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Handmade Cloning: A Handy Technique for Reproductive Cloning

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Abstract

Handmade cloning (HMC) is an alternative form of somatic cell nuclear transfer (SCNT) where there is no need of micromanipulator making the procedure simple and cheap for production of cloned animals. Since last 10 years significant improvement has been occurred in the HMC process to enhance the efficiency of live births and numbers of livestock animals have been produced using this technique. The HMC has wide application such as transgenic animal production, interspecies cloning (thus saving the endangered species), regenerative therapy, besides the conservation of genetically potential livestock animals. The transgenic animal production using HMC has great potential of producing pharmaceuticals/proteins through milk of livestock animals like cow and goat in the process of biopharming, thus making the production more robust once the transgenic animal is established. Although HMC has wide application, the postnatal abnormalities and decreased life span of cloned animals still possess a challenge for us which needs to overcome through appropriate epigenetic reprogramming. Intensive research work on this aspect and possibility of automation of the technique in future will make the reproductive cloning easy to adapt with higher efficiency. In this review, we have discussed the latest findings in improvisation of the technique which will facilitate the researchers for further study.

Key words: Blastocyst, demioocyte, donor cell, embryo, handmade cloning

INTRODUCTION

The somatic cell nuclear transfer (SCNT) technique gained momentum since the production of sheep Dolly and widely used to clone the livestock animals [1]. The cloned embryo is produced asexually using the somatic cell (2n chromosome) as donor of nucleus fused to the enucleated oocyte of the female [1, 2]. The SCNT is basically of two categories: Traditional cloning (using micromanipulator), and Handmade cloning (HMC). The HMC technique is the form of SCNT, in which there is no need of micromanipulator (the main costly equipment used in traditional SCNT) for the cloning process, and all the steps are done by hand. The technique has gained popularity due to the low cost involved in producing the cloned animal and not much expertise required to perform this technique. This method can be used not only in intra-species cloning but also in inter-species cloning (iSCNT) for endangered species conservation, transgenic animal production, regenerative therapy, and creating disease model [3]. This technique was pioneered by Gabor Vajta and it is also termed as hand-guided technique [4]. The HMC technique has been used to clone various species of animals with at par or even better efficiency as compared to traditional micromanipulator-based technique.

If we look back to the history of nuclear transfer technique the first experiment began more than 60 years back in amphibia [5]. Subsequently, fertile cloned frogs were produced successfully which paved the way for cloning of mammals [6]. First cloned mouse was produced in 1981 using stem cell as donor cell derived from inner cell mass and transferred to enucleated zygote [7]. The creation of Dolly (differentiated mammary epithelial cell as donor of nucleus) revolutionized the World of cloning and subsequently various animal species have been cloned.

Tatham et al. (1995) were the first group to perform the zona-free nuclear transfer approach where the enucleation was done by density gradient centrifugation of zona-free oocytes [8]. Subsequently enucleation method was improvised by Peura et al. (1998) [9]. Later on contribution by several workers led to the establishment of handmade cloning with successful pregnancies and live births in different livestock species such as bovine [10-12, 4]; porcine [13-15], and murine [16]. The term handmade cloning was given because all the steps can be performed by hand, without the use of micromanipulator [10]. Gabor Vajta has contributed significantly to the HMC technique along with WOW culture system on which he won the patents [17, 18].

Some researchers have reviewed on HMC [19-21, 3, 4] technique and assessed the advantages and disadvantages in present scenario. The present review focuses on the latest improvements in the HMC technique and studies performed to improvise the efficiency of cloned live births. This review will help the researchers to identify the lacunae in HMC technique and motivate to further plan the work on HMC to achieve the best out of it.

Steps in HMC technique

This technique is unique in its use of zona-free oocytes to enucleate and fuse with the donor cell [Fig. 1]. Two enucleated demioocytes are fused to the donor cell to compensate the loss of cytoplasm (15 – 50 % loss) occurred during protrusion-cone guided cutting of oocytes for enucleation [22]. Reduced cytoplasmic volume of recipient oocyte affects blastocyst development [23].

Oocyte selection, in vitro maturation and enucleation

Oocyte quality is a major factor in successful development of embryo in *in vitro* condition. Oocyte selection by morphology observation is a common practice where number of cumulus cell layer surrounding the oocytes, their compactness, and ooplasm homogeneity is observed [24].

