Oocyte maturation

Basic and clinical aspects of in vitro maturation (IVM) with special emphasis of the role of FF-MAS

Christian Grøndahl

This review has been accepted as a thesis together with fourteen previously published papers, by the University of Copenhagen, August 14, and defended on November 21, 2007.

Hagedorn Research Institute, Gentofte, Denmark.

Correspondence: Søndersøvej 41, 3500 Værløse, Denmark.

E-mail: chgr@novonordisk.com

Official opponents: Flemming Skovby, Anne Grethe Byskov, and Alex Tsafriri, Israel.

Dan Med Bull 2008;55:1-16

2. ABSTRACT

In Vitro maturation (IVM) of human oocytes is an emerging infertility treatment with great promise. To be successful this future assisted reproductive technology must entail both nuclear and cytoplasmic maturation of the oocytes and give rise to human embryos that have the same developmental potential as embryos resulting from the golden standard of human IVF.

The aspiration of immature oocytes from small to medium size antral follicles followed by their maturation In Vitro present an attractive alternative to the hormonal stimulation of patients in In Vitro fertilization (IVF) treatment, since administration of exogenous hormones is a costly treatment and may cause severe health problems. Of the long list of side effect and health concern ovarian hyper-stimulation syndrome (OHSS) is by far the most severe although long term effect on cancer prevalence is another concern. Another potential group of patients that could benefit from future IVM treatments are the young women undergoing anticancer therapy (radiation- or chemotherapy). Thus, ovarian and oocyte cryopreservation techniques are emerging, however such treatments can only be fully realized when IVM becomes an efficient means of obtaining healthy birth. At present, the In Vitro maturation techniques are highly successful in mice, variable successful in domestic species and still regarded experimental in the human clinic due to suboptimal fertilization rates and embryo quality. This review discusses comparative studies of the processes of oocyte maturation In Vivo and In Vitro, in various mammalian species including human.

Among the substances that have been reported to influence oocyte maturation there is an interesting endogenous signaling molecule: FF-MAS (4,4-dimethyl-5α-cholest-8,14,24-trien-3β-ol), an intermediate in the cholesterol biosynthetic pathway present in all cells. This review gives special focus to FF-MAS, the effect seen in animal and human studies so far and its potential use in treatment of human infertility is being discussed, including both the safety and efficacy issues that need to be addressed. It is being reviewed how FF-MAS and related MAS analogues by our group and other scientific groups have been observed to mediate a dose-dependant response on both the nuclear maturation and especially the cytoplasmic maturation during oocyte maturation In Vitro thus giving rise to pre-embryos of higher developmental potential. Studies are reviewed regarding the family of meiosis activating sterols, its In Vivo regulation by gonadotropins (especially LH) and suggestions to the signaling pathways as the putative MAS receptor eliciting the important cytoplasmic maturation signaling cascade that involves mos/MAP kinase. The pharmacological effect of synthetic FF-MAS has been observed in various models and species, including murine, porcine and humane oocytes. Finally, the chromosome status of IVM human oocytes has been the focus of a large prospective clinical trial, documenting that FF-MAS acting on human oocytes during In Vitro maturation presents a safe procedure evaluated on numerical chromosome aberration rates in metaphase-II oocytes.

In conclusion the In Vitro maturation of human oocytes is already now a valuable clinical treatment alternative for a subset of infertile patients, especially the Polycystic Ovarian Syndrome (PCOS) patients. IVM has the promise of being tomorrow's gold standard in treatment of human infertility if most of the important components of oocyte maturation are understood and can be adequately addressed In Vitro. Considering the present low frequency of successful fertilization and pre-implantation development following In Vitro maturation of human oocytes, the addition of FF-MAS or MAS analogues to the maturation medium to improve the cytoplasmic maturation and to yield higher quality pre-embryos may prove highly beneficial.

3. THESIS TOPICS, FOCUS AND OBJECTIVES

This review deals with both basic and applied aspects of oocytes maturation.

In human infertility the golden treatment standard of In Vitro Fertilization (IVF) involves ultrasound guided transvaginal aspiration of cumulus-oocytes-complexes (COCs) that have been In Vivo matured using gonadotropins.

An alternative approach to the collection of mature oocytes obtained after a combined GnRH analogue and gonadotropin treatment is the aspiration of immature cumulus-oocytes-complexes (COC) in the first week of the menstrual cycle from smaller follicles and the completion of nuclear and cytoplasmic maturation in the culture dish (for review see Smitz et al. 2004).

A group from Cornell University IVF, N.Y. was the first to report on the successful maturation and fertilization of immature human oocytes in their IVF program (Veeck et al. 1983). Clinical pregnancies from oocytes extracted out of small follicles from human ovaries submitted for pathological examination were first described by Cha and co-workers (Cha et al. 1991; Cha and Chian, 1998). The clinical aspects of IVM were pioneered by the group of Trounson and Wood at Monash University, Australia, with a series of In Vitro Maturation (IVM) treatments performed without ovarian stimulation in patients at risk for ovarian hyper stimulation syndrome. Unfortunately, the clinical pregnancy rate was too low to become clinically viable as first line treatment (Trounson et al. 1994a). Recently, however, even with modest success rates, IVM has been proposed as first line treatment in combination with natural-cycle IVF (Chian et al. 2004b).

The safety of the IVM technique, in terms of the normality and development of the resulting children, seems to be comparable with IVF/ICSI babies although the reports are still based on fairly modest numbers (Nagy et al. 1996; Mikkelsen et al. 1999; Mikkelsen, 2005).

Actual scientific reports suggest that this new technique remains challenging and that in many areas the standard operating procedures have still to be optimized (Jaroudi et al. 1999; Papanikolaou et al. 2005). Among the many unresolved issues are: the necessity to pre-treat patients with gonadotropins, the target range of follicle diameters to aspirate, the aspiration pressure for oocyte collection, the maturation medium composition, the optimal time for maturation, the time for insemination or sperm injection, and the steroid substitution regimen to prepare a receptive endometrium in the transfer cycle.

After an IVM procedure, nuclear maturation rate, fertilization and cleavage rates are generally acceptable, but the further developmental capacity or the cytogenetics of these pre-embryos appear often to be compromised (Nogueira et al. 2000). IVM embryos have an overall chance of implantation between 7 and 12% upon 2-8 cell transfers (Chian, 2003; Lin et al. 2003). To compensate for this poor

embryo quality some IVM groups transferred 3 or more embryos to achieve acceptable pregnancy rates (Chian et al. 2004a, 2004b).

Another strategy to improve embryo quality is to design a culture medium for IVM that can compensate for the reduced maturation time (24 to 36 hours) that oocytes undergo spontaneously when aspirated out of the follicles compared to the situation where they would remain within the ovary (typically more than 48 hours). Designing the best In Vitro maturation conditions to allow for developmentally competent embryos has been a long lasting concern. Studies on requirements for maturation In Vitro have been performed in the mouse (Biggers et al. 1967; Brower et al. 1982; Eppig et al. 2000), in the bovine (Bavister et al. 1992; Brackett and Zuelke, 1993; Cetica et al. 2002) and in the rhesus monkey (Schramm et al. 2003). In the rhesus monkey IVM was observed to cause development failure by impairment of the embryonic genome activation. Very few studies in IVM have been conducted in humans due to the scarcity of human material (see review by Trounson et al. 2001). Consequently, the choice of culture medium by human embryology laboratories has been based on the reports of animal IVM experiments (Wright and Bondioli, 1981; Weston et al. 1996). Hence comparative studies of In Vivo and In Vitro maturation and In Vivo fertilization, IVM, ICSI and IVC in both animal species and man are discussed in this review.

Among the many substances that have been reported to influence oocyte maturation there is a promising endogenous signaling molecule: FF-MAS (4,4-dimethyl-5α-cholest-8,14,24-trien-3β-ol), an intermediate in the cholesterol biosynthetic pathway present in all cells. First discovered in 1995 by Byskov and co-workers this FF-MAS molecule whether naturally extracted or pharmaceutically synthesized has been shown to trigger the resumption of meiosis (i.e. the nuclear maturation) of mouse oocytes In Vitro (Byskov et al., 1995, Grøndahl et al. 1998, IV). The compound was capable of initiating meiosis in isolated naked or cumulus cell-enclosed oocytes from several mammalian species (Byskov et al. 1995, Hegele-Hartung et al., 1999).

Studies suggest that FF-MAS is under gonadotropin regulation. However, it remains to be fully clarified which gonadotropins physiologically mediate endogenous FF-MAS production. A core theme of this review is to explore and discuss the pharmacological effects of FF-MAS on meiosis. Progressively during our activities with FF-MAS and IVM our specific contribution has been to focus on dissecting FF-MAS' effect specifically on cytoplasmic maturation and the subsequent embryo quality in various models and species including human, effects that were not obvious in the original discovery of FF-MAS.

Hence the objectives of this review can be summarized to the following:

To advance the basic understanding of IVM by comparative studies in the equine and porcine species where only limited original studies have been performed, to study IVM in both outbreed and inbred mice as model for human fertility and ultimately perform direct clinical studies on human oocytes. Furthermore, it has been the objective to investigate methods of preparing both synthetic FF-MAS as well as the pharmaceutical optimization of synthetic analogue MAS ligands. In addition the objective has been to document any pharmacological effect and physiological role of MAS sterols on the process of oocyte maturation. It has been the objective to document the meiosis activating effect in various models and more specifically to investigate and document the pronounced effect on cytoplasmic maturation and the subsequent effect on embryo quality and development potential. Finally, it has been the objective to document the safety aspects of applying FF-MAS to human oocytes in culture and to optimize both the dosage and the exposure time in clinical and pre-clinical studies.

The overall vision behind this research being that IVM in the future could present a milder, more affordable, patient friendly future infertility treatment without compromising safety of mother or child or at the expenses of reduced treatment outcome; and finally to contribute to the uncovering of whether FF-MAS is a part of the physiological regulation of mammalian oocyte maturation.

4. INTRODUCTION TO MEIOSIS, OOCYTES MATURATION AND FF-MAS

Meiosis is the unique cell division-process that produce gametes – two cell divisions with no intervening DNA replication resulting in halving of the chromosome complement – a common theme that is highly conserved throughout evolution. The unique aim of meiosis is two-fold: 1) to create haploid gametes; and 2) to allow for genetic recombination and thus the generation of genetically unique gametes.

The details between male and female gametogenesis are remarkably different. The mammalian oocyte begins meiosis during fetal life but does not complete meiosis I until ovulation and meiosis II is only completed upon fertilization. Male meiosis begins at puberty and runs as a continuous process through meiosis I and II in less than a week; however the final specialization to a full fertilizable and mobile gamete takes additional several weeks (Hunt and Hassold, 2002).

The first meiotic division in the oocyte is a particular important event step. If that division occurs correctly, then a normal egg (i.e. mature metaphase II oocyte) has a good change of being produced that can be fertilized and go on to normal development. If meiosis I division goes wrong the result is usually an error-prone embryo that either is unable to complete development or occasionally gives rise to individuals with chromosomal alterations. Other abnormalities may be encountered e.g. during the quiescent prophase I (leading to chromosome aberrations).

NUCLEAR MATURATION

Oocyte nuclear maturation comprises nuclear modifications that take place during resumption of meiosis producing a haploid chromosome complement from the previous diploid state. Oocytes are arrested at the prophase I displaying a visible nucleus referred to as germinal vesicle (GV). When meiosis resumes, the oocytes undergo germinal vesicle breakdown (GVBD) i.e., dissolution of the nuclear envelope. Chromatin condenses where then homologues chromosomes are paired aligning on the meiotic spindle at metaphase I. During anaphase and telophase bivalents separate as the homologous chromosomes separate and separation is complete when metaphase II occurs, recognizable by presence of 1st polar body (PB). Meiosis is completed following successful fertilization visualized as the presence of 2nd PB. The legal nomenclature in several countries defines the resulting zygote or pre-embryo formation strictly to when the 2 pronuclei dissolves (i.e., the chromosomes become localized centrally at syngamy or synkaryosis).

CYTOPLASMIC MATURATION

The regulatory signals for the molecular processes involved in oocyte growth and maturation are concerted during a sophisticated 2-way cell communication between the gamete and the surrounding somatic cells of the follicle in response to circulating levels of gonadotropins and based on a developmental program intrinsic to the oocyte. Mammalian oocyte maturation is a very complex process involving the resumption of the meiotic cell cycle as well as cytoplasmic modulations including organelle migration and molecular changes. The acquisition of developmental competence involves the synthesis and storage of a wide range of molecules during oocyte growth followed by reprogramming and timely utilization of these stored messages during final maturation, fertilization and early embryogenesis.

The endpoint of oocyte maturation In Vivo is the release from the follicle of a mature (metaphase II) gamete, that upon sperm penetration, activation and maternal-embryonic transition of genome activation can support normal embryonic and fetal development to term and the birth of live offspring. Cytoplasmic maturation is shortly described as the unity of processes modifying the oocyte cy-

toplasm that are essential for fertilization and pre-implantation embryonic developmental competence. It comprises production and presence of specific factors, the relocation of cytoplasmic organelles and post-transcriptional modifications of mRNAs accumulated during oogenesis. Thus some elements of cytoplasmic maturation can be visualized (e.g. the line-up of cortical granules) whereas many other elements of cytoplasmic maturation are molecular and very challenging to visualize or monitor. Ultimately the quality and completeness of cytoplasmic maturation can be viewed as the developmental potential of the resulting pre-emplantation embryo.

FF-MAS

Among the substances that have been reported to influence oocyte maturation there is a promising endogenous signaling molecule: FF-MAS (Follicle Fluid-Meiosis Activating sterol, 4,4-dimethyl-5α-cholest-8,14,24-trien-3β-ol), an intermediate in the cholesterol biosynthetic pathway present in all cells. FF-MAS has been known for some time to be able to trigger the resumption of meiosis in oocytes from mice In Vitro. This review gives special focus to FF-MAS, the effects seen in animal and human studies so far and its potential use in treatment of human infertility is being discussed, including both the safety and efficacy issues that need to be addressed.

5. MATERIAL AND METHODS

All of the 14 review papers but two (Paper I and II) investigate mammalian oocytes during in vitro culture.

Paper I is a comparative study of the process of oocyte maturation In Vivo thus being entirely descriptive of the physiological oocyte maturation process as a baseline in a seldom studied species (equine) both to document fundamental differences and to identify common themes to other domestic animals or human. Paper II is a descriptive study of the In Vivo fertilized equine embryo to document an important embryonic process the Maternal-Embryonic Transition (MET) for the first time in the equine species and describe the ultra structural changes associated with this development milestone.

All FF-MAS used in present studies are pharmaceutical grade quality produced by Medicinal Chemistry at Novo Nordisk A/S through chemical synthesis from Litocol Acid obtained from fermentation. FF-MAS has in all studies been formulated mainly in 0.1 or 0.2% ethanol-based formulations or alternatively in human serum albumin.

