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PhD Thesis

"Impact of different gonadotrophins preparation on human oocyte morphology in intracytoplasmic sperm injection "

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BACKGROUND AND AIM OF THE PROJECT

The standard long protocol GnRH agonist regimen is a well established strategy for controlled ovarian stimulation (COS) in young normogonadotrophic women undergoing IVF/ICSI (Hughes et al., 1992; Tan et al., 1992). In such a protocol, GnRH agonist administration is primarily followed by 'monotherapy' with highly purified urinary FSH (FSH-HP) or recombinant human FSH (r-FSH). Thus, endogenous LH secretion is suppressed with no exogenous LH activity.

Nevertheless, this approach is effective in almost all women, suggesting that residual circulating levels of endogenous LH are usually adequate to support multiple follicular growth and oocyte development (Loumaye et al., 1997; Sills et al., 1999). About 12–14% of these patients treated with a depot GnRH agonist, an abnormal ovarian response profile to this protocol is observed (De Placido et al., 2001; 2004) . In particular, an initial follicular growth [at least six follicles ≥6 mm in diameter on the fifth day of stimulation in association with a ≥2-fold increase in estradiol (E2) levels with respect to the value observed on the day of pituitary suppression assessment] is followed by a plateau phase, in which no significant change in follicular diameter and a suboptimal increase in E2 levels are observed between days 5 and 8 of stimulation, despite the use of age and body mass index (BMI)-appropriate dosage increase of r-FSH. This condition (steady response) usually leads to an increase in the r-FSH daily dose and results in a suboptimal ovarian response with a large consumption of r-FSH. It has been recently hypothesised that suboptimal ovarian response to r-FSH may also be due to excessive pituitary LH suppression. In such cases, an improvement in the ovarian outcome from LH-containing gonadotrophin preparations would be expected (Laml et al., 1999; Levy et al., 2000; De Placido et al., 2001,

2004). This hypothesis is consistent with previous data demonstrating that in young normogonadotrophic patients undergoing a GnRH agonist long protocol, cycles displaying the above-mentioned pattern of initial steady response to r-FSH are rescued by administering HMG, an extractive preparation of FSH and LH in a 1:1 ratio, from the eighth day of stimulation. The use of hMG as a source of LH supplementation results in an increase in the FSH dosage as well. Interestingly, women who benefited from exogenous LH supplementation showed endogenous hormonal levels during the early stimulation that were comparable with those observed in age and BMI matched subjects showing an adequate response to monotherapy with r-FSH. Taken together, these data represented the first clinical evidence of the need for LH supplementation in specific patient groups, despite the lack of a priori indications. Furthermore, these findings were consistent with previous experimental and clinical lines of evidence indicating that immunoreactive LH is not necessarily representative of LH bioactivity (Mitchell et al., 1995). With the recent availability of recombinant human LH (r-LH), clinicians have the opportunity of administering the two gonadotrophins independently. Thus, exogenous LH administration may be calibrated independently of r-FSH, preliminary prospective randomized trial demonstrated that a daily r-LH dose of 150 IU resulted in a significant increase in the mean number of oocytes retrieved when compared with 75 IU in women displaying steady response to r-FSH (De Placido et al., 2004).

In conclusion, the aim of the present project is to study the influence of different ovarian stimulations.

Based on this preliminary studies, the aim of this present projects is to study the:

- The efficacy of a supplementation of r-LH versus an r-FSH GnRH agonist long protocol in normogonadotrophic women with a normal and 'steady response' to monotherapy with r-FSH on the maturity and number of recruited oocyte undergoing IVF cycles.

- The efficacy of a supplementation of r-LH on oocyte cytoplasmic maturity in terms of 'funnel effect' in normogonadotrophic women with a normal and 'steady response' to monotherapy with rFSH.

- The importance of the oocyte scoring on zygotes and embryo quality in patients undergoing IVF/ICSI cycles.

CHAPTER 1

LH effect on oocyte morphology and quality in normogonadotrophic "normal responders" to r-FSH patients

INTRODUCTION

Physiology of gonadotrophins

About 10% of the anterior hypotalamus is constituted by gonadotrophin cells. They produce two gonadotrophins, the luteinising hormone (LH) and the follicle stimulating hormone (FSH). Analogously to the thyroid stimulating hormone (TSH) and to the human chorionic gonadotrophin (hCG), LH and FSH are two glycoprotein hormones characterized by two subunit α e β. The α subunit is common to all these glycoprotein hormones, while the specificity is conferred by the subunit β, coded by various genes. The synthesis and release of the gonadotrophins are a phenomena regulated in a dynamic manner. This can be seen particularly in woman whose changes in levels rapidly fluctuate during a menstrual cycle. The hypothalamic releasing factor of gonadotrophins [*gonadotrophin releasing hormone* (GnRH)], consist of a 10 aminoacids peptide synthesized in the preoptic area, which regulates the synthesis and the secretion of both gonadotrophins. GnRH secretion is pulsatil within a 60-120 minutes interval, such impulses, determine those of LH and FSH. The GnRH acts through a receptor combined with a G protein which, once divided, activates the phosfolipase and proteinkhinase C pathway, in addition to intracellular calcium. GnRH pulsatile secretion is very important since it determines the priming of the gonadotrophin cells, while its continuous production causes desensibilization. The GnRH analogous (GnRH-a) are used when continous administration is required, in order to suppress the pulsatility action of the gonadotrophins

in: children with premature puberty, in men with prostate cancer, women with endometriosi (I-IV stage) and in the treatment of the uterine fibroids. GnRH analogous are also used in some stimulation induction protocols in order to reduce endogenous gonadotrophin production and thus allow exogenous hormonal control. The estrogens on the hypothalamic and hypofisary level in order to regulate the gonadotrophic secrection. Indeed, a chronic exposure to estrogens has an inhibitory effect either on the hypothalam or hypofisary function. Increasing levels of estrogens, as can be observed during the pre-ovulatory peak stimulate a positive feedback, thus increasing the range and the frequency of gonadotrophin secrection. On the contrary, progesterone reduces pulsation frequency, and adversely enhances gonadotrophin response at GnRH.

Eventhough GnRH is the principal factor in LH and FSH regulation and secrection, its synthesis is controlled by activin and inhibin. The aforementioned belong to the family of the growth factors (TGF- β). Inhibin acts selectively in opposition to FSH secrection, while activin stimulates its synthesis. Gonadotrophins, binding to receptors expressed on the ovary and testicle, respectively, induce the germinal cell development and maturity, thus increasing the biosynthesis of the sexual hormones. In women, FSH regulates follicle development and stimulates the estrogen production in the ovary. Instead, LH is responsible for ovulation and maintenance of the luteal phase. In man, LH induces the synthesis and the secrection of testosterone via Leydig cells, while FSH stimulates the development of seminiferous tubules and regulates spermatogenesis.

1.2 Role of FSH and LH in folliculogenesis

FSH has a key role in reproductive function: in males it is essential for Sertoli cell function and spermatogenesis, and in females it stimulates the growth of a large pre-ovulatory follicle that, because of its FSH-dependent maturation, is able to.ovulate and to form a corpus luteum in response to the mid-cycle surge of LH.

Follicle development from the primordial to the pre-ovulatory stage takes several months (Gougeon, 1996). The initiation of growth of the primordial follicles takes place continuously and it appears to be independent of pituitary gonadotrophins. In vitro studies show that other factors are probably involved in the early stages of follicular development and apoptosis (programmed cell death): activin, transforming growth factor- β, bone morphogenetic proteins, growth and differentiation factor-p, oestrogens, androgens, insulin and insulin-like growth factor-I (Miro and Hillier 1996; Touraine et al., 1999; McGee and Hsueh 2000; Zeleznik 2004). The role of these factors *in vivo* remains uncertain.

Early antral follicles become FSH responsive and constitute a pool of available follicles that can be stimulated to growth. The final destiny of the majority of these follicles will be atresia, except for a single follicle that will grow until final maturation to the pre-ovulatory stage.

At the end of the luteal phase, early antral follicles (2-5 mm in diameter) are present. Granulosa cells of these early antral follicles seem more sensitive to FSH stimulation.

During the luteo-follicular transition, due to the demise of the corpus-luteum and the subsequent decrease in oestrogen production, the FSH serum concentration rises (perimenstrual rise), maintaining a plateau in the first days of the follicular phase. FSH must reach a threshold: a

critical concentration of FSH must be achieved to initiate the process of follicular development. In anovulatory women changes of 10-30% are sufficient to initiate follicular development (Brown, 1978).

From mid-follicular through late-follicular phase, follicular selection is achieved: granulosa cells acquire aromatase activity. This enzymatic activity leads to a rise in oestradiol (and inhibin) and, as a consequence, a progressive fall in FSH serum concentration (negative feed-back). The mid-follicular fall in FSH concentration determines the atresia of less mature follicles that are unable to grow without adequate FSH concentrations. The maturing selected follicle continue to develop because it acquires less dependency on FSH by increased sensitivity to FSH itself. It has been demonstrated that FSH induces LH receptor formation on pre-ovulatory follicle granulosa cells (Zeleznik et al., 1981). The action of LH on its receptors activates adenyl cyclase: the consequent production of cAMP represents an additive response to FSH (Goff et al., 1977). Therefore, the maturing follicle reduces its dependency on FSH by acquiring LH receptors and LH responsiveness (Fauser and Van Heusden 1997; Willis et al., 1998; Campbell et al., 1999; Sullivan et al., 1999; Filicori et al., 2002). On the other hand, lower concentrations of FSH bring less mature follicles to atresia. The granulosa cells from early antral follicles are only responsive to FSH; granulosa cells from FSH-stimulated follicles are responsive to either FSH or LH (Zeleznik and Hillier, 1984).

LH plays a key role in the intermediate-late phases of folliculogenesis. According to the 'two-cells-two- gonadotrophin's model (Fevold, 1941; Hillier et al., 1994) LH plays a key role not only in theca cell steroidogenesis stimulation, but also in follicle growth and maturation. LH exerts its activity in theca cells, which form the involucres of the

growing follicles and express enzymatic pathways of androgen synthesis. The theca wall surrounds the granulosa cells, whose activities and proliferation are directly regulated by FSH. This hormone induces the expression of the aromatase enzyme which in turn, converts theca-deriving androgens into oestradiol. This theory reinforced the notion that granulosa and theca cells are distinct compartments regulated by FSH and LH respectively. However, it was subsequently found, in different line research, that LH receptors are detectable on the granulosa compartment (Shima et al., 1987) at the intermediate follicular phase (Erickson et al., 1979; Filicori et al., 2003a), at a time when blood concentrations of LH increase. In particular both oestradiol and FSH seem to cooperate in inducing the expression of these receptors. Therefore, it appears that LH regulates both granulosa and theca cells. FSH and LH induce the local production of the soluble molecule inhibin B and growth factors. Among these, insulin-like growth factors (IGF)-1 and -2, which are expressed by both granulosa and theca cells throughout folliculogenesis, are important in promoting follicular maturation (Zhou and Bondy, 1993; Huang et al., 1994). Thus, gonadotrophins and the autocrine-paracrine system contribute to the complex mechanisms governing follicular growth and selection. The finding that both gonadotrophins also regulate granulosa cell activity suggests that LH is involved in inducing and maintaining this paracrine system of biochemical factors by acting on the theca and granulosa compartments. These findings may explain the observation that FSH activity can be totally substituted by LH once granulosa cells express adequate amounts of LH receptors (Zeleznik and Hillier, 1984; Filicori et al., 2002).

Hence, LH seems to play two roles during folliculogenesis. The first one consists of an induction of androgen production exerted in the theca

compartment. The second one, consists of inducing the local production of various molecules that begins during the intermediate follicular phase (Willis et al., 1998; Filicori et al., 2003a) and involves granulosa cells. These factors promote the growth of granulosa cells which in turn, regulate oocyte maturation. These two mechanisms are closely related and probably support one another. This observation also leads to hypothesise that these paracrine activities are able to enhance LH activity on theca compartment: according to the so called 'spare receptor hypothesis' (Chappel and Howles, 1991), when inhibin B and IGF-l are adequate1y secreted, androgen synthesis and release are optimal even if less than 1% of LH receptors are occupied. LH has been implicated in a third process during folliculogenesis. In particular, it has been suggested that this hormone may contribute negatively on the selection of non-dominant follicles. This idea was based on the observation that, following the mid-cycle LH surge, granulosa cell mitosis is blocked and oocyte meiosis is resumed (Shoham et al., 1995). Preclinical evidence showed that developing follicles have specific requirements for exposure to LH beyond which, normal maturation ceases (Hillier et al., 1994). This finding gave rise to the concept of an 'LH ceiling', meaning that, each follicle would have an upper lirnit of stimulation. The LH ceiling may be higher in larger follicles and lower in smaller ones. As a consequence, an increasing LH concentration would promote leading follicle progression (being below its ceiling) and degeneration of secondary ones (by overcoming their ceiling). According to others, a dynamic interplay between LH secretion and receptor expression by different ovarian compartments, governs the selection of dominant follicles. Due to the fact that small follicles in granulosa cells do not express LH receptors, LH may indirectly promote their degeneration.

