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CRISPR Technology in Animal Embryos: A Literature Review

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ABSTRACT. CRISPR, also known as "Clustered Regularly Interspaced Short Palindromic Repeats", is an immune defense mechanism that was first identified in the 1980s in the form of repeated sequences in the DNA of bacteria and archaea, used to protect against attacks by viruses and plasmids by cutting the invader's nucleic acids, thus interrupting its replication cycle. At a later stage, scientists adapted the CRISPR-Cas system for gene editing, making it possible to precisely cut DNA sequences at specific locations and thus enabling precise changes to the genome. Since the discovery of this new potential for genome editing, CRISPR has represented a major leap forward in technology, especially since it does not depend on the long and costly process of modifying proteins to confer specificity to the target, and can thus be used in various areas, such as medicine, agriculture and biotechnology, even going so far as to alter embryos. This article carries out a critical analysis of the scientific literature on the application of the CRISPR-Cas technique to animal embryos. In the review in question, all 17 results found in the database search were promising in the field of gene editing in animals. Several species have been subjected to the technique,

with success in correcting a significant range of genes that cause animal and human diseases of high epidemiological relevance, for example Duchane Muscular Dystrophy, phenylketonuria, cataracts, among others. It has been determined that gene editing in animal embryos using CRISPR-Cas technology is a precise, effective and promising tool, with the capacity to offer significant benefits by making it possible to correct known genetic mutations, introduce new characteristics, strengthen resistance to diseases, investigate human pathologies, develop innovative therapies for such pathologies and deepen understanding of the mechanisms controlling embryonic development, paving the way for significant advances in the field of biomedicine.

Key words: CRISPR-Cas9; Animal embryo; Gene editing.

INTRODUCTION

In recent years, Personalized Genomic Medicine has emerged with the potential to revolutionize medical practice by taking into account the individual genetic information of each patient to personalize the diagnosis, treatment and prevention of diseases. This method is based on the use of genetic tests, the identification of biomarkers and the development of therapies for genetic diseases (Iriart, 2019). Following advances in gene mapping and the development of genomic medicine, a tool has emerged that can provide a new approach used in studies of functional genomics, transgenic organisms and gene therapy in recent decades, called CRISPR-Cas technology (Zhang et al., 2014).

CRISPR/Cas9 was first observed in bacteria and archaea by Ishino in 1987, as a sequence present in DNA, which had nucleotides that form short stretches repeated at regular, palindromic intervals, and it was later discovered that it performed a protective function against invading viruses and plasmids, like an acquired immune system, and was called clustered regularly interspaced short palindromic repeats (CRISPR) (Sganzerla, 2020; Ahumada-Ayala, 2023).

According to Zhang et al., (2014), the CRISPR system occurs through the recognition of an invading DNA based on CRISPR RNA (crRNA), generating a memory.

Subsequently, in a secondary invasion, there is cleavage of the invading DNA mediated by Cas endonucleases with specific detection for the silencing of nucleic acids of exogenous origin.

Doudna and Charpentier, in 2012, were the first researchers to use CRISPR as a genomic editing technique, increasing the precision of this tool by adding guide RNA (sgRNA), work that was recognized in 2020 with a Nobel Prize for Chemistry. The following year, Ran and other researchers carried out the first tests of the CRISPR system on mammalian eukaryotic cell genomes, using Cas9 nickase as sgRNA. This created a powerful tool that allows the deliberate manipulation of the DNA of any organism (Ran et al., 2013; Doudna et al., 2014; Martinez-Oliva, 2020).

This system, involving interference via sgRNA, has been used as a versatile biotechnological mechanism for genome editing that can induce specific alteration of a DNA sequence. This occurs by means of a small sgRNA that drives designed, programmable and highly specific nucleases to a site in the genome to generate a double strand break (BSD) in the target DNA with the same precision as an sgRNA would do in a bacterium. And, together, there is a subsequent repair through the cell's own systems by the mechanism of homology-directed repair (HDR) or non-homologous end joining (NHEJ) generating insertions, substitutions or deletions in genes of interest. It is therefore used to

redesign genetic information, leading to a transformative advance in the life sciences (Zhang et al., 2014; Paul and Montoya, 2020).

