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An update: Reproductive handmade cloning of water buffalo (*Bubalus bubalis*)

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ABSTRACT

The first birth of a cloned animal produced through the Handmade cloning (HMC) technique was reported more than 15 years ago in cattle. This method of somatic cell nuclear transfer (SCNT) has subsequently been evolving as a much simpler alternative to the classical micromanipulator-based SCNT. Several farm animal species such as cattle, buffalo, pigs, sheep, and goats have been successfully cloned using HMC. In buffalo, HMC technique is now well established, and several births of cloned calves have been reported by us. Several factors such as source of somatic cells, quality of recipient oocytes, cell cycle stage prior to SCNT, electrofusion and culture conditions, and epigenetic status of somatic cells, have been optimized leading to the production of good quality cloned embryos. The preservation through cloning of proven breeding bulls that have died by producing live offspring using somatic cells isolated from frozen semen as donor cells and birth of a cloned calf from urine-derived cells are impressive examples of the success of HMC in buffalo. In conclusion, HMC is a valued reproductive technique in buffalo that offers the opportunity to make multiple copies of highly valuable animals, particularly proven breeding bulls. In this review, there is a discussion of the advancement of the HMC technique in buffalo and factors responsible for the efficient production of cloned embryos.

1. Introduction

Since the birth of first cloned farm animal - Dolly, the sheep, more than 20 species have been cloned using somatic cell nuclear transfer (SCNT) procedures (reviewed by Keefer, 2015). The method used to produce 'Dolly' involves the use of sophisticated and expensive equipment, including micromanipulator, for enucleation of oocytes and transfer of somatic cells into enucleated oocytes. Also, highly trained operators are required for making manipulation tools and to perform SCNT (Vajta, 2007). Peura et al. (1998) first demonstrated the concept of micromanipulator-free SCNT in which embryonic blastomeres were used as donors, and recipient oocytes were enucleated using microblade followed by fusion of two enucleated oocytes with one blastomere. Another group subsequently refined this approach of micromanipulator-free SCNT significantly and extensively used it to produce cloned cattle and pigs using differentiated somatic cells, and named this alternative SCNT as Handmade cloning (HMC; Vajta, 2007). Domestic farm animals (cow, pigs, buffalo, goats, and sheep) have been cloned using HMC technique (Fig. 1). Since the initiation of HMC, the technology has

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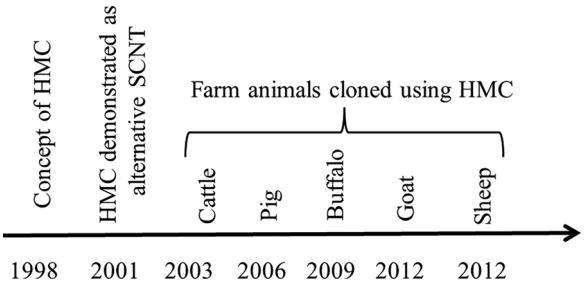

Fig. 1. Domestic farm animals cloned using HMC: Peura et al. (1998) demonstrated zona-free method of SCNT, later called HMC (Vajta et al., 2001); cattle was the first cloned animal produced by HMC (Tecirlioglu et al., 2003) followed by pigs (Du et al., 2007), buffalo (Shah et al., 2009), goats (http://www.icar.org.in/node/5371), and sheep (Zhang et al., 2013).

now evolved as a simple and cost-effective alternative to the conventional micromanipulator-assisted method of SCNT.

