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REVIEW

Zona-free embryo culture: is it a viable option to improve pregnancy rates?

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Abstract Sporadic reports published during the previous decade have documented pregnancies achieved with transfer of zona-free human embryos. Although the overall efficiency seems to be good and some authors have suggested systematic application for special infertility problems, there have been only a few attempts to compare the benefits of zona-free embryo culture and transfer with the traditional approach using zona-intact embryos. So far, the majority of instances in which zona-free culture has been applied have occurred accidentally. This review summarizes the known functions of the zona pellucida, analyses natural and artificial situations where its function is compromised, including zona hardening and difficult hatching that seem to be related to in-vitro embryo culture, and discusses possible methods and timing for artificial zona removal. With the availability of in-vitro systems capable of replacing important functions of the zona pellucida, routine use of zona-free culture for the whole in-vitro period, after or even before fertilization, is a realistic possibility with potential additional benefits. Based on the increasing amount of animal studies, a systematic comparison is suggested that may eventually diminish the handicaps of the in-vitro situation and lead to simplification of manipulations as well as higher success rates after embryo transfer.

KEYWORDS: assisted hatching, hatching, microwell, pronase, zona functions, zona hardening

Introduction: the origin, structure and biological function of the zona pellucida

Mammalian eggs, zygotes and early embryos are surrounded by a thick extracellular elastic coat called the zona pellucida. The zona is produced by the developing egg — and in some species also by the granulosa cells — in the early phase of oogenesis (Epifano et al., 1995; Gook et al., 2008; Liu et al., 1996; Prasad et al., 1999; Rankin and Dean, 1996; Wassarman and Albertini, 1994). The material of the zona pellucida is a relatively simple but highly specialized three-dimensional multilayered structure. Depending on

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species, it is composed of three or four long interconnected sulphated glycoprotein fibrils that exhibit a structural repeat and are bound to each other in a non-covalent way (Familiari et al., 2008; Greve and Wassarman, 1985; Izquierdo-Rico et al., 2009). Accordingly, the zona can be dissolved relatively easily using solutions with low pH (<5), elevated temperature, low ionic strength buffers, denaturants, strong oxidizing or reducing compounds and a number of proteolytic enzymes including trypsin, chymotrypsin, ficin and pronase (Mintz, 1962; Moor and Cragle, 1971; Wassarman, 1988), although some of these manipulations may cause irreversible damage in the enclosed oocyte or embryo.

The view of the zona pellucida under the light microscope indicates a passive compact layer; however, ultrastructural investigations have proven that it can vary from a porous, net-like structure permeable to relatively large molecules such as antibodies and small viruses to a nearly compact, smooth layer. After fertilization *in vivo*, the number and size of pores may transitionally increase and the compact structure becomes dominant only at the blastocyst stage. Nevertheless, wide variations can be observed at all phases (Michelman et al., 2007; Wassarman and Litscher, 2008). According to recent polarized microscopic investigations, the birefringence of the inner layer of the zona in oocytes and early embryos is correlated with developmental competence (Ebner et al., 2009; Montag and van der Ven, 2008; Rama Raju et al., 2007).

