Uterine vascularization and pregnancy outcome in patients undergoing intracytoplasmatic sperm injection: The role of nitric oxide

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Abstract *Purpose:* To evaluate whether, after pituitary desensitisation, the impedance to flow in the uterine vessels may be an indicator of an ICSI programme outcome, and to test the relationship between intrafollicular nitric oxide and oocyte/embryo quality.

Methods: Thirty-eight women, on the basis of impedance to flow at the level of uterine artery, evaluated on the first day of COH, were divided in patients with normal (Pulsatility Index—PI, ≤ 2.5 ; Group I, $n = 11$) and elevated ($PI > 2.5$; Group II, $n = 27$) PI values. The patients were submitted to hormonal, ultrasonographic and Doppler evaluations. Plasma and follicular fluid concentrations of nitrites/nitrate (NO_2^-/NO_3^-) were assayed.

Results: In the Group I, the impedance to flow remained lower than in Group II and was associated to good quality embryos and to a higher pregnancy rate. Follicular fluid NO_2^-/NO_3^- levels were inversely correlated with the embryo quality. The uterine artery PI and the pregnancy rate were inversely correlated. The PIs analysed on the day 1 of stimulation, were positively correlated with those registered on day 8 and on the day of ovum pick-up.

Conclusions: A Doppler analysis done on day 1 of controlled ovarian hyperstimulation may be an useful indicator of ART outcome.

Keywords ICSI · Ultrasonography · Doppler · Nitric oxide · Pregnancy rate

Introduction

Helping infertile couples to have healthy children is one of the primary tasks of assisted reproductive technologies. In order to fulfil this task, reproductive medicine constantly needs to obtain information on physiology and pathophysiology of infertility and to develop efficient strategies for controlled ovarian hyperstimulation (COH).

The current use of different ovulatory agents and laboratory techniques allows high rates of oocyte retrieval and embryo transfer. However, pregnancy rates remain disappointingly low. This may be related to oocyte/embryo quality and/or endometrial receptivity.

Transvaginal colour flow Doppler ultrasound by facilitating the detection of small vessels and the measurements of impedance to flow in the utero-ovarian circulation, may represent an important tool for studying the female reproductive system and the pelvic hemodynamics.

It has been suggested that uterine blood flow has an important role in endometrial receptivity; and it has been demonstrated that embryos fail to implant in women with impaired uterine perfusion as expressed by high values of pulsatility index (PI) [1–4].

An increased vascularization of ovarian follicles during the course of their development occurs in experimental animals [5]. In women, an enhanced vascularization seems to be responsible for the selection and maturation of follicles both in spontaneous and stimulated IVF cycles [6–8]. Gonadotrophins, steroids, prostaglandins and other vasoactive molecules are involved in the regulation of ovarian blood flow [9]. The importance of nitric oxide (NO) as an

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intra- and intercellular modulator has been recognized in many biological processes, including ovarian physiology [10]. Nitric oxide is a labile and diffusible molecule which forms stable oxidized metabolites (nitrite/nitrate; $\text{NO}_2^-/\text{NO}_3^-$) detectable in many biological fluids. In vivo, NO is formed from L-arginine either by a constitutive calcium-dependent, or a pro-inflammatory cytokine-inducible, NO-synthase [11]. Although the precise role of NO has not been elucidated, it has been suggested that NO participates in periovulatory modulation of ovarian blood flow and seems to be involved in follicular maturation and ovulation [10, 12, 13]. A critical level of NO seems to be mandatory for the establishment of pregnancy [14–16]. In fact, although Chwalisz et al. [17] demonstrated that NO is required for normal embryonic development, Barroso and co-workers [18] showed that high NO concentrations, in mice, may be cytotoxic and could alter the embryo development in vitro, and its implantation in vivo. Similar results were obtained in humans [15, 16].

The aim of the present study was to evaluate if, in patients with normal ovarian reserve, the impedance to flow in the uterine vessels may be used as an indicator of an ICSI programme outcome when studied after pituitary desensitisation and before starting COH. In addition, we tested the possible relationship between uterine vascularization and circulating NO and between intrafollicular NO and oocyte/embryo quality.

