Human Reproduction vol 11 no 11 pp 2387–2391, 1996

Nuclear maturity and oocyte morphology after stimulation with highly purified follicle stimulating hormone compared to human menopausal gonadotrophin

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Several studies have shown that high concentrations of luteinizing hormone (LH) in the follicular phase of stimulation can have a negative effect on oocyte quality, pregnancy rate and incidence of miscarriage. The aim of the present study was to examine the effects of highly purified follicle stimulating hormone (FSH HP) on ovarian stimulation and particularly on nuclear maturity and morphological appearance of the oocyte in intracytoplasmic sperm injection (ICSI) therapy and to compare the results with human menopausal gonadotrophin (HMG) stimulation. For this purpose, 50 patients for ICSI (HMG: 30; FSH HP: 20) and 26 patients for in-vitro fertilization (IVF; HMG: 14, FSH HP: 12) were stimulated with either HMG or FSH HP using a short-term protocol. Patients were divided into the two groups according to the first letter of their family name. No differences were observed among the groups in relation to patient age, duration of stimulation, number of aspirated oocytes or maturity of the oocyte–cumulus complex. After removal of the cumulus–corona cells in the ICSI oocytes, a significantly higher proportion of oocytes in the FSH HP group were nuclear mature (metaphase II) than in the HMG group (FSH HP: 88.8%, HMG: 80.6%; $P = 0.009$). Furthermore, in the FSH HP group, significantly fewer oocytes with dark cytoplasm were observed (FSH HP: 14.4%, HMG: 22.4%; $P = 0.02$). Fertilization, cleavage and pregnancy rates (FSH HP 38%, HMG: 34% per retrieval) were comparable in both groups. Based on the results obtained, it can be concluded that the short-term FSH HP treatment protocol synchronizes oocyte maturation better than comparable stimulation with HMG.

Key words: follicle stimulating hormone/human menopausal gonadotrophin/intracytoplasmic sperm injection/nuclear maturity

Introduction

The most commonly used agent worldwide for ovarian stimulation is human menopausal gonadotrophin (HMG), which is a 1·1 mixture of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Several studies suggest that high concentrations of LH during the follicular phase of stimulation can have a negative impact on oocyte quality, pregnancy rate and incidence of miscarriage (Stanger and Yovich, 1985; Howles *et al.*, 1986; Homburg *et al.*, 1988; Regan *et al.*, 1990; Shoham *et al.*, 1990). Several centres therefore substituted HMG partially or completely by pure FSH (Venturoli *et al.*, 1986; Diedrich *et al.*, 1987). In spite of the misgivings about LH, no comparative in-vitro fertilization (IVF) study has to date been able to demonstrate the superiority of pure FSH over HMG (Bentick *et al.*, 1988; Edelstein *et al.*, 1990; Tanbo *et al.*, 1990; Check *et al*, 1995), with the exception of a controversial meta-analysis (Daya, 1995).

It is possible that no differences were found because, although HMG and FSH may indeed be different, the difference is in fact clinically irrelevant. It could, however, also be due to the fact that the sensitivity, specificity and precision of measurement of the parameters studied were too weak. In the studies cited, oocyte maturity was assessed indirectly using the criteria of Veeck (1988), that is, without prior removal of the cumulus oophorus. Thus, the nuclear maturity and the morphological appearance of the oocytes themselves could not be examined.

In contrast to conventional IVF, oocytes are routinely separated from the cumulus oophorus in microinsemination techniques (Imthurn *et al.*, 1995). As the morphological structure of the denuded oocytes can be assessed in a more detailed and precise manner, this procedure permits a more clearly defined differentiation of these cells.

Both the degree of maturity and the morphology of the oocytes are significantly more directly dependent on the stimulation used than, for example, fertilization. Hence, these parameters are better markers than the fertilization rate to score the quality of the stimulation. The magnitude of the fertilization rate not only correlates with oocyte quality, but also, among other factors, with the quality of the spermatozoa, the culture conditions and, particularly in the case of micro-insemination, with technique and experience. At present, no studies are available that have investigated the nuclear maturity and morphology of denuded oocytes and compared the results with respect to the stimulation used.

The aim of the present study was to examine the effects of FSH HP on ovarian stimulation and particularly on nuclear maturity and oocyte morphology in intracytoplasmic sperm injection (ICSI) therapy and to compare the results with those obtained when HMG is used.

