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In vitro maturation of bovine cumulus–oocyte complexes in undiluted follicular fluid: effect on nuclear maturation, pronucleus formation and embryo development

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Abstract

Since resumption of meiosis and cytoplasmic maturation of bovine oocytes takes place in close association with follicular fluid, it would be logical to assume that this might be a perfect maturation medium. To test the hypothesis, abattoir-derived cumulus–oocyte complexes (COCs) were in vitro matured in undiluted (i) mixed follicular fluid (FF) from 3 to 15 mm follicles from abattoir ovaries, (ii) preovulatory follicular fluid (POF) from the dominant follicle from a cyclic unstimulated heifer, (iii) preovulatory follicular fluid (OPU) from synchronised and superovulated heifers 60 h after prostaglandin and 20 h after GnRH treatment, and in (iv) TCM-199 with 5% serum. Subsequent to IVM, the COC were subjected to IVF and IVC, and embryo development was followed until the blastocyst stage at Day 8 after insemination. The MII rates in the TCM-199 (69%), POF (69%) and OPU (72%) groups were not different from each other but different from the FF (41%) group ($P < 0.05$). In spite of the high MII rates, none of the follicular fluids supported embryo development: the FF, POF and OPU blastocyst rates were alike (3%, 3%, 2%) and different ($P < 0.05$) from the rates in the TCM-199 (19%). During IVM in follicular fluids but not in TCM-199, the expanded cumulus masses became trapped in a coagulum. Although it could be prevented by the presence of heparin during IVM, it did not improve the blastocyst rates. In conclusion, undiluted preovulatory follicular fluids supported nuclear maturation but not further embryonic development as judged by the high MII and low blastocyst rates.

Keywords: Coagulation; Nuclear maturation; MII; Cytoplasmic maturation; Fertilisation; Cleavage; Blastocyst

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1. Introduction

In the antral follicle, the cumulus–oocyte complex (COC) and the granulosa cells are enclosed in follicular fluid which originate from granulosa cell secretions and from a transudate similar but not identical to blood plasma. During follicle growth, the endocrine and other biochemical profiles of the follicular fluid and the microvascularity of the follicular wall undergo changes, which are of importance for subsequent oocyte maturation, ovulation and corpus luteum formation [1–4]. The specific function of follicular fluid is not known. It has, however, been speculated that follicular fluid could protect the oocyte from factors that could induce premature resumption of meiosis [5], guard the oocyte from proteolytic attack and facilitate its extrusion during ovulation [6], and enhance spermatozoa attraction, motility and acrosome reaction [7,8].

Assuming that preovulatory follicular fluid could play an active role in the final oocyte maturation, it would be logical to consider it to be the medium of choice for in vitro maturation as well. In fact addition of diluted follicular fluid to maturation media has been used as an alternative to serum for in vitro production of embryos in the equine [9], bovine [10–12], human [13], porcine [14,15] and caprine [16] species, while the developmental potential of oocytes exposed to pure follicular fluid during IVM has been described in the bovine [17–21] and equine species [22,23].

The conclusions from the above cited articles can be briefly summarised as follows: the oocyte maturation rates in follicular fluid were dependent on the estrous stage; undiluted follicular fluid from large follicles supported oocyte maturation better than fluid from small or medium sized follicles; full embryo development to the blastocyst stage was supported by the presence of diluted follicular fluid during IVM, although follicular fluid was not superior to diluted serum.

As stated above, the nuclear and cytoplasmic maturation in vivo takes place in the presence of preovulatory follicular fluid. Consequently, this study was designed to evaluate the developmental competence to the blastocyst stage of bovine oocytes matured in vitro in undiluted bovine follicular fluids of different origin: (i) mixed follicular fluid (FF) from a pool of 3 to 15 mm follicles from abattoir ovaries, (ii) preovulatory follicular fluid (POF) from the dominant follicle from a non-stimulated normally cycling heifer, (iii) preovulatory follicular fluid (OPU) from synchronised FSH-treated heifers 60 h after prostaglandin and 20 h after GnRH treatment, and (iv) the control oocytes were matured in TCM-199 with 5% serum. The experimental groups were chosen because it was shown that in vitro oocyte maturation and further development would be compromised in mixed follicular fluid [21], while reports on the developmental capacity after IVM in preovulatory follicular fluid has never before been published.

