Microvascular morphodynamics of swine periovulatory ovarian follicles as studied by SEM of vascular corrosion casts

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Summary

The growth of ovarian follicle and corpus luteum development is dependent on angiogenesis, the proliferation of new capillaries from pre-existing vessels. The morphological changes driven by angiogenesis ensure the adequate metabolic support to the follicular-luteal complex and are essential for fertility. However, the morphofunctional relation between neoangiogenesis and fertility are not yet fully understood, especially in the time between the LH surge and ovulation (periovulatory period). In this stage, somatic follicular cells rapidly differentiate in luteal steroidogenic cells. The metabolic switch, from an estrogen-to-steroidogenic activity, is sustained by extensive blood vessel remodelling. The purpose of this work was to describe the sequence of vascular remodelling events, rapidly occurring during the periovulatory period. Preovulatory, early periovulatory and late periovulatory follicles were obtained at a precise timing from prepubertal gilts stimulated by a validated hormonal protocol (eCG+hCG). The swine model was chosen due to a long periovulatory window (40-44 h, as in human). The three-dimensionality of the blood vessel network, the presence of angiogenesis and the patterns of angiogenic figures (sprouting or non-sprouting angiogenesis), were analysed by means of scanning electron microscopy of vascular corrosion casts. Results showed the presence of three concentric vascular plexuses (inner, medium and outer) in all groups. In the inner network, a high angiogenic activity was evidenced in preovulatory follicles, as demonstrated by a dense carpet of capillaries, with sprouting angiogenic figures. In early periovulatory follicles, the layer compactness decreased in consequence of an elongation of capillaries. The pattern of angiogenic figures was similar to the previous group. Close to the ovulation, in late periovulatory follicles, evident modifications in the vessel architecture were observed. The inner layer became undulated, as several folds were visible on its surface. Many gaps showed the characteristics of the underlying medium layer. Sprouting and non-sprouting angiogenesis, as intussusception, were visible. From the data obtained it is possible to conclude that the follicular angiogenesis in pig is high during the preovulatory phase, while the periovulatory period is constituted by a first step of quiescence followed by a second one of active angiogenesis, characterized by sprouting and, interestingly, non-sprouting angiogenesis. The quiescence status, in early periovulatory follicles, represents the starting point for the subsequent metamorphosing process, necessary to transform them into functional corpora lutea.

Key words: angiogenesis, vascular corrosion casts, scanning electron microscopy, ovarian follicles, pig.

Introduction

The formation of new blood vessels by migration and proliferation of pre-existing vessels is defined angiogenesis. In adults, vasculature is in a quiescent phase, with a turnover of endothelial cells lasting years. Physiological angiogenesis is prominent only in the female reproductive system, while the pathological one is related to tumour growth and metastatization (Plendl, 2000). In the ovary, new vessels are cyclically and rapidly formed during the reproductive lifespan due to angiogenesis in developing follicles and corpora lutea (Findlay, 1986; Reynolds *et al.*,

2002; Fraser, 2006). Angiogenesis is regularly followed or accompanied by a likewise rapid regression and disruption of the newly formed vessels, or angioregression, both in follicles and in corpora lutea (Suzuki *et al.*, 1998; Plendl, 2000; Tamanini and De Ambrogi, 2004). The cyclic growth and regression is regulated by a cyclic and pulsatile secretion of gonadotropins, which leads to ovulation and steroidogenesis (Geva and Jaffe, 2000; Motta *et al.*, 2003). The angiogenic waves involved in this phenomena are coordinated by extra-ovarian gonadotropins, as demonstrated in vivo and in vitro for LH (Luteinizing Hormone), FSH (Follicle Stimulating Hormone) and hCG (human Chorionic Gonadotropin) (Reisinger et al. 2007), and/or locally produced steroids (Rubanyi *et al.*, 2002). These morpho-functional changes, mainly occurring in the ovarian microvasculature domain, are essential to guarantee the adequate metabolic supply to the so called follicular-luteal complex (FLC). Moreover, since they appear to play important roles in folliculogenesis, ovarian hormone production and ovulation, it is evident the essential role of the angiogenic process to ensure fertility (Nottola *et al.*, 1997; Plendl, 2000; Macchiarelli, 2000; Motta *et al.*, 2003; Macchiarelli *et al.*, 2006; Jiang *et al.*, 2003, 2008).