Methods are also there to select the oocytes on the basis of their ability to degrade brilliant cresyl blue stain (BCB) by the enzyme glucose-6-phosphate dehydrogenase (G6PDH), thus loosing the colour. Full grown oocytes have decreased G6PDH level, thus stain more with BCB (BCB+) and are useful for further use. This method has been used for IVF embryo production in various species [25-27]. Mohapatra et al. (2015) used BCB+ oocytes for use in HMC buffalo embryo production [28]. The BCB+ blastocysts showed better developmental ability, epigenetic status as compared to BCB- blastocysts.

The oocytes are matured *in vitro* for 22-24 hr using maturation medium which contains the hormones like FSH, LH, and estradiol [29]. Subsequently cumulus cells are removed using hyaluronidase and zona is removed using pronase and zona-free oocytes are processed for enucleation. The enucleation in HMC cloning is performed through protrusion cone (polar body) – guided cutting of oocytes using microblade. Few studies compared chemically assisted handmade enucleation (CAHE) (using demecolcine) with polar body – oriented handmade enucleation (OHE) [30, 31]. The study by Li et al. (2009) showed OHE method to be potential for HMC nuclear transfer in pigs where they used transgenic fibroblast cells as donor cells and 0.4 µg/ml demecolcine used. However, the study by Akshey et al. (2011) in HMC goat embryos had some contradictory conclusions; where CAHE method showed to be superior to the OHE method with 0.5 µg/ml demecolcine used for 2 hr in the donor cells. One interesting study by Du et al. (2008) showed treatment of *in vitro* matured porcine oocytes with high hydrostatic pressure (HHP) (20 MPa for 2 hr) enhanced the blastocyst formation rate and cell number per blastocyst [32]. It was also observed that HHP enhanced the cryotolerance and supported fetal development. Study on effect of HHP on gene expression profiles of porcine HMC embryos revealed almost 44 transcripts expression being altered and the HHP mainly affected the imprinting gene expression [33].

Selection of donor cells

Donor cell selection is very important with regard to the type of cell, cell cycle stage, and the quality. Various researchers have worked with different cell types and observed their efficacy for HMC embryo production. Comprehensive study has been done to determine the efficiency of HMC in porcine using more than 2 lakh reconstructed embryos with significant findings [34]. This study showed that adult donor fibroblast cells resulted in higher blastocyst formation rate; whereas efficiency of piglets born was high with fetal donor cells accompanied with lower rate of developmental abnormalities. Besides; their study on effect of genetic modifications of donor cells on cloning efficiency showed transgenic and gene knock out fetal fibroblasts have more developmental abnormalities and less efficiency in HMC as compared to normal donor cells. Donor cell from different sources have been used for HMC embryo production with convincing results. Jena et al. (2012) compared the efficiency of fetal and adult fibroblast cells on HMC goat embryo production and found very similar effect on the developmental competency of the embryos [29]. Comparative study with donor cells from various sources of buffalo (fetal fibroblasts, newborn fibroblasts, adult fibroblasts, and cumulus cells) for production of HMC buffalo embryos revealed cumulus cells to be superior in terms of the blastocyst formation rate [35]. One study used somatic cells isolated from urine as donor cells and successfully produced cloned buffalo calf using HMC technique [36]. Lymphocytes isolated from peripheral blood was used as donor cells for HMC cloned buffalo embryo production where the blastocyst formation rate was lower as compared to fibroblast cells as donor cells; however the total cell number per blastocyst and apoptotic index were very similar [37]. The same research group also used somatic cells isolated from milk as donor cell and found lowered blastocyst formation rate as compared to that of skin cell-derived blastocysts but still they can be used as donor cells in future research work on HMC [38]. Selection of donor cells at G0/G1 stage of cell cycle is important for appropriate reprogramming of the cells during embryo development. Study on effect of roscovitine to improve synchronization of donor cell in G0/G1 stage revealed roscovitine treated donor cells contributing higher blastocyst formation rate (62.9 %) as compared to nontreated cyclic cells [39]. Besides, the cryosurvival rate of blastocysts , and number of cells in inner cell mass (ICM) was also enhanced with 30 µM roscovitine. Similar study performed by Akshey et al. (2011) showed higher blastocyst formation rate using roscovitine treated donor fetal fibroblast cells to develop HMC goat embryos [40]. Liu et al. (2012) observed effect of digitonin (permeabilizing agent) and *Xenopus laevis* egg extract on donor porcine fibroblast cells and HMC embryo development [41]. Interestingly, both the digitonin and egg extract had significant effect on blastocyst formation (increased). In this process, the donor cells were treated with the extract and digitonin for 3 or 5 days and then used for HMC embryo formation. HMC in goats was performed using three types of donor cells like adult fibroblast cells, putative embryonic stem cells, and lymphocytes [42]. The putative embryonic stem cells were superior to others in terms of cleavage and blastocyst formation rate. Study on effect of donor cell confluency (70-80, 80-90, and >95 %) on HMC blastocysts development showed >95 % confluency gives better results where the donor cells were adult skin cells from a Nellore cow [43].