In the studies of nuclear maturation of mouse oocytes cultured in vitro the models have often involved inhibition of meiotic maturation by including Hypoxanthine (Hx) or dbcAMP in the culture medium in assays modified from Byskov et al. (1995). In the human studies or in the studies of cytoplasmic maturation in both outbreeds and inbreed mice models no inhibition of meiotic maturation has been attempted. All human studies have been made according to GXP and following approval of protocols and approved inform consents by the scientific-ethical committees of the respective countries and following the Helsinki declaration of clinical investigation in human subjects. No fertilization or embryo transfer of FF-MAS exposed human oocytes have been attempted in the studies comprised in this review. In all studies a 0.05 p-level of significance has been applied where peer-reviewed statistical analysis has been performed. For details of Material and Methods (M&M) reference is made to the specific thesis papers M&M sections. Some study specific details are also mentioned in the section 6A-J when these operational details are further discussed.

6. RESULTS AND CRITICAL ASSESSMENT OF CONCLUSIONS IN PAPERS I-XIV

A. ULTRASTRUCTURAL CHANGES AND ENDOCRINE DYNAMICS DURING MEIOTIC MATURATION IN VIVO IN THE EQUINE OOCYTES (PAPER I)

This study was undertaken to describe the ultrastructure and the endocrine dynamics of the equine maturing oocyte In Vivo. The In Vivo equine oocyte maturation has not previous been described in details presumably due to the difficulties associated with obtaining this material.

Thus, the ultrastructural characteristics of the resumption of meiosis In Vivo of equine oocytes was subject for our investigation and several unique findings have been documented for the equine species (Grøndahl, et al. 1995, I). Oocytes and follicular fluid was aspirated at different time-points throughout the follicle growth phase until very near ovulation to document the In Vivo maturation process. Oocytes were subjected to fine structure morphological investigation and follicle fluid to biochemical measurements. In summary, the final maturation of equine oocytes includes a series of well-defined nuclear changes (oocyte nucleus migration, dissolution of the nuclear envelope, extrusion of the first polar body) paralleled by defined cytoplasmic changes (disruption of cumulus-oocyte intermediate junctions and trafficking of mitochondria to a central position). Moreover, a massive build up of cortical granules was noted as a significant element of equine cytoplasmic maturation quite similar to what has been observed in human pre-ovulatory oocytes In Vivo (Sathananthan, 1994). In the equine follicle milieu a sharp rise in Progesterone (P4) was noted to be highly correlated with fully mature metaphase-II oocyte inside the follicle and a high degree of synchrony between nuclear and cytoplasmic markers of full maturity. The concentration of Estradiol-17-beta (E2) was observed to be constantly elevated throughout the period and did not drop prior to ovulation.

These findings are in contrast to the steroid content in pre-ovulatory follicles of sheep (Hay and Moor, 1975), cattle (Dieleman et al. 1983) and human (Lumsden et al. 1986) where marked declines in E2 are seen prior to ovulation. These findings could be important in the design of future composition of In Vitro culture media for equine and other species.

Fundamentally differences have been observed between mammalian species in the oocytes development, biochemistry and physiology (Zamboni et al. 1972; Dvoràk et al. 1982; Van Blerkom, 1989; 1991; Sathananthan, 1984; 1994), however, also many common themes have been identified. Of pivotal differences between mouse and human oocytes can be mentioned the paternal centrioles/centrisomes, which are inherited by the early embryo and actively participate in the mitotic cell cycle in human zygote which is quite unlike the mouse embryo (Sathananthan, 1994). Kinetics of oocyte maturation is also vastly different among species. Approximately 40% of rodent and bovine oocytes will resume meiosis within 1-2 hours as measured by visible initiation of nuclear envelope breakdown after release from the follicle (Anderiesz et al. 2000a), whereas human, equine and primate oocytes resume spontaneous meiosis at a very low rate and with a difference in kinetics compared to other species (e.g., the human egg has been reported to still be in GV stage after 24 hours of culture in sharp contrast to e.g. mouse oocytes) (Edwards, 1965; Trounson et al. 1994a; Grøndahl et al. 1995 (I); Grøndahl et al. 1997 (III); Grøndahl et al. 1998 (IV); Sathananthan, 1994; Anderiesz et al. 2000b; Grøndahl et al. 2000a (V)). A common theme however seems to be that the process of oocyte maturation In Vitro of mammalian oocytes once initiated progress faster than the physiological process In Vivo (as we have seen in both equine, mice and humans) which creates further requirements on the completeness of the cytoplasmatic processes In Vitro.

B. FERTILIZATION AND ACTIVATION OF THE EMBRYONIC GENOME IN EQUINE ZYGOTES (PAPER II)

These studies were undertaken to characterize, in the equine species, one of the most important pre-implantation embryo development phases namely the timing of the maternal-embryonic transition (MET).

Pre-implantation embryos were surgically obtained from mares where the insemination and ovulation timing were closely monitored. Subsequently, the pre-embryos were subjected to short In

Vitro culture (labelled Uridine culture) as well as to ultrastructural morphological investigation.

In summary, we have found in the equine In Vivo derived embryo from naturally cycling mares the maternal to embryonic transition was first established to occur in 6-8 cell embryos of the 4th cell cycle and clearly paralleled with significant ultrastructural features of the nucleolus apparatus turning from the inactive stage of nucleolus precursor bodies to fully RNA-synthesis active reticulated nucleoli (Grøndahl et al. 1993; Grøndahl and Hyttel, 1996, Paper II). In humans this transition has similarly been observed to occur between the 4- to 8-cell stage, thus considerable different from mice (Tesarik et al. 1986; Braude et al. 1988; Sathananthan, 1994).

The ability to progress through the natural transition of maternally controlled development to that of embryonic governance is acquired during oocyte maturation and early development. Fertilization of the mammalian oocytes results in completion of the meiotic process that has been initiated at the time of oocyte formation in fetal life. Mammalian pre-implantation embryogenesis is initially dependent upon maternally-inherited molecules during early development (Mclaren, 1981). Upon sperm activation of the ooplasm inactivation of MPF occurs allowing the cell cycle to progress through the arrest at metaphase-II. Subsequently, the important cortical reaction (release of sub-zonal cortical granules) takes place that ideally prevent the situation of polyspermia to happen (Szöllösi, 1967; Szöllösi and Hunter, 1973; Ducibella et al. 1990; 1993; Ducibella, 1996). The acquired ability of the oocyte to release Ca^{2+} in response to the fertilizing spermatozoa constitute an important competency step during oocyte maturation, for review see (Carroll et al. 1996). The frequency and amplitudes of calcium waves (and thereby the quality of the signal) are dependent upon the maturational stage of the oocyte and are determent for embryo quality (Swann and Lawrence, 1996; Herbert et al. 1997). As example the rate of calcium release in response to fertilization is four times greater in fully mature oocytes compared to maturing oocytes (Carroll et al. 1996).

The very important developmental check-point, transition from maternal to embryonic genome transcription (MET), occurs at species specific cell-cycle and is pivotally influenced by transcripts and factors during oocyte maturation and early embryonic development (Telford et al. 1990; Hwang et al. 2001). In the mouse there is evidence that a major burst of embryonic transcription occurs at the 2-cell stage and that these transcripts are essential for continued development (Kidder and McLachlin, 1985). Transcription may already occur at a very low rate from the 1- or 2-cell stage in other mammalian species as reported in cattle (Plante et al. 1994; Hay-Schmidt et al. 2001), however the major burst of transcription occurs later at species specific stages of development as reported in human (Tesarik et al. 1986), bovine (Camous et al. 1986; Grøndahl et al. 1990) and comparatively reviewed by Telford (Telford et al. 1990). Also in non-human primates (rhesus monkeys) MET has been identified to occur between the 4-8 cell-stage. In rhesus monkeys inadequate cytoplasmic maturation In Vitro has led to poor developmental potential and impairments of the embryonic genome activation (Schramm et al. 2003). Early cleavage arrest has been reported in most species studied including man and the need for adequate changes in protein transcripts around the time of maternal-embryonic transition has been firmly substantiated (Davis, 1985; Crosby et al. 1988; Frei et al. 1989; Heikinheimo et al. 1995; Moor et al. 1998).

Our findings in the equine species are the first to describe this process in this rarely studied species; findings that may well be important and used in designing the optimal culture conditions for embryo culture as well as guidance to timing of back-transfer to the uterus to best mitigate the risk of early cleavage arrest In Vitro.

C. ICSI FERTILIZATION OF EQUINE IVM OOCYTES (PAPER III)

This study was undertaken in order to improve and pioneer In Vitro fertilization methods of the equine oocytes since capacitation of the equine sperm has proven particular difficult hence normal IVF has been challenging in the horse and consequently the comparative knowledge in this species is limited.

Intracytoplasmic sperm injection (ICSI) was performed on equine oocytes matured In Vitro. Oocyte were aspirated from abattoir ovaries and matured In Vitro for 36 hours in TCM-199 medium according to method previously described by Grøndahl et al. (1995b).

In summary, we found that when employing ICSI to equine In Vitro matured oocytes it was noted that the pronuclear formation was obtained in a similar manner and with normal kinetics as observed In Vivo maturation and In Vivo fertilization (Grøndahl et al. 1993; Grøndahl et al. 1997, III). However, the oocyte activation was still incomplete and even though cleavage was obtained further embryonic development was largely compromised. This is thought to be a sign of inadequate quality M-II following the In Vitro maturation. In mice ICSI has been particularly troublesome (Markert, 1983; Ron-El et al. 1994) until Kimura and Yanagimachi (Kimura and Yanagimachi, 1995) reported a breakthrough using piezo-driven injection techniques highlighting that physical properties of the mouse oocyte make them leak after sperm injection.

Historically, the rapidly evolving techniques of IVF since the essential work of Steptoe and Edwards (Steptoe and Edwards, 1978) have been very successful in humans as well as moderate successful in several farm animals as reviewed by Trounson (Trounson et al. 1994a) and Edwards (Edwards, 1995). Intracytoplasmic injection of spermatozoa into the ooplasm of the oocyte (ICSI) has dramatically improved the chances of treatment of male infertility (Palermo et al. 1992; Van Steirteghem et al. 1993), despite the fact that this technique bypasses the normal gamete interactions like sperm-egg fusion and membrane hyper-polarisation. Nevertheless the ICSI injections spurs calcium oscillations to occur which is followed by activation of the egg leading to fairly high percentage of two-pronuclear eggs (Tesarik et al. 1994). This technique has had variable success in domestic species, cow (Younis et al. 1989; Goto et al. 1990), comparative studies in different species (Iritani, 1991) and equine (Squires et al. 1996). We contributed to the development of the ICSI technique in the equine species in the form of the present paper (III). Today ICSI is widely used in the human clinic and at some IVF centers up to 50% of procedures for infertile couples make use of ICSI. These techniques have noticeably never been through thorough safety investigations (e.g. chromosome soundness evaluation) before the widespread use in the human clinic and, consequently, the involved safety of the ICSI procedure and wellbeing of the resulting children has exclusively been and is still monitored as retrospective follow-up studies.

Only if the quality and success rates of IVM of equine oocytes dramatically improve it is likely that this ICSI technique will be of use for this variant of husbandry breeding.

D. PHARMACOLOGICAL EFFECT OF MAS STEROLS ON IN VITRO MATURATION (PAPERS IV & VIII)

This study was primarily undertaken to investigate the pharmacology of synthetic FF-MAS on murine oocytes and establish a classical dose-response curve as well as testing related oxysterols effect on oocyte maturation.

Follicular fluid meiosis-activating sterol (FF-MAS, 4,4-dimethyl-5α-cholest-8,14,24-trien-3β-ol) has been known for some time to be able to trigger the resumption of meiosis in oocytes from mice In Vitro (Byskov et al. 1995), however, the mechanism is largely unknown. FF-MAS (and the close metabolite, Testis meiosis-activating sterol, T-MAS) are intermediates in the cholesterol biosynthetic pathway and present in all cholesterol-producing cells (see Figure 1).

Since this original observation, numerous studies using follicle derived or synthetic FF-MAS have been carried out to observe FF-MAS effectiveness to induce oocyte maturation.

We found that FF-MAS in a dose-dependant manner can induce resumption of meiosis of murine oocytes cultured In Vitro under different meiosis inhibiting principles (Hypoxanthine, IBMX, db-cAMP) and could be consistent with a physiological role for FF-MAS in oocyte maturation (Grøndahl et al. 1998 (IV)).

Moreover we published in a separate study that the range of meiotic active endogenous molecules are far greater than just FF-MAS and T-MAS and seen in this illustration from Grøndahl et al. (2001). We identified the chemical structure of 8 endogenous MAS molecules (see Figure 2) that are approximately equipotent in micromolar activity in their capacity to induce nuclear maturation (i.e. meiosis) in naked mouse oocytes.

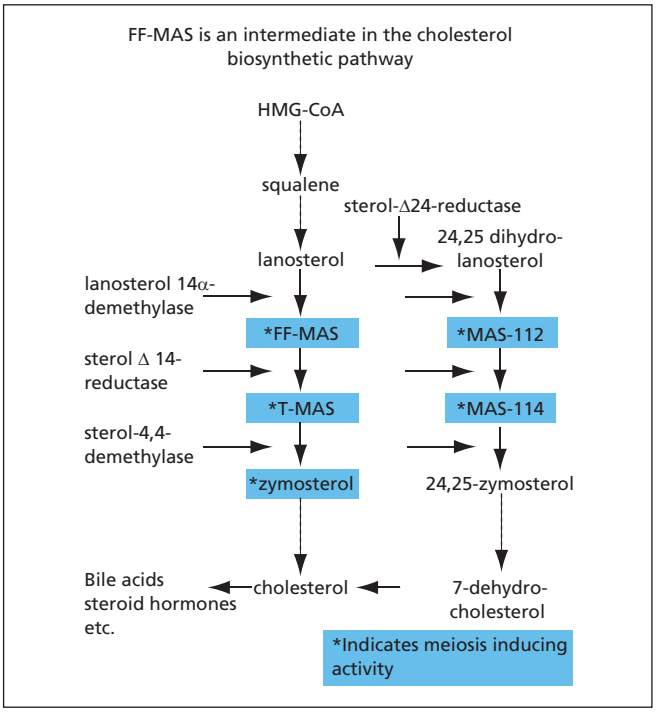

Figure 1. Pathways of FF-MAS and T-MAS synthesis In Vivo.

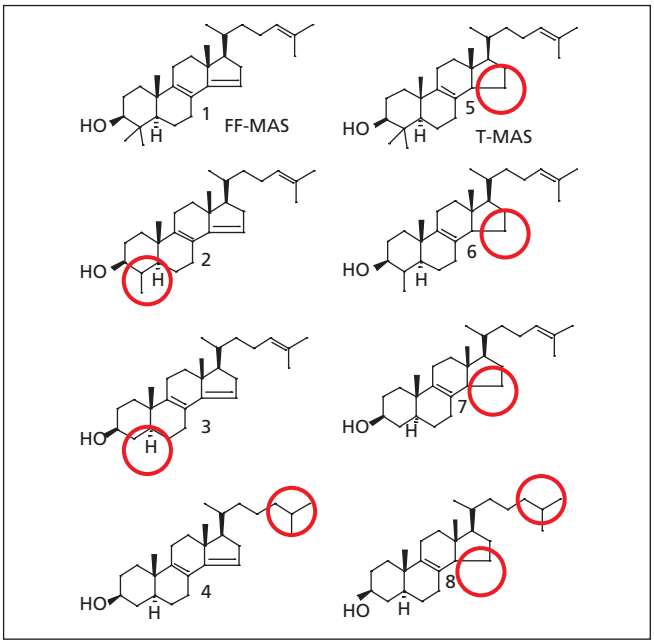

Figure 2. Endogenously isolated close metabolites of FF-MAS and T-MAS that have equipotent meiosis activating activity. The red circle illustrate the area in the molecule that differs from the closest metabolite (Modified from Grøndahl et al., 2001).

