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Review

Encapsulation of micromanipulated mammalian embryos

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Micromanipulation of gametes is one of the reproductive biotechnologies that have an obvious contribution to animal production, human welfare, industry and research. The current reproductive techniques that employ micromanipulation are chimerism, embryo bisection, intracytoplasmic sperm injection (ICSI), gene injection and nuclear transfer (the so cold cloning). In all these techniques it is necessary to perforate, slit or even to remove zona pellucida (ZP). The ZP is a glycoprotein layer that protects embryos not only from predators but also protects them from toxic substances in culture media and/or uterine environment. This article summarized the influences of micromanipulation and encapsulation with artificial ZP (AZP) made of sodium alginate on *in vitro* development of micromanipulated embryos. The article also described how embryos with defective ZP (DZP) could be microencapsulated with an AZP.

Key word: Encapsulation, DZP, sodium alginate, embryos.

INTRODUCTION

Preimplantation mammalian embryos cannot run away from predators or move away to a safer environment, nor they can withstand toxic substances in culture media or uterine environment, so they come equipped with a protective layer to overcome these problems. All mammalian eggs are equipped with a glycoprotein layer (ZP) which is made during oocyte maturation (Gilbert, 1997). This ZP protects embryos from predators (Gilbert 1997), toxic substance in culture media (Fukuda et al., 1987; Nagano et al., 1995, Elsheikh et al., 2003) and paraffin oil used to cover drops during *in vitro* culture (Flemming et al., 1987; Flood and Shirley, 1991). Furthermore, ZP is essential for maintenance of cell to cell contact at the 4 cell stage (Suzuki et al., 1995; Elsheikh et al., 1997).

The micromanipulation techniques, such as chimerism, embryo bisection, intracytoplasmic sperm injection (ICSI), gene injection and nuclear transfer necessitate perforation, slitting or even removal of ZP. Microsurgical treatment of ZP is know to have an inhibitory effect on *in vitro* development of porcine embryos (Niemann et al., 1983), bovine embryos (Westhusin et al., 1989) and mouse embryos (Elsheikh et al., 1997; 2003). Even in transgenic poultry normal development is impaired when there is an opening remaining from injection of transformed cells into chick embryo (Niemann 1998). Although, in all *in vitro* culture systems, chelators such as ethylene diamine tetra acetic acid (EDTA), amino acids and proteins are supplemented to culture media to modify and reduce the effects of toxic substances (Fukuda et al., 1987; Nasir Efahani et al., 1990; Nagano et al., 1995; Elsheikh et al., 2003), the deleterious effects of defective zona pellucida (DZP) on embryo development cannot be overcome if the DZP is not covered by a protective layer of sodium alginate commonly referred to as artificial zona pellucid (AZP) (Elsheikh et al., 1997). The AZP improves the development of embryo with DZP not only by preventing invaders or toxic substance to enter the embryo but also by maintaining cell to cell contact at the 4–cell stage (Elsheikh et al., 1997). This article summarized the influences of DZP and AZP on micromanipulated embryo development *in vitro*.

TYPES OF DZP

Partial or complete removal of the ZP is an unavoidable step in embryo micromanipulation techniques. The ZP might be removed completely perforated or slit during embryo manipulation.

