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Serum and follicular fluid levels of soluble Fas, soluble Fas ligand and apoptosis of luteinized granulosa cells in PCOS patients undergoing IVF

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BACKGROUND: There are limited data about the levels of soluble apoptotic factors and their modulation with therapeutic regimens in IVF cycles. The aim of the current study was to determine follicular fluid, and serum levels of soluble Fas (sFas) and soluble Fas ligand (sFasL) in PCOS patients undergoing IVF/ICSI cycles; also to investigate the effects of metformin on these factors and on apoptosis of luteinized granulosa cells. METHODS: We investigated the serum and follicular fluid levels of sFas and sFasL in patients with PCOS ($n = 28$) and compared them with those of the patients with infertility due to male factor ($n = 12$) undergoing IVF cycles. Effects of metformin therapy on these parameters and apoptosis of luteinized granulosa cells were also investigated among the patients with PCOS. RESULTS: Serum levels of sFas were significantly lower in the PCOS group compared to those in women with infertility due to male factor. Metformin therapy in PCOS patients preceding IVF cycles increased serum levels of sFas and decreased follicular fluid levels of sFasL compared to those on placebo. Follicular fluid from PCOS patients demonstrated luteinized granulosa cell DNA fragmentation in agarose gel, whereas a similar pattern was not observed among PCOS patients undergoing metformin therapy. CONCLUSION: Decreased serum levels of sFas and luteinized granulosa cell DNA fragmentation is observed in patients with PCOS undergoing IVF cycles. Metformin therapy preceding IVF demonstrates an antiapoptotic effect with increased serum levels of sFas, decreased follicular fluid levels of sFasL and prevention of luteinized granulosa cell DNA fragmentation.

Key words: apoptosis/IVF/metformin/PCOS/sFas

Introduction

Polycystic ovary syndrome (PCOS), a widely prevalent disorder among reproductive-aged women, is still an enigma regarding its physiopathology (Homburg, 2002). A combination of abnormally functioning genes are involved in the pathophysiology of PCOS, either contributing to, or as a consequence of, the arrested development of follicles. Apoptosis—programmed cell death—is an essential mechanism for follicular atresia and arrested development of follicles in both normal physiology and PCOS (Homburg, 1998).

Diverse hormonal signals by gonadotrophins, growth factors, steroid hormones and death factors, such as Fas ligand (FasL), converge on selective intracellular pathways to regulate follicular apoptosis (Kaipia and Hsueh, 1997). Fas and Fas ligand are membrane proteins that exist in both transmembrane and soluble forms. The former triggers apoptosis when bound by FasL, whereas the latter inhibits Fas-mediated apoptosis by preventing death signal transduction (Ueno *et al.*, 1999). Soluble Fas (sFas) is detected in human sera and fluids of the reproductive system, including seminal plasma, oviductal fluid and follicular fluid (Srivastava and Fichorova, 1998). Presence of apoptotic and proliferative factors in both follicular fluid and serum samples of women undergoing IVF cycles further confirms apoptosis as a regulatory mechanism for oocyte maturation and survival (Sarandakou *et al.*, 2003).

Among women with PCOS, 80% of obese and 30–40% of women with normal weight present with hyperinsulinaemia (Dunaif *et al.*, 1989). Insulin modulates insulin-like growth factor (IGF)-binding protein and biologically active IGF-I levels which are important factors in ovarian cellular mitosis, steroidogenesis and apoptosis. Metformin, an oral biguanide,

improves hyperandrogenism by decreasing insulin levels. Clinical outcomes of metformin therapy prior to IVF are controversial with few studies on this subject (Stadtmauer *et al.*, 2001; Fedorcsak, 2003; Kjotrod *et al.*, 2004). Metformin is suggested to be beneficial in PCOS patients undergoing gonadotrophin therapy and IVF as well as ovulation induction (Stadtmauer *et al.*, 2002).

Analysis of follicular fluid in combination with serum reflects autocrine and paracrine factors regulating ovarian folliculogenesis along with systemic endocrine dynamics (Lambert-Messerlian *et al.*, 1997). To our knowledge there are limited data in the literature about the levels of soluble apoptotic factors in IVF cycles and whether they have any prognostic value on the clinical outcome. In this prospective, randomized, placebo-controlled study, we investigated the serum and follicular fluid levels of sFas and sFasL in patients with PCOS undergoing IVF/ICSI cycles and compared them with those of infertility due to male factor. We also investigated whether metformin therapy prior to IVF–ICSI cycles alters any of these parameters and luteinized granulosa cell apoptosis among patients with PCOS.

