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ANIMAL CLONING AND GENOME EDITING IN BUFFALO, WITH SPECIAL REFERENCE TO INDIA

Clonación de animales y edición del genoma del búfalo, con especial referencia a la India

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ABSTRACT

India owns the best buffalo breeds, particularly Murrah, which is famous worldwide for high milk production. India's white and pink revolution cannot be imagined without the contribution of buffalo, and to achieve this, the best productive animals need to be produced through scientific interventions. Animal cloning is a technique used to produce multiple copies of the best animals without normal reproduction. In India, buffalo cloning has already happened, and India's first cloned buffalo was produced in 2009. Later, several buffalo clones were produced, and attempts are ongoing to produce a stock of more elite animals. Buffalo cloning has made its way from scientific manuscripts to farmers' farms. Recently, genome editing has emerged as a powerful tool to manipulate the genomes of several animal species. Various livestock models have already been produced, and it can be foreseen that more model animals will be produced in the future using the CRISPR-Cas system. These success stories have opened multiple prospects for Indian researchers. In this article, we provide an overview of the progress of buffalo cloning in India and new initiatives on genome editing.

Keywords: Buffalo, Embryo, Cloning, genome editing, CRISPR.

RESUMEN

India posee las mejores razas de búfalos, en particular Murrah, famosa en todo el mundo por su alta producción de leche. La revolución blanca y rosa de la India no se puede imaginar sin la contribución del búfalo y, para lograrlo, es necesario producir los animales más productivos mediante intervenciones científicas. La clonación animal es una técnica utilizada para producir múltiples copias de los mejores animales sin una reproducción normal. En la India ya se ha clonado búfalos, y el primer búfalo clonado del país se produjo en 2009. Posteriormente, se produjeron varios clones de búfalos y se están realizando intentos para producir una población de más animales de élite. La clonación de búfalos ha llegado desde los manuscritos científicos hasta las granjas de los agricultores. Recientemente, la edición del genoma se ha convertido en una poderosa herramienta para manipular los genomas de varias especies animales. Ya se han producido varios modelos de ganado y se puede prever que en el futuro se producirán más animales modelo utilizando el sistema CRISPR-Cas. Estas historias de éxito han abierto múltiples perspectivas para los investigadores indios. En este artículo, ofrecemos una descripción general del progreso de la clonación de búfalos en la India y nuevas iniciativas sobre edición del genoma.

Palabras clave: Búfalo, Embrión, Clonación, edición del genoma, CRISPR.