The process of vascular remodelling in the FLC is particularly intense in the periovulatory period, when the ovulated follicle should rapidly differentiate into a highly vascularised structure: the corpus luteum. This structure, capable to synthesize high level of progesterone necessary to maintain the pregnancy, requires the support of an extensive blood vessel network, in order to ensure an adequate trophic supply to follicular/luteal cells (Hunter *et al.*, 2004). The rapid development of the ovulatory follicle in the subsequent curpus luteum is, indeed, sustained by an abundant but finely tuned angiogenesis. However, in this so peculiar moment, the sequence of angiogenic events, determined by specific morphological patterns of angiogenic figures (sprouting or non-sprouting angiogenesis), are not well known. Based on the above consideration, it becomes extremely interesting, from a morpho-functional point of view, to better understand the dynamics of blood vessels remodelling involved in the rapid events transforming the ovarian follicle into a corpus luteum. A better knowledge of such dynamics will be a valuable tool to discriminate among the factors of female infertility.

To this aim, the purpose of this work is to describe the sequence of vascular remodelling events, rapidly occurring in a well defined temporal moment: the periovulatory period, comprise between the LH surge and the ovulation of the oocyte.

Since angiogenesis is a phenomenon not only time-regulated but evolving in a three-dimensional pattern in space, to finely evaluate the morphological dynamics of the vascular network, the best technical approach is represented by Scanning Electron Microscopy applied to Vascular Corrosion Cast (SEM of VCC).

Materials and Methods

Experimental model

The polyovular swine model has been chosen because of the presence of: 1) multiple developing follicles and subsequent corpora lutea, allowing to follow the angiogenic dynamics in several follicular-luteal complexes; 2) a long periovulatory period (lasting 40–44 h, as in woman) which facilitates the analysis of the temporal evolution of angiogenesis and 3) a validated hormonal protocol, ensuring a precise estrous synchronization.

Since nowhere the scientific background about ovarian angiogenesis in pig has mainly involved normal cycling animals, the application of a validated hormonal protocol allowed an accurate selection of follicles at different moments of development. This was also helped by the presence of a long periovulatory window.

Ovarian collection

About fifteen prebubertal Large White gilt of approximately 100 Kg were injected i.m. with a single dose of 1250 IU of eCG (Folligon; Intervet) to promote follicular growth in 60-72 h (Shimizu *et al.*, 2002). After 60 h animals were injected with 750 IU of hCG (Corulon; Intervet) to induce ovulation (Martelli *et al.*, 2006; 2009). On the basis of the hormonal protocol, the treated animals were divided in three groups of five animals each, in order to obtain: a) a control group of preovulatory follicles (60 h after eCG administration), b) early periovulatory follicles (18 h after hCG administration) and c) late periovulatory follicles (36 h after hCG administration).

Ovaries were recovered by laparatomy from

anaesthetized animals by an injection of azaperone (6 mL/gilt) and atropine sodium salt (2 mg/gilt) and maintained under thiopenthal sodium (1.5 g/gilt). All protocols had prior approval from the Ethical Committee of the University of Teramo.

