Effect of rescuing donated immature human oocytes derived after FSH/hCG stimulation following in vitro culture with or without Follicular Fluid Meiosis Activating Sterol (FF-MAS)—an embryo chromosomal and morphological analysis

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Abstract *Purpose:* Studies in mice and humans have shown that Follicular Fluid – Meiosis Activating Sterol (FF-MAS) induces meiotic maturation of immature oocytes *in vitro*. A multicenter, prospectively randomised study evaluated chromosomal status of embryos from FSH/hCG primed human immature oocytes, cultured with or without FF-MAS.

Methods: Denuded immature oocytes ($n = 365$) were randomly allocated into inert control, FF-MAS 5 μM or 20 μM . Seventy ± 2 hours after ICSI on matured oocytes, all cleaved embryos were fixed for fluorescence in situ hybridisation analysis.

Results: Only 15% of oocytes resulted in cleaved embryos. GV oocytes matured at significantly lower rates (14% and 7%) in the two FF-MAS groups compared to the inert control group (47%). High rates of chromosomal abnormalities were found in all groups.

Conclusion: Immature oocytes showed poor development with high rates of embryo chromosomal abnormalities. Ex-

posure to FF-MAS in the concentrations, duration and/or formulation used in this study did not improve the results.

Keywords Immature oocytes · FF-MAS · Human · Aneuploidy · In vitro fertilisation

Introduction

In a standard in vitro fertilisation (IVF) cycle, approximately 10–15% of aspirated oocytes will be at the germinal vesicle (GV) or metaphase I stage (MI) [1–2]. Although many of these oocytes manage to resume meiosis *in vitro* and mature to metaphase II (MII) stage within 24 hours, they have a poor developmental prognosis [1–4]. It has been suggested that present standard IVF culture systems support nuclear maturation but not cytoplasmic competence, resulting in embryos with reduced developmental potential [5–7].

Follicular-Fluid Meiosis Activating Sterol (FF-MAS) which is found in high concentrations (around 1.6 μM) in follicular fluid in mammals [8], has been shown to be involved in the control of meiosis [8–10]. In mouse oocytes, addition of FF-MAS to the culture medium induce dose-dependent resumption of meiosis [10–11], and increase the synchrony of cytoplasmic and nuclear maturation [12–13].

In women with polycystic ovarian syndrome the frequency of fully matured MII oocytes was found to be significantly higher in FF-MAS mediated *in vitro* matured oocytes compared to a control group [14]. The aim of this prospective randomised trial was to investigate the safety and feasibility of rescuing immature FSH/hCG primed immature oocytes by exposing denuded GV/MI stage human oocytes to one of two different concentrations (5 μM or 20 μM) of FF-MAS and assessing the rate of chromosomal abnormalities in the cleaved embryos. In addition, the effect of FF-MAS

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Table 1 Patient demography by treatment group

	Inert control <i>n</i> = 123	FF-MAS 5 μ M <i>n</i> = 121	FF-MAS 20 μ M <i>n</i> = 121
Age (years)			
Mean (SD)	31.2 (3.3)	31.7 (3.1)	31.9 (3.2)
Range	24.6–37.5	24.6–38.0	26.0–38.0
BMI			
Mean (SD)	23.5 (3.9)	23.4 (4.2)	23.6 (4.3)
Range	18.6–37.8	17.6–37.8	17.6–37.8
Menstrual cycles (days)			
Mean (SD)	28.8 (1.8)	28.9 (1.6)	28.9 (1.8)
Range	25.0–35.0	25.0–34.0	25.0–35.0
Smoking (number of subjects)			
Yes – past	28 (22.8%)	30 (24.8%)	25 (20.7%)
Yes – currently	35 (28.5%)	26 (21.5%)	30 (24.8%)
No	60 (48.8%)	65 (53.7%)	66 (54.5%)
Alcohol (units per week)			
Mean (SD)	1.9 (2.2)	2.1 (2.4)	2.2 (2.4)
Range	0.0–15.0	0.0–15.0	0.0–10.0

exposure upon maturation and cleavage rates together with embryo morphology was studied.

Material and methods

The study was designed as a multi-center, prospectively randomised, double-blind, controlled in-vitro study. Oocytes were donated by patients from three centres, Rigshospitalet, Copenhagen, Denmark, Sahlgrenska University Hospital, Göteborg, Sweden and Fertilitetscentrum, Carlanderska Hospital, Göteborg, Sweden. Patient demographics are described in (Table 1). No statistical differences were found between the groups.

