstein (São Paulo). 2017;15(3):369-375.
3. Mali P, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. Nature Methods 2013 10:10. 2013;10(10):957-963.
4. Bae S, Park J, Kim JS. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. 2014;30(10):1473-1475.
5. Fu Y, Foden JA, Khayter C, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature Biotechnology 2013 31:9. 2013;31(9):822-826.
6. Rasoulianboroujeni M, Kupgan G, Moghadam F, et al. Development of a DNA-liposome complex for gene delivery applications. Materials Science and Engineering: C. 2017;75:191-197.

7. Liu F, Huang L. Development of non-viral vectors for systemic gene delivery. Journal of Controlled Release. 2002;78(1-3):259-266.
8. Teixeira HF, Bruxel F, Fraga M, et al. Cationic nanoemulsions as nucleic acids delivery systems. Int J Pharm. 2017;534(1-2):356-367.
9. Tros de Ilarduya C, Sun Y, Düzgüneş N. Gene delivery by lipoplexes and polyplexes. European Journal of Pharmaceutical Sciences. 2010;40(3):159-170.
10. Schuh RS, Poletto É, Pasqualim G, et al. In vivo genome editing of mucopolysaccharidosis I mice using the CRISPR/Cas9 system. Journal of Controlled Release. 2018;288:23-33.
11. Schuh RS, Gonzalez EA, Tavares AMV, et al. Neonatal nonviral gene editing with the CRISPR/Cas9 system improves some cardiovascular, respiratory, and bone disease features of the mucopolysaccharidosis I phenotype in mice. Gene Ther. 2020;27(1-2).
12. Vera LNP, Schuh RS, Fachel FNS, et al. Brain and visceral gene editing of mucopolysaccharidosis I mice by nasal delivery of the CRISPR/Cas9 system. J Gene Med. 2022;24(4):e3410.
13. Fujii W, Kawasaki K, Sugiura K, Naito K. Efficient generation of large-scale genome-modified mice using gRNA and CAS9 endonuclease. Nucleic Acids Res. 2013;41(20):e187.

14. Cradick TJ, Qiu P, Lee CM, Fine EJ, Bao G. COSMID: A Web-based Tool for Identifying and Validating CRISPR/Cas Off-target Sites. Mol Ther Nucleic Acids. 2014;3(12):e214.

15. Animals NRC (US) C for the U of the G for the C and U of L. Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. December 2011.

16. Schuh RS, Poletto É, Fachel FNS, Matte U, Baldo G, Teixeira HF. Physicochemical properties of cationic nanoemulsions and liposomes obtained by microfluidization complexed with a single plasmid or along with an oligonucleotide: Implications for CRISPR/Cas technology. J Colloid Interface Sci. 2018;530:243-255.

17. Fraga M, Schuh RS, Poletto É, et al. Gene Therapy of Mucopolysaccharidosis Type I Mice: Repeated Administrations and Safety Assessment of pIDUA/Nanoemulsion Complexes. Curr Gene Ther. 2021;21(5):464-471.

18. Bonamassa B, Hai L, Liu D. Hydrodynamic Gene Delivery and Its Applications in Pharmaceutical Research. Pharm Res. 2011;28(4):694-701.

19. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther. 1999;10(10):1735-1737.

20. Zhang JS, Liu F, Conwell CC, Tan Y, Huang L. Mechanistic studies of sequential injection of cationic liposome and plasmid DNA. Molecular Therapy. 2006;13(2):429-437.
21. Ishiwata H, Suzuki N, Ando S, Kikuchi H, Kitagawa T. Characteristics and biodistribution of cationic liposomes and their DNA complexes. Journal of Controlled Release. 2000;69(1):139-148.
22. Zhang B. CRISPR/Cas gene therapy. J Cell Physiol. 2021;236(4):2459-2481.
23. Tsai SQ, Joung JK. Defining and improving the genome-wide specificities of CRISPR–Cas9 nucleases. Nature Reviews Genetics 2016 17:5. 2016;17(5):300-312.
24. Chen M, Mao A, Xu M, Weng Q, Mao J, Ji J. CRISPR-Cas9 for cancer therapy: Opportunities and challenges. Cancer Lett. 2019;447:48-55.
25. Tao J, Wang Q, Mendez-Dorantes C, Burns KH, Chiarle R. Frequency and mechanisms of LINE-1 retrotransposon insertions at CRISPR/Cas9 sites. Nature Communications 2022 13:1. 2022;13(1):1-17.
26. Lin Y, Cradick TJ, Brown MT, et al. CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. Nucleic Acids Res. 2014;42(11):7473-7485.
27. Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target effects in CRISPR/Cas9-mediated genome engineering. Mol Ther Nucleic Acids. 2015;4(11):e264.

28. Ran FA, Hsu PD, Lin CY, et al. XDouble nicking by RNA-guided CRISPR cas9 for enhanced genome editing specificity. Cell. 2013;154(6):1380-1389.

29. Zischewski J, Fischer R, Bortesi L. Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. Biotechnol Adv. 2017;35(1):95-104.