Other groups have investigated the pharmacological effects (i.e. physiological or supra physiological doses interacting In Vitro) of FF-MAS (Hegele-Hartung et al. 1999; Hegele-Hartung et al. 2001; Downs et al. 2001; Tsafriri et al. 2001; Griffin et al. 2004) and confirmed the nuclear maturation activity in the low micromolar range (1-10 micromolar). Initially it was hypothesized that the primary effect of MAS was that of the pivotal meiosis (i.e. nuclear maturation) inducing signal (Byskov et al. 1995; Byskov et al. 1998; Byskov et al. 1999; Andersen et al. 1999). Many groups have confirmed the effect of MAS sterols on oocyte nuclear maturation. Our group has later focused on FF-MAS effect on oocyte quality and several studies are pointing towards the hypothesis that MAS sterols are important molecules for cytoplasmic maturation and the quality of the oocyte and subsequent embryo potential (a discussion that will be further substantiated in later sections). Even though FF-MAS in several models has been shown to induce nuclear maturation it is still debated whether FF-MAS constitute a pivotal meiosis mediating signal downstream from gonadotropins (Downs et al. 2001; Baltsen, 2001; Tsafriri et al. 2002; Grøndahl et al. 2003, X). Therefore, the potential therapeutic value of MAS based therapeutic are foremost thought to be in the field of treatment of human infertility. Possible uses could arise in the potential field of novel non-hormonal contraception if the postulation of MAS being an obligatory molecule for meiosis can be further substantiated and safe and non-toxic MAS antagonist can be discovered. In conclusion, a proven pharmacological effect on meiosis has been observed both by us and other groups establishing 0.1 micromolar as non-effective dose, 0.7-1 micromolar as the lowest effective dose (see Figure 3) and 20 micromolar as the highest efficacy dose with doses of 50 or 100 micromolar being supra-physiological and approaching toxicity limits (Grøndahl et al.1998; unpublished data Grøndahl et al.).

These findings have assisted us and others to guide the dose range of synthetic FF-MAS used for subsequent clinical studies in humans considering 0.1 micromolar FF-MAS as an ineffective dose and the most interesting active dose-range to be 1-10 micromolar FF-MAS. Fifty or 100 micromolar has been observed to have negative effects on oocyte homeostasis and on meiosis (IV; VIII, XIII)

E. EFFECT OF FF-MAS ON HUMAN OOCYTES FROM WOMEN DIAGNOSED AS HAVING POLYCYSTIC OVARIAN SYNDROME (PCOS) (PAPER V)

This study was undertaken as a pilot clinical study to get important knowledge on how the human oocytes reacted to FF-MAS and to get some initial data on both kinetics and pharmacology of synthetic FF-MAS formulated in an ethanol-based formulation. Hence our group was the first to investigate and report the use of synthetic FF-MAS for improving human IVM with the aim to optimize culture design and media with regards to In Vitro culture of immature human oocytes aspirated from small antral follicles in patients diagnosed with polycystic ovarian syndrome (PCOS) (V). Numerous

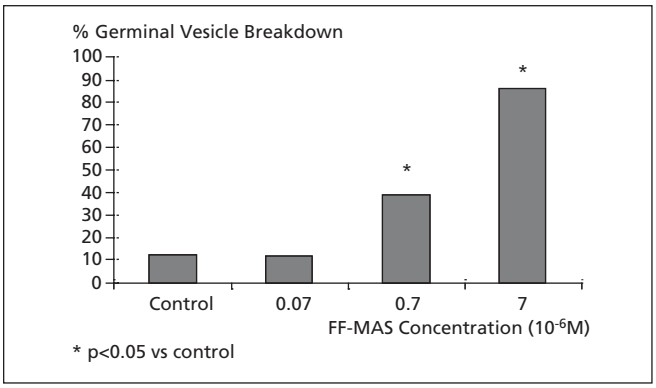

Figure 3. Pharmacological effect of synthetic FF-MAS in mouse oocytes cultured In Vitro (From Grøndahl et al., 1998, IV).

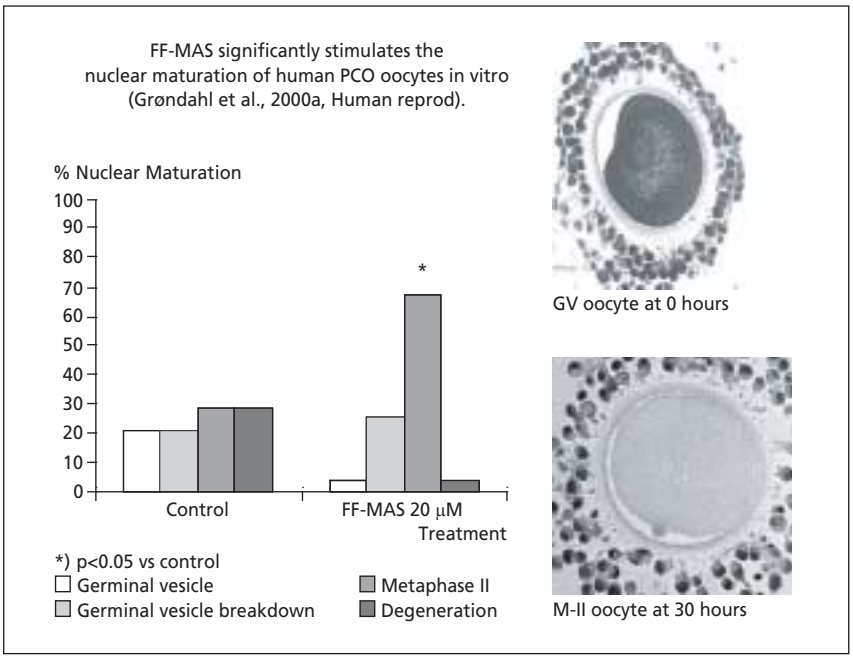

Figure 4. Effect of 20 micromolar FF-MAS on human oocytes cultured In Vitro. Illustration of immature human oocytes at the point of culture initiation and subsequent following 30 hours of culture. Histogram shows meiotic maturation following 30 hours of In Vitro culture (Modified from Grøndahl et al. 2000a, V).

groups have been reporting on successful IVM on PCOS patients, however still with low clinical efficiency (Trounson et al.1994b; Beckers et al. 1999; Chian et al. 1999; 2000; Cha et al. 2000; Chian et al. 2000, Mikkelsen and Lindenberg 2001; Lin et al. 2003).

In collaboration with a Danish IVF clinic that obtained approval from both the local (Copenhagen) and central (Danish) scientific-ethical committees to perform immature oocyte retrievals in patients diagnosed with polycystic ovarian syndrome (PCOS) the objective was formulated to optimize the culture conditions including exploring the use of synthetic FF-MAS. Importantly FF-MAS exposed oocytes were not attempted fertilized or otherwise being part of a clinical treatment of patients.

The patients were asked to give their written consent to donate (randomly chosen) half of the aspirated oocytes for the optimization of the culture protocol provided that 4 or more oocytes were isolated. The other half of the oocytes were used for the benefit of the patients pursuing pregnancy and remained outside the present study.

The immature follicles (sized 8-12 mm in diameter) were aspirated using a single barrel needle (COOK) typically on day 7-9 in the cycle and after the patients had received a low dose FSH priming for 3 days. The oocytes were cultured in a very simple and completely defined medium (TCM-199) without any supporting cells or serum, human serum albumin (hSA) being the only protein source added. Moreover, no FSH, LH or other hormones or growth factors were added – for two separate reasons: 1) the placebo control group should act as a true baseline of spontaneous oocyte maturation and 2) the exclusive effect of exposing with FF-MAS on nuclear maturation should be observed. Synthetic FF-MAS was made to high purity (> 99% pure) from Litocol acid as previously described (Murray et al. 2000) and formulated in final concentration of 0.2% v/v ethanol in TCM-199 medium. The control groups did also receive 0.2% v/v ethanol. Thirty-one treatment cycles were performed and 86 human oocytes were donated to enter the study and randomly assigned to 1 of 7 treatment protocols: 0 hour controls (fixed immediately upon isolation), 22 hours culture with or without FF-MAS (20 micromolar), 30 hours with or without FF-MAS (20 micromolar) and 40 hours with or without FF-MAS (20 micromolar), approximately 12 oocytes per group.

We observed that the culture time of 30 hours was superior to both 22 hours (majority of eggs still immature (GV) or maturing (M-I)) as well as 40 hours, irrespective of FF-MAS treatment (potentially 40 hours was too long culture period under our culture condition, since the degeneration rate (morphological criteria) was observed to increase).

Furthermore, we observed a significant beneficial effect of FF-MAS (20 micromolar) at 30 hours culture both on the frequency of M-II oocytes (29% in controls vs. 67% in FF-MAS treated) as well as apparent effect on degeneration (29% in controls vs. 4% in FF-MAS treated, see Figure 4). Caution should be applied in concluding on these results since the entire study was based on a small oocyte number (86 oocytes in total, divided into 7 groups). Moreover these were oocytes from PCOS patients and it is known that the endocrine background and the oocyte physiology are different vs. the more normal infertile patient population.

Forty hours of culture was seen in our study to be too long and clear signs of oocytes aging/degeneration were noted and all the oocytes had lost their cumulus cells.

In comparison several research groups have reported beneficial effect upon human oocyte nuclear maturation In Vitro by applying even longer culture times e.g. 48-74 hours, in media containing sera, pituitary hormones, growth factors, steroids or cells (Lefevre et al. 1987) (48 hours), (Prins et al. 1987) (36-74 hours), (Cha et al. 1991) (32-48 hours), (Trounson et al. 1994a) (21-54 hours), (Barnes et al. 1995, 1996) (36-46 hours), and (Russell et al. 1997) (52 hours).

One other investigation has been published on the effect of exposing human oocytes In Vitro to meiosis activating sterol, however MAS in the form of extracted and purified FF-MAS from human follicle fluid (Cavilla et al. 2001). The study population was a mixture of PCOS patients and patients from couples undergoing ICSI treatment for male infertility, and the patients responded quite different. For the ICSI patients FF-MAS (in 10 and 30 micromolar concentration) was seen to increase nuclear maturation (to M-II) similar to what Grøndahl et al. had observed with synthetic FF-MAS (concentration 20 micromolar, Grøndahl et al. 2000a, V)), albeit in a PCOS population. Cavilla et al. observed the oocytes from PCOS patients to benefit from 30 micromolar by higher survival rates (lower degeneration rates), but noticeably no significant impact on nuclear maturation to M-II completion was noted. Finally, an apparent tendency towards improved subsequent development in the FF-MAS treated groups following ICSI by sperm donor with proven fertility was noted as encouraging,

In summary, we found that oocytes from PCOS patients tolerated to be cultured in a very basic TCM-199 based medium without any hormones, sera or other additives except hSA as protein source and pyrovate as energy source, that 30 hours were the optimal culture

time to obtain M-II and that FF-MAS had beneficial effects towards nuclear maturation, cytoplasmic maturation and oocyte degeneration a knowledge we have utilized in subsequent studies. We have later adapted a more complex medium that contains a series of survival factors for the subsequent IVM trials necessary for culture of oocytes from non-PCOS patients which will be addressed later in section 6J (Smitz et al. 2007, XIV).

F. SIGNALING PATHWAYS OF FF-MAS IN MOUSE OOCYTES (PAPERS VI & VII)

These studies were undertaken to investigate the signaling pathways of FF-MAS, to corroborate or disprove the hypothesis that FF-MAS is part of the physiological maturation signal downstream of FSH and LH surges and to reveal any potential ligand-receptor mechanism.

We have hypothesized that FF-MAS and related endogenous sterols could act as a secondary messenger downstream from the gonadotropins surge inducing signals in mammals involved in meiosis signaling and oocyte maturation. This effect could be on the nuclear character (chromatin condensation etc) and on the cytoplasmic maturation (biochemical, MPF activation etc) as well as on processes interlinking nuclear and cytoplasmic maturation e.g. spindle assembly. Different line of evidence has been generated to support the existence of a receptor action in the induced-maturation pathway.

The FF-MAS induced maturation has been found to be pivotally dependent upon protein synthesis (Grøndahl et al. 2000b, VI). This is similar to progesterone mediated maturation in Xenopus oocytes that requires initiation of protein synthesis. In Xenopus following progesterone induction and preceding GVBD several proteins start to be synthesized from stored mRNA pools including Cyclin A and B1 and *c-mos* protein (Nebreda and Hunt, 1993).

We have furthermore established that the FF-MAS-induced (however not the spontaneous) oocyte maturation in mice can be blocked by even low concentrations of cholera toxin (Grøndahl et al. 2000b, VI). This could suggest that a G-coupled receptor mechanism is involved in the FF-MAS signaling. This is analogous to observations in Xenopus lavis oocytes where progesterone-induced oocyte maturation is sensitive to cholera toxin (Schorderet-Slatkine et al. 1978). Noticeably, Xenopus oocytes are non-responsive to FF-MAS and mammalian oocytes non-responsive to progesterone (Grøndahl and Ottesen, 1997).

Moreover to support the hypothesis of the existence of a MAS receptor we have published how labeled FF-MAS show displaceable binding to the oolemma of oocytes from mice, cows and monkeys (marmoset) in contrast to non-specific binding of close cholesterol analogues (Faerge et al. 2001b, VII).

In comparison in fish and amphibians the endogenous meiosis signaling has been identified and a firm receptor-paradigm of the signaling pathway is being constructed. A Japanese group has put forward a general model of MPF formation during oocyte maturation in amphibians (Yamashita et al. 2000). In this model progesterone (secreted from the supporting cells in the oocyte micro-environment upon gonadotropins regulation) mediates a membrane receptor induced signaling that via mos, MEK and MAP-kinase modulating pre-MPF (pre-maturation promoting factor) to MPF in Xenopus lavis oocytes. The responsible progesterone receptor on the Xenopus oocyte surface has recently been cloned (Bayaa et al. 2000) and it is noticeably the non-genomic signaling pathway that mediates meiosis. In fish and other amphibians than Xenopus the initiation of meiosis is mediated by 17α-20β-dihydroxy-4-pregnen-3-one (17, 20-DP) and again involves activation of this pathway. In mammals a putative receptor-mechanism is hypothesized to involve three pathways 1) the mos/MEK/MAP-kinase pathways leading to MPF formation and stabilization 2) direct translational activation of masked Cyclin B mRNA translation to Cyclin B and 3) a pathway of decreasing cAMP and A-kinase activation (Yamashita et al. 2000; Schmitt and Nebreda, 2002).