Vascular corrosion casts and scanning electron microscopy

The ovaries of each animal were processed for the vascular corrosion casts (Jiang *et al.*, 2002) to obtain almost three-dimensional images at relatively high resolution (Murakami, 1971; Lametschwandtner *et al.*, 1990; Macchiarelli, 2000) and to highlight differences in the vascularization of the FLC, both in terms of angiogenesis that angioregression, depending on the stage of ovarian cycle. Moreover, the application of this technique allowed the evaluation of index of preservation, growth and regression of follicular cells, as the number of blood layers, shape and arrangement. In brief, to wash out all blood, swine ovaries were cannulated and perfused with a heparinised saline solution. All samples were, then, slowly perfused through the ovarian artery by a solution of Mercox, until polymerization started (Murakami *et al.*, 1971). The resin-injected ovaries were placed for 3-4 h in a warm water bath to complete polymerization, corroded in a NaOH (10%) solution for 24-48 h al 60°C, and gently washed for a few hours under tap water. Then, they were immersed in distilled water for 2-3 days at 60° C to completely remove macerated tissues and washed again under running tap water. Ovarian vascular casts were air dried, mounted onto aluminium stubs and coated with platinum. SEM observation were performed at low accelerating voltage (3-12 kV).

To examine the inner wall of follicular vessels, samples were frozen at -20°C, and then cracked with a razor blade. After immersion in alcohol (100%), samples were dried at 60°C overnight before further coating. The distinction between arteries and veins was made on arteries morphology (tortuous configuration and fusiform depressions on the surface of the casts due to endothelial cells prints) and veins morphology (straight configuration with rounded shallow endothelial depressions). The capillary network extension was evaluated qualitatively (Macchiarelli *et al.*, 1991; Nottola *et al.*, 1997; Jiang *et al.*, 2002; 2003) and quantitatively according to morphometric methods (Lametschwandtner *et al.*, 1990; Minnich *et al.*, 2001).

Results

General vascular architecture of swine ovarian follicles

SEM observation of VCC allowed the identification of numerous vascular plexuses, in the follicular architecture, mainly of ovoid shape and with different size, that appeared well-perfused by the casting medium. Vessels were classified according to their diameters and shapes of endothelial cell nuclei (Macchiarelli *et al.*, 1991, 1992, 1993, 1995, 1998). Indeed, arteries showed a tortuous configuration and presented fusiform endothelial cell print depression on the surface of the casts, while veins showed a rather straight course and possessed rounded depressions (Takada *et al.*, 1987). Characteristic figures of follicular angiogenesis (i.e. budding, growth and division of capillaries from pre-existing blood vessels) were easily distinguishable in all the samples (Macchiarelli *et al.*, 2006).

In detail, in all the groups analysed, ovarian follicles showed three concentric vascular plexuses connected to each other by anastomotical bridges: an inner, a medium and an outer vascular network (Figure 1A, 1B, 1C). Since the microvascular architecture presented a cup-like aspect, with an empty central cavity (before corrosion occupied by the granulosa layer, oocyte and antrum cavity), in the inner layer it was possible to verify the presence of regional-specific differences in the distribution pattern of angiogenic figures. In fact, three area (apical, equatorial and basal) were identified in the casted ovarian follicles and the number of angiogenic structures were counted in each of them.

Group 1

In preovulatory follicles (control group) the inner network evidenced a peculiar carpet-like aspect, due to a very high density of short and small capillaries (~7 µm in diameter), presenting angiogenic figures such as budding and sprouting (Figure 2A). The number of angiogenic structures was significantly higher in the equatorial region (13.25 ± 1.45^{a}), respect to the basal (3.01 ± 0.88) and apical (1.05 ± 0.49) ones. The medium vascular plexus was mainly constituted by large vessels,

