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Morphometrical analysis to study preantral follicular survival of VEGF-treated bovine ovarian cortex tissue following xenotransplantation in an immune deficient mouse model

Running title: 'Stereology on xenotransplanted ovary'

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Abstract

The increasing number of cancer survivors the past decades, has sparked the attention for fertility preservation strategies. Due to mainly ethical constraints, human material is scarce. The bovine model is suggested as a valuable alternative. Therefore, the following aims are defined: 1) to xeno-graft bovine ovarian cortex tissue in immune deficient mice as a study-model for female fertility preservation strategies, 2) to stereologically quantify vascularization in Vascular Endothelial Growth Factor (VEGF)-treated and non-treated tissue, 3) to study preantral follicular survival *in situ*, after xenotransplantation.

Bovine ovarian tissue strips were incubated with or without VEGF prior to grafting into female, neutered BALB/c-nu mice (n = 16). Non-transplanted cortical tissue was fixated as a control. Two (2w) and four (4w) weeks after transplantation, grafts were retrieved and assessed by von Willebrand Factor and caspase-3 immunostaining. Data were analyzed using a linear mixed model.

While in the $VEGF^+$ grafts 31% of the follicles were considered 'alive' 2w after transplantation, this was only 17% in the $VEGF^-$ grafts ($P < 0.05$). No difference could be detected 4w after transplantation ($P = 0.76$). Less follicles were considered 'alive' after transplantation (22%) as compared to the control (47.5%) ($P < 0.05$). The vascular surface density was significantly less in the grafts, irrespective of the transplantation period or the use of VEGF.

Although the transplantation process overall negatively influenced the number of viable follicles and the density of vascularization, VEGF exposure prior to transplantation can benefit follicle survival when considering a 2w transplantation period.

Keywords (max 6)

Bovine ovarian cortex

50 Ovarian tissue xenotransplantation

Vascular endothelial growth factor

Early preantral follicles

Stereology

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1. Introduction

Female mammals have a large ovarian reserve established during fetal life (e.g. in humans, ruminants) or the perinatal period (as in rodents) (Hirshfield, 1991), stored in the
60 ovarian cortex. During fetal life and shortly after birth, the density of the preantral follicles (PAFs) is higher as compared to adult individuals (Fortune et al., 2000). Follicles develop synchronously in follicular waves through a process of recruitment, selection and dominance. Only a few of them are selected to develop into the dominant stage, increasingly secreting estradiol, which contributes to estrus behavior in mammals displaying an estral cycle (Aerts
65 and Bols, 2010a; b) and ultimately leads to the ovulation of a developmental competent oocyte. However, the vast majority of these recruited cohorts of follicles will degenerate through a natural process of atresia (McGee and Hsueh, 2000; Johnson, 2003).

The chance of developing cancer for women of reproductive age, was recently estimated at 2.46% (Altekruse SF, 2010). In the period 1999-2006, the five-year-survival rate
70 of women at age 0-44 diagnosed with any site of cancer was 83.7% (Seli and Agarwal, 2012). Thus, the fortunate increase of cancer survivors establishes a growing group of patients with a remaining desire to have children. Ovarian structures are damaged when cytotoxic treatments (e.g. chemotherapy) or irradiation therapies are applied (Sonmezer and Oktay, 2004; Lee et al., 2006; Schmidt et al., 2011). For those patients not facing surgical sterility, it has been
75 estimated that survivors of childhood cancer have an overall reduction of 19% in the likelihood of ever being pregnant (Green et al., 2009).

It is still impossible to culture human PAFs (or from any other large mammal) *in vitro* for a significant amount of time (Smitz and Cortvrindt, 2002), let alone to produce *in vitro* embryos, originating from PAF-derived oocytes. Therefore, cryopreservation of ovarian tissue
80 prior to gonadotoxic treatment and autotransplantation after recovery is the only option to date to preserve fertility post treatment (Donnez et al., 2006; Donnez and Dolmans, 2010).