1.3 LH and ovarian stimulations

The gold standard for ovarian stimulation in young, normogonadotrophic women is the gonadotrophin-releasing hormone agonist (GnRH-a) long protocol (Hughes et al., 1992). Exogenous FSH is administered only when a GnRH-a mediated suppression of the hypothalamus-pituitary-gonad axis is achieved. Moreover, monotherapy with recombinant human FSH (r-FSH), which is free of LH activity, is used in most cases. The degree of pituitary suppression also depends on the GnRH-a formulation, dose and way of administration (Westergaard et al., 2001). Following suppression, LH concentrations usually range between 0.5 and 2.5 IU/l. These concentrations often fall by <0.5 IU/l during the intermediate late stages of stimulation. Thus, multiple follicular growth is often induced in a low endogenous LH environment, an adequate ovarian response is achieved in almost all patients.

A hiatus appears to exist between IVF practice and information deriving from folliculogenesis models and hypogonadotrophic hypogonadism. In particular, in the former situation, follicular growth normally occurs even when LH concentrations are below 0.5 IU/l. In contrast, there is evidence that in women with hypogonadotrophic hypogonadism, LH concentrations of 1.2 IU/l are required in order to achieve adequate ovarian response to r-hFSH. These discrepancies can be attributed to biological events that involve steroidogenic dynamics and follicular growth. Regarding stimulated cycles, the 'spare receptor hypothesis' (Chappel and Howles, 1991) can be invoked: even when pituitary desensitization is achieved, circulating concentrations of LH are able to occupy an adequate percentage of receptors and to elicit sufficient androgen release. FSH-dependent paracrine activities, including the production of inhibins and IGF-l, may favour adaptation mechanisrns by

enhancing theca sensitivity to LH. In this context, a low LH environment would be advantageous during stimulation, LH-induced suppression of small follicles is not required. Furthermore, during the early-intermediate stages of follicular growth, low LH concentrations have been associated with a more physiological endometrial proliferation, which in turn seems to synchronize this compartment for successive embryo implantation (Kolibianakis et al., 2004).

At the present time, due to the low number of published studies, no definite conclusion can be drawn regarding r-LH adjunctive treatment effectiveness. It has been suggested that the use of LH adjunctive therapy in late follicular phase could be helpful in 'hyporesponsive' patients. These patients need more FSH ampoules and more days of treatment to achieve adequate follicular growth compared with normoresponsive patients. This favourable effect of exogenous LH in this subgroup of patients might be due to an excessive suppression in endogenous LH concentrations due to GnRH agonist or antagonist co-treatment (Ferraretti et al., 2004; De Placido et al., 2005). A possible beneficial role has also been suggested in patients over 35 years old (Marrs et al., 2004). LH co-treatment has also been studied in poor responder patients, but apparently with no benefit (Chung et al., 2005).

1.4 MII Oocyte assessment

Metaphase II oocytes collected from patients following ovarian stimulation show a variety in morphological characteristics. The most important processes that should be completed in a well-coordinated and synchronized manner are the nuclear and cytoplasmic maturation. Both types of maturation are highly susceptible to failures in hormonal supply and/or *in-vitro* culture conditions (e.g. pH, temperature, oxygen) that may cause alterations in oocyte morphology, with some of these anomalies being visible at light-microscopic level (Gaulden et al., 1992; Van Blerkom et al., 1997; Hu et al., 2001). Nuclear maturity is shown by the resumption of meiosis and the progression to metaphase II, the natural arresting point prior to ovulation. Two molecules have been identified supporting the transition from G2 to M phase of the cell cycle, cyclin B and $p34^{cdc2}$. Soon after $p34^{cdc2}$ dephosphorylation, they associate and form the maturation promoting factor (MPF) (Norbury and Nurse, 1992). Active MPF activate nuclear maturation (e.g. germinal vesicle breakdown) and condensation of metaphase I chromosomes. A second peak in MPF activity will then drive the oocyte to metaphase II (Eppig, 1996). Although oocyte are competent to complete nuclear maturation, they could still be deficient in cytoplasmic maturation. Both of which are important for oocyte activation, adequate fertilization, as well as, further preimplantation development. Studies on human and animal models show that the gamete has to obtain competence to release intracellular stores of calcium (hamster; Fujiwara et al., 1993) and cortical granules (mouse; Eppig, 1996); glutathione concentration has to be increased (pig; Yoshida, 1993), and special patterns of active mitochondria have to be expressed (Wilding et al., 2001; Barritt et al., 2002). Asynchrony of these processes may result in different morphological abnormalities, depending on

whether nuclear or cytoplasmic maturation has been affected. In this respect, it has been suggested that dysmorphic features occurring in early meiotic maturation, may be associated with a higher frequency of aneuploidy and fertilization failure, while, those occurring in late maturation may cause a higher incidence of developmental failure. In fact, more than half of the gametes collected show morphological abnormalities, some of which seem to be correlated with an impaired outcome (e.g. aggregation of endoplasmic reticulum, vacuolization, increased ooplasmic viscosity, giant eggs). Therefore, it is strongly recommended to include oocyte quality in all scoring systems applied in IVF laboratories.

The most distinctive parameter for oocytes that did not resume meiosis, is the presence of the prophase I germinal vesicle (GV), which identifies the cohort with a diploid chromosomal set. After GV breakdown, oocytes reach metaphase l stage characterized by the absence of the first polar body extrusion. Though some of these intermediate oocyte may even be fertilizable (De Vos et al., 1999), the formation of the first polar body is the only prognostic factor to assume that a gamete has completed nuclear maturation, i.e. showing a haploid set of chromosomes.

First polar body

As a result of a normal hormonal ovulation induction, at the time of oocyte retrieval, most of the oocytes should have a regular size and a correct nuclear maturation, showned by the extrusion of the first polar body in the perivitelline space. Though some first polar bodies in humans remain intact for more than 20h after ovulation, first polar bodies in eutherian mammals generally have a shorter life (Ortiz et al., 1983). Taking this time dependency into consideration one may postulate that first polar body morphology provides adequate information on the

actual post-ovulatory age of the corresponding oocyte. (Xia, 1997) analysed fertilization rate and embryo quality in oocytes with varying perivitelline space size and first polar body appearance. In this study, optimal oocytes were characterized by an intact first polar body and a small perivitelline space. Ebner (2000) group tried to focus exclusively on the status of the first polar body. The data obtained indicate that oocytes with an intact well-shaped first polar body yield better fertilization rates and embryo quality. This in turn gives higher implantation and pregnancy rates (Ebner et al., 1999). However, in other works neither fertilization rate, embryo quality or outcome were found to be affected (Ciotti et al., 2004; De Santis et al., 2005). It has to be taken into account that in this paper intracytoplasmic sperm injection (ICSI) was performed up to 9h after oocyte collection, a situation that is hardly comparable to previous work (Xia, 1997; Ebner et al., 2000; 2002a). Verlinsky's group (Verlinsky et al., 2003) genetically analysed embryos deriving from different polar body classes. As expected, no correlation was observed between polar body shape and genetic constitution; however, the only polar body group bearing a theoretical risk of chromosomal disorder, considering the larger volume of ooplasma in huge polar bodies, was not analysed.

Interestingly, oocytes showing a first polar body some times may not have finished nuclear maturation. In fact, it has been found that human oocytes with a polar body but without a birefringent spindle may still be at telophase I or prometaphase I stage (Eichenlaub-Ritter et al., 2002; De Santis et al., 2005).

MATERIALS AND METHODS

2.1 Patients

Among candidates for IVF–embryo transfer or ICSI cycles, only patients aged 18–37 years, with menstrual cycles ranging from 24 to 35 days (intra-individual variability ±3 days), basal FSH (day 3 of a spontaneous menstrual cycle) concentrations ≤9 IU/l, and hysteroscopic evidence of a normal uterine cavity within the last 6 months were included. In addition, only women undergoing a GnRH agonist long protocol followed by rFSH administration were enrolled.

The following exclusion criteria will be adopted: body mass index [BMI = weight (kg)/height (m^2)] <18.0 and >28.0, biochemical and/or ultrasonographic evidence of polycystic ovarian syndrome (PCOS), stage III–IV endometriosis according to the rAFS (1985), chromosomal abnormalities, endocrinological and/or autoimmune disorders, more than two previously unsuccessful IVF or ICSI cycles, and presence of only one ovary. Moreover, patients who displayed one to four follicles of diameter >10mm or no follicle >10mm but serum E_2 levels ≥180 pg/ml on day 8 of stimulation.

A prospective randomized controlled trial will be conducted. Patients with a normal response to rFSH will be randomized to two treatment groups : the first one undergoing an rFSH protocol (group A), the second one undergoing an r-LH protocol (group B). Cinicians will perform oocyte collection and I will observe oocyte morphology of each group.

2.1 Ovarian stimulation protocols

All patients will undergo a GnRH agonist long protocol, a well established strategy for COS in young, normogonadotrophic women (Hughes et al., 1992). Pituitary desensitization was induced with the administration of the GnRH agonist triptorelin (Decapeptyl 3.75 mg depot; Ipsen S.P.A, Italy) on the first day of the menstrual cycle. After 15 days, patients undergo transvaginal ultrasound evaluation and biochemical evaluations. Subjects showing serum E_2 level $\leq$40 pg/ml, endometrial thickness $\leq$5mm and arrested follicular development started gonadotrophin administration. Women with delayed suppression (including subjects who developed ovarian cysts after the GnRH agonist administration) will be excluded from the study. A fixed daily dose of 150 IU of rFSH (Gonal-F; Serono Pharma, Italy) was administered. On the fifth day of stimulation, serum E_2 levels were measured and the daily rFSH dose was reduced by 75 IU in patients showing concentrations >180 pg/ml. On the eighth day of stimulation serum E_2 concentrations will be measured and follicular growth monitored with a transvaginal scan, and thereafter on alternate days until hCG will be administered. Patients satisfying inclusion and exclusion criteria and characterized by a tripling of serum E_2 levels between days 5 and 8, and with more than four follicles >10mm in diameter on day 8, will continue current rFSH dose and the other group will add r-LH to their stimulation protocol.

2.3 Oocyte maturity evaluation

Oocytes had been retrieved under conscious sedation by transvaginal ultrasound-guided aspiration 35-36 h after the hCG injection. After incubation for 2-4 h in drops of IVF medium under oil at 37°C and 5% CO2 incubator, oocyte are going to be expose to 80 IU/ml hyaluronidase (MediCult, Copenhagen, Denmark) facilitating mechanical removal of the cumulus cells.(Ebner et al., 2001). At this time every oocyte will be separate in numbered drops and will be evaluated, under inverted microscope equipped with a Hoffman, for maturation stage metaphase II (MIl), metaphase I (MI) or germinal vesicle (GV).

2.4 Statistical analysis

The results are reported as the mean ± SD. Data were analysed with the SPSS version 12.0 (SPSS Inc., USA). One-way ANOVA was used to determine the effect of the stimulation protocol on continuous variables. The *post hoc* The Mann–Whitney *U*-test was applied to test differences between groups for continuous variables with non-parametric distributions. χ^2 statistics were used to compare discontinuous data. A p value <0.05 was considered statistically significant.

RESULTS

Demographic, anthropometric and hormonal characteristics were comparable between groups (Table 1). A total of 247 oocyte in the and 194 oocyte in group B were collected. Patients treated with r-hFSH plus r-hLH showed a significantly higher percentage of mature oocytes (p <0.025) (Table 2). In order to evaluate if r-hLH may differently affect oocyte quality according to age, patients were "a posteriori" divided in two subgroups: women ≤30 years constituted the group 1, whereas patients >30 represented group 2. Thus, four subgroups have been identified: subgroup 1A (n = 12) included younger women treated with r-FSH; subgroup 1B (n = 11) was constituted by young patients stimulated with r-FSH plus r-LH. Women >30 years treated with r-FSH and r-FSH plus r-LH were included in the groups 2A (n = 10) and 2B (n = 9), respectively. Demographic, anthropometric and hormonal characteristics among groups were similar (data not shown). Comparisons of percentages of mature oocytes between groups 1A and 1B and between 2A and 2 B are shown in tables 3 and 4, respectively.

