Superficial ovarian cortex vascularization is inversely related to the follicle reserve in normal cycling ovaries and is increased in polycystic ovary syndrome

F. Delgado-Rosas¹, M. Gaytán¹, C. Morales², R. Gómez¹, and F. Gaytán³,⁴

¹Instituto Valenciano de Infertilidad, Valencia, Spain ²Department of Pathology, University of Córdoba, Córdoba, Spain ³Department of Cell Biology, Physiology and Immunology, School of Medicine, University of Córdoba, 14004 Córdoba, Spain

Correspondence address. E-mail: bc1galuf@uco.es

BACKGROUND: The superficial ovarian cortex constitutes the micro-environment where resting and early growing follicles reside. As small follicles do not possess an independent capillary network, both their survival and early growth depend on their proximity to the cortical vessels. Little is known about the possible changes in superficial ovarian cortex vascularization in normal women throughout reproductive life or in pathological conditions such as polycystic ovary syndrome (PCOS) involving abnormal early follicle growth. We studied the vascularization of the superficial and deep cortical stroma (DCS) in normal cycling ovaries from 21 to 50 years of age and in infertile women with PCOS.

METHODS: We used archival ovarian samples and specific CD34 immunostaining to determine blood vessel density and to analyse correlation with age and with the ovarian follicle reserve.

RESULTS: Normal cycling ovaries showed an age-related increase in the superficial cortical stroma vascularization that was inversely correlated with the density of small (primordial and primary) follicles. In contrast, blood vessel density in the DCS significantly decreased in women aged ≥40 years. Ovaries from PCOS showed a 2-fold increase in blood vessel density in both superficial cortical stroma and DCS with respect to age-matched controls.

CONCLUSIONS: The increased vascularization of the superficial cortical stroma in normal ovaries in relation to age and in ovaries from PCOS could have profound effects on cortical metabolic rate, primordial follicle survival/activation and early follicle growth, and may underline changes in follicle dynamics in mid-aged women and in PCOS.

Key words: ovarian ageing / ovarian cortex / vascularization / PCOS / follicle reserve

Introduction

Fecundity in women is closely related to age, and decreased fertility is evident from 35 years of age onwards (Menken et al., 1986). It is well established that the age-related decline in reproductive capacity in women is mainly due to ovarian ageing. Although neuroendocrine and uterine factors are also implicated, assisted reproductive technologies have clearly demonstrated that reduced fertility can be partially overcome by oocyte donation from younger women (Navot et al., 1994; Budak et al., 2007), thus indicating that age-related ovarian changes are determinant factors responsible for decreased fertility.

One of the most peculiar aspects of ovarian biology is that primordial follicles, which constitute the follicle reserve and reside in the superficial cortical zone, are recruited to the growing follicle pool in a timing process during a very long period of about 50 years. During this long period, resting follicles are exposed to age-related micro-environmental changes. In this context, knowledge of the ovarian ageing process is essential to the understanding of the mechanisms leading to the depletion of the ovarian follicle reserve and to design strategies to preserve fertility.

The superficial ovarian cortical stroma constitutes the environment in which the majority of resting follicles reside at the primordial follicle...
state and where essential events of early follicle development occur. Indeed, activation of primordial follicles leaving the resting pool and entering into the growing phase becoming primary follicles, as well as the transition from primary to secondary follicles take place at the superficial ovarian cortex. The cortical stroma is an apparently simple tissue composed of spindle-shaped, fibroblast-like cells that are densely packed in the primate ovary. Far beyond being a mere supportive tissue for resting and early growing follicles, cortical stromal cells (CSCs) are the source of theca cells, an essential component of the growing follicles beyond the secondary state, and stromal blood vessels proliferate and give rise to the perifollicular capillary network from the secondary follicle stage onwards. The close histological relationship between resting and early growing follicles and the superficial cortical stroma provides the physical basis for the existence of a regulatory cross-talk between follicles and CSCs. However, most of the previous studies have been focused on the interrelationship between the oocyte and its immediate surrounding somatic cell type (i.e. granulosa cells), and the possible regulatory roles of the superficial cortical stroma have been scarcely considered.