Materials and methods

The study was performed with patients attending Centrum IVF Clinic between April 2003 and May 2004. Informed consent in writing was obtained from each patient; consent forms and protocols were approved by the Local Ethics Committee.

Individual follicular fluid and serum samples were collected from 40 women undergoing IVF treatments. Patients with PCOS (n = 28) had the diagnostic criteria of oligomenorrhoea or amenorrhoea as a surrogate for oligo-anovulaton since menarche. These patients also had at least one of the following indications of hyperandrogenism: (i) a hirsutism score of >7, according to Ferriman and Gallway classification (Ferriman and Gallway, 1961); (ii) an elevated level of serum testosterone (≥ 3.15 nmol/l). All other causes of hyperandrogenism were ruled out before the diagnosis of PCOS. Diagnosis of PCOS follows the criteria approved by the Rotterdam Consensus Conference since oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism fulfil two out of three criteria for the revised diagnosis of PCOS by the Conference (Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). PCOS patients were divided into two groups, one group receiving metformin (n = 14), and the other receiving placebo (n = 14) for 8 weeks before IVF–ICSI cycle. Subjects treated with hormonal medications or insulin-lowering agents within 3 months were excluded from the study. Twelve had infertility due to male factor which was described as $<500\,000$ sperm with progressive motility and $>90\%$ abnormal sperm. Ovulation was confirmed by mid-luteal progesterone measurements. Progesterone levels <3 ng/ml were accepted as evidence for anovulation.

IVF–ICSI stimulation procedure

All patients underwent controlled ovarian stimulation (COS) with a combination of GnRH agonist and recombinant (r)FSH, using a long protocol (n = 40) for IVF–ICSI treatment. During the standard long protocol for pituitary suppression, GnRH agonist (0.1 mg Decapeptyl; Ferring, Germany) was administered s.c. from day 21 of the previous cycle followed by FSH (Gonal F; Serono, Turkey) for ovarian stimulation. The standard initial dose of gonadotrophin was 2–4 ampoules of rFSH (150–300 IU) per day and doses were adjusted based on individual responses. hCG (10 000 IU, Pregnyl; Organon, Turkey) was administered when a minimum of three leading follicles ≥ 18 mm mean diameter and serum levels of E_2 <5500 pg/ml were detected. Oocyte retrieval was performed by vaginal ultrasound-guided follicular puncture, 35–36 h following hCG administration. In addition to patients with infertility due to male factor, patients with PCOS were offered ICSI, a procedure which has the advantage of bypassing the barriers that may be of oocyte origin, and may thus overcome the lower fertilization rates in PCOS patients during their IVF cycles (Hwang *et al.*, 2005). Oocytes were inspected for the presence of pronuclei 16–18 h after ICSI procedure. The embryos were evaluated according to blastomere size equality and the relative proportion of anucleate fragments. A maximum number of two embryos was transferred on the third day. Pregnancy was confirmed when serum hCG concentrations were rising on at least two separate occasions between the 12th and 14th days after the embryo transfer. Clinical pregnancy was diagnosed by ultrasonography during the 7th week of pregnancy.

In PCOS patients, for the randomization process, women were given an envelope labelled as either metformin or placebo according to the code provided by computer-generated randomization in blocks. Patients in the metformin group received metformin (Glucophage Retard; Ilsan-Iltaş Pharmaceuticals, Istanbul, Turkey) 850 mg twice a day if they had a body mass index (BMI) of <28 kg/m^2 or three times a day (850 mg) for a BMI >28 kg/m^2 for 8 weeks before their first IVF–ICSI cycle. Placebo identical to the metformin capsule was obtained from the same company. Patients were instructed not to modify their usual eating habits throughout the study. All patients received their treatments until a positive pregnancy test.