BUFFALO CLONING JOURNEY

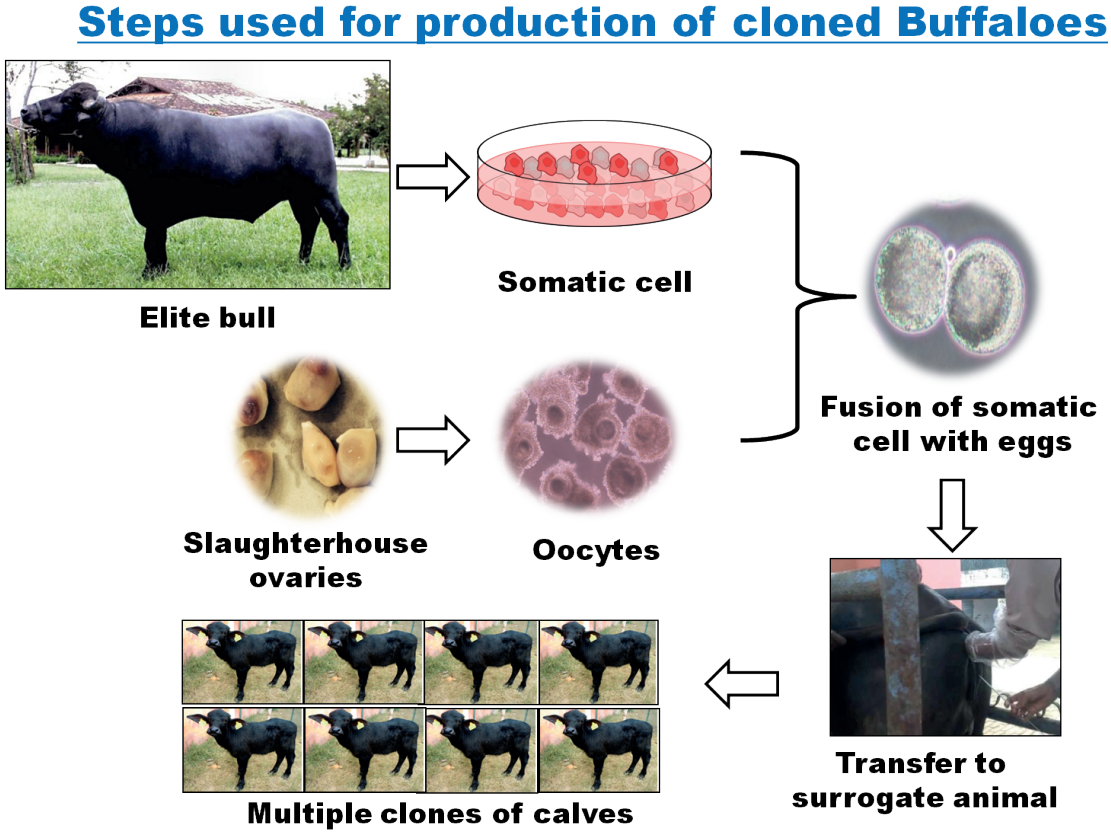
Ten years ago, on February 6, 2009, a team of scientists at the ICAR-National Dairy Research Institute (NDRI), Karnal, created history in the field of animal cloning research in India. They produced the world's first cloned riverine buffalo calf, Samrupa, using an inexpensive, simple somatic cell nuclear transfer (SCNT) technique called handmade cloning [1]. This simple technique is less demanding regarding equipment, skill, and time. It does not require sophisticated tools like micromanipulators and needle grinders to perform microscopic manipulation of oocytes and somatic cells [2]. The world's first cloned mammal, 'Dolly', the sheep, was born 13 years earlier at Scotland's Roslin Institute using SCNT. SCNT is an advanced assisted reproduction technique in which a somatic cell is transferred or fused with an enucleated oocyte. The reconstructed embryo, thus produced, develops to the blastocyst stage, at which it transplants into a surrogate mother, who carries the pregnancy and delivers the offspring. Worldwide, more than 20 animal species have been cloned using different SCNT methods, including handmade cloning. Currently, China is at

the forefront of cattle and pig cloning, whereas India is heading buffalo cloning and has made significant achievements.

In comparison to other farm animals, limited studies have been done in the field of buffalo cloning. Researchers at NDRI made the first attempt to clone buffaloes, Karnal, during 1994-97, in which cells from fertilized or *in vivo*-produced embryos were injected into enucleated oocytes to generate cloned embryos [3]. This method of nuclear transfer using micromanipulators was similar to a method that was used to produce the Dolly. Unfortunately, no blastocyst-stage embryo could be produced during that time. The possible reasons could be 1) lack of technical advances at that time, since highly skilled micromanipulation methods were used; 2) improper culture conditions for SCNT embryos; 3) not much research had been done in SCNT during the late nineties; therefore, the researchers did not have much information on SCNT. Later, in 2009, a simplified method of SCNT, named handmade cloning, was used for reprogramming differentiated somatic cells of adult animals. This method was used successfully at NDRI, Karnal, for producing blastocyst-stage embryos, and India's first cloned farm animal was born in 2009 [4]. Subsequently, many cloned buffaloes were produced [1]. Later, in 2017, several years after the birth of India's first cloned buffalo, another research institute, ICAR- Central Institute for Research on Buffalo (CIRB), Hisar, transferred the buffalo cloning technology to the field, away from the cloning laboratory [5]. CIRB also produced seven cloned copies from a single breeding bull.

