Power doppler ultrasound assessment of the relationship between age and ovarian perifollicular blood flow in women undergoing *in vitro* fertilization treatment

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Abstract *Purpose:* To examine the relationship between age and ovarian perifollicular blood flow (PFBF) in women undergoing IVF.

Methods: Serial transvaginal power Doppler ultrasound (PDU) scans to assess ovarian PFBF were performed prospectively throughout the follicular phase of ovarian stimulation in women undergoing IVF. The ultrasound assessment days were categorized according to day of hCG trigger.

Results: A total of 1050 ovarian follicles from 34 women undergoing one IVF treatment cycle were used for data analysis. The median age of the women was 38.5 years, ranging from 28 years to 44 years. There was a significant negative correlation between age and ovarian PFBF on the day of hCG trigger or trigger day minus 1, but not beforehand during the follicular phase.

Conclusions: There was a significant negative correlation between age and ovarian PFBF in women undergoing IVF which was only observed very late in the follicular phase of ovarian stimulation.

Keywords Age · Doppler ultrasound · Follicular vascularity · IVF · Ovarian perifollicular blood flow

Introduction

Female fertility declines with age due to a decrease in oocyte quality [1]. Chromosomal aneuploidy is the major cause of the age-related decline of oocyte quality. An increase in maternal age results in an increased embryonic aneuploidy rate resulting in an increase in pregnancy loss both prior to and after implantation. The increased embryonic aneuploidy rate results predominately from meiotic non-disjunction errors in the oocytes of these older women [2].

As the actual cause of this age-related increase in oocyte chromosomal meiotic non-disjunction errors is unclear at this point in time, a number of hypotheses have been proposed [1]. One such speculative cause is an increasingly compromised microcirculation around the leading follicle causing reduced oxygen levels in the follicular fluid [3, 4]. The oocyte has no direct vascular supply as there is no internal circulation in the follicle. The oocyte is dependent on oxygen diffusion from the microvasculature in the thecal layer around the follicle through the basement membrane of the follicle, granulosa cells, follicular fluid, and surrounding zona pellucida [3].

Angiogenesis is the process by which new capillary blood vessels develop from pre-existing mature vessels and leads, therefore, to neovascularization [5]. The tissues of the female reproductive tract undergo cyclical physiological angiogenesis in the processes of ovarian folliculogenesis, corpus luteum formation, and endometrial development [6]. In the ovary, primordial and preantral follicles have no special vascular supply of their own and derive their blood supply from the stromal blood vessels. However, the subsequent growth

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of the primary follicles leads to the development of a vascular network in the theca layer with increased follicular blood flow [7, 8].

The introduction of transvaginal power Doppler ultrasound (PDU) has enabled detailed non-invasive studies of the vascularity of ovarian follicles [9]. Ovarian perifollicular blood flow (PFBF) assessment during IVF using PDU has been demonstrated to be a good marker of oocyte competence, embryo viability, and subsequent implantation potential [10] with a number of studies showing a higher pregnancy rate if embryos resulting from the fertilization of eggs from better perfused follicles are transferred [11–14].

A number of cross-sectional studies using Doppler ultrasound have assessed the effect of age on ovarian stromal blood flow with contradictory findings [15–18]. However, we are not aware of any previously published studies assessing specifically the effect of advancing maternal age on ovarian follicular vascularity using PDU.

The finding that oocyte meiotic non-disjunction errors increase in frequency in older women, together with the proposal that defective follicular vascularity of the leading follicle may be responsible, led us to the hypothesis that ovarian follicular blood flow declines with advancing maternal age. In order to test this hypothesis and increase our understanding about the effect of ageing on the ovarian follicle, we decided to perform a longitudinal study aiming to examine the relationship between age and follicular phase ovarian PFBF in women undergoing IVF.

Materials and methods

Subjects

This prospective longitudinal observational study recruited 34 women undergoing IVF treatment between May 2002 and March 2003 at IVF-Australia, Royal Hospital for Women, Sydney, Australia. All women had regular spontaneous ovulatory menstrual cycles ranging from 25 to 32 days and underwent IVF treatment because of unexplained, male or tubal factor infertility. Inclusion criteria were (1) presence of both ovaries; (2) lack of ovarian cysts; (3) no history of partial or complete surgical resection of the ovary; and (4) no history or signs of ovarian endometriosis. The study was approved by the South-Eastern Sydney Area Health Service Research Ethics Committee—Eastern Section and the Human Research Ethics Committee at the University of New South Wales.