The CRISPR gene editing technique has expanded significantly due to its high specificity, simplicity, low cost and versatility. It quickly became one of the methods for editing genes, making it possible to investigate specific mutagenic genes for diseases and to create precise models, both cellular and animal. As a result, it represents a major scientific advance with the potential to revolutionize various areas of science and medicine (Nicholson and Pepper, 2016).

Applications of the CRISPR system occur in different areas of biology and have been demonstrated in various reports, such as agriculture, gene therapy, vector control, human reproduction and others (Santos and Wiethölter, 2021). Corroborating with Dias and Dias (2018) describe that the CRISPR-Cas technique in animal embryos is currently being widely used by researchers, due to its wide range of possibilities as a gene editing technique. Several researchers indicate that studies have demonstrated effective corrections of altered genes by inserting CRISPR-Cas into the zygote, and subsequent transmission of the repaired trait to subsequent generations of the animal.

However, although there are several approaches and techniques that have emerged using CRISPR-Cas since its discovery as an immunological mechanism in bacteria and first application as a biotechnological tool, the validation of this "editing and correction system" requires *in vivo* tests, depending on methods that produce a defined, unalterable expression and with minimal gene silencing triggers, to observe its real functionality, efficiency and safety (Vilarino et al., 2017; Koltun et al., 2024).

Based on the growing relevance of CRISPR-Cas in gene editing, the proposed article aims to review the most recent studies on its application in animal embryos, considering its implications in various areas, such as the environment, politics, the economy and society.

MATERIALS AND METHODS

The integrative review is the broadest methodological approach to reviews, allowing the inclusion of experimental and non-experimental studies for a complete understanding of the phenomenon analyzed.

In order to achieve the objectives set out in the literature review, PRISMA was used to gather and compare articles that fully address the purpose of this article, using the *PubMed, Scielo* and *Lilacs*. The following descriptors were used for the search, in various combinations in Portuguese and English: "CRISPR-Cas9",

"Animal Embryo", "Gene Editing", "Animal Embryo" and "Gene Editing".

The inclusion criteria established were: original articles available in full and online, published in English and Portuguese between January 2014 and January 2024, which provide evidence of the use of CRISPR-Cas therapy in animal embryos.

Exclusion criteria included literature review articles, articles on CRISPR-Cas in somatic cells, articles on CRISPR-Cas in plants, case reports, incomplete articles and reflections, theses, dissertations and repeated articles in different databases.

Finally, all the scientific papers chosen were analyzed using the Google Docs program to create tables and graphs with the data presented, with satisfactory or unsatisfactory results.

RESULTS

The results of the search revealed the identification of 178 studies in various databases, distributed as follows: 84 in PubMed, 45 in SciELO and 49 in LILACS. After the first analysis of the articles identified, 110 articles were excluded because they did not fit the inclusion criteria: 3 were excluded because they were studies using CRISPR in plants; 18 because they were studies using CRISPR in human somatic cells; 56 because they were literature review studies 65 because they did not address the topic (Figure 1).

Therefore, 36 articles were read in their entirety, 19 of which were excluded because they did not meet the objectives and methodology proposed, and 17 were analyzed to produce this review, as shown in Table 1.

