Ovariectomy improves neovascularization and microcirculation of freely transplanted ovarian follicles

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Abstract

To investigate the influence of ovariectomy on the physiological process of neovascularization of ovarian follicles, we harvested follicles with a theca interna at 48 h after pregnant mare's serum gonadotropin (PMSG) treatment of Syrian golden hamsters, for subsequent transplantation onto striated muscle tissue of chronically implanted skinfold chambers of non-ovariectomized PMSG-synchronized hamsters and bilaterally ovariectomized hamsters. Non-ovariectomized non-PMSG-treated animals served as controls. During a 2 week period after transplantation, neovascularization of these freely transplanted grafts was quantified *in vivo* by assessment of the newly developed microvascular follicular network, its microvessel density, the diameter of microvessels and their volumetric blood flow using fluorescence microscopic techniques. At day 3 after transplantation, capillary sprouts could be observed in all groups studied, finally developing

a complete glomerulum-like microvascular network within 5–10 days. In ovariectomized animals, however, vascularization of follicular tissue was found to be accelerated and enhanced when compared with the follicles in both groups of non-ovariectomized animals. This was associated with significantly higher capillary blood perfusion, which may in part represent the graft's adaptive response to pro-angiogenic stimuli due to elevated gonadotropin levels, but might in particular be mediated by gonadotropin-induced release of vasoactive substances. Interestingly, small preantral follicles lacking a theca interna failed to vascularize in either of the groups. In conclusion, the study demonstrates that ovariectomy improves neovascularization and microcirculation of freely transplanted ovarian follicles.

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Introduction

The ovary is one of the adult organs in which angiogenesis, the sprouting of new vessels from preexisting blood vessels, occurs as an integral part of physiology (Reynolds *et al.* 1992). Primary follicles consist of an oocyte which is surrounded by an avascular layer of cuboidal granulosa cells. During the female reproductive cycle those primary follicles mature to preovulatory follicles with a dense capillary network originating in the theca cell layer. A prerequisite for this cyclic growth is the tight regulation of angiogenesis, i.e. new blood vessel formation, which is strictly delimited and finely tuned by a balance of stimulatory and inhibitory angiogenic factors (Augustin 2000). Therefore, ovarian follicular tissue offers an unique system to study a number of distinct angiogenic processes. Since Roy & Greenwald (1996) developed successful methods to isolate and classify intact follicles from ovaries of several mammals, it even has become possible to investigate angiogenesis dependent on the stage of follicular development and free of other intraovarian regulating factors.

Corpora lutea survive grafting to tissues such as the rabbit cornea and are capable of inducing neovascularization with reattachment of preexisting vessels by anastomosis to the host vessels (Gospodarowicz & Thakral 1978). Furthermore, it has been shown that preovulatory follicles implanted under the renal capsule of pseudopregnant animals luteinize spontaneously (Ellsworth & Armstrong 1974). Thus, it is likely that transplanted follicles are not only in a position to maintain their physiological function of steroid production and luteinization but also their control of angiogenesis. In view of the importance of angiogenesis in ovarian function, the search for those factors that are responsible for selective vascularization of the follicle and the corpus luteum is central for understanding and controlling both normal and abnormal ovarian function. The regulators of this physiological angiogenesis are unclear, although basic fibroblast growth factor (Neufeld *et al.* 1987, Reynolds & Redmer 1998), vascular endothelial growth factor (Phillips *et al.* 1990, Shweiki *et al.* 1993, Kamat *et al.* 1995) and angiogenin (Heungshik *et al.* 1999) have been implicated as possible controlling factors.

Beside angiogenic substances, steroids are intimately involved in the function of the reproductive tract and are produced in large quantities by the ovarian follicle and the corpus luteum. The gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are essential for the cyclic growth of follicles, but have not been studied in detail in terms of their role in the process of angiogenesis. For this purpose, we analyzed *in vivo* microscopically the process of neovascularization and the microcirculation of ovarian follicles which were harvested 48 h after pregnant mare's serum gonadotropin (PMSG) treatment and transplanted into ovariectomized hamsters. Those grafts were compared with follicular grafts transplanted into non-ovariectomized, PMSG-treated animals. Follicular grafts transplanted into non-ovariectomized non-PMSG-treated animals served as controls.

