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Basal level of anti-Müllerian hormone is associated with oocyte quality in stimulated cycles

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BACKGROUND: Serum anti-Müllerian hormone (AMH) levels provide a powerful means for predicting ovarian response, which is reflected not only by the size of the primordial follicle pool but also by the quality of the oocytes. Considering a mutual interdependence between AMH-expressing somatic cells and gametes, this prospective morphological study was set up to evaluate whether extreme AMH levels represent diminished oocyte quality and developmental incompetence. METHODS: A total of 141 consecutive ICSI patients were subdivided into three groups using the 25th and 75th percentiles of the serum AMH levels (cycle day 3). In these three groups, morphology of all oocytes and fertilization rate, embryo quality and blastocyst formation were evaluated, and FSH, LH and estradiol (E_2) levels were also measured. RESULTS: Cycle cancellation rate was correlated with AMH levels ($P < 0.05$). AMH groups 1 (<1.66 ng/ml) and 3 (>4.52 ng/ml) showed oocytes of lower quality [dark central granulation, aggregation of smooth endoplasmic reticulum (sER)] compared with the median group 2 (1.66–4.52 ng/ml). Basal serum FSH did not allow for adequate prognosis in terms of gamete appearance. Fertilization and further cleavage up to blastocyst stage was not affected by AMH levels. CONCLUSIONS: AMH seems to be superior to FSH in predicting both oocyte number and quality.

Key words: AMH/central granulation/cycle cancellation/FSH/oocyte quality

Introduction

Müllerian-inhibiting substance (MIH), more commonly referred to as anti-Müllerian hormone (AMH), was identified as a member of the transforming growth factor-β superfamily (Pepinski *et al*., 1988). In the male, the dimeric glycoprotein AMH/MIH is produced by Sertoli cells during fetal sex differentiation, in which it represses the development of Müllerian ducts (Cate *et al*., 1986). In the female, however, it is a post-natal product of the granulosa cells from pre-antral and small antral follicles (Durlinger *et al*., 2002; Gruijters *et al*., 2003; Weenen *et al*., 2004).

Although AMH is barely detectable at birth in the female, hormone production peaks after puberty (Rajpert-De Meyts *et al*., 1999). The longitudinal course of AMH expression is characterized by a steady decline towards menopause when serum concentration becomes undetectable (de Vet *et al*., 2002; Mulders *et al*., 2004).

In contrast to normal menstrual cycles in which AMH levels remained constant between cycle days 2 and 6 (La Marca *et al*., 2004), stimulated cycles are characterized by a progressive decrease until the late follicular phase (Fanchin *et al*., 2003a). This reduction in plasma AMH level during controlled ovarian stimulation (COH) may easily be explained by the ongoing growth of AMH-producing small follicles causing the differentiation of its granulosa cells, thus probably altering their ability of producing AMH (Baarends *et al*., 1995). Follicular atresia, estrogens and androgens may additionally contribute to the decrease in AMH levels (Baarends *et al*., 1995; Teixeira *et al*., 2001).

Inter-cycle reproducibility of serum AMH measurements in three consecutive menstrual cycles was found to be superior to that of FSH, inhibin and early antral follicle count (AFC) (Fanchin *et al*., 2005a).

Undoubtedly, serum AMH levels correspond to the number of antral follicles and, therefore, provide a powerful means for predicting ovarian response to COH (Seifer *et al*., 2002; van Rooij *et al*., 2002; Fanchin *et al*., 2003b). However, it has to be taken into account that ovarian response is reflected not only by the size of the primordial follicle pool but also by the quality of the oocytes (te Velde and Pearson, 2002), a factor that has been neglected in previous AMH studies. This is all the more regrettable because the fate of a germ cell strongly depends on the health of the corresponding follicle, not least of its granulosa cells, the site of AMH production.

On account of this mutual interdependence of AMH-expressing somatic cells and gametes, this prospective morphological study was set up to evaluate whether extreme AMH

levels represent diminished oocyte quality and developmental incompetence.

Materials and methods

Between August and December 2005, all ICSI patients who had their AMH levels analysed were incorporated into this prospective study. Because AMH measurement had to be paid for by the patients, only 141 of 200 (70.5%) consecutive ICSI patients participated during the 5-month study period. On the third day of the menstrual cycle, an AMH/MIS enzyme immunoassay (Instrumentation Laboratory & Beckman-Coulter, Vienna, Austria) was used for the determination of serum AMH (ng/ml). The analytical sensitivity of this enzyme-linked immunosorbent assay (ELISA) was estimated at 0.1 ng/ml corresponding to 0.7 pmol/l (multiplier for conversion from ng/ml to pmol/l is ×7.14). The intra-assay coefficient of variation (CV) is 12.3% and the inter-assay CV 14.2%.

