Cumulus Cell Contribution to Cytoplasmic Maturation and Oocyte Developmental Competence In Vitro

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Objective: Although several reports indicated the bidirectional communication between the oocyte and granulosa cell, and the potential negative impact of premature cumulus removal, clinical data are controversial. The present analysis report the results of a prospective randomized study done on sibling oocytes to evaluate whether or not preincubation with intact cumulus is needed in ICSI.

Methods: It was a prospective randomized study, and conducted at Alexandria IVF–ICSI center. This study was performed using 926 M2 oocytes from 141 cycles of patients undergoing ICSI. Oocytes were randomized between three protocols: protocol A (immediate denudation, immediate injection), protocol B (delayed denudation, delayed injection), and protocol C (early denudation, delayed injection). Main outcome measure(s) were maturation, fertilization, and cleavage rate after ICSI.

Results: Aspects of nuclear, cytoplasmic maturation and oolemma properties were improved when oocytes were preincubated with intact cumulus before ICSI.

Conclusions: Preincubation, before ICSI improved results. Duration of preincubation should be program specific.

KEY WORDS: cumulus cell; cytoplasmic maturation; ICSI; nuclear maturation; oocyte.

INTRODUCTION

Oocytes and their companion somatic cells maintain a close association throughout oogenesis (1). This association is fundamental for normal oocyte and follicular development (2,3). During this development,

oocytes acquire the molecular program for embryogenesis (3), and undergo major changes in number, morphology, and distribution of cytoplasmic organelles (2,3). The default in follicular somatic cells is the formation of mural granulosa (4). Proliferation, development, differentiation, and functional heterogeneity of these cells are attributed to the presence of the oocyte (4,5). Mouse oocytes also regulate granulosa steroidogenic gene(s) expression (6). The regulatory interaction is not unidirectional; follicular somatic cells also play a major role in regulation of oocyte growth, maintenance of meiotic arrest (7), induction of oocyte maturation, and acquisition of full embryonic developmental competence during oocyte maturation (4). Granulosa and cumulus cells play different roles in oocyte protein phosphorylation (8). Cumulus mainly transmits meiosis stimulatory signals (9) whereas distant mural granulosa (10) and theca (11) cells principally conduct inhibitory signals (12). Surprisingly, although several reports indicated this bidirectional communication (8,9,13,14), and the potential negative impact of premature cumulus removal (15), clinical data from the assisted reproductive technology (ART) are very controversial. In IVF, earlier reports indicated that cumulus removal could enhance fertilization (15,16), whereas other data indicated the opposite (8,17,18). They even demonstrated that early cumulus removal impaired maturation (17), and that late removal impaired mainly blastocyst formation (17). The duration of preincubation before insemination was also reportedly variant (15,18,19). Contrary to IVF, ICSI allows a unique opportunity to assess an essential aspect of cytoplasmic maturation that occurs after GVBD (20). There is no agreement, however, on timing of cumulus removal after oocyte retrieval in ICSI (20,21). Several groups reported that timing of injection is not important (21), whereas others indicated that preincubation is beneficial (20). In the

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present analysis, we report the results of a prospective randomized study done on sibling oocytes to evaluate whether or not preincubation with intact cumulus is needed in ICSI.

MATERIAL AND METHODS

This study was performed using 926 M2 oocytes from 141 cycles of patients undergoing ICSI for male factor infertility.

Stimulation Regimen

A long agonist protocol was used for ovarian stimulation. Buserelin acetate (suprefact; Hoechst AG, Frankfurt am Main, Germany) was given in a dose of 0.5 mg subcutaneously daily starting on Day 21 of the preceding cycle. Human menopausal gonadotropin (hMG) [pergonal 500; Serono S.P.A, Rome, Italy, packed by E.I.P.I.C.O., 75 IU of follicle stimulating hormone (FSH) and 75 IU of luteinizing hormone (LH) per ampule] was started after menstruation, when serum plasma estradiol dropped below 40 pg/mL. The dose of hMG was 4 ampules per day, patients were monitored by E2 and ultrasound (22,23). The ovulatory dose of hCG (10,000 IU, Pregnyl; Organon, Egypt) was administered when at least three mature follicles (mean diameter > 17 mm) were seen with concomitant estradiol of 150 pg/mL per follicle above 17 mm.