Although this strategy is already in a clinical phase, it is still based on empirical results (Donnez et al., 2011; Donnez et al., 2013). Unfortunately, the progress in the research field of fertility preservation (FP) is slow, basically due to the scarcity of human material which is caused by ethical and practical restrictions. In contrast, the access to bovine slaughterhouse ovaries is nearly infinite. Fortunately, many similarities are present between human and bovine reproductive physiology. Both are mono-ovular species, have the same size of ovary and in both PAFs are randomly distributed in clusters at the outer periphery of the ovarian cortex. At the cellular level, bovine and human follicles and oocytes show the same size and organel organization and have comparable developmental characteristics. As a consequence, xenotransplantation (Paris et al., 2004) of bovine tissue can be considered as a relevant model for human follicular development (Baerwald et al., 2003; Malhi et al., 2005; Adams et al., 2012) using the mouse as a convenient bio-incubator, to overcome problems related to the lack of long-term *in vitro* culture (Senbon et al., 2004; Bols et al., 2010).

One of the most important problems determining the 'success' of transplantation is rejection of the transplanted tissue (Aubard, 2003). PAFs have a relatively low metabolic rate and are used to the lower oxygen tension in the outermost avascular cortex. Altogether, PAFs are assumed to be relatively tolerant to ischemia and the first to benefit from renewed ingrowing vascularization. Nevertheless, following transplantation, follicles are subjected to an ischemic episode whereby approximately 50% of the transplanted follicle population suffers reperfusion injury and apoptosis (Liu et al., 2002; Hernandez-Fonseca et al., 2005; Aerts et al., 2008). Therefore, many research efforts focus on the development of a transplantation strategy to optimize graft and follicle survival (Abir et al., 2011; Bukovsky and Caudle, 2012). While vascularization is crucial, vascular endothelial growth factor (VEGF) is pivotal for angiogenesis and subsequent graft survival and thus follicular development. It has already been proposed earlier as an angiogenic substance in many other

transplantation techniques (Abir et al., 2011; Araujo et al., 2011b; Shikanov et al., 2011; Commin et al., 2012; Labied et al., 2013) and is considered a very important molecule in ovarian physiology (Araujo et al., 2011a). To assess the vasculature density, different
110 methods can be applied such as fluorescence combined with computerized imaging (Greene and Rieder, 2001) or immunohistochemistry (IHC) staining linked with a stereological quantification (Gundersen et al., 1988). The latter method relies upon extensive use of random and systematic random sampling trying to make a representative 3D image out of 2D pictures.

In an attempt to stimulate graft survival through a better vascularization, oxygen and
115 nutrient supply, we hypothesized that survival of PAFs in fetal bovine tissue will benefit from a treatment with VEGF as a trigger for angiogenesis. We have used xenotransplantation of bovine cortical tissue as a model for human reproductive research and applied stereology as a quantification method for vascularization density. Hence, the specific aims of this manuscript were: 1) to develop an easy-to-use xenograft technique, using fetal bovine ovarian cortex
120 tissue transplanted retroperitoneally in ovariectomized SCID mice, 2) to stereologically quantify vascularization in VEGF-treated and non-treated tissue after retrieval and 3) to study and quantify the follicular survival of bovine fetal PAFs *in situ*, following xenotransplantation.

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2. Materials and methods

This study was approved by the Ethical Committee on Animal Experimentation from the University of Antwerp (approval number 2007-05). Experimental animals were handled according to the Ethical guidelines of FELASA. Sixteen female BALB/c-nu mice (Janvier Labs) of 6 to 8 weeks of age were housed in groups of 4 animals, under controlled temperature (25-27°C), air (HEPA-filter) and lighting (12 hours light (20LUX) : 12 hours dark) conditions. Commercial food and water were given ad libitum. All chemicals were purchased from Life Technologies, unless stated otherwise. Experiments were executed in culture media at room temperature (RT): 20 - 24°C.

