Follicle environment and quality of in vitro matured oocytes

Marc-André Sirard

Received: 31 January 2011 / Accepted: 25 February 2011 / Published online: 11 March 2011
© Springer Science+Business Media, LLC 2011

Abstract In mammalian reproduction, the oocyte depends on the ovarian follicle for most of its growth. They form a bipolar partnership and the status of one will impact the functioning of the other. When oocytes are removed from their follicle by ovulation, they have normally completed all the steps required to begin their journey into the oviduct and drive the early embryonic development. When oocytes are removed from their follicle before natural ovulation, the process by which they acquire all the important components for their journey might not be completed and their ability to mature, fertilize or develop into embryos or to term might be compromised. Animal models have been useful to define the important steps required for the oocyte's growth phase, and in the mouse, when the oocyte has reached its full size, the program is ready. This is not the case in larger mammals where the completion of growth does not ensure that the oocyte is fully capable of undergoing all the steps to the embryo and to term. The final steps of oocyte preparation also involve a progressive condensation of the chromatin that may facilitate normal maturation but may also indirectly reduce the lifespan of the oocyte. In such a scenario, the oocyte would have an expiration date when fully competent. In humans, a number of indications may justify the

aspiration of oocytes from unstimulated patients and the development of an in vitro maturation (IVM) process that would allow fertilization and subsequent development. This objective could be realized by a better understanding of the essential follicular contribution required before removing the oocyte. Therefore, this review will focus on the large animal models where IVM has been used and studied for more than 25 years. The status of the follicle at the time of oocyte recovery and the status of the oocyte's chromatin will be described in detail as they have a significant impact on the outcome.

Keywords Oocyte · In vitro maturation · Chromatin · Competence · Follicle

Introduction

The process of making an oocyte is more complicated than it seems. The oocyte is not simply the largest cell of all; it is the only cell that does not die when transmitted to the next generation. This cell must have special characteristics to achieve such a role. One of the important parameters of the oocyte is the capacity to go through several cell divisions without any transcription going on. This unique feature requires that the RNA required for keeping the cell alive and dividing must be accumulated and stored. Normally, newly synthesized RNA lasts 2–3 h in a somatic cell and is then degraded. In large mammals, some oocyte RNA may survive as long as 7 days to ensure the new embryonic genome has all the time needed to establish a new individual. This capacity to live without transcription and to rely on very stable RNA is unique to the oocyte, although long-lasting RNA is produced in our nerve cells to

Capsule This paper summarizes pertinent data in large mammals concerning the characteristic of the oocyte according to the follicle enclosing it and the ensuing competence to develop into an embryo.

M.-A. Sirard (✉)
Centre de Recherche en Biologie de la Reproduction,
Département des Sciences Animales, Université Laval,
Québec, Canada G1K 7P4
e-mail: marc-andre.sirard@fsaa.ulaval.ca

cover the 1–2 m distance between the nucleus in the spine and the peripheral axons [1].

Therefore, the oocyte has some unique mechanisms to synthesize and store mRNA. These are best known in vertebrate model species such as frogs (*Xenopus*) mainly because the oocyte size enables chemical analysis [2]. This RNA accumulation also involves a limited functional period as these molecules are not accumulated in primary oocytes but only in growing antral follicles. The preparation for such a critical period is based on the cooperation of the surrounding cells, the cumulus and the granulosa, for the accumulation of essential oocyte components and, more importantly, for the timing of ovulation. Indeed, once the program of nuclear maturation begins, transcription must cease for several cell cycles [3]. If the oocyte is not activated by a spermatozoon, it will age rapidly and die [4, 5]. It is of utmost importance that the oocyte is perfectly synchronized with the ovulation process in order to maximize its survival chance once outside the follicle.

Spontaneous maturation of mammalian oocytes

Surprisingly, when oocytes are removed before ovulation from an antral follicle, the separation triggers a pseudo-maturation event leading in general to the completion of the first meiotic division and the second arrest at the metaphase II stage. This has been described as early as 1939 by Pincus [6] and generalized to most domestic mammals, by Edwards, in 1965 [7]. This process has been called spontaneous maturation and is believed to be induced by the removal of an inhibitory factor present in the follicular context [8]. The mechanism of such inhibition has been elusive for more than 40 years, although the impact of cAMP on the inhibitory process has been known and used during this period [9]. A recent paper by the group of Eppig [10] has now demonstrated the importance of cGMP in the control of phosphodiesterase 3 involved in the crucial decrease of intra-oocyte cAMP and meiotic resumption. This may also explain the reason cumulus-enclosed oocytes cannot generate this inhibitory signal outside of the follicle and the difference between induced and spontaneous maturation.