Because blood samples were stored (at –20°C) for a more economical analysis (which was performed every 2 weeks), hormonal levels were not known at the time stimulation was started.

The mean age of the female patients was 32.9 ± 4.5 years (range 20–41 years). Because this study exclusively enrolled ICSI cases, the vast majority of couples suffered from male factor infertility (81.6%), and nine patients even showed azoospermia (6.4%). The remaining 17 couples either had a history of failed fertilization with IVF (n = 12) or had an ICSI on demand (n = 5).

Two stimulation protocols were applied to harvest a maximum number of oocytes without risking ovarian hyperstimulation syndrome (OHSS). Therefore, an individual dose was adjusted based on the AFC. The vast majority of patients (n = 111) were stimulated using a GnRH antagonist protocol. In this regimen, recombinant FSH (Puregon®, Organon, Vienna, Austria) was started on day 2 of the cycle. In addition, a GnRH antagonist (Cetrotide®, Serono, Vienna, Austria) was administered after 5–6 days of stimulation depending on the presence of a 12–13 mm follicle in the ultrasound scan.

In the long protocol group (n = 30), down-regulation of the pituitary was achieved with the GnRH agonist buserelin (Suprecur®, Aventis Pharma, Vienna, Austria). Stimulation was initiated with hMG (Menogon®, Ferring, Kiel, Germany).

In all patients, ovulation was induced with 5000–10 000 IU hCG (Pregnyl®, Organon), provided the lead follicle had reached a diameter of 19 mm and serum estradiol (E_2) appeared adequate. Oocyte retrieval was carried out transvaginally under ultrasound guidance 36 h after hCG administration.

After a 2–4 h incubation, oocytes were removed from their cumulus complex using the enzyme hyaluronidase (80 IU/ml; MediCult, Copenhagen, Denmark) and hand-drawn glass pipettes. Following this denudation, ICSI was performed as published previously (Ebner *et al*., 2001).

Maturation and morphological features of the oocytes (Ebner *et al*., 2003a) were investigated immediately before ICSI. Analysed anomalies included dark central granulation of the cytoplasm, refractile bodies, dark incorporations, vacuoles, aggregation of smooth endoplasmic reticulum (sER) and perivitelline space granularity.

Fertilization rate, which was assessed 19–21 h after injection, was characterized by the presence of two pronuclei. Early embryo development as assessed by the number of blastomeres and the percentage of fragmentation was evaluated 42–44 h after injection. Special care was taken to identify embryos with pitted cytoplasm, uneven cleavage or multi-nucleated blastomeres. Day 3 morphology (66–70 h after injection) was checked in all patients regardless of whether intrauterine transfer was planned on day 3 (n = 80) or day 5 (n = 52). In the latter group, however, signs of compaction (90–100 h after ICSI) as well as blastocyst formation and quality (114–120 h after injection) were checked according to internal guidelines (Ebner *et al*., 2003b). Unfortunately, fertilization failed or embryonal development stopped in nine couples (6.4%), and thus no transfer could be done at all.

This study exclusively focussed on preimplantation development, whereas implantation and pregnancy rates were not addressed because the patient collective appeared rather inhomogeneous in this respect (e.g. application of two stimulation protocols or different days of transfer).

For statistical evaluation, patients were subdivided into three groups using the 25th and 75th percentiles. Consequently, groups 1 (low AMH) and 3 (high AMH) were composed of 35 patients, whereas group 2 was composed of 71 patients. Data were analysed using SPSS software (SPSS, Chicago, IL, USA). The statistical methods used were Mann–Whitney U-test, t-test and χ^2-test. Significance was defined as $P < 0.05$.

Results

The mean (±SD) value of day 3 AMH for all 141 patients was 3.39 (±2.22) ng/ml. The range, however, was from 0.13 to 10.37 ng/ml with a median of 3.0 ng/ml.

The mean level of AMH of the nine patients (1.86 ± 1.28 ng/ml) who had no embryo transfer differed significantly ($P < 0.05$) from that of those with a transfer (3.46 ± 2.24 ng/ml). The prognostic importance of AMH values is further emphasized by the fact that group 1 (AMH <1.66 ng/ml) comprised of older patients ($P < 0.05$) with a longer period of childlessness ($P < 0.05$) as indicated in Table I. In addition, a close relationship between low AMH and elevated basal FSH levels in group 1 ($P < 0.001$) could be observed. As a consequence, a higher dosage had to be applied in this group ($P < 0.001$) as compared with the others to collect lower number of oocytes ($P < 0.001$) in the presence of lower levels of E_2 ($P < 0.05$).