a) Removal of ZP

Recently handmade cloning (HMC) of mammalian is car-

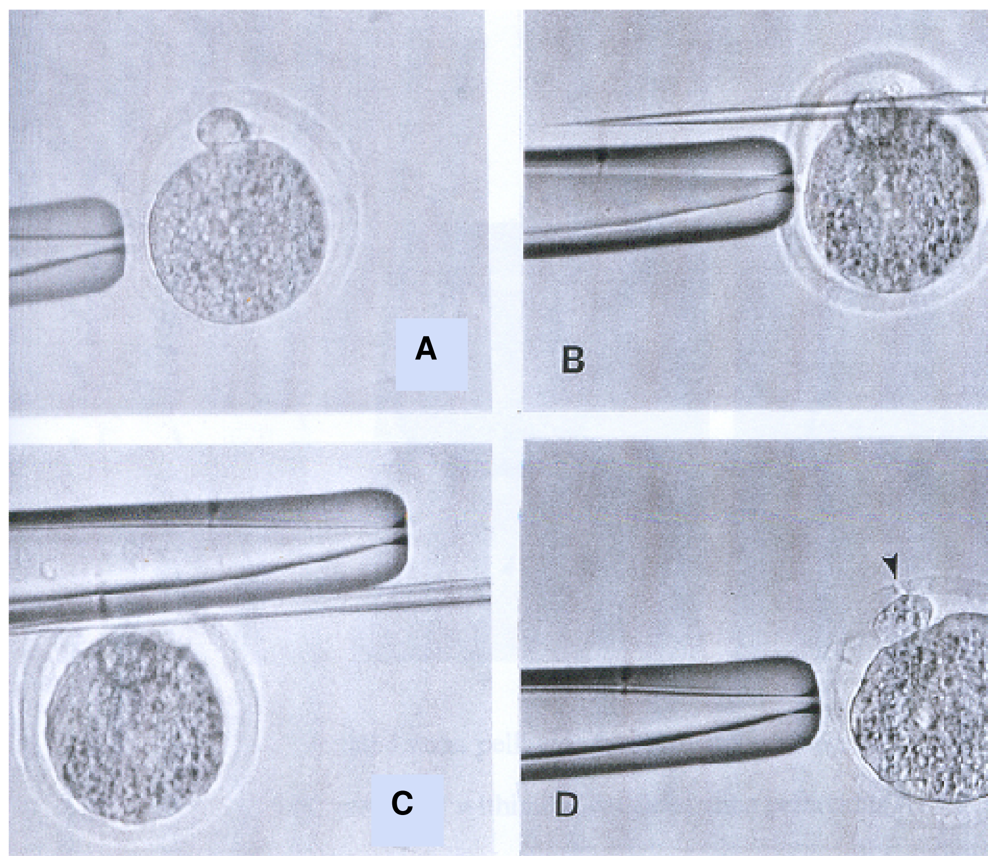

Figure 1. Slitting of ZP with a micro glass needle.

ried out without the use of micromanipulators (Taniguchi et al., 1991; Elsheikh et al., 1997; Vajita et al., 2001, 2005; Elsheikh et al., 2006, 2007). The HMC is a simple and promising technique to produce cloned animals; however the reconstituted embryos produced are ZP free (ZPF). The ZPF embryos are reported to have problems at the 4–cell stage (Suzuki et al., 1995, Elsheikh et al., 1997). Also some researchers removed the ZP to produce chimeric animals by aggregation (Markert and Petters 1978). The ZPF embryos also suffered from toxic substances in culture media compared to ZP intact (ZPI) embryos (Elsheikh et al., 1997).

b) Slitting of ZP (ZPS)

Many researchers prefer the ZPS technique described by Tsunoda et al. (1986) to perform manipulator based nuclear transfer or to assist sperms to fertilize the oocytes. In this technique a slit in ZP is made by a micromanipulator. The slit size varies according to the size of the needle used and the percentage of ZP circumference slit. Some researchers prefer to use a microneedle of 5 µm diameter and slit length of about 20% of the ZP circumference (Elsheikh et al., 1997, 2003). This slit in ZP (Figure 1) allows toxic substance, to enter into the embryo and impairs normal development, thus, this slit needs to be sealed.

c) Perforation of ZP (PZP)

Most of the manipulator based cloning technique employs the technique described by McGrath and Solter (1983). In this procedure the ZP is perforated by a micro glass needle to enucleate the oocytes (Prather et al., 1987; Bondioli et al., 1990; Cheong et al., 1993; Tanaka et al., 1995; Campbell et al., 1997; Wilmut et al., 1997). The PZP allows the entrance of toxic substances and microbes to the embryos. Also when the embryos with PZP reach the blastocyst stage they start to escape through this opening (Figure 2). Therefore, this opening must be sealed.

d) Bisected embryos

Some researchers are interested in production of identical twins by embryo bisection. The bisection is done either after removal of ZP (Tarkowski and Rossant, 1976; Elsheikh and Kanagawa, 2003) or after slitting of ZP (Barton and Surani, 1983). The embryos produced by bisection are ZPF and need to be protected by AZP.