Materials and Methods

Preparation of the hamster dorsal skinfold chamber

The experiments were conducted in accordance with the German legislation on protection of animals and the *NIH Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, Washington, USA).

The hamster dorsal skinfold chamber was used as the host site for ovarian follicle transplantation and for *in vivo* analysis of follicular neovascularization. The preparation contains one layer of striated muscle and skin and allows for intravital microscopic observation of the microcirculation in awake animals over a prolonged period of time. The chamber technique with its implantation procedure (Endrich *et al.* 1980) and its broad versatility (Menger & Lehr 1993) have been described previously in detail. In brief, under pentobarbital sodium anesthesia (50 mg/kg body weight i.p.) two symmetrical titanium frames are implanted on the extended dorsal skinfold of 8- to 10-week-old Syrian golden hamsters. One layer of skin, subcutaneous tissue and striated muscle is removed in a circular area of 15 mm in diameter and the remaining layers, consisting of striated muscle, subcutaneous tissue and skin, are covered with a removable cover slip incorporated into one of the titanium frames. In addition, a permanent catheter is passed from the dorsal to the ventral side of the neck and inserted into the jugular vein for application of fluorescent dyes. The animals were allowed to recover from anesthesia and surgery for at least 48 h. After i.v. application of 5% fluorescein isothiocyanate (FITC)-labeled dextran 150 000 (Sigma, Deisenhofen, Germany), the chamber allows for continuous observation and repetitive measurements of the microcirculation by means of intravital fluorescence microscopy.

Follicle isolation and transplantation

For follicle donation, 8- to 10-week-old female hamsters were used and pretreated with PMSG (Sigma) dissolved in PBS (1000 U/ml). PMSG was given s.c. at 0800 h at a single dose of 2 U/10 g body weight. After 48 h, donor ovaries were aseptically removed and placed in 30 mm diameter plastic Petri dishes, filled with 37 °C DMEM medium (10% fetal calf serum, 0.1 mg/ml gentamycin) and the fluorescent dye bisbenzimidazole H33342 (200 µg/ml) (Sigma). After removing the surrounding tissue, the ovaries were microdissected under a stereomicroscope using 27 gauge needles. According to size, the follicles were visually collected and transferred into 37 °C bisbenzimidazole H33342-free DMEM medium.

The first group of animals which received follicular grafts consisted of PMSG-synchronized female hamsters ($n=5$). A second group of animals comprised bilaterally ovariectomized hamsters ($n=9$) which were allowed to recover from ovariectomy for at least 1 week prior to preparation of the dorsal skinfold chamber and follicle transplantation. A third group of non-ovariectomized animals without PMSG-treatment served as controls ($n=9$).

For follicle transplantation the cover glass of the skinfold chamber was removed and one to three follicles were placed onto the striated muscle within the chamber. A hand-picking procedure guaranteed single connective tissue-free follicles for transplantation.

Intravital microscopy

For *in vivo* microscopic observation, the awake animals were immobilized in a Plexiglas tube and the skinfold preparation was attached to the microscopic stage. The stage was placed on a computer-controlled microscope desk, which allowed repeated scanning of each individual follicle for intravital microscopy. After i.v. injection of 0.2 ml 5% FITC-labeled dextran 150 000 (contrast enhancement by staining of plasma) and 0.1 ml 0.1% rhodamine 6G (Sigma) (direct staining of white blood cells), *in vivo* microscopy was performed using a modified Leitz Orthoplan epi-illumination microscope with a 100 W HBO mercury lamp attached to a Ploemo-Pak illuminator with blue, green and ultraviolet filter blocks (Leitz, Wetzlar, Germany). The microscopic images were recorded by a charge-coupled device video camera (CF8/1 FMC; Kappa GmbH, Gleichen, Germany) and transferred to a video system for off-line evaluation. With the use of $4\times$, $6.3\times$, $10\times$ and $20\times$ long-distance objectives (Leitz) magnifications of $\times 86$, $\times 136$, $\times 216$ and $\times 432$ were achieved on a 14 inch video screen (PVM 1444; Sony, Tokyo, Japan).

