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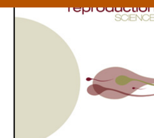

Review article

The potential for modification in cloning and vitrification technology to enhance genetic progress in beef cattle in Northern Australia

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Recent advances in embryology and related research offer considerable possibilities to accelerate genetic improvement in cattle breeding. Such progress includes optimization and standardization of laboratory embryo production (*in vitro* fertilization – IVF), introduction of a highly efficient method for cryopreservation (vitrification), and dramatic improvement in the efficiency of somatic cell nuclear transfer (cloning) in terms of required effort, cost, and overall outcome. Handmade cloning (HMC), a simplified version of somatic cell nuclear transfer, offers the potential for relatively easy and low-cost production of clones. A potentially modified method of vitrification used at a centrally located laboratory facility could result in cloned offspring that are economically competitive with elite animals produced by more traditional means. Apart from routine legal and intellectual property issues, the main obstacle that hampers rapid uptake of these technologies by the beef cattle industry is a lack of confidence from scientific and commercial sources. Once stakeholder support is increased, the combined application of these methods makes a rapid advance toward desirable traits (rapid growth, high-quality beef, optimized reproductive performance) a realistic goal. The potential impact of these technologies on genetic advancement in beef cattle herds in which improvement of stock is sought, such as in northern Australia, is hard to overestimate.

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1. Introduction

Over 75% of farming land in Australia is dedicated to beef production, with 79,000 cattle farmers collectively running 28.5 million cattle across 200 million hectares (Meat and Livestock Australia, 2013). Northern (tropical savannah) cattle producers account for around half of the national herd, with approximately 90% of these produced using a free range production strategy, as opposed to feedlots (Bortolussi et al., 2005a). Cattle in the northern region typically forage for feed over sprawling properties. Central Queensland acts as a major breeding area, hosting the majority of cattle stud operations (Bortolussi et al., 2005b).

With only 3% of cattle numbers, Australia is a relatively modest producer of beef on a global scale. However, the small population means that domestic consumption is low, which allows the country to export around 60% of its production each year, second only in live and beef export volume to Brazil, and trading to 110 countries worldwide (Bindon and Jones, 2001). The gross value of Australian cattle and calf production is estimated at AUS $7.9 billion annually (Meat and Livestock Australia, 2013). Domestic producers are required to fulfil strict accreditation rules for export to some markets, especially Europe, where transmissible encephalopathies and infectious diseases are a continuing food safety concern. In this regard, the disease-free status and traceability of Australian beef provides a competitive advantage. In addition, Australian farmers are facing increased competition from Latin American producers in the previously favored markets of Korea, Japan, and the United States.

Northern Australia is currently experiencing a widespread drought, which is adversely affecting the productivity and profitability of the associated beef industry, for which reparative measures are an imperative. Faced with this major threat to the livelihood of its members, technological interventions to improve the genetic stock of beef cattle will play a significant role in the strategic response of the cattle-farming sector. It has been recommended that in extensive breeder regions, there should be renewed focus on, among other measures, breeder performance and bull selection based on inherent fertility (McCosker et al., 2010). Reproductive technologies may be the way Australia maintains its world-class bovine production status in the future (Farquharson and Banks, 2002). This is particularly relevant to Queensland and Northern Territory, where *Bos indicus* cattle are generally selected due to their hardiness, tick resistance, and ability to cope with heat. The beef produced from these animals is of lower quality than the premium beef produced from *Bos taurus* animals (Angus, Hereford, and Charolais breeds) that results from more intensive southern production systems and is targeted to high-value markets including Korea, Japan, and Russia (Strydom et al., 2011). Cattle from *B. indicus* breeds, including Brahman, Braford, and Santa Gertrudis, are either exported live to destinations such as Indonesia or are sent to southern Australia to be fattened on grain and processed for export as 'hamburger' meat (Price Waterhouse Coopers, 2011).

2. *In vitro* fertilization

In Brazil, *in vitro* fertilization (IVF) is rapidly superseding multi-ovulation embryo transfer as the artificial breeding strategy of choice for cattle (Viana, 2012). A similar trend may be observed in other South American nations and is predictable in several countries, notably the BRICS states (Brazil, Russia, India, China, South Africa), with the exclusion on religious grounds of India. The reasons for this include reduced reliance on hormone administration, less likelihood of rendering valuable donor females infertile, and the opportunity for faster rates of genetic gain (Mapletoft, 2012; Walton, 2013). Using IVF for cattle breeding, it is possible to produce routinely 50 calves per donor per year, and sometimes up to three times this rate, at a vastly reduced cost compared to current embryo transfer methods (Haag and Dorshorst, 2013; Johnson, 2014). Additionally, the use of sexed semen allows the added benefit of gender selection (Xu et al., 2009; Hayakawa, 2012; Morotti et al., 2014).