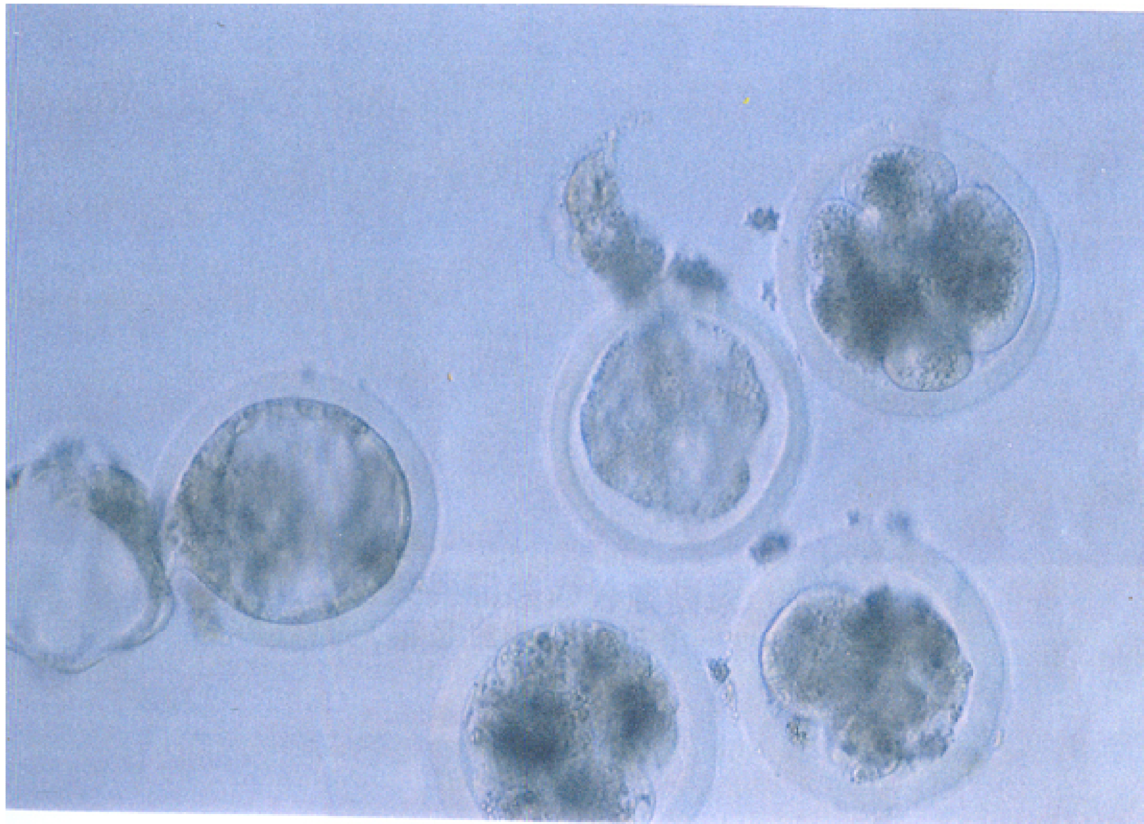

Figure 2. Escape of the blastocyst through the slit in ZP.

e) Aggregation chimera

Some researchers are interested in production of chimeric animals by blastomere aggregation which necessitates ZP removal (Mintz et al., 1973; Markert and Petters, 1978). These chimeric embryos are subjected to the bad effect of ZP removal.

CELL TO CELL CONTACT IN ZP FREE EMBRYOS

The number of cell contacts among blastomeres at the 4-cell stage varies from embryo to another depending on the condition of ZP (Suzuki et al., 1995; Elsheikh et al., 1997). Elsheikh et al. (1997) reported that about 86% of ZP intact (ZPI) 4–cell stage mouse embryos had 6 points of contact among their blastomeres, while only 14% of them had 5 points of contact. When ZP free (ZPF) mouse embryos were cultured to the 4–cell stage they developed varieties of contacts (Elsheikh et al., 1997). About 23% of them had 6 points of contacts 19% had 5 points of contacts, 35% had 4 points of contacts and 23% had 3 points of contacts (Figure 3). The association of cells at the 4–cell stage was changed when ZPI and ZPF mouse embryos were encapsulated with AZP and cultured *in vitro* to the 4–cell stage (Elsheikh et al., 1997). All ZPI mouse embryo encapsulated with AZP (100%) developed 6 points of contacts, and 88% of ZPF mouse embryos with AZP developed 6 points of contact (Figure 4). This cell to cell contact is not only important at the 4–cell stage but it is also important for cellular differentiation and development to blastoyst (Suzuki et al., 1995).