Controlled ovarian stimulation

All patients underwent one of three ovarian stimulation protocols: IVF long protocol (IVFLP), IVF short protocol

(IVFSP), and IVF gonadotrophin releasing hormone (GnRH) antagonist protocol (IVFANT).

In the IVFLP, patients were pre-treated with the oral contraceptive pill (OCP) (Brevinor 21, Pharmacia Australia, Rydalmere, NSW, Australia) from day 5 of the menstrual cycle preceding the treatment cycle for 21 days. Fifteen days after starting the OCP, GnRH agonist was introduced either as a nasal spray (Nafarelin acetate, Pharmacia Australia, Rydalmere, NSW, Australia) 200 µg twice daily, or as subcutaneous injection (Leuprorelin acetate, Abbott Australasia, Cronulla, NSW, Australia) 1 mg daily for at least 10 days until pituitary downregulation was confirmed by serum estradiol (E2) <120 pmol/l. Recombinant follicle stimulating hormone injections (Gonal F, Serono Laboratories, Frenchs Forest, NSW, Australia or Puregon, Organon Laboratories, Lane Cove, NSW, Australia) were then commenced for ovarian stimulation with the starting dose being determined according to the patients age and the presence or absence of polycystic ovaries on ultrasound.

In the IVFSP, GnRH agonist was administered from menstrual cycle day 1 and FSH injections were commenced on cycle day 2 or 3. In both of these protocols, daily FSH injections and GnRH agonist were continued until the day of human chorionic gonadotropin (hCG) injection (Profasi, Serono Laboratories, Frenchs Forest, NSW, Australia).

In the IVFANT protocol, daily FSH injections commenced from cycle day 1 or 2 and continued until the day of hCG injection. A single 3 mg dose of GnRH antagonist (Cetrorelix acetate, Serono Laboratories, Frenchs Forest, NSW, Australia) was administered on cycle day 6 or 7. If ovarian follicular growth did not allow ovulation induction with hCG injection on the 5th day after injection of GnRH antagonist, cetrorelix acetate 250 µg was administered once daily beginning 96 hours after the injection of cetrorelix acetate 3 mg until the day of ovulation induction.

Intramuscular injection of hCG 10,000 i.u. was given for all three protocols when at least one follicle was ≥18 mm in mean diameter. Transvaginal sonography (TVS) guided ovum pick up was performed 36 h after the hCG injection. The eggs collected were either inseminated (IVF) or subjected to intracytoplasmic sperm injection (ICSI) with prepared sperm 2–4 h following collection and fertilisation was confirmed 16–18 h later. Embryos were transferred transcervically into the uterine cavity under ultrasound control 2 or 3 days following ovum pick up. Clinical pregnancy was confirmed with the visualisation of a gestational sac on ultrasound at week 6 of amenorrhoea.

Power Doppler ultrasound examinations

All patients underwent serial transvaginal Doppler ultrasound scanning from day of first FSH injection until day

of hCG trigger using the Eccocee SSA—340A ultrasound machine (Toshiba Corporation, Japan) with power and colour Doppler facilities, equipped with a 6 MHz curvilinear transvaginal probes. The spatial peak temporal average intensity of ultrasound for B-mode and Doppler examinations was less than 100 mW/cm^2 , which is within the safety limits recommended by the Bioeffects Committee of the American Institute of Ultrasound in Medicine [19]. The sensitivity for the detection of blood flow was set at 0.09 cm/s for all the cases. During power Doppler ultrasound colour gain for all the cases was set at the level A3 [10], where A stands for amplitude and A3 denotes highest amount of persistence, 10 shows the level of colour gain when the noise (over-gaining) just disappears. All scans were performed by a single operator (SMS) at the same time each morning (between 7 am and 9.30 am), thus controlling the effects of circadian variation on blood flow [20].

Ultrasound assessment days during the follicular phase included first ultrasound day (pre-FSH injections), second ultrasound day (days 6–7 of FSH injections), third ultrasound day (days 8–9 of FSH injections), fourth ultrasound day (days 10–11–12 of FSH injections), and fifth ultrasound day (days 13–14–15 of FSH injections). First ultrasound day (pre-FSH injections) was on the day of pituitary downregulation for IVFLP, menstrual cycle days 2 or 3 for IVFSP, and menstrual cycle days 1 or 2 for IVFANT. The ultrasound assessment days were categorized according to day of hCG trigger and included day of hCG trigger or trigger day minus 1 (TD0/−1), trigger day minus 2 or 3 (TD-2/−3), trigger day minus 4 or 5 (TD-4/−5), trigger day minus 6 or 7 (TD-6/−7), trigger day greater than minus 7 (TD>−7), or pre-FSH injections (first ultrasound day).