The past decade, however, has seen a decrease in the intensity of effort, investment levels, and output in domestic animal embryology. While this has been a global trend, it is especially noticeable in developed countries, including Australia which was regarded previously as a leader in this area. Advancements in embryo culture *in vitro* (e.g., Vajta et al., 2010) and embryo/oocyte cryopreservation (e.g., Vajta and Nagy, 2006) have resulted in a dramatic improvement in the efficiency in human-assisted reproduction. However, the impact of these technologies has so far translated modestly to Australian beef cattle, much less than in Brazil (Viana, 2012), and with negligible application of novel embryo cryopreservation methods (Vajta, 2012).

The Australian beef herd is relatively genetically 'advanced'. Hence, use of IVF has less impact than, for example, in the BRICS countries. Nonetheless, IVF has value in its potential both to provide and to preserve elite male and female genetics. This enables breeders to develop a holistic, customized reproductive management program, an additional benefit of which is to safeguard against an infectious disease outbreak, e.g., foot-and-mouth disease (CRC for Beef Genetic Technologies, 2012). Cloning can enhance the rate of genetic gain in a population compared to IVF, but cloning will always be relatively expensive compared to 'natural' reproduction, with laboratory and surrogate pregnancy costs incurred. An indicative cost is AUS $15,000 per birth, but methodology changes (as reviewed below) might reduce this to AUS $5000 for each animal. At present, the

average sale price of a beast for its meat value is greater for an IVF Brahman bull than for its non-IVF counterpart, AUS $7800 compared to $5600 (McCosker, 2013). However, the profit margins are such that the entire herd would never be cloned nor would this be desired – in order to avoid a ‘mono-culture’. Rather, current practice nationally is to invest in one or two bulls with elite genetics, defined as a show-ring animal or one that rates very highly on a standard selection index and to purchase a large number of animals with poorer genetic qualities at sale yards (Miller, 2012), in northern Australia from ‘breeding’ properties in Central Queensland. At present, an elite genetic individual has a cost of around AUS $50,000, so purchase of 10 clones of varying but elite genetics rather than one non-clone has value.

Apart from the increasing application of ovum pick-up and *in vitro* fertilization in cattle breeding (Wu and Zan, 2012), the most important but so far underexploited possibility is the application of somatic cell nuclear transfer (Wilmut et al., 1997) in cattle breeding (Vajta, 2007a). This technique enables production of a theoretically infinite number of copies of an existing animal of proven genetic values (the genetic identity comprises all nuclear material, i.e., greater than 99% of the mammalian DNA) and consequently dramatic improvement of the genetic value of livestock in just one generation. Societal debate over the ethical issues surrounding genetic cloning, welfare implications and losses associated with abnormal epigenetic reprogramming, and food safety concerns has delayed development of this field, yet slow but considerable progress has been achieved over the last 16 years (Vajta, 2007a; Oback, 2008; Niemann and Lucas-Hahn, 2012), although some reviews do not acknowledge entirely this advancement (Galli et al., 2012). Indeed, the introduction of a simplified cloning technique called handmade cloning (HMC) (Vajta et al., 2001, 2003) that requires only a stereomicroscope and a simple electrofusion machine for manipulations has opened up a new perspective for large-scale industrial application (Vajta et al., 2005; Vajta, 2007b; Taylor-Robinson et al., 2014). In order to fully realize this possibility, further research is required: (1) to optimize each step of the procedure and (2) to combine cloning with a highly efficient cryopreservation technique specifically developed for bovine handmade cloned embryos.