Vascularization is a prominent characteristic of tissues, as blood vessels are responsible for an adequate supply of oxygen, nutrients and regulatory signals. This is particularly relevant in the superficial ovarian cortex, since resident primordial and early growing follicles do not possess an independent vascular network and are, therefore, dependent on their proximity to stromal vessels. Although many studies have been focused on ovarian angiogenesis, providing evidence on the essential role of vascularization for antral follicle and corpus luteum development and function (Gaytán et al., 1999; Dickson and Fraser, 2000; Reynolds et al., 2002; Fraser and Wulff, 2003; Fraser, 2006), little attention has been devoted to the vascularization of the superficial cortical stroma. In this context, age-related changes in superficial cortex vascularization may have profound effects on both the survival of primordial follicles and on the early stages of follicle growth. Yet the relationship between resting and early growing follicles and the superficial cortical microvascular bed in the primate ovary, as well as the possible changes in the superficial cortical stroma vascularization during ovarian ageing are unknown. Changes in overall ovarian vascularization have been reported in normal ovaries in relation to age and in polycystic ovary syndrome (Ng et al., 2004, 2005) by using ultrasound technology. However, superficial cortical microvessels cannot be detected by Doppler-based techniques and detailed studies on human ovarian cortex vascularization are lacking. The recent availability of endothelial cell markers now allows histological evaluation of the microvascular bed in tissues.

The aim of this study was to analyse superficial ovarian cortex vascularization in normally cycling women in relation to age and to the ovarian follicle reserve. We also studied superficial ovarian cortex vascularization in polycystic ovary syndrome (PCOS) that shows alterations of the initial follicle growth (Stubb et al., 2007) and ovarian vascularization (Abd El Aal et al., 2005; Ng et al., 2005).

Materials and Methods

Ovarian samples

Ovarian samples corresponded to archival material from the files of the Department of Pathology of the University of Córdoba that had been collected from 1980 to 2000. The ovaries from 46 cycling women were selected from a larger series. Ovarian samples corresponded to oophorectomized women due to gynaecological (other than ovarian) pathology, such as endometrial and cervical carcinoma, pelvic primitive neuroendocrine tumours, malignant tumours of the contralateral ovary, uterine fibroids, pelvic pain or ovarian wedge resections to discard ovarian pathology, and the women were not undergoing hormonal therapy at the time. All ovarian samples were re-evaluated by an experienced pathologist (C.M.), were cycled (as evidenced by the presence of several generations of functional and/or regressing corpora lutea) and did not show pathological alterations. Ovarian samples were divided into six groups according to age: 21–25 years (23 ± 1 years, mean ± SEM for n = 6); 26–30 years (27.2 ± 0.48 years, mean ± SEM for n = 6); 31–35 years (32.9 ± 0.4 years, mean ± SEM for n = 10); 36–40 years (38.7 ± 0.4 years, mean ± SEM for n = 12); 41–45 years (42.9 ± 0.5 years, mean ± SEM for n = 8) and 46–50 years (47.6 ± 0.6 years, mean ± SEM for n = 5). Samples from polycystic ovary syndrome, aged 20–30 years (24.3 ± 1, mean ± SEM for n = 15), corresponded to ovarian wedge resections as a treatment for infertility that had been diagnosed as Stein–Leventhal syndrome. All ovarian samples were re-evaluated, confirming that they corresponded to non-ovulatory PCOS. Only one ovary remained was found in one patient. All tissues had been fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. Sections (5 μm thick) were cut and placed on poly-L-lysine-coated slides and used for immunohistochemistry. Additional sections were stained with haematoxylin and eosin.

Specific blood vessel immunostaining

Blood vessel immunostaining was carried out with monoclonal CD34 antibodies (NCL-END clone QBEND/10, Novocastra Laboratories Ltd, Barcelona, Spain), following previously described methods (Gaytán et al., 1999), with slight modifications. In summary, trypsin pretreatment (0.1% trypsin at 37°C for 10 min) was used instead of microwave heating for antigen retrieval, as this procedure provided a higher intensity of the signal, which is particularly important for superficial cortex microvessels. In these conditions, CD34 selectively labels endothelium of either newly formed blood vessels (in the theca layer of early antral follicles or in the granulosa-lutein layer of newly formed corpora lutea), or mature blood vessels (i.e. arteries in the deep ovarian cortex; Fig. 1). The signal was absent in negative control sections by replacing the first antibody by PBS or non-immune serum. Bound CD34 antibodies were revealed by the ABC complex method as described previously (Gaytán et al., 1999).