Activation of mos/MAP-kinase during meiotic maturation has been established for many species first in Xenopus oocytes (Nebreda and Hunt, 1993) later in mammals e.g. mice (Verlhac et al. 1994) and human (Heikinheimo et al. 1995; 1996).

A study by Coticchio and Flemming observed that spontaneous and FSH-induced maturation in mouse oocytes is mediated by different intracellular pathways (Coticchio and Flemming, 1998). The authors found overt differences via investigating the IP3 pathway and interfering with Ca^{2+} dynamics in maturing oocytes. In a later study both FSH, epidermal growth factor and FF-MAS was observed to differentially influencing nuclear maturation and polar body extrusion (Coticchio et al. 2004).

We and others have been demonstrating that the signaling pathway of spontaneous and induced meiotic maturation is different observing that for instance the mos /MAP kinase pathway is involved in both the FF-MAS and the FSH signaling In Vitro in contrast to spontaneous resumption of meiosis where GVBD can occur without MAP kinase activation (Grøndahl et al. 2000b, VI; Faerge et al. 2001a, VII; Leonardsen et al. 2000b; Sun et al. 1999a;1999b).

This observation could be *key* in the mechanistic understanding of the role of FF-MAS in oocyte maturation. Since FF-MAS clearly signals through mos and MAP-kinase activity this could elicit a plethora of important cytoplasmic and nuclear events including regulation of microtubule dynamics, spindle assembly and chromosome condensation all determinants for oocyte quality and embryo developmental potential (Verlhac et al. 1993; 1994; 1996; Dedieu et al. 1996; Sun et al. 1999a; 1999b). Microtubule dynamics and spindle quality was observed to be beneficially influenced by FF-MAS in murine oocytes (Hegele-Hartung et al. 1999). Furthermore FF-MAS has been observed to protect oocytes from precocious chromatid segregation in mice (Cukurcam et al., 2003) which could be associated with positive influence on the spindle structure and microtubule configurations related to the mos-gene family/ MAP kinase signaling. Also in human oocytes mos/MAP-kinase has been identified (in the specific isoform p42ERK2) and although not widely studied in humans yet, MAP-kinase is thought to play a similar role in oocyte maturation as in other vertebrates (Sun et al. 1999a).

In conclusion we have found strong evidence for that the signaling pathway of FF-MAS involves the activation of the mos-gene *family*/MAP-kinase signaling cascade. Further studies are needed to investigate the eventual ligand-receptor interaction on this activation. Finally, we hypothesize that LXRalpha and especially LXRbeta receptors are involved in FF-MAS signaling since we have observed binding and conformational changes to these receptors of FF-MAS and other meiosis activating compounds, presently published in the form a WO patent application (Grøndahl et al. WO 200404459). Janowski reported that oxysterols made use of LXRalpha in their signaling (Janowski et al. 1996) however our group could not confirm that the oxysterols in that publication had any meiosis activity (Grøndahl et al. 1998, IV).

Hence the full signaling pathway of FF-MAS and the influence of gonadotropins and growth factors still remain to be fully elucidated.

G. THE ENDOGENOUS FORMATION AND BIOCHEMICAL SYNTHESIS OF FF-MAS (PAPERS IX & X)

These studies were undertaken to investigate 1) the physiological nature of FF-MAS and 2) to identify the regulation under which FF-MAS is governed including the relationship to the gonadotropins FSH and LH.

Furthermore the synthetic generation of FF-MAS has been pioneered as well as alteration of the molecule to discover more drug-like analogues i.e. by increasing the potency as well as improving the formulation and stability characteristics of MAS analogues.

The first isolation and identifications of FF-MAS (and the closely related meiosis active metabolite T-MAS) were obtained from samples of human follicle fluids from IVF patients and crude testicular extracts from bulls (Byskov et al. 1995). FF-MAS is the product of

demethylation of lanosterol by the action of the enzyme lanosterol 14alpha-demethylase (LDM) and converted to T-MAS by sterol delta14-reductase (Aoyama and Yoshida, 1986). T-MAS is further converted to zymosterol and later cholesterol in several steps under the action of among others delta7-reductase (Schroepfer, 1982; Yoshida and Aoyama, 1984). LDM is a key enzyme in cholesterol biosynthesis and the gene encoding for LDM is identified to be CYP51 (Rozman et al.1996; Strömstedt et al. 1996) a member of the cytochrome P450 gene family and highly conserved among yeast, plants and animals (Aoyama et al. 1996). The human and rat CYP51 gene has recently been cloned (Strömstedt et al. 1998) and show high degree of homology.

Thus, FF-MAS and T-MAS are produced in all cholesterol-producing cells by this conversion of lanosterol, however, they accumulate only in the gonads probably due to the tissue-specific blocking of the enzymes converting these sterols downstream to zymosterol (Leonardsen et al. 2000a).

Quite a range of endogenous sterols that are meiosis active have been described in human follicular fluid with similar potency as FF-MAS (Grøndahl et al. 2001) as earlier discussed (see section 6.D and Figure 2). Synthetic FF-MAS and MAS analogues have been engineered and a preliminary structural-activity relationship of synthetic sterol analogues has been modeled (Murray et al. 2000; Murray et al. 2002, IX; Murray & Blume unpublished). In summary, lipophilic substituents on the 25 and 26 position of the sterol side chain are generally tolerated. Thus, these analogues have retained efficacy to induce meiosis in the oocyte assay (see Figure 5).

Moreover, it was observed that in compounds bearing hydroxy-group on position 16, the delta^{8} or the delta^{8,14} double bond moieties is not essential for activity. Again it was substantiated that only very minor chemical alteration completely changed the meiotic activity as seen in a previous report (Grøndahl et al. 1998, IV). Synthetic analogues that are potent antagonists to the meiotic resumption of meiosis have also been generated (Grøndahl et al. Int. Patent WO98/28323) as have analogues that possess both a superior efficacy as well as superior potency profile vs. the endogenous sterols (i.e. compounds that are active in the nano-molar range see Marin Bivens et al. 2004b, XII). Highly surprising is has been possible to screen on the simple parameter of meiosis activating activity (i.e. inducing meiosis) and to get very potent candidates that are highly efficacious in inducing cytoplasmic maturation in both outbreed (fertile) and inbreed (sub-fertile) mouse models as later discussed in details (see section 6.I)

The endogenous regulation of FF-MAS and the influence of gonadotropins on FF-MAS dynamics has been the focus of two reports, one paper in mice (Baltsen, 2001) and one paper in rabbits (Grøndahl et al. 2003, X), respectively, however with somewhat different kinetic findings. In mice, Baltsen observed that the ovaries responded to an ovulatory stimulation of FSH and hCG by increasing their content of 4, 4 di-methyl-sterols. This increase was dose-dependently correlated to gonadotropins administrated. The peak of FF-MAS was observed to build up 10-15 hours after hCG administration peaking in the time before ovulation and therefore the author questioned whether this could support the role of FF-MAS for mediating the resumption of meiosis. In pre-pubertal rabbits, we found that the stimulation of FSH was not seen to elicit any significant burst of FF-MAS synthesis. However as early as 1 hour after hCG stimulation (following 48 hours of FSH priming) a significant 4-times increase of FF-MAS was observed i.e. much faster than was reported in mice by Baltsen. This increased FF-MAS content in the rabbit ovaries (concentration in total ovarian tissue, for detailed M&M see Grøndahl et al. 2003, X) was maintained throughout the observation periods (4 and 12 hours after hCG administration) (Figure 6).

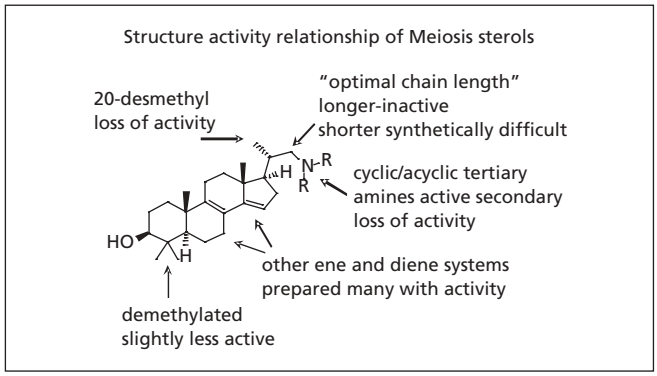

Figure 5. Proposed structure-activity relationship of MAS sterols (Modified from Murray et al.2000; 2002; Murray and Blume personal communication).

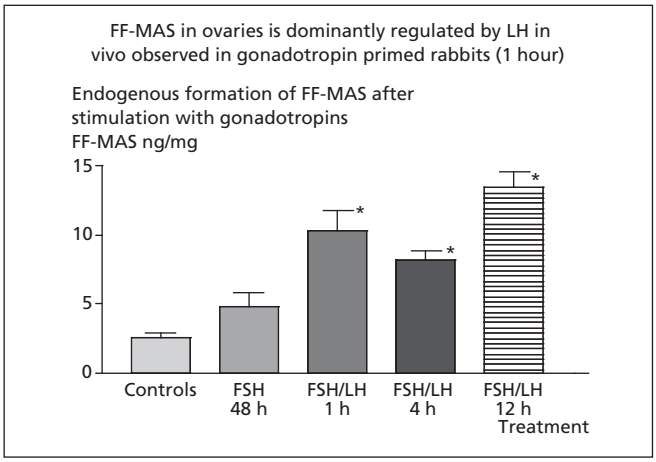

Figure 6. Observations of endogenous levels of FF-MAS in rabbit ovaries following gonadotropin treatment. (From Grøndahl et al. 2003, X).

The timing of resumption of meiosis In Vivo is known to species dependent. It is believed that the mid-cyclic LH rise in most if not all species (either in the form of a peak or an LH build-up) triggers the oocyte maturation to occur In Vivo (Adashi, 1994). Interestingly, it has been observed that recombinant LH acts as a survival and differentiating factor that increases oocyte maturation when acting on the entire follicle in mouse pre-antral follicle culture In Vitro (Cortvrindt et al. 1998). Moreover, Zhang et al. (1993) reported enhanced fertilization and cleavage rate of IVM human oocytes when cultured in the presence of menopausal gonadotropin comprising mainly LH activity.

In conclusion, we postulate that the FF-MAS rise following hCG injection could be interpreted as some physiological role of MAS in relation to LH and therefore MAS could be argued to have the possibility to influence the final maturational steps in particular cytoplasmic maturation that occurs post GVBD a hypothesis that both our own data and data published by Baltsen support.

H. FF-MAS EFFECT ON OOCYTE MATURATION IN PORCINE OOCYTES – DOSES AND SPECIES SPECIFIC FEATURES (PAPER XIII)

It has been documented in a range of studies that FF-MAS increases the quality of oocyte maturation In Vitro in mice, and thus the developmental potential of oocytes exposed to FF-MAS during In Vitro maturation is improved.

This study (Paper XIII) was undertaken to investigate the effect of FF-MAS in porcine oocyte maturation and pronuclear formation to obtain data to guide the use of FF-MAS in humans or even to be deployed as a niche in biomedical animal model production. It should be stressed that it is highly unlikely that IVM, IVF and IVC ever will find use in commercial pig reproduction.

Porcine cumulus-oocyte complexes (COCs) were obtained by follicle aspiration of abattoir ovaries and cultured In Vitro for 48 hours in medium with or without 10% porcine follicle fluid (pFF) and

supplemented with 1, 3, 10, 30, or 100 micromolar FF-MAS. The resulting matured oocytes were In Vitro fertilized and evaluated after 18 hours of In Vitro culture.

The results demonstrated that porcine COCs could be matured and fertilized in the absence of pFF and benefited from the presence of FF-MAS. Previous studies in porcine oocytes have observed an inhibitory effect on meiosis of both co-culturing granulosa cells and also by addition of follicular fluid (Tsafriri and Channing, 1975).

FF-MAS lowered the degeneration rate of zygotes (i.e. one-cell fertilized eggs) and lowered the errors of fertilization (polyspermia rate) regardless of the presence or absence of pFF. However this effect on cytoplasmic maturation is highly dose dependent and positive effect of FF-MAS (lowering polyspermia rate; lowering degeneration rate) was only seen in the dose range 1-10 micromolar and not in 30 and 100 micromolar concentrations.

This is the first report on effect of FF-MAS in porcine oocytes and gives important information to doses and effects in non-rodent species.

In comparison in mice micromolar concentration of synthetic FF-MAS (range 0.1-20 micromolar, optimal range 1-10 micromolar (thus the same concentration window), EC_{50}~ 1 micromolar, Emax (maximal efficacy) = 86% GVBD at 7 micromolar) has robustly been observed to overcome the meiotic inhibition from hypoxanthine (Hx, endogenous purine), or isobuthyl-methyl-xanthine (IBMX) or dbcAMP and significantly induce GVBD and PB extrusion in the C57black/ DBA F1 mice. These findings and the effective dose-range have been confirmed by several groups (Downs et al. 2001; Tsafriri et al. 2002), although some discrepancies were noted on the specific meiosis inhibitors and the effect in cumulus-enclosed oocytes. Recently, choice of mice strains has been observed to influence the action of Hx and the specific effect of FF-MAS (Griffin et al. 2004). We have been hypothesizing that the spontaneous maturation vs. (MAS)-induced maturation are vastly different, both with regards to requirements of protein synthesis inside the oocyte, the signaling messages and the resulting cytoplasmic maturation as well as the quality of the oocyte and the developing zygote post-fertilization.

Also a range of studies has been published investigating the influence on the meiotic process that enzymatic inhibition of the cholesterol biosynthesis may cause, thus aiming for inducing an endogenous accumulation of meiosis active sterols (Leonardsen et al. 2000a; Vaknin et al. 2001; Tsafriri et al. 2002).

FF-MAS has also been published to signal in rat oocytes In Vitro (Hegele-Hartung et al. 2001). Importantly, entire ovaries have been cultured as ex vivo perfused organ culture ad modum Brännström (Brännström et al. 1995) and FF-MAS has been observed to overcome this purely physiological meiosis inhibition exerted by the full functional follicle in rats in dose-dependent manner (Hegele-Hartung et al. 2001).

In conclusion we have found that FF-MAS has been effective in influencing the quality of oocytes maturation and thus the developmental potential of oocytes exposed during maturation in a range of species including porcine and murine oocytes in effective doses ranging from 1-10 (20) micromolar. Thirty (30) micromolars has been established to be too high and 0.1 micromolar has been established to be a non-effective dose in all species tested at least with regards to inducing nuclear maturation.