Oocyte Preparation

Following retrieval, oocytes were washed three times in Ham's F10, serum free medium and finally incubated in IVF medium droplets under oil (Scandinavian IVF science, Gothenburg, Sweden). Then oocytes were randomly allocated to three different protocols concerning the time of cumulus corona removal. In protocol A, cumulus cells were immediately removed enzymatically after retrieval, using ready-made 10% hyaluronidase (Scandinavian IVF science, Gothenburg, Sweden). Cumulus oocyte complexes were repeatedly pipetted in the hyaluronidase for a maximum of 15 s. Before cumulus was completely dissociated, oocytes were moved from hyaluronidase into HEPES-buffered medium (Sperm prep Scandinavian IVF science, Gothenburg, Sweden) where corona removal was performed mechanically by repeated pipetting through finely pulled glass pipettes, until no more

cells were seen attached to the zona pellucida. Then injection (ICSI) was performed immediately following corona removal. In protocol B, intact undenuded cumulus oocyte complexes were first incubated for 4 h in a humidified atmosphere of 5% CO₂ at 37°. Surrounding cells were then removed as in protocol A, and sperm injection (ICSI) was immediately performed following corona removal.

In protocol C, cumulus corona cells were immediately removed after oocyte retrieval. Denuded oocytes, free of granulosa cells, were then incubated in humidified atmosphere of 5% CO₂ at 37° for 4 h before injection (ICSI). Expanded cumulus corona complexes were determined, both at retrieval (protocol A, B, and C) and after 4 h of pr(protocol B). After cumulus corona removal, three oocyte criteria were evaluated: nuclear maturation, pattern of oolemma penetration during ICSI (24), and incidence of perivitelline space granularity (25). Oolemma penetration was identified as normal breakage, when the injection needle created an invagination that ruptured at the approximate center of the egg; sudden breakage, when the membrane broke without creating a funnel; and difficult breakage, when the membrane did not break or broke after several penetration attempts (24). Perivitelline space granularity (PVCg) was determined by the presence of a dark coarse granules in the perivitelline space (25). Semen was prepared by double wash and resuspension. Testicular sperm samples were put in petri dishes containing Ham's F10, and tissue samples were squeezed using the plunger of 20 mL syringe. The medium was aspirated, transferred into a conical tube, and prepared by double wash and resuspension.

Microinjection Procedure

Sperm petri dishes were prepared with two 2–3 µL droplet of ready-made polyvinylpyrrolidone (PVP) solution (Medicult a/s, Copenhagen O, Denmark) and another droplet of PVP mixed with prepared sperm; all were covered by light mineral oil. Oocyte microinjection dishes were prepared from four 3–4 µL droplets of a HEPES-buffered medium (Spermprep, Medicult a/s, Copenhagen O, Denmark) and one 2–3 µL droplet of PVP solution. The holding and the injection pipettes were ready-made (Humagen Fertility Diagnostics, Charlottesville). Motile morphologically normal sperm were selected for microinjection and were moved to the PVP droplet in the oocyte microinjection dish. At the time of microinjection, oocytes were transferred to the microinjection dish

Table I. Comparison of Oocyte Morphology Between the Three Different Protocols Applied

Parameters	Protocol A	Protocol B	Protocol C
PVSG (%)	51	61	48
Difficult penetration (%)	39 [†] *	19 [†]	23.3*
Rigid membrane (%)	5.9*	10.7	23.3*
Degenerated oocytes	9/152 (5.9%)	2/149 (1.3%)*	12/150 (8%)*

Note. Protocol A – immediate denudation, immediate injection; Protocol B – delayed denudation, delayed injection; Protocol C – early denudation, delayed injection.

[†] $P < 0.06$.

* $P < 0.05$.

(4 oocytes per dish). Only metaphase II oocytes were injected. Selected sperm were immobilized, then aspirated, tail first, into the injection pipette. Before the injection of the sperm, a small amount of cytoplasm was aspirated to be reinjected again together with the sperm.

Observation and Culture

After micromanipulation, the oocytes were washed and incubated in IVF medium. Microdroplets of IVF medium (100 μ L each) were put in a petri dish (Falcon, 3004, NJ) and covered by mineral oil. Three or four injected oocytes were put together in each microdroplet. Fertilization was checked 16–18 h later. Fertilized oocytes were transferred into new petri dishes and cultured together again, in groups of three to four, in IVF medium. Embryos were evaluated 48 h after retrieval. Embryos with <20% anucleate fragments, equal regular blastomeres, and variable thickness of the zona pellucida were classified as Class A embryos.