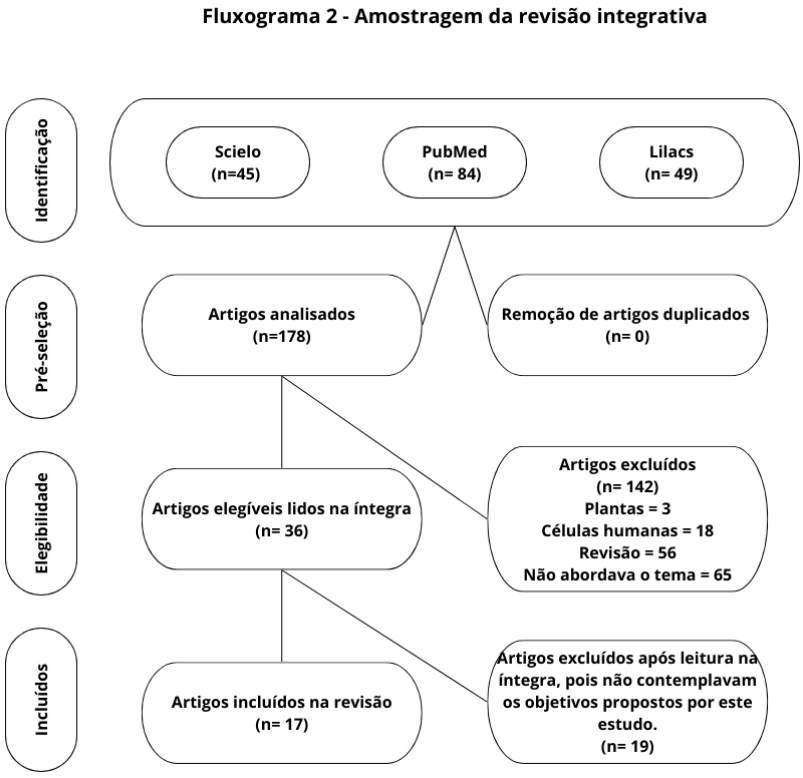

Figure 1. Summarizes the main characteristics of the 17 original articles that make up the sample of studies, with a survey focusing on different species and the main genes published.

Table 1. Table of results included articles.

Authors	Species	Objective of the study	Results
Wu Y, et al., (2013)	Mice	Developing the dominant cataract disorder through a mutation in the *Crygc* gene.	24 rats were cured of cataracts using the CRISPR-Cas9 system.
Moro LN, et al., (2020)	Horses	Eliminate the myostatin gene (*MSTN*).	The average efficiency was 39 clonal cell lines edited by evaluating two gRNA targeting the gene *MSTN*.
Villarino M, et al., (2017)	Sheep	Breeding sheep with pancreatogenesis deficiency due to deletion of the *PDX1* gene, critical for pancreas development.	Two of the four oocytes microinjected in metastasis with CRISPR/Cas9 showed mutations in the *PDX1* locus, showing complete interruption of the locus and consequently no development of the pancreas.
Zou Q, et al., (2015).	Beagle dog	Eliminating the myostatin gene (*MSTN*), a negative regulator of skeletal muscle mass.	The MSTN protein was not detected by immunohistochemistry in the muscles of chicks #5 and #11 out of a total of 27.
Zhang T, et al., (2020)	Coelho	To generate rabbits with a G307S point mutation in the Cystathionine β Synthetase (CBS) gene on lipid metabolism in rabbits.	a *CBS* G307S rabbit model exhibited severe dyslipidemia indicating that the G307S mutation in the *CBS* gene is a causative factor of dyslipidemia.
Sper RB, et al., (2017)	Pigs	Generate pigs with H2B-eGFP fusion protein with integration in the allele at the ACTB locus.	Three transgenic pigs (parental generation) were generated from a recipient after the transfer of 119 embryos and the transgene transmission rate was 55.8%
Koppes EA, et al., (2020)	Pigs	Development of PAH (phenylalanine hydroxylase) null pigs, which is the classic form of PKU disease.	Pig 116-1 from the set of transferred embryos was born with the mutation in the PAH gene and showed high levels of phenylalanine in the blood and other symptoms of PKU, such as hypopigmentation and delayed juvenile growth.
Yu HH, et al., (2016)	Pigs	Generation of a porcine model with myopathy due to mutation of the DMD gene.	This result demonstrates that *DMD* knockout was achieved in the *DMD* modified pig and *DMD* targeting efficacy was 50% in two piglets.
Sui T, et al., (2016)	Coelho	Generation of a rabbit model with XLH generated via knockout of the *PHEX* gene	According to a PCR carried out on each pup, 19 of the 26 (73.1%) newborn pups carried a *PHEX* mutation, and 11 pups (42.3%) carried the *PHEX* biallelic mutation.
Ryu J, et al., (2022)	Rhesus monkey	Creation of a Rhesus monkey model with Usher Syndrome type B1 (USH1B), through mutations in the MYO7A gene.	A live-born offspring (Mya) was generated with a homozygous mutation in the MYO7A gene and showed profound hearing loss and abnormal retinal function.
Xu Y, et al., (2018)	Coelho	Generation of a knouchout (KO) rabbit model of the PAX4 gene responsible for The production of insulin producing Beta cells.	PAX4 KO rabbits have been successfully generated with large deletions in the PAX4 gene and phenotypes typical of diabetes mellitus (DM).