Determination of blood vessel and small follicle densities

Vascularization was assessed by determining the volume density of blood vessels (i.e. the proportion of the cortical tissue occupied by blood vessels). This parameter is independent of the orientation of blood vessels and includes changes in both number and size of blood vessels. Volume density was determined by point counting with the aid of a 212 test point grid incorporated to the microscope. Three different sections per ovary, taken at least 20 μm apart, and 25 microscopic fields per section with ×20 objective were analysed. Microscopic fields were selected at random in the cortical zone following a systematic procedure throughout the ovary section. The test area corresponded to 0.16 mm² and, therefore, a total area of 12 mm² of stromal tissue per ovary was used for counting procedures. Both ovaries were studied when available. Superficial cortical stroma was recognizable by the presence of densely packed stromal cells, the presence of primordial and early growing follicles and low density of small blood vessels. The number of test points on immunostained vascular profiles divided by the...
number of test points on superficial cortical stroma corresponded to the volume density of blood vessels. Although it was not the main objective of this study, blood vessel density was also determined in the deep cortical stroma (DCS) in five normal women per age group and in eight with PCOS, in order to compare it with superficial cortex vascularization. The DCS was composed of less densely packed stromal cells together with more abundant and larger blood vessels. Volume density of deep cortical blood vessels was estimated by applying the same counting rules and discarding the areas corresponding to the theca layer of antral follicles, corpora lutea or avascular corpora albicantia. The medullary zone containing densely packed large blood vessels, as a continuum with the ovarian hilus, was not considered.

In order to analyse the possibility of cyclic changes in superficial ovarian cortex vascularization, we carried out a pilot study comparing volume density of superficial cortical blood vessels in the follicular and luteal phases of the menstrual cycle. For this, five women aged 30–40 years in each phase of the cycle were selected. No significant differences were found in the volume density of blood vessels between follicular and luteal phases (1.66 ± 0.24 versus 1.76 ± 0.15%; mean ± SEM for n = 5, Mann–Whitney U-test) and, therefore, the phase of the cycle was not taken into account.

The ovarian follicle reserve was estimated as the density of follicles per unit area of cortical stroma, in sections stained with haematoxylin and eosin. Counts were performed in three different sections per ovary taken at least 20 μm apart and 25 microscopic fields per section (a total area of 12 mm² per ovary), by counting the number of small (primordial, transitional primary and classical primary) follicles and analysed in order to establish a correlation with blood vessel density in the superficial ovarian cortex, showed the expected age-related decay. Small follicles were abundant in the superficial ovarian cortex from 21 to 30 years of age, less abundant from 31 to 35 years of age, scarce from 36 to 40 years of age and very scarce from 40 years of age onwards (Fig. 2a and b). Quantitatively, there were no significant differences in the density of small follicles up to 30 years of age, but the density decreased thereafter in parallel to age (Fig. 2c and d).

**Results**

**Age-related changes in the density of small follicles in normal ovaries**

The ovarian follicle reserve, estimated as the density of small (i.e. primordial, transitional and primary) follicles and analysed in order to establish a correlation with blood vessel density in the superficial ovarian cortex, showed the expected age-related decay. Small follicles were abundant in the superficial ovarian cortex from 21 to 30 years of age, less abundant from 31 to 35 years of age, scarce from 36 to 40 years of age and very scarce from 40 years of age onwards (Fig. 2a and b). Quantitatively, there were no significant differences in the density of small follicles up to 30 years of age, but the density decreased thereafter in parallel to age (Fig. 2c and d).