2. Materials and methods

2.1. In vitro embryo production

The protocol for the in vitro procedures has been described in details earlier [24,25]. Unless otherwise indicated media and chemicals were from Sigma (Sigma–Aldrich

Denmark A/S, Vallensbaek, Denmark), plastic ware was Nunclon quality (Nunc, Roskilde, Denmark) and media were prepared with MilliQ water (MilliRO Plus and MilliQ PF Plus Water Purifications Systems, from Millipore A/S, Hedehusene, Denmark).

The ovaries, originating mainly from culled Danish dairy cows and heifers, were collected from a local slaughterhouse (NV-OX, Slagelse, Denmark) and transported in thermo boxes in sterile saline to the laboratory. The temperature from ovary collection throughout all manipulations outside the incubator was kept between 32 and 35 °C.

Antral follicles with surface diameters between 3 and 15 mm were evacuated by use of a vacuum pump (KNF Neuberger N86 KN.18, VWR International, Copenhagen, Denmark) connected with an 18-G short bevelled needle (18-G × 1.5, 1.2 × 40, Terumo Neolus Luer) into a 50-ml tube containing 1 ml hepes-buffered TCM-199 wash medium (M 2520) with 20 U/ml heparin to prevent clotting. After retrieval and wash, the COCs were washed once in IVM medium before being transferred to the same medium for in vitro maturation for 22.5 to 24 h at 38.8 °C in 5% CO_2 in humidified air. The IVM medium was hepes-buffered TCM-199 (M 2520) with 2 U/ml PMSG-hCG (Suigonan Vet®, Intervet Scandinavia, Skovlunde, Denmark), 0.2 mM pyruvate (P 3662), 50 ng/ml EGF (E 4127), 50 μg/ml gentamycine (G 1264) and 5% estrous cow serum (ECS; produced by the Danish Veterinary Institute, Copenhagen, Denmark). Usually, 50–60 COCs would be incubated in 0.7 ml IVM medium in a 4-well dish without oil overlay, but because only a limited amount of follicular fluid was available, the COCs concentration was around 95 in 0.5 ml medium or follicular fluid. In our system, this is known to lower the blastocyst rates from 31 ± 5 to 24 ± 8%; however, the blastocyst rates would still be reasonable.

After IVM, the cumulus expansion and viscoelasticity were routinely scored on an arbitrary scale from 0 to 3. Then, the COCs were co-incubated for 20 to 22 h with frozen–thawed washed semen from one bull (Bubka, RDM 34572) from Taurus A.I. Station (Aalborg, Denmark) at 38.8 °C in 5% CO_2 in humidified air. COCs (50–60) were placed in 0.5 ml TALP medium [26] without oil overlay and washed spermatozoa were added at a final concentration of 2.5×10^6/ml. The TALP medium contained 25 mM sodium bicarbonate, 6 mg/ml BSA (A 4919), 10 mM lactate (L 4263), 0.2 mM pyruvate (P 3662), 30 μg/ml heparin (H 3149), 50 μg/ml gentamycine (G 1264) and PHE (20 μM penicillamine (P 4875), 10 μM hypotaurine (H 1384), 1 μM epinephrine (E 4250)).

After IVF, cumulus cells and excess spermatozoa were removed by vortex agitation for 1.5 min. After wash, the inseminated oocytes were transferred to 0.1 ml IVC droplets of B2 medium, supplied with 10% ECS (Upgraded B2 INRA Medium, Laboratoire C.C.D., Paris, France). The B2 droplets contained a 1:200 (v/v) suspension of bovine oviduct epithelial cells, prepared from oviducts on the day of ovary collection, and added to the B2 droplets the day before start of IVC. The embryos were cultured until Day 8 post-insemination (pi) at 38.8 °C in 5% CO_2 in humidified air. Embryo development was evaluated at Days 2 and 8 pi for cleavage and blastocyst formation. The results were expressed per inseminated oocyte placed into IVC.