Serum and follicular fluid collection and measurement of sFas, sFasL levels

Blood samples were drawn just before follicle aspiration. Follicular fluid aspirates from all mature follicles (≥ 14 mm diameter) were collected at the time of transvaginal ultrasound-guided oocyte retrieval and immediately centrifuged at 4°C. The cell-free supernatants were aliquoted and stored at -80°C until assay. As a reflection of follicular asynchrony during ovarian stimulation for IVF, wide interfollicular variations in steroid and cytokine concentrations have been reported (Barak *et al.*, 1992). Therefore, we collected pooled follicular fluid aspirations from each patient in an attempt to assess whole ovarian production as previously recommended, rather than to evaluate each follicle separately (Orvieto *et al.*, 1995).

Follicular fluid and serum levels of sFas and sFasL were analysed by a sandwich enzyme-linked immunosorbent assay (human sFasL and sFas; Biosource International, CA, USA). Sensitivities, intra- and inter-assay coefficients of variation were 0.1 ng/ml, 6.1 and 7% for sFasL and 20 pg/ml, 4.4 and 5.9% for sFas respectively.

Serum and follicular levels of sFas, sFasL and the parameters participating in IVF outcome, including serum levels of E_2 on day 3 of the menstrual cycle and on the day of hCG injection, number of antral follicles and oocytes >17 mm in diameter, total number of oocytes retrieved, metaphase I and II oocytes, rate of fertilization, number of embryos transferred, rates of biochemical and clinical pregnancies were compared between the study groups.

DNA isolation and fragmentation analysis

Cellular DNA was extracted from luteinized granulosa cells, precipitated and dissolved as described previously with some modifications (Sanbrook *et al.*, 1989). Briefly, granulosa cells were collected by centrifugation at 800rpm ($100 \times g$) for 5 min at room temperature. All cells were treated with 3 ml lysis buffer (400 mmol/l NaCl,

10 mmol/l Tris–HCl, 2 mmol/l EDTA, pH 8.0), 200 μl 10% sodium dodecyl sulphate and 150 μl proteinase K (10 mg/ml), mixed gently and incubated overnight at 37°C. The next day 1 ml of ammonium acetate (saturated) was added onto the mixture and incubated for 15 min at room temperature. The mixture was then centrifuged at 4000rpm (2500 × g) for 30 min at room temperature. Supernatant was transferred into another tube and 2.5 vols of absolute ethanol was added. DNA was dissolved in Tris–EDTA buffer (pH 8.0). Extracted DNA fragments were electrophoretically separated on 2% agarose gel containing ethidium bromide (0.5 mg/ml) in Tris Acetate-EDTa buffer at 90 V for 1 h. DNA samples were visualized in a UV transilluminator.

Statistical analysis

Serum and follicular levels of sFas, sFasL and the parameters participating in IVF outcome were not normally distributed. Mann–Whitney *U*-test, Kruskal–Wallis *H*-test and Fisher's exact tests were used where appropriate. SPPS for Windows 10.0 software was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

The mean age, duration of infertility and body mass index of women were similar between the study groups (Table I).

Before the current study, we could not find any data about SD of apoptotic factors in follicular fluid in PCOS, in order to calculate the number of samples needed in each group. At the end the study, power was calculated as 88% by NCSS/PASS program.

Table I. sFas, sFasL levels and clinical outcome measures for IVF in study groups

Patients	Male factor infertility	PCOS
	(n = 12)	(n = 28)
Age (years)	31.8 ± 2.8	31.8 ± 2.8
Duration of infertility (years)	10.5 (6–15)	8.1(4–14)
Body mass index (kg/m^2)	25.3 (18.7–27.5)	24 (20–31.2)
Estradiol (pg/ml)	71 (4–96)	60 (31–112)
Antral follicle(s)	5.9 (2–11)[a]	8 (6–13)
Estradiol on hCG day (pg/ml)	2615 (1280–4310)	3222 (1382–6439)
≥17 mm follicle	7 (4–12)	7.5 (3–6)
No. of retrieved oocytes	13.5 (10–25)	17.5 (11–30)
No. of metaphase II oocytes	10.5 (8–17)	12 (6–23)
No. of metaphase I oocytes	4 (3–8)	6 (0–10)
Fertilization rate (%)	75 (67–85)	89.1 (62–100)
No. of embryos transferred	2 (2–3)	2 (1–2)
sfasL–follicular fluid (ng/ml)	0.027 (0.007–0.048)	0.030 (0.002–0.054)
sfas–folliclar fluid (ng/ml)	0.091 (0.066–0.127)	0.083 (0.065–0.109)
sfasL–serum (ng/ml)	0.039 (0.006–0.60)	0.036 (0.002–0.093)
sfas–serum (ng/ml)	0.132 (0.119–0.167)[a]	0.098 (0.058–0.161)
Estradiol–follicular fluid (ng/ml)	3832 (2837–4827)	4048 (3279–4817)

[a]$P < 0.05$: infertility due to male factor versus polycystic ovarian syndrome (PCOS).