Characteristic	**Group A** (n = 22)	**Group B** (n = 20)
Age (years)	32.6 ± 3.4	33.9 ± 3.7
Body Mass Index [weight(kg)/ height(m)^2]	24.6 ± 3.2	24.2 ± 3.4
Duration of infertility (years)	3.1 ± 0.91	2.9 ± 1.1
Basal FSH (IU/l)	6.9 ± 2.8	6.2 ± 1.9
Basal LH (IU/l)	4.7 ± 1.4	4.9 ± 1.2
Basal Estradiol (pg/ml)	52.7 ± 15.9	48.1 ± 17.4

Table 1. Characteristics of the two study groups

Numbers are mean ± SDs, except where differently indicated. One-way ANOVA was adopted for continuous variables. No statistically difference was revealed between groups for each of the analyzed.

	Group A (r-FSH)	Group B (r-FSH + r-LH)
N° oocyte retrieved	247	194
Percentage of MII oocyte* (n)	76.5 (189)	23.5 (165)

Table 2. MII Oocyte distribution in the two groups

*X^2 $p = 0.03$

	Group 1A (r-FSH)	Group 1B (r-LH)
N° oocyte retrieved	114	99
Percentage of MII oocytes* (n)	75.4 (86)	24.6 (80)

Table 3. Impact of different gonadotrophins on patients ≤30 years

*X^2 $p = 0.43$

	Group 2A (r-FSH)	Group 2B (r-LH)
N° oocyte retrieved	133	95
Percentage of MII oocytes* (n)	77.4 (103)	89.5 (85)

Table 4. Impact of different gonadotrophins on patients >30 years

*X^2 $p = 0.03$

CHAPTER 2

LH effect on oocyte cytoplasmic quality in terms of "funnel effect" in normogonadotrophic "normal responders" to r-FSH patients

INTRODUCTION

Oocyte cytoplasm

Cytoplasmic maturation involves numerous metabolic and structural modifications in preparation for subsequent fertilization (Eppig et al., 1994).

Extensive cytoplasmic granularity may either be homogeneous, affecting the whole gamete, or centrally located. The latter was found to be negatively correlated with ongoing pregnancy rate (Kahraman et al., 2000). In contrast, slight or moderate granularity has been accepted as a normal feature of oocytes. It is conceivable that an increased viscosity of the cytoplasm may constrain cell organelles and/or pronuclei in their movement preventing the zygote from achieving alignment of both pronuclei or alignment of pronuclei with respect to the polar bodies, thereby severely impairing polarity and further preimplantation development (Edwards and Beard, 1997; Garello et al., 1999). Though it has been reported that granular areas are more viscous than the surrounding cytoplasm (Payne et al., 1997) there is a lack of markers of increased cytoplasmic viscosity. MII oocytes of good morphology should show a clear and colourless cytoplasm with moderate granulation and no inclusions; however, more than half of all human (Ebner et al., 2003a), as well as primate oocytes (Suzuki et al., 2004), show at least one morphological abnormality. Intracellular pressure and ooplasm fluidity can only be estimated by the extent to which the ooplasm rises within the injection

pipette immediately following penetration of the oolemma. Ooplasm of higher viscosity is more likely to adhere to the spike of the injection pipette (Ebner et al., 2001) and the persistence of the injection funnel after ICSI may reflect a deficiency in cytoplasmic texture. The injection funnel is likely to be responsible for sealing the breach during injection (Kimura and Yanagimachi, 1995; Palermo et al., 1996). The absence of such a protective mechanism in oocytes with sudden breakage (without any injection funnel at all) causes an increase in oocyte degeneration (Palermo et al., 1996; Ebner et al., 2001). Increased viscosity of the cytoplasm (persistent funnel) may keep it from leakage, whereas oocytes with more aqueous cytoplasm may tend to leak more frequently after intracytoplasmic oocyte injection (ICSI). Altered flux characteristics of cytoplasm does not allow the oocyte to restore its original spherical shape after the sprematozoon injection as fast as it is seen in gametes with a more aqueous ooplasm. This phenomenon is possibly due to a decreased fluidity of the cytoplasm, which may result in a reduced intracellular pressure. Since differences in ooplasm viscosity have been observed (Payne et al., 1997) it is possible that alterations in viscosity may delay abuttal of pronuclei by severely impairing microtubule organization. In contrast, a more fluid cytoplasm would promote optimal conditions in preparation for further preimplantation development. As a consequence, viscous oocyte had a negative influence on developmental competence since zygotes quality (optimal pronuclear pattern, presence of a halo) was severely impaired (Ebner et al., 2003b).

MATERIALS AND METHODS

Patients

For this study, only patients aged 18–37 years, with menstrual cycles ranging from 24 to 35 days (intra-individual variability ±3 days), basal FSH (day 3 of a spontaneous menstrual cycle) concentrations ≤9 IU/l, and hysteroscopic evidence of a normal uterine cavity within the last 6 months were included. In addition, only women undergoing a GnRH agonist long protocol followed by rFSH administration were enrolled. Exclusion criteria and ovarian stimulation protocol had already been specified in chapter 1.

ICSI procedure

Oocytes had been retrieved as shown in chapter 1. The three best oocyte will be injected with the spermatozoon while the remaining one will undergo a MOCK ICSI. With this procedure we can observe the presence of a 'funnel effect' without injecting a spermatozoon. The ICSI procedure is carried out by using an injection pipette with an angle of approximately 30° with a 2 μm spike. A 140° bend at 1 mm from the pipette permitted the terminal end of both pipettes to operate almost parallel to the bottom of the injection dish. The oocyte was held in place by the holding pipette via suction on the *zona pellucida* at 9 o'clock, maintaining the inferior pole of the oocyte in contact with the dish as an additional point of support while injecting the pipette at 3 o'clock.

Funnel effect assessment

For this study, one embryologist performed the ICSI, while another observed the procedure on a video and recorded the membrane patter of each oocyte. The spike and initial part of the bevel then pierced the inner

surface of the *zona* and came in contact with the oolemma. At this point, we observed two distinct patterns of membrane reaction. In some cases, the tip penetrating the membrane brakes the oolemma without creating a funnel, which we termed 'easy breakage'. Sometimes, the penetration proceeded normally to 9 o'clock, creating a funnel-shaped invagination and the membrane ruptured at the approximate centre of the oocyte, which we termed 'normal breakage'.

Statistical analysis

The results are reported as the mean ± SD. Data were analysed with the SPSS version 12.0 (SPSS Inc., USA). Statistical analysis was performed as described in the chapter 1. A p value <0.05 was considered statistically significant.

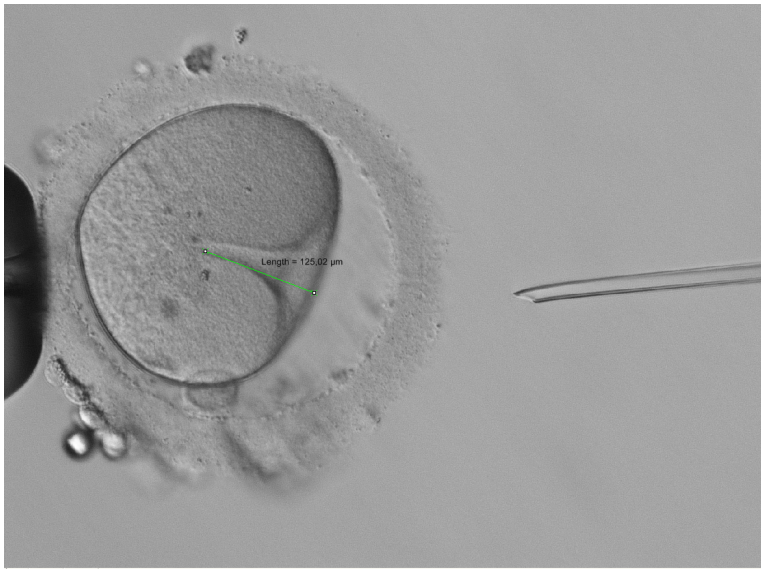


Fig. 1 Presence of a funnel effect in an injected MII oocyte

RESULTS

Characteristics of study were comparable as reported in Table 1.

In a total of 135 and 159 oocytes in the groups A and B, respectively, the group B displayed a statistically significant increase in the percentage of oocytes with funnel effect (Table 5).

Also in this case the hypothesis that r-LH differently affects oocyte quality according to age has been evaluated and whole study population was divided into two subgroups: women ≤30 years constituted the group 1, whereas patients >30 represented the group 2. Moreover, four subgroups have been identified: subgroup 1A (n = 12): younger women treated with r-FSH; subgroup 1B (n = 11): young patients stimulated with r-FSH plus r-LH. Women >30 years treated with r-FSH plus r-LH were included in the groups 2A (n = 10) and 2B (n = 9), respectively. Demographic, anthropometric and hormonal characteristics among groups were similar (data not shown). Comparisons of percentages of the oocytes presenting funnel effect between groups 1A and 1B and between 2A and 2B are shown in table 6 and 7, respectively.

	Group A (r-FSH)	Group B (r-LH)
N° injected oocyte	135	159
Percentage of oocyte presenting funnel effect* (n)	66 (89)	76.7 (122)

Table 5. Presence of a funnel effect in MII injected oocyte

*X^2 $p = 0.04$

	Group 1A (r-FSH)	Group 1B (r-LH)
N° injected oocyte	72	80
Percentage of oocyte presenting funnel effect* (n)	70.8 (51)	76.3 (61)

Table 6. Presence of a funnel effect in patients ≤ 30 years

*X^2 $p = 0.57$

	Group 2A (r-FSH)	Group 2B (r-LH)
N° injected oocyte	63	69
Percentage of oocyte presenting funnel effect* (n)	66.7 (42)	78.5 (62)

Table 7. Presence of a funnel effect in patients >30 years

*X^2 $p = 0.43$

DISCUSSION CHAPTERS 1 AND 2

In the first chapters of this work, we investigated on the effects of different types of ovarian stimulation on folliculogenesis. More specifically, two protocols have been tested: monotherapy with r-FSH *versus* an association of r-FSH and r-LH. In this context, oocyte quality has been our endopoint. In the first phase of the study, we considered only the percentage of mature oocytes. This choice was related to the fact that high percentage of MII oocytes is considered a reliable marker of adequate folliculogenesis and a good prognostic factor for IVF. The main finding of this experimental phase was the statistically significant increase in the percentage of MII oocytes in the group treated with r-LH. This observation should be carefully interpreted due to the relatively small study population. Nevertheless, it may represent interesting information in the complex puzzle of clinical research on LH activity during ovarian stimulation. In fact, most recent RCTs failed to show that r-LH supplementation can significantly increase implantation rate when compared with r-FSH monotherapy, at least in unselected patients. Nevertheless, taken together these studies showed a relevant trend toward an increase in implantation rate in women undergoing r-FSH plus r-LH (Marrs et al., 2004; Humaidan et al., 2004; Lisi et al., 2005). Implantation represents the effect of different steps in which a plethora of confounders can be introduced. As a consequence, huge sample size could be required to definitively understand the impact of r-LH. In this context, the evidence that r-LH can improve the quality of oocytes can be relevant. Following confirmation of our data, researches can be encouraged to continue to compare the two protocols to reach sample size able to point out the real impact of improved oocytes on outcome of

IVF.

In the second chapter, another endopoint was proposed. In particular, the so called “funnel effect” has been proposed as functional marker of gamete quality. The execution of ICSI requires the penetration of the *zona pellucida* and oolemma with a sharp glass tool - the injection pipette. Once the tip of this pipette reaches the perivitelline space and enters the membrane, two distinct membrane response patterns can be observed. The first one is characterized by the invagination of the oolemma followed by a spontaneous breakage. This is the most frequently encountered response and is considered a normal behaviour for the membrane. The second pattern consists of a sudden tearing of the membrane that retracts without forming a funnel. Explanation for the different behaviours of the oolemma can be attributed to the characteristic membrane structure of the human oocyte. In the last years the idea that funnel effect is representative of adequate cytoplasm maturation has been proposed. Currently, funnel effect is considered a reliable indicator of maturation stage of both oolemma and cytoplasm (Nagy et al, 1995). In the present study, for the first time data concerning the relationship between the stimulation protocol (LH supplementation) and the funnel effect is reported. The main finding was the statistically significant increase in the percentage of oocytes with funnel effect in the group treated with r-FSH plus r-LH. Also in this case the observation needs to be confirmed in a larger sample size. Anyway, the idea that more frequent observation of a funnel effect represents the consequence of a more physiological stimulation is consistent with previous literature. Several studies demonstrated that LH activity is crucial in regulating last stages of both folliculogenesis and oogenesis (Filicori et al., 2003). More specifically, it has been proven that embryos obtained from women

stimulated with both FSH and LH show a significant increase in the ploidy when compared with those deriving from FSH monotherapy (Weghofer et al., 2008). This observation clearly supports the idea that LH-dependent changes in the oocytes cytoskeleton occur and that these changes clearly conditions gamete competence, including its capability in performing meiosis. The mechanism by which hormonal stimulation can modify cytoplasm characteristics is difficult to explain. It could be argued that hormonal conditions affect the cytoskeleton by modifying the quantity of cytokeratin, which supports the hypothesis that the hormonal milieu can in fact influence oolemma behaviour (Amsterdam and Aharoni, 1994).