Patients and methods

The study protocol was approved by the institutional ethics review committee. In all, 38 women attending the Modena University Infertility Clinic, and included in an ICSI programme, participated the study after giving informed consent. On the basis of impedance to flow at the level of uterine artery, evaluated on the first day of COH after pituitary desensitisation, the patients were arbitrarily divided in those with normal ($\text{PI} \leq 2.5$; Group I, $n = 11$) and elevated ($\text{PI} > 2.5$; Group II, $n = 27$) pulsatility index values.

To avoid any bias due to utero-ovarian vascular interference a very homogeneous group of patients was selected. All the women were, in fact, recruited among couples who suffered from pure male infertility (severe oligozoospermia: sperm concentration $< 5 \times 10^5/\text{mL}$) [19] and who were characterized by: age > 30 years and < 38 years; regular menstrual cycles (28 ± 4 days); patent tubes on X-ray hysterosalpingography, hormonal and ultrasonographic confirmed ovulatory cycles, normal weight with a body mass index [$\text{BMI} = \text{weight (kg)}/8^2 (\text{m}^2)$] ≥ 20 and ≤ 25 . Patients with intercurrent illness were excluded from the study. Other exclusion criteria were: uterine malformations, fibroids, polyps, endometrial synechiae; endometriosis; ovarian functional

cyst; polycystic ovarian syndrome; unilateral ovarian resection or ovariectomy. Furthermore, patients who took intense regular exercise, heavy smokers (> 10 cigarettes/day), and with hypertension (systolic blood pressure > 140 mmHg and/or diastolic pressure > 90 mmHg) were excluded from the study. Women had not received hormonal treatments for at least 4 months before the ICSI attempt.

To assess ovarian reserve, on day 3 of the cycle antecedent the ICSI attempt, peripheral blood was obtained from all patients between 8.00 and 11.00 a.m., after an overnight fast, and basal plasma estradiol (E_2), testosterone (T), follicle stimulating hormone (FSH), and luteinizing hormone (LH) concentrations were determined by a radio-immunoassay (RIA) (Radim, Pomezia, Italy). Androstenedione (A) was measured by RIA with the Quantitative Measurement of Androstenedione in Serum and Plasma kit (Diagnostic System Laboratories, Inc., Webster—TX, USA). Serum growth hormone (GH) concentrations were similarly measured by radioimmunoassay (Sorin Biomedica, Saluggia, Italy). The concentrations of insulin-like growth factor (IGF-1) were determined by double-antibody radioimmunoassay (Immuno Nuclear Corp., Stillwater—CA, USA). Results of hormonal values were converted to SI units using the following conversion factors: LH (IU/L) = mIU/mL $\times 1.0$; FSH (IU/L) = mIU/mL $\times 1.0$; E_2 (pmol/l) = pg/mL $\times 3.761$; T (nmol/L) = ng/mL $\times 3.467$; A (nmol/L) = ng/dL $\times 0.0349$; GH ($\mu\text{g/L}$) = ng/mL $\times 1$; IGF-1 (nmol/L) = ng/mL $\times 0.131$.

In addition, on day 3 of the cycle antecedent the ICSI attempt, the number of small antral ovarian follicles (maximum diameter < 1.0 cm) was evaluated by transvaginal ultrasonography and the uterine vascularization was assessed by uterine artery Doppler analysis.

Controlled ovarian hyperstimulation was achieved by an injection, on day 20 of the cycle, of i.m. GnRH-a triptorelin (Decapeptyl 3.75; Ipsen, Milano, Italy) and, after pituitary desensitisation (plasma oestradiol concentrations < 100 pmol/L; ovaries with no follicles > 5 mm in diameter and endometrial thickness < 5 mm), subcutaneous recombinant-alpha-follitropin (Gonal-f; Serono, Rome, Italy. 225 IU in the first three days of the cycle, then in an individually assessed dosage) administration.