Microcirculatory analysis

By means of a computer-assisted microcirculation analysis system (CapImage; Zeintl, Heidelberg, Germany)

quantitative off-line analysis of the videotapes included the determination of the diameter of the transplanted follicles (in μm), the size of the transplanted follicles (in mm^2) and the vascularized area of the transplanted follicles (size of the growing microvascular network as the percent of the initial follicular size) at a magnification of $\times 216$. Microvessel density, i.e. the length of red blood cell (RBC)-perfused microvessels per observation area (in cm/cm^2) and the diameters of follicular microvessels (in μm) were determined at a magnification of $\times 432$. Follicular growth was assessed by the actual diameter of follicular tissue at the respective time points of analysis and is given as a percent of the initial diameter at the day of transplantation. Upon ultraviolet epi-illumination the dye bisbenzimidazole H33342 is characterized by a bright blue fluorescence with only little bleaching which persists through several cell generations. The specific fluorescence/background fluorescence ratio is high enough over a sufficient time period of weeks to precisely delineate the stained follicular graft from the non-stained surrounding host tissue.

Centerline RBC velocity (V_{RBC}) in the individual microvessels was measured by frame-to-frame analysis at a magnification of $\times 432$. Volumetric blood flow (VQ) of individual microvessels was calculated from V_{RBC} and diameter (D) for each microvessel as $VQ = \pi \times (D/2)^2 \times V_{\text{RBC}}/K$, where $K (=1.3)$ represents the Baker–Wayland factor (Baker & Wayland 1974) considering the parabolic velocity profile of blood in microvessels.

Rhodamine 6G-stained white blood cells were classified in accordance with their interaction with the endothelium of newly formed microvessels as rolling cells when they were moving with a velocity less than two-fifths of the centerline velocity (given as percentage of non-adherent leukocytes passing through the observed vessel segment within 20 s) and as adherent cells when they did not move or detach from the endothelial lining within an observation period of 20 s (given as number of cells per microvascular network area).

Experimental protocol

For the first group, a total of 11 follicles at 48 h after PMSG treatment were transplanted onto the striated muscle of skinfold chambers of five synchronized female hamsters. For the second group a total of 17 follicles were transplanted onto the muscle of skinfold chambers of nine female ovariectomized hamsters. As control series, 21 follicles were transplanted onto the striated muscle of skinfold chambers of nine non-ovariectomized, non-PMSG-treated animals. The macroscopic appearance of the skinfold chamber preparation and the implanted grafts were documented daily. Intravital multi-fluorescence microscopic analysis of growth, angiogenesis and microcirculation of the follicular grafts was performed on days 3, 5, 7, 10 and 14 after transplantation. Measurements on

vascular density and microhemodynamics included only newly formed microvessels which could be clearly distinguished by their glomerulum-like arrangement from the autochthonous host striated muscle microvessels, which display the typical parallel arrangement of muscle capillaries (Menger & Lehr 1993). Vascular density was measured within five regions of interest per graft and observation time point. Regions of interest were selected along the line from the left to the right margin of the individual follicle. Microvascular diameters and hemodynamic parameters were determined by analyzing ten microvessels per region of interest. Microvessels were selected randomly inasmuch as those microvessels were chosen for analysis of diameter which crossed a vertical line drawn over the center of the video screen. In all microvessels selected, both vessel diameter and V_{RBC} were determined for subsequent calculation of VQ.

Statistics

After multivariate analysis of interaction between time and group, differences between groups were tested separately at each time point by ANOVA followed by the appropriate post hoc comparison. To test for time effects separately for each group, multivariate ANOVA for repeated measures was applied. This was followed by the paired Student's *t*-test, including correction of the α -error according to Bonferroni probabilities for repeated measurements (SigmaStat; Jandel Corporation, San Rafael, CA, USA). All values are expressed as means $\pm$ S.E. The criterion for significance was set at $P < 0.05$.