Focussing on oocyte quality, Table II summarizes that there was a significantly higher number of good-quality ova in group 2 as compared with the groups with lower ($P < 0.05$) or higher AMH values ($P < 0.01$). In more detail, dark central granulation of the cytoplasm was the most dominant anomaly in group 1 ($P < 0.01$), whereas cytoplasmic incorporations were observed at a lower frequency than in the other groups ($P < 0.001$). Vacuoles and refractile bodies were seen more often in the normal group 2 ($P < 0.01$). In the group with the highest AMH levels, however, aggregations of the sER were predominant ($P < 0.01$). FSH levels and stimulation protocols were found to be unrelated to oocyte morphology.

It could be clearly shown that fertilization rates in groups 1–3 (69.8, 68.0 and 73.1%) were not associated with AMH and neither was embryo morphology on day 2 (50.5, 46.7 and 54.0%) and day 3 (46.4, 47.3 and 52.6%) of preimplantation development because the percentage of good-quality embryos was comparable in all three groups ($P > 0.05$).

Morphology on day 4 and blastocyst formation on day 5 could only be evaluated for groups 2 and 3, because the limited number of oocytes in group 1 kept us from blastocyst culture. Only those embryos considered for prolonged culture till day 5 were included in compaction stage and blastocyst scoring. No significant differences ($P > 0.05$) between groups 2 and 3 were

Table I. Comparison of demographic data and controlled ovarian stimulation (COH) details with respect to anti-Müllerian hormone (AMH) group affiliation

	Group 1 (AMH <1.66)	Group 2 (AMH 1.66–4.52)	Group 3 (AMH >4.52)
Age (years)	34.8 ± 3.7*	32.0 ± 4.6**	30.5 ± 4.4
Wish (years)	6.0 ± 5.2***	3.35 ± 2.5	3.4 ± 2.3
Cycle number	2.2 ± 1.8	1.9 ± 1.3	2.0 ± 1.5
FSH (IU/ml)	10.5 ± 3.8****	7.7 ± 2.1	6.9 ± 2.0
LH (IU/ml)	5.7 ± 2.6	5.8 ± 2.5	5.8 ± 2.9
Dose (IU)	2396 ± 790****	1712 ± 623**	1449 ± 439
Duration (days)	9.6 ± 1.8	9.7 ± 1.7	10.3 ± 2.4
Endometrium (mm)	9.9 ± 1.3	10.2 ± 1.9	10.2 ± 1.8
E_2 (pg/ml)	802.6 ± 377.8*****	1279.5 ± 630.9**	1728.8 ± 912.2
Number of oocytes	4.6 ± 3.1****	9.1 ± 5.1*****	12.9 ± 5.7
Number of metaphase II oocytes	4.0 ± 2.8****	7.4 ± 4.1**	10.4 ± 5.1

All values are mean ± SD; AMH, ng/ml; estradiol (E_2), day of ovulation induction; FSH and LH, cycle day 3. *P < 0.05 versus group 2 and P < 0.001 versus group 3, **P < 0.05 versus group 3, ***P < 0.05 versus other groups, ****P < 0.001 versus other groups, *****P < 0.001 versus group 3.

Table II. Number and quality of oocytes with respect to anti-Müllerian hormone (AMH) group affiliation

	Group 1 (AMH <1.66)	Group 2 (AMH 1.66–4.52)	Group 3 (AMH >4.52)
Number of oocytes	155	627	462
MII	139 (89.7)	538 (85.8)	390 (84.4)
Normal oocytes	47 (33.8)	242 (45.0)***	139 (35.6)
Anomalies	92 (66.2)	296 (55.0)	251 (64.4)
Central granulation	43 (46.7)***	88 (29.7)	75 (29.9)
Vacuolization	9 (9.8)	39 (13.2)**	13 (5.2)
Aggregation sER	4 (4.4)	5 (1.7)**	16 (6.4)
Incorporation	14 (15.2)****	61 (20.6)	85 (33.8)
Refractile body	7 (7.6)	77 (26.0)***	39 (15.5)
PVS granula	7 (7.6)	13 (4.4)	8 (3.2)
Multiple anomalies	8 (8.7)	13 (4.4)	15 (6.0)

MII, metaphase II; PVS, perivitelline space; sER, smooth endoplasmic reticulum. *P < 0.05 versus group 1, **P < 0.01 versus group 3, ***P < 0.01 versus other groups, ****P < 0.001 versus other groups.

found in the rates of compaction (58.3 versus 59.5%) and blastocyst formation (55.9 versus 62.7%). In addition, the percentage of good-quality blastocysts was similar (51.8 versus 50.4%).

Discussion

No international assay standard for AMH measurement exists, which may contribute to the discordance between different studies and make comparison between laboratories quite difficult (Peñarrubia *et al*., 2005).

