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CODEN: IJBNHY
ISSN: 2278-778X
Review Article
International Journal of Bioassays

Advances in equine cloning by Somatic Cell Nuclear Transfer (SCNT) technique in horses: A review

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Received: October 15, 2016; **Received:** November 23, 2016; **Accepted**: November 27, 2016
Available online: 1st December 2016

Abstract: Cloning a horse means using the genetic material (DNA) from a donor horse to produce a genetically identical foal. This technique involves collecting the DNA from the donor and inserting that DNA into an egg from another mare whose, DNA content has been removed, fusing donor nucleus with enucleated recipient oocytes, which then develops as an embryo, *in vitro* culture of embryo and lastly transfer cultured embryo into the uterus of a recipient mare. The modification of the *in vitro* culture conditions which can be suitable for equine oocyte activation, oocyte maturation and embryo development are the fundamental steps for a successful *in vitro* procedure for somatic cell nuclear transfer (SCNT) in the horse to avoid the embryo losses. In general, few studies are available in the literature on equine *in vitro* embryo production and it is only recently that reports have been published on completely *in vitro* production of equine preimplantation embryos by means of *in vitro* oocyte maturation. The present review discusses the latest developments in the field of equine cloning technique with the employment of SCNT. The basic understanding of SCNT for *in vitro* culture conditions is relevant to the increased efficiency of cloning. The available genotype can be used by SCNT which can enhance the vigour of a particular infertile or low fertile animal to produce normal fertility.

Key words: Cloning; Horse; *in vitro* production; SCNT

Introduction

Cloning is defined as making an exact genetically identical copy of another cell or organism through asexual reproduction. Farmed animals species mainly, sheep, cattle, pigs, goat, rabbits and horses were amongst the first mammalian species to be cloned. These species were selected because of their economic importance and well developed assisted reproduction techniques, such as large numbers of oocytes from the slaughterhouse, *in vitro* production of embryos and their transfer into surrogate mothers. Cloning mammals by somatic cell nuclear transfer (SCNT) has become a common technology in recent years, following the cloning of Dolly sheep[1]. Till date most domestic and laboratory species have been cloned, including the horse[2]. The number of cloned horses being born per year is small. There were approximate 100–200 cloned horses worldwide[3]. Somatic cell nuclear transfer (SCNT) or 'cloning' is currently being offered as a commercial method of horse reproduction in different countries[4].

The refinement of the *in vitro* culture conditions suitable for equine oocyte maturation and embryo development[5,6], the development of an adequate horse oocyte activation protocol[7] and the application of zona-free manipulation for embryo reconstruction[8,9], are all fundamental steps for the development of a successful *in vitro* procedure for somatic cell nuclear transfer in the horse. Both expanded and compact cumulus–oocyte complexes were used as well as a modified zona-free method for embryo reconstruction that is known to increase the fusion rate in other species[9]. Different sources of donor somatic cells were compared in various experiments for their ability to develop to the blastocyst stage and to term[10,11].

History of cloning

The birth of three mule foals cloned from fetal cells, using *in vivo* matured oocytes, which were transferred to the oviducts of recipient mares, immediately after nuclear transfer and activation[12] and one horse foal cloned from adult somatic cells[13] were reported in 2003 and additional cloned foals have also been recognized by various workers[14,15]. In contrast to *in vivo* technique, the cloning of horse embryos up to the blastocyst stage carried out completely *in vitro*[16]. Complete *in vitro* production of equine pre-implantation embryos by means of *in vitro* oocyte maturation, fertilization by intracytoplasmic sperm injection

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(ICSI) and *in vitro* culture and develop into live offspring[17,18]. Fulka and Okolski[19] reported the first successful in *vitro* maturation of horse oocytes. The first embryo production from *in vitro* matured horse oocytes, when oocytes collected at the abattoir were matured *in vitro* and transfer to the oviducts of inseminated mares and recovery by uterine flushing 7 days later.[20] A study was designed by Gambini *et al.*[21] to determine whether embryo aggregation improves in vitro and in vivo cloning efficiency in the equine through an assessment of the quantity and quality, the Oct-4 level expression and growth of embryos, the establishment of pregnancies, and the birth of foals following embryo transfer. They obtained that the embryo quality was reduced when aggregation was not performed and early pregnancy rates were higher following the transfer of blastocysts derived from aggregated groups.