**Age-related changes in superficial ovarian cortex vascularization in normal ovaries**

Superficial cortex vascularization was assessed by determining the volume density of blood vessels, identified by specific immunostaining with monoclonal anti-CD34 antigen. The superficial cortical stroma was poorly vascularized and a gradient in the density of blood vessels was evident with respect to the DCS. Blood vessels in the superficial cortex corresponded mostly to microvessels with a narrow lumen that seemed to be homogenously distributed throughout without any apparent relationship to resting follicles located at the inner superficial cortical zone (Fig. 3a–c). The volume density of superficial cortical blood vessel increased with age (Fig. 3d), showing a 30% increase from 30 to 35 years of age, about a 50% increase from 35 to 45 years of age and a 9% increase from 45 to 50 years of age (Fig. 3e). Multivariate regression analysis demonstrated that a strong inverse correlation (R = 0.93, P < 0.001, n = 46) existed between the volume density of blood vessels and the density of small follicles (Fig. 4a). The presence of abundant small follicles in young women was coincident with a low volume density of blood vessels and, as a consequence, most follicles were not closely related to capillaries (Fig. 4b).
Superficial cortical stroma vascularization and resting follicle numbers in polycystic ovaries

Based on previous reports indicating the existence of abnormal early follicle growth (Stubbs et al., 2007) and increased blood flow (Abd El Aal et al., 2005; Ng et al., 2005) in PCOS, we analysed both small follicle and superficial cortical blood vessel density in anovulatory polycystic ovaries. As all polycystic ovaries corresponded to young (20–30-year-old) women, age effects cannot be analysed, and data from PCOS ovaries were compared with age-matched normal ovaries (aged 21–30 years). PCOS ovaries showed abundant small follicles and blood vessels in the superficial ovarian cortex (Fig. 5a). As a consequence, direct contacts between blood vessels and small (primordial and primary) follicles (Figs 5b and c), as well as early secondary follicles (ESFs) (Fig. 5d) were frequently observed. Quantitatively, PCOS ovaries showed a two-fold increase in the volume density of blood vessels (Fig. 5f) with respect to age-matched normal ovaries, whereas no significant differences existed with respect to the density of small follicles (Fig. 5e).

DCS vascularization in normal and polycystic ovaries

Blood vessel density was also determined in the DCS, where most growing follicles were located, for comparison with the superficial ovarian cortex. In normal ovaries, blood vessels were abundant in the DCS when compared with the superficial ovarian cortex (Fig. 6a). Quantitative data showed a slight, but significant, decrease in the volume density of blood vessels from 40 years of age onwards (Fig. 6b). Notably, PCOS ovaries showed a two-fold increase in the volume density of blood vessels in the DCS (Figs 6c and d).

Discussion

This is the first study addressing changes in the human superficial ovarian cortex vascularization during reproductive life and in
Figure 3  Age-related changes in the volume density of blood vessels in the superficial ovarian cortex. Representative CD34 immunostained sections showing increasing number of vascular profiles (white arrows) and decreasing numbers of small follicles (black arrows) from women aged (a) 23, (b) 37 and (c) 40 years. (d) Volume density of blood vessels (Vvbv) plotted against chronological age. (e) Changes in the volume density of blood vessels in the different age groups. *P < 0.05 versus the previous age group (Kruskall–Wallis and Mann–Whitney U-tests for the numbers are indicated in parentheses).

Figure 4  Relationship between small follicles and blood vessels in the superficial ovarian cortex of normal ovaries. (a) Multiple linear regression between the logarithm of the number of small follicles per cm² of superficial cortical stroma (NSF/cm²), the volume density of blood vessels (Vvbv) and chronological age. (b) Representative high magnification of the superficial ovarian cortex from a young (23-year-old) women showing the scarcity of blood vessels (white arrows) surrounding small follicles (black arrows).
anovulatory polycystic ovary syndrome. Worthy to note that the superficial ovarian cortex constitutes the poorest vascularized zone in the human ovary. Previous studies in human (Suzuki et al., 1998; Gaytán et al., 1999; Wulff et al., 2001) and primate (Fraser and Wulff, 2003; Fraser, 2006) ovaries have reported that the highest vascular density corresponds to the corpus luteum and, to a lesser extent, to large antral follicles, whereas the ovarian stroma (mostly corresponding to DCS) shows a considerably lower vascularization (Suzuki et al., 1998). We report herein that a significant gradient in stromal vascularization also exists between the deep and the superficial cortical zones, with the latter showing small and scarce blood vessels. This agrees with a previous study on the vascularization of the bovine ovary (Herrmann and Spanel-Borowski, 1998). This characteristic micro-architecture of the ovarian vasculature should have a physiological significance. A possibility is that such a poor vascularization of the superficial ovarian cortex is related to the maintenance of the resting stage of primordial follicles (Herrmann and Spanel-Borowski, 1998) and to the very low growth rate of primary follicles and ESFs (Gougeon, 1996).