Peng J, et al., (2015)	Pigs	Generate pigs that express recombinant human albumin (rHSA) by inserting cDNA into the porcine albumin (Alb) locus.	The HSA cDNA was successfully inserted into the Alb locus of 16 F0 pigs. And rHSA was detected in the plasma of all F0 pigs.
Chen Y, et al., (2015)	Rhesus monkey	Targeting the monkey dystrophin gene to create mutations that lead to Duchenne muscular dystrophy (DMD).	14 live monkeys with mutations in the DMD gene were generated showing loss of function of the dystrophin protein.
Reyes LM, et al., (2014)	Pigs	Creation and characterization of knockout pigs for MHC class I, fundamental to the function of the immune system in infection and transplantation.	Three SLA-deficient piglets were successfully generated, lacking these molecules on the cell surface. The SLA-deficient piglets showed reduced proportions of CD8+ T cells, suggesting an important role for SLA molecules in T cell development and function.
Wang X, et al., (2015)	Goats	Production of genetically modified goats targeting two functional genes (MSTN and FGF5).	In total, 26 goats (26/98, 26.5%) showed a deficiency of one or two genes, indicating efficient genomic modifications in infant goats.
Yao J, et al., (2021)	Swine	Correction of the MITF c.740T>C mutation that causes Waardenburg Syndrome type 2A (WS2A) in a miniature pig model.	The MITF c.740T>C mutation was corrected monoallelically in porcine fibroblast cells using ssODN as a repair model.
Hendricks-Wenger A, et al., (2021)	Swine	Pig production disabled in *RAG2/IL2RG* with Panc01 cells grafted for studies ex vivo from IRE in pancreatic tumors human.	The RAG2/IL2RG pigs endured successfully stopped the growth of tumors xenografts Panc01 and showed similar histology pancreatic cancer tumors human for the evaluation of IRE.

DISCUSSION

Based on the analysis and although the table shows a variety of gene studies in different animals, the MSTN gene and the DMD gene stand out as the most frequent and relevant, appearing in 5 studies: Moro LN, et al., (2020); Zou Q, et al., (2015); Wang X, et al., (2015); Yu HH, et al., (2016) and Chen Y, et al., (2015). This is because they are genes that play crucial roles in muscle development and function, each in different ways: MSTN as an inhibitor of muscle development and DMD as a producer of dystrophin, which is an essential enzyme for muscle function.

Duchenne Muscular Dystrophy (DMD) is the disease that is most relevant because it is relatively common among all the others studied, affecting around 1 in every 5,000 boys are born in the world. It is a genetic disease linked to the X chromosome, this gene encodes the dystrophin protein which has the function of stabilizing the muscle fibre during contraction and has a considerably high mutation rate (Bez et al., 2023).