METHOD OF BUFFALO CLONING

The method of producing cloned embryos appears to be quite simple and straightforward. In principle, the donor genome is fused with the cytoplasm of an enucleated ooplasm, followed by activation of the recombined ooplasm to stimulate embryonic development to the blastocyst stage, at which the embryos can be either transferred to suitably synchronized recipients or cryopreserved for future use. Two different versions of SCNT have been used to produce cloned embryos in buffalo [1]. The first is the traditional micromanipulation-based method of SCNT, which was used to produce the first cloned mammal, 'Dolly.' This method is well-established and has been extensively used in most cloning laboratories. To date, 23 animal species, including buffalo, have been successfully cloned using this method. An alternative method called Hand-made cloning (HMC), which was developed as a simplified alternative to micromanipulation-based SCNT [2], has been successfully used for the production of cloned offspring in many farm animal species such as cattle, pig, sheep, and goat, and buffalo. A significant advantage of HMC over the micromanipulation-based method is that there is no requirement for micromanipulators and their tools-making instruments for enucleation and fusion and for a highly skilled workforce to operate those instruments [2]. This significantly reduces the cost of establishing a cloning laboratory, so such laboratories can be set up in developing countries where the availability of funds and technical expertise



are major constraints. In addition to the multiple advantages of HMC, one practical problem is that two oocytes are used for the reconstruction of a single embryo, which increases the requirements of oocytes to generate cloned embryos, and the use of two recipient oocytes to generate a single cloned embryo may be responsible for higher mitochondrial heteroplasmy. We adopted this simplified method of SCNT developed by Vajta et al. (2001) and incorporated several modifications in the basic procedures, resulting in efficient enucleation, fusion, and activation, leading to a high blastocyst development rate [1]. Published reports suggest that with a higher blastocyst production rate, HMC can be used as an alternative method of SCNT.

APPLICATION OF BUFFALO CLONING IN INDIA

Potential applications of buffalo cloning in India are 1) make multiple copies of elite buffaloes such as high milk-producing females or proven breeding bulls; 2) creation of transgenic buffaloes that harbor human genes in their genome and can serve as bioreactors to produce therapeutic proteins like insulin and blood clotting factors, preferably in milk and 3) creation of disease model buffaloes that are designed to express certain human diseases. Despite multiple applications, buffalo cloning needs improvement since, on average, less than 6-8 % of the transferred cloned embryos produce healthy offspring [6]. Faulty or improper reprogramming of differentiated somatic cells is considered a major problem behind the low success of cloning technology. Joint research efforts are going on at two laboratories in India, namely CIRB, Hisar, and NDRI, Karnal, to unravel the exact cause of faulty reprogramming and to improve buffalo cloning efficiency.

CURRENT STATUS OF BUFFALO CLONING RESEARCH

Since Dolly died at the age of 6 years, the fear has been spread that clones could not survive as long as their original donors due to premature aging and genetic abnormalities. Researchers who produced Dolly spent more than 15 years unraveling the issue of aging in cloned animals. They recently published a paper in the Nature Journal that proved premature aging in clones was a wrong perception and that four genomic copies of Dolly have normal growth, health, and aging processes [7]. This study tells us that premature aging in cloned animals has been exaggerated. The same aging principle can be applied to buffalo since the basic reprogramming mechanisms are similar across the species. In addition to the wrong perception of the premature aging of cloned animals, people are also worried about the safety of products such as milk, meat, and semen obtained from cloned animals. In 2006, the U.S. Food and Drug Administration (FDA) examined the results of extensive studies that were conducted in different countries such as the U.S., U.K., Japan, China, and New Zeeland, and recommended that products obtained from cloned animals are

FIGURE 1. The cloned buffalo, named Garima, born at ICAR-National Dairy Research Institute, Karnal on 6th June, 2009 with the birth weight of 43 kg, has produced seven healthy and normal progeny. This cloned female has been growing well and does not have any physiological abnormalities

similar in terms of constituents and nutrients to the products of non-cloned animals [8]. Therefore, consumers are expected to benefit from consistent milk, meat, and semen from cloned animals, including buffalo.

During the last ten years, research and improvement in buffalo cloning techniques have produced several buffalo clones that are normal, healthy, and fertile [1,6]. These advances were made possible by significant improvements in blastocyst production rate and reduced health risks to born clones. Buffalo cloning can allow breeders and farmers to produce identical copies of their best animals, particularly proven breeding bulls, to exploit maximum production potential from them. By considering the potential utility of buffalo cloning in India, the Indian Council of Agriculture Research has been working on a mega research project to improve buffalo's production potential using semen of clones of elite breeding bulls, and our team is heading towards this goal.