In both chapters patients were divided according to age. Interestingly, both percentage of mature oocytes and percentage of gametes with funnel effect were significantly higher only in subgroups of women l >30 years. This observation is consistent with previous comparative trials which demonstrated that LH supplementation does not modify pregnancy rates in younger patients but significantly increase this parameter in women >35 years (Humaidan et al., 2004; Marrs et al., 2004). The reason why LH is more effective in elderly women is still unclear. Explanations fall into two hypothetic categories: the first is based on the concept that LH is crucial in sustaining several granulosa activities, including growth factors and cytokines; aging leads to a significant reduction of both quality and quantity of granulosa cells, which in turn can affect LH-dependent activities and increase the LH requirement (Alviggi et al., 2009). The second hypothesis is related to the LH-dependent androgen production in theca cells. Androgens are crucial in follicular recruitment, whereas low androgen levels can negatively affect follicular growth and competence (Hillier, 1994). Also

in this case, aging reduced androgen production. This effect could be balanced by exogenous LH.

Finally, the observation that findings concerning both oocyte maturity and funnel effect were consistent in the same population seems to reinforce the idea that LH can improve overall oocyte quality, mainly in advanced reproductive age.

In conclusion, more extended studies on the possible connection between hormonal profile and the different membrane behaviours are required. The analysis of a further series of couples with oocytes consistently displaying a particular membrane pattern, and the assessment of the hormonal milieu of individual follicular fluids would probably help to identify the reason for the different membrane behaviours.

CHAPTER 3

The importance of oocyte scoring as a predictive value for zygotes and embryo quality in patients undergoing IVFcycles.

INTRODUCTION

1.1 Oocyte physiology

During the 85 days preceding ovulation, a 100 fold increase of oocyte volume occurs. This phenomenon is related to the storage of different products, including small molecules, amino acids and nucleotides, which derives from cumulus-oophorus cells and reach the oocyte by means of junction systems and follicular fluid. Granulosa deriving signals are also involved in the regulation of oocyte transcriptional activities, which become maximal at the stage of the tertiary follicle. As a consequence, the gamete progressively accumulates heterogeneous materials, including polypeptides and RNA macromolecules, which will be crucial for successive steps of development including the initial stages of zygote life. Given that both gonadotrophins directly regulate granulosa cells activities, it could be argued that LH indirectly regulate variables involved in the final step of oocyte maturation and early embryo survival (Elder and Dale, 2000; Fair, 2003).

Various investigators showed that molecular communication between oocytes and granulosa cells exists during folliculogenesis (hypothesis of an oocyte-granulosa cell regulatory loop), essential for inducing and coordinating differentiation in the oocyte and in the somatic compartment. Several molecules are involved in this specific pathway, particularly: oocyte growth differentiation factor 9 seems to control the physiological syncronization of cytoplasmic and nuclear maturation. The

mRNA of epidermal growth factor (EGF) was also found in granulosa, cumulus, and oocyte cells, and it acts on granulosa and cumulus cells with an anti-apoptotic effect. Growth differentiation factor-9 synthesis is activated by EGF in the oocyte, with a positive effect on cumulus cells. The expression of EGF seems to be induced by LH in the theca cells, and as a molecular cascade it involves granulosa, cumulus, and oocyte synthesis of EGF, preserving these cells from an apoptotic destiny.

In vitro fertilization (IVF) is a common technique in medically assisted reproduction that has been continuously improved during the last few decades. Despite this progresses only a minority of the in vitro-embryos have the ability to implant and to give a viable pregnancy, probably due to intrinsic characteristics of the gametes. In order to guarantee high pregnancy rates, the transfer of a single embryo with high implantation potential would be the ideal strategy. For this purpose, the most widely supported strategy is to rely on the identification of oocytes prior to fertilization, scoring of pronuclear (PN), stage zygotes (Ebner et al., 2003; Germond and Senn, 1999). and the grade of the embryos at the time of embryo transfer.

Denudation of cumulus cells creates an opportunity to observe morphological deviations from the so-called normal. A good quality metaphase II oocyte is defined as an oocyte with clear, moderately granular cytoplasm, small perivitelline space, and a clear to colourless zona pellucida (Veeck, 1988). Most frequently observed morphological variations of the oocyte are cytoplasmic and include changes in colour, granularity and homogeneity of the cytoplasm, and cytoplasmic incorporations. Extracytoplasmic variations are deviations from normal of perivitelline space, zona pellucida colour and oocyte shape (Van Blerkom, 1990). However, most of the studies published so far

examining the effect of clinical parameters on oocyte quality have been dependant upon oocyte maturity rather than morphology. Few articles examined the effect of different stimulation protocols with different types of gonadotrophins on the morphology of the oocyte (Imthurn et al., 1996; Yu Ng et al., 2001; Rashidi et al., 2005). Imthurn et al. (1996) examined the effect of highly purified follicle stimulating hormone (HP - FSH) on nuclear maturity and morphological appearance of the oocyte in couples undergoing ICSI and compared the results with human menopausal gonadotrophin (HMG) stimulation. Patient characteristics did not differ between the two groups. A significantly higher proportion of oocytes in the FSH-HP group were nuclear mature (metaphase II) than in the HMG group. The percentage of oocytes with cytoplasmic abnormalities was similar between the groups. However, when a subgroup analysis of these cytoplasmic abnormalities was performed, it was evident that significantly fewer oocytes with dark cytoplasm were encountered in the FSH-HP group, whereas the group of oocytes showing course-grained granulations was similar in both groups. Similar fertilization, cleavage and pregnancy rates were obtained for both groups. More recently Ng studied the effect of HMG versus FSH stimulation on oocyte maturity as well as on extracytoplasmic and cytoplasmic morphology of the oocyte (Ng et al., 2001). The percentage of mature oocytes (metaphase II) was similar between the groups. The incidence of oocytes with extracytoplasmic abnormalities such as *zona* abnormality or first polar body abnormality, as well as oocytes with cytoplasmic abnormalities was also similar. Rashidi et al. (2005) also showed that nuclear maturity, oocytes with abnormal zona, polar body or cytoplasmic morphology were similar between the group of patients stimulated with either HMG or recombinant FSH.

1.2 Oocyte morphology

Cytoplasm

In addition to cytoplasmic inclusions, apparent differences in cytoplasmic texture and density have been described (Kahraman et al., 2000; Meriano et al., 2001). Extensive cytoplasmic granularity may either be homogeneous, affecting the whole gamete, or centrally located. The latter was found to be negatively correlated with ongoing pregnancy rate (Kahraman et al., 2000). In contrast, slight or moderate granularity has been accepted as a normal feature of oocytes. It is conceivable that an increased viscosity of the cytoplasm may constrain cell organelles and/or pronuclei in their movement preventing the zygote from achieving alignment of both pronuclei or alignment of pronuclei with respect to the polar bodies, thereby severely impairing polarity and further preimplantation development (Edwards and Beard, 1997; Garello et al., 1999).

Zona pellucida

Low expression of the zona proteins by the growing human oocyte may indicate reduced developmental potential. A German group (Shen et al., 2005) observed that oocytes with zona splitting, probably caused by mechanical stress during retrieval or denudation, were exclusively associated with non-conception cycles. Probably, in these cases the patterning of proteins may be temporarily interrupted during formation of the extracellular coat. Though these types of oocyte usually show an ovoid shape it has to be noted that the zona is responsible for the dysmorphism. If both zona pellucida and oocyte are involved in distortion, corresponding embryos run the risk of developmental incompetence. If this shape dysmorphism runs throughout

preimplantation development, theoretically, it will result in an a typical elongated embryo. This deviation from normal morphology of human 4-cell embryos (Edwards et al., 1970), which is characterized by a 'crosswise' appearance of the blastomeres (with three blastomeres lying side by side), may lead to a reduction in cell-to-cell contact points as a prerequisite for an optimal development of the fetus (Suzuki et al., 1995). This decrease in the number of available tight junctions could cause delayed formation of the blastocoele and full expansion of the blastocyst not before day 6, as indicated by data from zona-free ICSI (Ding et al., 1999, Ebner et al., 2004). However, at least one successful pregnancy has been reported after replacement of extensively elongated embryos (Esfandiari et al., 2005).

It can be stated that some major anomalies of the oocyte are of prognostic value in terms of further development, such as fertilization (meiotic spindle absence, vacuolization), cleavage (meiotic spindle absence, fragmented first polar body), development to blastocyst stage (fragmented first polar body, vacuolization, cytoplasmic viscosity) and implantation (aggregation of sER, zona defects). Therefore, the prognostic role of oocyte morphology should not be underestimated.

Inclusions

Regardless of the consistency of the ooplasm, numerous types of cytoplasmic inclusion may be visible under light microscope. In contrast to conventional IVF, minor dysmorphisms, such as incorporations or refractile bodies, are unlikely to have any impact on fertilization rate and embryo quality in ICSI patients (De Sutter et al., 1996; Balaban et al., 1998; Ebner et al., 2001). On the other hand, vacuoles as well as aggregations of smooth endoplasmic reticulum (sER) were found to be

the most apparent and dynamic cytoplasmic features that wilI impair developmental capacity of human oocytes.

Vacuoles

Vacuoles are membrane-bound cytoplasmic inclusions filled with fluid that is virtually identical with perivitelline space liquid (Van Blerkom, 1990). They may vary in both size and number, and it is assumed that vacuoles arise either spontaneously (Van B1erkom, 1990) or by fusion of pre-existing vesicles derived from the sER and/or Golgi apparatus (El Shafie et al., 2000).

In previous studies, the incidence of vacuoles in metaphase II oocytes varied between 5.7% (De Sutter et al., 1996) and 12.4% (Alikani et al., 1995). Multiple vacuoles accounted for only a small part of approximately 1% (De Sutter et al., 1996; Loutradis et al., 1999). Interestingly, there is only one ICSI study (De Sutter et al., 1996) that found a severely reduced fertilization rate in vacuolized oocytes (40%) compared with gametes without vacuolization (69.6%).

In theory, it is very likely that a larger vacuole or multiple vacuoles will cause a more detrimental effect on the oocyte than a small one, since a larger portion of the cytoskeleton (e.g. microtubuli) cannot function as it is supposed to. In addition, Van Blerkom (1990) suggested that large vacuoles may displace the metaphase II spindle from its polar position, which in turn can result in fertilization failure, cleavage anomalies and/or an abnormal cytokinesis pattem (Nayudu et al., 1989; Van Blerkom et al., 1990). This is in line with a recent paper (Ebner et al., 2005) describing a significant correlation between vacuole diameter and the presence of two pronuclei at the zygote stage. Interestingly, no fertilizations above a vacuole size of 14 μm occurred in this study.

sER cluster

In contrast to vacuolization, the mechanisms responsible for sER clusters are unknown. Such aggregations can easily be distinguished from vacuoles since they are not separated from the rest of the ooplasmic volume by a membrane. Transmission electron microscopic analysis showed that some minor clusters (2-5 1μm) may be overseen using light microscopy (Otsuki et al., 2004). Carefully avoiding direct injection of the sperm into the sER aggregation gives satisfactory fertilization results. Although cell division rates were almost identical in sER cluster-positive and negative cycles, biochemical pregnancy rate in the first group was significantly higher (22.2%) compared with unaffected cycles (3.5%). The same negative impact was found for clinical pregnancy rate, since it was found to be reduced from 28.2% in unsuspicious oocytes to 5.6% in cluster-positive oocytes. In the Otsuki paper (2004), only one pregnancy deriving from gametes with a sER defect has been reported, and, unfortunately, the baby was diagnosed with Beckwith-Wiedemann syndrome.