Significantly lower serum levels of sFas were detected in women with PCOS compared to those in the male factor group ($P < 0.05$) (Table I).

When we compared the serum and follicular fluid levels of both sFas and sFasL among PCOS patients receiving either metformin or placebo, the metformin group had decreased levels of sFasL in follicular fluid and increased levels of sFas in serum ($P < 0.05$) (Table II).

DNA fragmentation of granulosa cells in follicular fluid samples was analysed among PCOS patients with and without metformin therapy. We did not observe granulosa cell DNA fragmentation in follicular fluid samples among PCOS patients undergoing metformin therapy (Figure. 1). However, follicular fluid from PCOS patients demonstrated luteinized granulosa cell DNA fragmentation in agarose gel (Figure. 2).

Clinical pregnancy and implantation rates for patients with infertility due to male factor and PCOS were 66.7 and 51%, and 35.6 and 26.5% respectively (not significant; Table III).

There was a tendency for higher number of retrieved oocytes (18 versus 13, $P = 0.07$), increased implantation and clinical pregnancy rates among PCOS patients on metformin therapy, though not statistically significant in comparison to those of PCOS patients on placebo. Clinical pregnancy and implantation rates for metformin and PCOS (placebo) groups were 52.6 versus 47% and 28 versus 25% respectively (not significant).

Discussion

The Fas–FasL interaction is one of the fundamental measures for the induction of apoptosis; however, the exact role for soluble forms of these proteins in the reproductive system is still unknown. Fas and FasL expressions are demonstrated in the ovary and Fas is suggested to trigger apoptosis of theca and granulosa cells in PCOS (Cataldo *et al.*, 2000). An alteration in Fas-mediated apoptosis is suggested as a possible promoter of ovarian vascular remodelling in PCOS (Webber *et al.*, 2003). The incidence of apoptosis in granulosa cells from FSH-primed polycystic ovaries is increased compared to those from FSH-primed normal ovaries during *in vitro* maturation (Mikkelsen *et al.*, 2001). Dysregulation in the secretion of ovarian growth factors, deficit in the paracrine control of folliculogenesis and increased androgen production associated with PCOS may lead to significantly lower serum levels of sFas in women with PCOS undergoing IVF cycles compared to those in the male factor group in the current study. The systemic apoptotic tendency in patients with PCOS did not affect the overall clinical outcomes of IVF including clinical pregnancy, implantation and abortion rates compared to the control group; however, the power of the study may not be sufficient to comment on clinical outcomes.

Metformin reduces insulin resistance, insulin secretion and hyperinsulinaemia by increasing insulin sensitivity (Dunn and Peters, 1995). Although there are controversial data about the outcomes of metformin, the rationale for this treatment modality is to reduce insulin resistance and sequelae of hyperinsulinaemia including hyperandrogenism (Crave *et al.*,

Table II. sFas, sFasL levels and clinical outcome measures for IVF among patients with polycystic ovarian syndrome (PCOS) receiving metformin and placebo