Altogether 187 women donated 365 oocytes to this trial. Patients were down-regulated with GnRH (Synarel[®], Searle, or Suprefact[®], Aventis Pharma,) for at least 14 days or using short protocols with GnRH antagonists (Orgalutran[®], Organon, Oss, The Netherlands or Cetrotide[®], Serono, Geneva, Switzerland). Patients were stimulated with recombinant FSH (Puregon[®], Organon, Oss, The Netherlands or Gonal-F[®], Serono, Geneva, Switzerland). hCG (10 000IE Profasi[®], Serono; Pregnyl[®], Organon) was administered when ≥ 3 follicles of 17 mm were registered by ultrasound. After denudation oocytes at GV or MI stage were randomised sequentially to one of the three treatments.

The oocytes were cultured in IVF medium (IVF-20, Vitrolife, Gothenburg, Sweden) supplemented with either 5 or 20 mM FF-MAS, or water for injection (inert control). Oocytes that reached metaphase II (MII) at 26 h or 48 h were subjected to intracytoplasmic sperm injection (ICSI). At 20 h post ICSI they were checked for pronuclei and thereafter

transferred to 0.5 ml fresh IVF medium without treatment. Embryo evaluation was performed at 26, 44, 50 and 68 $\pm$ 1 h after sperm injection. A “good quality embryo” was defined as having ≥ 4 cells at 44 hours or ≥ 6 cells at 68 hours, with 0–10% fragmentation, equally sized blastomeres and homogeneous cytoplasm [15].

At 70 $\pm$ 2 h post ICSI all blastomeres from all cleaved embryos were fixated individually on silanized slides (Oncor, Gathersburg, USA, cat.no. S1308) in a HCl/Tween-20 solution (0.01N/0.1%) [15–16]. The cytogenetic analysis of the blastomeres was performed centrally by an independent laboratory (Quest Diagnostics Inc., San Juan Capistrano, CA, USA). Fluorescence in situ hybridisation (FISH) analysis for chromosomes 13,16,18, 21, 22, X and Y was performed by sequential hybridization using Vysis MultiVysion PB and CEP X /CEP Y (alpha satellite) (Vysis, Downers Grove, IL, USA). A genetically overall normal embryo was defined as an embryo with at least 50% of its analysed cells having a single nucleus with a normal constitution.

Approval from The Danish and Swedish Scientific and Ethical Committees were obtained. Written informed consent was obtained from all patients and from both partners. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP).

After approximately three months of enrolment an interim safety analysis was performed by an independent data monitoring board. Based on the conclusive results from the interim analysis, the trial enrolment was discontinued. All data were analysed using SAS version 6.12 (SAS Institute Inc., Cary, US) run on a UNIX platform. Comparison between the groups concerning the incidence of maturation and fertilisation rate was performed using Fishers exact test at the 0.05 level of significance.

Table 2 Cytogenetic analysis results

	Control (<i>n</i> = 115)		FF-MAS 5 μ M (<i>n</i> = 117)		FF-MAS20 μ M (<i>n</i> = 119)	
	GV	MI	GV	MI	GV	MI
Randomised oocytes (minus censored) ^d	83	32	84	32	81	38
Fixated embryos (> 1 blastomere 68h post ICSI)	24	17	2	3	1	5
Fixated blastomeres	106	73	13	11	3	2
Cytogenetically evaluated blastomeres	80	51	7	5	0	5
Multinucleated blastomeres (% of analysed)	41 (51)	18 (35) ^a	5 (71)	1 (20)	–	5 (100) ^b
Normal blastomeres (% of analysed) ^c	4 (5)	8 (16)	0 (0)	0 (0)	–	0 (0)
Overall normal embryos ^c	2	2	0	0	0	0
Overall abnormal embryos	19	3	2	2	0	3
Non-classifiable embryos	3	2	0	1	1	2
Percentage overall normal embryos	10(2/21)	13 (2/15)	0	0	–	0
Uniformly normal embryos	2	1	0	0	–	0

^avs. ^b*p* = 0.008.^cStatistical comparison among the groups not appropriate due to the low numbers.^dOocytes lost during the trial due to technical problems or protocol violation based on deviation of the FF-MAS concentration in the culture media were excluded from the analyses.