30. Bao XR, Pan Y, Lee CM, Davis TH, Bao G. Tools for experimental and computational analyses of off-target editing by programmable nucleases. Nature Protocols 2020 16:1. 2020;16(1):10-26.

31. Liang D, Gutierrez NM, Chen T, et al. Frequent gene conversion in human embryos induced by double strand breaks. bioRxiv. June 2020:2020.06.19.162214.

32. Long C, Amoasii L, Mireault AA, et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. Science (1979). 2016;351(6271):400-403.

33. Nelson CE, Hakim CH, Ousterout DG, et al. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science (1979). 2016;351(6271):403-407.

34. Kim K, Park SW, Kim JH, et al. Genome surgery using Cas9 ribonucleoproteins for the treatment of age-related macular degeneration. 2017.

35. Bengtsson NE, Hall JK, Odom GL, et al. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. Nature Communications 2017 8:1. 2017;8(1):1-10.

36. Han HA, Pang JKS, Soh BS. Mitigating off-target effects in CRISPR/Cas9-mediated in vivo gene editing. J Mol Med. 2020;98(5):615-632.

37. Tsai SQ, Zheng Z, Nguyen NT, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol. 2015;33.

38. Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. 2013.

39. Wienert B, Wyman SK, Richardson CD, et al. Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. Science (1979). 2019;364(6437):286-289.

40. Kang SH, Lee W jae, An JH, et al. Prediction-based highly sensitive CRISPR off-target validation using target-specific DNA enrichment. Nature Communications 2020 11:1. 2020;11(1):1-11.

ANEXO

NORMAS PARA SUBMISSÃO DE TRABALHOS

Journal of Inborn Errors of Metabolism and Screening

Instruções aos autores

Forma e preparação de manuscritos

Declarações e Convenções

No final de seu artigo, as seguintes seções devem aparecer: Agradecimentos, seguidos por sua Declaração de Conflito de Interesses, reconhecimento de financiamento, referências e para manuscritos com 5 ou mais autores cada autor deve assumir a responsabilidade por pelo menos um componente do trabalho (por exemplo: Concepção e desenho; Aquisição de dados; Análise e interpretação de dados; Procedimentos técnicos; Análise estatística; Escrita do manuscrito; Revisão crítica; Aprovação final).

Agradecimentos

Todos os colaboradores que não atenderem aos critérios de autoria devem ser listados na seção "Agradecimentos". Exemplos daqueles que podem ser reconhecidos incluem uma pessoa que forneceu ajuda puramente técnica, assistência por escrito ou um chefe de departamento que forneceu apenas apoio geral. Os autores devem divulgar se tiveram alguma assistência por escrito e identificar a entidade que pagou por essa assistência.

Declaração de conflito de interesses

É política do Journal of Inborn Errors of Metabolism and Screening exigir uma declaração de conflito de interesses de todos os autores, permitindo que

uma declaração seja transportada dentro das páginas paginadas de todos os artigos publicados.

Por favor, inclua qualquer declaração no final do seu manuscrito após qualquer reconhecimento e antes das referências, sob o título "Declaração de Conflito de Interesses". Se nenhuma declaração for feita, o seguinte será impresso sob este título em seu artigo: "Nada declarado". Alternativamente, você pode declarar que "os autores (as) declaram que não há conflito de interesse".

Ao divulgar qualquer conflito de interesses, as informações de divulgação devem ser específicas e incluir qualquer relação financeira que todos os autores do artigo tenham com qualquer organização patrocinadora e os interesses com fins lucrativos que a organização representa e com qualquer produto com fins lucrativos discutido ou implícito no texto do artigo.

Quaisquer envolvimentos comerciais ou financeiros que possam representar uma aparência de conflito de interesses precisam ser adicionalmente divulgados na carta de acompanhamento que acompanha o seu artigo para auxiliar o Editor a avaliar se a divulgação suficiente foi feita dentro da Declaração de Interesses Conflitantes contida no artigo.

Ética em pesquisa

Todos os artigos que relatam estudos em animais e humanos devem incluir se o consentimento por escrito foi obtido do Comitê de Ética local ou do Conselho de Revisão Institucional. Por favor, certifique-se de fornecer o nome completo e instituição do comitê de revisão e um número de referência do Comitê de Ética.

Aceitamos manuscritos que relatam estudos em humanos e / ou animais para publicação somente se ficar claro que as investigações foram realizadas com um alto padrão ético. Estudos em humanos que possam ser interpretados como experimentais (por exemplo, ensaios controlados) devem estar em conformidade com a Declaração de Helsinque http://www.wma.net/en/30publications/10policies/b3/index.html e os textos tipográficos devem incluir uma declaração de que O protocolo de pesquisa foi aprovado pelo comitê de ética apropriado. De acordo com a Declaração de Helsinque de 1975, revista Hong Kong 1989, encorajamos os autores a registrar seus ensaios clínicos (em http://clinicaltrials.gov ou outros bancos de dados

adequados identificados pelo ICMJE, http://www.icmje.org/ sobre-icmje / faqs / clinical-trials-registration/). Se o seu teste foi registrado, por favor, cite esta informação na Página de Título. Ao relatar experimentos com animais, indique na página de título que a diretriz / lei sobre o cuidado e uso de animais de laboratório foi seguida.