	PCOS + metformin (n = 14)	PCOS + placebo (n = 14)	P
Age (years)	31.8 ± 5.3	30.8 ± 3.4	NS
Duration of infertility (years)	10 (6–13)	6 (4–14)	NS
Body mass index (kg/m^2)	24 (20.9–28)	24 (20–31.25)	NS
Estradiol (pg/ml)	62 (31–96)	58 (42–112)	NS
Total testosterone (0.35–3.15 nmol/l)	3.2 ± 0.53	3.3 ± 0.63	NS
Prolactin (62–1075 pmol/l)	463.6 ± 311.4	474.1 ± 258.1	NS
Fasting glucose (mg/dl)	88.2 ± 14.5	84.3 ± 12.2	NS
Insulin (IU/l)	16.3 ± 6.78	16.9 ± 6.69	NS
Antral follicle(s)	9 (6–13)	8 (6–12)	NS
Estradiol (pg/ml)	3200 (1382–6400)	3245 (1526–5137)	NS
≥17 mm follicle	9 (6–16)	5 (3–9)	NS
No. of retrieved oocytes	18 (11–30)	13 (11–27)	0.007
No. of metaphase II oocytes	12 (9–23)	13 (6–18)	NS
No. of metaphase I oocytes	6 (1–7)	6 (0–10)	NS
Fertilization rate (%)	83 (75–100)	90 (62–100)	NS
No. of embryos transferred	2 (2–2)	2 (1–2)	NS
sFasL–follicular fluid (ng/ml)	0.019 (0.002–0.03)	0.037 (0.021–0.054)	0.004
sFas–folliclar fluid (ng/ml)	0.087 (0.068–0.097)	0.079 (0.065–0.109)	NS
sFasL–serum (ng/ml)	0.031 (0.002–0.93)	0.042 (0.014–0.066)	NS
sFas–serum (ng/ml)	0.126 (0.08–0.161)	0.87 (0.058–0.109)	0.026
Estradiol–follicular fluid (ng/ml)	3976 (3110–4862)	4020 (3381–4659)	NS

NS = not significant.

1995). There are only limited studies reporting the effects of metformin on IVF outcomes (Stadtmauer *et al.*, 2001; Onalan, 2005).

In the current study we demonstrated decreased levels of sFasL in follicular fluid and increased levels of sFas in serum samples among PCOS patients receiving metformin compared to those receiving placebo. Patients with PCOS on placebo had luteinized granulosa cell DNA fragmentation in follicular fluid samples. Similar DNA fragmentation was not observed in PCOS patients on metformin therapy, which may imply an antiapoptotic effect of metformin on soluble factors both in follicular fluid and serum. This effect can be a result of reduced androgen production by metformin that may inhibit apoptosis; however, the role of androgens in follicle development, whether atretogenic or trophic, is debatable probably due to interspecies differences in ovarian follicular development with different experimental conditions (Jonard and Dewailly, 2004). As in the β-cell in the pancreas (Kefas *et al.*, 2004), metformin can also inhibit apoptosis in granulosa cells, which may result in the induction of biosynthesis and release pathways preventing the functional exhaustion.

There was a tendency for higher number of retrieved oocytes, increased implantation and clinical pregnancy rates among PCOS patients on metformin; however, the differences were not statistically significant in comparison to those of PCOS patients on placebo in the current study. Apoptosis is suggested to predominate in the granulosa cell layer of preantral and antral follicles rather than the dominant follicles (≥10 mm) in normal ovaries with androgen-dominant follicles uniformly displaying characteristics of apoptosis (Yuan and Giudice, 1997). The Fas–FasL system may play a role in preventing oocyte atresia during folliculogenesis. Since levels of sFas and FasL may not be critical in controlling the quality of oocytes once they overcome meiotic arrest and achieve the metaphase II stage of development (Jose de los Santos *et al.*, 2000), the Fas–FasL system may not have negative impact on IVF outcomes including fertilization of mature oocytes, embryo quality or pregnancy rate. *In vitro* and *in vivo* studies investigating the direct or indirect effects of metformin on ovarian cell apoptosis may further delineate its mechanism of action and studies with larger samples may shed light on the consequences for clinical outcomes.

To our knowledge this is the first study demonstrating serum and follicular fluid levels of sFas and sFasL in patients with PCOS undergoing IVF cycles and elucidating the effects of prior metformin therapy on these parameters and apoptosis of luteinized granulosa cells in follicular fluid.