I. THE EFFECT OF FF-MAS AND MAS ANALOGUE MEDIATED OOCYTE MATURATION ON FERTILIZATION AND PRE-EMBRYO DEVELOPMENT IN NORMAL AND SUB-FERTILE MICE STRAINS (PAPERS XI & XII)

Most previous studies of FF-MAS have been made on oocytes that have been arrested in their meiotic development and the observations have been focused to overcome the meiotic inhibition from hypoxanthine (Hx, endogenous purine), or isobuthyl-methyl-xanthine (IBMX) or dbcAMP. The present studies (XI and XII) were designed primarily to study cytoplasmic maturation and to evaluate the effect of FF-MAS and MAS analogues on cytoplasmic maturation in cumulus enclosed oocytes in different mice strains without any attempts of inhibiting meiosis (Marín-Bivens et al. 2004a, XI; 2004b, XII). Moreover, focus was to look at germinal vesicle (GV) to metaphase-II (M-II) transition in models where this process is known to be impaired.

The idea was to zoom in on the influence on cytoplasmic maturation observed as the resulting developmental potential of oocytes exposed exclusively during In Vitro maturation. Some of the strains used have an excellent fertility and display normal progression and kinetics of meiosis (e.g. C57BL/6J × SJL/J)F_1, whereas other strains have a phenotype of disturbed meiosis and reduced fertility (e.g. inbred strains, that exhibit retarded kinetics of germinal vesicle breakdown (GVBD) and low incidence of progression to M-II (Eppig and Wigglesworth, 1994)). The design involved In Vitro maturing cumulus cell-enclosed oocytes in medium supplemented with FF-MAS and assessing the progression of meiosis to M-II. This was subsequently followed by observation of the M-II and their competence to undergo development to the 2-cell stage and blastocyst stage after In Vitro fertilization.

We found the results to be very interesting and de facto constitute a Proof-of-Concept for FF-MAS as promoting cytoplasmic maturation and increasing embryo quality. On a background of normal fertility FF-MAS had no effect on the kinetic of meiosis (i.e. from GV to GVBD), in contrast to previous studies in arrested oocytes

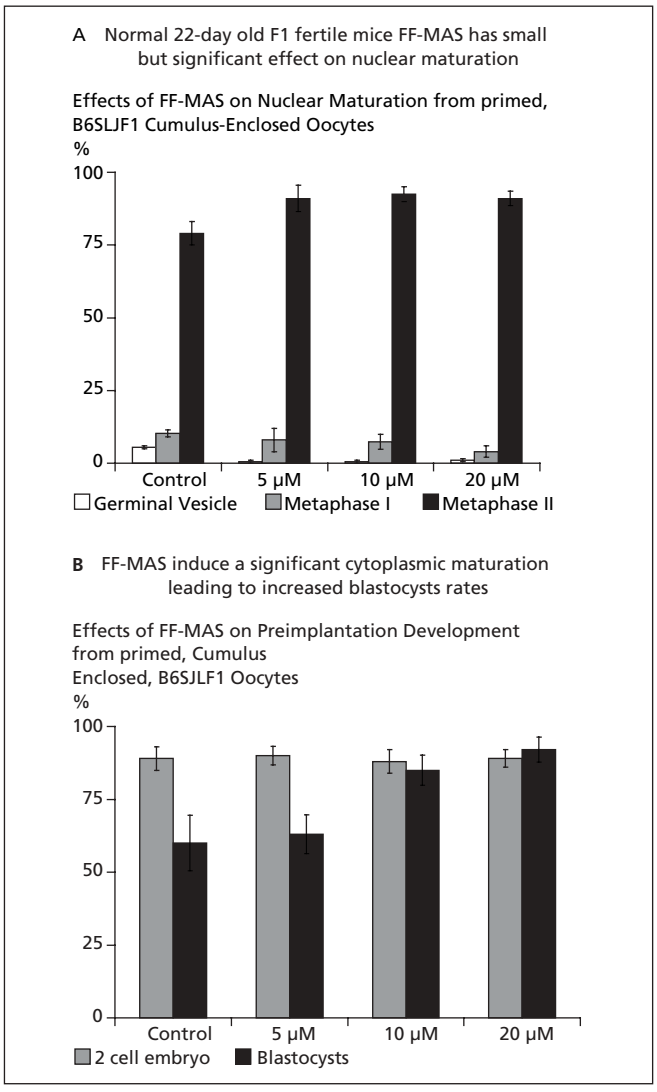

Figure 7A and B. Effect of FF-MAS in normal fertile mice on nuclear maturation i.e. completion of maturation to M-II (7A) and cytoplasmic maturation i.e. blastocyst rate (7B). Modified from Marin-Bivens et al (2004a, XI).

(Hegele-Hartung et al. 1999); however FF-MAS significantly increased the frequency of M-II. No effect was noted in the frequency of 2-cells in these models of outbred mice (see Figure 7); however a significant beneficial effect was noted in developmental potential of 2-cells to blastocyst. As a consequence of increased frequencies of maturation to M-II and completion of the 2-cell to blastocyst transition, the percentage of oocytes (total starting number) that developed all the way to the blastocyst stage increased dramatically from 42% (controls) to 75% with maturation promoted by FF-MAS (in concentration 10 and 20 micromolar). Moreover the percentage of 2-cells that developed to blastocysts increased significantly from 60% in controls to 85-90% with 10 and 20 micromolar FF-MAS (see Figure 7A, B). Thus, both nuclear and cytoplasmic maturation were improved by treatment with FF-MAS in normal fertile F1 mice.

Oocytes and embryos from C57BL/6J inbred mice generally are much less successful in undergoing In Vitro fertilization and preimplantation development than those from hybrid or outbred mice (Roudebush and Duralia, 1996). Only 32% of the oocytes cultured in control medium matured to M-II, whereas FF-MAS (10 micromolar) increased the M-II rate to 79%. Frequency of 2-cell stage embryos increased from 38% in the control group to 60% in the FF-MAS-treated group (in inbred sub-fertile mice), paralleled by an increase in blastocyst transition after oocyte maturation in medium containing FF-MAS. This study concluded that the overall percentage of the total number of oocytes that developed to the blastocyst stage was significantly increased (4% to 17%) by exposure of FF-MAS (10 micromolar) exclusively during the pivotal time of final oocyte maturation.

In summary, FF-MAS was observed to induce both nuclear as well as important elements of cytoplasmic maturation that could be observed as readouts in embryo development both in fertile mouse models and in sub-fertility models.

In human infertility both age related and/or meiosis related mechanisms are thought to be involved. Moreover, dramatic stimulatory protocols have been seen to upset the synchrony of the oocyte and somatic cell compartment and therefore hamper the synchrony between nuclear and cytoplasmic maturation (Hunter, 1977; Eppig et al. 1992; Moor et al. 1998; Eppig et al. 2002).

The efficacy results documented in the present study pertaining to both fertile and sub-fertile mouse strains with significant effect on blastocyst rates and overall efficacy from GV stage oocytes to viable blastocysts (Marín-Bivens et al. 2004a, XI) present a strong pre-clinical proof-of-concept. If similar data in the future were to be reproduced, in a controlled clinical setting with human oocytes and embryos, then a solid clinical proof-of-concept would be established.

The objective of the following study (Marín-Bivens et al. 2004b, XII) was to investigate whether analogues of FF-MAS initially identified on the basis of their greater potency to overcome meiotic inhibition in medium cultured with hypoxanthine could also promote the completion of meiotic maturation and improve developmental competence to complete pre-implantation development.

Analogues were prepared by sterol chain introduction of amines to generate more water soluble, more potent, drugable and patentable analogues. MAS analogue '933 was found to be active in the nanomolar range and at least 10 × more potent than FF-MAS i.e. active at 0.1 micromolar concentration significantly influencing M-II, 2-cell rates and blastocyst rates. In a separate aim of this study, FF-MAS and MAS analogue '933 ability to promote meiotic competence in immature oocytes from very young research animals (in mice of only 18 or 20 days of age respectively) was investigated.

Both FF-MAS and MAS analogue '933 promoted the completion of the physiological processes that would have occurred later when the oocyte was growing in a large follicle – a development that gonadotropin treatment (eCG) In Vivo failed to promote. This could be clinically very relevant since many reports exist of failure of human oocytes to complete meiotic maturation during clinical IVF protocols (Rudak et al., 1990; Levran et al., 2002; Neal et al., 2002).

In conclusion, considering the low frequency of successful fertilization and development following In Vitro maturation of human oocytes, the addition of FF-MAS or MAS analogue '933 to the maturation medium may prove highly beneficial.

J. CLINICAL PHASE I SAFETY TESTING OF APPLYING SYNTHETIC FF-MAS IN HUMAN IVM (PAPER XIV)

Given the beneficial effect seen by exposure of the endogenous signaling molecules FF-MAS in all pre-clinical models tested, The Fertility Team at Novo Nordisk together with leading clinical IVM researchers set out to move into the human clinic with this molecule based on the following key observations: 1) FF-MAS (and related MAS sterols) induces meiosis in mouse oocytes In Vitro in both denuded and cumulus enclosed oocytes, shown by an increased onset of germinal vesicle breakdown and an increased incidence of metaphase II stage oocytes after exposure to FF-MAS (Grøndahl et al. 1998, IV ; 2000b, VI; Grøndahl, 2002, VIII; Marín-Bivens et al. 2004a, XI). 2) FF-MAS stimulation of oocytes during In Vitro maturation mediates a higher fertilization rate in mouse oocytes In Vitro after IVF compared to In Vitro spontaneously matured oocytes and In Vivo matured oocytes (Hegele-Hartung et al. 1998; Marín-Bivens et al. 2004a, XI and 2004b, XII). 3) FF-MAS stimulates the In Vitro resumption of meiosis and its corresponding cytoplasmic maturation in murine and human oocytes (Hegele-Hartung

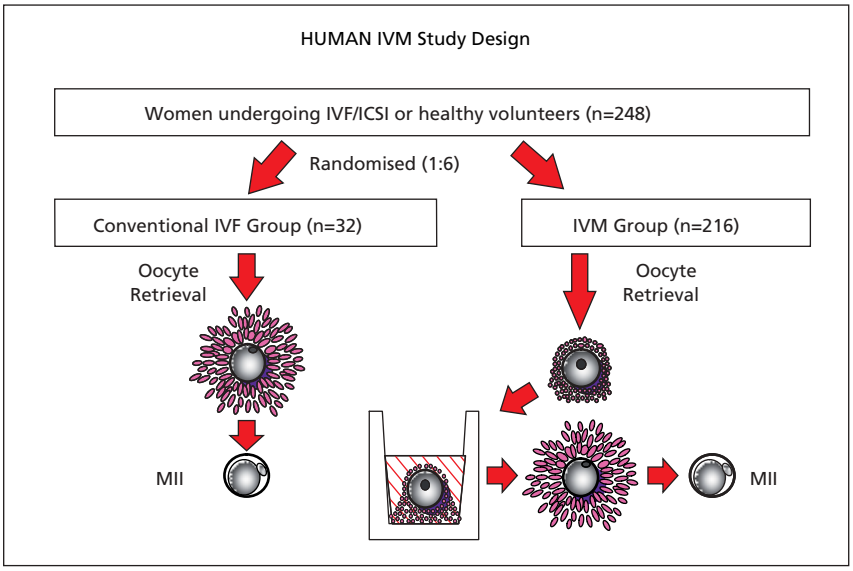

Figure 8. Women with a medical indication for IVF or ICSI, or healthy volunteers, were randomized into either conventional IVF or the IVM group in a ratio 1:6. Oocytes in the IVM groups were randomized into 1 of 6 groups (4 active FF-MAS groups and 2 controls). Modified from Smitz et al (2007, XIV).

et al. 1999, Grøndahl et al. 2000a, V; Marín-Bivens et al. 2004a, XI and 2004b, XII). 4) FF-MAS when exposed to oocytes during In Vitro maturation mediates both a higher fertilization rate and yield significant higher blastocyst rates in mice in both fertile and sub-fertile models (Marín-Bivens et al. 2004a, XI and 2004b, XII). 5) FF-MAS when exposed to oocytes during In Vitro maturation mediates a higher fertilization rate after IVF and yields higher numbers of live born pups after 2-cell transfer in mice (Hegele-Hartung et al. 1998; Hegele-Hartung and Grøndahl unpublished data; Grøndahl, 2002, VIII).

The design of this study was a bi-centric, parallel-group, controlled, partially blinded and prospective clinical trial (Smitz et al. 2007, XIV). The study objective pursued was In Vitro safety and efficacy evaluation of applying FF-MAS In Vitro to immature human oocytes based on the primary endpoint: incidence of Metaphase-II (M-II) oocytes with numeric chromosome abnormalities using full or partial karyotyping. The secondary endpoint was to assess the efficacy of FF-MAS in obtaining M-II oocytes after In Vitro culture compared to FSH/hCG-stimulated In Vivo matured human oocytes. No fertilization was attempted on FF-MAS exposed oocytes (or any oocytes) in this trial.

The very basic study design is depicted in Figure 8.

Numeric chromosome abnormalities were assessed using three different methods of karyotyping: Giemsa count, Partial Karyotyping using FISH (Fluorescent in situ Hybridization using 7 commercial probes from MultiVision) or full karyotyping using SKY (Spectral Karyotyping) as shown in Figure 9.