The fact that meiotic resumption is triggered upon physical removal of the oocyte and its cumulus cells does not mean that this maturation is physiological. It appears to be a default mechanism acting in replacement of an induced signal normally associated with the LH surge. Nevertheless, the process of physical removal from the follicle induces early oocyte nuclear maturation unless the follicle is at that time totally ready for ovulation [7]. In fact, recent studies in our laboratory support this conclusion as almost 100% of cow oocytes removed following a time-regulated protocol achieve developmental competence [11].

The reasons why fully grown oocytes are not capable of becoming viable embryos in large mammals are still elusive. One possible hypothesis is that the fully totipotent oocyte may represent a threat to the ovary if not expelled. In support of this hypothesis is the phenotype of *cMos* double KO mice, where the oocytes become self-activated in the follicle and all females die of ovarian cancer before reaching their first anniversary [12]. If the oocyte presents a threat, the LH receptor represents a way to expulse the oocyte in an environment less favourable to cancer growth, the oviduct. Whatever the real explanation, in most mammals, the oocyte has reduced capacity to develop when the follicle containing it is still growing (see next section).

The links between follicle and oocyte

The follicular growth and differentiation process is very dynamic and changes can be seen on a daily basis in terms of size or follicular populations. The process is also continuous as it does not really stop or reverse direction. When a follicle starts to grow, it must encounter the right endocrine and paracrine support to keep growing or it simply dies. The last portion of the growth, the antral phase, is even more dynamic and, in mono-ovulating animals, a competition is set between follicles to become the dominant one [13]. Once the dominant is formed, it keeps growing but begins a final growth phase to allow endocrine signalling (mainly through estradiol and inhibins) and ovulation. Inside these follicles, the oocyte must prepare for the transcriptional silence that will last several days; but if it occurs too soon, the oocyte will not survive the wait and if it occurs too late (as precocious LH triggers ovulation), the oocyte might be missing important components that are made at the last minute.

The impact of chromatin status at the time of oocyte aspiration on further capacity to develop into an embryo

The oocyte is almost constantly changing, as is the follicle. This is quite obvious as its size progresses but even when the oocyte reaches its final size, the transformation continues. The oocyte progressively decreases its transcriptional activities [14]. This is illustrated by both uridine incorporation experiments and a progressive change in the nuclear and nucleolar structures during the dominant-preovulatory period [15, 16]. One of the major transformations is the chromatin sequence of visible changes that seems to follow a universal pattern in mammals studied so far. From mouse and rabbit to sheep, goat, cow, horse and human, the progressive configuration from diffuse to more condensed and from non-surrounded nucleolus to surrounded nucleolus seems to be associated with a slow shutdown of the transcriptional machinery [15]. A similar

change in chromatin configuration has also been observed in human oocytes [17]. This simple transformation might indicate that the oocyte finally has all the RNA required to proceed. If the oocyte has not completed such RNA accumulation, it may have impaired capacity (Fig. 1).

Another unresolved question is whether the oocyte can make the last required mRNA molecules and prepare for transcriptional silencing once outside the follicle. This has been demonstrated to some extent in the mouse but some somatic cells must be present for the full capacity to develop. The culture of entire follicles has been studied and progress has been made, especially in species where tissues are readily available, such as mouse and cow [18, 19].

An alternative procedure is to maintain the oocyte in meiotic arrest outside the follicle with the hope that the oocyte will finalize its RNA storage and that chromatin changes will occur before the arresting condition is removed. Again, the culture of mouse oocytes in meiotic arrest has been possible for years using phosphodiesterase inhibitors or cAMP agonists [20]. In large mammals, few laboratories [21, 22] have succeeded in maintaining meiotic arrest without negative effects on the developmental potential, but up to now, no increase was obtained with this approach. In theory, the functional use of the natural process, i.e., phosphodiesterase 3 inhibition, by the addition of a specific inhibitor such as cilostamide should allow the culture of meiotically arrested oocytes, but in reality, the oocyte cannot be maintained for more than a few hours with such treatment [23]. In addition, all these chemical inhibitors result in ultrastructural changes [24]. Recent development in trying to mimic the endogenous induction of meiotic maturation in vitro has met with some success in cow and mouse [25].