2.2. Fixation and evaluation of oocytes/zygotes

Before fixation, all cumulus cells were removed from the IVM-oocytes or -zygotes by vortex agitation in 1 ml wash medium for 5 or 1.5 min, respectively. The oocytes/zygotes

were mounted on a slide between two vaseline stripes and a coverslip, fixed in a mixture of methanol and glacial acetic acid (3:1, v/v), left for 1–3 days at room temperature, stained for 2 min with 1% orcein (O 7380) in 40% acetic acid and then evaluated for stage of nuclear maturation or pronucleus formation by bright field or phase contrast microscopy (magnification: 400×). The stages of nuclear maturation [27–29] were assessed as germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase I (MI), anaphase I and telophase I (AITI) or metaphase II (MII). The pronuclear (PN) stages [30] were assessed as 0 PN (unfertilised), 1 PN (unfertilised), 2 PN (normal fertilisation) and >2 PN (polyspermy).

2.3. Collection and preparation of follicular fluid

2.3.1. FF

Mixed follicular fluid was retrieved from slaughterhouse ovaries. Follicles with diameters between 3 and 15 mm were evacuated by aspiration; the pooled follicular fluid was then centrifuged at 4000 rpm, the supernatant aliquoted and stored at −20 °C, until use. All experiments were performed with the same batch.

2.3.2. POF

Preovulatory follicular fluid was retrieved by ultrasound-guided aspiration from a preovulatory follicle from a single non-stimulated normally cycling heifer, which was monitored for follicle growth, corpus luteum size and physical signs of heat. The follicular fluid was collected from the dominant follicle over three cycles. The follicles sizes were 18 mm × 15 mm, 18 mm × 15 mm and 20 mm × 10 mm, respectively. The follicular fluid was centrifuged at 4000 rpm after each collection and the supernatant stored frozen at −18 °C. After the last collection, the frozen follicular fluid was thawed, pooled, and aliquoted and stored at −20 °C, until use.

2.3.3. OPU

Preovulatory follicular fluid from stimulated heifers was retrieved in connection with ovum-pick up using a standard protocol [31]. Briefly, six heifers were synchronised by intravaginal placement of CIDR progesterone-releasing and CIDIROL oestradiol benzoate devices for 8 days; after removal, 2 ml of prostaglandin $F_{2\alpha}$ analogue (PG; Estrumate® Vet, Shering-Plough) was injected i.m. Ten days after heat, the dominant follicle was aspirated. Then, the heifers were superstimulated for 5 days with two daily doses i.m. of 1.5 ml porcine pituitary FSH (Folltropin®). On the third day of FSH treatment, 2 ml of PG was injected, followed by 3 ml of GnRH (Receptal® Vet, Hoechst Roussel Vet) on day 5 of FSH treatment. The follicular fluid was collected 60 h after PG and 20 h after GnRH treatment. Finally, it was pooled, centrifuged at 4000 rpm, the supernatant aliquoted and stored at −20 °C, until use.

3. Experimental design

3.1. Experiment 1: developmental capacity of oocytes matured in follicular fluid

The COCs were divided equally and randomly to one of the four IVM groups: TCM-199 or undiluted FF, POF or OPU follicular fluid. After IVM, the degree of cumulus expansion

and viscoelasticity were evaluated and samples of COC removed for MII fixation, while the rest were inseminated. After IVF, samples of inseminated oocytes were removed for PN fixation, while the rest were placed into IVC. Because highly viscous cumulus masses were invariably observed in the follicular fluid groups after IVM, and because decreased sperm penetration was noticed in these groups, in one replicate samples of matured COCs were subjected to hyaluronidase and pronase digestion to test whether a mechanical hindrance or zona hardening could offer an explanation.

3.2. Digestion with hyaluronidase or pronase

The matrix of expanded cumulus cells, which is rich in hyaluronic acid, can be digested with hyaluronidase, while the zona pellucida can be digested with pronase.

The hyaluronidase (H 4272) was from bovine testes with an activity of 750–1500 U/mg. The pronase (P 5147), a type XIV bacterial protease (EC 3.4.24.31), was non-specific proteases with an activity of 4 U/mg. Working solutions were prepared in protein-free hepes-TL medium [32]. The hyaluronidase concentration was 1 mg/ml, the pronase concentration 5 mg/ml. Until use, they were stored at −20 °C. For digestion, one vial of each enzyme per experimental group were thawed and heated to 33 °C; then the COCs were added and watched every 30 s for the progression of digestion of either intercellular matrix or zona pellucida.