The ICSI cycles were cancelled when estradiol plasma levels were $< 1,100$ pmol/L and/or < 3 follicles were recruited by cycle day 8. Similarly, the ICSI cycles were cancelled in those patients at risk of developing ovarian hyperstimulation syndrome (≥ 15 follicles/ovary and/or estradiol plasma levels $\geq 9,000$ pmol/L).

When at least two follicles > 17 mm in diameter were present, triptorelin and recombinant-alpha-follitropin were withdrawn and 10,000 IU human chorionic gonadotropin (hCG; Profasi; Serono) were administered i.m.. Ultrasonographic oocyte recovery was performed transvaginally 35–36 h after hCG injection. The retrieved oocytes were

classified as mature, immature and atretic on the basis of the morphology and the appearance of the oocyte cumulus-corona complex according to the criteria of Acosta et al. [20]. Intracytoplasmic sperm injection was performed as described by Van Steirthegeheim et al. [21]. To study the impact of embryo quality on implantation, the embryos were graded morphologically before replacement. The embryos were scored as follows: grade A, equal size blastomeres, no fragmentation; grade B, equal or unequal sized blastomeres, <20% fragmentation; grade C, equal or unequal sized blastomeres, 20–50% fragmentation; grade D, equal or unequal sized blastomeres, >50% fragmentation. Embryo transfer (ET) was performed 72 h after oocyte retrieval. Between 1 and 3 embryos were replaced at 6–12-cell stage. Transcervical transfer was carried out using a Frydman catheter (SCS International; Genoa, Italy). The remaining cleaved embryos with <20% fragmentation were allocated to a cryopreservation protocol. Vaginal progesterone (Esolut; Angelini, Rome, Italy) was daily prescribed as luteal phase support until the serum β -hCG assay. A clinical pregnancy was diagnosed by ultrasonographic evidence of embryonic heart activity.

During the ovarian stimulation regimen the patients were submitted to hormonal, ultrasonographic and Doppler evaluations. Plasma and follicular fluid concentrations of nitrites/nitrate ($\text{NO}_2^-/\text{NO}_3^-$) were assayed.

Ultrasound and Doppler examinations

Transvaginal ultrasonographic assessments of endometrial thickness was performed on the day of hCG administration using a 6.5 MHz vaginal transducer (AU 4 Idea, Esaote; Milano, Italy). Measurements of follicular number and size were performed daily beginning on day 8 of the cycle until the day of oocyte retrieval. A modified ovarian synchrony index (no follicles >17 mm/no follicles 10–14 mm $\times$ 100; OSI) was calculated on the day of hCG administration [22].

Doppler flow measurements of uterine and perfollicular arteries were performed transvaginally with a 6.5 MHz (AU 4 Idea) colour Doppler system. The Doppler examination of uterine arteries was performed on day 3 of the cycle antecedent the ICSI attempt, at the beginning of recombinant-alpha-follicle stimulating hormone administration, on day 8 of COH and on the day of oocyte retrieval. The Doppler analysis of perfollicular arteries was done on the day of OPU [23]. All the patients were studied between 08.00 and 11.00 h to exclude the effects of circadian rhythmicity on blood flow [24]. They rested for at least 15 min before being scanned, and completely emptied the bladder to minimize any external effects on blood flow [25]. A 50 Hz filter was used to eliminate low frequency signals originating from vessel wall movements. The maximum ultrasonographic energy was <80 mW/cm². The intensity is within the safety limits suggested by the American Institute for Ultrasound in