METHODS USED TO MODIFY GENOME OF DOMESTIC ANIMALS

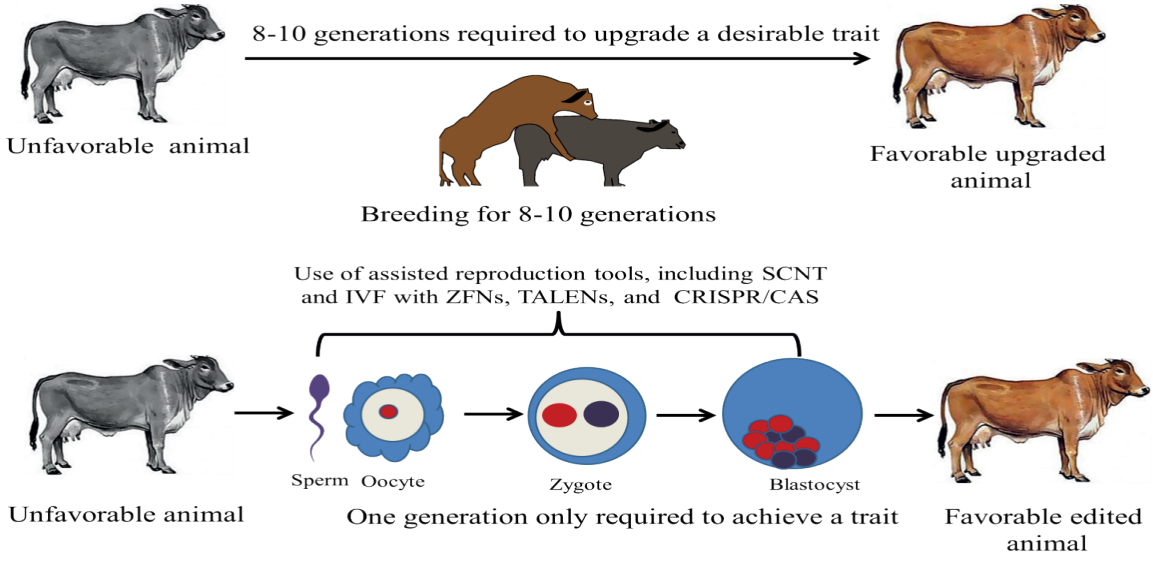
Conventionally, genetic changes in the livestock genome can be achieved by selective breeding, in which continual mating of favorable elite animals with unfavorable animals over many generations leads to the up-gradation of the specific alleles in the genome of the targeted animal population [9]. In dairy husbandry, semen from bulls of high milk-producing breeds (e.g., Holstein Friesian bulls) have been used to inseminate the low milk-producing breeds (e.g., Tharparkar cows) with the aim of producing upgraded female calves with im-

proved milk production trait. A well-known 'Karan Fries' breed of cattle was developed by the crossing of Holstein Friesian with Tharparkar at the National Dairy Research Institute, Karnal, using the principle of conventional breeding (http://www.ndri.res.in/ndri/Design/livestock_farm.html, accessed on September 10, 2021). With the selective breeding, milk production in India has increased from 55 million tonnes in 1991-92 to 187 million tonnes in 2018-19, almost triple (http://www.nddb.org/information/stats/milkprodindia, accessed on September 10, 2021), titled India becomes world's largest producer of milk. It is perceived that selective breeding continues to be a vital approach to producing desirable animals. Moreover, breeding allows the genome modification of those genetic traits which are naturally available in the population.

The employment of engineered nucleases would save the eight to ten generations of back-crossing required in conventional breeding to modify animals and allow the addition of new genes or the deletion of unfavorable genes. In FIG. 2, we have schematically shown the comparison between traditional breeding and current genetic engineering methods; both can be used to produce desirable animals. Before the birth of the famed 'Dolly' (born in 1996), the world's first genetically modified bovine, Herman the Bull, was produced by manipulation of the early embryo stage with the human lactoferrin gene [10]. Eighty-three calves were produced from this modified bull, and all calves inherited the lactoferrin production gene [11]. This work has been opening unlimited promises to livestock research, such as manipulating genes that affect milk and meat production, the production of essential proteins in milk, and producing model animals for biomedical or veterinary applications. Multiple research success stories have already demonstrated that genetic engineering with reproductive technologies (e.g., SCNT and IVF) can be used to manipulate the livestock genome [12]. However, wide-scale applications have always been a challenge due to the extremely poor efficiency of these techniques.