Results

A very low number (54/365, 14.8%) of all randomised oocytes matured, fertilised and cleaved (Table 3). In the FF-MAS groups no blastomeres/embryos were of normal diploid status, while in the control group three oocytes developed into uniformly normal embryos. The rate of multinucleation was high, ranging from 20–100% (Table 2).

The number of germinal vesicle oocytes maturing to metaphase II stage was significantly (*p* < 0.0001) lower in the FF-MAS 5 μ M and 20 μ M groups compared to the inert control group. For metaphase I stage oocytes there was

no significant difference in maturation rate, but significant differences in fertilisation/cleavage rates between the MI control and the MI FF-MAS groups (Table 3).

Due to the low numbers good quality embryos at 44 and 68 hours post ICSI, statistical evaluation was not considered appropriate.

Discussion

The primary endpoint of this safety study was to assess possible cytogenetic effects of adding FF-MAS to standard IVF

Table 3 Maturation, fertilisation and embryo development

	Control (<i>n</i> = 123)		FF-MAS 5 μ M (<i>n</i> = 121)		FF-MAS20 μ M (<i>n</i> = 121)	
	GV	MI	GV	MI	GV	MI
No of oocytes	88	35	87	34	83	38
Maturation to MII at 4 h (%)	2 (2)	12 (34)	0 (0)	16 (47)	2 (2)	18 (47)
Maturation to MII at 26/48 h (%) ^a	41 ¹ (47)	25 (71)	12 ² (14)	21 (62)	6 (7)	27 (71)
Fertilisation at 20/26 h (% of ICSI) ^b	27 (66)	20 (80)	4 (33)	7 (35)	2 (33)	12 (44)
Cleaved (% of fertilised) ^c	24/27 (89)	18/20 (90)	2/4 (50)	4/7 (57)	1/2 (50)	5/12 (42)
4–6 cells 44 h post ICSI ^d	7	7	0	1	0	1
GQE 44 h post ICSI ^d	1	2	0	0	0	0
≥ 6 cells 68 h post ICSI ^d	5	1	0	1	0	0

¹Two oocytes matured at 48 h.²One oocyte matured at 48 h.^aMaturation at 26 h: GV control vs. FF-MAS 5 μ M, *p* = < 0.0001, GV control vs. FF-MAS 20 μ M, *p* = < 0.0001. MI control vs. MI treatments = NS.^bFertilisation 20/26 h: GV control vs. GV FF-MAS 5 μ M and vs. GV FF-MAS 20 μ M = NS. MI control vs. FF-MAS 5 μ M, *p* = 0.024, MI control vs. FF-MAS20 μ M, *p* = 0.010.^cCleaved: GV control vs. GV FF-MAS 5 μ M and vs. GV FF-MAS 20 μ M = NS. MI control vs. FF-MAS 5 μ M = NS, MI control vs. FF-MAS20 μ M, *p* = 0.0057.^dStatistical comparison among the groups not appropriate due to the low numbers.

media when culturing hCG primed immature oocytes. Although, due to the low number of embryos that matured, fertilised and cleaved in this study, only a small proportion of all randomised oocytes could actually be analysed by FISH (Table 2), the results showed a high chromosomal and nuclear error rate in general.

The secondary goals, to investigate the effect of FF-MAS upon maturation and embryo development, showed that denuded immature human oocytes aspirated 36–38 h after hCG do not benefit from exposure to FF-MAS. At the time of this study, FF-MAS was only available in an ethanol-based solution. This had been used in other studies with denuded human oocytes [17–18], without any demonstrated negative effect on embryo development or chromosomal status. In the study by Cavilla et al. [17] increased maturation and fertilisation rates of denuded human GV oocytes was shown after supplementation with FF-MAS (in an 0.5% ethanol formulation) to the culture medium. However, in a recent similar study by Loft et al (2004), where cumulus-enclosed oocytes were used, it was found that the ethanol control group had a significantly lower number of chromosomally normal blastomeres than the inert control group [19]. This was not seen in a later study [20], where a new formulation of FF-MAS (with HSA replacing ethanol) was used.

Thus, it is concluded that a majority of hCG exposed oocytes that have not reached MII at oocyte pick-up are defect. This may be the main reason for the poor success rates, and for the lack of positive results from adding FF-MAS, and suggests that FSH/hCG exposed oocytes retrieved in the GV or MI phase should not be used in ART programmes.

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