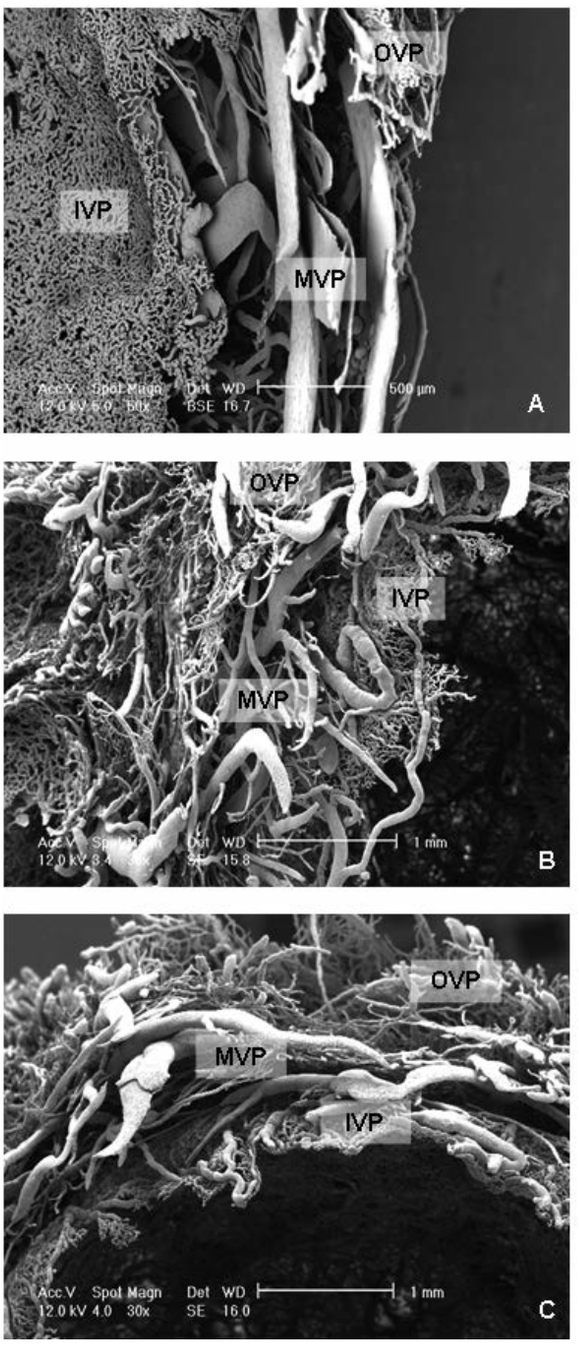

Figure 1. Representative pictures of the inner (IVS), medium (MVS) and outer (OVS) vascular plexus as observed in gilts by SEM of VCC at low magnification in preovulatory (A), early periovulatory (B) and late periovulatory ovarian follicles.