1.3 Computerized biology

The computerized biology is a field in which computer mathematics and statistic techniques can be applied to biological problems. This method could be useful in order to find a repeatable system for an objective oocyte selection. Such discipline proposes to understand the structure and the function of biomolecules to understand their structure and the relationships among them. The mathematical, statistic and computer methods for the coding images and recognition of a specific pattern, would be able to collect all the informations and have the possibility to compare them, with a possible impact expecially on the biomedical research.

Currently, in IVF techniques, the choice of the three best oocyte to use for IVF insemination is based on the operator experience. This project is part of a collaboration with the Molecular Biodiversity Laboratory, IBM Italy. We bought and managed to set the new system for the acqiuring images (Nis-Elements AR Nikon) together with the identification of the best oocyte morphology parameters that could best fit the aim of the project.

The aim of the project is to elaborate a functional and efficient software that can help the embryologist to select the best oocytes. The goal is to find a correlation between these oocytes, zygotes and embryonic morphology, moreover with clinical outcome.

MATERIALS AND METHODS

Oocyte morphology assessment

For this study we considered an unselected population. Ovarian stimulation protocol, oocytes retrieval and ICSI procedure had already been specified in chapter 1 and 2.

Morphological abnormalities of a metaphase II (MII) oocyte after retrieval and denudation of the corona-cumulus layer were collected in a data base (Table 8) and examined.

TIME OF OBSERVATION:	1	2	3	4	5	6	7	8	9	10
POLAR BODY										
Intact										
Fragmentated										
PERIVITELLINE SPACE										
Small										
Large										
Absent										
Granular										
CYTOPLASM										
Clear/homogeneous										
Dark										
Disomogeneous										
Granular										
INCLUSIONS										
Yes										
No										
VACUOLES										
No										
Small										
Large										
POLARITY										
Yes										
No										
ZONA PELLUCIDA										
Normal										
Yellowish										
Thin										
Thick										
Disomogeneous										
REFRACTILE BODIES										
Yes										
No										
OTHER:										

Table 8. Oocyte scoring table

Zygote scoring

In vitro fertilization (IVF) human pronucleate zygotes are scored on the basis of pronuclear alignment, size, number, equality and distribution of nucleoli, cytoplasmic heterogenicity and presence or absence of cytoplasmic halos (Scott et al., 1998; Kattera et al 2004; Ebner et al., 2003; Demirel et al., 2001) Unfortunately, there is no standard zygote grading system used in assisted reproduction laboratories. Two main systems for assessing pronuclear morphology were developed by Scott and Smith (1998) and Tesarik (2000).

At 16–18h after insemination, fertilization was assessed by observing the presence of 2PN and two polar bodies under an inverted microscope equipped with Hoffman modulation contrast. Zygotes observed under Hoffman contrast (X200 magnification) were photographed to use the scoring system as a tool to evaluate the effects of different pattern. The zygotes were scored according to the Tesarik et al. classification. The system took account of nuclear size and alignment and nucleoli (nucleolar precursor bodies, NPB) number and distribution. Briefly, $2PNP_0$ zygotes had equal numbers of NPB aligned at the pronuclear junction. The absolute number was not counted but was between three and six. $2PNP_1$ zygotes had small sized, unequal and scattered nucleoli. $2PNP_2$ zygotes had large sized and scattered nucleoli. $2PNP_3$ had small sized, allined at the pronuclear junction. $2PNP_4$ had unequal number but equal sizes of nucleoli (between three and six) aligned at the pronuclei junction. $2PNP_5$ zygotes had unequal number and sizes of NBP but with one nucleus having alignment at the pronuclear junction and the other with scattered nucleoli. Zygotes with separated, very different sizes or periphery located pronuclei were classified as “not classified” (Figure 2).

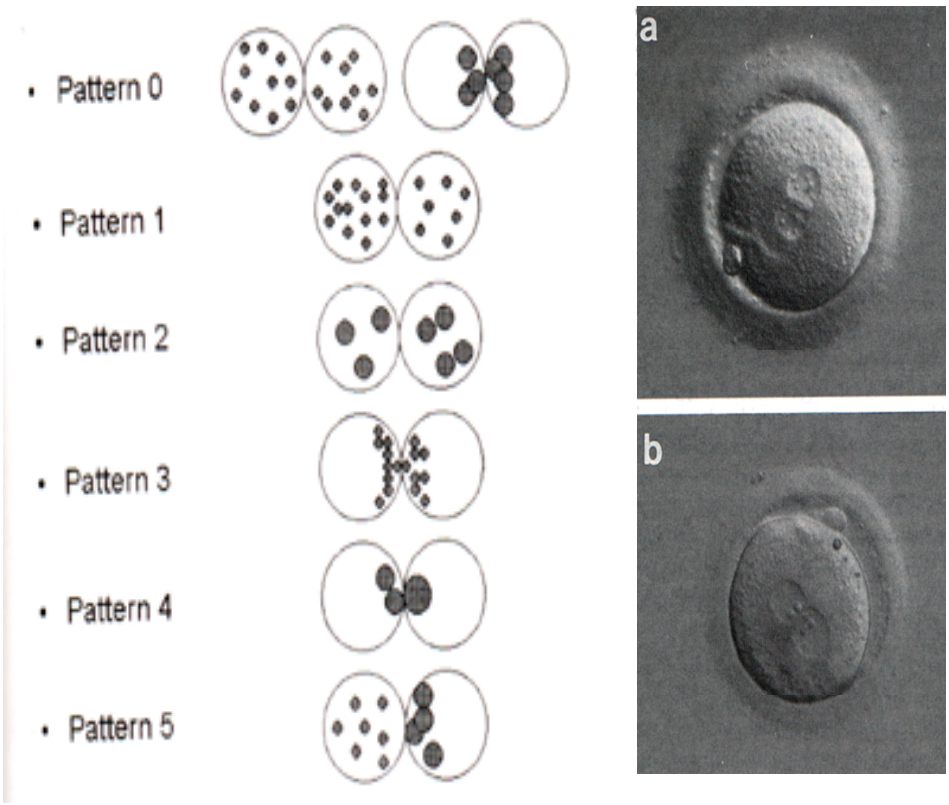


Figure 2. Zygote scoring

Day 2 embryo scoring

The morphology of an embryo was noted 20-24h (day2) after insemination. The embryos were scored as "good or bad" quality according to degree of cytoplasm fragmentation and the number of blastomeres. Grade "good quality embryos" contained the best embryos: at least 2 blastomeres (2-4 blastomeres) and maximum 10% of cytoplasm fragmentation. Grade B embryos have 7-9 cells also but with over 20% of cytoplasmic fragmentation. Grade C has 4-6 cells embryos with maximum 20% fragmented cytoplasm and finally grade D, contained the worst (morphologically lower) embryos with 4-6 cells and over 20% of fragmentation (Figure 3).

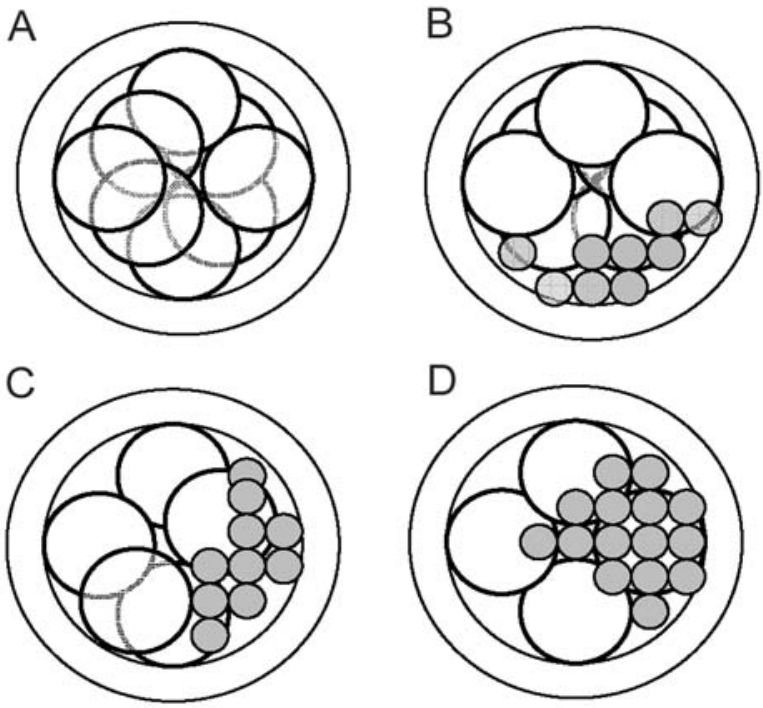


Figure 3. Embryo scoring

Acquiring images system

This software allows us to acquire, to elaborate and to file the images by applying a camera on the inverted microscope, used for the ICSI procedures, which can be connected with a monitor. In this part of the project we managed to set different ranges for each oocyte parameters in order to create a correct data base to collect these different measurements (Table 9 and 10). This step is followed by the observation of the oocyte "tracking" (the follow-up of the oocyte during each of the IVF steps).

Statistical analysis

The results are reported as the mean ± SD. Data were analysed with the SPSS version 12.0 (SPSS Inc., USA). One-way ANOVA was used to determine the effect of the stimulation protocol on continuous variables. Correlations between continuous variables were performed with Pearson correlation coefficient test. The *post hoc* The Mann–Whitney *U*-test was applied to test differences between groups for continuous variables with non-parametric distributions; χ^2 statistics were used to compare discontinuous data. A p value <0.05 was considered statistically significant.

Oocytes morphological parameters	Range	Value	Manual measurments
Polar body			
Diameter if intact	14-16 µm	1	Measure the diameter as a distance between two points
Fragmentated			
Zona pellucida lenght			
	15 - 20 µm	2	Measure the thickness in 4 points: 3, 6, 9, 12 o'clock and write the average number
Oocyte Diameter			
	115 - 165 µm	1	Trace the oocyte area by using different points on the oocyte at 3and 6 o'clock, under the pipette,at 12 o'clock above the pipette. Write the circumferance's diameter.
Refractile bodies			
Absence		3	
Presence	diameter <5 µm		Consider 3 different points and trace the circumferance Write the dimeter.
Presence	diameter >5 µm		

Table 9 Software database

Perivitelline Space	Range	Value	Manual Measurements
	14-16 µm	2	Measure the thickness as a distance between points nearby the polar body
Vacuoles			
Absence		3	
Presence	diameter <9 µm		Measures as refractile bodies
Presence	diameter >10 µm		
Polarity			
Presence		1	Measure the area by fixing different points of the region
Absence			
Citoplasm			
Uniform/Clear/medium granulosity		1	Automatic measurement
Uniform/Dark/high granulosity			

Table 10 Software database

RESULTS

A total of 190 oocytes were analysed and morphologically evaluated. The entire population was divided into two groups based on the oocyte cytoplasmic morphology.

Group 1. Oocytes with clear and homogeneous cytoplasm

Group 2. Oocytes with dark and granular cytoplasmic

Of the 190 oocyte collected, 38.9% (n = 74) were retrieved in group 1 and 61.05% (n = 116) in group 2 (Figure 4).

Group 1 and group 2 oocytes were evaluated for the presence of the polarity. For group 1, 63.5% (n = 47) oocytes showed a polarity while only the 31% (n = 36) in group 2 oocytes. (Table 11)

Clear and homogenous cytoplasm is significantly correlated with the presence of polarity phenomenon. (p <0.001).

Based on these results we analysed the combination between this two parameters (clear-homogeneous cytoplasm and polarity) and zygotes quality. We considered 6 different pattern one of which classified all non developed zygotes. Nevertheless, the association didn't show any statistical significance (p = 0.87), we could observe that oocytes with clear and homogeneous cytoplasm and polarity presence showed a higher percentage of good zygotes quality. In particular:

- group 1 (oocytes with clear and homogeneous cytoplasm and presence of polarity) correlates with the 29.8% (n = 14) of good quality zygotes: pattern 0 (Table 12; Figure 13);
- group 2 (oocytes with dark granularity cytoplasm and presence of polarity) correlates with the 11.1% (n = 4) of good quality zygotes: pattern 0 (Table 12; Figure 14).

(Pronuclear and embryo scoring were assessed too.)

We than evaluated the impact of clear-homogeneous oocyte cytoplasm on day 2 embryo quality.

Embryo quality was divided in two groups:

1. Embryos of good quality (grade 1 and 2)
2. Embryos of bad quality (grade 3,4 and 5)

We observed a total of 190 embryos. 166 (87%) embryos were classified in group 1, 95 (57%) of which presented clear-homogeneous cytoplasm and 71 (43%) presented dark-granular cytoplasm. A total of 24 (13%) embryos were classified in group 2. 18 (75%) group 2 embryos showed clear-homogeneous cytoplasm, while 6 (25%) showed a dark-granular cytoplasm.