For logistical reasons, cryopreservation has always been regarded as a key element of large-scale application of assisted reproductive technologies. However, traditional ways of embryo freezing are inefficient following *in vitro* manipulations and culture of cattle embryos. Vitrification is an alternative approach (Rall and Fahy, 1985), with ice crystal-free solidification of solutions. Introduction of purpose-developed tools to ensure cooling and warming rates in excess of 20,000 °C/min have improved considerably the efficiency of this technology (Vajta et al., 1998; Kuwayama et al., 2005a). These techniques have already resulted in live offspring from cryopreserved cloned embryos in cattle and pigs (Tecirlioglu et al., 2003; Du et al., 2007). However, further improvement and alternative technical solutions are still required to reach the competency needed for large-scale industrial use. There is a pressing need to increase the efficiency and reliability of both HMC and vitrification by optimization of all steps and/or by finding alternative routes for the procedures. It should then be possible to combine the two technologies by adjusting steps and parameters to increase the overall efficiency to a level which is commercially viable. These should be simple and efficient ways to promote rapid propagation of laboratory production and on-farm application of the relevant stages of the procedure, respectively.

These techniques are outlined below.

3. Handmade cloning

The principle of HMC is that, in contrast to the traditional approach, the zona pellucida (analogous to an egg shell) is removed at the very beginning of the procedure by enzymatic digestion. This radical step allows handmade manipulations, but requires special care in handling oocytes and embryos, as well as a modified dish for embryo culture.

The steps of HMC may be summarized as follows:

1. ovaries are collected from slaughtered animals and transported to the laboratory;
2. oocytes are aspirated from the visible 2–7 mm diameter follicles;
3. following a 22 h maturation, cumulus cells are removed by vortexing;
4. denuded oocytes are incubated for a further 1–2 h in demecolcine;
5. the zonae pellucidae are digested by pronase;
6. through the joint effect of demecolcine and pronase, an extrusion cone occurs on the surface, which serves as an orientation point for enucleation by hand with a disposable blade;
7. karyoplasts containing the chromatin are discarded, whereas cytoplasts are used as recipients;
8. somatic cells, derived from another cattle, calf, or fetus, are cultured on monolayers;
9. following trypsinization, these cells are attached individually to cytoplasts that have been submerged briefly into phytohemagglutinin to make their surface sticky, then the pairs of cells are transferred to between the electrodes of a fusion chamber;
10. with a low-voltage AC current, pairs are floated to one of the wires, with the somatic cell far from the wire. Subsequently, another cytoplast is also floated to touch both the somatic cell and the first cytoplast. The triplets are subjected to electrofusion, when reconstructed embryos are formed;
11. reconstructed embryos are subjected to chemical activation;
12. reconstructed embryos are then cultured *in vitro* for 1 week in microwells of a special dish (well of the well – WOW) (Vajta et al., 2000);
13. emerging blastocysts are transferred into recipients to produce animals (almost) identical with the somatic cell donor (Vajta, 2007b).

Compared to traditional, micromanipulator-based cloning, HMC requires negligible specific investment apart from the basic items of equipment of a standard

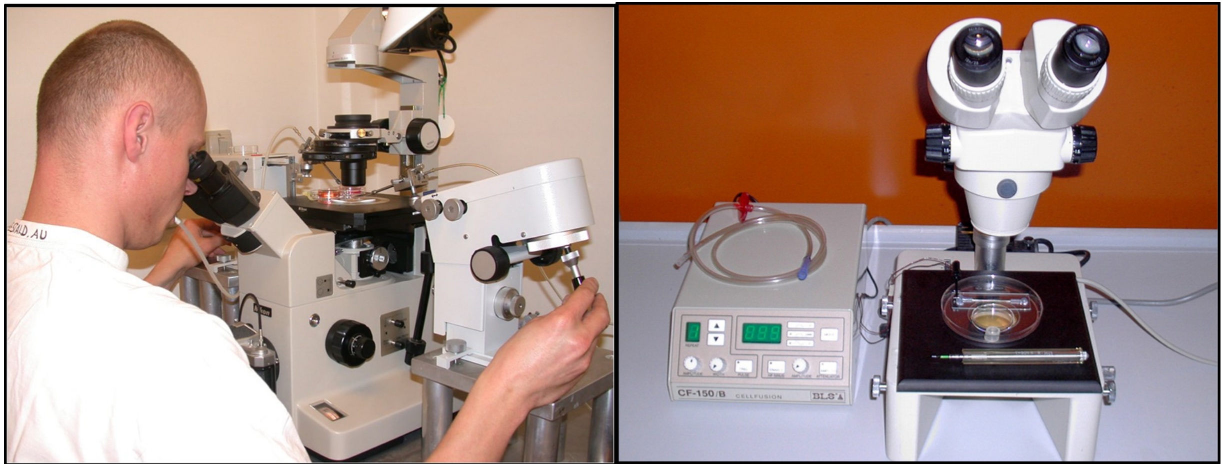

Fig. 1. Equipment required for traditional cloning (left) and for handmade cloning (right).

mammalian embryology laboratory. The only specialist instrument is a relatively inexpensive electrofusion machine (~€3500; BLS, Hungary). In traditional cloning, a high-quality inverted microscope equipped with a sophisticated micromanipulator and preferably with ultraviolet illumination is required, whereas all HMC manipulations can be performed under a low-specification binocular microscope (Fig. 1). The electrofusion machine (shown in the right panel only) is necessary for both procedures.