2.1 Collection of bovine, fetal ovaries, processing of ovarian cortex fragments

Intact reproductive tracts (n=4) of female bovine foetuses with a minimum crown-rump length of 66cm (i.e. between 190-280 days of gestation with an average of 233.5 days), were obtained in the slaughterhouse and transported to the laboratory in warm (25°C) collection medium consisting of MEM (0.95 g/l, Sigma), HEPES (10 mM) and PenStrep (100 IU/ml). The ovaries were washed three times in alcohol and warm physiologic solution supplemented with kanamycin (0.25%) and transferred to isolation medium consisting of MEM (0.95 g/l, Sigma), HEPES (10 mM) and BSA (3 mg/ml, Sigma).

For an overview of the experimental set-up, see Figure 1. Two thin tissue strips were cut from each ovary with a scalpel. Both were divided in three pieces of 0.5 by 0.5 mm. Two parts of the first tissue strip were transplanted for 2 weeks, two parts of the other one for 4 weeks. One part of each tissue strip ($VEGF^-$) was put in 500 µl culture medium (Cm) consisting of MEM + glutamine supplemented with Penicillin-Streptomycin (100 ng/ml), a mixture of Insulin, Transferrin and Selenium (ITS, 0.01 µg/ml, 0.55 µg/ml and 6.7 ng/ml respectively), BSA (1.25 mg/ml, Sigma) and FSH (50 ng/ml, Merck-Serono). The second part

of each strip (VEGF$^+$) was transferred to 500 µl CmV, consisting of Cm supplemented with mVEGF (5 ng/ml, Sigma). Both VEGF$^-$ and VEGF$^+$ pieces were incubated for 24h at 38.5°C and 5% CO_2. A third part of both strips (control (CTL)) was immediately fixated as a non-transplanted control in 2% paraformaldehyde and 2% glutaraldehyde in distilled water at RT
155 for 24h, followed by an overnight incubation in PBS at RT to remove the fixative. Subsequently, the CTL pieces were embedded in paraffin blocks and cut into 5 µm thick histological sections.

2.2 Xenotransplantation procedure

160 The next day, four BALB/c-nu mice (20.6 g ± 1.7) of 8-10 weeks of age were anesthesized intraperitoneally (IP) with xylazine (1 mg/kg; Rompun 2%, Bayer, Germany), ketamine (90-100 mg/kg; Ketamine 1000, CEVA, Belgium) and buprenorphine (0.1 mg/kg; Temgesic, Schering-Plough, USA) for analgesia. The surgical procedure for neutering and xenotransplantation took on average 35 minutes. Mice were allowed to recover on a warmed
165 plate. They were individually marked by small cuts in the ear. Epinephrine (0.5 mg/ml; Sterop, Belgium) was prepared in case a profuse bleeding should occur during surgery.

Positioning of the grafts can be briefly described as follows. A midline laparotomy was performed and both ovaries were removed to create a GnRH supporting environment for developing follicles. Subsequently, a pouch was created between the m. obliquus abdominus
170 internus and the peritoneum on both sides of the abdominal incision at the linea alba. In the pouch on the right-hand side, a cortical graft incubated in CmV was transplanted (VEGF$^+$), whereas on the left-hand side of the incision, a non-treated piece of cortex (Cm) was grafted (VEGF$^-$).

175 *2.3 Graft retrieval and histological processing of the tissue*

Two, respectively four weeks after xenotransplantation, the mice were euthanized by cervical dislocation. Both grafts were collected from each mouse (Figure 2) and fixated in 2% (w/v) paraformaldehyde and 2% (w/v) glutaraldehyde in distilled water at RT for 24h, followed by an overnight incubation in PBS to remove the fixative. Subsequently, the grafts
180 were embedded in paraffin and cut in 5 μm thick histological sections.

2.4 Immunohistochemistry

After rehydration, the sections were treated differently for activated caspase-3 or von Willebrand Factor (vWF). Because staining protocols are very similar, they are jointly
185 described.