RESULTS

Incubation for 4 h with intact cumulus resulted in a significant ($P < 0.05$) increase in M2 oocytes from 80.5 (protocol A and C) to 91.9% (protocol B). The total number of M2 oocytes was 947 oocytes. When examined at retrieval, 78.9% had expanded cumuli that increased to 89.9% after 4 h of incubation ($P < 0.05$). As shown in Table I, perivitelline space granularity, a phenomenon accompanying oocyte maturation, did not change as time passed after retrieval neither with nor without intact cumulus. Incubation, however, changed patterns of membrane penetration. Difficulty in penetration of the oolemma was significantly reduced with preincubation ($P < 0.05$) (Table I). Oolemma rigidity (immediate breakage) increased significantly when oocytes were injected 4 h after denudation (protocol C, Table I) (24). Preincubation

with intact cumulus (protocol B) resulted in a significant decrease ($P < 0.05$) in the incidence of oocyte degeneration after ICSI (Table I). Delayed injection improved fertilization, cleavage, and blastocyst formation only when oocytes were preincubated with intact, but not with denuded cumulus (Table II). One hundred Class B embryos were not transferred, and an informed consent was obtained to allow their observation in culture ($n = 38, 29$, and 33 , for protocol A, B, and C, respectively). The percentage of blastocyst formation was 15.8, 48.3, and 18.2% for protocol A, B, and C respectively ($P = 0.065$) (Table II). In 23 patients, only protocol A embryos were transferred, in 42 patients only protocol B embryos were transferred, and in 17 patients only protocol C embryos were transferred. Pregnancy rates were 8.7, 26.2, and 11.8%, respectively (Table II). In 59 patients, a “mix” embryo transfer was done, principle for selection was ? 4 equal blastomeres with no fragmentation, pregnancy rate was 13.6%.

DISCUSSION

Regulation of oocytes and accompanying somatic cell gene expression and bidirectional control (4) requires gap junction gene expression (22) and structural integrity (4). Without gap junction, putative regulatory molecules will only function if diffusible and not labile, which is not always the case (25). Expression of connexin (22) and maintenance of gap

Table II. Comparison Between the Three Different Protocols Applied in Terms of Fertilization Blastocysts, and Pregnancy Rate

Parameters	Protocol A	Protocol B	Protocol C
M2 oocytes	319	313	315
Fertilization (%)	43.4*	72.5*	46
Blastocysts (%)	16.7 [†]	42.9 [†]	18.8
Pregnancy (%)	8.7	26.2	11.8

[†] $P < 0.06$.

* $P < 0.05$.

junction is temporarily related to oocyte maturity; they decrease in mature oocytes (26). Therefore, role of somatic cells in control of germ cell maturation is obvious before GVBD (7,27,28). After the ovulatory stimulus, gap junctions between somatic cells nearby the oocyte are still present whereas distant junction are lost (29,30) resulting in a net gain of stimulatory rather than inhibitory signals (12). On cellular level, these data provide the rationale behind a potential beneficial effect exerted by preincubation with intact cumulus before ICSI. In the present study, perivitelline space granularity (PVSG), a sign of maturation, was not, however, increased when oocytes were preincubated with intact cumulus (protocol B) nor without (protocol C). Oolemma properties, however, were significantly affected by preincubation. Difficult penetration decreased significantly in oocytes preincubated with the cumulus as compared to control. Such an effect was not detected when oocytes were incubated denuded. Oolemma breakage patterns were previously reported, variant and variations of results were also related to patterns of membrane penetration (23). Also in mice, maturation of both oolemma and cytoplasm are acquired together, and they are affected by cumulus cells during meiotic progression (28). Our data showed significant nuclear maturation upon incubation with cumulus, probably induced by meiosis stimulating signals conducted through intact cumulus-oocyte gap junctions (31). Cytoplasmic maturation was improved when oocytes were allowed more time after retrieval with cumulus intact cells as evidenced by significantly higher fertilization and good quality early embryos. A conclusion about other advanced aspects of cytoplasmic maturation could not be reached from the present work. Improvement of blastocyst formation and pregnancy rates were not significantly different from other groups. Collectively our data are more in favor of preincubating oocytes with intact cumulus before ICSI. It is not clear why our results should differ from those reported by some other groups (21). One explanation could be that we are retrieving more immature oocytes, hence preincubation time is crucial for further nuclear and cytoplasmic maturation. Both aspects require extended cumulus gene expression (22). Another possibility is that subtle changes in some of the interconnected variables of patient biodata, stimulation protocol, hCG criteria, or laboratory protocols could be in favor of preincubation. Whatever the reason is, it seems to be a rational conclusion that preincubation period, or absence of, before ICSI should be protocol and program specific.

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