In addition, information on AMH threshold values possibly separating good from bad prognosis patients is scarce in literature. In fact, some authors (Silberstein *et al*., 2006) used the median of all AMH values measured (at the time of hCG administration, 2.7 ng/ml) to distinguish between two study groups in terms of implantation (P < 0.001). Others (Eldar-Geva *et al*., 2005) published a threshold basal AMH level of 18 pmol/l (~2.52 ng/ml), which allowed for significant prediction of ongoing pregnancy (P < 0.01). A threshold of 4.9 pmol/l (~0.69 ng/ml) discriminated between cancelled and ongoing cycles (Peñarrubia *et al*., 2005) and using a threshold value of 8.1 pmol/l (~1.13 ng/ml), plasma AMH assessment could predict poor ovarian reserve on a subsequent IVF cycle, with a sensitivity of 80% and a specificity of 85% (Tremellen *et al*., 2005).

Taking into account that both extreme forms of ovarian response, i.e. low and hyper-response, may be associated with diminished oocyte quality (Aboulghar *et al*., 1997; Akande *et al*., 2002), it was decided to subdivide all patients using the 25th (1.65 ng/ml) and 75th (4.53 ng/ml) percentiles to identify the medium (considered normal) cohort (1.66–4.52 ng/ml). Data from literature suggest that the increase of AMH serum level in PCOS (group 3) is the consequence of the androgen-induced excess in small antral follicle number and that each follicle produces a normal amount of AMH. It has been hypothesized that an increased AMH tone within the cohort could be involved in the follicular arrest of polycystic ovary syndrome (PCOS) by interacting negatively with FSH at the time of selection (Pigny *et al*., 2003).

Present data strongly support previously published manuscripts dealing with the prognostic value of AMH on cycle cancellation (Peñarrubia *et al*., 2005), ovarian response (Seifer *et al*., 2002; van Rooij *et al*., 2002; Muttukrishna *et al*., 2005; Tremellen *et al*., 2005; Silberstein *et al*., 2006) as well as its negative correlation to female age (Mulders *et al*., 2004; van Rooij *et al*., 2005) and basal FSH (de Vet *et al*., 2002; Fanchin *et al*., 2003b).

In addition, this is the first article that could demonstrate a clear relationship between the actual AMH level and the quality of the oocytes. In detail, approximately half of the oocytes deriving from patients with a normal AMH value (group 2) were found to be unaffected compared with only one-third in the low and high AMH groups. On account of the fact that AMH is produced by the granulosa cells at earlier follicular stages, lower levels of the hormone may be associated with a failure in granulosa cell expression, which could irreversibly harm the gamete. This is in line with this finding that the predominant negative feature in the low AMH group is the dark central granulation of the cytoplasm, a phenomenon that is

thought to arise rather early in oocyte maturation (Van Blerkom and Henry, 1992). Although cytoplasmic inclusions that (except vacuolization) do not impair further development (Ebner *et al*., 2006) were the most frequent anomalies in group 2, aggregations of sER were characteristic of group 3. The latter also supports a recently published paper noting a correlation between sER clusters and higher levels of E_2 (Otsuki *et al*., 2004).

Interestingly, neither fertilization rate nor embryo quality could be estimated using basal AMH levels. This is in contrast to the work of Silberstein *et al*. (2006) who found embryos of better morphology and cleavage behaviour in patients with AMH levels ≥2.7 ng/ml as compared with patients with values below this threshold. This may in part be explained by the fact that these authors performed AMH measurement on the day of ovulation induction (not on cycle day 3), a time when AMH values are usually declining because of the presence of growing follicles (Fanchin *et al*., 2003a) and may thus fail to reflect the actual competence of the oocyte or embryo. In addition, embryo scoring criteria were slightly different in this study, because we also analysed multi-nucleation in the blastomeres as well as pitting of the cytoplasm. However, further development to blastocyst stage was not predictable on the basis of AMH measurement alone, but it should be kept in mind that group 1, composed of the worst-quality oocytes, had not a single blastocyst transfer because of the limited oocyte number.

The drawback of reduced oocyte quality could have serious consequences for both rates of implantation (Silberstein *et al*., 2006) and clinical pregnancy (Hazout *et al*., 2004) in patients with low AMH. This problem will not apply to hyper-responding patients (e.g. polycystic ovaries) whose higher number of available embryos may compensate for gamete deficiency. However, present data are perfectly consistent with the hypothesis of Fanchin *et al*. (2005b) who analysed the follicle's individual ability to produce AMH. They reported not only a quantitative relationship between peripheral AMH and the surrounding follicular status but also a positive correlation between serum and follicular fluid AMH levels, indicating that serum AMH measurement may reflect qualitative ovarian responsiveness.

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