The expiry date concept

The nuclear morphological changes described above could indicate that when the oocyte has begun the process of final

preparation, it rapidly decreases the production of new mRNA. Does this process result in a stable or viable period for the oocyte or does it imply that the oocyte has a limited time to be ovulated or an expiry date? Histological evaluation of ovaries indicates that such condensed chromatin structure is present in atretic follicles that either ovulate or disappear within 2–4 days. Therefore, although the true answer is not known, the evidence indicates no example where these types of oocytes can last longer. A few studies were performed to extend the duration of the dominant follicle in cattle and humans. In the cow, postponing ovulation by only 1 day with the use of progesterone implants results in a decreased fertility rate associated with the pre-ovulatory follicle [26]. In our laboratory, we have performed several experiments on coasting in cows (preventing ovulation by endogenous progesterone) to show that 72 h after the last FSH surge, the quality of the oocyte decreases rapidly [27].

As any living cell, the oocyte needs proteins to survive, and proteins are being turned over at defined rates in somatic tissues. This protein turnover requires RNA for replacement and functional ribosomes for making them. Three possible hypotheses can be proposed: (1) either the RNA required for housekeeping is decreasing and the survival of the oocyte is progressively compromised, or (2) stored RNA for the oocyte's needs (requirements) after the LH surge are not maintained and the oocyte can go through some step of fertilization and early cleavage, or (3) specific competence RNA is degraded and results in a compromised embryo incapable for example to activate its own embryonic genome. In either case, the result is lower fertility. New transcriptomic contrasts are being used to explore if indeed some mRNA amounts are modulated during developmental competence acquisition using the bovine model (ongoing in our laboratory).

IVM in a suitable animal model for human

In vitro maturation is possible because the spontaneous maturation of oocytes occurs in vitro. The process of IVM therefore is facilitated by the simplicity of inducing nuclear maturation (meiotic resumption) but complicated by the difficulties to induce the proper cytoplasmic and molecular maturation essential for developmental competence [28]. The use of in vitro maturation has been developed in cows in the mid-eighties, not as a clinical requirement but as a research tool [29]. The principal reason is that bovine ovaries are available in slaughterhouses and represent a readily accessible source of experimental material. The initial experimental conditions for the culture of immature bovine oocytes have survived 25 years of improvement. Indeed, the method used in most laboratories to culture

Fig. 1 The growing oocyte has very limited competence (green) but becomes more competent (red) in the antral, selection, or dominant phase, depending on the species. The condensed chromatin status of the GV nucleus also seems indicative of the readiness of the oocyte

bovine eggs remains the use of TCM-199 (a somatic culture medium with 200 components) and the addition of 10% FBS (fetal bovine serum), pyruvate, FSH, estradiol, and antibiotics [29]. With this simple system either in open media or under oil, a reasonable blastocyst rate of 30% can be achieved quite reliably. The same system was applied for human IVM by RC Chian after a few years of using it on bovine eggs in our laboratory [30]. More recently, defined systems were developed to remove serum and to have much less components present; this system uses a modification of SOF media [31] and results in similar blastocyst rates with only BSA as a biological supplement.

The use of slaughterhouse ovaries implies that the animals are not treated before oocyte collection. This implies that the ovaries are distributed randomly across the estrous cycle which has 15 days of luteal phase and 6–7 days of follicular phase in cows. During the luteal phase, 2 or 3 follicular waves are generated and each of them is characterized by the appearance of a dominant follicle and the demise of subordinate follicles since the cow is a mono-ovulator, as are humans [13]. This context implies that follicles present in slaughterhouse animals contain all types and sizes of antral follicles. This is interesting for research but such diversity is not a good system to optimize culture conditions for oocytes at so many different stages of differentiation. It can be concluded from several studies over the years that small growing antral follicles do not result in blastocyst formation after IVF [32, 33]. Follicles larger than 8–9 mm that have LH receptors are classified as dominant and contain oocytes of better competence unless their health status is seriously compromised [34]. But the biggest surprise came from the analysis of cumulus morphology, which indicated that compact cumulus is not a very good sign of developmental competence while the beginning of slight expansion as seen in early atretic follicles is a better indicator of competence. This observation led to a study where each follicle was assessed for atresia and each oocyte was individually assessed for developmental competence. The results indicated early follicle atresia was associated with better competence of the oocyte [32].