During the ultrasound scanning of each ovary, the power Doppler colour box was positioned over each ovarian follicle and the cross-sectional image of the follicle with the maximum colour indication in the follicular circumference was frozen and then the PFBF was graded. Ovarian PFBF was graded using the modified grading system (Grade 0: 0%, Grade 1: 1–25%, Grade 2: 26–50%, Grade 3: 51–75%, Grade 4: 76–100%) based on Chui et al. 1997 [11].

Therefore, Grade 0 follicles do not have any detectable blood flow around the follicular circumference, Grade 1 follicles have blood flow visible in 1% to 25% of the follicular circumference; Grade 2 follicles have blood flow visible in 26% to 50% of the follicular circumference, Grade 3 follicles have blood flow visible in 51% to 75% of the follicular circumference, and Grade 4 follicles have blood flow visible in 76% to 100% of the follicular circumference. The ovarian PFBF grades were categorized as grades 1-2-3-4 (i.e. presence of ovarian PFBF), 2-3-4 (presence of better ovarian PFBF), and 3-4 (presence of high grade ovarian PFBF). Ovarian PFBF grades 3-4 have previously been defined as high grade PFBF by previous authors [11–14].

Thereafter, the size of the follicle was calculated from the mean of two maximum diameters. The study follicles were also divided into one of 3 categories according to the size range: small (5–10 mm), medium (11–14 mm) and large ($\geq 15 \text{ mm}$).

Statistical analysis

Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test and subsequently analysed using either the unpaired Student's *t*-test (normal data distribution) or Mann Whitney *U* test (skewed data) to compare two means (normal data distribution) or medians (skewed data) where appropriate. Categorical variables were analysed using the Chi-square (χ^2) test or Fishers exact test where appropriate. Either Paired Sample *t*-test (for continuous variables) or Wilcoxon Signed Ranks Test (for categorical variables) was used to assess the intra-observer variability in the measurement of ultrasound variables. The strength and direction of the relationship between age and ovarian PFBF was measured using Pearson's *r* correlation. A *P* value <0.05 was considered statistically significant. Statistical analysis was performed using Software Package for Social Sciences (SPSS) version 13.0.

Results

Thirty four women undergoing one IVF treatment cycle were enrolled and 1050 ovarian follicles were used for data analysis. Indications for assisted reproduction were unexplained infertility ($n = 9$), male infertility ($n = 10$), tubal factors ($n = 3$), and mixed causes ($n = 12$). The mean ($\pm$ SD) patient age was $37.6 (\pm 4.5)$ years. The age of the women ranged from 28 to 44 years with a median age of 38.5 years.

We then examined the relationship between age and ovarian PFBF on the various follicular phase assessment days according to the day of hCG trigger. Table 1 demonstrates whether the percentage of small-medium-large ($>4 \text{ mm}$) ovarian follicles with PFBF grades 1-2-3-4, 2-3-4, and 3-4 on each of the categorized trigger assessment days correlated with age. There was no correlation between age and ovarian PFBF (grades 1-2-3-4, 2-3-4, and 3-4) in small-medium-large ovarian follicles at the time of pre-FSH injections (first ultrasound day), TD>−7, TD-6/-7, TD-4/-5, and TD-2/-3. However, there was a significant negative correlation between age and ovarian PFBF (grades 1-2-3-4, 2-3-4, and 3-4) in small-medium-large ($>4 \text{ mm}$) ovarian follicles on TD0/−1.