IN VITRO DEVELOPMENT OF EMBRYOS WITH AZP

Most culture media used to culture embryos are supplemented with chelators such as proteins, amino acids and EDTA which modulate the effects of toxic substances in culture media. When these chelators are excluded from culture media the embryo development is altered (Elsheikh et al., 2003). When ZPF mouse embryos were cultured in protein supplemented media 66% of them developed to blastocysts, and when they were cultured in protein free media only 55% of them reached the blastocyst stage (Elsheikh et al., 2003). The *in vitro* development of ZPF mouse embryos to blastocyst was improved when they were coated with an AZP which allowed 85% of them to develop to the blastocyst stage (Elsheikh et al., 1997). Also the development of ZPS mouse embryos was improved to reach 86% by encapsulation with AZP. Embedding of micromunipulated

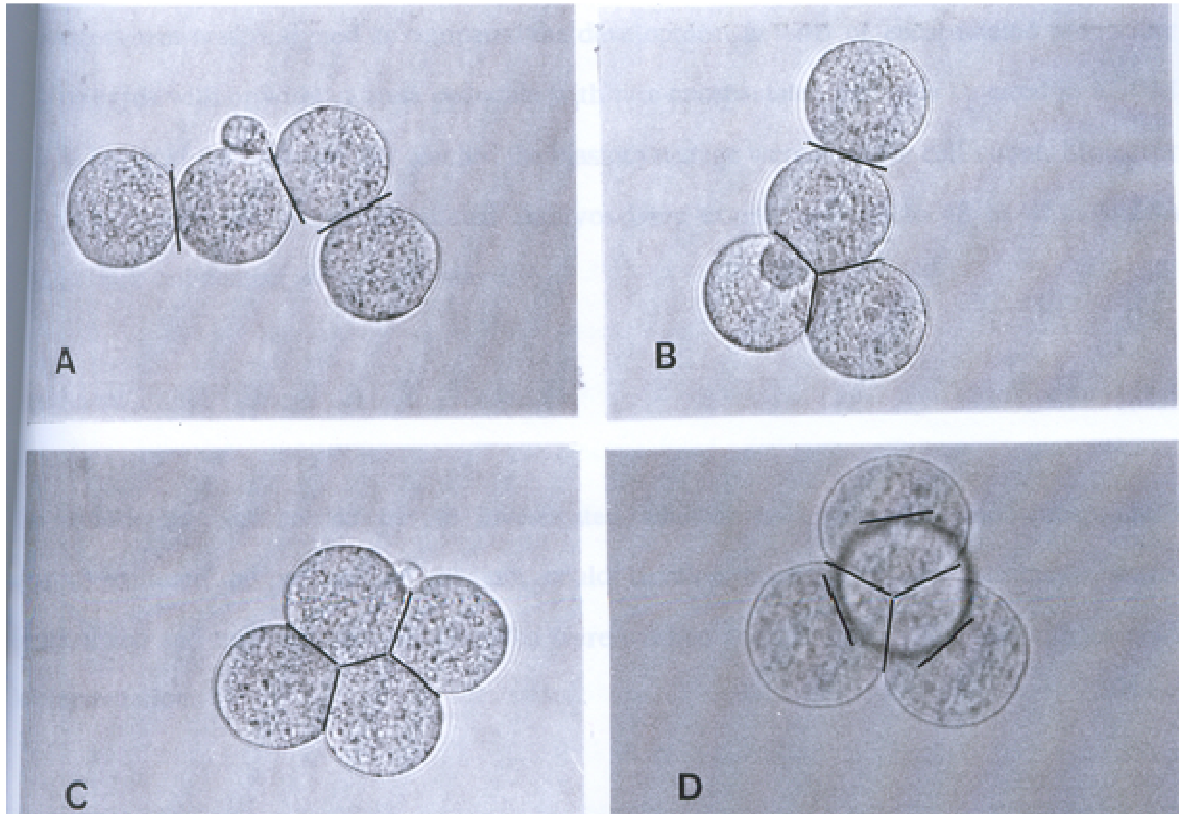

Figure 3. Points of contacts among blastomeres of a 4-cell stage ZPF mouse embryo. A) Three contacts, B) four contacts, C) five contacts, and D) six contacts.