The most relevant age-related changes in the superficial ovarian cortex were the decrease in the follicle reserve and the increase in vascularization. The age-related decrease in the ovarian follicle reserve is clearly established (Faddy et al., 1992; Gougeon et al., 1994; Lobo, 2005), and mathematical modelling has been used to explain the rate of decay of the ovarian follicle reserve, either by exponential biphasic models showing a sudden acceleration of follicle loss at

Figure 5 (a) Superficial ovarian cortex in polycystic ovaries. In sections immunostained for CD34, both blood vessels (white arrows) and small follicles (black arrows) are abundant. As a consequence, direct contact (arrows) between small follicles and capillaries were frequently observed (arrows in b). (c) Blood vessels can be observed surrounding primordial follicles even in haematoxylin- and eosin-stained sections, or (d) closely apposed to ESF. Quantitative data show (e) equivalent follicle density and (f) increased volume density of blood vessels in polycystic ovaries with respect to age-matched controls. *P < 0.001 versus controls (Mann–Whitney U-test for the numbers is indicated in parentheses).
about 37–38 years of age \cite{Faddy92, Gougeon94, Faddy96} or by a simple power function showing a constantly accelerating rate \cite{Hansen08}. In these models, resting follicle loss seems to be faster with increasing age and the ovarian follicle reserve is highly depleted from 37 to 38 years of age onwards. In the present study, the ovarian follicle reserve was estimated as the follicle density in the superficial ovarian cortex, since the ovarian volume was not available. However, relative age-related changes in the density of resting follicles reported herein were similar to those reported in absolute follicle numbers using updated stereological methods \cite{Hansen08} at the same age intervals. The age-related increase in the density of microvessels in the superficial cortical stroma raises several possibilities with respect to the mechanisms leading to increased vascularization: (i) age-dependent depletion of resting follicles induces changes in the superficial ovarian cortex leading to increased angiogenesis or (ii) ovarian age-dependent changes induce both depletion of resting follicles and increased superficial cortex vascularization by independent mechanisms.

With respect to the first possibility, age-related depletion of the resting and early growing follicle population could modify tissue homeostasis leading to structural and functional changes in the cortical stroma. Increased superficial cortex vascularization could be related to either the loss of resting follicles by itself or repeated interactions with growing follicles. Although the existence of bidirectional regulatory loops between the oocyte and granulosa cells is clearly established \cite{Albertini01, Eppig01}, possible factors mediating communication between the oocyte/primordial follicles and the surrounding cortical stroma are largely unknown. Growing follicles are a source of angiogenic factors involved in the theca layer vascularization that could affect the vascularization of the surrounding stroma and, in addition, blood vessels in the theca layer may persist in the stroma after follicle atresia. However, although previous studies have reported an age-dependent increase in the proportion of growing versus resting follicles, the absolute number of growing follicles decreases with age \cite{Gougeon94, Al-Sunaidi06} and, therefore, an age-related decrease in the local concentration of angiogenic factors (released by growing follicles) is expected. In contrast to the superficial ovarian cortex, a slight, but significant, decrease in the vascularization of the DCS (where most growing follicles are located) was found at advanced ages. This is more concordant with the age-dependent decrease in the number of growing

**Figure 6** Vascularization of the DCS in (a) normal and (b) polycystic ovaries. Quantitative data show a significant decrease in the DCS vascularization from 40 years of age onwards in (c) normal women, and (d) a 2-fold increase in the volume density of blood vessels in PCOS compared with age-matched controls. $^aP < 0.001$ versus previous age group; $^bP < 0.001$ versus controls (Mann–Whitney $U$-test for the numbers is indicated in parentheses).
follicles and agrees with previous ultrasound studies indicating an age-related decrease in ovarian stromal blood flow in women aged \( \geq 41 \) years (Ng et al., 2005). In this sense, the age-dependent increase in vascularization that was limited to the superficial cortical stroma, is difficult to explain as a result of interactions with growing follicles.