Materials and methods

The study was examined and approved by the local ethical committee. A total of 93 ICSI and IVF cycles were stimulated with either HMG or FSH HP using a short-term protocol Patients were divided into the two groups one after the other according to the first letter of their

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family name (A–L: HMG, M–Z: FSH HP). Patients who presented for a second treatment during the duration of the study were then treated with the relevant alternative preparation, so that it could be conducted in 10 cases as a cross-over. The primary indication for IVF was always a mechanical factor, and for ICSI severe male subfertility, except for one case of unexplained infertility. For IVF, the spermiogram was normal (World Health Organization, 1993).

A total of 53 patients in 53 cycles were stimulated with HMG, and 40 patients in 40 cycles with FSH HP. For conventional IVF, 37 ovarian stimulations were performed, and 56 stimulations for ICSI. The average age of the HMG group was not significantly different from that of the FSH HP group (HMG: 33.1 ± 0.7 years, FSH HP: 34.0 ± 0.6 years; *t*-test). In addition, no significant difference was observed in the age of the ICSI subgroups (HMG: 33.2 ± 0.8 years, FSH HP: 34.0 ± 0.7 years)

Ovarian stimulation has already been described in detail elsewhere (Imthurn *et al.*, 1992). Briefly summarized, norethisterone acetate (Primolut-Nor®; Schering, Zurich, Switzerland), at a dose of 10 mg daily for a duration of 10–25 days, was prescribed to programme the stimulation. The start of stimulation always commenced with daily injections of 0.1 mg s.c. D-triptoreline (Decapeptyl®; Ferring, Duebendorf, Switzerland). Treatment with HMG (Pergonal®, Serono, Aubonne, Switzerland) or FSH HP (Metrodin HP®; Serono) began concomitantly (day 1) with the administration of gonadotrophin-releasing hormone agonist, or 1 or 2 days later (day 2 or 3). Individualization of the dose was carried out according to the expected stimulation response. HMG was injected i.m. by a medically trained person in the University Hospital of Zurich, or in the patient's home city. FSH HP was usually injected s.c. by the patient herself. The dose of the hormone was adapted on stimulation day 6 according to the serum oestradiol concentrations (radioimmunoassay; BioMérieux, Marcy l'Etoile, France). Starting on day 8, and then if necessary daily, vaginal sonography and further oestradiol assays were carried out. Serum progesterone concentrations (radioimmunoassay; Amersham, Zurich, Switzerland) were assessed on day 6 and on the day of ovulation induction. Induction of ovulation was achieved by the administration of 10 000 IU human chorionic gonadotrophin (HCG) i.m., if at least two follicles had attained a diameter of 18 mm and the serum oestradiol concentration reached at least 3 nmol/l. If one or both of these criteria could not be met, the stimulation was discontinued. The triptoreline injections were stopped at the time of the HCG administration. Transvaginal follicular aspiration was carried out 34–36 h later. Aspiration cycles with <3 oocytes were excluded from the study analysis, as a representative assessment of an adequate number of oocytes was not possible in these cases.

Immediately after the oocyte retrieval, the oocyte–cumulus complex (OCC) was assessed by the reproductive biologist using inversion microscopy (Diaphot TMD; Nikon, Kuesnacht, Switzerland) in accordance with the criteria of Veeck (1988), and categorized as being mature, immature or atretic stage. The biologist performing the assessment was not informed which stimulation had been used for which patients. In the case of ICSI, nuclear maturity was assessed after removal of the cumulus oophorus with hyaluronidase. Oocytes were judged to be nuclear mature only if a polar body could be observed in the perivitelline space (metaphase II). In addition, the clarity of the cytoplasm and the granularity of the intracellular granulations were rated for the denuded ICSI oocytes, and cytoplasmic anomalies such as vacuoles or fragments were looked for.

The insemination and microinsemination techniques and the culture conditions have also been previously described in detail (Macas *et al.*, 1990; Imthurn *et al.*, 1995). Further examination of the oocytes was carried out 16–18 h after insemination or ICSI, in order to check fertilization and to record the number of pronuclei. Surplus diploid pronuclear stage zygotes were cryopreserved at this time. A further 24 h later, usually three, and exceptionally four, embryos were transferred to the cavum uteri. Immediately before the transfer, the number of blastomeres was recorded and the morphological quality of the embryos scored (Plachot and Mandelbaum, 1990). The regularity of the blastomeres, clarity of the cytoplasm and presence of intracellular vacuoles and fragments in the perivitelline space were noted.

