

Review Article

Indian J Med Res 148 (Supplement), December 2018, pp 115-119
DOI: 10.4103/ijmr.IJMR_2096_17



Approaches used to improve epigenetic reprogramming in buffalo cloned embryos

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Received December 31, 2017

The reproductive cloning in buffalo in India has been started using a simplified somatic cell nuclear transfer technique named handmade cloning. Since the birth of first cloned female buffalo in 2009, a number of buffalo clones have been produced in India by utilizing different types of donor cells such as ear cells, embryonic stem cells, semen somatic cells and urine somatic cells. The use of buffalo cloning on a large scale is restricted due to low pregnancy rates and poor calf survival. Considerable attempts have been made to improve the overall buffalo cloning efficiency, particularly by modifying epigenetic reprogramming of cloned embryos. Previous studies have demonstrated that chemical epigenetic modifiers such as trichostatin A and 5-aza-2'-deoxycytidine, m-carboxycinnamic acid bishydroxamide can be used to treat donor somatic cells and reconstructed fused embryos to correct the epigenetic reprogramming to enhance the overall cloning efficiency in terms of live birth rates.

Key words Birth rate - buffalo - cloned - embryos - epigenetics - reprogramming

Introduction

Somatic cell nuclear transfer (SCNT) is one of the most successful tools of assisted reproductive techniques, which has been used to produce identical copies of elite domestic animals such as cattle, buffalo and sheep, goats and pigs¹, and for drug production (biopharming), regenerative medicine (nuclear stem cell lines to cure diseases) and conservation of genetic resources (cloning of endangered species)². Buffalo SCNT has enormous potential applications; however, the technique has been suffering from low live offspring birth rate (<2%) after transfer of cloned blastocysts^{3,4}. Many abnormalities such as high rate of early abortion, large

offspring syndrome, placental defects and health issues of born clones have been reported in cloned animals^{3,5}. These developmental abnormalities are conceivably accountable for poor cloning efficiency and it is believed that abnormal epigenetic reprogramming of differentiated somatic cells is the main cause of poor cloning efficiency across the species, including buffalo.

Incorrect or faulty epigenetic reprogramming of donor cells by recipient oocytes during SCNT is one of the key factors responsible for the low cloning efficiency; therefore, for achieving correct nuclear reprogramming, the epigenetic status of donor cells or cloned embryos needs to be manipulated in such a

way that generated cloned blastocysts would be more closely similar to *in vivo* or *in vitro* fertilized (IVF) embryos. To correct the nuclear reprogramming and to improve the developmental competence of cloned embryos, various approaches have been employed either in somatic cells or in cloned embryos; however, significant success in the term of live birth rate has not been achieved. Here we discussed about *in vitro* studies in which epigenetic modifiers were used to correct reprogramming of SCNT buffalo embryos.

Methods of buffalo cloning

The buffalo can be cloned using two broadly used SCNT techniques, first is a classical SCNT in which micromanipulator instrument is used⁶; whereas, other method is a micromanipulator-free SCNT called Handmade cloning (HMC)^{7,8}. The HMC is preferred due to the low cost of embryo production and less requirement of highly skilled workforce to perform embryo manipulations. The HMC procedure includes five steps which are: (i) removal of genetic material (enucleation) from zona-free oocytes using a small blade, (ii) electro-fusion of donor cells with nucleus-free oocytes, (iii) activation of fused embryos, (iv) *in vitro* culture of activated embryo for 7 or 8 days, and (v) *in vivo* transfer of generated blastocysts into foster mothers to deliver cloned calves. Basic differences between classical SCNT and HMC are shown in Table I. The births of healthy calves have been reported in cattle, pig and goat and buffalo using HMC technique³.

Status of buffalo cloning in India

In India, a simplified HMC, which was earlier demonstrated by Vajta *et al*⁹ in cattle has been adopted. The basic method was modified to support the developmental competence of buffalo oocytes and embryos, which resulted in the efficient enucleation, fusion and activation and high blastocyst production rates^{7,10}. The first live birth of cloned buffalo was reported in 2009 using optimized buffalo HMC method¹¹. Following the birth of first cloned buffalo, several cloned buffaloes were produced in India, and attempts are continued to produce more clones³. Optimized HMC technique has yielded much higher blastocyst production rate than that of IVF technique (35-45% blastocyst rates in case of HMC; whereas, 10-15% in case of IVF embryos); however, the large number of produced blastocysts could not be translated into live clones³. Previous studies in buffalo suggested

Table I. Classical somatic cell nuclear transfer (SCNT) vs. simplified handmade cloning