Reconstructed embryo formation and culture media

The donor cell is fused to one demioocyte by phytohemagglutinin and this fused cell is again fused to another demioocyte by providing electric pulse. Selokar et al. (2012) optimized the elctrofusion parameters and post fusion holding time required for HMC embryo production in buffalo [44]. The triplet alignment with 4 V AC and single step fusion using 3.36 kV/cm DC pulse for 4 µs is suitable for reconstruction of cloned buffalo embryos. The AC pulse of 4V for alignment and 2.1 kV/cm for 5 µs of DC pulse for electrofusion is suitable for HMC goat embryo production [29]. The reconstructed embryo is further activated using calcium ionophore and 6-

dimethylaminopurine, thereafter it is kept in culture media for embryo development. Study on HMC goat embryo production showed electric pulse activation (2.31 kV/cm, 15 µs) is better than calcium ionophore activation of reconstructed embryos with regard to the cleavage and blastocyst formation rate [40]. Early-cleaved HMC embryos are found to be superior in developmental competence than late-cleaved embryos (> 24 hr post *in vitro* culture) [45]. The blastocyst formation rate, and total cell number per blastocyst were higher and apoptotic index was lower in early cleaved embryos which indicate their superiority in developmental competence and quality. A recent study on creation of miniature pigs using HMC technique adopted a unique way of aggregating the cloned embryos and culturing those embryos [46]. Three cloned embryos (each of 4-cell stage) were aggregated to develop into one blastocyst for transfer to the uterus for further development *in vivo*. This strategy showed blastocyst formation rate almost double (73.6 %) than that derived from single cloned embryo.

Epigenetic reprogramming of donor cell

The abnormalities observed of the live births from traditional SCNT as well as HMC cloned animals are largely attributed to abnormal epigenetic reprogramming of the donor cell chromatin material during the development of the cloned embryo. The epigenetic modifications can be performed *in vitro* by using some epigenetic modifiers in cultured donor cells as well as cultured embryos. Various studies have been performed to improve the developmental competence of cloned embryos through use of epigenetic modifiers. Previous study on donor cell treatment with valproic acid (VPA, a histone deacetylase inhibitor) for 24 hr duration altered the cell proliferation rate and apoptosis rate. Blastocyst formation rate increased and apoptosis was decreased of HMC embryos produced from VPA-treated donor cells showing them as good choice of epigenetic modifier for HMC embryo production [47]. However, recently same researcher and coworkers observed the effect of VPA treated to donor fibroblast cells, on embryo development rate which showed the lowering down of apoptotic index, although the embryo development rate was not significantly affected [48]. Few other epigenetic modifiers are also in use in HMC. Suberoylanilide hydroxamic acid (a histone deacetylase inhibitor) is found to be superior on epigenetic reprogramming as compared to valproic acid improving the acetylation level of HMC cloned porcine embryos [49]. Study on activity of trichostatin-A (TSA, a histone deacetylase inhibitor) and 5 – aza – 2' – deoxycitidine (5-aza-dC, inhibitor of DNA methyl transferase) to improve developmental competence of HMC buffalo embryos showed their superior action when used in combination instead of using alone [50]. The combination (50 nM TSA + 7.5 nM 5-aza-dC) can either be treated with donor cells or the reconstructed embryos. Similar study on effect of TSA on blastocyst formation rate in HMC porcine embryos revealed dramatic increase (80 %) as compared to control group (54 %) and 37.5 nM TSA treated 22-24 hr after activation was optimal for this purpose [51]. These above studies on TSA provide evidences of somatic cell genome reprogramming by TSA treatment supporting embryo development in *invitro* condition and also full term *invivo* development. Another HDAC inhibitor called scriptaid had better effect in HMC embryo production [52]. Use of scriptaid after 10 hr of reconstruction of buffalo HMC embryos increased the cleavage rate as well as blastocyst formation rate significantly with 1000 nmol/litre concentration. Study by Chawalit et al. (2012) showed that TSA and ascorbic acid had different mechanism of enhancing the blastocyst development rate [53]. Ascorbic acid shows its effect through the traditional antioxidant pathway whereas TSA shows its effect through epigenetic reprogramming. Ascorbic acid supplementation enhanced blastocyst formation rate and total cell number per blastocyst with decreased apoptotic indices with optimal concentration of 50 µg/ml used [54]. The study by Liu et al. (2017) showed the histone acetylation pattern of cloned embryos to be different between the traditional nuclear transfer and HMC technique as time progresses, with the HMC cloned porcine embryos showing higher blastocyst formation rate and higher cell numbers per blastocyst [55]. The use of siRNA knockdown assay targeting the DNA methyl transferase 1 (DNMT1) gene of one-cell stage HMC embryos showed some significant findings such as increased blastocyst formation rate, although this silencing process did not alter the DNA methylation level [56].