Results

General remarks

Follicles with diameters larger than 250 μm showed typical signs of angiogenesis, whereas smaller follicles failed to vascularize in either of the groups. Initial signs of angiogenesis were irregularities at the wall of the host vasculature (mostly capillaries) with formation of sinusoidal sacculations and sprouts (Fig. 1A). Within a few days, a dense network of newly formed capillaries, interconnecting with each other, developed (Fig. 1B) and covered the initial follicular area (Fig. 2A–D). Hematoxylin–eosin histology of isolated single follicles revealed the absence of thecal cell layers in the smaller follicles (Fig. 3A), while follicles with diameters $>250 \mu\text{m}$ exhibited an intact multilayered theca interna (Fig. 3B), underlining the role of thecal cells as either the origin of humoral factors or paracrine interactions with granulosa cells, both of which are important for stimulation of vascular growth. Hematoxylin–eosin histology of transplanted and vascularized follicles demonstrated that the grafts were normal in their appearance (Fig. 4A and B).

Figure 1 Intravital fluorescence microscopy of follicular angiogenesis. The process of vascularization is characterized by formation of sinusoidal sacculations and capillary sprouts (A), which interconnect with each other forming a microvascular network (B). Blue light epi-illumination with contrast enhancement by 5% fluorescein isothiocyanate (FITC)-labeled dextran 150 000 i.v. Magnification $\times 220$.

Quantitative analysis of follicular microcirculation

All of the transplanted follicular grafts with a diameter $<250\ \mu\text{m}$ on the day of transplantation failed to vascularize and, thus, did not provide a microcirculation.

In ovariectomized hamsters areas of follicular tissue of grafts with a diameter $>250\ \mu\text{m}$ were vascularized to approximately 50 and 100% at days 3 and 5, while the highest values of $130 \pm 16\%$ and $132 \pm 11\%$ were found at days 7 and 10 after transplantation (Figs 5B and 6A). Vascularization of follicles $>250\ \mu\text{m}$ transplanted into both PMSG-synchronized and non-PMSG-treated, non-ovariectomized animals was less rapid, exhibiting mean values of the total vascular area of approximately 70 and 100% at days 5 and 7 respectively (Figs 5D and 6A). Mean diameters of follicular tissue at day 0 after transplantation did not differ between groups (non-ovariectomized,

non-PMSG-treated animals: $899 \pm 55\ \mu\text{m}$; non-ovariectomized, PMSG-treated animals: $876 \pm 100\ \mu\text{m}$; ovariectomized animals: $946 \pm 97\ \mu\text{m}$). Sequential analysis of the diameter of the follicular grafts over time revealed a progressive increase, indicating follicular growth post-transplantation (Fig. 6B).

Microvessel density of the newly developing networks progressively increased over the days after transplantation ($\sim 300\ \text{cm}/\text{cm}^2$ at day 7) and did not differ between follicles transplanted in ovariectomized and ovary-bearing animals (Fig. 6C). Moreover, mean values of microvessel density remained almost unchanged between days 7 and 14 after transplantation, except for a slight decrease in non-ovariectomized, non-PMSG-treated animals (Fig. 6C).

In follicular grafts of synchronized non-ovariectomized animals, analysis of capillary V_{RBC} revealed a progressive increase from $29 \pm 5\ \mu\text{m}/\text{s}$ at day 3 to $170 \pm 34\ \mu\text{m}/\text{s}$ and $242 \pm 27\ \mu\text{m}/\text{s}$ at days 7 and 10 after transplantation (Fig. 7A). In the non-ovariectomized, non-PMSG-treated animals, capillary V_{RBC} remained at lower values of $\sim 120\ \mu\text{m}/\text{s}$ from day 7–14 after transplantation. In contrast, follicles transplanted into ovariectomized animals initially revealed a steeper rise of capillary V_{RBC} from $55 \pm 17\ \mu\text{m}/\text{s}$ at day 3 to $236 \pm 50\ \mu\text{m}/\text{s}$ at day 5, but without further changes until day 10. Comparison between the ovariectomized and the two other groups did show marked differences in capillary V_{RBC} at days 5 and 7 after transplantation (Fig. 7A). Diameters of the follicular grafts' capillaries were found significantly reduced from $15\text{--}17\ \mu\text{m}$ at day 3 to $8\text{--}9\ \mu\text{m}$ at day 14 after transplantation without marked differences between the three groups (Fig. 7B). Calculation of the individual capillary blood perfusion in non-ovariectomized, PMSG-treated animals revealed a 2- to 3-fold increase of blood flow to $\sim 14\ \text{pl}/\text{s}$ until vascularization of the grafts was completed (day 7–10). In non-PMSG-treated animals, capillary VQ ranged between 5 and $8\ \text{pl}/\text{s}$ throughout the observation period (Fig. 7C). In ovariectomized animals, grafts presented with peak values of individual capillary blood flow ($26 \pm 6\ \text{pl}/\text{s}$) already at day 5 after transplantation (Fig. 7C). Subsequently, capillary blood flow values decreased, but remained slightly higher when compared with corresponding values of follicular grafts in non-ovariectomized animals (Fig. 7C).