3.3. Experiment 2: developmental capacity of oocytes matured in follicular fluids and removal of the cumulus cells before IVF

Oocytes were in vitro matured in TCM-199, FF, POF or OPU follicular fluid, and samples were removed for MII fixation as in Experiment 1. Then, the cumulus cells were removed mechanically by vortex agitation before IVF. After IVF, samples of oocytes were removed for PN fixation, while the remaining inseminated oocytes were placed into IVC and allowed to develop as in Experiment 1.

3.4. Experiment 3: developmental capacity of oocytes matured in follicular fluids supplied with heparin and its effects on cumulus expansion and viscoelasticity

All IVM media (TCM-199, FF, POF and OPU) were supplied with 0.1 mg/ml heparin (H 3119) before initiation of IVM. After IVM, samples of oocytes were removed for MII fixation, while the remainder underwent IVF, PN fixation and IVC as described in Experiment 1.

3.5. Experiment 4: developmental capacity of oocytes matured in TCM-199 with or without heparin

To assess whether addition of heparin would affect the in vitro maturation and embryo development, IVM was performed in TCM-199 with or without the presence of 0.1 mg/ml heparin. MII and PN fixation, IVF and IVC were as described in Experiment 1.

4. Statistics

Sample means and standard deviations (mean ± S.D.) were calculated per replicate for each of the developmental stages: MII, <2 PN, 2 PN, >2 PN, CL and BL in the four maturation groups: TCM, FF, POF and OPU. The data were analysed by one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparisons test; the level of significance was $P < 0.05$. The effects of the different treatments within each individual maturation group (Experiments 1, 2 or 3) were analysed by Fishers exact test by pair wise comparisons. With three treatment groups, it is possible to make three pair wise comparisons among the sample means. In order to avoid errors of inference by this method, each test can be performed at a more stringent level. In our case, the level of significance was chosen to be $P < 0.02$ instead of $P < 0.05$. The software program GraphPad InStat was used for the statistical calculations (Version 3.00 for Windows NT, GraphPad Software, San Diego, CA).

5. Results

The combined results (Experiments 1, 2 and 3) are shown in Table 1. The apparent differences in percentages in Table 1 were due to the fact that the means were calculated per replicate while the numbers were representing the total over replicates. Nuclear maturation in mixed undiluted follicular fluid (FF: 41 ± 15%) was significantly lower than in the undiluted preovulatory follicular fluids (POF: 69 ± 23%, OPU: 72 ± 21%) and also lower than in the TCM-199 (69 ± 18%), whereas the three latter groups did not differ significantly. Normal fertilisation was highest in the TCM-199 group (56 ± 11%), although not significantly different from the values in the FF (41 ± 23%) and POF (47 ± 17%), but different from the OPU (34 ± 14%); the three follicular fluid groups were, however, not significantly different from each other. Although the polyspermy rates were not significantly different for the TCM-199 and the follicular fluid groups, there seemed to be a

Table 1
Nuclear maturation and embryo development of bovine oocytes subsequent to IVM in TCM-199 or in undiluted bovine follicular fluids

	Replicates	TCM-199	FF	POF	OPU
MII	10	69 ± 18% (83/121)[a]	41 ± 15% (31/78)[b]	69 ± 23% (55/79)[a]	72 ± 21% (71/100)[a]
<2 PN	12	30 ± 17% (52/167)[a]	57 ± 23% (87/146)[b]	52 ± 17% (78/148)[b]	59 ± 14% (74/125)[b]
2 PN	12	56 ± 11% (93/167)[a]	41 ± 23% (56/146)[ac]	47 ± 17% (68/148)[ac]	34 ± 14% (43/125)[bc]
>2 PN	12	14 ± 17% (22/167)	2 ± 5% (3/146)	1 ± 3% (2/148)	7 ± 9% (8/125)
CL	7	64 ± 12% (274/422)[a]	39 ± 9% (120/296)[b]	38 ± 15% (135/336)[b]	23 ± 15% (67/249)[b]
BL	9	19 ± 11% (112/581)[a]	3 ± 3% (11/415)[b]	3 ± 3% (13/424)[b]	2 ± 2% (8/323)[b]