The luteal phase was routinely supported with micronized progesterone starting from the day of aspiration (vaginal suppositories 100 mg 3×2 daily; Utrogestan®, Golaz, Ecublens, Switzerland).

The results are presented as mean values ± SEM, and were analysed statistically using Student's *t*-test (unpaired) and the χ^2-test. Results were considered significant when P values were <0.05. Unless a P value is given in the Results, differences between stimulation protocols or between methods of insemination were not significantly different.

Table I. Comparison of various clinical parameters during stimulation with human menopausal gonadotrophin (HMG) or highly purified follicle stimulating hormone (FSH HP)

	HMG	FSH HP
Duration of stimulation (days)	11.0 ± 0.2	11.0 ± 0.2
ICSI (n = 56)	11.1 ± 0.3	11.2 ± 0.2
IVF (n = 37)	10.9 ± 0.3	10.8 ± 0.3
Number of ampoules (75 IU)	31.2 ± 2.0	32.5 ± 1.8
ICSI (n = 56)	30.2 ± 2.4	33.4 ± 2.2
IVF (n = 37)	33.4 ± 3.9	30.9 ± 3.1
Oestradiol concentration day 6 (nmol/l)	2.1 ± 0.2	1.7 ± 0.3
ICSI (n = 56)	2.1 ± 0.3	1.7 ± 0.4
IVF (n = 37)	2.0 ± 0.4	1.9 ± 0.3
Preovulatory oestradiol concentration (nmol/l)	12.6 ± 0.8	10.6 ± 0.8
ICSI (n = 56)	12.7 ± 1.1	10.5 ± 1.1
IVF (n = 37)	12.6 ± 1.4	10.8 ± 1.0
Progesterone concentration day 6 (nmol/l)	1.9 ± 0.1	1.8 ± 0.2
ICSI (n = 56)	1.8 ± 0.2	1.8 ± 0.2
IVF (n = 37)	2.1 ± 0.3	1.8 ± 0.3
Preovulatory progesterone concentration (nmol/l)	4.5 ± 0.3	4.1 ± 0.2
ICSI (n = 56)	4.7 ± 0.3	4.2 ± 0.3
IVF (n = 37)	4.0 ± 0.3	3.8 ± 0.3
Cancelled cycles (n = 93)	8%	10%
No. of oocyte retrievals for ICSI[a]	30	20
No. of oocyte retrievals for IVF[a]	14	12

[a]Cycles with >2 oocytes aspirated.
IVF = in-vitro fertilization, ICSI = intracytoplasmic sperm injection.

Results

Four stimulations in each of the HMG and the FSH HP groups had to be discontinued due to poor ovarian response (HMG: 8%, FSH HP: 10%; Table I). A further five HMG and four FSH HP cycles were not analysed, as in each case the yield was only one or two oocytes. Thus, 44 HMG and 32 FSH HP cycles in 50 ICSI (HMG: 30, FSH HP: 20) and 26 IVF (HMG: 14, FSH HP: 12) treatments were included in the study.

The number of ampoules required for HMG and FSH HP stimulation (HMG: 31.2 ± 2.0, FSH HP: 32.5 ± 1.8 ampoules), as well as the duration of stimulation (HMG: 11.0 ± 0.2, FSH HP 11.0 ± 0.2 days) were almost identical in both groups. There were also no differences within the ICSI group. The oestradiol and progesterone concentrations on day 6 of the stimulation and before HCG administration are presented in Table I. No significant differences were observed either in the

Table II. Maturity and morphological quality of the oocyte–cumulus complexes and of the denuded oocytes after stimulation with human menopausal gonadotrophin (HMG) or highly purified follicle stimulating hormone (FSH HP)