Statistical analysis underlined a significant association between clear-homogeneous cytoplasm and good embryo quality [$p = 0.09$ (Table 13)]

	Group 1	Group 2
Percentage of polarity presence	63.5(47)	31(36)
Percentage of polarity absence	36.5(27)	69(80)

Table 11. Evaluation of the presence of polarity in clear-homogeneous (group 1) and dark-granular (group 2) oocytes

X^2 $p = 0.0001$

	Group 1	Group 2
Percentage of pattern 0(2PNp0)	29.8(14)	11.1(4)
Percentage of pattern 1-5	70.2(33)	88.9(32)

Table 12. Pattern 0 and pattern 1-5 in clear-homogeneous and dark-granular oocytes

X^2 $p = 0.0755$

	Group A ("good" embryo)	**Group B ("bad" embryo)**
Percentage of embryo from oocyte with clear-homogeneous cytoplasm	57(95)	75(18)
Percentage of embryo from oocyte with dark-granular cytoplasm	43(71)	25(6)

Table 13. Good embryos (group A) and bad embryos (group B) deriving from clear-homogeneous and dark-granular cytoplasm

X^2 $p = 0.1512$

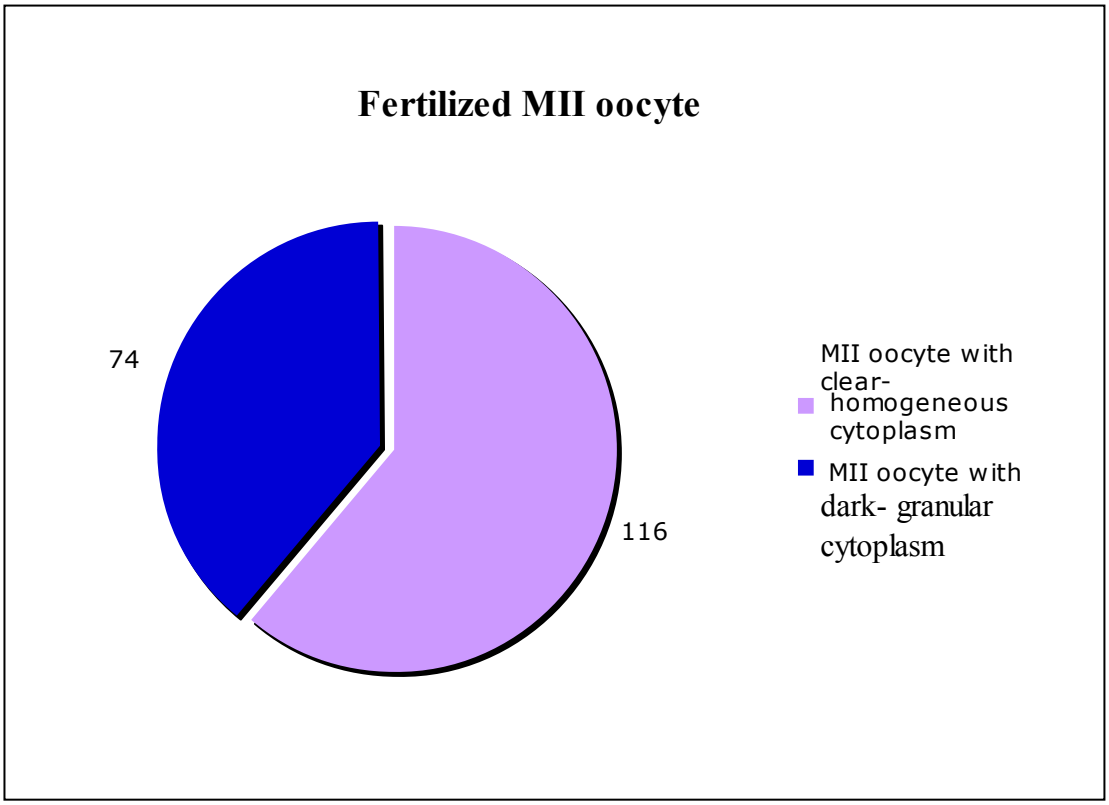


Figure 11. Number of MII oocyte with either clear-homogeneous nor dark-granular cytoplasm

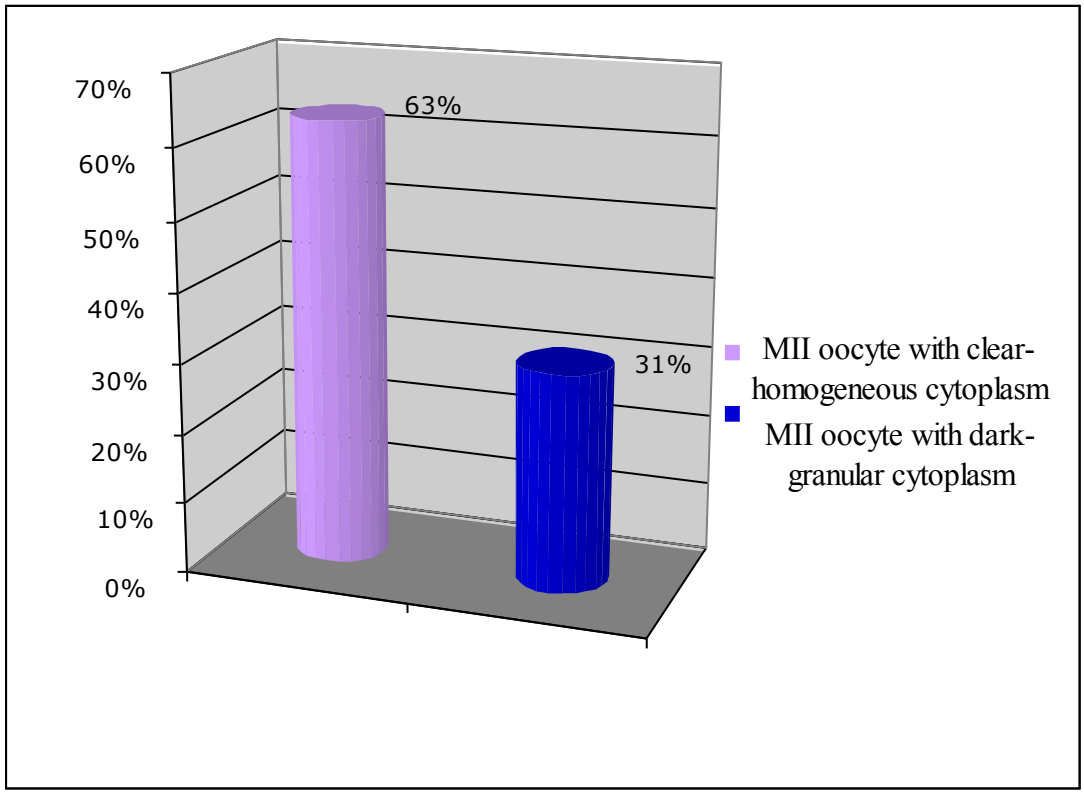


Figure 12. Presence of polarity in MII injected oocyte with clear-homogeneous or dark-granular cytoplasm

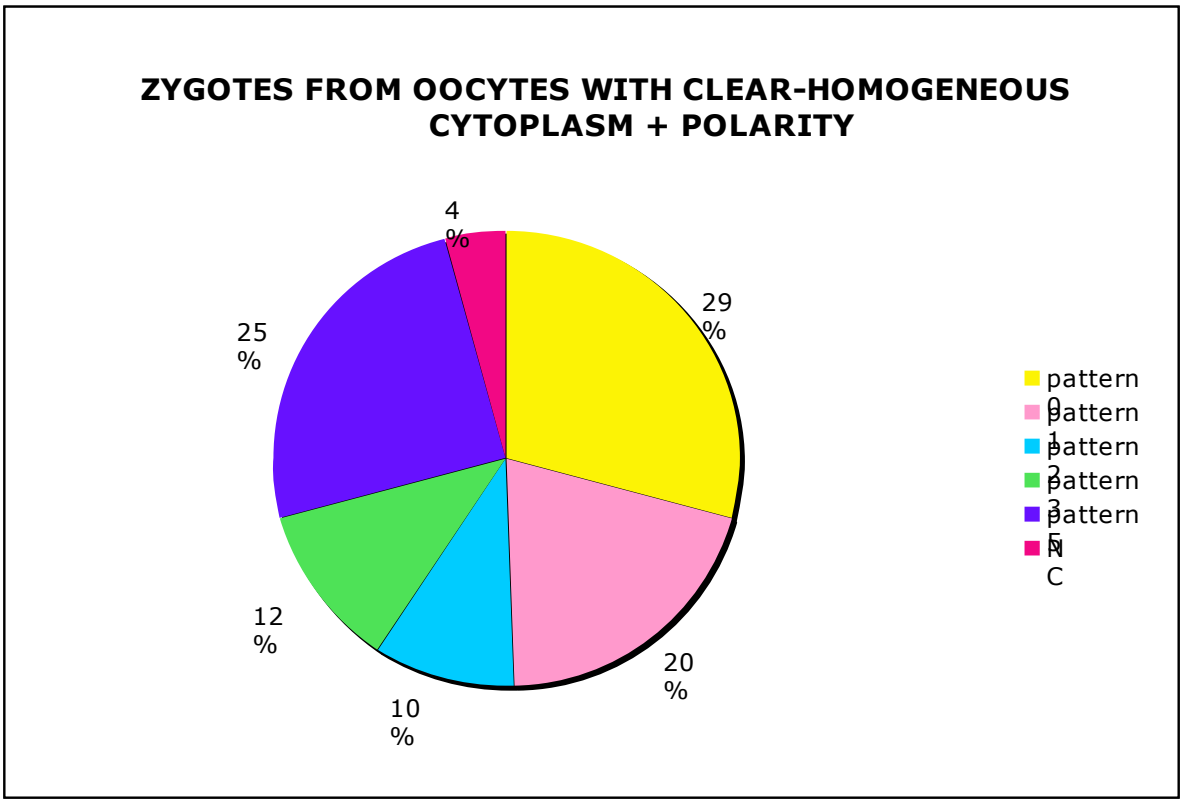


Figure 13. Zygotes from injected MII oocyte with clear-homogeneous cytoplasm and polarity

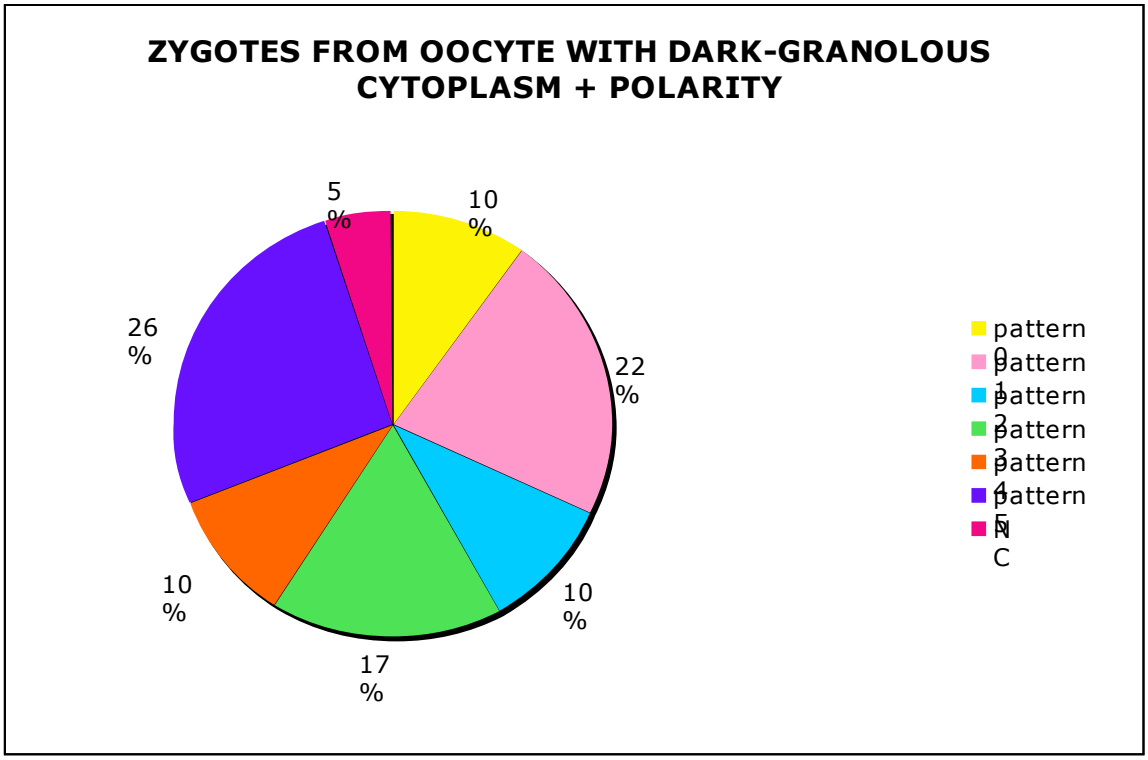


Figure 14. Zygotes from injected MII oocyte with dark-granolous cytoplasm and polarity.