Medicine [26]. Colour flow images of the ascending branches of the uterine arteries were sampled lateral to the cervix in a longitudinal plane. The angle of insonation was altered to obtain the maximum colour intensity. When good colour signals were obtained, blood flow velocity waveforms were recorded by placing the sample volume across the vessel and entering the pulsed Doppler mode. The pulsatility index ($\text{PI} = \text{S} - \text{D}/\text{mean}$), defined as the difference between peak systolic (S) and end diastolic (D) flow velocity divided by the mean flow velocity, was calculated electronically by the machine. The PI has been shown to reflect blood flow impedance and may be used when the end diastolic frequency shift is absent or reversed. For each examination the mean value of three consecutive waveforms was obtained. No significant differences between the PI of the left and right uterine arteries were observed. Therefore, the average value of both arteries was used. The perfollicular arteries, on day of oocytes retrieval, were identified around the follicles (>1.0 cm in maximum diameter). Recorded spectra were analysed and PI was obtained. Arteries demonstrating the lowest downstream impedance were selected for measurements, assuming that these are the branches supplying the developing follicles directly. In the results the PIs of both uterine and perfollicular arteries have not been corrected for heart rate. An indication of within-patient precision of the Doppler procedures was obtained by analysing the flow velocity waveforms recorded on three occasions either from uterine and perfollicular arteries at 1 minute intervals. An analysis of variance of the results from 10 patients gave a mean coefficient of variation of 5.1% for uterine and 6.2% for perfollicular arteries and showed no significant differences between the replicate analyses. Ultrasonographic and Doppler analyses were performed by one examiner (C.B.).

Hormonal and biochemical assay

Peripheral blood was obtained between 08.00 and 11.00 h, after an overnight fast, on day 1, day 8, and on day of ovum pick-up (OPU). Blood was immediately centrifuged and serum stored at -70°C , until assays. Hormones (E_2 , T, A, GH, IGF-1) were assayed as previously described.

Nitric oxide production was assessed by monitoring (on day 1, day 8, and day of ovum pick-up) plasma levels of stable oxidation products of NO metabolism [nitrites/nitrates ($\text{NO}_2^-/\text{NO}_3^-$)]. Since very little or no NO_2^- is normally found in the serum, we did not attempt to differentiate between NO_2^- and NO_3^- amounts, therefore we report our results as $\text{NO}_2^-/\text{NO}_3^-$. The $\text{NO}_2^-/\text{NO}_3^-$ were assayed with the Greiss reaction with procedures previously described [27, 28].

$\text{NO}_2^-/\text{NO}_3^-$ levels were also assayed in follicular fluid (FF) in those patients who reached ovum pick-up: after the transvaginal needle aspiration of the accessible follicles, to

Table 1 Demographic, clinical, hormonal, ultrasonographic and Doppler data on day 3 of the cycle preceding the ICSI attempt in patients with normal and elevated uterine artery PIs. Values are expressed as mean $\pm$ standard deviation

	Group I ($n = 11$)	Group II ($n = 27$)	Normal range
Age (yrs)	33.0 $\pm$ 1.9	33.6 $\pm$ 3.3	
Duration of infertility (yrs)	3.7 $\pm$ 2.2	4.1 $\pm$ 3.2	
BMI (kg/m ²)	21.2 $\pm$ 1.1	21.5 $\pm$ 1.3	>20 and <25
FSH (IU/L)	5.6 $\pm$ 2.4	6.0 $\pm$ 1.5	1.5–10
LH (IU/L)	5.2 $\pm$ 2.1	4.5 $\pm$ 2.0	1.5–10
Estradiol (pmol/L)	164 $\pm$ 47	164 $\pm$ 41	<180
Testosterone (nmol/L)	73 $\pm$ 34	52 $\pm$ 34	100–300
Androstenedione (nmol/L)	4.9 $\pm$ 2.6	3.7 $\pm$ 1.6	4–10
GH (μ g/L)	1.9 $\pm$ 0.3	1.3 $\pm$ 0.2	1.0–2.0
IGF-1 (nmol/L)	24.9 $\pm$ 3.9	23.7 $\pm$ 6.4	15–40
Ovarian Follicles <10 mm (n)	7 $\pm$ 2	6 $\pm$ 2	$\geq$ 4
Uterine artery (PI)	2.64 $\pm$ 0.13	2.57 $\pm$ 0.22	<3.0

Notes. Normal range was obtained from normally ovulating patients attending the infertility clinic.

homogenate the fluids and to reduce the possible interfollicular differences, the FFs of follicles ≥ 17 mm were pooled and immediately centrifuged ($2,000 \times g$ for 20 min). The supernatant was stored at -70°C until bioassay. Similarly, aliquots of FFs obtained by the aspiration of all accessible follicle were pooled, centrifuged, stored at -70°C and, subsequently, assayed. The analyses were performed with the same methods used for serum assays [15].