In conclusion, the current study suggests that sFas and sFasL are detected in both serum and follicular fluid samples from IVF cycles. Patients with PCOS undergoing IVF cycles

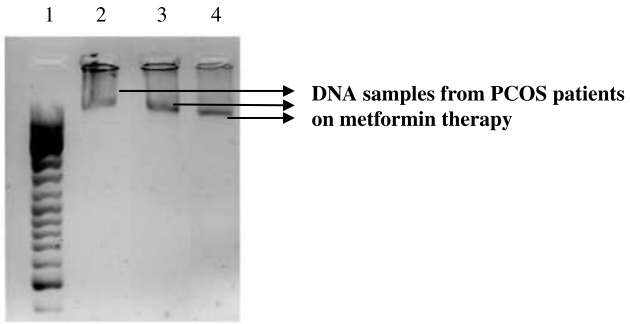

Figure 1. Agarose gel electrophoresis [2% agarose gel containing ethidium bromide (0.5 mg/ml) in TAE buffer] of genomic DNA extracted from granulosa cells obtained from PCOS patients on metformin therapy. Lane 1: DNA ladder from 50 to 1031 bp. Lanes 2, 3 and 4: genomic DNA samples from PCOS patients on metformin therapy.

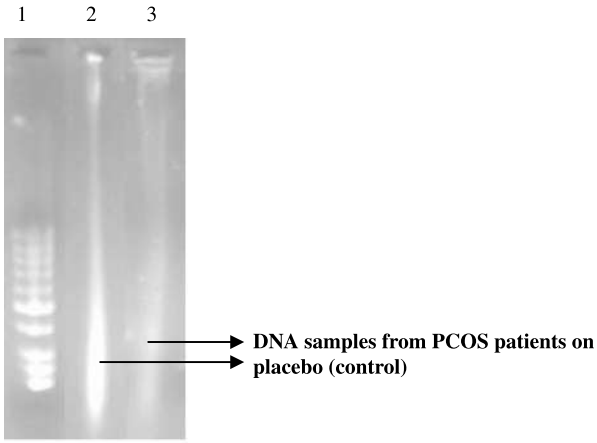

Figure 2. Agarose gel electrophoresis [2% agarose gel containing ethidium bromide (0.5 mg/ml) in TAE buffer] of genomic DNA extracted from granulosa cells from PCOS patients (control group) Lane 1: DNA ladder from 50 to 1031 bp. Lanes 2 and 3: Genomic DNA samples from PCOS patients on placebo (control).

Table III. Pregnancy outcome for IVF in study groups

	Male factor (n = 12)	Polycystic ovarian syndrome	
		Metformin (n = 14)	Placebo (n = 14)
Cinical pregnancy (%)	66.7	52.6	47
Implantation rate (%)	35.6	28	25
Abortion rate (%)	3.6	4.7	3.7

have lower serum levels of sFas. Metformin therapy in PCOS patients preceding IVF cycles decreases follicular fluid levels of sFasL, increases serum levels of sFas and prevents luteinized granulosa cell DNA fragmentation, demonstrating an antiapoptotic effect.

References

Barak V, Mordel N, Zajicek G, Kalichman I, Treves AJ and Laufer N (1992) The correlation between interleukin 2 and soluble interleukin 2 receptors to oestradiol, progesterone and testosterone levels in periovulatory follicles of in-vitro fertilization patients. Hum Reprod 7,926–929.

Cataldo NA, Dumesic DA, Goldsmith PC and Jaffe RB (2000) Immunolocalization of Fas and Fas ligand in the ovaries of women with polycystic ovary syndrome: relationship to apoptosis. Hum Reprod 15,1889–1897.

Crave JC, Fimbel S, Lejeune H, Cugnardey N, Dechaud H and Pugeat M (1995) Effects of diet and metformin administration on sex hormone-binding globulin, androgens, and insulin in hirsute and obese women. J Clin Endocrinol Metab 80,2057–2062.

Dunaif A, Segal KR, Futterweit W and Dobrjansky A (1989) Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes 38,1165–1174.

Dunn CJ and Peters DH (1995) Metformin. A review of its pharmacological properties and therapeutic use in non-insulin-dependent diabetes mellitus. Drugs 49,721–749.

Fedorcsak P, Dale PO, Storeng R, Abyholm T and Tanbo T (2003) The effect of metformin on ovarian stimulation and in vitro fertilisation in insulin resistant women with polycystic ovary syndrome: an open-label randomised cross-over trial. Gynecol Endocrinol 17,207–214.

Ferriman D and Gallway JD. (1961) Clinical assessment of body hair growth in women. J Clin Endocrinol and Metab 21,1440–1448.