CRISPR EDITING

Engineered nucleases, including zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) system have been used for targeted modifications of the genomes of many organisms. Before engineered nucleases were developed, efficient genome manipulations, particularly knocking-out of genes, could only be achieved by using the inefficient homologous recombination (HR) in pluripotent embryonic stem (ES) cells. Thousands of mice lines were produced using the



Conventional breeding and new genome editing tools can use to manipulate the genome of domestic animals. In conventional breeding, unfavorable animals population (lower producers) were repeated inseminated with the semen of favorable animals (Bulls of high milk producers) for many generations (8-10) resulting in the up-gradation of the specific trait (milk production). In genome editing, new genes can add to a genome (e.g. lactoferrin in milk) or existing genes can delete (e.g. beta-lactoglobulin from milk) to a genome, possibly in one generation. Working mechanisms of molecular scissors are shown in fig 1 in details).

FIGURE 2. Conventional breeding and new genome editing as tools to manipulate the genome of domestic animals. In conventional breeding, unfavorable animal population (lower milk producers) inseminated with the semen of favorable animals (Bulls of high milk producers) for many generations (8-10), which resulting in the up-gradation of the specific trait (milk production). In genome editing, new genes can add to genome (e.g. lactoferrin in milk) or existing genes can delete (e.g. beta-lactoglobulin from milk) from genome, possibly in one generation

HR approaches; however, the translational to other species, including livestock, was limited by the absence of genuine ES cells in these species. In recent years, genome manipulation has gained tremendous momentum due to the discoveries of engineered nucleases, popularly named genome nucleases or scissors, namely ZFNs, TALENs, and CRISPR. In principle, these designer nucleases create double-strand breaks (DSBs) at specific desired sites in the genomic DNA. DSBs are repaired either by the non-homologous end joining (NHEJ) or the homologous directed repair (HDR) procedures, which have been exploited to manipulate the genome [13]. NHEJ is an error-prone procedure involving interrupted joining of the broken ends of DNA, creating insertions and deletions (indels) of nucleotides at cleavage sites, leading to disruption of gene function. HDR is a more accurate procedure, which requires homologous DNA sequences as templates for repair; therefore, a designed nucleotide sequence can be integrated into the genome by supplying it as an exogenous DNA template for HR events. FIG. 3 represents how designer nucleases work.

POSSIBLE OPPORTUNITIES TO INDIA

India possesses a large number of domestic animals: about 192.49 million cattle, 109.85 million buffaloes, 74.26 million sheep, and 148.88 million goats. Milk and milk products, meat and meat products, eggs and eggs products are integral to the Indian diet. To satisfy the demand for food for the ever-growing population, the country needs the continuous and sustainable production of milk, meat, and other animal products. Climate change, emergence and re-emergence of disease pathogens, and non-availability of fodders are new challenges that might be overcome through scientific intervention for sustainable animal production. We believe that the CRISPR-Cas system and other sophisticated genome engineering techniques could be able to manage some of these concerns. A few futuristic examples are: 1) the production of bulls carrying disease resistance (e.g., FMD, rinderpest, and black quarter) or stress-tolerant (e.g., extreme heat or extreme cold) genes. The semen of such bulls would be valuable to produce tolerance offsprings. 2) The generation of bulls, which produce only spermatozoa with the X chromosome, produced sorted semen, producing female offspring. Male offspring are unprofitable to farmers due to the ban on cattle slaughter and mechanization of agriculture. 3) The production of veterinary vaccines (e.g., against FMD and rinderpest) or human therapeutic proteins (e.g., insulin) in the mammary glands of dairy animals for clinical uses.