The mystery of early atresia

The early observation that the process of follicle selection would create subordinate follicles containing more competent oocyte was made in the bovine model [32, 35], but over the last decade, similar observations were made especially in mono-ovulating species like the horse [36]. The first question that arises from such observation is why would oocytes from dying follicles be better? The simplest answer is that the process of early atresia mimics several of the pre-ovulatory changes such as a rise in progesterone

and androgen by the down-regulation of aromatase and a progressive decrease of follicular support from the granulosa layer, which may be perceived by the oocyte as pre-ovulatory signals [37].

Atresia in human follicles

The process of follicular selection is quite similar in human where a cohort of several follicles is formed at the beginning of the follicular phase. As only one follicle normally becomes dominant, the rest of the cohort will undergo atresia in the following few days. If the oocytes are aspirated early in the selection process, the growing follicles could be containing incompetent oocytes, as in the cow [33] but if the atresia process has begun or has been activated by an hCG injection, then their competence could be better as observed in cow. There is a large body of literature indicating that a GnRH injection will ovulate a dominant and destroy the subordinates, creating a new follicular wave either for synchronization or timed ovarian stimulation [38]. If the dominant does not ovulate in response to LH, like in the luteal phase, it means that the atresia process is so advanced that the dominant effect is no longer functional and, as above, a new cohort of follicular development will begin. Therefore, depending on the follicular size of such dominant at the time of hCG injection, several outcomes are possible:

- 1) The dominant is early and still growing (10–12 mm) but has LH receptors, and the oocyte maturation is triggered to lead to at least one oocyte with a metaphase I or II status 34 h later; the subordinate follicles will have a theca cell response (rise in androgen) and will begin the atretic process. By 34 h, the oocytes enclosed in the early atretic follicles are probably still good and may have begun a pseudo chromatin condensation, improving their ability to go through GVBD in vitro and to be fertilized.
- 2) The dominant is already at the plateau phase (12–13 mm) and will respond by producing one metaphase egg, but the subordinates will be one step further in atresia and expanded cumuli from subordinates can be expected with oocytes normally at the GV stage or more, as well as oocytes from less atretic follicles with compact cumulus and also at the GV stage.
- 3) The dominant is moving to a pre-ovulatory (above 13 mm) status and the hCG triggers ovulation like a regular natural cycle. The subordinate follicles contain oocytes at more advanced stages of atresia and although they mature easily in vitro, their competence has been compromised.

These parameters not surprisingly correspond to what has been observed in clinics performing hCG-triggered IVM with leading follicles of different sizes (Lim et al., this issue).

The human context for IVM

Human IVM trials were initially undertaken almost 20 years ago by scientists with extensive knowledge of the bovine system [39, 40], but the indications are limited as IVF gives much better results. It is interesting to go back to the early studies which can for example support the value of IVM using oocytes from luteal phase [41]. Since then, several experiments have used immature GV-stage oocytes collected at the same time as mature oocytes following COS. The general feature associated with these follicles is that the immature oocytes probably originate from late-recruited follicles (with repetitive FSH treatments, they are continuously recruited) and therefore belong to very rapidly growing follicles category. Our analysis of those in the bovine model has indicated that although they are meiotically competent, they have very little chance to develop further as their chromatin is still diffuse [16, 32].

Therefore, it is not possible to make any comparison of IVM from stimulated regular cycles and IVM of later-stage follicles. The few groups who have worked with IVM can be divided in 3 different approaches:

- 1) IVM with hCG and oocyte aspiration 34–38 h later. This was initially tried on PCOs patients and progressively on more normal patients or patients at risk for HSS at McGill University in Canada [42]. As mentioned before, the approach used was copied from the concept developed in cow [43].
- 2) IVM after a few days of FSH, no hCG. This was initially tried by Mikkelsen [44] with some success but as mentioned above, if no coasting is used, the FSH effect may not be very helpful.
- 3) IVM after a few days of FSH, coasting with hCG. This approach was recently developed by Fadini and his group in Milan [45]. The results they obtained are quite encouraging and confirm the observations made in the bovine model [11].