	HMG	FSH HP	
Number of oocytes	12.9 ± 1.0	10.3 ± 0.7	
ICSI (n = 50)	13.3 ± 1.2	10.8 ± 0.9	
IVF (n = 26)	12.1 ± 1.9	9.6 ± 1.3	
Oocyte–cumulus complex (mature)	92.0%	92.7%	
ICSI (n = 613)	91.2%	93.5%	
IVF (n = 285)	94.1%	91.3%	
Oocytes in metaphase II (n = 613)	80.6%	88.8%	P = 0.009
Dark cytoplasm (n = 613)	22.4%	14.4%	P = 0.02
Coarse-grained granulations (n = 613)	17.3%	18.1%	
Cytoplasmic anomalies (n = 613)	8.3%	8.4%	

IVF = in-vitro fertilization, ICSI = intracytoplasmic sperm injection

total sample or in the ICSI subgroup. Although higher mean preovulatory oestradiol values were measured in the HMG group, the difference was not statistically significant [HMG: 12.6 ± 0.8 nmol/l (range 2.2–27.0), FSH HP: 10.6 ± 0.8 nmol/l (range 4.2–22.0].

A total of 568 and 330 oocytes were aspirated in the HMG and FSH HP groups respectively (Table II). No statistically significant difference between the HMG and the FSH HP group was observed in relation to the average number of aspirated oocytes [HMG: 12.9 ± 1.0 (range 3–28), FSH HP: 10.3 ± 0.7 oocytes (4–20)]. The proportion of mature OCC in the HMG group was 92.0%, and 92.7% in the FSH HP group. When analysed according to the method of insemination, 93.0% of the OCC with IVF (HMG: 94.1%, FSH HP: 91.3%), and 92.0% of the OCC with ICSI (HMG: 91.2%, FSH HP: 93.5%) were judged to be mature.

After removal of the cumulus oophorus, a polar body in the perivitelline space could be observed in 83.5% (HMG: 321, 80.6%; FSH HP: 191, 88.8%) of a total of 613 ICSI oocytes (HMG: 398, 13.3 ± 1.2; FSH HP: 215, 10.8 ± 0.9). This means that 91% of the OCC judged to be mature also exhibited nuclear maturity. In 17.6% of the ICSI oocytes (HMG: 17.3%, FSH HP: 18.1%), coarse-grained intracellular granulations were discernible, 19.6% exhibited a dark cytoplasm (Figure 1; HMG: 22.4%; FSH HP: 14.4%; P = 0.02) and 8.3% showed cytoplasmic anomalies (HMG: 8.3%; FSH HP: 8.4%). On average, 9% of the oocytes with ICSI were damaged (HMG: 9.1%; FSH HP: 8.3%).

The fertilization rate with IVF was 69.5% (HMG: 67.1%; FSH HP: 73.0%) and with ICSI 71.2% (HMG: 69.2%; FSH HP. 74.6%; Table III). Pronuclear stage zygotes were cryopreserved after HMG stimulation in 41% (5.7 ± 0.7 pronuclear stages) and after FSH HP (4.4 ± 0.6 pronuclear stages) in 28% of the respective cycles. Of the remaining zygotes, 94.2% (HMG: 99.0%; FSH HP: 93.9%) cleaved into predominantly 4-cell embryos (HMG: 79.6%; FSH HP: 74.3%). A difference in the rate of cleavage between the two groups was not observed. This means that the incidence of embryos with less than, or with more than, four blastomeres was comparable (<4 blastomeres: HMG: 15.5%; FSH HP: 22.8%; >4 blastomeres: HMG: 4.9%; FSH HP: 2.9%). The incidence of morphologically regular embryos was practically the same in both groups (HMG: 74.6%; FSH HP: 76.1%). No transfer was carried out