Figure 1 demonstrates the scatterplots of age and the percentage of medium-large ($>10 \text{ mm}$) and large ($>14 \text{ mm}$) ovarian follicles with PFBF grades 1-2-3-4, 2-3-4, and 3-4 on TD0/−1. A significant negative correlation was also

Table 1 Correlation of the percentage of ovarian follicles >4 mm with perifollicular blood flow (PFBF) grades 1-2-3-4, 2-3-4, and 3-4 on each of the categorized trigger assessment days with age

Assessment day	PFBF grades 1-2-3-4	PFBF grades 2-3-4	PFBF grades 3-4
Pre-FSH (first ultrasound day)	No pearson's $r = -0.038$ $p = 0.908$	No pearson's $r = -0.265$ $p = 0.406$	No pearson's $r = -0.265$ $p = 0.406$
TD> - 7	No pearson's $r = -0.340$ $p = 0.410$	No pearson's $r = -0.314$ $p = 0.448$	No pearson's $r = -0.313$ $p = 0.450$
TD - 6/- 7	No pearson's $r = -0.131$ $p = 0.719$	No pearson's $r = -0.132$ $p = 0.717$	No pearson's $r = 0.143$ $p = 0.694$
TD - 4/- 5	No pearson's $r = -0.260$ $p = 0.391$	No pearson's $r = -0.209$ $p = 0.492$	No pearson's $r = 0.010$ $p = 0.975$
TD - 2/- 3	No pearson's $r = -0.441$ $p = 0.114$	No pearson's $r = -0.508$ $p = 0.064$	No pearson's $r = -0.350$ $p = 0.219$
TD 0/- 1	Yes pearson's $r = -0.779$ $p < 0.001$	Yes pearson's $r = -0.601$ $p = 0.014$	Yes pearson's $r = -0.635$ $p = 0.008$

Note. TD, trigger day; No, no correlation with age present; Yes, correlation with age present.

found between age and ovarian PFBF (grades 1-2-3-4, 2-3-4, and 3-4) in both medium-large (>10 mm) and large (>14 mm) ovarian follicles on TD0/- 1.

Reliability assessment

For intra-observer error, there was no significant difference between the consecutively repeated measurements of ovarian PFBF (Wilcoxon Signed Ranks Test $p > 0.05$). In addition, there was no inter-observer variability in this study because all the measurements were performed by the same operator (SMS).

Discussion

This is the first published study, to the best of our knowledge, to examine the relationship between age and ovarian follicular blood flow assessed by PDU in the natural or IVF

treatment setting. This study demonstrated a significant negative correlation between age and ovarian PFBF in women undergoing IVF which was only observed very late in the follicular phase (around the time of the hCG trigger) of ovarian stimulation.

The neovascularization occurring in the ovarian follicle during the menstrual cycle is termed 'physiological angiogenesis' as it occurs in a physiological state [21]. It is generally recognized that wound healing and associated 'pathological' angiogenesis is retarded with ageing as evidenced from studies on older women [22] and animal models [23]. However, the effect of ageing on the vascular dynamics in the ovary has not been well studied [21].

There have been a number of published studies using Doppler ultrasound evaluating the effect of age on ovarian stromal blood flow in both fertile and infertile women [15–18]. These studies were cross-sectional in design and assessed blood flow on a single occasion during the study period. Age did not affect ovarian stromal blood flow assessed by color Doppler ultrasound in the early follicular phase of the menstrual cycle in a population-based study of fertile pre-menopausal women [17]. Conversely, studies using the three-dimensional PDU technique have demonstrated reduced ovarian stromal vascularity with increasing maternal age in general population pre-menopausal to perimenopausal to post-menopausal women [15], fertile pre-menopausal women in the early follicular phase [18], and infertile pre-menopausal women at the time of down-regulation during IVF treatment [16].

However, ovarian stromal blood flow (blood flow in the vessels that supply blood to the follicles in the ovaries) may not reflect the vascularity of the developing antral follicle(s) recruited during FSH stimulation. Antral follicles (seen on ultrasound), unlike the preantral and primordial follicles, do not derive their blood flow directly from ovarian stromal vessels but develop, through the process of neovascularization, a vascular sheath consisting of arterioles, venules and capillaries in the surrounding theca layer [7, 8]. Although the precise regulation of this cyclic angiogenesis and consequent neovascularization in the human ovary remains unclear, a number of angiogenic factors and inhibitors are likely to be involved including vascular endothelial growth factor (VEGF) [8, 10, 24]. In addition, a number of IVF studies have shown that each mature follicle has unique blood flow characteristics and perifollicular capillary bed development [4, 11, 26, 27] supporting the concept that not all follicles are 'created equal' in terms of vasculature [25].