It is now quite clear that several clinics are trying the FSH-coasting approach with or without LH to obtain immature oocytes for a real IVM procedure. This is in contrast with option 1 where often the largest follicle will deliver an already matured oocyte; only the “other” requires IVM.

The problem of PCO is different as the follicles are already in an early “atretic” status mainly as a consequence of growth arrest and higher androgen content.

Conclusions and perspectives

The accumulation of information in several animal species is indicating that the oocyte has limited lifespan once the follicle enclosing it triggers the signal of either upcoming ovulation or atresia. This limitation may be associated with the chromatin configuration allowing less and less RNA to be made and preparing for the transcriptional shutdown characteristic of early mammalian embryos. This information should be considered when designing IVM conditions to ensure that the oocytes have reached the adequate chromatin status before removing them from their follicles.

References

1. Holt CE, Bullock SL. Subcellular mRNA localization in animal cells and why it matters. *Science*. 2009;326:1212–6.
2. Radford HE, Meijer HA, de Moor CH. Translational control by cytoplasmic polyadenylation in *Xenopus* oocytes. *Biochim Biophys Acta*. 2008;1779:217–29.
3. Memili E, Dominko T, First NL. Onset of transcription in bovine oocytes and preimplantation embryos. *Mol Reprod Dev*. 1998;51:36–41.
4. Lacham-Kaplan O, Trounson A. Reduced developmental competence of immature, in-vitro matured and postovulatory aged mouse oocytes following IVF and ICSI. *Reprod Biol Endocrinol*. 2008;6:58.
5. Miao YL, Kikuchi K, Sun QY, Schatten H. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Hum Reprod Update*. 2009;15:573–85.
6. Pincus G. Ovum culture. *Science*. 1939;89:509.
7. Edwards RG. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*. 1965;208:349–51.
8. Tsafiri A, Pomerantz SH. Oocyte maturation inhibitor. *Clin Endocrinol Metab*. 1986;15:157–70.
9. Downs SM, Eppig JJ. Cyclic adenosine monophosphate and ovarian follicular fluid act synergistically to inhibit mouse oocyte maturation. *Endocrinology*. 1984;114:418–27.
10. Zhang M, Su YQ, Sugiura K, Xia G, Eppig JJ. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*. 2010;330:366–9.
11. Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biol Reprod*. 2002;66:38–43.
12. Hashimoto N, Watanabe N, Furuta Y, Tamemoto H, Sagata N, Yokoyama M, et al. Parthenogenetic activation of oocytes in c-mos-deficient mice. *Nature*. 1994;370:68–71.
13. Ginther OJ, Beg MA, Donadeu FX, Bergfelt DR. Mechanism of follicle deviation in monovular farm species. *Anim Reprod Sci*. 2003;78:239–57.
14. Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol Reprod Dev*. 1995;42:437–42.
15. Tan JH, Wang HL, Sun XS, Liu Y, Sui HS, Zhang J. Chromatin configurations in the germinal vesicle of mammalian oocytes. *Mol Hum Reprod*. 2009;15:1–9.
16. Lodde V, Modena S, Maddox-Hyttel P, Franciosi F, Lauria A, Luciano AM. Oocyte morphology and transcriptional silencing in relation to chromatin remodeling during the final phases of bovine oocyte growth. *Mol Reprod Dev*. 2008;75:915–24.