Mutations in the DMD gene cause the disorganization of several proteins associated with dystrophin, compromising its distribution and cell signaling. Both factors culminate in rapid and severe muscle loss characterized by respiratory failure, cardiac myopathy and loss of skeletal muscle function (Bez et al., 2023). Duchenne muscular dystrophy has no cure, but there are several treatments that can control it and improve quality of life, such as corticosteroids that slow down the progression of muscle weakness.

Therefore, research into gene therapies is underway to find new treatments and a cure for DMD (Elangkovan and Dickson, 2021).

In addition, the animal with the highest frequency in relation to the articles analyzed is pigs, present in 7 studies: Sper RB, et al., (2017); Koppes EA, et al., (2020); Yu HH, et al., (2016); Peng J, et al., (2015); Reyes LM, et al., (2014); Yao J, et al., (2021); Hendricks-Wenger A, et al., (2021).

The choice of pigs as an animal model in these gene editing studies is due to several factors, including: physiological and anatomical similarity to humans, making them valuable models for biomedical research; they are large animals which allows for more complex studies and the collection of larger tissue samples; and the fact that pigs are susceptible to a variety of diseases that affect humans, such as cardiovascular disease, diabetes and certain types of cancer.

Finally, the study conducted by Xu, et al., (2018) aimed to create a knockout (KO) rabbit model of the PAX4 gene that is essential for the development of insulin-producing pancreatic beta cells using genomic editing. The result of this study shows that rabbits were generated with deletions in this PAX4 gene, developing phenotypes typical of Diabetes Mellitus (DM), which is one of the main causes of death and morbidity, affecting millions of people worldwide. This PAX4 KO rabbit model represents an important advance in understanding the development of type 1 DM, and also allows us to investigate mechanisms underlying the destruction of pancreatic beta cells, test new therapies, identify biomarkers for diagnosis and prognosis, and develop prevention strategies for type 1 DM (Xu et al., 2018).

The articles analyzed demonstrate the relevance of gene editing in animal models for the study of various diseases, which proves that the CRISPR-Cas tool in animal embryos opens up a range of possibilities for scientific and medical progress. From an ethical point of view, hereditary gene editing in embryos raises significant concerns. Genome modifications can have irreversible impacts on animal species by permanently altering the genome of generations of the same, affecting biodiversity and ecological balance. It is essential to carefully assess the impact environmental and long-term consequences before starting any experiment (Actis, 2021; Menchaca, 2023).

In addition, the technique presents inherent technical risks. Off-target editing can generate unwanted mutations in regions adjacent to the gene of interest. Another risk is genetic mosaicism, where only a few cells of the embryo are modified, leading to unpredictable and long-term effects that are not yet fully understood. One example is the study conducted by Ryu, et al. (2022), who used the CRISPR-Cas9 technique to edit the MYO7A gene in Rhesus monkey embryos, with the aim of creating a model of Usher Syndrome type 1B (USH1B). Although the gene editing was successful, one of the resulting monkeys showed genetic mosaicism, leading to developmental complications and dysfunctions due to the absence of the desired mutation in all the animal's cells.

In short, CRISPR-Cas in animal embryos offers promising potential for research and medicine, but requires careful and responsible use. Careful evaluation of the ethical aspects and technical risks is essential to ensure the responsible development of this powerful technology.

CONCLUSION

In conclusion, of the studies observed, all describe positive results that were expected for each study model. However, genetic manipulation for research purposes to understand diseases and develop therapeutic alternatives can lead, even if not described in most of the articles, to potential risks of the technique with negative impacts on the animal under study.

Therefore, expanding research into different animals and diseases is crucial in order to identify unwanted effects in the search for corrections and reproducibility. Caution and responsibility are of fundamental importance, considering the ethical aspects and risks involved. Finally, public debate and strict regulation are essential to guarantee the ethical and responsible use of this powerful technology.

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