Tissue sections were incubated in 0.01 M citrate buffer (pH 6) and heated in the microwave (20 min, 90W, 96°C) to retrieve antigenicity for the activated caspase-3 staining and cooled down for 20 min (RT). For the vWF staining, sections were incubated in 0.05% trypsin at 37°C for 30 min to retrieve antigenicity and were allowed to cool down for 10 min
190 (RT). Sections were rinsed two times for 5 min with 0.05 M Tris-buffered saline (TBS) (pH 7.4), afterwards endogenous peroxidase activity was depleted by incubating the sections in 3% (v/v) H_2O_2 in TBS (10 min, RT). Non-specific staining was blocked with normal goat serum (NGS) (1/4, Dako, Glostrup, Denmark) diluted in TBS, enriched with 0.3% Triton-X 100 (v/v) (Tx - Sigma) and 1% (w/v) BSA (Sigma) for 30 min at RT. Subsequently, sections
195 were incubated overnight (RT) with activated caspase-3 (Sigma) diluted in TBS, enriched with 0.3% (v/v) Tx and 5% (w/v) BSA (1/225). For the vWF staining (Dako – cross-reacting for bovine and murine vWF), sections were incubated overnight (4°C), diluted in the same buffer as NGS (1/250). The next day, following two wash steps of 5 min each with Dako Wash Buffer, the sections were incubated for 30 min at RT with ant-rabbit Envision (Dako).
200 Following another two wash steps for 5 min each with TBS and one step with distilled water,

positive reactions were determined by incubating the sections for 10 min (RT) with the chromogen 3-amino-9-ethylcarbazole (Dako). The sections were counterstained with Carazzi's haematoxilin (Klinipath, Olen, Belgium), rinsed and mounted with Faramount Medium (Dako).

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2.5 Analysis of the immunohistochemistry

The grafts were analyzed using systematically randomly retained (i.e. every seventh section after slicing the tissue block at a random start position) sections that were processed for immunohistochemistry. For the analyses of the vWF and activated caspase-3 stained tissue

210 sections, an Olympus BX50 microscope connected to a computer running the software program CAST 2 (Olympus Belgium) was used. One single investigator performed the analyses blinded to treatment ($VEGF^+$, $VEGF^-$ or CTL) and transplantation period (2 or 4 weeks). If 70% of the surrounding granulosa cells were defined as 'activated caspase-3-immunoreactive (IR)' (stained red), the entire follicle was considered 'dead'. The number of

215 'dead' follicles versus the total number of follicles was counted and their ratio was calculated (Figure 3). No valid recordings from the $VEGF^+$ treatment were obtained in OV5, therefore data from this ovary are excluded for all further analyses. Ovaria numbers 6 to 8 were renumbered into ovaria 5 to 7.

2.5.1 Qualitative analysis – Activated Caspase-3

220 Within each graft, several sections were analyzed microscopically and the number of activated caspase-3-immunoreactive (IR) ('dead') and activated caspase-3-immunonegative (live) follicles were counted (Figure 3). To assess the total number of activated caspase-3-IR and caspase-3-immunonegative follicles in one graft, the numbers counted in separate sections were summarized.

225 *2.5.2 Quantitative analysis - vWF - stereology*

The surface density of the vasculature was estimated by using a cycloid grid at a magnification of 20x (Figure 3). The surface densities (S_v) were calculated using the following stereological equation (Howard and Reed; Mandelbaum et al., 1998):

$$S_v = 2.\Sigma I / (l_{(p)}.\Sigma P)$$

230 wherein ΣP refers to the number of points hitting the reference space (i.c. the ovarian tissue), ΣI is the number of intersections between the vWF-IR endothelial cells and the cycloids, and $l_{(p)}$ stands for the test line length associated with a test point.

To approximate a coefficient of error (CE) of the ratio estimate of the surface density of blood vessels in the transplant, the following equation can be used (Borini et al., 2004):

235 $$CE = [(k/k\text{-}1).((\Sigma I^2/\Sigma I\Sigma I) + (\Sigma P^2/\Sigma P\Sigma P) - (2 . \Sigma IP/\Sigma I\Sigma P))]^{1/2}$$

wherein k is the number of sample fields, I are the intersections and P stands for the points that were counted. The number of sample fields and the density of the stereologic grid were adjusted to result in a CE smaller than 5%.