Beside the assessment of microarchitecture and microhemodynamics of the newly developed follicular networks, analysis of rhodamine 6G-stained leukocytes revealed that almost all of the leukocytes ($>98\%$) passed the networks without any transient tethering or rolling interaction. Moreover, firm adherence of leukocytes within the follicular networks was only rarely observed, indicating the absence of inflammation within the follicular grafts. Lack of edema formation within the skinfold chamber upon follicle transplantation and vascularization further reflected the acceptance of the graft tissue by the host.

Figure 2 Intravital fluorescence microscopy of a follicular graft directly after transplantation (A) as well as at days 3 (B), 5 (C) and 7 (D) after transplantation into the hamster dorsal skinfold chamber of a PMSG-stimulated, non-ovariectomized animal. Note the initial lack of nutritive capillaries within the freshly transplanted graft (A, asterisk). On day 3 after transplantation parts of the follicular graft display newly formed microvessels (B, arrowheads), while some areas still lack vascularization (B, arrows). On day 5 a dense glomerulum-like network of newly formed microvessels covers the entire graft (C, asterisk), which further extends in size until day 7 after transplantation (D, asterisk). Blue light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150 000 i.v. Magnification $\times 70$.

Discussion

The major finding of the present study is that the process of vascularization and the microvascular perfusion of mature follicular grafts are markedly accelerated and enhanced when transplanted in bilaterally ovariectomized animals compared with non-ovariectomized PMSG-synchronized or non-PMSG-treated controls. We further demonstrate that in the case of transplantation of immature follicles (stages <6 (Roy & Greenwald 1985)) vascularization fails regardless of the condition of the host animal.

Methodological remarks

The development of complete microvascular networks might, at least in part, be referred to as angiogenesis which is driven by stress, such as hypoxia, with subsequent

up-regulation of vascular endothelial growth factor (VEGF) as a stress-induced angiogenic growth factor (Plate *et al.* 1992, Shweiki *et al.* 1992). Hypoxia as a driving force for induction of angiogenesis must be considered in all animals studied, because the follicular grafts were transplanted freely and, thus, lack an initial vascular supply, solely depending on nutrient transport by diffusion (Neeman *et al.* 1997). To minimize the variation of oxygen demand between the individual follicular grafts, the population of follicles which we transplanted for subsequent analysis of neovascularization was of comparable developmental stage/size in both ovariectomized and non-ovariectomized animals.

The observation that follicles which consist of an oocyte surrounded by several layers of granulosa cells and the theca layer vascularize is in contrast to results of Gospodarowicz & Thakral (1978), reporting that follicles

Figure 3 Hematoxylin-eosin stained cross sections of formalin-fixed isolated follicular grafts as harvested from an animal at 48 h after pregnant mare's serum gonadotrophin (PMSG) treatment. (A) A tri- to quadrilaminar follicle without any trace of theca cell layers, representing the stage of follicles which failed to vascularize upon transplantation (the layer to the right of the granulosa cells is embedding medium). Magnification $\times 400$. (B) A mature follicle, which is characterized by several layers of granulosa cells and a well-developed multilayered thecal shell. Magnification $\times 150$.