Condition	Classical SCNT	Handmade cloning
Use of micromanipulator	Yes	No
Zona-free	No	Yes
Manual enucleation	No	Yes
Activation and culture methods	Similar	Similar
Problems associated with zona hatching	Yes	No
Problems associated with mitochondrial heteroplasmy	No	Yes
Comparative cell number in produced blastocysts	Less	High
Problems associated with genomic reprogramming	Yes	Yes
Skilled workforce manpower to perform experiment	Yes	No
Involved cost	High	Less

Source: Ref. 8

that cloned embryos exhibited hypermethylated, abnormal histone acetylation and methylation and altered genes expression pattern as compared to IVF embryos¹²⁻¹⁵. These reports suggest that basic research is needed to understand the epigenetic machinery of somatic cells and cloned embryos to improve the success rate of SCNT.

Ways to correct epigenetic reprogramming

Different approaches have been used to correct the epigenetic reprogramming in cloned embryos such as treatment of somatic cells, oocytes and fused embryos with epigenetic modifiers or with oocytes or stem cells extract, overexpression or suppression of important regulatory genes in somatic cells or one cell stage cloned embryos (Figure). Buffalo somatic cells and/or fused embryos were treated with different epigenetic modulating agents, such as trichostatin A (TSA), 5-aza-2'-deoxycytidine (5-aza-dC), valproic acid (VPA) and m-carboxycinnamic acid bishydroxamide (CBHA)^{14,16-18}. These studies showed that treatment of buffalo donor cells and/or one cell stage fused embryos or both with epigenetic modulators, alone or in combination, resulted in higher blastocyst production rates and lower level of apoptosis in generated cloned

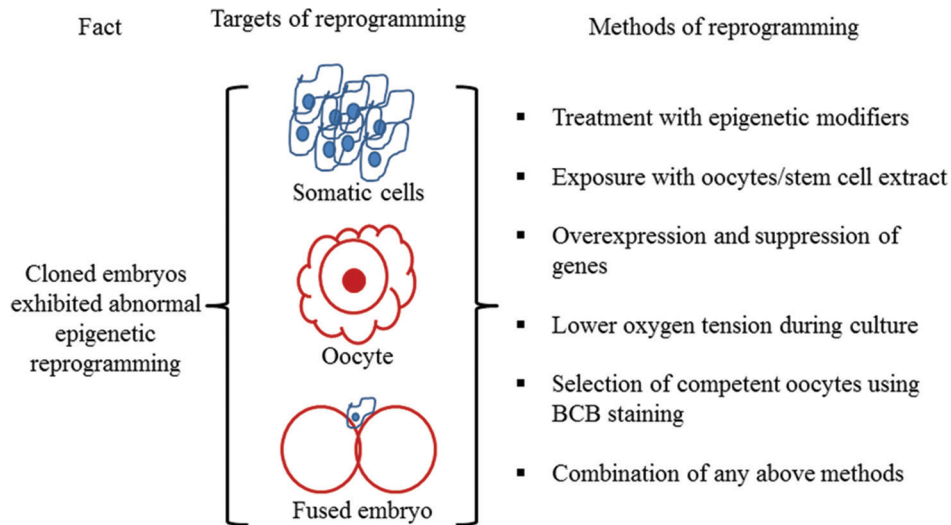


Figure. Different approaches used to correct an epigenetic reprogramming in buffalo cloned embryos. Somatic cell, oocyte, and fused embryo can be treated with any mentioned method either individually or in combination to improve reprogramming. BCB, Brilliant Cresyl Blue.
Source: Ref. 3.

blastocysts^{14,15}. Future studies are needed to transfer improved blastocysts produced following the treatment of epigenetic modifiers into recipients to improve live birth rates.

Use of epigenetic modifiers in buffalo cloning

Though different epigenetic modifiers were used to correct reprogramming in buffalo embryos; but best results were obtained with the combination of TSA and 5-aza-dC treatments (Table II). Panda *et al*¹⁹ reported that treatment of fused embryos with 500 and 1000 nmol/l of scriptaid, a histone deacetylase inhibitor, significantly increased the blastocyst production rate. Furthermore, blastocysts derived from treated groups had higher cell number (339.9±1.4 and 343.4±2.4, respectively) than that of untreated group (150.7±2.0)¹⁹. Saini *et al*¹⁴ examined the effects of treatment of donor cells with TSA and 5-aza-dC and found that donor cell treatments altered the expression of epigenetic-related genes, namely *HDAC1*, *DNMT1* and *DNMT3a*. Treatment with these two epigenetic modifiers also increased the acetylation level of lysine at position 9 or 14 in histone 3 (H3K9/14ac), lysine at position 5 in histone 4 (H4K5ac), lysine at position 18 in histone 3 (H3K18ac) and decreased tri-methylation of lysine at position 27 in histone 3 (H3K27me3) in the cells. Simultaneous treatment of donor cells with TSA (50 nM) and 5-aza-dC (7.5 nM) resulted in improved blastocyst rates, lower apoptotic index and higher level of H3K27me3 in cloned blastocysts¹⁴. Subsequently,