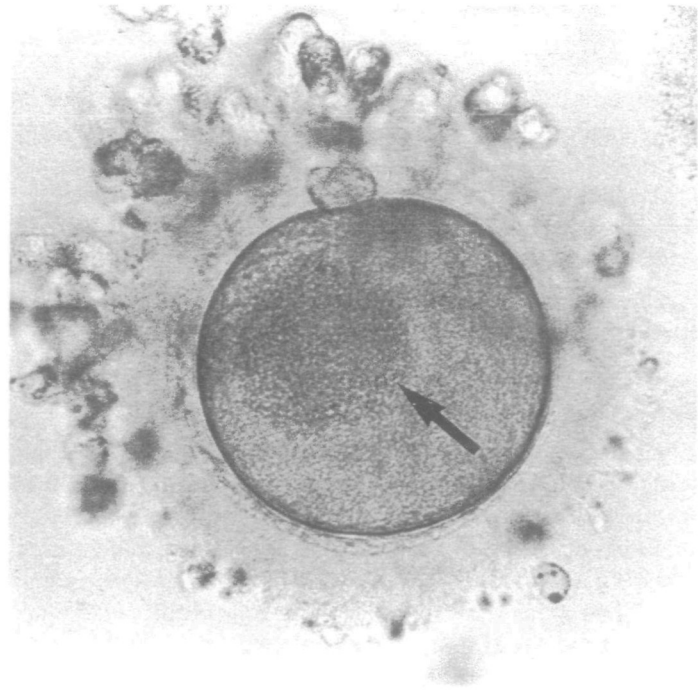

Figure 1. Denuded oocyte displaying dark cytoplasm (arrow). Original magnification. ×400.

Table III. Fertilization, cleavage and pregnancy rates after stimulation with human menopausal gonadotrophin (HMG) or highly purified follicle stimulating hormone (FSH HP)

	HMG	FSH HP
Damaged oocytes after ICSI (n = 512)	9.1%	8.3%
Fertilization		
ICSI (n = 467)	69.2%	74.6%
(n = 50)	6.7 ± 0.7	6.6 ± 0.7
IVF (n = 285)	67.1%	73.0%
(n = 26)	8.1 ± 1.3	7.0 ± 1.1
Cleavage rate (n = 330[a])	99.0%	93.9%
Morphologically regular embryos (n = 311)	74.6%	76.1%
Implantation rate per transferred embryo	15.6%	16.0%
Pregnancy rate per retrieval[b] (n = 76)	34%	38%
Delivery rate per retrieval (n = 76)	23%	22%

[a]After cryopreservation of 143 pronuclear stage oocytes
[b]Includes preclinical pregnancies
IVF = in-vitro fertilization, ICSI = intracytoplasmic sperm injection

in three cases in the HMG group, twice because no fertilization was achieved, and once because all pronuclear stage zygotes were cryopreserved due to a high risk of severe ovarian hyperstimulation syndrome. There was one case of no transfer in the FSH HP group due to lack of fertilization.

A total of 10 patients were stimulated once with HMG and once with FSH HP. In seven cases, a total of 14 ICSI treatments were carried out. Stimulation in the first cycle was undertaken with FSH HP on three occasions, and with HMG on four occasions. Even with this crossed study design, a significant difference could be observed in the metaphase II oocytes between HMG and FSH HP stimulation. On average, 10.9 ± 1.2 oocytes (HMG: 11.0 ± 1.7; FSH HP: 10.7 ± 1.9) were aspirated. After removal of the cumulus oophorus, a polar body in the perivitelline space was discerned in 88.3% of the HMG stimulation cases, and in 97.3% of the FSH HP stimulation cases ($P = 0.03$). No significant difference could be observed in any of the other parameters studied.

Per transfer, 2.6 ± 0.1 (0–4) embryos were replaced. A pregnancy rate of 34% per retrieval, including preclinical pregnancies, was obtained in the HMG group. Five patients aborted (33%), so that a birth rate of 23% was achieved. Twelve pregnancies were diagnosed in the FSH HP group (38% per retrieval). Again, five patients aborted, resulting in a delivery rate of 22%. Three of these pregnancies were twins. Multiple pregnancies greater than twins did not occur.

Discussion

Comparative investigations in IVF stimulation with HMG and 'pure' FSH have to date not been able to show any superiority for one or the other stimulation method (Bentick *et al.*, 1988; Edelstein *et al.*, 1990; Tanbo *et al.*, 1990). These studies used sample sizes of only 14 to a maximum of 20 cycles per group, which were clearly too small to test non-specific end-points such as duration of stimulation, preovulatory oestradiol concentrations or pregnancy rates. However, even with greater group sizes, it is almost impossible to find statistically significant differences using these markers (Out *et al.*, 1996).

Our results confirm the previously published findings with 'pure' FSH and recombinant FSH (rFSH) in comparison with HMG (Bentick *et al.*, 1988; Edelstein *et al.*, 1990; Tanbo *et al.*, 1990; Check *et al.*, 1995; Out *et al.*, 1996), as we also found no differences in non-specific parameters such as oestradiol concentrations, number of aspirated oocytes, or fertilization and pregnancy rates, either in the ICSI sample studied, or in the IVF group. The present investigation, however, revealed significant differences between HMG and FSH HP in specific end-points that have never been examined in any previous study.