All samples from each subjects were analysed in duplicate in the same assay.

Statistical analysis

A statistical analysis was performed using the Mann–Whitney test, chi-square test, Fisher–Irwin exact test, Wilcoxon test and a one-way analysis of variance where indicated. The relationship between the parameters analysed was assessed using the linear regression method. $P \leq 0.05$ was considered to be the limit of statistical significance. Data are presented as mean $\pm$ SD, unless otherwise indicated.

Results

The patients resulted similar for age, duration of infertility and BMI (Table 1). Hormonal, ultrasonographic and Doppler

evaluations on day 3 of the cycle preceding the ICSI attempt confirmed normal ovarian reserve in both groups of patients (Table 1). No significant differences, in the studied parameters, were observed among the groups.

Thirty-four patients completed the study. The cancellation rate, due to a poor response, was 4/38 (10.5%). These four cycles were suspended on day 8 of COH and belonged to Group II. To avoid bias and confounders, the suspended cycles were extrapolated and considered apart (Group III, $n = 4$). Hormonal, and Doppler evaluations after pituitary desensitisation (plasma oestradiol concentrations <100 pmol/L; ovaries with no follicles >5 mm in diameter and endometrial thickness <5 mm) are reported in Table 2. Except for the obvious significant Doppler differences, no further differences were observed in hormonal and nitrites/nitrates concentrations. How these parameters varied during COH are reported in Figures 1–4: oestradiol was significantly lower in Group III than in other groups on the day 8; the serum GH, IGF-1 and androgens concentrations were not different among the groups; the lowest uterine artery PIs were observed in the Group I patients on day 8 and on the day of OPU; the nitrite/nitrate circulating levels were not significantly different among the groups during COH.

The response to controlled ovarian hyperstimulation was evaluated on the day of hCG administration. The duration of

Table 2 Hormonal and Doppler data on day 1 of COH, after pituitary desensitisation, in patients with normal and elevated uterine artery PIs

	Group I ($n = 11$)	Group II ($n = 23$)	Group III ($n = 4$)
Estradiol (pmol/L)	184 $\pm$ 97	158 $\pm$ 43	116 $\pm$ 56
Testosterone (nmol/L)	53 $\pm$ 33	58 $\pm$ 25	54 $\pm$ 41
Androstenedione (nmol/L)	4.2 $\pm$ 2.1	4.7 $\pm$ 1.1	3.9 $\pm$ 1.8
GH (μ g/L)	1.4 $\pm$ 0.2	1.2 $\pm$ 0.5	1.1 $\pm$ 2.0
IGF-1 (nmol/L)	22.7 $\pm$ 2.9	20.3 $\pm$ 4.3	21.2 $\pm$ 3.3
NO ₂ ⁻ /NO ₃ ⁻ (μ mol/L)	11.8 $\pm$ 7.9	7.5 $\pm$ 7.2	9.5 $\pm$ 8.9
Uterine artery (PI)	2.09 $\pm$ 0.34	2.77 $\pm$ 0.19 ^a	2.92 $\pm$ 0.22 ^b

Notes. Values are expressed as mean $\pm$ standard deviation.

^aGroup I vs Group II: $p = 0.032$.

^bGroup I vs Group III: $p \leq 0.001$.

Fig. 1 Serum estradiol and androgens concentration during ovarian controlled hyperstimulation. On day 8, the estrogens circulating levels were significantly lower in Group III than Group I and Group II. Serum

testosterone and androstenedione concentrations were not significantly different among the groups

recombinant-alpha-follitropin (rec-FSH) administration, the number of rec-FSH ampoules, and rec-FSH units/day did not differ significantly between the groups (Table 3). The number of recruited follicles on the day of hCG administration was similar in Group I and Group II (Table 3). The number of large (>17 mm maximum diameter) and small (10–14 mm maximum diameter) follicles, as well as the OSI (an index of follicular growth homogeneity) and endometrial thickness are reported in Table 3: the number of large (>17 mm) follicles was not significantly different, whereas the number of

small (10–14 mm) follicles and the OSI resulted significantly different between the groups.