In summary of this 248-patient study investigating 1627 human

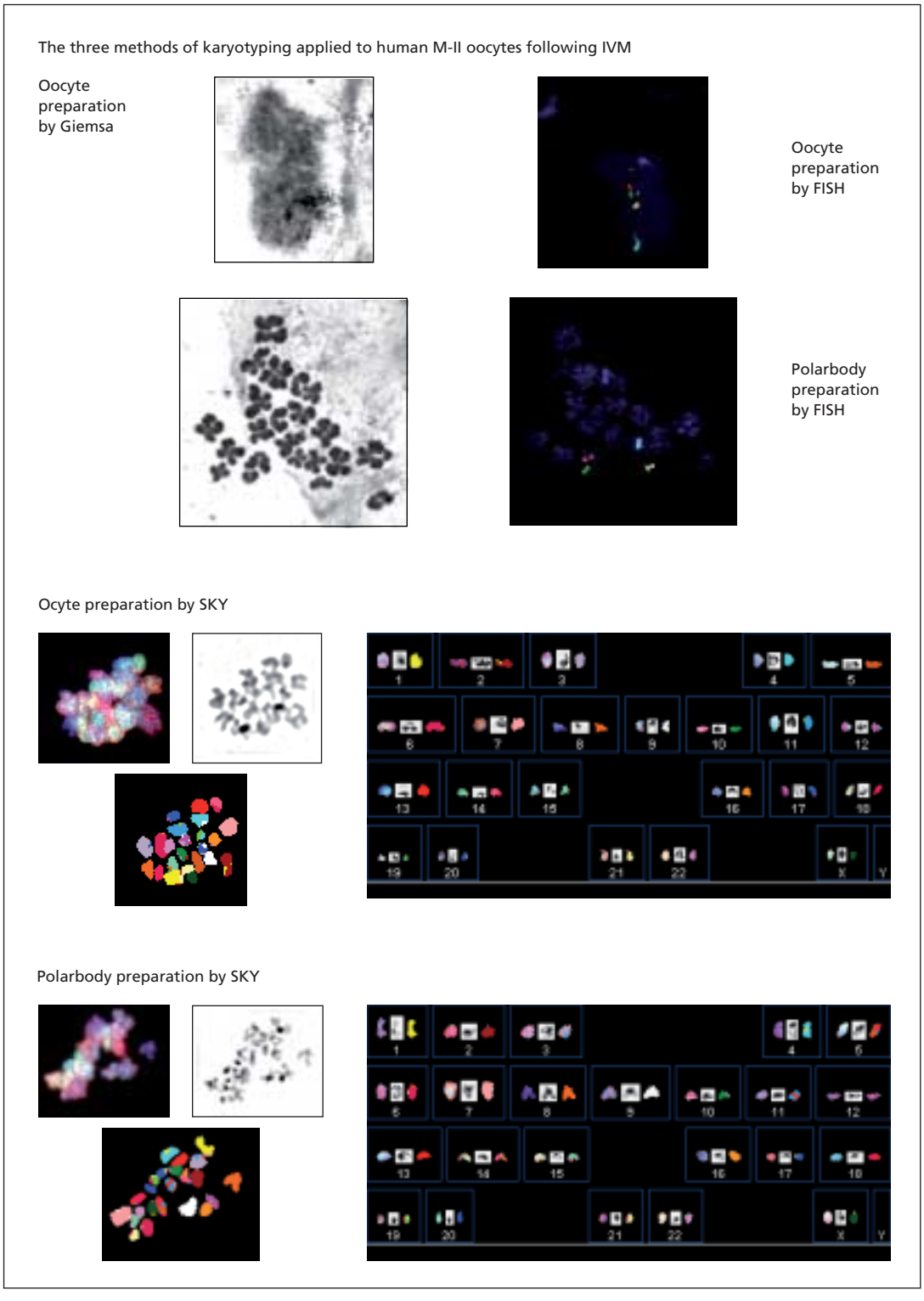

Figure 9. The 3 methods of karyotyping: Giemsa, FISH (MultiVision) and SKY™

oocytes (from non-PCOS IVF patients and healthy volunteers, age range 25-37 years), IVM with or without the presence of FF-MAS was found to yield M-II oocytes that did not display any increase in the rate of numeric chromosome abnormalities as compared to In Vivo FSH/ hCG stimulated controls. Furthermore, similar numbers of fully mature MII oocytes could be obtained from IVM protocols with MAS as could from FSH/hCG protocols (Smitz et al., 2007, XIV). It was confirmed that 30 hours of culture was optimal as seen previously in oocytes from PCOS-patients (Grøndahl et al. 2000a, V) based on the chromosome abnormality endpoint. However, due to the utilization of a complex defined culture medium and the inclusion of oocyte survival factors like rFSH, Insulin, IGF-1, nuclear maturation to M-II was high (> 70%) in all groups and this secondary endpoint gave no possibility for differentiation between groups, however could further substantiate the safety objective of the study.

In the IVM groups the short low-dose rFSH protocol (modified from Wynn et al. 1998) induced follicle growth in all patients. The overall oocyte retrieval rate was 61% and a mean number of 6.2 cumulus-oocytes complexes per patient were available for IVM.

Overall high chromosome normality rates were found in both the IVF and IVM groups after 30 hours of culture (e.g. 85% up to 93% of oocytes were observed to be chromosomal normal haploid cells). Tendencies of reduced aneuploidy rate were seen in the 1 and 10 micromolar FF-MAS groups after 30 hours of culture (down to 7% aneuploidy rate vs. 15% in controls), however no significant differences were observed.

Other studies have focused on the safety of FF-MAS added to In Vivo matured FSH stimulated oocytes retrieved after normal IVF stimulation and subsequent cultured with FF-MAS 4 hours prior to fertilization (Loft et al. 2004; Loft et al. 2005). In these studies FF-MAS was found to be safe as a medium additive exposed before fertilization based on chromosome abnormalities in the resulting pre-embryos. Moreover, the albumin formulation was observed to be superior to the ethanol based formulation. In the albumin formulated study the chromosome aberration rate on the resulting pre-embryos ranged between 39-53% with no significant difference among controls or FF-MAS groups.

In contrast FF-MAS was seen to cause chromosome aberrations when acting on FSH stimulated In Vivo maturated oocytes both before and 24 hours following fertilization (Bergh et al. 2004). Hence it can be concluded that FF-MAS exclusively should be exposed to oocytes prior to fertilization and not after zygote formation when the pre-embryo is transforming to make mitosis divisions.

Direct studies of human gametes have previously shown that as many as 20-25% of oocytes, but only 3 to 4% of sperm cells, are chromosomal abnormal (Martin et al. 1986; Pellestor, 1991; Hunt and Hassold, 2002). Our findings in the present paper displaying lower rates of chromosome abnormal oocytes could partly be due to the study population of both mainly younger patients and also younger fertile healthy volunteers.

Aneuploidy as such has been named the biggest single problem in human reproduction, being the single most common cause of miscarriage and affecting up to 50% of transferred embryos in the clinic (Cohen, 2002; Munne and Cohen, 1998; Munne et al. 2002; Ziebe et al. 2003).

A large proportion of the IVM or FSH/hCG primed IVF oocytes were observed to be chromosomally normal (between 85 to 93% normal haploid MII oocytes) in the present 248-patient study (age range 25-37 years) of IVF and IVM human oocytes (Smitz et al. 2007, XIV). In contrast, less than half of the IVF embryos (exactly 31% uniformerly normal embryos out of 144 human randomly selected IVF embryos) was observed to be normal in the study with material from 4 large hospital IVF clinics in Scandinavia (age range 25-37 years) (Ziebe et al. 2003).

Thus, surprisingly by combining the observations from the two different FF-MAS safety programmes on the one hand the present IVM study (Smitz et al. 2007) and on the other hand the parallel IVF studies we have conducted (Ziebe et al. 2003; Bergh et al. 2004; Loft et al. 2004, 2005), respectively, it appears that it is primarily during the fertilization process and the early pre-implantation cell divisions that a major part of the chromosome errors are introduced.

Moreover, a great deal of mosaics was noted in Ziebe et al. 2003 which questions the validity of using pre-implantation diagnostics on a single blastomeric cell as a accurate tool, when very often more than 2 cell lines exist in the embryo, given the possibility of both false positive (the biggest clinical challenge) and potentially false negative outcomes. Finally, it needs to be further substantiated to what degree an early cleaving human embryo can deal with a low grade of aneuploidy in a subset of the blastomers.

In conclusion, further clinical studies in human IVM involving fertilization and embryo transfer are needed to evaluate whether FF-MAS also acts at the human oocytes by contributing favorably to cytoplasmic maturation and oocyte quality or developmental potential, analogously to the data that have been obtained in several models of mouse oocytes (Marin-Bivens et al. 2004a, XI and 2004b, XII). This is now possible to pursue in subsequent clinical trials based on the safety assessment of human IVM documented herein.

7. CONCLUSIONS

In Vitro maturation (IVM) of human oocytes is at present an emerging infertility treatment with great promise.

In summary we have established new knowledge both within the basic understanding of IVM as well as in the applied areas of clinical IVM.

To conclude on the contribution of advancing the basic understanding of IVM by comparative studies in the equine species where only limited original studies have been performed we have in detail described the ultrastructural changes in the maturing oocyte during In Vivo maturation and measured biochemical changes in the surrounding follicular fluid. The In Vivo baseline of maternal embryonic transition (MET) in the equine species and the ultrastructural changes associated with this transition have been documented for the first time. Finally, in the comparative study of equine oocytes culture conditions and elements of IVM and ICSI have been pioneered.

Furthermore, our group has studied FF-MAS and related MAS sterols during IVM in both outbred and inbred mice as model for human infertility. It has been documented that FF-MAS posses a clear dose-depending effect on activating meiosis when cultured under meiosis-inhibiting conditions (using hypoxanthine, IBMX or dibutyril cyclic AMP). Moreover it has been documented that in models of sub-fertility where the transition from GV to M-II is somewhat impaired FF-MAS has direct meiosis or nuclear maturation activating effects. The dose range of activity has been reproduced by many groups and in several species to be 1-10 (20) micromolar with 0.1 micromolar to be an ineffective dose and doses higher than 20 micromolar have been documented to be less than optimal (Paper IV, V, VI, VII, X).

One of the most important contributions from our group has been the profiling of FF-MAS and related MAS analogues as important molecules capable of positively influencing the cytoplasmic maturation and hereby increases the quality of the resulting embryo and its developmental potential as published in paper XI and XII. This is in contrast to the original discovery and scientific communication around FF-MAS and its designated nomenclature Follicle Fluid Meiosis Activating Sterol as primary influencing meiosis i.e. the nuclear maturation. Our findings clearly point towards a dual role of the MAS sterols influencing both nuclear and in particular cytoplasmic maturation and we hypothesize that the cytoplasmic maturation may represent the physiological role of MAS sterols and potentially the one that could bring the clinical benefits to IVM.

Finally, it has been the objective to document the safety aspects of applying FF-MAS to human oocytes in culture and to optimize both

the dosage and the exposure time in clinical and pre-clinical studies. We can conclude based on the primary endpoint of a large prospective clinical phase I safety trial where numerical chromosome abnormalities were studied on M-II oocytes following IVM or in vivo FSH and hCG stimulation (IVO) that no significant difference was observed between IVM and IVO (Paper XIV).

The maturation In Vitro of human oocytes is already now a valuable clinical treatment alternative for a subset of infertile patients, especially the PCOS patients and also in some clinics as a combination to natural cycle IVF. IVM has the promise of being tomorrow's gold standard in treatment of human infertility if many more of the important components of oocyte maturation are understood and can be adequately addressed In Vitro. Further research is definitely needed into In Vitro maturation of human oocytes and the endogenous molecule FF-MAS or engineered MAS analogues. Whether FF-MAS, or related compounds, may assist in making human IVM widely used by enhancing the cytoplasmic maturation of human oocytes in clinical settings remains an exciting prospect for treatment of infertile couples.

References

Adashi, E.Y. (1994) Endocrinology of the ovary. Hum Reprod, 9, 815-827.

Anderiesz, C., Ferraretti, A., Magli, C. et al. (2000a) Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development In Vitro. Hum Reprod, 15, 1140-1148.

Anderiesz, C., Fong, C.Y., Bongso, A. et al. (2000b) Regulation of human and mouse oocyte maturation In Vitro with 6- dimethylaminopurine. Hum Reprod, 15, 379-388.

Andersen, C.Y., Baltsen, M. and Byskov, A.G. (1999) Gonadotropin-induced resumption of oocyte meiosis and meiosis-activating sterols. Curr Top Dev Biol, 41, 163-185.

Aoyama, Y., Noshiro, M., Gotoh,O. et al. (1996) Sterol 14-demethylase P450 (P45014DM) is one of the most ancient and conserved P450 species. J Biochem, 119, 926-933.

Aoyama, Y. and Yoshida, Y. (1986) Evidence for the contribution of a sterol 14-reductase to the 14 alpha-demethylation of lanosterol by yeast. Biochem Biophys Res Commun, 134, 659-663.

Avery, B., Færge, I., Grøndahl, C. et al. (1999) Nuclear maturation and embryo development of bovine oocytes, matured in a semi-defined medium supplemented with meiosis activating sterol (MAS). Theriogenology, 51, 367.

Baltsen, M. (2001) Gonadotropin-induced accumulation of 4,4-dimethylsterols in mouse ovaries and its temporal relation to meiosis. Biol Reprod, 65, 1743-1750.

Barnes, F.L., Crombie, A., Gardner, D.K. et al. (1995) Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum Reprod, 10, 3243-3247.

Barnes, F.L., Kausche, A., Tiglias, J. et al. (1996) Prodution of embryos from In Vitro-matured primary human oocytes. Fertil Steril, 65, 1151-1156.

Bavister, B., Rose-Hellekant, T., Pinyopummintr, T. (1992) Development of in-vitro In Vitro fertilized bovine embryos into morulae and blastocysts in define culture media. Theriogenology, 37:127-145

Bayaa, M., Booth, R.A., Sheng,Y. et al. (2000) The classical progesterone receptor mediates Xenopus oocyte maturation through a nongenomic mechanism. Proc Nat Acad Sci USA, 97, 12607-12612.

Beckers, N.G., Pieters, M.H., Ramos, L. et al. (1999) Retrieval, maturation, and fertilization of immature oocytes obtained from unstimulated patients with polycystic ovary syndrome. J Assist Reprod Genet, 16, 81-86.

Bergh, C., Loft, A., Lundin, K., Ziebe, S., Nilsson, L., Wikland, M., Grøndahl, C. and Arce, J.C. (2004) Chromosomal abnormality rate in human preembryos derived from In Vitro fertilization cycles cultured in the presence of Follicular-Fluid Meiosis Activating Sterol (FF-MAS). Hum Reprod 19:2109-17.

Bergh, P.A. and Navot, D. (1992) Ovarian hyperstimulation syndrome: A review of pathophysiology. J Assist Reprod Genet, 9, 429-438.

Biggers, J.D., Whittingham, D.G. and Donahue, R.P (1967) The pattern of enrgy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci USA 58:560-567

Brackett, B. and Zuelke, K. (1993) Analysis of factors involved in the In Vitro production of bovine embryos. Theriogenology, 39:43-64.

Braude, P., Bolton, V., and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature, 332, 459-461.

Brower, P. and Schultz, R. (1982) Intercellular communication between granulose cellsand mouse oocytes: existence and possible nutritional role during oocyte growth. Dev Biol 90: 144-153.

Brännström, M. and Flaherty, S. (1995) Methodology and characterization of an In Vitro perfusion model for the mouse ovary. J Reprod Fertil, 105, 177-183.

Byskov, A.G., Andersen, C.Y., Nordholm, L. et al. (1995) Chemical-structure of sterols that activate oocyte meiosis. Nature, 374, 559-562.

Byskov, A.G., Andersen, C.Y., Hossaini, A. et al. (1997) Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. Mol Reprod Dev, 46, 296-305.

Byskov, A.G., Baltsen, M. and Andersen, C.Y. (1998) Meiosis-activating sterols: background, discovery, and possible use. J Mol Med, 76, 818-823.

Byskov, A.G., Andersen, C.Y., Leonardsen, L. et al. (1999) Meiosis activating sterols (MAS) and fertility in mammals and man. J Exp Zool, 285, 237-242.

Camous, S., Kopecny,V., and Flechon, J.E. (1986) Autoradiographic detections of the earliest stage of 3H-uridine incorporation into the cow embryo. Biol Cell, 58, 195-200.

Carroll, J., Jones, K.T., and Whittingham,D.G. (1996) Ca^{2+} release and the development of Ca^{2+} release mechanisms during oocyte maturation: a prelude to fertilization. Rev. Reprod, 1, 137-143.

Cavilla, J.L., Kennedy, C.R., Baltsen, M. et al. (2001) The effects of meiosis activating sterol on in-vitro maturation and fertilization of human oocytes from stimulated and unstimulated ovaries. Hum Reprod, 16, 547-555.

Cetica, P., Pintos, L., Dalvit, G., Beconi, M. (2002) Activity of key enzymes involved in the glucose and triglyceride catabolism during bovine oocyte maturation i vitro. Reproduction 124:675-681.