However, when calculating the volume of an organ, shrinkage of the organ due to 240 tissue preparation has to be taken into account. By measuring the circumference (µm) of the transverse tissue blocks before and after paraffin embedding, tissue shrinkage was estimated to be 34.5%. We used this coefficient to correct the data, assuming that shrinkage of the ovarian tissue is homogenous in all directions (circumferentially, axially and radially).

245 *2.6 Statistical analysis*

All statistical analyses were performed in the software package R, version 2.13.1 (http://www.r-project.org). Linear mixed models were fitted using the add-on package lme4. Posthoc testing was performed using the package multcomp. Significance level was set at $P < 0.05$.

250 *2.6.1 Qualitative data*

The effect of VEGF treatment on the survival of PAF in pieces of ovarian cortex before transplantation and the effect of transplantation on the survival of the follicles were analyzed by fitting a generalized linear mixed regression model with treatment and transplantation period as fixed effects, and the percentage survival deducted from the number
255 of activated caspase-3-IR ('dead') and activated caspase-3-immunonegative ('live') follicles, as outcome variable. Because there is a considerable variability and thus dependence between grafts retrieved from the same ovary and the same fetus, this needed to be accounted for by including random effects for fetus and ovary, nested within a specific fetus. To determine if the effect of VEGF on the percentage of surviving follicles in the graft was dependent on the
260 transplantation period, an interaction between treatment and graft duration was added to the model and tested for significance using a likelihood ratio test. The interaction term was significant ($P < 0.05$), which means that the treatment effect depends on the transplantation period. Therefore, separate models were fitted for 2w and 4w incubation time, and separate effect sizes were estimated.

265 *2.6.2 Quantitative data*

For the analysis of the vWF data, all initial values were corrected for the shrinkage due to tissue processing. That is, all initial values were multiplied by a factor 1.345 and the effect sizes were calculated on these corrected values. The effect of the VEGF treatment on vascularization was modeled using a linear mixed model. The logarithm of the vascularization
270 surface density ($/mm^2$) was used as outcome variable. The dependence between the measurements from the same ovary was accounted for by including a random intercept term for ovary and a random slope for VEGF treatment. The fixed effects included treatment ($CTL/VEGF^-/VEGF^+$), graft duration time (2w/4w) and their interaction. Since the interaction between treatment and time was significant ($P = 4.3 \times 10^{-6}$), separate models were fitted for
275 2w and 4w of incubation. Posthoc testing with a Tukey correction was performed to

determine a difference in outcome between the CTL/VEGF^{-}/VEGF^{+}. All effect sizes obtained through the linear mixed model were back-transformed to the linear scale. Geometric means for each treatment group were obtained by exponentiation of the predicted group means from the linear mixed model. The pairwise differences between the treatment groups and their 95%
280 confidence intervals were obtained by back-transforming the predicted differences in mean outcome (and the boundaries of the 95% confidence interval) from the linear mixed model.

3. Results

3.1 The effect of VEGF on the survival percentage

285 All analyses were performed blinded to treatment and incubation period. While the entire follicle was considered 'dead' if 70% of the surrounding granulosa cells were defined as activated caspase-3-IR, the percentage surviving was determined by counting the number of 'dead' follicles relative to the total number of follicles present (Figures 4 and 5).

Two weeks after transplantation, the percentage surviving follicles differed 290 significantly between the $VEGF^-$ biopsies and the $VEGF^+$ grafts ($P < 0.05$). All but one (OV5) have consistently higher percentages of surviving follicles in VEGF+ grafts compared to $VEGF^-$. On average, in $VEGF^-$ grafts approximately 17% of the follicles were considered 'alive', whereas in the $VEGF^+$ grafts, this was only the case in 31% of the follicles. Four weeks after transplantation, there were no significant differences between $VEGF^-$ and $VEGF^+$ 295 grafts for the percentage of surviving follicles ($P = 0.76$). On average, both treatments result in 22% 'survived' follicles. The odds ratios and 95% confidence intervals are given in Table 1.