According to existing knowledge and technologies available in India, the buffalo will be a preferred choice among domestic animals for exploring CRISPR-Cas applications. Ex-

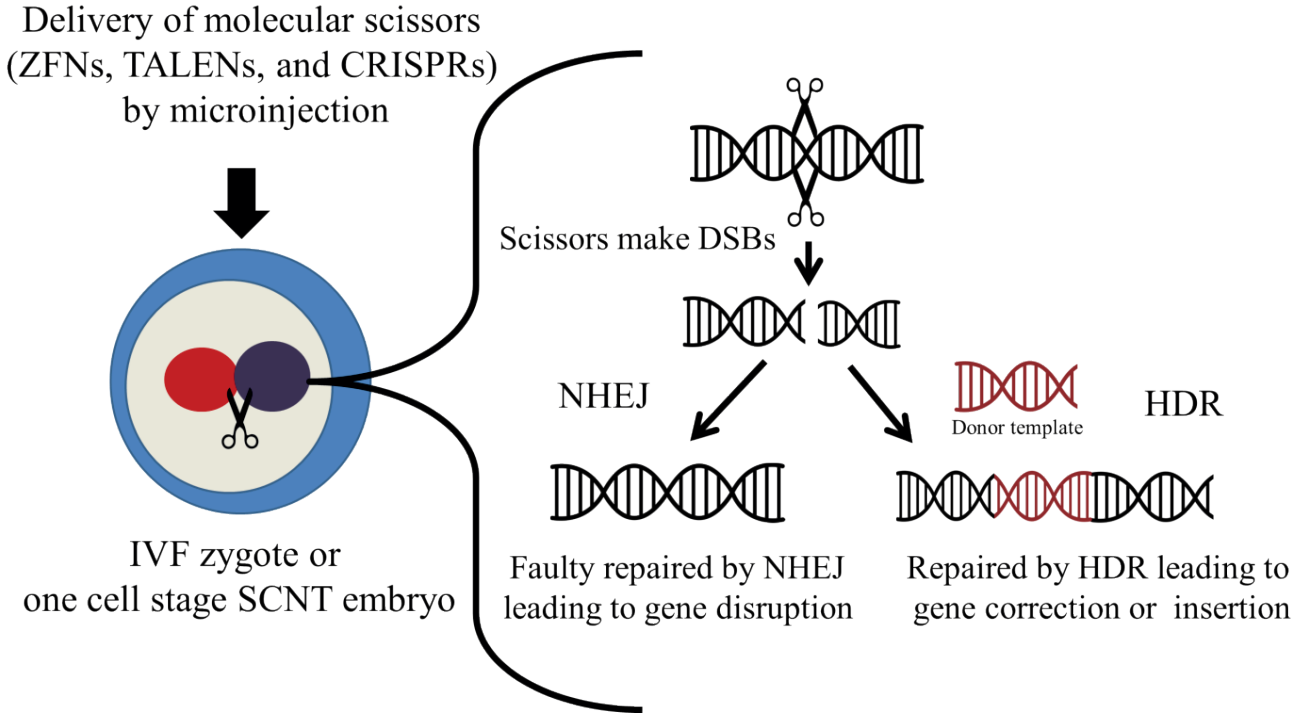


FIGURE 3. How molecular scissors/designer nucleases work in embryos: Molecular scissors are delivered in a one cell stage embryos, they specifically cleavage the target DNA causing a double-strand break (DSB). The DSB is subsequently repaired either by the error-prone non-homologous end joining pathway (NHEJ), or the homology-directed repair (HDR) processes. NHEJ leads to gene disruption due to insertions or deletions (indels) of nucleotides, whereas HDR leads to gene correction or insertion if provided with donor template

perimental working with buffalos has several advantageous aspects: 1) ample availability of oocytes for manipulations, 2) most assisted reproductive techniques, such as IVM, IVF, IVC, ET, and MOET, have been demonstrated, 3) SCNT has successfully been proved and is currently being used in the cloning of breeding bulls and elite females, and 4) scientific expertise and technical personnel are available, including staff for efficient manipulation of oocytes and embryo transfer. Our lab recently initiated a proof of principle study aiming to produce manipulated buffaloes expressing improved production traits and health. Two major limitations need to be addressed to reach this aim: 1) poor efficiency of buffalo cloning success rate, and 2) the complete annotation of the buffalo genome, which still needs to be fully accomplished. Basic and applied aspects of research are required to achieve successful gene editing in the buffalo species.

CONFLICT-OF-INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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