Análise estatística

Quando as análises estatísticas tiverem sido realizadas, certifique-se de que a metodologia foi descrita com precisão. Em estudos comparativos, os cálculos de potência são geralmente necessários. Em trabalhos de pesquisa que requerem estatísticas complexas, o conselho de um estatístico especialista deve ser buscado no estágio de projeto / implementação do estudo.

Preparação de manuscritos

O texto deve ter espaço duplo e ter um mínimo de 3 cm para as margens esquerda e direita e 5 cm para o cabeçalho e o rodapé. O texto deve ser de 12 pontos padrão.

O resumo deve consistir de não mais do que 200 palavras resumindo o conteúdo do artigo. Por favor, não subdivida o resumo em: Introdução, Objetivo, Resultados, Conclusão ou similar. O resumo deve ser independente, e deve-se presumir que o leitor tenha algum conhecimento do assunto, mas não tenha lido o artigo. Pelo menos **3 palavras-chave devem ser fornecidas após o resumo.**

Folha de rosto

A página do título deve fornecer as seguintes informações: (1) título do artigo (em negrito), tão curto e informativo quanto possível, sem qualquer abreviação; (2) um primeiro nome, iniciais (se aplicável) e sobrenome de cada autor; (3) nome e endereço do departamento ou instituição ao qual o trabalho deve ser atribuído; (4) nome, telefone, número de fax e endereço de e-mail do autor correspondente e para quem as provas devem ser enviadas.

Citações e Referências

Os manuscritos do JIEMS devem ser preparados de acordo com as diretrizes de estilo da American Medical Association (AMA) (10ª edição). Os autores são responsáveis pela exatidão das referências. Digite as referências (espaço duplo) no final do manuscrito. Cite as referências no texto na ordem de aparição. Exemplos abaixo.

Livro:

Katz DL, Clinical Epidemiology & Evidence-Based Medicine: Fundamental Principles of Clinical Reasoning & Research, Thousand Oaks, CA: SAGE; 2001.

Capítulo:

Schulz, J, HIV testing. In Kirton C, ed., ANAC's Core Curriculum for HIV/AIDS Nursing, 2nd ed. Thousand Oaks, CA: SAGE; 2003.

Periódico:

Baldo G, Mayer FQ, Martinelli BZ, et al. Enzyme replacement therapy started at birth improves outcome in difficult-to-treat organs in mucopolysaccharidosis I mice. Mol Genet Metab. 2013; 109(1): 33-40.

Língua Inglesa

O texto deve estar no idioma inglês. Se o idioma nativo dos autores não for o inglês, recomendamos a revisão e a edição por um colega cujo idioma nativo seja o inglês ou o uso de um serviço de edição em inglês.

Diretrizes para enviar trabalhos de arte, figuras e outros gráficos

Figuras: As figuras devem ser submetidas como arquivos separados. Os formatos para figuras são TIFF ou JPEG. Eles devem ser numerados consecutivamente usando algarismos arábicos. Figuras em formato Word, PowerPoint ou Excel não podem ser publicadas. Apenas os dados da sequência de ácidos nucleicos podem ser apresentados em formato Word.

A arte de linha deve ter uma resolução de pelo menos 1200 dpi (pontos por polegada) e fotografias eletrônicas; radiografias, tomografia

computadorizada, ressonância magnética, todas as imagens médicas e todas as imagens digitalizadas devem ter uma resolução de pelo menos 300 dpi. Se as fontes forem usadas no trabalho artístico, elas deverão ser convertidas em caminhos ou contornos ou elas deverão ser incorporadas nos arquivos. Imagens coloridas devem ser criadas / digitalizadas, salvas e enviadas como arquivos CMYK. Observe que as ilustrações geradas a partir de programas do conjunto de aplicativos do Office, como o CorelDRAW e o MS Word, e obras de arte baixadas da Internet (arquivos JPEG ou GIF) não podem ser usadas. Cite as figuras e numere-as na ordem em que são discutidas no texto do manuscrito.

Legendas de figuras: Legendas devem ser enviadas para todas as figuras. Elas devem ser breves e específicas, e devem aparecer em um local separado da página do manuscrito para seguir as referências. Use marcadores de escala na imagem para micrografias eletrônicas e indique o tipo de mancha usada.

Tabelas: Crie tabelas usando o recurso de criação e edição de tabelas do seu software de processamento de texto (por exemplo, MS Word ou Excel). Agrupe todas as tabelas no final do manuscrito ou forneça-as em um arquivo separado. Cite tabelas consecutivamente no texto e numere-as nessa ordem. Digite cada uma em uma folha separada e inclua o título da tabela, cabeçalhos de coluna apropriados e legendas explicativas (incluindo as definições de quaisquer abreviações usadas). Não inclua tabelas no corpo do manuscrito. Eles devem ser auto-explicativas e devem complementar, em vez de duplicar, o material no texto.

Outras informações

Quaisquer correspondências, consultas ou pedidos adicionais de informação devem ser enviados para o seguinte e-mail: jiems@jiems-journal.org