The aim of every stimulation is to yield as many mature and as few immature oocytes as possible; that is, to attain the best possible synchronization of follicular maturation. We have investigated nuclear maturity for the first time as one of the new specific indicators, and have found a significantly higher proportion of metaphase II oocytes with FSH HP stimulation than with the HMG protocol. This observation was confirmed by the 14 crossed ICSI cycles. In spite of the small sample size, the proportion of nuclear mature oocytes after FSH HP was also found to be higher than after HMG stimulation.

With HMG stimulation, the number of mature oocytes aspirated was not reduced, but the number of immature oocytes and consequently the total number of oocytes were increased. The greater the number of oocytes that develop, the higher is the oestradiol concentration. The preovulatory oestradiol concentrations did, indeed, show a tendency to be higher after HMG stimulation ($P = 0.09$) than after FSH HP. This is an unwanted effect, as the risk of ovarian hyperstimulation syndrome correlates with the magnitude of serum oestradiol concentrations, and thus increases with raised oestradiol concentrations (Schenker and Weinstein, 1978).

When the results of the OCC assessment are considered, it is striking that no significant difference between the two stimulation methods was observed for this parameter, which is also a measure of oocyte maturity; indeed, the results were practically identical. The reason why significant differences could be observed for nuclear maturity, but not, however, for the assessment of the maturity of the OCC, is probably related to the fact that assessment of maturity based solely on the extent, clarity and colour of the cumulus cells and the corona radiata can be misleading. This is because the expansion of the cumulus cells can be influenced by various factors and does not necessarily have to correspond to the nuclear maturity of the oocyte (Elder and Avery, 1992).

No differences between the stimulation protocols could be found for the assessment of oocyte morphology in relation to cytoplasmic anomalies and granulations. This, however, was not the case concerning the clarity of the cytoplasm. Dark cytoplasm, which was observed significantly less often after FSH HP stimulation, is interpreted as a sign of incipient atresia (Veeck, 1988) or, according to our own experience, of cytoplasmic immaturity. If, in addition to the decreased number of cytoplasmic dysmature eggs, the higher proportion of nuclear mature oocytes is also taken into account, it may be concluded that FSH HP stimulation synchronized follicular maturation better than HMG stimulation. The extent to which the morphology of the oocyte really does influence the results of ICSI, however, is controversial. Thus, De Sutter *et al.* (1996) did not find any relationship between the cytoplasmic appearance of an oocyte and the ICSI fertilization rate. Alikani *et al.* (1995), however, reported a higher rate of miscarriage in women with dysmorphic oocytes.

In our study, we investigated new indicators for the assessment of the quality of stimulation. We also used a different hormone preparation. FSH HP differs from 'pure' FSH not only in a further reduction of the proportion of LH present, but above all in a massive reduction in the content of foreign protein, whose effect on ovarian stimulation has not been defined and therefore whose presence is undesired. The present study, however, cannot answer the question as to which component is responsible for the better results in comparison to HMG stimulation. If a low LH and foreign protein content is considered as being favourable, the development of rFSH represents even greater progress (Howles *et al.*, 1994; Out *et al.*, 1995), as rFSH contains absolutely no LH and no protein with an undefined effect.

A second limitation of our study was that, although the biologist who assessed morphology and nuclear maturity of the oocyte was unaware which stimulation regimen had been used, the clinician taking the decision about the timing of administration of HCG was not blinded. However, the criteria for HCG administration were the same for both the HMG and the FSH HP regimen. That these criteria were followed equally in the two groups is shown by the fact that the duration of stimulation was exactly the same for both regimens: 11.0 ± 0.2 days. Of course, this does not exclude an unconscious observer bias, but makes it highly improbable.

In summary, it can be concluded that the FSH HP short-term stimulation protocol synchronizes oocyte maturation better than a comparable stimulation with HMG. The proportion of nuclear mature oocytes is higher, and the number of cytoplasmic dysmature oocytes lower. The present study, however, did not elucidate the question as to whether the difference is caused by the lower LH content, or by the reduction in the amount of undefined protein.

Acknowledgement

This study was in part supported by Laboratoires Serono S A., Zug (Switzerland).

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Received on March 22, 1996, accepted on August 6, 1996