With regard to the second possibility, the increase in the superficial ovarian cortex vascularization could be related to age by mechanisms independent of changes in the ovarian follicle reserve. Although an age-related impairment of angiogenesis seems to be the general rule in most tissues (Edelbergh and Reed, 2003), vascularization may increase in specific environments in response to local changes. A fact that has received little attention is that ovarian blood vessels exhibit a rate of ageing faster than that of other organs. Early vascular alterations consisting of hyalinization and thickening of the vessel wall in mid-sized arteries in the medulla and deep cortex are present from 30 years onwards and increase with age (Shimada et al., 1993). This determines a decrease in the lumen diameter and, presumably, a decreased blood flow to the superficial cortical areas, as blood supply to the ovary is an end artery system. In this context, increased superficial cortex vascularization could be a reactive response of terminal microvessels to ischaemia, in an already poorly vascularized area. Hypoxia is a trigger of vascularization through induction of angiogenic factors via hypoxia-inducing factor 1 (Geva and Jaffe, 2000). This agrees with a previous study reporting proliferation of small blood vessels in the superficial ovarian cortex following deep cortex blood vessel damage in human ovaries exposed to chemotherapy (Meirow et al., 2007).

Otherwise, the clear-cut inverse relationship between superficial cortex vascularization and the ovarian follicle reserve raises the question of whether increased superficial cortex vascularization has any effect on follicle dynamics, by influencing the rate of depletion of resting follicles, either by promoting the entrance into the growing phase or increasing follicle deletion by atresia. Regardless of the mechanisms underlying increased superficial ovarian cortex vascularization, a richer microvascular bed may increase the delivery of regulatory signals to resting and early growing follicles, thus affecting their functional status. The activation of primordial follicles and the early stages of follicle growth seem to be dependent on as yet not well-characterized local stimulatory and inhibitory factors (Fortune, 2003). In the last decades, regulatory roles for different hormones and growth factors such as testosterone, anti-Müllerian hormone (AMH), kit-ligand, growth differentiation factor 9, bone morphogenetic protein 15, activin and follistatin have been proposed (reviewed by Fortune, 2003), but their precise roles on the timing activation of primordial follicles and on the earliest stages of follicle development are not fully understood. It has been proposed that increased recruitment of primordial follicles into the growing pool is the main mechanism responsible for the depletion of the ovarian reserve from 30 years of age onwards (Gougeon et al., 1994) and this is roughly coincident with the increase in superficial cortex vascularization. Furthermore, recent studies have reported that several angiogenic factors that play pivotal roles in follicle and corpus luteum vascularization such as vascular endothelial growth factor (VEGF; Fraser et al., 2000; Geva and Jaffe, 2000; Wulf et al., 2002; Fraser, 2006) and basic fibroblast growth factor (bFGF; Geva and Jaffe, 2000) have also apparently direct effects on primordial follicle survival and pre-annel follicle growth in the rodent (Danforth et al., 2003; Roberts et al., 2007), bovine (Yang and Fortune, 2007) and human (Quennell et al., 2004) ovary, thus establishing a new link between ovarian vascularization and follicle dynamics.