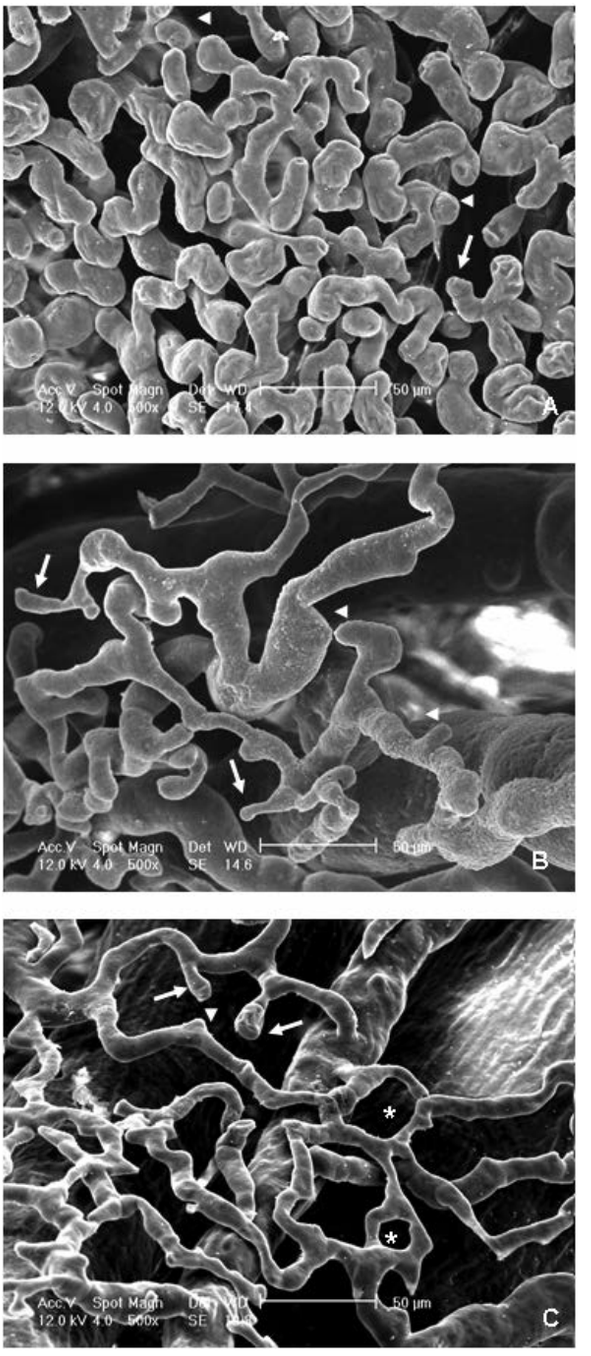

Figure 2. SEM of VCC micrographs at high magnification of the inner vascular plexus in preovulatory (A), early periovulatory (B) and late periovulatory pig ovarian follicles. Angiogenic figures are indicated: budding (arrowhead), elongation of capillaries (arrow) and intussusception (asterisk).

with a diameter of about 46 µm, necessary to support the inner plexus. The outer network was characterized by thinner vessels (~11 µm in diameter), distributed at different levels (Figure 1A).

Group 2

In early periovulatory follicles, the inner network showed comparable thickness and blood vessel diameter (~6 µm) to group 1. However, differently to preovulatory follicles, an evident elongation of capillaries, associated to a consequent decrease in the layer compactness, was observed. Budding and sprouting were the angiogenic figures present (Figure 2B); their number was not statistically different, respect to group 1, in the apical (1.05±0.49 vs 1.97±0.78) and basal (3.01±0.88 vs 4.02±1.01) regions of the inner layer. On the contrary, it was observed a statistically significant decrease in the equatorial area (13.25 ± 1.45^{a} vs 4.76 ± 0.99^{b}). In the medium layer, blood vessels resulted similar to its analogous in preovulatory follicles (44.40 vs 46.23 µm, respectively). Arterioles and venules from the middle plexus showed a low density, but they were visible underneath the gaps of the inner network. The vessels from the outer plexus showed similar characteristics to group 1 (Figure 1B).

Group 3

Close to the ovulation, the inner layer architecture in late periovulatory follicles was similar to groups 1 and 2, in terms of thickness and vascular diameters (~8 µm). However, differently to them, this layer showed an undulated aspect, with ridges, folds and numerous large gaps dispersed among. It was intriguing to understand that these phenomena of infolding, observed for the first time at this stage, were determined by the vessels from the middle network that lifted in folds the inner plexus. The inner layer displayed several angiogenic figures, differently distributed among the regions (the uncasted apical region was not determinable). Their number in the equatorial region, statistically decreased respect to group 2 (4.76 ± 0.99^{b} vs 11.45 ± 1.57^{a}, respectively), while did not show differences in the basal region (4.02±1.01 3.91±1.56). The figures were mainly constituted by sprouting and non-sprouting (as intussusceptions) angiogenesis (Figure 2C). In particular, the inner vascular plexus showed the formation of numerous meshes and transcapillary pillars raging from 3 to 20 µm. The intussusceptive pillar appeared shortly distant from the bifurcation or in the centre of the vessel in some circumstances, while in others holes appeared within the capillary bed, otherwise long parallel rows of pillars appeared in longitudinal folds of the endothelium. Due to the presence of several folds, the thickness of the middle layer was bigger, even if the vessel diameter was similar to the other groups (~ 65 µm). In the outer plexus, vessel diameter and distribution were similar to groups 1 and 2 (~ 11 µm) (Figure 1C).

Discussion

Angiogenesis is the physiological process by which new blood vessels originate from a preexisting microvasculature. It has a key role in embryonic development, adult organ growing, functioning and survival but especially in the reproductive physiology as the normal menstrual cycle and pregnancy (Robinson *et al.*, 2009). An active angiogenesis, however, accompanies also several pathological conditions such as tumour (Cuevas and Boundreau, 2009), inflammation (Jackson *et al.*, 1997), rheumatoid arthritis (Szekanecz *et al.*, 2009). The pathogenesis of numerous other diseases is also referable to insufficient growth and maturation of the vascular network (Ferrara et al, 2005). Despite in the last years scientific literature acquired many information on the morphological expression of angiogenesis in different experimental models as well as on its regulatory molecular mechanisms, this biological process is still only partially known.