Somatic cell nuclear transfer (SCNT) methodology

a) **Collection and *In vitro* maturation of oocytes:** Oocytes can be harvested from the ovaries of live donors by ovum pick up (OPU) or from the ovaries of slaughtered mares[20], Once the collection of oocytes is completed and selected on the basis of cumulus morphology, they are transferred to maturation medium and allowed to mature for 24 h.[18] The oocytes with expanded cumulus are more capable to complete maturation than were oocytes with a compact cumulus. The maturation rate ranges from 51.1% to 60% for compact and expanded cumulus, respectively[22]. The *in vitro* maturation conditions significantly influence the development of fertilized oocytes to the blastocyst stage. Oocytes are matured *in vitro* for 24-28 h at 38.5° C in 5% CO_2. The most common medium used for maturation of equine oocytes is TCM 199, probably because of its widespread use for bovine[23].

b) **Preparation of donor cells:** Donor cells were obtained by biopsy, harvesting at slaughter or after death. In the horse, foetal fibroblasts[12], adult fibroblasts and granulosa cells have been used[14,24]. The variation in the success rate of horse SCNT, because of the specific cell line source of donor nuclei[14]. The cell cycle of the donor cell is crucial for success and G0 or G1 stages have been used in horses. These stages can be achieved through serum starvation, contact inhibition or the use of kinase inhibitors like Roscovitine[25,26].

c) **Preparation of enucleated oocytes:** After 22–24 h of maturation, the oocytes are discarded of cumulus cells by pipetting[27].

d) **Fusing donor nucleus with enucleated recipient oocytes:** The development process was started by fusion of enucleated recipient oocytes and the donor nucleus. Fusion can be accomplished by the administration of a brief electrical pulse or by chemical fusion to initiate fertilization which then develops into an embryo.

e) **Embryo culture:** The culture of zygotes *in vivo* in the mare oviduct allowed 30% higher development[28]. The embryos produced *in vitro* and various media such as DMEM-F12, CZB[28] and modified SOF[29] were used for pre-implantation development of fertilized horse oocytes. In most of these systems, yet, the blastocyst rates remained low, ranging from 4% to 16% of injected oocytes[8].

f) **Embryo transfer into recipient:** Finally, the embryo transfer into oviduct of recipient mare. The mare examined for pregnancy diagnosis and thereafter, weekly throughout the first trimester of pregnancy and later at monthly intervals until foaling[14,30]. The birth of a dozen equids following oviduct transfer of early cleaving embryos[12] or culture *in vitro* to the blastocyst stage for transfer directly to the uterus of a recipient mare[10,14].

Limitations of SCNT

a) Limited interest demonstrated by the horse industry in comparison to the cattle industry in the development and application of reproductive biotechnologies.

b) Inadequate information available on assisted reproductive techniques, such as oocyte maturation, activation and *in vitro* culture of early pre-implantation embryo in Equine.

c) Limited availability of horse oocytes compared with other large domestic species because, anatomy and physiology of the mare's ovary

d) The maturation rate of horse oocytes is also quite variable, averaging between 25% and 70%[31].

e) Low ability of oocytes to fuse with donor cell nucleus and the limited developmental capability of nuclear transfer embryos *in vitro.*

f) Pregnancy losses with equine SCNT embryos occur throughout gestation. 57% of nuclear transfer pregnancies from adult fibroblasts were lost between days 17 and 35.[22,32]

g) Three live foals were resulted from transfers of more than 100 cloned embryos[10,29]. Thus, embryo losses occur in equine cloning.

Benefits of cloning

Cloning of horses to preserve endangered breeds such as the Przewalski's horse in an outbreak of fatal disease, such as African horse sickness can be practiced. Preservation of genetic material from individual animals can be possible which would otherwise not be able to reproduce[33,34], research and as companion animal for some horse owners[35]. SCNT has the potential to impact animal breeding in as fundamental a manner as artificial insemination. SCNT will likely be used to improve production characteristics of food producing animals by providing breeding animals, just as any

breeding program would select the most elite animals for breeding, and not as production animals. Cloning has the relative advantage of allowing for the propagation of animals with known phenotypes to serve as additional breeding animals. This is critically important in breeding programs to declare the merit of a sire or dam. It also allows the propagation of animals whose reproductive function may be impaired.[36,37] Equine reproductive technologies, such as cloning and intracytoplasmic sperm injection (ICSI) have become routine laboratory practice in many parts of the world.[38]

Conclusion

The equine cloning is associated with high rates of embryonic loss, fetal abnormalities, dystocia. These problems are likely to reduce as SCNT techniques improve. There are many steps in cloning mammals by somatic cell nuclear transfer. The basic understanding of these events particularly *in vitro* culture conditions leading to improve the efficiency of cloning. SCNT can be used to increase the available genotype of a particular animal with low fertility with the clones appear to be exhibiting normal fertility. However, the cost of derivation of SCNT lines is higher than iPS cells. The major cost contributor in SCNT is an egg donation. It allows the propagation of valuable deceased animals from which tissue samples have been appropriately collected or preserved, which may have profound implications for species or breeds nearing extinction.

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Cite this article as:

Rajesh Wakchaure, Subha Ganguly. Advances in equine cloning by Somatic Cell Nuclear Transfer (SCNT) technique in horses: A Review. *International Journal of Bioassays* 5.12 (2016): 5124-5127.

DOI: http://dx.doi.org/10.21746/ijbio.2016.12.002

Source of support: Nil

Conflict of interest: None Declared