We are aware of only one published study that has previously examined the effect of age on ovarian PFBF using Doppler ultrasound. Balakier and colleagues [28] longitudinally assessed perifollicular blood flow every 2 days starting from days 6–7 of ovarian stimulation and continuing until the day after hCG trigger in 52 infertile women undergoing

Fig. 1 Scatterplots of age and the percentage of ovarian follicles >10 mm and >14 mm with perfollicular blood flow (PFBF) grades 1-2-3-4, 2-3-4, and 3-4 on day of hCG trigger or trigger day minus 1 (TD0/-1)

IVF using color Doppler ultrasound (CDU). There was no difference in PFBF peak velocity or resistance index between women in the younger and older age groups. This study had two important differences in methodology compared to our study which may account for the different findings.

Firstly, Balakier et al. [28] used CDU to locate follicular vessels and pulsed Doppler to assess velocity profiles or impedance to blood flow whereas our study used PDU which is three-fold more sensitive than conventional CDU at detecting flow with low volumes and velocity, and hence improves

the visualization of small vessels such as those surrounding ovarian follicles [29, 30]. Secondly, the authors combined the longitudinal data on PFBF into two categorized time-frames: blood flow before and after hCG trigger. Therefore, the authors did not assess PFBF specifically on trigger day 0 or – 1 as the data on these two days were combined with the data on all of the previous days of ovarian stimulation.

There have been a number of published studies demonstrating that qualitative indices of ovarian PFBF (grades 1–4) during IVF correlate with pregnancy. These studies used either PDU [11–13] or CDU [31] and demonstrated that between 52% and 64% of the follicles have high grade (Grades 3 or 4) PFBF. Unfortunately, one cannot compare the proportion of high grade follicles between these studies in terms of maternal age as the mean age of the women were similar (32.5–35 years) and there were important methodological differences (i.e. patient and follicle selection, PDU or CDU, timing of Doppler ultrasound either just prior to hCG trigger or after hCG trigger on the day of egg collection) between the studies which could account for any differences in PFBF. Interestingly, the study by Coulam et al. [31] also examined quantitative indices of PFBF (peak systolic velocity) and found both qualitative and quantitative measurements of PFBF to predict pregnancy after IVF.

The exact cause for the age related decline in ovarian follicle vascularity demonstrated in the present study is unknown but could include perfollicular endothelial cell under-expression of, receptor defects in, or receptor deficits in angiogenic growth factors such as VEGF, leptin, interleukins, angiopoietins, insulin-like growth factor, basic fibroblast growth factor, and endothelin [9]. The full extent of the relationship between follicular vascularity and levels of angiogenic factors has yet to be determined.

The finding of an age related decline in ovarian follicle blood flow very late in the follicular phase is interesting when one considers that the oocyte becomes arrested at the diplotene stage of prophase 1 of the first meiotic division during fetal life and remains so until very late in the final stages of folliculogenesis just prior to ovulation when meiosis is resumed as a result of the LH surge [32]. Indeed, a less than optimal microvasculature around a follicle need not be a long-lived condition but need only be present at the critical point just prior to anaphase 1 segregation of the bivalent halves [3]. Therefore, the finding of an age related reduction in follicular blood flow around the time of the resumption of meiosis in the oocyte adds support to the notion that deficient blood flow around the follicle may be an important contributing factor to the maternal age related increase in meiotic nondisjunction errors seen in oocytes [3, 4].

There is other indirect evidence to support the hypothesis that defective ovarian follicular microcirculation may cause the increase in oocyte meiotic nondisjunction errors seen with advancing female age. Older women undergoing IVF

have been shown to have higher follicular fluid VEGF levels, consistent with a hypoxic environment within follicles of older women [33]. Ovarian perfollicular vascularity correlates with follicular fluid oxygen content [9, 34]. Oocytes derived from follicles with low oxygen content have a significantly higher frequency of defects in chromosome number and spindle organization. Such a hypoxic intra-follicular environment could result from a failure of an appropriate microvasculature to develop around the growing follicle [4].

It has been suggested that the effects of hypoxia on the oocyte could include reduced levels of metabolism and lower intracellular pH which in turn could influence the organization and stability of the meiotic metaphase spindle. Alterations in spindle organization or stability could result in chromosomal displacements (scattering) and segregation disorders (anaphase lag, nondisjunction) [3]. However, the question of whether the oocyte chromosomal abnormalities evident in older women are due to the hypoxic environment consequent to reduced follicular blood flow still needs to be answered.