The actual overall efficiency of HMC in cattle can be characterized by the following parameters: on a single day, one embryologist can produce from 200 abattoir-derived, *in vitro* matured oocytes approximately 60–70 reconstructed embryos. Out of these, 25–30 blastocysts may develop after embryo culture. By transferring two embryos to each cow, 4–6 pregnancies may be achieved and 2–4 healthy calves may be obtained. This rate of efficiency is at least comparable with that achieved by conventional nuclear transfer (Vajta et al., 2003, 2005).

The several advantages of HMC over the traditional technology of somatic cell nuclear transfer are summarized in Table 1.

Table 1
Advantages of handmade cloning (HMC) compared to traditional technology.

Criterion	Comments
Equipment	One order of magnitude less expensive than that required for micromanipulation-based cloning
Procedure	Simple, rapid, easy to learn, and perform
Efficiency	Required time, workforce, and investment are lower than in traditional cloning; transferable embryo per oocyte rates are similar, although two oocytes are used for reconstruction of one embryo
Embryo cryopreservation	Healthy offspring produced in cattle and pigs
Pregnancy and calving rates	At least equal to those reported for traditional cloning (from few available data – about 20 calves worldwide)

4. Vitrification

The definition of vitrification in cryobiology is solidification of a water-based solution (in this case containing the sample) without the formation of any ice crystals. It can also be regarded as an extremely increased viscosity of solutions at low temperatures. In order to induce this phenomenon under practical conditions, there are two requirements: relatively high concentrations of cryoprotectants, permeable or non-permeable substances that interfere with crystal formation and protect biological structures; and increased cooling and warming rates (>20,000 °C/min or higher) (Vajta, 2012).

In order to ensure these extremely rapid temperature changes, specific tools have been developed, including the Open Pulled Straw (OPS) (Vajta et al., 1998; Fig. 2) and the Cryotop (Kuwayama et al., 2005a); the review by Vajta and Nagy (2006) provides a general description. Most other commercially available instruments are analogues of these two basic tools. Both techniques enable loading of samples in very small (less than 1 μl) volumes and permit direct contact between the solution and the liquid nitrogen or the warming solutions, into which these tools are simply submerged.

Numerous publications, as reviewed by Vajta (2012), indicate that the optimal cryoprotectant mixture is composed of dimethyl sulfoxide and ethylene glycol, with sucrose or trehalose as a non-permeable component. Although these chemicals have a relatively low toxicity, the required high concentration and osmotic effect may require very careful addition and removal before cooling and after warming, respectively.

Both the OPS and the Cryotop techniques result in excellent (close to 100%) survival after warming of human or bovine zona-intact blastocysts, and the *in vivo* developmental competence in humans does not seem to be compromised compared with that of fresh embryos (Kuwayama et al., 2005b). However, HMC embryos are entirely free of zona pellucida, and this situation may require the application of a different, most probably more delicate, approach with adjusted equilibration and dilution