The interest of our research group to unravel the morpho-functional mechanisms of angiogenesis in the female reproductive system is due to its important role exerted to guarantee a reproductive success. In fact, defects in ovarian angiogenesis may contribute to ovarian dysfunction and infertility. For example, in infertile women, decreased ovarian vascularity, consequence of a down-regulation of angiogenesis, has been associated with lower pregnancy rates. Polycystic ovarian syndrome (PCOS), ovarian hyperstimulation syndrome (OHSS) and ovarian cancer are pathologies associated, on the contrary, with an up-regulation of angiogenesis, inducing vessels hyper-permeability (Geva and Jaffe, 2000).

From what reported above it is now clear that a correct sequence of follicular growth and development, accompanied by a perfectly balanced angiogenesis, can ensure the ovulation and, indeed, fertility.

The peculiar moment in the follicular development, studied here, is that immediately following the LH surge and ending with the ovulation: the so named "periovulatory period". During this phase, lasting 40-44 hours in pig, important spatio-temporal remodelling in the follicular microvascular architecture occurs. The ovarian follicle, a limited blood supplied structure, should transform in a well vascularized organ, the corpus luteum, to sustain the pregnancy.

The use of SEM applied on vascular corrosion casts, allowed to obtain quasi three-dimensional images at relatively high resolution (Murakami, 1971; Lametschwandtner *et al.*, 1990; Macchiarelli, 2000) and to highlight differences in the vascularization of the FLC, both in terms of angiogenesis that angioregression, depending on the stage of ovarian cycle.

Results obtained showed the extremely high angiogenic plasticity of the periovulatory follicle. The preovulatory period that precedes the LH surge (follicles from group 1) is characterized by a discrete angiogenic activity, as evidenced by the compact layer of capillaries in the inner network, presenting phenomena of sprouting angiogenesis, especially in the equatorial region. At the beginning of the periovulatory period (follicles from group 2) a quiescence in the angiogenesis is detected. In fact, respect to group 1, it was observed a decrease in the inner layer compactness, accompanied by a parallel elongation of capillaries, and in the number of angiogenic figures of the equatorial region. Approaching to the ovulation (follicles from group 3), an intense vascular remodelling restart. The vascular area of the inner vascular plexus increases so evidently, as sustained by sprouting and non-sprouting (as intussusception) angiogenesis, to refold into the antrum. The infolding was due to the action of vessels from the middle network. From what observed by SEM observation at higher magnification, the transcapillary pillars formation was not detected before the late periovulatory phase, suggesting that intussusception did not occur before this developmental stage. Differently to sprouting, the non-sprouting (intussusceptive) angiogenesis occurs almost in absence of endothelial cell proliferation, is achieved at low vascular permeability levels, and requires only 4-5 hours for completion (Djonov *et al.*, 2003). It it easy to argument that intussusception is the optimal physiological solution to sustain a rapid angiogenesis during the quick transformation from the ovulatory follicle into a functional corpus luteum (Djonov *et al.*, 2003; Burri *et al.*, 2004).

In conclusion, these data allow to hypothesize a metamorphosing nature of the early periovulatory follicle that, over a few hours, transforms into a highly vascularized structure, probably in order to sustain the corpus luteum development after ovulation.

Since angiogenesis is a phenomenon which evolves in a three-dimensional pattern, the application of SEM of VCC confirm to be the technique of choice to: i) determine the indexes of preservation, growth and regression of FLCs; ii) evidence the number, shape and arrangement of blood layers and iii) to clarify the patterns of angiogenic figure (sprouting and/or non-sprouting) present during the different stages of development in FLCs (Macchiarelli *et al.*, 2006).

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