3.2 The effect of transplantation on the percentage of surviving follicles

300 The results are displayed in Figure 6. To determine the effect of transplantation, we combined the $VEGF^-$ and $VEGF^+$ groups (VEGF+/-) and compared the percentage survival follicles to the percentage in the CTL (non-transplanted) group for both 2 and 4 weeks transplantation period. A generalized linear model analysis showed that the interaction between incubation time and treatment group was significant ($P = 3.7 \times 10^{-7}$), which means 305 that the difference between CTL and VEGF+/- groups was not the same following 2 or 4 weeks of transplantation. Subsequently, separate models were fitted for both transplantation periods. At 2 weeks, the CTL group counted on average 49.4% surviving follicles, whereas in

the VEGF+/- group 22% of the follicles were considered ‘alive’. At 4 weeks incubation, these percentages were 46% and 22% respectively. The odds ratios and 95% confidence intervals are given in Table 1.

3.3 The influence of VEGF-treatment and incubation time on graft vascularization

To compare surface density of vascularization over treatments, sections were incubated with vWF and evaluated blinded to treatment and incubation period. A significant interaction between treatment and time implies that the difference in density between the 3 treatments (VEGF$^+$, VEGF$^-$ and CTL) is not the same at 2w and 4w. Separate models were fitted, indicating that the effect of treatment was significant (2w: $P = 0.007$ and 4w: $P = 0.0086$), and the vascular density is not the same across treatments. Posthoc testing showed that both at 2w and 4w, the surface density (/mm^2) of the blood vessels from the CTL groups was significantly higher compared to the VEGF$^+$ and the VEGF$^-$ grafts, with a larger difference after 4w transplantation (Figure 7). At 2w, the surface density in the VEGF$^-$ graft was on average a factor 0.4 lower compared to the CTL ($P < 0.05$), while this was 0.38 for VEGF$^+$ grafts ($P < 0.05$). At 4w, the surface density in the VEGF$^-$ grafts was on average 0.28 times lower compared to the CTL ($P < 0.05$) and 0.33 times lower for VEGF$^+$ grafts ($P < 0.05$). But no significant differences were observed when the VEGF$^+$ grafts were compared to the VEGF$^-$ after 2w ($P = 0.85$), nor after 4w ($P = 0.38$) of transplantation. The 95% confidence intervals are shown in Table 2.

330 **4. Discussion**

As described above, we have used a new xenografting technique transplanting bovine fetal ovarian tissue to a retroperitoneal location, as a model to study human fertility preservation strategies. Immune histochemical staining for activated caspase-3 and vWF followed by morphometry confirms that transplantation of ovarian cortical tissue impairs
335 follicular survival irrespective of transplantation period or VEGF treatment. A 24h incubation with VEGF prior to transplantation increases the short-term survival rate. However, the surface density of the graft vasculature did not change irrespective of the transplantation period.

The bovine-murine xenotransplantation model combines the 'best of both worlds' for
340 studies on (human) reproductive physiology. Transfer of bovine ovarian cortex tissue as a relevant model for human (pre-implantation) reproductive research, in an (immune deficient) mouse as a highly convenient *in vivo* bio-incubator, shows to be a manageable model under laboratory conditions. Due to a lack of standardized, non-invasive quantitative assessment methods, follicle density in the grafts could not be assessed prior to processing or incubation.
345 However, literature describes an abundance of PAFs in fetal ovaries (Markstrom et al., 2002; Aerts and Bols, 2010b) and a random distribution of clustered PAFs in adult ovaries (Schmidt et al., 2003; Araujo et al., 2014). Taken into account that approximately 50% of the follicles present will undergo apoptosis following transplantation (Aerts et al., 2008), the large stock of PAFs in fetal ovaries will ensure that a sufficient number remains present to asses following
350 xenografting. Because blood vessels and the peritoneum are both of mesodermic origin, angiogenic properties are assigned to the peritoneum (Nisolle et al., 2000; Bonvini et al., 2012). All mice were neutered before transplantation. Thus, high endogenic levels of gonadotrophine releasing hormone (GnRH) were installed by removing estrogen mediated negative feedback through the hypothalamic-pituitary axis (Markstrom et al., 2002;