DISCUSSION

In this study we included all women that underwent ICSI procedure. We focused our attention on oocyte quality in order to find a morphological parameter that could predict a good zygote and embryo quality. A data base was created to collect all oocytes morphological informations, while demographic, anthropometric and hormonal characteristics for this unselected population were collected in a different data base.

Cytoplasm aspect is an important parameter because includes all of the organelles that play an important role on the activation of the oocytes competence at the time of the spermatozoon entrance. Extensive cytoplasmic granularity may either be homogeneous, affecting the whole gamete, or centrally located. The latter was found to be negatively correlated with ongoing pregnancy rate (Kahraman et al., 2000) Moreover, an increased viscosity of the cytoplasm may constrain cell organelles or pronuclei in their movement preventing the zygote from achieving alignment of both pronuclei, thereby severely impairing polarity and further preimplantation development (Edwards and Beard, 1997; Garello et al., 1999).

Based on the literature background, we focused our interest on cytoplasm morphology and on the presence of polarity in MII oocytes and on their impact on zygotes and embryo quality. We analysed 190 oocytes and we observed that 116 oocytes had a clear-homogeneous cytoplasm and 74 had a dark-granular one.

One of the other oocytes parameters observed was the presence of polarity. This phenomenon is probably the result of an intense ribosomal activity in a specific region of the oocyte that could have a positive significance. This last statement needs still to be supported by other studies because in literature only two studies had been published until

now. We, than, decided to see if the presence of polarity could be related to the cytoplasm morphological aspect. With this investigation we saw that the number of oocytes with dark-granular cytoplasm that showed polarity was lower than the group of oocytes with clear-homogeneous cytoplasm and this result was statistically significant.

To support the theory that clear-homogeneous cytoplasm and presence of polarity could be considered two predictive parameters we followed the development of these injected oocytes. Injected MII oocyte show different zygotes pattern on day 1 and recent studies showed that pronuclear zygote morphology might predict the outcome of IVF (Payne et al., 2005).

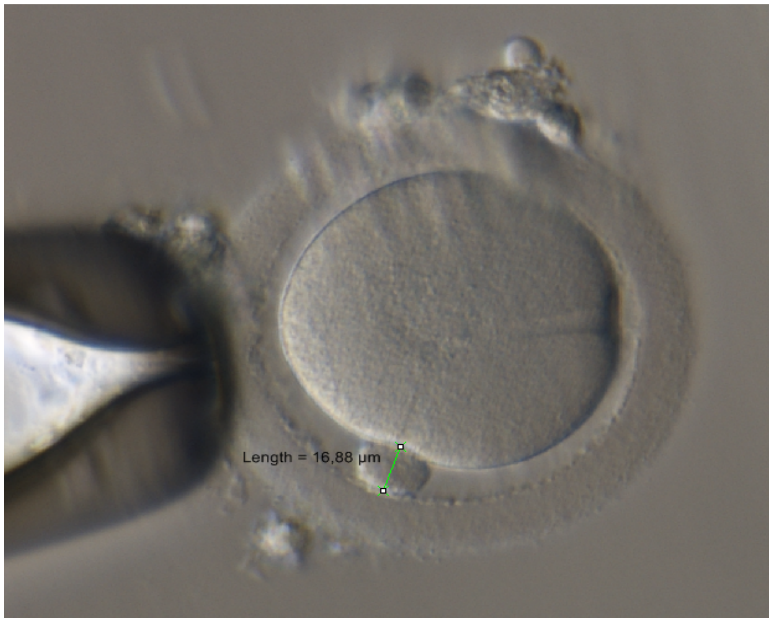
Our preliminary data demonstrates that the best pattern, $2PNP_0$ zygotes, derives from oocyte with clear-homogeneous cytoplasm and polarity. But this data didn't show any statistically significance. Neverthless, the relationship between these parameters can be considered a starting point from which we can create a good oocyte selection system but further observations need to be done in order to have a higher number of sample to analyse.

In most of the oocyte morphology studies embryo quality is the ending point because embryo scores are still accepted as the best predictor of pregnancy for IVF (Fisch et al., 2003; Laasch and Puscheck, 2004),

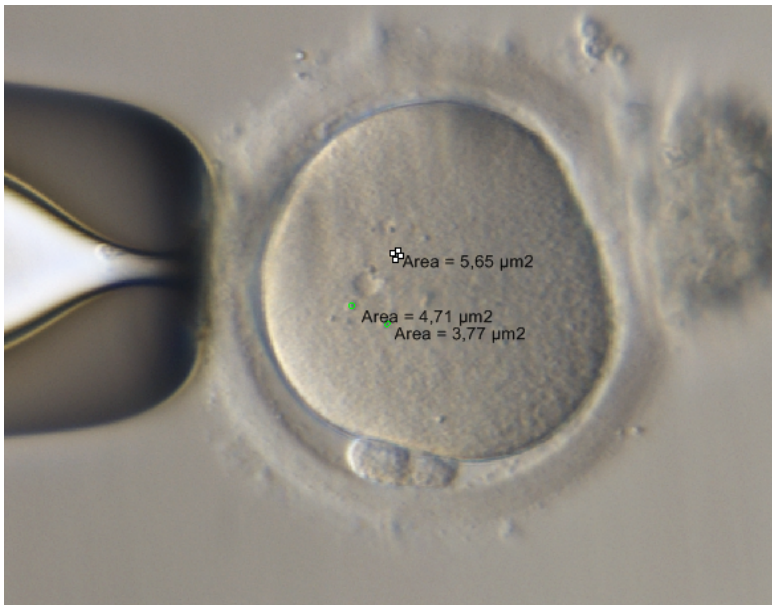
The quality of embryos has traditionally been evaluated based on the cleavage rate and blastomere morphology. However, these criteria by themselves lack sufficient sensitivity and specificity in the prediction of pregnancy rates. In the last phase of the study we observed embryos development. We divided them into two main groups: “good” and “bad” quality embryos. Even in this results we didn't find any statistically significance probably due to the poor number of observations.

In conclusion, the data presented in this study show that the oocyte scoring might be a more immediate predictive value for the entire IVF cycle. A good quality cytoplasm can predict a polarity presence and when both good quality cytoplasm and presence of polarity appear in the same oocyte, they can predict the best zygote quality. About 50% of recruited oocyte presents both characteristics and the embryologist need to be able to find them and to choose them in order to give the patient the highest possibility of a positive result for their IVF cycle. Moreover, we can state that clear-homogeneous cytoplasm can be considered a good embryo quality parameter. So, based on oocyte scoring system we could decrease the number of injected oocyte and sovranumerary oocyte can either be thawed or used for research but further investigations should be done in order to increase its predictive value.

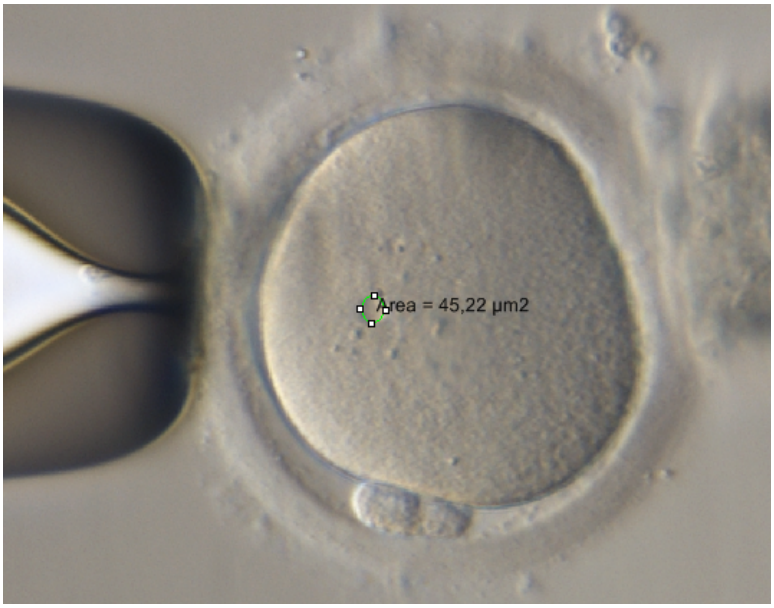
Some images captured by the software



Polar body measurement



Inclusion measurement



Refractile body measurement

REFERENCES

Amsterdan, A. and Aharoni, D. (1994) Plasticity of cell organization during differentiation of normal and oncogene transformed granulosa cells. Microsc. Res. Technique, 27, 108-124.

Balakier H, Bouman D, Sojecki A (2002) Morphological and cytogenetic analysis of human giant oocytes and giant embryos. Human Reproduction 17, 2394-240.

Barritt J, Kokot M, Cohen J (2002) Quantification of human ooplasmic mitochondria. Reproductive BioMedicine Online 4, 243-247.

Brown JB (1978) Pituitary control of ovarian function - concepts derived from gonadotrophin therapy. Australian and New Zealand Journal o/Obstetrics and Gynaecology 18, 46-54.

Campbell BK, Dobson H, Baird DT, Scaramuzzi RJ (1999) Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle-selection in sheep. Journal of Reproduction and Fertility 117, 355-367.

Chappel SC, Howles C (1991) Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory processo Human Reproduction 6, 1206-1212.

Chung K, Krey L, Katz J, Noyes N (2005) Evaluating the role of exogenous luteinizing hormone in poor responders undergoing in vitro fertilization with gonadotropin-releasing hormone antagonists. Fertility and Sterility 84,313-318.

Ciotti PM, Notarangel0 L, Morselli-Labale AM (2004) First polar body morphology before lCSI is not related to embryo quality or pregnancy rate. Human Reproduction 19,2334-2339.

Demirel LC, Evirgen O, Aydos K. (2001) The impact of source of spermatozoa used for ICSI on pronuclear morphology. Human Reproduction; 11: 2327-2332.

De Placido G, Alviggi C, Perino A (2005) Recombinant human LH supplementation versus recombinant human FSH (rFSH) step-up protocol during controlled ovarian stimulation in normogonadotrophic women with initial inadequate ovarian response to rFSH. A multicentre, prospective, randomized controlled trial. Human Reproduction; 20: 390-396.

De Placido G, Alviggi C, Mollo A (2004) Effects of recombinant LH (rLH) supplementation during controlled ovarian hyperstimulation (COH) in normogonadotrophic women with an initial inadequate response to recombinant FSH (rFSH) after pituitary downregulation. Clinical Endocrinology (Oxjord) 60, 637-643.

De Placido G, Mollo A, Alviggi C (2001) Rescue of IVF cycles by HMG in pituitary down-regulated normogonadotrophic young women characterized by a poor initial response to recombinant FSH. Human Reproduction 16,1875-1879.

De Santis L, Cino I, Rabellotti E (2005) Polar body morpho10gy and spind1e imaging as predictors of oocyte qua1ity. Reproductive BioMedicine Online 11, 36-42.

De Vos A, Van de Ve1de H, Ioris H (1999) In vitro matured metaphase-I oocytes have a lower ferti1ization rate but similar embryo quality as mature metaphase-II oocytes after intracytoplasmic sperm injection. Human Reproduction 14, 1859-1863.

Ding I, Rana N, Dmowski WP (1999) Intracytoplasmic sperm injection into zona- free human oocytes results in normal fertilization and blastocyst development. Human Reproduction 14,476-478.

Ebner T, Moser M, Tews G (2004) Intracytoplasmic sperm injection in zona-free oocytes. Journal of the Turkish German Gynecological Association S, 294-298.

Ebner T, Moser M, Sommergruber M (2003a) Selection based on morphologicai assessment of oocytes and embryos at different stages of preimp1antation development. Human Reproduction Update 9, 251-262.

Ebner T, Moser M, Sommergruber M (2002a) First po1ar body morpho10gy and blastocyst formation rate in ICSI patients. Human Reproduction 17,2415-2418.

Ebner T, Yaman C, Moser M (2001) A prospective study on oocyte survival rate after ICSI: influence of injection technique and morphologica1 features. Journal of Assisted Reproduction and Genetics 18,601-606.