Homburg R (1998) Involvement of growth factors in the pathophysiology of polycystic ovary syndrome. Gynecol Endocrinol 12,391–397.

Homburg R (2002) Should patients with polycystic ovarian syndrome be treated with metformin? A note of cautious optimism. Hum Reprod 17,853–856.

Hwang JL, Seow KM, Lin YH, Hsieh BC, Huang LW, Chen HJ, Huang SC, Chen CY, Chen PH and Tzeng CR (2005) IVF versus ICSI in sibling oocytes from patients with polycystic ovarian syndrome: a randomized controlled trial. Hum Reprod, in press.

Jonard S and Dewailly D (2004) The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest. Hum Reprod Update 10,107–117.

Jose de los Santos M, Anderson DJ, Racowsky C and Hill JA (2000) Presence of Fas-Fas ligand system and bcl-2 gene products in cells and fluids from gonadotropin-stimulated human ovaries. Biol Reprod 63,1811–1816.

Kaipia A and Hsueh AJ (1997) Regulation of ovarian follicle atresia. Annu Rev Physiol 59,349–363.

Kefas BA, Cai Y, Kerckhofs K, Ling Z, Martens G, Heimberg H, Pipeleers D and Van de Casteele M (2004) Metformin-induced stimulation of AMP-activated protein kinase in beta-cells impairs their glucose responsiveness and can lead to apoptosis. Biochem Pharmacol 68,409–416.

Kjotrod SB, von During V and Carlsen SM (2004) Metformin treatment before IVF/ICSI in women with polycystic ovary syndrome; a prospective, randomized, double blind study. Hum Reprod 19,1315–1322.

Lambert-Messerlian G, Taylor A, Leykin L, Isaacson K, Toth T, Chang Y and Schneyer A (1997) Characterization of intrafollicular steroid hormones, inhibin, and follistatin in women with and without polycystic ovarian syndrome following gonadotropin hyperstimulation. Biol Reprod 57,1211–1216.

Mikkelsen AL, Host E and Lindenberg S (2001) Incidence of apoptosis in granulosa cells from immature human follicles. Reproduction 122, 481–486.

Onalan G, Pabuçcu R, Goktolga U, Ceyhan T, Bagis T and Cıncık M (2005) Metformin treatment in patients with PCOS undergoing in vitro fertilization: a prospective randomized trial. Fertil Steril, in press.

Orvieto R, Voliovitch I, Fishman P and Ben-Rafael Z (1995) Interleukin-2 and ovarian hyperstimulation syndrome: a pilot study. Hum Reprod 10, 24–27.

Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group (2004) Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 19, 41–47.

Sanbrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sarandakou A, Malamitsi-Puchner A, Baka S, Rizos D, Hassiakos D and Creatsas G (2003) Apoptosis and proliferation factors in serum and follicular fluid from women undergoing in vitro fertilization. Fertil Steril 79,634–636.

Srivastava, MLJ, Fichorova R, De los Santos and Anderson DJ (1998) Soluble Fas (sFas) and soluble Fas ligand (sFasL) in human reproductive tract fluids. Proceedings of the 45th Annual Meeting of the Society for Gynecologic Investigation, Chicago, IL 1998 Abstract T203.

Stadtmauer LA, Toma SK, Riehl RM and Talbert LM (2001) Metformin treatment of patients with polycystic ovary syndrome undergoing in vitro fertilization improves outcomes and is associated with modulation of the insulin-like growth factors. Fertil Steril 75,505–509.

Stadtmauer LA, Wong BC and Oehninger S (2002) Should patients with polycystic ovary syndrome be treated with metformin? Benefits of insulin sensitizing drugs in polycystic ovary syndrome—beyond ovulation induction. Hum Reprod 17,3016–3026.

Ueno T, Toi M and Tominaga T (1999) Circulating soluble Fas concentration in breast cancer patients. Clin Cancer Res 5,3529–3533.

Webber LJ, Stubbs S, Stark J, Trew GH, Margara R, Hardy K and Franks S (2003) Formation and early development of follicles in the polycystic ovary. Lancet 362,1017–1021.

Yuan W and Giudice LC (1997) Programmed cell death in human ovary is a function of follicle and corpus luteum status. J Clin Endocrinol Metab 82,3148–3155.

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