355 Hernandez-Fonseca et al., 2005; Aerts and Bols, 2010b), which are considered to be favorable for PAF development. We chose to use BALB-c/Nu SCID mice as we aimed to establish a standardized, easy-to-use method and because a comparable situation occurs when cryopreserved ovarian tissue is transplanted to the original donor where no tissue rejection is to be expected. Even though the quality of the oocytes of rescued follicles remains to be 360 determined (Mermillod et al., 2008; Zuccotti et al., 2011), these results confirm that transplantation of ovarian cortical tissue impairs follicular survival. However, when a short-term transplantation period (2w) is considered and the ovarian graft is previously exposed to VEGF, this damage can be reduced by 14% (69% versus 83% ‘dead’ follicles). If a longer transplantation period (4w) is considered, differences are flattened out and 78% of the 365 follicles can be considered ‘dead’ in both treatments.

While other staining methods for apoptosis are described in literature (Santos et al., 2013), we specifically wanted to assess only the earliest stages of apoptosis, visualized by activated caspase-3 (Duan et al., 2003). Comparable to previous results (Aerts et al., 2008), we report a high rate of ‘dead’ follicles in the control tissue. Already between 50.6% (control 370 tissue of 2 weeks) and 54% (control tissue of 4 weeks) of the follicles embedded in the cortex tissue underwent apoptosis, irrespective of the incubation and transplantation process. This high follicle mortality rate can partly be explained by naturally occurring selection mechanisms decreasing the number of follicles from birth to adulthood (O'Connor et al., 1998; Jensen et al., 2006). Together with the average 78% mortality in the combined 375 VEGF+/- group, this might suggest that the net percentage of ‘dead’ follicles due to a combination of incubation and transplantation is relatively low. While it should be stressed that the proportion of ‘dead’ follicles is always compared to the internal controls (CTL, not transplanted tissue) of the same ovarian cortex strip (Figure 1), the main future challenge probably lies in the preservation and rescue of early PAFs.

380 In cancer research, anti-VEGF treatments are of interest because they counteract growth of fast growing and high-demanding tumor cells (Olsson et al., 2006; Goel and Mercurio, 2013). On the opposite, grafting experiments strive for a higher graft acceptance and vascularization in order to have a faster nutrient and oxygen supply to the transplanted tissue (Israely et al., 2003). Moreover, in ovarian physiology, the presence of VEGF and the 385 increased expression of VEGFR-2 seem to be pivotal for follicular development (Araujo et al., 2011b; Dath et al., 2011). Vascular endothelial growth factor promotes ovarian graft survival in mice and literature suggests that VEGF A, more specifically isomer 165, stimulates the development and oocyte competence of goat PAF (Araujo et al., 2011b). While Buchele et al. (2014) report a donor-derived neo-angiogenesis, previous studies emphasize 390 host-derived angiogenesis, indicating the importance of determining the origin of vascularization. Previous research assessed vascularization through IHC staining of PECAM-1 (Rahimi et al., 2010) or CD34 (Dath et al., 2011). Because our aim was to assess vascularization of the graft, irrespective of the species origin, we quantified the vWF positive cells, with a cross reaction for endothelial cells of murine or bovine origin. The 395 vascularization density is geometrically quantified in 3D (Gundersen et al., 1988) using geometric ‘probes’ (in this case cycloids) in the grafted ovarian cortex, a very labor-intensive, though robust and reliable tool. As could be expected, a significantly greater vasculature density is seen in the CTL tissue compared to the transplanted tissue. Comparing VEGF-treated and non-treated grafts, no significant influence could be seen on vascular 400 density, irrespective of the duration of transplantation period. This is in contradiction with the results obtained by Labied et al. (2013) who transplanted cryopreserved ovine ovarian cortex for 3 days or 3 weeks, embedded in a collagen matrix with or without VEGF. In the present study, VEGF was administered to the treated grafts via the culture medium 24h prior to transplantation. Because fetal tissue is soft and relatively easy to penetrate, we assumed that

405 VEGF would be distributed in the tissue through diffusion. Probably, the way of VEGF administration and the duration of exposure can strongly influence VEGF availability to the relevant cells.