Cha, K.Y., Koo, J.J., Ko,J.J. et al. (1991) 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, 55, 109-113.

Cha, K.Y. and Chian, R.C. (1998) Maturation In Vitro of immature human oocytes for clinical use. Hum Reprod Update, 4, 103-120.

Cha, K.Y., Han, S.Y., Chung, H.M. et al. (2000) Pregnancies and deliveries after In Vitro maturation culture followed by In Vitro fertilization and embryo transfer without stimulation in women with polycystic ovary syndrome. Fertil Steril, 73, 978-983.

Chian, R.C., Buckett, W.M., Too, L.L. et al. (1999) Pregnancies resulting from In Vitro matured oocytes retrieved from patients with polycystic ovary syndrome after priming with human chorionic gonadotropin. Fertil Steril, 72, 639-642.

Chian, R.C., Buckett, W.M., Tulandi, T. et al. (2000) Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. Hum Reprod, 15, 165-170.

Chian, R.C., (2003) In Vitro maturation of human oocytes. RBM Online, 8:148-66.

Chian, R.C., Lim JH, Tan SL. (2004A) State of the art in in-vitro oocyte maturation. Curr Opinion Obstet Gyn, 16:211-9.

Chian RC, Buckett WM, Abdul Jalil AK, Son WY, Sylvestre C, Rao D, et al.. (2004B) Natural-cycle In Vitro fertilization combined with In Vitro maturation of immature oocytes is a potential approach in infertility treatment. Fertil Steril, 82:1675-8.

Cohen,J. (2002) Sorting out chromosome errors. Science, 296, 2164-2166.

Cortvrindt, R., Hu,Y., and Smitz, J. (1998) Recombinant luteinizing hormone as a survival and differentiation factor increases oocyte maturation in recombinant follicle stimulating hormone-supplemented mouse preantral follicle culture. Hum Reprod, 13, 1292-1302.

Coticchio, G. and Flemming S. (1998) Inhibition of phosphoinositide metabolism or chelation of intracellular calcium blocks FSH-induced but not spontaneous meiotic resumption in mouse oocytes. Dev Biol, 203, 201-209.

Coticchio, G., Rossi, G., Borini, A., Grøndahl, C., Macchiarelli, G., Flamigni, C., Flemming, S., Cecconi, S. (2004) Mouse oocyte meiotic resumption and polar body extrusion In Vitro are differentially influenced by FSH, epidermal growth factor and meiosis activating sterol. Hum Reprod, 19(12), 2913-18.

Crosby, I.M., Gandolfi,F., and Moor,R.M. (1988) Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil, 82, 769-775.

Cukurcam, S., Hegele-Hartung, C., Eichenlaub-Ritter, U. (2003) Meiosis Activating sterol protects oocytes from precocious chromosome segregation. Human Reprod. 18(9), 1908-17.

Davis,D.L. (1985) Culture and storage of pig embryos. J Reprod Fertil, 33, 115-124.

Dedieu, T., Gall, L., Crozet, N. et al. (1996) Mitogen-activated protein kinase activity during goat oocyte maturation and the acquisition of meiotic competence. Mol Reprod Dev, 45, 351-358.

Dieleman, S.J., Kruip, T.A.M., Fontijne, P. et al. (1983) Changes in oestradiol, progesterone, and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. J Endocrinol, 97, 31-42.

Downs, S.M., Ruan, B., and Schroepfer, G.J. (2001) Meiosis-activating sterol and the maturation of isolated mouse oocytes. Biol Reprod, 64, 80-89.
Ducibella,T., Duffy,P., Reindollar,R. et al. (1990) Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin- stimulated meiotic maturation and aging In Vivo. Biol Reprod, 43, 870-876.
Ducibella, T., Kurasawa, S., Duffy, P. et al. (1993) Regulation of the polyspermy block in the mouse egg – maturation-dependent differences in cortical granule exocytosis and zona-pellucida modifications induced by inositol 1,4,5-trisphosphate and an activator of protein-kinase-c. Biol Reprod, 48, 1251-1257.
Ducibella, T. (1996) The cortical reation and development of activation competence in mammalian oocytes. Hum Reprod Update, 2, 29-42.
Dvoràk, M., Tesarik, J., Pilka, L. et al. (1982) Fine structure of human two-cell ova fertilized and cleaved In Vitro. Fertil Steril, 37, 661-667.
Edwards, R.G. (1965) Maturation In Vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature, 208, 349-351.
Edwards, R.G. (1995) Cell cycle factors in the human oocyte and the intracytoplasmic injection of spermatozoa. Reprod Fert Develop, 7, 143-153.
Eppig, J.J., Schroeder, A.C., and O'Brien, M.J. (1992) Developmental capacity of mouse oocytes matured In Vitro: effects of gonadotrophic stimulation, follicular origin and oocyte size. J Reprod Fertil, 95, 119-127.
Eppig, J.J. and Wigglesworth, K. (1994) Atypical maturation of oocytes of strain i/lnj mice. Hum Reprod, 9, 1136-1142.
Eppig, J.J., Obrien, M., and Wigglesworth, K. (1996) Mammalian oocyte growth, and development in-vitro. Mol Reprod Dev, 44, 260-273.
Eppig, J.J. and Obrien, M.J. (1996) Development in-vitro of mouse oocytes from primordial follicles. Biol Reprod, 54, 197-207.
Eppig, J.J., Hosoe, M., O'Brien, M., Pendola, F., Requena, A., Watanabe, S. (2000) Conditions that affect acquisition of developmental competence by mouse oocytes In Vitro: FSH, Insulin, Glucose and Ascorbic Acid. Mol Cell Endocrinol 163:109-116.
Eppig, J.J., Wigglesworth,K., and Pendola,F.L. (2002) The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc Nat Acad Sci USA, 99, 2890-2894.
Faerge, I., Terry, B., Kalous, J., Wahl, P., Lessl, M., Ottesen, J., Hyttel, P. and Grøndahl, C. (2001a) Resumption of meiosis induced by meiosis-activating sterol has a different signal transduction pathway than spontaneous resumption of meiosis in denuded mouse oocytes cultured In Vitro. Biol Reprod, 65, 1751-1758. (VII)
Faerge, I., Grøndahl, C., Ottesen, J.L., and Hyttel, P. (2001b) Autoradiographic localization of specific binding of meiosis-activating sterol to cumulus-oocyte complexes from marmoset, cow, and mouse. Biol Reprod, 64, 527-536.
Faerge I., Strejcek, F., Laurencik, J., Rath, D., Nieman, H., Schellander K., Rosenkranz, C., Maddox Hyttel P. and Grøndahl, C. (2006) The effect of FF-MAS on Porcine Cumulus-Oocyte complex maturation, fertilization and pronucleus development In Vitro. Zygote 14:189-199 (XIII)
Frei, R.E., Schultz, G.A., and Church, R.B. (1989) Qualitative and quantitative changes in protein synthesis occur at the 8-16-cell stage embryogensis in the cow. J Reprod Fertil, 86, 637-641.
Goto, K., Kinoshita, A., Takuma,Y. et al. (1990) Fertilization of bovine oocytes by injektion of immobilized, killed spermatozoa. Vet Rec, 127, 517-520.
Griffin, A.M., Grøndahl, C. and Flemming, S.D. (2004) Action of hypoxanthine and Meiosis-activating Sterol on oocyte maturation in the mouse is strain specific. RBM Online 8(6) 673-681.
Grøndahl, C., Madison, V., Hyttel, P., et al. (1990) Onset of RNA synthesis in bovine embryos after maturation, fertilization and development In Vitro. Assisted Reproductive Technology/ Andrology 1, 135-142.
Grøndahl, C., Nielsen, C.G., Eriksen, T. et al. (1993) In Vivo fertilisation and initial embryogenesis in the mare. Equine Vet J, 15, 79-83.
Grøndahl, C., Hyttel, P., Grøndahl, M.L. et al. (1995a) Structural and endocrine aspects of equine oocyte maturation in-vivo. Mol Reprod Dev, 42, 94-105. (I)
Grøndahl, C., Høst, T., Brück,I. et al. (1995b) In Vitro production of equine embryos. In Sharp, D.C. and Bazer, F.W. (eds), Equine Reproduction VI. Society for the Study of Reproduction, Madison, WI, pp. 299-307.
Grøndahl, C. and Hyttel, P. (1996) Nucleologenesis and ribonucleic acid synthesis in preimplantation equine embryos. Biol Reprod, 55, 769-774. (II)
Grøndahl, C., Høst, T., Hossaini, A., et al. (1997). ICSI of In Vitro matured equine oocytes. Biol Reprod, 57, 1495-1501. (III)
Grøndahl, C. and Ottesen, J.L (1997) Meiosis activating sterol (MAS) effect on maturation In Vitro of Xenopus laevis oocytes. Biol Reprod, 56, p.97 abstract 58.
Grøndahl, C., Ottesen, J.L., Lessl, M. et al. (1998) Meiosis-activating sterol promotes resumption of meiosis in mouse oocytes cultured In Vitro in contrast to related oxysterols. Biol Reprod, 58, 1297-1302. (IV)
Grøndahl, C., Hansen, T.H., Marky-Nielsen, K. et al. (2000a) Human oocyte maturation In Vitro is stimulated by meiosis-activating sterol. Hum Reprod, 15, 3-10. (V)
Grøndahl, C., Lessl, M., Faerge, I. et al. (2000b) Meiosis-activating sterol-mediated resumption of meiosis in mouse oocytes In Vitro is influenced by protein synthesis inhibition and cholera toxin. Biol Reprod, 62, 775-780. (VI)
Grøndahl, C., Baltsen, M., Breinholt, J et al. (2001). Cascade of related endogenous MAS sterols in human follicular fluid possing meiotic inducing activity. Fertil Steril 76(3), P-457 (Abstract).
Grøndahl, C. (2002) FF-MAS and its role in mammalian oocyte maturation. Ernst Schering Res Found Workshop, Eds:: Eppig and Hegele-Hartung. Book chapter: 177-193. (VIII)
Grøndahl, C., Breinholt, J., Wahl, P. et al. (2003) Physiology of meiosis-activating sterol: endogenous formation and mode of action. Hum Reprod, 18, 122-129. (X)
Grøndahl et al. Int. Patent WO98/28323.
Grøndahl et al. Int. Patent WO200404459.
Hay, M.F. and Moor, R.M. (1975) Distribution of delta5-3beta-hydroxysteroid dehydrogenase activity in the graafian follicle of sheep. J Reprod Fertil, 43, 313-322.
Hay-Schmidt A., Viuff, D., Greve, T. and Hyttel, P. (2001) Transcriptional activity in in vivo developed early cleavage stage bovine embryos. Theriogenology. 56 (1):167-76
Hegele-Hartung, C., Lessl, M., Ottesen, J. L., and Grøndahl, C. (1998) Oocyte maturation can be induced by a synthetic meiosis activating sterol MAS leading to an improvement of IVF rate in mice. Hum Reprod 11. 193 (Abstract).
Hegele-Hartung,C., Kuhnke,J., Lessl,M., Grøndahl, C., Ottesen, J., Beier, H.M., Eisner, S. and Eichenlaub-Ritter, U. (1999) Nuclear and cytoplasmic maturation of mouse oocytes after treatment with synthetic meiosis-activating sterol In Vitro. Biol Reprod, 61, 1362-1372.
Hegele-Hartung, C., Grutzner,M., Lessl,M. et al. (2001) Activation of meiotic maturation in rat oocytes after treatment with follicular fluid meiosis-activating sterol In Vitro and ex vivo. Biol Reprod, 64, 418-424.
Heikinheimo, O., Lanzendorf, S. E., Baka, S. G et al. (1995) Cell-cycle genes c-mos and cyclin-b1 are expressed in a specific pattern in human oocytes and preimplantation embryos. Hum Reprod 10(3), 699-707.
Heikinheimo, O., Billeter, M., Toner, J. P et al. (1996). Messenger-ribonucleic-acid kinetics in human oocytes – effects of in-vitro culture and nuclear maturational status. Fertil Steril 65(5), 1003-1008.
Herbert, M., Gillespie, J.I., and Murdoch, A.P. (1997) Development of calcium signalling mechanisms during maturation of human oocytes. Mol Hum Reprod, 3, 965-973.
Hillensjo, T., Brannstrom,M., Chari,S. et al. (1985) Oocyte maturation as regulated by follicular factors. Ann NY Acad Sci, 442, 73-79.
Hunt, P.A. and Hassold,T.J. (2002) Sex matters in meiosis. Science Washington, 296, 2181-2183.
Hunter, R.H.F. (1977) Physiological factors influencing ovulation, fertilization, early embryonic development and establishment of pregnancy in pigs. Brit Vet J, 133, 461-470.
Hwang, S.Y., Oh,B., Knowles,B.B. et al. (2001) Expression of genes involved in mammalian meiosis during the transition from egg to embryo. Mol Reprod Dev, 59, 144-158.
Iritani, A. (1991) Micromanipulation of gametes for In Vitro assisted fertilization. Mol Reprod Dev, 28, 199-207.
Janowski, B.A., Willy, P.J., Devi,T.R. et al. (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXRalpha. Nature, 383, 728-731.
Jaroudi, K.A., Hollanders, J.M., Elnour,A.M. et al. (1999) Embryo development and pregnancies from in-vitro matured and fertilized human oocytes. Hum Reprod, 14, 1749-1751.
Kidder, G.M. and McLachlin, J.R. (1985) Timing of transcription and protein synthesis underlying morphogenesis in preimplantation mouse embryos. Develop Biol, 112, 265-275.
Kimura, Y. and Yanagimachi, R. (1995) Intracytoplasmic sperm injection in the mouse. Biol Reprod, 52, 709-720.
Lefevre, B., Gougeon, A., and Testart, J. (1987) In-vitro oocyte maturation: some questions concerning the initiation and prevention of this process in humans. Hum Reprod, 2, 495-497.
Leonardsen, L., Stromstedt, M., Jacobsen, D. et al. (2000a) Effect of inhibition of sterol Delta 14-reductase on accumulation of meiosis-activating sterol and meiotic resumption in cumulus-enclosed mouse oocytes In Vitro. J Reprod Fertil, 118, 171-179.
Leonardsen, L., Wiersma, A., Baltsen M, et al. (2000b). Regulation of spontaneous and induced resumption of meiosis in mouse oocytes by different intracellular pathways. J Reprod Fertil 120, 377-383. 2000.
Levran, D., Farhi, J., Nahum, H., Glezerman, M. and Weissman, A. (2002) Maturation arrest of human oocytes as a cause of infertility: case report. Hum Reprod, 17, 1604-1609.
Lin, Y.H., Hwang, J.L., Huang, L.W. et al. (2003) Combination of FSH priming and hCG priming for in-vitro maturation of human oocytes. Hum Reprod, 18, 1632-1636.
Loft, A., Bergh, C., Ziebe, S. et al. (2004) A randomised, double-blinded, controlled trial on the effect of addind Follicular-Fluid Activating Sterol in the ethanol formulation to donated human cumulus-enclosed oocytes prior to fertilisation. Fertil Steril, 81, 42-50

Loft, A., Ziebe, S., Erb, K., Rasmussen, P.E., Agerholm, I., Hauge, B., Bungum, M., Bungum, L., Grøndahl, C., Lyby, K. (2005) Impact of follicular-fluid meiosis activating sterol in an albumin formulation on the incidence of human pre-embryos with chromosome abnormalities. Fertil Steril, 84, Suppl 2: 1269-1276.