Results expressed as mean ± S.D. Means with different letters in superscript (a, b, c) within rows differ significantly ($P < 0.05$). FF: mixed follicular fluid from slaughterhouse ovaries. POF: in vivo aspirated preovulatory follicular fluid from the dominant follicle from a cyclic unstimulated heifer. OPU: in vivo aspirated preovulatory follicular fluid from synchronised and superovulated heifers. MII: metaphase II. PN: pronucleus. <2 PN: unfertilised. 2 PN: normal fertilisation. >2 PN: polyspermy. CL: Day 2 cleavage rates from inseminated oocytes. BL: Day 8 blastocyst rates from inseminated oocytes.

tendency towards lower polyspermy in the latter groups (14 ± 17% versus 2 ± 5%, 1 ± 3% and 7 ± 9%) while the proportion of unfertilised oocytes was significantly higher in the follicular fluid groups (FF: 57 ± 23%, POF: 52 ± 17%, OPU: 59 ± 14%) than in the TCM-199 group (30 ± 17%).

The cleavage rates in the follicular fluid groups FF (39 ± 9%), POF (38 ± 15%) and OPU (23 ± 15%) were not different from each other but very different from the TCM-199 group (64 ± 12%).

The blastocyst rates in the follicular fluid groups FF (3 ± 3%), POF (3 ± 3%) and OPU (2 ± 2%) were also very different from the TCM-199 group (19 ± 11%) but they were not different from each other.

The cumulus expansion after IVM was more pronounced in the TCM-199 (Grade 3) than in the follicular fluid groups (Grade 2), while the opposite was true for the viscoelasticity. The cumulus masses in the follicular fluid groups were embedded in a highly elastic web that looked like coagulated proteins. This made it extremely difficult to separate single oocytes and to remove cumulus cells after IVF by vortex agitation; it was also difficult to count the exact number of embedded oocytes placed into IVC, and they quickly degenerated to a point where no trace of them was left. This explains why the oocyte numbers in the follicular fluid groups appeared to be 25% lower than in the TCM-199 group.

When the cumulus cells were removed before IVF (Experiment 2; Tables 2–4), it did not change the fertilisation rates in any of the groups: TCM-199 (56% versus 58%), FF (42%

Table 2
Effects of the different experiments on the fertilisation rates (2 PN) of bovine oocytes in vitro matured in TCM-199 or in undiluted bovine follicular fluids

Experiment	Replicates	TCM-199	FF	POF	OPU
1	8	56% (58/104)	42% (40/95)[a]	47% (42/89)	36% (24/67)
2	2	58% (14/24)	55% (10/18)[a]	43% (10/23)	48% (11/23)
3	2	54% (21/39)	18% (6/33)[b]	44% (16/36)	23% (8/35)

Means with different letters in superscript (a, b) within columns differ significantly ($P < 0.02$). Experiment 1: standard IVM/IVF; Experiment 2: removal of cumulus cells before IVF; Experiment 3: presence of 0.1 mg/ml heparin during IVM. 2 PN: normal fertilisation. FF: mixed follicular fluid from slaughterhouse ovaries. POF: in vivo aspirated preovulatory follicular fluid from the dominant follicle from a cyclic unstimulated heifer. OPU: in vivo aspirated preovulatory follicular fluid from synchronised and superovulated heifers.

Table 3
Effects of the different experiments on the cleavage rates of bovine oocytes in vitro matured in TCM-199 or in undiluted bovine follicular fluids

Experiment	Replicates	TCM-199	FF	POF	OPU
1	3	70% (133/191)[a]	49% (58/119)[a]	50% (69/138)[a]	36% (34/94)[a]
2	2	56% (72/128)[b]	30% (16/53)[b]	33% (26/80)[b]	5% (2/40)[b]
3	2	67% (69/103)[ab]	37% (46/124)[ab]	34% (40/118)[b]	27% (31/115)[a]

Means with different letters in superscript (a, b) within columns differ significantly ($P < 0.02$). Experiment 1: standard IVM/IVF; Experiment 2: removal of cumulus cells before IVF; Experiment 3: presence of 0.1 mg/ml heparin during IVM. FF: mixed follicular fluid from slaughterhouse ovaries. POF: in vivo aspirated preovulatory follicular fluid from the dominant follicle from a cyclic unstimulated heifer. OPU: in vivo aspirated preovulatory follicular fluid from synchronised and superovulated heifers.