The domestic buffalo (*Bubalus bubalis*), a multipurpose livestock species in Asia, provides milk, meat, and draught power. Buffalo are divided into two subspecies, the riverine type (having 50 chromosomes) and the swamp type (having 48 chromosomes). The riverine buffalo breeds, which include Murrah, Nili-Ravi and Jafarabadi, are known for milk production and are used extensively to upgrade the genetic potential of non-descript lesser milk producing buffalo. In Europe, there has also been an increasing interest in buffalo farming aimed at fulfilling the demand for milk and meat (Presicce, 2007). As a result, several riverine buffalo herds have been introduced in many European countries and in swamp buffalo-dominated areas (Presicce, 2007). Due to the ever increasing demand of milk and meat in Asia, there is an urgent need to multiply genetically elite male and female buffalo to meet this demand. HMC technique has the potential of being applied to multiply elite male and female buffalo more rapidly than what can be achieved through conventional breeding. The HMC technique has been developed for buffalo and several clones of the Murrah breed have been produced which is the most desirable riverine dairy breed of buffalo (reviewed by Singla et al., 2015 and Selokar et al., 2018). There have been two publications in which there have been reports of live births in swamp buffalo using micromanipulator-based SCNT (Shi et al., 2007; Tasripoo et al., 2014). In this review paper, the development of buffalo HMC and the factors responsible for efficient production of cloned embryos are discussed.

2. HMC developments in buffalo

The first report on production of buffalo embryos through HMC was published by Shah et al. (2008). In this study, different culture surfaces *viz.* micro-drop, well-of-well (WOW) and flat surface and culture mediums, namely modified Charles Rosenkrans 2 medium (mCR2), modified synthetic oviductal fluid (mSOF), and commercially available Research Vitro Cleave medium (RVCL)) were compared for examining their efficacy in production of blastocyst-stage cloned embryos (Shah et al., 2008). Although the WOW culture surface had been reported to be the most efficient and preferred surface for the culture of zona free embryos and handmade cloned embryos in the several animal species and humans (Vajta, 2007), it was not found to be efficient in supporting the *in vitro* development of zona-free buffalo embryos produced through HMC (Shah et al., 2008). This disparity may be due to different methods used for making micro wells on the culture dishes, use of different culture media and conditions and species specificity. The combination of flat culture surface and commercially available RVCL medium was found to support high blastocyst production rate (30%–40%), comparable to that reported in cattle and pigs (Shah et al., 2008). In a later study, there was examination of the effects of different electrofusion parameters such as AC voltage alignment, DC pulses, fusion methods and the orientation, and position of somatic cell-cytoplasts on the developmental competence of cloned embryos (Selokar et al., 2012a). The results of this study suggested that the single step fusion method using 4 V AC alignment followed by dielectrophoresis induction by a single pulse of DC volt (3.36 kV/cm which is equivalent to 160 V in ECM-2001, BTX electrofusion machine) for 4 μsec was most efficient for electrofusion of one somatic cell with two enucleated oocytes (Selokar et al., 2012a). Subsequently, these optimized conditions of electrofusion (Selokar et al., 2012a) and *in vitro* culture conditions (Shah et al., 2008) have been used in several studies (Mohapatra et al., 2015; Duah et al., 2016; Jyotsana et al., 2016; Saini et al., 2016, 2017) aimed at producing cloned buffalo embryos.

After optimization of HMC, several different types of donor cells and approaches were used to produce cloned calves resulting in birth of 15 cloned calves (reviewed by Selokar et al., 2018). A few notable achievements using HMC in buffalo are i) restoration of a progeny-tested dead bull by isolating somatic cells from frozen-semen doses and using these as donor cells for producing cloned offspring (Selokar et al., 2014), ii) production of a cloned calf from urine-derived cells, a first reported study in animals (Madheshiya et al., 2015) iii) production of a wild buffalo (*Bubalus arnee*) using interspecies SCNT, in which somatic cells of a wild buffalo cells were fused with enucleated oocytes obtained from riverine buffalo (http://www.icar.org.in/en/node/8465) and iv) production of cloned embryos from milk-derived cells (Golla et al., 2012), blood-derived lymphocytes (Jyotsana et al., 2016) and non-viable somatic cells (Duah et al., 2016).

3. Steps of buffalo HMC

The HMC method (Vajta et al., 2001 and Du et al., 2005) is a simplified version of micromanipulation-based SCNT, which does not