To further analyse the possible relationship between superficial cortex vascularization and the ovarian follicle reserve, we studied polycystic ovaries that show abnormal initial follicle growth (Webber et al., 2003; Maciel et al., 2004; Stubbs et al., 2007) and increased ovarian vascularization as determined by Doppler-based blood flow measurements (Agrawal et al., 1998; Pan et al., 2002; Abd El Aal et al., 2005; Ng et al., 2005). In this study, polycystic ovaries showed a 2-fold increased volume density of blood vessels in both superficial cortical stroma and DCS with respect to age-matched controls, while the density of small follicles was equivalent. It should be taken into account that, in contrast to normal ovaries, increased vascularization in PCOS ovaries was not related to age, as ovarian samples corresponded to 20–30-year-old women and possible effects of ageing in PCOS ovaries cannot be observed. In this sense, changes in cortical vascularization could be mediated by different mechanisms in PCOS and normal ageing ovaries. The increased cortical vascularization in PCOS ovaries agrees with previous studies reporting increased blood flow (Agrawal et al., 1998; Pan et al., 2002; Abd El Aal et al., 2005; Ng et al., 2005). Worthy to note, serum and intraovarian concentrations of several angiogenic factors such as VEGF and bFGF are increased in PCOS (Agrawal et al., 1998; Abd El Aal et al., 2005, Artini et al., 2006). A previous study has reported the expression of VEGF and endothelial gland-vascular endothelial growth factor/Prokinection 1 (EG-VEGF/PROK 1) mRNA in normal and polycystic ovaries (Ferrara et al., 2003). Notably, EG-VEGF mRNA was expressed in the ovarian stroma and in small follicles in both normal and PCOS ovaries (Ferrara et al., 2003). The increased vascular density in both superficial cortical stroma and DCS in PCOS could be related to increased levels of angiogenic factors. However, which specific angiogenic factors are responsible for ovarian cortical stroma vascularization is not known.

In contrast to that which happens in normal ageing ovaries, increased superficial cortex vascularization in PCOS ovaries was coincident with a high density of resting follicles. This strongly suggests that increased vascularization of the cortical stroma is not due to the depletion of resting follicles, at least in polycystic ovaries, and raises the question of whether increased vascularization could influence follicle dynamics in PCOS. Previous studies have reported that PCOS ovaries show increased numbers of early growing follicles (Webber et al., 2003; Maciel et al., 2004; Stubbs et al., 2007), although it is unclear whether the higher numbers of early growing follicles in PCOS is due to accelerated activation and early follicle growth, as suggested by the higher expression of proliferation markers (Webber et al., 2003; Stubbs et al., 2007) or to stockpiling of early growing follicles due to a slower growth rate of primary/secondary follicles (Maciel et al., 2004). Increased concentrations of angiogenic factors may affect follicle dynamics by different mechanisms. Indeed, VEGF may affect follicle survival and early growth either directly (Yang and Fortune, 2007) or indirectly by increasing angiogenesis and/or vascular permeability (Geva and Jaffe, 2000), or through effects on AMH expression (Thomas et al., 2007). In any case, the coexistence of a high-density of primordial follicles together with increased vascularization in PCOS ovaries allows a closer relationship between resting follicles and cortical blood vessels. In fact, and in spite of the lack of an independent vascularization, direct contacts between
primordial/early growing follicles and cortical capillaries were frequently observed in this study in PCOS ovaries.

In summary, this study has addressed age-related changes in superficial ovarian cortical stroma vascularity in normal cycling and PCOS ovaries. Although the physiological significance of these changes remains to be established, increased superficial cortex vascularity may have profound effects on primordial follicle activation and early follicle growth in both normal cycling women at mid-age and in PCOS.

**Acknowledgements**
The authors are grateful to Pilar Cano and to Esteban Tarradas for technical assistance.

**Funding**
This work was supported by grants BFU 2008-00984 from the Ministerio de Ciencia e Innovación, and FI08/00830 from the Subdirección General de Educación y Fomento de Investigación and ISCIII, Spain.

**References**


Dickson SE, Fraser HM. Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate. _J Clin Endocrinol Metab_ 2000;86:2339–2344.

Edelbergh JM, Reed MJ. Aging and angiogenesis. _Fract Biosci_ 2003;8:1199–1209.

Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. _Reproduction_ 2001;122:829–838.


Maciel GAR, Baracat EC, Benda JA, Markham SM, Hensinger K, Chang RJ, Erickson GF. Stockpiling of transitional and classic primary follicles in ovaries of women with polycystic ovary syndrome. _J Clin Endocrinol Metab_ 2004;89:5321–5327.


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;19:2132–2137.

Ng EHY, Chan CCW, Yeung WSB, Ho PC. Comparison of ovarian stromal blood flow between fertile women with normal ovaries and infertile women with polycystic ovary syndrome. _Hum Reprod_ 2005;20:1881–1886.

Pan HA, Wu MH, Cheng YC, Li CH, Chang FM. Quantification of Doppler signal in polycystic ovary syndrome using three-dimensional power


Submitted on October 19, 2008; resubmitted on November 29, 2008; accepted on January 6, 2009