Transgenic animal production using HMC technique

Transgenic technology is gaining much popularity day-by-day due to its huge application in transgenic animal production and biopharming. At present so many researchers are producing transgenic livestock animals using the well-established HMC technique coupled to transgenesis. This is a suitable method already used and established technique to produce transgenic pigs [57]. Recently double gene knockout pigs were generated using CRISPER/Cas9 knockout system and HMC technique [58]. The porcine fetal fibroblast cells were targeted to knockout two genes such as α1,3-galactosyltransferase (GGTA1) and cytidine monophosphate-N-acetyl neuraminic acid hydroxylase (CMAH) simultaneously. The transgenic pigs developed, can be used for organ transplantation (xenotransplantation) as they seem to show reduced humoral rejection. Another study produced HMC transgenic piglets by using recombinant donor cells carrying the functional nematode (*Caenorhabditis elegans*) *fat-1* gene (n-3 fatty acid desaturase) [59]. The enzyme n-3 fatty acid desaturase which is lacking in mammals converts n-6 fatty acids to n-3 PUFAs which will increase the nutritional value of pork due to the synthesis of omega-3 fatty acids. Transgenic sheep was developed by HMC using recombinant ovine fibroblast cells as donor cells carrying the mfat-1 gene (codon optimized fat-1 gene) [60]. Kragh et al. (2009) developed a porcine model of Alzheimer's disease using HMC and transgenic technology [61].