do not have the capability to induce neovascularization. Although follicles were not further characterized by Gospodarowicz & Thakral (1978) and might occasionally have comprised only small primary follicles, these contradictory results on induction of neovascularization of follicles may also be due to differences in species (hamster vs rabbit) or differences in host tissue sites chosen for transplantation (striated muscle vs cornea). Supposedly, sparsely vascularized sites, like the cornea as a trans-

Figure 4 Hematoxylin-eosin stained cross section of a vascularized follicle at day 10 after transplantation onto striated muscle tissue (solid arrows) of the dorsal skinfold chamber of an ovariectomized animal (A), displaying the normal appearing follicular graft with antral cavity formation. Magnification $\times 100$. (B) Higher magnification ($\times 300$) reveals intact granulosa cells (asterisks) and a high density of newly formed microvessels (arrowheads) within the graft; open arrows = theca cells.

parent avascular substratum, might be associated with poor integration of follicular tissue, because stimulation by circulating FSH may be significantly lower at this site, while highly vascularized tissues, such as the muscle, would achieve better functional results. However, since we have noted that neovascularization of follicular grafts is completed within 7–10 days, which is not different from that observed for corpora lutea in the rabbit cornea (Gospodarowicz & Thakral 1978), the process of angiogenesis may not necessarily depend on the vascularity of the host tissue.

Follicular angiogenesis

In the present study, follicle vascularization is characterized by the successive development of a glomerulum-like

Figure 5 Intravital fluorescence microscopy of single follicular grafts at day 0 (A and C) and day 3 (B and D) after transplantation into an unstimulated ovariectomized animal (A and B) and a non-ovariectomized PMSG-synchronized animal (C and D). Note the marked difference in extent of follicular vascularization. The microvascular network of the follicular graft in the unstimulated, ovariectomized animal is fully developed (B, asterisk), while parts of the follicular graft in the non-ovariectomized, PMSG-synchronized animal still lack vascularization (D, arrows). Blue light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150 000 i.v. Magnification $\times 70$.

microvascular network with increasing microvessel density up to day 7. Microvessel diameters within the growing networks continuously decreased, finally ranging below $10\ \mu\text{m}$ after maturation. In accord with numerous reports on angiogenesis, in both malignant and benign tissue, the newly developing vessels exhibit much greater diameters than they normally have, because sprouts and new vessels initially demonstrate saccular configuration (Fig. 1A). In malignant tissue, these vessels even when fully developed have a high heterogeneity in terms of vessel caliber (Vajkoczy *et al.* 1999), while in benign tissue microvessels reveal a progressive decrease in diameter (Menger *et al.* 1992), similar to that observed in the present study.

It is generally accepted that early folliculogenesis is mainly regulated by putative intraovarian factors without involving FSH (Tonetta & DiZerega 1989). Peters *et al.* (1973) demonstrated that the initiation of follicular growth is independent of FSH, because elevating FSH levels by

unilateral ovariectomy of 2-day-old mice did not change the number of normally developing follicles. In line with this, we could show that premature follicles, lacking a thecal shell, fail to induce angiogenesis, even when transplanted into ovariectomized animals which regularly exhibit elevated FSH levels (Bex & Goldman 1975). The present observation of lack of vascular growth of the preantral follicles is thus compatible with previous reports that FSH is not able to stimulate morphological changes in the granulosa cell–oocyte complexes (Li *et al.* 1995, Yokota *et al.* 1997).

Serum gonadotropins strictly regulate the female reproductive system and are essential for the cyclic growth of follicles after the onset of antrum formation (Kumar *et al.* 1997). Therefore, it has been assumed that one effect mediated by these hormones seems to be the local activation of angiogenic factors in developing follicles and corpora lutea (Makris *et al.* 1984, Gospodarowicz *et al.*

Figure 6 Quantification of (A) vascularized area (as percent of the initial follicular size), (B) follicular growth (as percent of the initial follicular size), and (C) microvessel density of follicles after free transplantation into hamster dorsal skinfold chambers, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Follicles were harvested from animals 48 h after PMSG-stimulation and transplanted into non-ovariectomized, non-PMSG-treated animals (●), non-ovariectomized, PMSG-treated animals (■), and ovariectomized, non-treated animals (□). Means $\pm$ S.E. * P <0.05 vs non-ovariectomized, non-PMSG-treated animals at corresponding time points; # P <0.05 vs non-ovariectomized, PMSG-treated animals at corresponding time points; ^a P <0.05 vs day 0; ^b P <0.05 vs days 0 and 3; ^c P <0.05 vs days 0, 3 and 5; ^d P <0.05 vs days 0, 3 and 10.