This study has a number of potential limitations. It is a prospective observational study which, given the research question as to whether advancing age is associated with decreasing ovarian follicular blood flow in women undergoing IVF, is the ideal study design. However, observational studies are disadvantaged methodologically by an inability to control completely for confounding factors and biases [35, 36]. The women in our study underwent one of three different IVF protocols. The significance of this in relation to our study findings is uncertain but may represent a potential confounding factor if the type of ovarian stimulation protocol independently influences ovarian PFBF. However, we have observed no difference in PFBF between different IVF stimulation protocols (unpublished data).

It would have been preferable to have performed the serial ultrasound assessments for all patients on the same day of FSH injection (i.e. days 6, 8, 10, 12, and 14) rather than on days 6 or 7, 8 or 9, 10 or 11 or 12, and 13 or 14 or 15 to reduce the likelihood of information bias [36]. Unfortunately this was not possible due to logistical reasons. In addition, the operator performing the ultrasound assessments in this study was not blinded and therefore there is the potential for the introduction of measurement bias [36].

In conclusion, this study has demonstrated a significant negative correlation between age and ovarian PFBF in women undergoing IVF which was only observed very late in the follicular phase (around the time of the hCG trigger) of ovarian stimulation. As this is the first study assessing the effect of age on ovarian follicular vascularity using PDU, it is important that further larger prospective longitudinal studies, ideally in both the natural and assisted conception cycle settings, to confirm these findings. If confirmed, important further research into whether clinical

interventions could improve the vascularity of the follicle in older women and hence improve outcome would need to be performed.