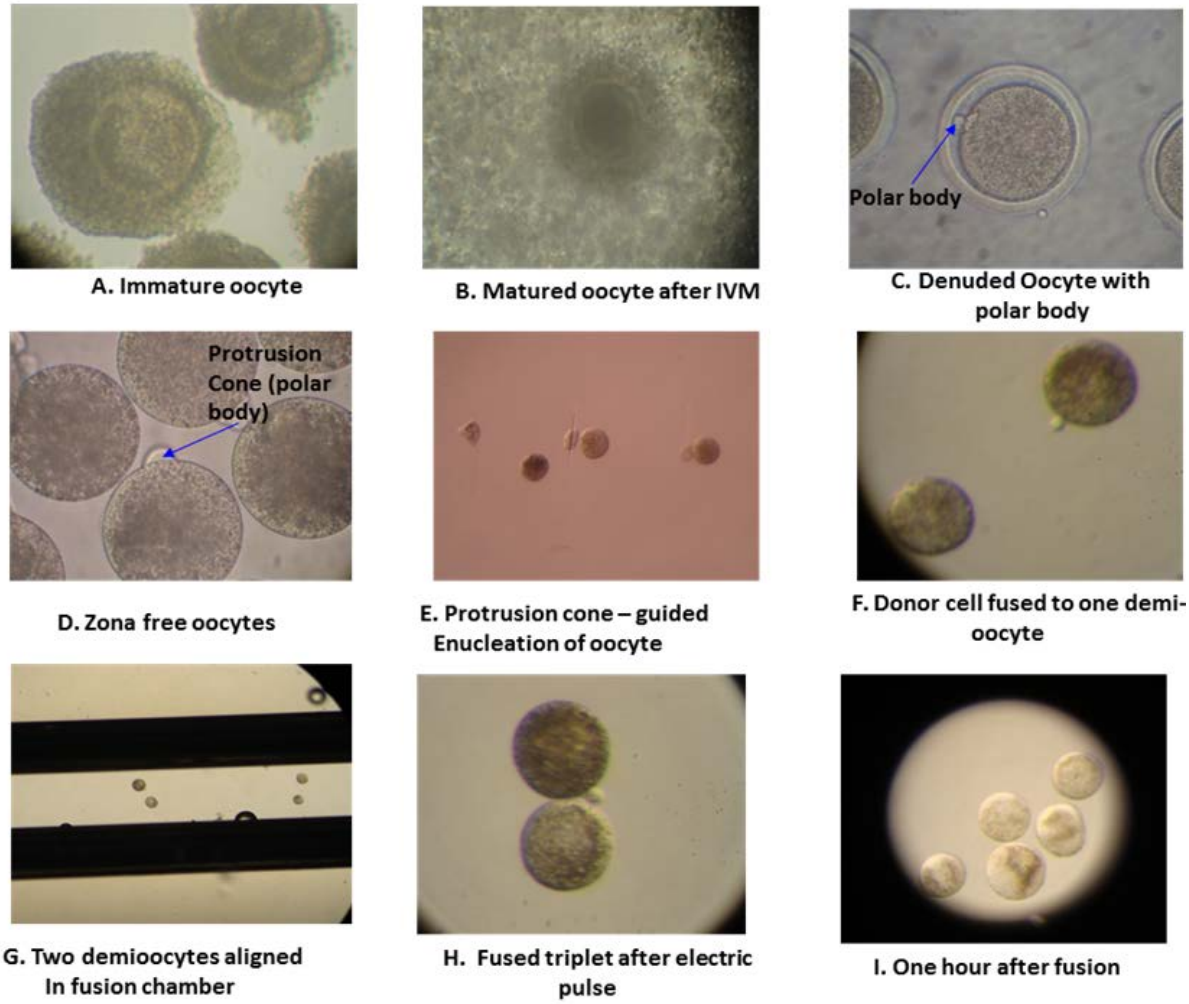

Fig. 1: Different steps of HMC technique. Oocytes isolated from goat ovary are used for HMC cloned embryo production. A. Immature oocyte isolated from ovary, B. Matured oocyte after treatment with In vitro maturation medium (IVM), C. Denuded oocyte after removal of cumulus cells by hyaluronidase treatment, D. Zona free oocytes after pronase treatment, E. Enucleation of oocyte by microblade, F. Donor cell fused to one demioocyte by phytohemagglutinin treatment, G. Alignment of two demioocytes (one fused to donor cell), H. Fused triplet, I. Embryo after one hour of fusion with electric pulse.

Advantages and disadvantages of HMC

The HMC has advantages in many aspects and the major advantage is the less cost involved in equipments required for the cloning process. Besides, the procedure is simple and needs less expertise, efficiency of blastocyst formation and live births is at par or even more than traditional cloning, ease of cryopreservation of embryos, and possibility of automation of the technique [19, 4].

The absence of zona pellucida may not protect the embryo from toxic materials present in the culture media [62], and may not prevent blastomere separation [63]. Another disadvantage may be the use of heterogeneous cytoplasm in HMC technique where two demioocytes from two individual oocytes are taken for one cloned embryo production, although no drawbacks reported so far.

FUTURE PERSPECTIVES

The HMC technique is gaining popularity due to its wide application and promising results. Interspecies cloning using HMC has paved the way to save endangered species. Another important aspect of HMC is to produce transgenic animals for use as disease model and pharmaceutical production (Biopharming). Besides, HMC technique can be used for production of patient-specific stem cells required in regenerative therapy. The potency and utility of the technique can further be enhanced exponentially if automation can be achieved in the days to come.

CONFLICT OF INTEREST STATEMENT

No conflict of interest by the authors.

AUTHOR CONTRIBUTIONS

MKJ has contributed by writing and revisions of the review. DM has contributed in revisions and modifications for the final manuscript.

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