On the day of ovum pick-up the perifollicular vascularization was similar in Group I and Group II ($PI = 0.81 \pm 0.08$ vs 0.82 ± 0.10). However, considering the follicles >17 mm, the PIs of perifollicular arteries were lower in Group I (0.66 ± 0.07) than in Group II (0.74 ± 0.10 ; $p = 0.049$). The intrafollicular nitrites/nitrates concentrations were significantly reduced in those patients who presented a lower uterine artery PI on the day 1 of COH (Fig. 4). The reduction

Table 3 Response to controlled ovarian hyperstimulation, in patients with normal and elevated uterine artery PIs

	Group I (n = 11)	Group II (n = 23)	P-value
No of rec-FSH ampoules	35.7 ± 8.7	34.6 ± 10.6	NS
No of days of rec-FSH treatment	12.5 ± 3.0	11.7 ± 2.4	NS
Rec-FSH units/day	2.9 ± 0.7	2.9 ± 0.8	NS
No of recruited follicles	12.1 ± 7.0	13.4 ± 4.0	NS
Follicles >17 mm	3.4 ± 1.2	3.3 ± 0.8	NS
Follicles 10–14 mm	5.5 ± 1.8	7.9 ± 1.3	0.034
Ovarian Synchrony Index (%)	64.1 ± 13.4	48.5 ± 16.5	0.041
Endometrial thickness (mm)	11.1 ± 0.9	10.5 ± 1.5	NS

Notes. Values are expressed as mean ± standard deviation. NS: Not significant.

Fig. 2 Growth hormone and IGF-1 circulating levels were not different among the studied patients. During the COH the values resulted relatively stable

was significantly different considering both the homogenate of the follicular fluid of all the accessible follicles and the pool of the follicular fluids of the follicles >17 mm in the maximum diameter.

The number and quality of oocytes collected, and the fertilization rate (number of oocytes fertilized/number of oocytes collected $\times 100$) did not differ significantly between the two groups (Table 4). The number of embryos transferred was similar in both groups (2.1 ± 0.7 vs 1.9 ± 0.8). In the Group I were obtained 9 pregnancies, whereas only 1 pregnancy was obtained in the Group II. In all the studied pa-

tients the pregnancy rate per cycle was 26.3%, whereas the pregnancy rate per embryo transfer was 29.4%. However, the pregnancy rate per cycle (81.8% vs 3.7%; $p \leq 0.0001$) and the pregnancy rate per embryo transfer (81.8% vs 4.3%; $p \leq 0.0001$) was significantly higher in those patients who presented a lower uterine artery PI on the day 1 of COH. Among the ten pregnancies obtained, nine resulted in live births. One patient spontaneously aborted at the 8th week of gestation.

Among the study population, follicular fluid $\text{NO}_2^-/\text{NO}_3^-$ levels were inversely correlated with the mature oocytes

Table 4 Response to controlled ovarian hyperstimulation, in patients with normal and elevated uterine artery PIs

	Group I (n = 11)	Group II (n = 23)	P-value
No of oocytes collected	8.0 ± 2.7	8.1 ± 3.4	NS
Oocyte motphology (%)			
Mature	76.8 ± 18.5	74.8 ± 20.7	NS
Immature	16.4 ± 13.8	20.1 ± 18.2	NS
Atretic	6.1 ± 7.6	4.8 ± 7.1	NS
Fertilization rate (%)	75 ± 27	74 ± 31	NS
Embryo morphology (%)			
Grade A + Grade B	69 ± 27	50 ± 41	0.034
Grade C + Grade D	32 ± 27	46 ± 39	NS

Notes. Values are expressed as mean $\pm$ standard deviation. NS: Not significant.