References

1. ESHRE Capri Workshop Group. Fertility and ageing. *Hum Reprod Update* 2005;11: 261–76.
2. Macklon NS, Geraedts JPM, Fauser BCJM. Conception to ongoing pregnancy: The ‘black box’ of early pregnancy loss. *Hum Reprod Update* 2002;8:333–43.
3. Gaulden ME. Maternal age effect: The enigma and Downs syndrome and other trisomic conditions. *Mutat Res* 1992;296:69–88.
4. Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: Association with vascular endothelial growth factor levels and perfollicular blood flow characteristics. *Hum Reprod* 1997;12:1047–55.
5. Folkman J. Tumor angiogenesis. *Adv in Cancer Research* 1985;43:175–203.
6. Weston G, Rogers PA. Endometrial angiogenesis. *Best Pract Res Clin Obstet Gynaecol* 2000;14: 919–36.
7. Findlay JK. Angiogenesis in reproductive tissues. *J Endocrinol* 1986;111:357–66.
8. Gordon JD, Shifren JL, Foulk RA, Taylor RN, Jaffe RB. Angiogenesis in the human female reproductive tract. *Obstet Gynecol Survey* 1995;50:688–97.
9. Van Blerkom J. Intrafollicular influences on human oocyte developmental competence: Perifollicular vascularity, oocyte metabolism and mitochondrial function. *Hum Reprod* 2000;15 Suppl 2:173–88.
10. Gregory L. Ovarian markers of implantation potential in assisted reproduction. *Hum Reprod* 1998;13 Suppl 4:117–32.
11. Chui DK, Pugh ND, Walker SM, Gregory L, Shaw RW. Follicular vascularity—the predictive value of transvaginal power Doppler ultrasonography in an in-vitro fertilization programme: A preliminary study. *Hum Reprod* 1997;12:191–6.
12. Bhal PS, Pugh ND, Chui DK, Gregory L, Walker SM, Shaw RW. The use of transvaginal power Doppler ultrasonography to evaluate the relationship between perfollicular vascularity and outcome in in-vitro fertilization treatment cycles. *Hum Reprod* 1999;14:939–45.
13. Borini A, Maccolini A, Tallarini A, Bonu MA, Sciajno R, Flamigni C. Perifollicular vascularity and its relationship with oocyte maturity and IVF outcome. *Ann. NY Acad. Sci.* 2001;943:64–7.
14. Costello MF, Sjoblom P, Shrestha SM. Use of doppler ultrasound imaging of the ovary during IVF treatment as a predictor of success. In: Rao KA, Brinsdon PR, Sathananthan H, editors. *The infertility manual*. New Delhi: JAYPEE Brothers Medical Publishers; 2004: p. 344–9.
15. Pan HA, Cheng YC, Li CH, Wu MH, Chang FM. Ovarian stromal flow intensity decreases by age: A three-dimensional power doppler ultrasonographic study. *Ultrasound Med Biol* 2002;28:425–43.
16. Kupesic S, Kurjak A, Bjelos D, Vujisic S. Three-dimensional ultrasonographic ovarian measurements and in vitro fertilization outcome are related to age. *Fertil Steril* 2003;79:190–7.
17. Ng EH, Yeung WS, Fong DY, Ho PC. Effects of age on hormonal and ultrasound markers of ovarian reserve in Chinese women with proven fertility. *Hum Reprod* 2003;18:2169–74.
18. Ng EHY, Chan CCW, Yeung WSB, Ho PC. Effect of age on ovarian stromal flow measured by three-dimensional ultrasound with power Doppler in Chinese women with proven fertility. *Hum Reprod* 2004;19:2132–7.
19. Barnett SB, Ter Haar GR, Ziskin MC, Rott HD, Duck FA, Maeda K. International recommendations and guidelines for the safe use of diagnostic ultrasound in medicine. *Ultrasound in Med & Biol* 2000;26:355–66.
20. Zaidi J, Jurkovic D, Campbell S, Pittrof R, McGregor A, Tan SL. Description of circadian rhythm in uterine artery blood flow during the peri-ovulatory period. *Hum Reprod* 1995;10:1642–6.
21. Hazzard TM, Stouffer RL. Angiogenesis in ovarian follicular and luteal development. *Best Pract Res Clin Obstet Gynaecol* 2000;14:883–900.
22. Ashcroft GS, Greenwell-Wild T, Horan MA, Wahl SM, Ferguson MW. Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. *Am J Pathol* 1999;155:1137–46.
23. Rivard A, Fabre JE, Silver M, Chen D, Murohara T, Kearney M, Magner M, Asahara T, Isner JM. Age-dependent impairment of angiogenesis. *Circulation* 1999;99:111–20.
24. Gordon JD, Mesiano S, Zaloudek CJ, Jaffe RB. Vascular endothelial growth factor localization in human ovary and fallopian tubes: Possible role in reproductive function and ovarian cyst formation. *J Clinical Endocrin Metabol* 1996;81:353–9.
25. Peters H, McNatty KP, Elek P. The ovary: A correlation of structure and function in mammals. London: Granada, 1980.
26. Nargund G, Bourne T, Doyle P, Parsons J, Cheng W, Campbell S, Collins W. Associations between ultrasound indices of follicular blood flow, oocyte recovery and preimplantation embryo quality. *Hum Reprod* 1996;11:109–13.
27. Van Blerkom J. Can the developmental competence of early human embryos be predicted effectively in the clinical IVF laboratory? *Hum Reprod* 1997a;12:1610–14.
28. Balakier H, Stronell RD. Color Doppler assessment of folliculogenesis in in vitro fertilization patients. *Fertil Steril* 1994;62:1211–16.
29. MacSweeney JE, Cosgrove DO, Arenson J. Colour Doppler energy (power) mode ultrasound. *Clin Radiol* 1996;51:387–90.
30. Rubin JM, Bude RO, Carson PL, Bree RL, Adler RS. Power Doppler US: A potentially useful alternative to mean frequency-based color Doppler US. *Radiology* 1994;190:853–6.
31. Coulam CB, Goodman C, Rinehart JS. Colour Doppler indices of follicular blood flow as predictors of pregnancy after in-vitro fertilization and embryo transfer. *Hum Reprod* 1999;14:1979–82.
32. te Velde ER, Scheffer GJ, Dorland M, Broekmans FJ, Fauser BC. Developmental and endocrine aspects of normal ovarian aging. *Mol Cell Endocrin* 1998;145:67–73.
33. Friedman CI, Danforth DR, Herbosa-Encarnacion C, Arbogast L, Alak BM, Seifer DB. Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. *Fertil Steril* 1997;68:607–12.
34. Huey S, Abuhamad A, Barroso G, Hsu MI, Kolm P, Mayer J, et al. Perifollicular blood flow Doppler indices, but not follicular pO₂, pCO₂, or pH, predict oocyte developmental competence in in vitro fertilization. *Fertil Steril* 1999;72:707–12.
35. Daya S. Evaluation of treatment efficacy—randomisation or observation. *Evidence-based Obstet Gynaecol* 2001;3:111–3.
36. Barnhart K. The truth is out there: A guide to the pitfalls of interpreting evidence-based medicine in studies of human reproduction. *Fertil Steril* 2002;77:223–28.