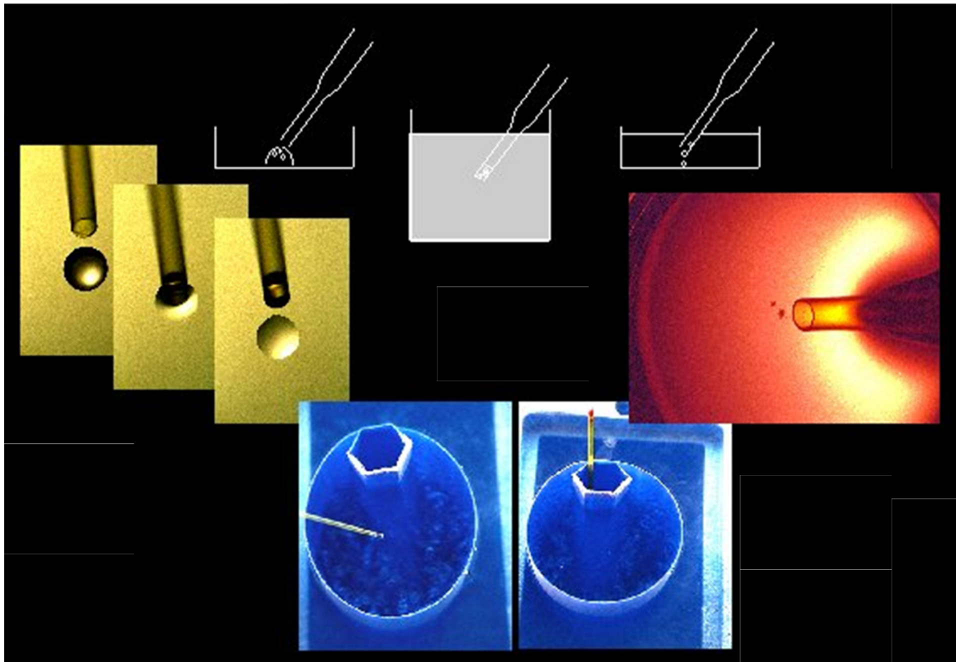

Fig. 2. Vitrification with the Open Pulled Straw (OPS) method. After a stepwise equilibration with cryoprotectants, embryos or oocytes are placed into a small (>1 μl) drop (left), then the drop is touched with the narrow end of the plastic OPS straw. According to the capillary effect, most of the drop, including the samples, enters the straw. Each straw is then immersed directly into liquid nitrogen (centre). Upon warming, the straws are immersed quickly into a warm sucrose solution (right); due to gravity, embryos or oocytes flood into the sucrose solution which is diluted stepwise afterwards.

parameters. Although HMC cattle embryos have been vitrified successfully by the OPS method, which resulted in healthy offspring after transfer (Tecirlioglu et al., 2003), the low number of transfers and offspring do not allow estimation of efficiency. Considering the intrinsic handicap of embryos produced by somatic cell nuclear transfer (Vajta, 2007a), modification of the vitrification procedure may be required to reach the commercially viable level of efficiency, i.e., pregnancy rates should be equal to or at worst only 5–10% lower than those achieved with fresh embryo transfers.

5. Future work

By performing vitrification research on both zona-free (HMC) embryos and zona-included or zona-free, parthenogenetically activated blastocysts, methodological improvements may be made. Crucial questions that remain to be answered include:

- how does a zona-free situation influence *in vitro* survival and development?
- can the differences observed in cloning efficiency of zona-free and zona-included embryos be compensated for by changing the equilibration-dilution parameters before cooling and after warming, respectively?
- is there any alternative option that may be more efficient for cryopreservation of zona-free HMC embryos?
- is in-straw dilution and semi-direct transfer (Vajta et al., 1999) of HMC embryos a realistic option?

These outstanding issues can be addressed by evaluation of *in vitro* survival and further development *in vitro*, selective staining of damaged apoptotic cells after vitrification, and by transfers after warming and *in vitro* dilution, or semi-direct transfers.

6. Conclusions

Artificial reproductive techniques will form a component of a responsive beef industry, and of these technologies those of HMC and vitrification offer great potential but require further optimization. A priority for future work is to achieve a consistent, predictable outcome and further improvement in the overall efficiency of both HMC and vitrification, and the cumulative efficiency of the two procedures if applied together. Through making basic modifications to some steps and improvements to others in HMC, and possibly by modifying or replacing the OPS straw as well as the above-mentioned parameters, it may be possible to achieve an even simpler, more reliable, and efficient combined technology with predictable *in vivo* outcomes.

Using the simplest and quickest methods for all procedures may facilitate the provision of information on the needs and limits of a successful somatic cell nuclear transfer and vitrification in cattle. These include: (1) establishment of somatic cell cultures from a given donor animal by performing biopsy under on-farm conditions: optimized oocyte maturation and enucleation procedures, improved fusion and activation parameters, and embryo culture environment; and (2) optimization of the carrier tool and

equilibration-dilution parameters before cooling and after warming, respectively.

For cloning to be of practical use to the northern Australian beef herds, it is envisaged that a centrally located facility would have the capacity to perform cloning and to maintain a recipient herd of around 50 cows, allowing centralization of veterinary care and other support. Elite individuals would be cloned in batches (of up to 50 in number), either on consignment or for sale into the existing market system in which farmers would seek to purchase small numbers of several different lines. This has the advantage of preserving genetic diversity in a herd while promoting genetic gain and offers a model for cost-effective production.

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