Fig. 3 The patients who expressed the lowest PIs after pituitary desensitisation (Group I) demonstrated significant lower values than other groups both on the day 8 and on the day of ovum pick-up

($r = -0.566$; $p = 0.039$), and with the embryo quality ($r = -0.831$; $p = 0.003$). The uterine artery PI was inversely correlated with the ovarian synchrony index ($r = -0.545$; $p = 0.045$) and the pregnancy rate ($r = -0.834$; $p = 0.002$). In addition, the uterine artery PIs analysed on the day 1 of COH, positively correlated with the PI values registered on the day 8 of COH ($r = 0.604$; $p = 0.043$) and on the day of OPU ($r = 0.875$; $p = 0.001$).

Discussion

Ovarian aging, ovarian surgery, environmental and genetic factors [29, 30] are associated with a reduction of the primordial follicle pool and loss of oocyte quality. This, during COH, leads to cycle cancellation, or, in cases in which oocyte retrieval is possible, a very low pregnancy rate [30]. Identifying these patients prior the initiation of hormonal stimulation for an Assisted Reproductive Technique (ART) would help us to provide an appropriate counselling and management. To fulfil this task, many studies have been conducted in the past few years and have underlined that advanced female age (≥ 40 years) [31], low ultrasonographic antral follicle count [32], elevated serum oestradiol [33], inhibin [34], FSH [35] and increased FSH/LH ratio [36] on day 3 of spontaneous cycles are associated with suboptimal response to stimulation. On the contrary, a rise in endogenous GH secretion is associated with significantly higher plasma oestradiol concentrations, more oocytes per stimulated cycle and higher pregnancy rate percentages [37].

Despite the extensive use of the above tests allows to adequately select the patients for COH/ART, the current ovulatory agents and laboratory techniques still result associated with high rates of oocyte retrieval/embryo transfer and relatively low pregnancy rates. This may be related to embryo quality and/or endometrial receptivity.

The measurement of impedance to uterine blood flow in IVF/ICSI cycles has provided an indirect measure of endometrial receptivity. The low uterine artery downstream impedance observed in patients who achieve pregnancies, has been, generally, evaluated on the day of hCG administration, on the day of OPU or just before the embryo transfer. Steer et al. [2] reported that 35% of women who failed to conceive in an in vitro fertilization (IVF) programme has a mean uterine artery PI value > 3.0 . Therefore, they suggested to proceed to embryo cryopreservation in those patients with a uterine artery PI > 2.99 for transfer in subsequent cycles. The best uterine receptivity was assumed to be expressed by a PI of 2.0–2.99. However, we demonstrated that the highest pregnancy rates may be obtained in those patients with a uterine artery PI < 2.5 on the day of embryo transfer [38]. It is unknown if the impedance to flow in the uterine vessels may be used also as an indicator of IVF/intracytoplasmic sperm injection (ICSI) outcome when analysed during COH.

In the present study, for the first time at the best of our knowledge, in a group of very homogeneous patients with normal ovarian reserve, the impedance to flow in the uterine vessels, after pituitary desensitisation, resulted as the most useful indicator of an ICSI programme outcome. No hormonal or biochemical parameters resulted correlated with COH parameters or pregnancy rate. The patients with a high uterine artery PI (≥ 2.5) at the beginning of rec-FSH administration presented the worst response with an elevated cancellation rate and a very low pregnancy rate. The vascular parameters were positively correlated with those observed on day 8 and on the day of ovum pick-up. We speculated that after the pituitary desensitisation, an increase of peripheral impedance in the uterine vascular bed is the consequence of a decreased blood flow and tissue perfusion which may, subsequently (as demonstrated by the maintenance of more elevated uterine artery PIs till the day of ovum pick-up), reduce the uterine receptivity.