The known in-vivo functions of the zona pellucida can be summarized as follows: (i) contribution to the successful completion of oocyte growth and follicle development (Wassarman and Litscher, 2008); (ii) protection of the oocyte during and after ovulation (Michelmann et al., 2007); (iii) contribution to the selection of spermatozoa for fertilization: only spermatozoa with an intact acrosome bind to the zona, the acrosome reaction is triggered by the zona pellucida and the accomplishment of the acrosome reaction is an integral part of fertilization (Austin and Bishop, 1958; Bedford, 2008); (iv) formation of a barrier against polyspermy through structural and biochemical changes induced by the release of the cortical granules from the egg at fertilization (Austin and Braden, 1956; Hunter, 1976; Sun, 2003); (v) prevention of blastomere separation in precompaction-stage embryos (Bronson and McLaren, 1970) and supporting maximum contact between blastomeres prior to compaction (Suzuki et al., 1995); (vi) inhibition of adhesion of blastomeres in the precompaction stage to the oviductal wall that may otherwise result in immediate arrest of development and rapid degeneration (Modlinski, 1970); (vii) prevention of chimera formation by aggregation of blastomeres of different embryos (Mintz, 1962); (viii) protection against viral, bacterial and fungal agents potentially present in the reproductive tract (Singh, 1987; Van Soom et al., 2010); (ix) protection of embryonic cells against invasion of immune cells of the maternal body (Cohen et al., 1990; Moore et al., 1968); (x) creation of a microenvironment by selective sequestration of oviduct-related soluble factors (Kapur and Johnson, 1986) and allowing accumulation of autocrine ligands in the perivitelline space (O'Neill, 2008; Stanger et al., 2001); and (xi) presumably, support of the rapid passage of the embryo through the oviduct, whereas unfertilized oocytes may be delayed or sequestered there, although this effect may differ from species to species (Croxatto, 2002).

The list of these important functions seem to support the opinion of many authors describing the role of the zona pellucida as 'essential', 'indispensable' or 'vital' for mammalian embryo development. However, thorough investigation of the individual points creates some doubts about these categorical statements.

Is the zona pellucida indispensable *in vivo*?

The fact that no evidence has been found for full-term development from an oocyte that has developed, matured and been fertilized *in vivo* without the zona pellucida may be the result of the extremely limited possibilities for investigating the process without profoundly interrupting it. Occasionally, recovered zona-free human oocytes embedded within an intact cumulus investment suggest genetic or immunological abnormalities (Calongos et al., 2009; Rankin and Dean, 1996; Rankin et al., 1996) rather than trauma-induced zona loss during aspiration. Repeated occurrence of the phenomenon in all oocytes at different retrievals from the same patient also contraindicates mechanical damage (Stanger et al., 2001). These oocytes (as well as others in which the zona was lost as the consequence of accidental in-vitro damage) can be fertilized by intracytoplasmic sperm injection (ICSI) and cultured to the blastocyst stage (Ding et al., 1999; Hsieh et al., 2001; Jelínková et al., 2000; Stanger et al., 2001; Takahashi et al., 1999). Advanced-stage pregnancy was also reported after transfer of such embryos (Stanger et al., 2001). Zona-free human and mouse oocytes can also be fertilized by simple in-vitro insemination, although the rate of polyspermy is high (Ivani and Seidel, 1991; Soupart and Strong, 1975). This polyspermy, however, may be caused by the in-vitro situation, while normal monospermic fertilization may occur *in vivo* where the spermatozoa/oocyte ratio is dramatically lower (Hunter, 1996). Transfer of porcine blastocysts produced by IVF of zona-free oocytes with low spermatozoa/oocyte numbers has resulted in live piglets (Wu et al., 2004). Even in-vitro maturation of zona-free oocytes is possible and may result in blastocysts in cattle after IVF and embryo culture (Xu et al., 1990).

Accordingly, the presence of the zona — although beneficial — does not seem to be indispensable to all but one phase of mammalian oocyte and embryo development *in vivo*. The formation and presence of the zona is not a prerequisite factor for follicle development, oocyte maturation, fertilization and some periods of embryo development *in vivo*. The critical phase is the development from the 1-cell embryo to the compacted morula phase. Animal experiments have demonstrated immediate block of cleavage and rapid degeneration-reabsorption of single zona-free rabbit blastomeres and 1- to 4-cell-stage murine embryos transferred zona-free into the oviduct (Modlinski et al., 1970; Moore et al., 1968). Development of zona-free 8-cell-stage embryos was also seriously handicapped in mice (Bronson and McLaren, 1970). From this point of view, it is rather surprising that in a prospective randomized study, transfer of day 3, i.e., 4- to 8-cell-stage zona-free human embryos has resulted in pregnancy rates at least as high as those achieved with their zona-enclosed counterparts