the same group¹⁵ optimized doses of TSA and 5-aza-dC to improve reprogramming in buffalo cloned embryos by examining whether combined treatments of epigenetic modifiers would offer any advantage over treatment with the individual epigenetic modifier. Irrespective of whether donor cells or fused embryos or both treated with 50 nM TSA+7.5 nM 5-aza-dC, the blastocyst rates significantly improved (Table I); low apoptotic index, which was similar to blastocysts produced through *in vitro* fertilization; and higher level of H3K18ac and lower H3K27me3 level in blastocysts than that of untreated group. This study demonstrated that similar beneficial effects could be obtained by treatment of donor cells or fused embryos or both with 50 nM TSA+7.5 nM 5-aza-dC¹⁵. Selokar *et al*¹⁶ used an epigenetic modifier, VPA, another class of histone deacetylase inhibitor, to treat the donor cells for correcting epigenetic reprogramming. This study demonstrated that treatment of donor cells with VPA did not improve the blastocyst production rate. Agrawal *et al*¹⁷ reported that 10 µM of CBHA could be used to improve the blastocyst rates and quality of cloned embryos.

Beneficial effects are mediated by decreasing DNA and histone methylation and increasing histone acetylation. Treatment of buffalo donor cells or fused embryos or both with epigenetic modulators can be one of the ways to improve the success rate of buffalo SCNT. In addition to the use of epigenetic modifiers, other approaches have also been used such as lower oxygen

Table II. Epigenetic modifiers used in buffalo handmade cloning experiments

Epigenetic modifier	Treatment type	Blastocyst rate (%)	Reference
Scriptaid (nmol/l)			
0	Fused embryos (6 h)	38	19
500		42 [#]	
1000		54 [#]	
TSA (50 nM) + 5-aza-dC (7.5 nM)*	Control	43	15
	Cells (24 h prior SCNT)	71 [#]	
	Fused embryos (10 h)	68 [#]	
	Cells + fused embryos	71 [#]	
Valproic acid (mM)			
0	Cells (24 h prior SCNT)	45	16
1.5		49	
3.0		48	
4.5		52	
CBHA (μM)			
0	Fused embryos (10 h)	48.63	17
5		52.00	
10		63.77 [#]	
20		48.32	
50		54.98	

*Mentioned doses of TSA (trichostatin A) and 5-aza-dC (5-aza-2'-deoxycytidine) were optimized by treating somatic cells, fused embryos or both using different concentrations of these drugs. [#]Represents the significant improvement in blastocyst production rates than that of the untreated control group. SCNT, somatic cell nuclear transfer; CBHA, m-carboxycinnamic acid bishydroxamide

tension during embryo culture²⁰, use of BCB staining to screen best competent oocytes for the production of cloned embryos²¹. These approaches also resulted in an improvement in the blastocyst production rates and quality of produced blastocysts; however, more attempts are required to translate *in vitro* improvements into *in vivo* developments.

Conclusion

Buffalo cloning is a powerful reproductive tool to multiply elite animals, particularly proven bulls; however, live offspring production efficiency is low which is mainly due to abnormal epigenetic reprogramming. Previous studies demonstrated that abnormal epigenetic reprogramming can be corrected using the treatment of somatic cells or fused embryos with epigenetic modifiers, namely TSA and 5-aza-dC. In Buffalo, limited attempts were made to transfer epigenetically improved blastocysts into recipient animals to examine their *in vivo* developments; therefore, further studies are required to determine whether the beneficial effects observed *in vitro* following treatment of epigenetic modifiers would translate to live births.

Acknowledgment: Authors thank the past members of buffalo cloning team of the Animal Biotechnology Centre (ABTC), National Dairy Research Institute (NDRI), Karnal, Haryana.

Financial support & sponsorship: This work was financially supported by the National Agriculture Innovative Project grants (C 2-1-(5)/2007, C-2067 and 075) to the ABTC, NDRI, Karnal, Haryana.

Conflicts of Interest: None.

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