Ramsay et al. [39, 40] found that human uterine blood flow can be increased by the administration of a NO donor drug and that the relaxation of vascular smooth muscle of uterine vessels may be partially mediated by NO or its derivatives [14]. In the studied patients no significant difference in nitrite/nitrate plasma concentration were observed. However, although other molecules as prostacyclin and endothelium-derived hyperpolarization factor may modulate [41, 42] the above mechanism, we are convinced that NO is deeply implicated and that the lack of a significant difference may be only due to the small number of patients evaluated.

Although, we did not study the ovarian vascularization after the Gn-RH-analog desensitization, we think that also this vascular district is associated with the same factors involved in the uterine arteries modulation since an inverse correlation was found between uterine artery PIs and OSI. In natural cycles, only one follicle is dominant and reaches a mean size of

Fig. 4 The intrafollicular nitrites/nitrates concentrations were (considering both the homogenate of the follicular fluid of all the accessible follicles, and the pool of the follicular fluids of the follicles >17 mm in

>17 mm maximum diameter, while the remaining follicles attain a maximum diameter <10 mm [43]. Thus, whilst in the natural cycle the ideal OSI is 100%, in stimulated cycles many follicles are dominant and it is not possible to obtain an ideal OSI. However, a progressive increase of OSI may reflect an increasing homogeneity of the follicular cohort. We speculated that, in the Group II, the high perifollicular resistances were responsible of an incongruent follicular growth, which most likely favoured a non-homogeneous follicular maturation and differentiation of the granulosa cells.

Although, apparently, no significant difference were registered in term of number and quality of oocytes collected, and the fertilization rate was similar in both groups, the perifollicular vascularization of the best follicles (≥ 17 mm in maximum diameter) was better in those patients who presented a lower uterine artery PI on the day 1 of COH. This was associated with a lower concentration of intrafollicular nitrites/nitrates and with a better percentage of good quality (type A + B) embryos. The follicular fluid $\text{NO}_2^-/\text{NO}_3^-$ levels were inversely correlated with the embryo quality.

Each embryo has its own developmental potential, and few cleaved embryos are competent to implant after IVF/ICSI and develop through gestation [44]. It is known that mature

Follicular fluid Nitrites/Nitrates concentrations

the maximum diameter), lower in those patients who presented a lower uterine artery PI on the day 1 of COH

oocytes often contain chromosomal and cytoplasmic structural defects that prevent the fertilized oocytes from adequate developmental growth. How and when such anomalies intervene is still not well understood. Although differences in follicle cell function and follicular biochemistry have been suggested to influence the developmental potential of human oocyte, no single factor—whether secreted into the circulation or present in the follicular fluid has been shown to provide definitive prediction of the developmental competence of the oocyte-embryo complex. It has been suggested that intrafollicular hypoxia (nitric oxide synthase is rapidly and reversibly induced by exposure to low partial pressure of oxygen) might negatively influence spindle organization and chromosomal segregation in the human oocyte [45]. However, the intra-ovarian regulatory system is composed of various substances including growth factors, cytokines, neuropeptides and vasoregulatory molecules and, among these, NO and its derivatives may serve an important role. In fact, several studies have demonstrated that elevated NO concentrations can reduce cellular ATP levels by inhibiting the cells' ATP-generating ability [11]. This cytostatic and cytotoxic mechanism induces a direct inhibition of mitochondrial respiration and DNA synthesis. Furthermore, elevated NO

concentrations react actively with oxygen, yielding strongly oxidizing molecules (nitrogen dioxide and peroxy nitrates) that are potentially more toxic than NO itself [46].

Conclusions

The above consideration allows us to speculate that, in an ICSI/IVF programme, a decreased utero-ovarian blood flow and tissue perfusion, after the pituitary desensitisation, may reduce the embryo quality and the uterine receptivity and that a Doppler analysis done on day 1 of COH may be an useful indicator of ART outcome.

Further extensive and prospective studies are necessary to elucidate the role of uterine vascularization and endometrial receptivity, to define the correlation between perifollicular blood flow and intrafollicular homeostasis (paracrine-autocrine or hypoxic-acidotic factors) and to determine how changes in follicular fluids modify the developmental capacity of the oocyte-embryo complex.

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