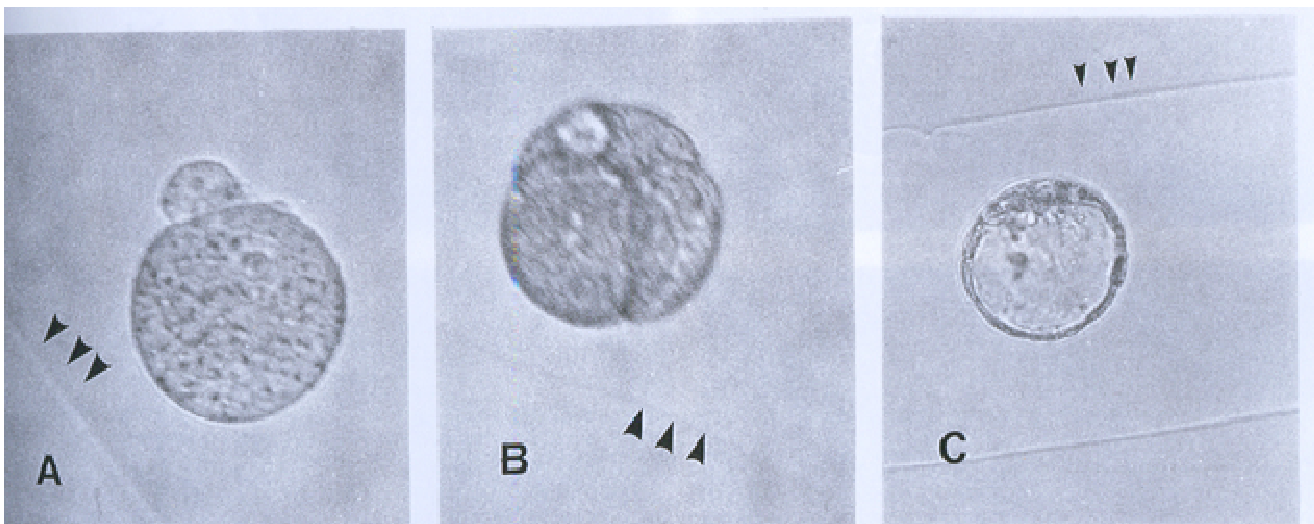

Figure 4. Encapsulated ZPF embryos. A) Pronuclear stage embryo, B) 4-cell stage embryo having six points of contacts among its blastomeres and D) A blastocyst that developed within the AZP. The arrow heads indicate the boundaries of the AZP.

porcine embryos (Niemann et al., 1983, micromunipulated bovine embryos (Westhusin et al., 1989), and bovine demi-embryos (Niemann et al., 1986) in agar improved their development. These findings indicate that sealing of a punctured or slit ZP with an AZP is critical for the development of micromanipulated embryos.

ENCAPSULATION PROCEDURE

Microencapsulation of embryos with an AZP made of sodium alginate has been described in several articles (Adaniya et al., 1987; Meredith et al., 1990; Cosby and Dunkelow, 1990; Adaniya et al., 1991; Hall and Yec, 1991; Krentz et al., 1993; Watanabe et al., 1995; Elsheikh et al., 1997). Embryos with punctured, slit, damaged or even removed ZP are washed several times in calcium free culture medium supplemented with protein, thereafter a single or groups of these embryos are immersed in 1.5% sodium alginate solution in physiological saline. The embryos are then aspirated into micropipette with small amount of sodium alginate and then expelled into 1.5% calcium chloride in phosphate buffered saline. Microgels will be formed around the embryo (s). The embryo within the microgels (Figure 4) can be cultured in any suitable culture medium to develop to blastocyst. Although embedding of embryos in agar improved embryo development in vivo (Westhusin et al., 1989), sodium alginate made AZP is easy to apply and was proofed to support excellent development *in vitro* (Elsheikh et al., 1997). The AZP improved the development of ZI embryos to blastocyst by 18% and the development of ZF embryos by 21% compared to the development of non encapsulated normal embryos.

CONCLUSION

This encapsulation procedure will help researchers who work in the field of animal cloning, chimerism, ICSI, gene injection and embryo bisection or any other biotechnology that necessitate slitting, puncturing or removing of ZP to achieve excellent *in vitro* development. Thus many cloned chimeric and transgenic animals can be produced for different purposes. This encapsulation procedure utilizes simple equipment and it is easy to apply.

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