17. Escrich L, Grau N, Meseguer M, Pellicer A, Escriba MJ. Morphologic indicators predict the stage of chromatin condensation of human germinal vesicle oocytes recovered from stimulated cycles. *Fertil Steril*. 2009;93:2557–64.
18. McLaughlin M, Telfer EE. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. *Reproduction*. 2010;139:971–8.
19. Telfer EE, McLaughlin M. In vitro development of ovarian follicles. *Semin Reprod Med*. 2011;29:15–23.
20. Downs SM. The biochemistry of oocyte maturation. *Ernst Schering Res Found Workshop* 2002;41:81–99.
21. First NL, Leibfried-Rutledge ML, Sirard MA. Cytoplasmic control of oocyte maturation and species differences in the development of maturational competence. *Prog Clin Biol Res*. 1988;267:1–46.
22. Lonergan P, Khatir H, Carolan C, Mermillod P. Bovine blastocyst production in vitro after inhibition of oocyte meiotic resumption for 24 h. *J Reprod Fertil*. 1997;109:355–65.
23. Mayes MA, Sirard MA. Effect of type 3 and type 4 phosphodiesterase inhibitors on the maintenance of bovine oocytes in meiotic arrest. *Biol Reprod*. 2002;66:180–4.
24. Faerge I, Mayes M, Hyttel P, Sirard MA. Nuclear ultrastructure in bovine oocytes after inhibition of meiosis by chemical and biological inhibitors. *Mol Reprod Dev*. 2001;59:459–67.
25. Albuz FK, Sasseville M, Lane M, Armstrong DT, Thompson JG, Gilchrist RB. Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum Reprod*. 2010;25:2999–3011.
26. Sirois J, Fortune JE. Lengthening the bovine estrous cycle with low levels of exogenous progesterone: a model for studying ovarian follicular dominance. *Endocrinology*. 1990;127:916–25.
27. Sirard MA, Picard L, Dery M, Coenen K, Blondin P. The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. *Theriogenology*. 1999;51:699–708.
28. Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. *Theriogenology*. 2006;65:126–36.
29. Sirard MA, Parrish JJ, Ware CB, Leibfried-Rutledge ML, First NL. The culture of bovine oocytes to obtain developmentally competent embryos. *Biol Reprod*. 1988;39:546–52.
30. Chian RC, Lim JH, Tan SL. State of the art in in-vitro oocyte maturation. *Curr Opin Obstet Gynecol*. 2004;16:211–9.
31. Ali A, Sirard MA. Effect of the absence or presence of various protein supplements on further development of bovine oocytes during in vitro maturation. *Biol Reprod*. 2002;66:901–5.
32. Blondin P, Sirard MA. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol Reprod Dev*. 1995;41:54–62.
33. Blondin P, Coenen K, Guilbault LA, Sirard MA. Superovulation can reduce the developmental competence of bovine embryos. *Theriogenology*. 1996;46:1191–203.
34. van de Leemput EE, Vos PL, Zeinstra EC, Bevers MM, van der Weijden GC, Dieleman SJ. Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory LH surge. *Theriogenology*. 1999;52:335–49.
35. Blondin P, Dufour M, Sirard MA. Analysis of atresia in bovine follicles using different methods: flow cytometry, enzyme-linked immunosorbent assay, and classic histology. *Biol Reprod*. 1996;54:631–7.
36. Hinrichs K. The equine oocyte: factors affecting meiotic and developmental competence. *Mol Reprod Dev*. 2010;77:651–61.
37. Sirard M-A, Trounson A. Follicular factors affecting oocyte maturation and developmental competence. In: Taog RG, editor. *Biology and pathology of the oocyte: its role in fertility and reproductive medicine*. Cambridge: Cambridge university Press; 2003. p. 305–15.
38. Twagiramungu H, Guilbault LA, Proulx J, Ramkumar R, Dufour JJ. Histological populations and atresia of ovarian follicles in postpartum cattle treated with an agonist of gonadotropin-releasing hormone. *J Anim Sci*. 1994;72:192–200.
39. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril*. 1991;55:109–13.
40. Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM, et al. Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum Reprod*. 1995;10:3243–7.
41. Cha KY, Do BR, Chi HJ, Yoon TK, Choi DH, Koo JJ, et al. Viability of human follicular oocytes collected from unstimulated ovaries and matured and fertilized in vitro. *Reprod Fertil Dev*. 1992;4:695–701.
42. Chian RC, Buckett WM, Tan SL. In-vitro maturation of human oocytes. *Reprod Biomed Online*. 2004;8:148–66.
43. Blondin P, Coenen K, Guilbault LA, Sirard MA. In vitro production of bovine embryos: developmental competence is acquired before maturation. *Theriogenology*. 1997;47:1061–75.
44. Mikkelsen AL, Smith S, Lindenberg S. Possible factors affecting the development of oocytes in in-vitro maturation. *Hum Reprod*. 2000;15 Suppl 5:11–7.
45. Fadini R, Dal Canto MB, Mignini Renzini M, Brambillasca F, Comi R, Fumagalli D, et al. Effect of different gonadotrophin priming on IVM of oocytes from women with normal ovaries: a prospective randomized study. *Reprod Biomed Online*. 2009;19:343–51.