Figure 7 Quantification of red blood cell velocity (V_{RBC}) (A), microvessel diameter (B) and individual capillary volumetric blood flow (VQ) of follicles (C) after free transplantation into hamster dorsal skinfold chambers, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Follicles were harvested from animals 48 h after PMSG-stimulation and transplanted into non-ovariectomized, non-PMSG-treated animals (●), non-ovariectomized, PMSG-treated animals (■) and ovariectomized non-treated animals (□). Means $\pm$ S.E. * P <0.05 vs non-ovariectomized, non-PMSG-treated animals at corresponding time points; # P <0.05 vs non-ovariectomized, PMSG-treated animals at corresponding time points; ^a P <0.05 vs day 3; ^b P <0.05 vs days 3 and 5; ^c P <0.05 vs days 3, 5 and 7; ^d P <0.05 vs day 5.

1985). For bilaterally ovariectomized hamsters, it has been shown that both serum LH and FSH concentrations are significantly elevated when compared with

unmanipulated animals (Bex & Goldman 1975). Studies in the rat (Disen *et al.* 1994) and non-human primate (Ravindranath *et al.* 1992) suggest that LH modulates expression of VEGF. Moreover, the rapid increase in

serum gonadotropin levels after ovarian autotransplantation in rats was found to be associated with a 40- to 60-fold increase in expression of the genes encoding the two angiogenic factors VEGF and transforming growth factor- β -1 (Dissen *et al.* 1994). In support of this, we now show for the first time by direct *in vivo* observation that in ovariectomized animals graft vascularization is not only accelerated, but also markedly enhanced when compared with that in non-ovariectomized controls. Unexpectedly, microvessel density of follicles did not differ between groups. However, considering the higher values of vascularized area, the overall vessel length per follicle in the ovariectomized group still exceeds that in the non-ovariectomized animals, indicating improved vascularization.

Notably, capillaries of the newly formed microvascular networks in ovariectomized animals presented with higher V_{RBC} and a higher individual blood flow throughout the experimental time period. These observations can in part be interpreted as an adaptive response to high pro-angiogenic stimuli due to supranormal gonadotropin levels. However, increased follicular blood flow might, in particular, be the response upon release of potent vasoactive substances, such as prostaglandins and histamine, known to be also released under high gonadotropin levels (Krishna *et al.* 1986, Brannian & Terranova 1991). With higher blood perfusion, delivery of gonadotropins to the follicles may be increased, facilitating enhanced follicular development (Krishna *et al.* 1986). In supposition of this view, grafts in the ovariectomized animals exhibited enhanced follicular growth.

Inflammation with leukocytic tissue infiltration is known as a potent inducer of corneal vascularization. Thus, it is important to note that enhanced leukocyte-endothelial cell interaction, i.e. increased numbers of leukocytes tethering to and firmly adhering to the endothelial lining of the vessel wall, was observed within neither the follicular capillary network nor the host microvasculature. The absence of a cellular inflammatory response upon follicular tissue transplantation disproves leukocytic activation and tissue infiltration as a significant inducer of follicular graft vascularization.

In conclusion, our study shows that free follicular grafts, when equipped with a thecal cell layer, are vascularized within 7–10 days after transplantation. Bilateral ovariectomy accelerates vascularization and improves microcirculation of the follicular grafts when compared with non-ovariectomized controls. The improvement of microvascular blood perfusion is based on an increase of capillary V_{RBC} , but not on a change in microvessel density and capillary diameter. This accelerated vascularization and increased microcirculation might be best interpreted in that ovariectomy with loss of steroid negative feedback and high gonadotropin secretion contributes to vascular growth and nutritive perfusion by stimulation of angiogenic factors and by release of vasoactive mediators.

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