Table 4
Effects on the different experiments on the blastocyst rates of bovine oocytes in vitro matured in TCM-199 or in undiluted bovine follicular fluids

Experiment	Replicates	TCM-199	FF	POF	OPU
1	5	22% (78/350)[a]	4% (3/238)	4% (8/226)	4% (6/168)
2	2	9% (12/128)[b]	0% (0/53)	3% (2/80)	0% (0/40)
3	2	21% (22/103)[a]	2% (2/124)	3% (3/118)	2% (2/115)

Means with different letters in superscript (a, b) within columns differ significantly ($P < 0.02$). Experiment 1: standard IVM/IVF; Experiment 2: removal of cumulus cells before IVF; Experiment 3: presence of 0.1 mg/ml heparin during IVM. FF: mixed follicular fluid from slaughterhouse ovaries. POF: in vivo aspirated preovulatory follicular fluid from the dominant follicle from a cyclic unstimulated heifer. OPU: in vivo aspirated preovulatory follicular fluid from synchronised and superovulated heifers.

versus 55%), POF (47% versus 43%), OPU (36% versus 48%), while the cleavage rates were significantly reduced in all of the groups: TCM-199 (70% versus 56%), FF (49% versus 30%), POF (50% versus 33%) and OPU (36% versus 5%). The blastocyst rates in the TCM-199 group were significantly reduced (22% versus 9%), while they were already at the lowest possible level in the follicular fluid groups: FF (4% versus 0%), POF (4% versus 3%) and OPU (4% versus 0%).

When heparin was present during maturation (Experiment 3), no coagulation was noted around the cumulus masses after IVM (Tables 2–4). It did not affect the blastocyst rates in the TCM-199 group (22% versus 21%) and it did not improve the developmental outcome (fertilisation, cleavage or blastocyst rates) in any of the follicular fluid groups either.

The presence of heparin during maturation in TCM-199 (Experiment 4) had no effect on embryo development (Table 5). Cleavage and blastocyst rates without or with heparin were 84% versus 81%, and 30% versus 31%, respectively.

When in vitro matured COCs were subjected to hyaluronidase digestion (Experiment 1), all cumulus cells except for the corona radiate cells were dispersed after 3 min in the TCM-199 group. In the follicular fluid groups, the intercellular matrix was digested after 4 min from around half of the oocytes while cumulus digestion from the remaining oocytes were unaffected with no progression during a 45-min observation period.

When in vitro matured COCs were subjected to pronase digestion (Experiment 1), the zona pellucida was completely dissolved after 4 min in all groups. In the follicular groups, the intercellular matrix was not affected at all by the pronase treatment during 45 min of observation while the cumulus masses were somewhat affected in the control group.

Table 5
Effect of 0.1 mg/ml heparin during IVM in TCM-199 on nuclear maturation and embryo development

IVM	COC/0.5 ml	MII	2 PN	>2 PN	CL	BL
TCM-199 without heparin	36	76% (31/41)	85% (33/39)	10% (4/39)	84% (261/310)	30% (92/310)
TCM-199 with heparin	36	90% (37/41)	77% (30/39)	3% (1/39)	81% (248/306)	31% (94/306)

Based on two replicates. MII: metaphase II. 2 PN: normal fertilisation. >2 PN: polyspermy. CL: cleavage rate. BL: Day 8 blastocyst rate.

6. Discussion

This study showed that very poor blastocyst rates were obtained after IVM in undiluted follicular fluid, meaning that the MII rates were not predictive for subsequent fertilisation and further embryo development.