Our results document the possibilities of a unique xenotransplantation technique to study vascularization density, through stereology, and monitor PAF apoptosis in bovine tissue 410 as a potential model for female fertility preservation strategies. The use of vascular endothelial growth factor showed a beneficial effect on short-term follicle survival rates. Unfortunately, no beneficial effects of VEGF on the vascularization of the grafted ovarian tissue in our experimental set-up could be reported.

415 **Conflict of interest declaration**

All authors state that funding of this research is provided by an independent operational fund of the University of Antwerp. There are no conflicts of interest, financially nor personal.

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600 **Table and figure legends**

Table 1: Odds ratios (OR) and 95% confidence intervals (95% CI) of the percentages ± standard error (SE) of 'survived' follicles in the retrieved grafts.

605 Table 2: Effect of VEGF treatment and incubation time on graft vascularization.

Figure 1: Experimental setup. (VEGF: Vascular endothelial growth factor; CTL: control tissue; Cm: Culture medium; CmV: Culture medium supplemented with VEGF; 2w: two weeks; 4w: four weeks)

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Figure 2: Overall view of the bovine/murine xenotransplantation model. Transfer of bovine ovarian cortex at a retroperitoneal location in a (immune deficient) mouse. Macroscopical graft aspect after a transplantation period of 2 weeks (A) and 4 weeks (B), with indications of neo-vascularization.

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Figure 3: A and B: Bovine fetal ovarian tissue immunohistochemically stained for activated caspase-3. A: Immunonegative secondary preantral follicle in grafted tissue (dotted circle) (200x). B: Overview of the bovine graft in the murine abdominal muscles (200x). Activated caspase-3-immunoreactive follicles (arrowheads) and immunonegative follicles (arrow). C:
620 The Software program CAST 2, displaying a number of cycloids (green curves). On this image section, follicles (dotted circles) and positive staining for vWF can be identified (red dots). Where the positive staining for vWF crosses a cycloid (yellow arrowheads), one 'count' is registered. D (200x) and E (400x): Bovine fetal ovarian tissue immunohistochemically

stained for von Willebrand Factor (vWF) (red dots). D: Murine abdominal muscles (arrow)

625 and early preantral follicles in grafted tissue (arrowheads).

Figure 4: Grafts treated with VEGF prior to transplantation procedure are marked as VEGF+. Grafts not treated with VEGF, are indicated as VEGF-. For each of the ovaria (OV), and for 2w incubation time, the percentage of surviving cells on each slide was plotted for the VEGF-

630 and VEGF+ grafts (X-axis). One observation corresponds to the percentage of surviving cells (Y-axis) in 1 slice. The mean percentage and its 95% confidence interval are shown by a red dot. (* $P < 0.05$; ** $0.01 < P < 0.001$; *** $P < 0.001$)

Figure 5: Grafts treated with VEGF prior to transplantation procedure are marked as VEGF+.

635 Grafts not treated with VEGF, are indicated as VEGF-. For each of the ovaria (OV), and for 4w incubation time, the percentage of surviving cells on each slide was plotted for the VEGF- and VEGF+ grafts (X-axis). One observation corresponds to the percentage of surviving cells (Y-axis) in 1 slice. The mean percentage and its 95% confidence interval are shown by a red dot. (* $P < 0.05$; ** $0.01 < P < 0.001$; *** $P < 0.001$)

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Figure 6: Scatterplot of the percentage survival (Y-axis) versus treatment (X-axis); depicted for 2w and 4w respectively. Both transplanted groups (VEGF+ and VEGF-) are summarized (VEGF+/-) against the non-transplanted control biopsies (CTL). (OV=ovarium)

645 Figure 7: The mean vascular density for the ovaria (OV) (for each treatment and time) are shown. Each time, a pairwise comparison is made between the treatments. In the scatterplot, each dot corresponds to the mean density of one OV. The red dot corresponds to the overall mean density (with 95% confidence interval) over all the OV.