Ebner T, Yaman C, Moser M (2000) Prognostic va1ue of first polar body morpho10gy on fertilization rate and embryo quality in intracytoplasmic sperm injection. Human Reproduction 15, 427-430.

Ebner T, Moser M, Yaman C (1999) E1ective transfer of embryos selected on the basis of first po1ar body morpho10gy is associated with increased rates of imp1antation and pregnancy. Fertility and Sterility 72, 599-603.

Edwards RG, Steptoe PC, Purdy JM (1970) Fertilization and cleavage in vitro of preovulatory human oocytes. Nature 227,1307-1309.

Eichenlaub-Ritter U, Shen Y, Tinneberg HR (2002) Manipulation of the oocyte: possible damage to the spindle apparatus. Reproductive BioMedicine Online S, 117-124.

Eppig JJ (1996) The ovary: oogenesis. In: Hillier SG, Kitchener HC, Neilson JP (eds) Scientific Essentials oj Reproductive Medicine 1st edn. WB Saunders Company Lld, London, 147-159.

Erickson GF, Wang C, Hsueh AJW (1979) FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium. Nature 279, 336-338.

Esfandiari N, Ryan EAI, Gotlieb L (2005) Successfu1 pregnancy following transfer of embryos from oocytes with abnorma1 zona pellucida and cytoplasm rnorpho10gy. Reproductive BioMedicine Online 11, 620-623.

Fauser BC, Van Heusden AM (1997) Manipulation of human ovarian function: physiological concepts and c1inical consequences. Endocrine Reviews 18, 71-106.

Ferraretti AP, Gianaroli L, Magli MC et al. (2004) Exogenous luteinizing hormone in controlled ovarian hyperstimulation for assisted reproduction techniques. Fertility and Sterility 82, 1521-1526.

Fevold H (1941) Synergysm of foll icle stimulating and luteinizing hormones in producing estrogen secretion. Endocrinology 28, 33-36.

Filicori M, Cognigni GE, Pocognoli P (2003a) Current concepts and novel applications of LH activity in ovarian stimulation. Trends in Endocrinology and Metabolism 14,267-273.

Filicori M, Cognigni GE, Ciampaglia W (2003b) What clinical evidence for an LH ceiling? Human Reproduction 18, 1556-1557.

Filicori M, Cognigni GE, Tabarelli C (2002) Stimulation and growth of antral ovarian follicles by selective LH activity administration in women. Journal /Clinical Endocrinology and Metabolism 87,1156-1161.

Fisch, J.D, Sher G, Adamowicz M. Keskintepe, L. (2003). The graduated embryo score predicts the outcome of assisted reproductive technologies better than a single day 3 evaluation and achieves results associated with blastocyst transfer from day 3 embryo transfer. Fertility and Sterility 80(6): 1352-1358.

Fujiwara T, Nakada K, Shirakawa H (1993) Development of inositol triphosphate-induced calcium release mechanism during maturation of hamster oocytes. Developmental Biology 156, 69-79.

Gaulden M (1992) Maternal age effect: the enigma of Down syndrome and other trisomic conditions. Mutation Research 296, 69-88.

Goff AK, Arrnstrong DT (1977) Stimulatory action of gonadotrophins and prostaglandins on adenosine-3',5'-monophosphate production by isolated rat granulosa cells. Endocrinology 101,1461-1467.

Gougeon A (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. Endocrine Reviews 17, 121-155.

Hillier SG, Whitelaw PF, Smytb CD (1994) Follicular oestrogen synthesis: the 'two-celI, two-gonadotrophin' model revisited. Molecular and Cellular Endocrinology 100, 51-54.

Hu Y, Betzendahl I, Cortvrindt R (2001) Effects of low O_2 and aging on spindles and chromosomes in mouse oocytes from pre-antral follicle culture. Human Reproduction 16,737-748.

Huang ZH, Clayton PE, Brady G (1994) Insulin-like growth factor-I gene expression in hurnan granulosa-Iutein cells. Journal oj Molecular Endocrinology 12, 283-29l.

Hughes EG, Fedorkow DM, Daya S (1992) The routine use of gonadotrophin-releasing horrnone agonists prior to in vitro fertilisation and gamete intrafallopian transfer: a meta-analysis of randornised controlled trials. Fertility and Sterility 58,888-896.

Imthum B, Macas E, Rosselli M, Keller PJ (1996) Nuclear maturity and oocyte morphology after stimulation with highly purified follicle stimulating hormone compared to human menopausal gonadotrophin. Human Reproduction 11,2387-2391.

Kattera S, Chen C. (2004) Developmental potential of human pronuclear zygotes in relation to their pronuclear orientation. Hum. Reprod.; 19: 294-299.

Kolibianakis EM, Zikopoulos K, Schiettecatte J (2004) Profound LH suppression after GnRH antagonist administration is associated with a significantly higher ongoing pregnancy rate in IVE Human Reproduction 19, 2490-2496.

Laasch C, Puscheck E. (2004) Cumulative embryo score, not endometrial thickness, is best for pregnancy prediction in IVF. J. Assist. Reprod. Genet. 21(2): 47-50.

Ludwig M, **Schöpper B, Al-Hasani S, Diedrich K. (2000)** "Clinical use of a pronuclear stage score following intracytoplasmic sperm injection: impact on pregnancy rates under the conditions of the German embryo protection law." Human Reproduction

Marrs R, Meldrum D, Muasher S (2004) Randomized trial to compare the effect of recombinant human FSH (follitropin alfa) with or without recombinant human LH in women undergoing assisted reproduction treatment. Reproductive BioMedicine Online 8,175-182.

McGee EA, Hsueh AI (2000) Initial and cyclic recruitment of ovarian follicles. Endocrine Reviews 21, 200-214.

Miro F, Hillier SG (1996) Modulation of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. Endocrinology 137, 464-468.

Mitchell R, Hollis S, Rothwell C, Robertson WR (1995) Age related changes in the pituitary-testicular axis in normal men; lower serum testosterone results from decreased bioactive LH drive. Clinical Endocrinology 42,501-507.

Nagy, Z.P., Liu, J., Joris, A., Wisanto, P., Devroey, P. and Van Steirteghem, A.C. (1995) The influence of the site of sperm deposition and mode of oolemma breakage at intracytoplasmic sperm injection (ICSI) on fertilization and embryo development rates. J. Assist. Reprod. Genet., 12, 76S (Abstracts from the EX World Congress on IVF and Alternate Assisted Reproduction, Vienna, Austria, April 3-7, 1995).

Ng Siu T, Chang Tai-Ho, Jackson TC 1999 Prediction of the rates of fertilization, cleavage, and pregnancy success by cumulus-coronal morphology in an in vitro fertilization program. Fertility and Sterility 72, 412-417.

Norbury C, Nurse P (1992) Animal cell cycles and their control. Annual Review oj Biochemistry 61, 441-470.

Ortiz ME, Lucero P, Croxatto HB (1983) Postovulatory aging of human ova: II spontaneous division of the first po1ar body. Gamete Research 7,269-276.

Payne JF, Raburn DJ, Couchman GM, Price TM, Jamison MG, Walmer DK. (2005) Relationship between pre-embryo pronuclear morphology (zygote score) and standard day 2 or 3 embryo morphology with regard to assisted reproductive technique outcomes. Fertility and Sterility

Palermo, G.D. and Rosenwaks, Z. (1995) Assisted fertilization for male factor infertility. Semin. Reprod. Endocr, 13, 39-52.

Palermo, G., Joris, H., Dcrde, M.P., Camus, M., Devroey, P. and Van Steirteghem, A.C. (1993) Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. Fertility and Sterility, 59, 826-835.

Rashidi BH, Sarvi F, Tehrani ES (2005) The effect of HMG and recombinant human FSH on oocyte quality: a randomized single blind clinical trial. European Journal of Obstetrics and Gynecology 120, 190-194.

Rosenbusch B, Schneider M, Glaser B (2002) Cytogenetic analysis of giant oocytes and zygotes to assess their relevance for the development of digynic triploidy. Human Reproduction 17, 2388-2393.

Rosenbusch B, Schneider M (1998) Maturation of a binuclear oocyte from the germinal vesicle stage to metaphase II: formation of two polar bodies and two haploid chromosomal sets. Human Reproduction 13, 1653-1655.

Scott L, Alvero R, Leondires M, Miller B. (2000) "The morphology of human pronuclear embryos is positively related to blastocyst development and implantation." Human Reproduction

Scott LA, Smith S. *(*1998) The successful use of PN embryo transfers the day following oocyte retrieval. Human Reproduction; 13:1003-1013.

Shen Y, Stalf T, Mehnert C (2005) High magnitude of light retardation by the zona pellucida is associated with conception cycles. Human Reproduction 20, 1596-1606.

Sills ES, Levy DP, Moomjy M (1999) A prospective, randomized comparison of ovulation induction using highly purified follicle-stimulating hormone alone and with recombinant human luteinizing hormone in in-vitro fertilization. Human Reproduction 14,2230-2235.

Suzuki H, Togashi M, Adachi J (1995) Developmental ability of zona-free mouse embryos is influences by celi association at the 4-cell stage. Biology of Reproduction 53, 78-83.

Shima K, Kitayama S, Nakano R (1987) Gonadotropin binding sites in human ovarian follicles and corpora lutea during the menstrual cycle. Obstetrics and Gynecology 69,800-806.

Shoham Z, Schacter M, Loumaye E (1995) The luteinizing hormone surge - the final stage in ovulation induction: modern aspects of ovulation triggering. Fertility and Sterility 64, 237-25 l.

Sullivan MW, Stewart-Akers A, Krasnow JS (1999) Ovarian responses in women to recombinant follicle-stimulating hormone and luteinizing hormone (LH): a role for LH in the final stages of follicular maturation. Journal 01 Clinical Endocrinology and Metabolism 84, 228-232.

Tesarik J, Junca AM, Hazout A. Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, non-invasive examination of PN morphology. Hum Reprod 2000; 15: 1396-9

Touraine P, Beau I, Gougeon A (1999) New natural inactivating mutations of the follicle-stimulating hormone receptor: correlations between receptor function and phenotype. Molecular Endocrinology 13,1844-1854.

Van Blerkom J (1990) Occurrence and developmental consequences of aberrant cellular organization in meiotically mature human oocytes after exogenous ovarian hyperstimulation. Journal of Electron Microscopical Technologies 16, 324-346.

Van Blerkom J, Antczak M, Schrader R (1997) The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Human Reproduction 12, 1047-1055.

Veeck LL (1988) Oocyte assessment and biological performance. Annals 01 the New York Academy 01 Sciences 541, 259-274.

Verlinsky Y, Lerner S, Illkevitch N (2003) Is there any predictive value of first polar body morphology for embryo genotype or developmental potential? Reproductive BioMedicine Online 7, 336-341.

Westergaard LG, Erb K, Laursen SB (200l) Human menopausal gonadotrophin versus recombinant follicle-stimulating hormone in normogonadotropic women down-regulated with a gonadotrophin-releasing hormone agonist who were undergoing in vitro fertilization and intracytoplasmic sperm injection: a prospective randomized study. Fertility and Sterility 76,543-549.

Wilding M, Dale B, Marino M (2001) Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. Human Reproduction 16, 909-917.

Willis DS, Watson H, Mason HD (1998) Premature response to luteinizing hormone of granulosa cells from anovulatory women with polycystic ovary syndrome: relevance to mechanism of anovulation. Journal o/Clinical Endocrinology and Metabolism 83,3984-3991.

Xia P (1997) Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. Human Reproduction 12, 1750-1755.

Yoshida M (1993) Role of glutathion in the maturation and fertilization of pig oocytes in vitro. Molecular Reproduction and Development 35,76-81. 1992).

Yu Ng Eh, Lan Lau EY, Biu Yeung WS, Ho Chung P (2001) HMG is as good as recombinant FSH in terms of oocyte and embryo quality: a prospective randomized trial. Human Reproduction 16, 319-325.

Zeleznik AJ (2004) The physiology of follicle selection. Reproductive Biology and Endocrinology 2,31-37.

Zeleznik AJ, Hillier SG (1984) The role of gonadotrophins in the selection of the pre-ovulatory follicle. Clinical Obstetrics and Gynecology 27,927-940.

Zeleznik AJ, Schuler HM, Reichert LE Jr (1981) Gonadotrophin-binding sites in the rhesus monkey ovary: role of the vasculature in the selective distribution of human chorionic gonadotrophin to the pre-ovulatory follicle. Endocrinology 109, 356-362.

Zhou l, Bondy C (1993) Anatomy of the human ovarian insulin-like growth factor system. Biology of Reproduction 48, 467-482.