A high rate of unfertilised oocytes and a low rate of polyspermy were typical for the follicular fluid groups. It was possible that the solid web of deposits in the expanded cumulus cells could have created a mechanical barrier to sperm penetration. Removal of the cumulus cells before IVF did, however, not change anything, except for lowering the developmental potential in the control group. Neither did premature zona hardening cause the poor development, since no difference was detected in zona pellucida digestion of the follicular groups and the control. Zona hardening occurs as a natural process after fertilisation to prevent polyspermy [33–35]. It is induced by the proteases released from the cortical granules, and results in biochemical and structural changes of the zona. Premature zona hardening has been reported to happen in human [36], rat [37,38], mouse [39], equine [40] and porcine [41] oocytes, especially if they are cultured under serum-free conditions [42,43], subjected to cryoprotectants [44] or parthenogenetically activated [45].

Analysis of human [46,47], equine [48,49] and bovine [50] follicular fluids have shown the presence of coagulant, inhibitory and fibrinolytic activity. It means that like blood, follicular fluid is a part of the haemostatic cascade system with creation of a fibrin clot as the end point [51–53]. The fact that heparin could prevent formation of deposits but not dissolve already formed deposits supported the belief that the material embedded in the cumulus masses was most likely fibrin and that the coagulation system had been activated locally around the cumulus masses.

How could the very poor embryo development after IVM in undiluted follicular fluids be explained? Was it because follicular fluid per se is not important for final oocyte maturation in vivo and that proper maturation mainly depends on cell-to-cell communications? Or was it due to a suboptimal oocyte/follicular fluid volume during maturation, asynchrony between the follicular fluid composition and the oocyte, instability of important molecules in the follicular fluid caused by the preparation and freezing, protein denaturation and ammonia formation, or just due to a general mishmash of the in vivo and in vitro maturation systems?

Regarding volume, under in vivo conditions, one oocyte at a time is matured in large quantities ($\sim$1 ml) of follicular fluid. Because of limited amounts of preovulatory follicular fluid, it was not possible to test if this would also be a requirement for the in vitro maturation. In our standard system using TCM-199, the optimal IVM-concentration was around 80 COC/ml while very poor blastocyst development was seen when 1–10 COC/ml were used.

The protein concentration in follicular fluid is of the same order of magnitude as in serum, meaning that oocytes tolerate a microenvironment high in protein. Nevertheless, protein denaturation and formation of ammonia from amino acids could perhaps reach levels toxic to the oocytes during IVM. In the study by Hammon and co-workers [54,55], it was showed that the ammonia concentrations decreased as the follicles became larger, and that the developmental capacity of oocytes exposed to follicular fluid levels of ammonium chloride during IVM, was not compromised.

In vivo and in vitro maturation differ in several ways, basically one is dynamic the other static; besides it has been suggested from studies of meiosis activating sterols (MAS) that at

the MAS-induced maturation pathways are different from the spontaneous in vitro maturation [56–58], although the role of MAS as the primary physiological trigger for meiotic resumption in vivo still needs to be validated [59].

Until the COC is ovulated, it is connected to the follicular wall by a stalk of cells meaning that the oocyte interacts dynamically with the vascular bed and the granulosa cells [60,61]. In vivo, resumption of meiosis is triggered by the LH surge [62–64] while mechanical removal of the oocyte initiates in vitro nuclear maturation [65,66].

In an experiment by Goudet et al. [67], immature equine oocytes were transferred into preovulatory follicles of donor mares and allowed to mature for 34 h, until collection. From the follicles where the original oocyte had reached MII, 38% of the transferred oocytes had also reached the MII stage, which was comparable to the in vitro maturation rates in this species. In this experiment, the oocytes were thus contained in the right volume, the right medium, the right dynamic system and kept at the right temperature but still the results were not comparable to those of the indigenous oocytes. It suggests that it may be the removal of the COC from the follicular wall that triggers the critical point of no return.

In conclusion, regardless of the high MII rates after IVM in preovulatory follicular fluid, reduced sperm penetration and very low blastocyst rates were attained subsequent to IVF and IVC. Since this could not be explained by zona hardening or protein deposits in the cumulus masses, the most likely explanations would be a compromised cytoplasmic maturation and/or modifications of the zona pellucida.

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