Lumsden, M.A., Kelly, R.W., Templeton, A.A. et al. (1986) Changes in the concentration of prostaglandins in preovulatory human follicles after administration of hCG. J Reprod Fertil, 77, 119-124.

Marín-Bivens, C., Grøndahl, C., Murray, A., Thorsten Blume, You-Qiang Su and Eppig, J.J. (2004a) Meiosis-activating sterol (MAS) promotes cytoplasmic maturation and the completion of nuclear maturation of mouse oocytes. Biol Reprod, 70, 1458-1464. (XI)

Marín-Bivens, C., Lindenthal, B., O'Brien MJ, Wigglesworth K, Blume T, Grøndahl C, Eppig JJ. (2004b) A synthetic analogue of meiosis-activating sterol (FF-MAS) is a potent agonist promoting meiotic maturation and preimplantation development of mouse oocytes maturing In Vitro. Hum Reprod, 19, 2340-4. (XII)

Markert, C.L. (1983) Fertilization of mammalian eggs by sperm injection. J Exp Zool, 228, 195-201.

Martin, R.H., Mahadevan, M.M., Taylor, P.J. et al. (1986) Chromosomal analysis of unfertilized human oocytes. J Reprod Fertil, 78, 673-678.

Mclaren, A. (1981) Germ Cells and Soma: A new look at an old problem. Yale University Press. New Haven, CT

Mikkelsen, A.L., Smith, S.D. and Lindenberg, S. (1999) In-vitro maturation of human oocytes from regularly menstruating women may be successful without follicle stimulating hormone priming. Hum Reprod, 14, 1847-1851.

Mikkelsen, A.L. and Lindenberg, S. (2001) Benefit of FSH priming of women with PCOS to the In Vitro maturation procedure and the outcome: a randomized prospective study. Reproduction, 122, 587-592.

Mikkelsen, A.L. (2005) Strategies in human in-vitro maturation and their clinical outcome. RBM Online 10, 593-9.

Moor, R.M., Osborn,J.C., Cran,D.G. et al. (1981) Selective effect of gonadotrophins on cell coupling, nuclear maturation and protein synthesis in mammalian oocytes. J Embryol Exp Morphol, 61, 347-365.

Moor, R.M., Dai, Y., Lee, C. et al. (1998) Oocyte maturation and embryonic failure. Hum Reprod Update, 4, 223-236.

Morishige, K., Kurachi, H., Amemiya, K. et al. (1993) Menstrual stage-specific expression of epidermal growth factor and transforming growth factor-alpha in human oviduct epithelium and their role in early embryogenesis. Endocrinology, 133, 199-207.

Munne, S. and Cohen, J. (1998) Chromosome abnormalities in human embryos. Hum Reprod Update, 4, 842-855.

Munne, S., Sandalinas, M., Escudero, T. et al. (2002) Chromosome mosaicism in cleavage-stage human embryos: evidence of a maternal age effect. Reprod Biomed Online, 4, 223-232.

Murray, A., Grønvald, F.C., Nielsen, J.K. et al. (2000) Synthesis of FF-MAS from litocholic Acid. Bioorganic & Medicinal Chemistry Letters, 10, 1067-1068.

Murray, A., Grøndahl, C., Ottesen, J.L. et al. (2002) Meiosis activating sterols derived from diosgenin. Bioorg.Med. Chem.Lett., 12, 715-717. (IX)

Nagy, Z.P., Cecile, J., Liu, J. et al. (1996) Pregnancy and birth after intracytoplasmic sperm injection of In Vitro matured germinal-vesicle stage oocytes: case report. Fertil Steril, 65, 1047-1050.

Neal, M.S., Cowan, L., Louis, J.P., Hughes, E., King, W.A., Basrur, P.K (2002) Cytogenetic evaluation of human oocytes that failed to complete meiotic maturation In Vitro. Fertil Steril, 77, 844-845.

Nebreda, A.R. and Hunt, T. (1993) The c-mos proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of Xenopus oocytes and eggs. Embo J, 12, 1979-1986.

Nogueira, D., Staessen, C., Van de Velde, H. and Van Steirteghem, A. (2000) Nuclear status and cytogenetics of embryos derived from In Vitro matured oocytes. Fertil Steril, 74:295-8.

Palermo, G., Joris, H., Devroey, P. et al. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet, 340, 17-18.

Papanikolaou, E.G., Platteau, P., Albano, C., Nogueira, D., Cortvrindt, R., Devroey, P. et al. (2005) Immature oocyte in-vitro maturation: clinical aspects. RBM Online 10:587-92.

Pellestor, F. (1991) Frequency and distribution of aneuploidy in human female gametes. Hum Genet, 86, 283-288.

Plante, L., Plante,C., Shepherd,D.L. et al. (1994) Cleavage and h-3 uridine incorporation in bovine embryos of high in-vitro developmental potential. Mol Reprod Dev, 39, 375-383.

Prins, G.S., Wagner, C., Weidel, L. et al. (1987) Gonadotropins augment maturation and fertilization of human immature oocytes cultured In Vitro. Fertil Steril, 47, 1035-1037.

Ron-El, R., Liu, J., Nagu Z. et al. (1994). Intracytoplasmic sperm injection in the mouse. Hum Reprod, 10, 2831-2834.

Roudebush,W.E. and Duralia, D.R. (1996) Superovulation, fertilization, and In Vitro embryo development in BALB/cByJ, BALB/cJ, B6D2F1/J, and CFW mouse strains. Lab Anim Sci, 46, 239-240.

Rozman, D., Strömstedt, M., Tsui, L.C., et al. (1996) Structure and mapping of the human lanosterol 14alpha-demethylase gene (CYP51) encoding the cytochrome P450 involved in the cholesterol biosynthesis; comparison of exon/intron organization with other mammalian and fungal CYP genes. Genomics, 38, 371-381.

Rudak, E., Dor, J., Kimchi, M., Goldman, B., Levran, D. and Mashiach, S (1990) Anomalies of human oocytes from infertile women undergoing treatment by In Vitro fertilization. Fertil Steril 54, 292-296.

Russell, J.B. (1998) Immature oocyte retrieval combined with in-vitro oocyte maturation. Hum Reprod, 13 Suppl 3, 63-70.

Sathananthan, A.H. (1984) Ultrastructural morphology of fertilization and early cleavage in the human. In Trounson,A. and Wood,C. (eds), In Vitro fertilization and embryo transfer. Churchil Livingstone, Edinburgh, 131-158.

Sathananthan, A.H. (1994) Ultrastructural changes during meiotic maturation in mammalian oocytes: Unique aspects of the human oocyte. Microscopy Research and Technique., 27, 145-164.

Schmitt, A. and Nebreda,A.R. (2002) Inhibition of Xenopus oocyte meiotic maturation by catalytically inactive protein kinase A. Proc Nat Acad Sci USA, 99, 4361-4366.

Schorderet-Slatkine, S., Schorderet, M., Boquet, P. et al. (1978) Progesterone-induced meiosis in Xenopus laevis oocytes: a role for cAMP at the "maturation-promoting factor" level. Cell, 15, 1269-1275.

Schramm, R.D., Paprocki,A.M., and VandeVoort,C.A. (2003) Causes of developmental failure of in-vitro matured rhesus monkey oocytes: impairments in embryonic genome activation. Hum Reprod, 18, 826-833.

Schroepfer, G.J. (1982) Sterol biosynthesis. Annu Rev Biochem, 51, 555-585.

Smitz, J., Picton, H, Platteau, P., Rutherford, A., Cortvrindt, R., Clyde, J., Nogueira, D., Devroey, P., Lyby, K. and Grøndahl, C. (2007) Principal findings from a multicenter trial investigating safety of FF-MAS for IVM of human cumulus enclosed oocytes. Fertil Steril 87:949-964. (XIV)

Smitz J, Nogueira D, Vanhoutte L, De Matos DG, Cortvrindt R. (2004) Oocyte In Vitro maturation. In: Gardner, Weissman, Howles, Shoham, eds. Textbook of assisted reproductive techniques: Laboratory and Clinical Perspectives: Second Edition. Martin Dunitz LTD, London; Chapter 10: 125-61.

Squires, E.L., Wilson, J.M., Kato, H. et al. (1996) A pregnancy after intracytoplasmic sperm injection into equine oocytes matured In Vitro. Theriogenology, 45, 306.

Steptoe, P.C. and Edwards, R.G. (1978) Birth after the reimplantation of a human embryo. Letter to Lancet, 2, 366.

Strömstedt, M., Waterman, M.R., Haugen, T.B. et al. (1998) Elevated expression of lanosterol 14alpha-demethylase (CYP51) and the synthesis of oocyte meiosis-activating sterols in postmeiotic germ cells of male Endocrinology, 139, 2314-2321.

Sun, Q.Y., Blumenfeld, Z., Rubinstein, S. et al. (1999a) Mitogen-activated protein kinase in human eggs. Zygote, 7, 181-185.

Sun, Q.Y., Rubinstein, S., and Breitbart, H. (1999b) MAP kinase activity is downregulated by phorbol ester during mouse oocyte maturation and egg activation In Vitro. Mol Reprod Dev, 52, 310-318.

Swann, K. and Lawrence,Y. (1996) How and why spermatozoa cause calcium oscillations in mammalian oocytes. Mol Hum Reprod, 2, 388-390.

Szöllösi, D. (1967) Development of cortical granules and the cortical reaction in rat and hamster eggs. Anat Rec, 159, 431-446.

Szöllösi, D. and Hunter, R.H.F. (1973) Ultrastructural aspects of fertilization in the domestic pig: sperm penetration and pronucleus formation. J Anat, 116, 181-206.

Tanghe, S., Van Soom, A., Nauwynck, H. et al. (2002) Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev, 61, 414-424.

Telford, N.A., Watson,A.J., and Schultz,G.A. (1990) Transition from maternal to embryonic control in early mammalian development: A comparison of several species. Mol Reprod Dev, 26, 90-100.

Tesarik, J., Kopecny, V., Plachot, M. et al. (1986) Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing In Vitro. J Reprod Fertil, 78, 463-470.

Tesarik, J., Sousa, M., and Testart, J. (1994) Human oocyte activation after intracytoplasmic sperm injection. Hum Reprod, 9, 511-518.

Trounson, A.O., Pushett, D., Maclellan, L.J. et al. (1994a) Current status of IVM/IVF and embryo culture in humans and farm animals. Theriogenology, 41, 57-66.

Trounson, A.O., Wood,C., and Kausche,A. (1994b) In Vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil Steril, 62, 353-362.

Trounson, A., Anderiesz,C., and Jones,G. (2001) Maturation of human oocytes In Vitro and their developmental competence. Reproduction, 121, 51-75.

Tsafriri, A. and Channing,C.P. (1975) An inhibitory influence of granulosa cells and follicular fluid upon porcine oocyte meiosis In Vitro. Endocrinology, 96, 922-927.

Tsafriri, A., Cao,X., Vaknin,K.M. et al. (2002) Is meiosis activating sterol (MAS) an obligatory mediator of meiotic resumption in mammals. Mol.Cell.Endocrinol., 187, 197-204.

Vaknin, K.M.; Lazar, S., Popliker, M.; Tsafriri, A. (2001) Role of meiosis-activating sterols in rat oocyte maturation: Effects of specific inhibitors and changes in the expression of lanosterol 14α-demethylase during the preovulatory period. Biol Reprod, 64, 299-309

Van Blerkom, J. (1989) Morphodynamics of nuclear and cytoplasmic reorganization during the resumption of arrested meiosis in the mouse oocyte. Progress in Clinical and Biological Research, 294, 33-51.

Van Blerkom, J. (1991) Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. Proceedings of the National Academy of Sciences of the United States Of, 88, 5031-5035.

Van Steirteghem, A.C., Liu, J., Joris, H. et al. (1993) Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive cycles. Hum Reprod, 8, 1055-1060.

Veeck, L.L., Wortham,J.W., Witmyer,J. et al. (1983) Maturation and fertilization of morphologically immature human oocytes in a program of In Vitro fertilization. Fertil Steril, 39, 594-602.

Verlhac, M.H., de Pennart,H., Maro,B. et al. (1993) MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. Develop Biol, 158, 330-340

Verlhac, M.H., Kubiak,J.Z., Clarke,H.J. et al. (1994) Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development, 120, 1017-1025.

Verlhac, M.H., Kubiak, J.Z., Weber, M. et al. (1996) Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. Development, 122, 815-822.

Weston, A.M., Zelinski-Wooten, M.B., Hutchison, J.S. et al. (1996) Developmental potential of embryos produced by in-vitro fertilization from gonadotrophin-releasing hormone antagonist- treated macaques stimulated with recombinant human follicle stimulating hormone alone or in combination with luteinizing hormone. Hum Reprod, 11, 608-613.

Wright, R.W. and Bondioli, K.R. (1981) Aspects of In Vitro fertilization and embryo culture in domestic animals. J Anim Sci, 53, 702-729.

Wu, T.C., Wang, L., and Wan,Y.J. (1993) Detection of estrogen receptor messenger ribonucleic acid in human oocytes and cumulus-oocyte complexes using reverse transcriptase- polymerase chain reaction. Fertil Steril, 59, 54-59.

Wynn, P., Picton, H.M., Krapez, J.A. et al. (1998) Pretreatment with follicle stimulating hormone promotes the numbers of human oocytes reaching metaphase II by in-vitro maturation. Hum Reprod, 13, 3132-3138.

Yamashita, M., Mita, K., Yoshida, N. et al. (2000) Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. Prog Cell Cycle Res, 4, 115-129.

Yoshida, Y. and Aoyama, Y. (1984) Yeast cytochrome P-450 catalyzing lanosterol 14 alpha-demethylation. I. Purification and spectral properties. J Biol Chem, 259, 1655-1660.

Younis, A.I., Keefer, C.L., and Brackett, B.G. (1989) Fertilization of bovine oocytes by sperm injection. Theriogenology, 31, 276.

Zamboni, L., Thompson, R.S., and Smith, D.M. (1972) Fine morphology of human oocyte maturation In Vitro. Biol Reprod, 7, 425-457.

Ziebe, S., Lundin, K., Loft, A., Bergh, C., Nyboe Andersen, A., Selleskog, U., Nielsen, D., Grøndahl, C., Kim, H. and Arce, J.C. (2003) FISH analysis of chromosome 13, 16, 18, 21, 22, X and Y in all blastomeres of embryos from 144 randomly selected donated human oocytes and impact on preembryo morphology. Human Reprod 18(12), 2575-2581.

Zhang, X., Zerafa, A., Wong, J. et al. (1993) Human menopausal gonadotropin during In Vitro maturation of human oocytes retrieved from small follicles enhances In Vitro fertilization and cleavage rates. Fertil Steril, 59, 850-853.