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GYNECOLOGIC ENDOCRINOLOGY AND REPRODUCTIVE MEDICINE

In vitro growth (IVG) of human ovarian follicles in frozen thawed ovarian cortex tissue culture supplemented with follicular fluid under hypoxic conditions

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Abstract

Background Despite its clinical success rates, transplantation after ovarian tissue cryopreservation (OTC) remains a matter of concern. Certain cancer subtypes may lead to the transfer of malignant cells when transplantation of affected ovarian tissue is conducted. IVG and subsequent isolation of vital follicles obtained from frozen thawed ovarian tissue for further in vitro maturation (IVM) would expand current fertility protection techniques while reducing the risk of retransplanting malignant cells.

Methods A total of 216 cortical biopsies from 3 patients were included in this study in 4 treatment groups. After freezing, thawing and 8 days of hypoxic tissue culture supplemented with different concentrations of human follicular fluid (HuFF) and follicle-stimulating hormone (FSH), follicles were isolated enzymatically and stained with calcein to determine follicular viability. Numbers and size of vital follicles were assessed by fluorescence microscopy (Ti2, Nikon) and specified by computer assisted, semi-automated measurement (NIS software, Nikon). To estimate the effect of in vitro culture on apoptosis, tissue sections were stained for nicked DNA (TUNEL) prior and after tissue culture.

Results Analysing 3025 vital follicles, we observed significant differences [$P < 0.01$] regarding follicle size when hypoxic tissue culture was supplemented with HuFF compared with the control group on day 1, individual follicles reached sizes > 100 µm.

Conclusions The results implicate that HuFF contains valuable factors contributing to significant IVG of follicles in human ovarian tissue and could be regarded as an additional tool in personalized fertility restoration prior to retransplantation of ovarian tissue.

Keywords Follicle · Ovarian tissue · Reproduction

What does this study add to the clinical work

This culture approach could facilitate a higher follicular yield while isolating ovarian follicles from unstimulated ovarian tissue bears the risk of obtaining only a small proportion of follicles suited for further single follicle culture.

Introduction

In reproductive medicine, the area of fertility preservation has seen a rapid evolvement of techniques in the last 20 years due to the fact, that cancer treatment influences negatively on female and male fertility [1]. Seeing an increase in the incidence of cancer and rising numbers of

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cancer survivors [2], high attention has been paid on the relevance of gonadotoxic potential and diverse side effects of treatments of different cancer types [3, 4]. In general, female cancer patients requiring fertility preservation have the option of oocyte cryopreservation and OTC for fertility preservation [5]. When first-line chemotherapy cannot be rescheduled, OTC is the only possibility for fertility protection in children and prepubertal girls [6–8]. Previous studies have reported pregnancies and live births after transplantation of cryopreserved and thawed ovarian tissue, proofing that OTC is a safe alternative method of fertility preservation [9–17].

However, despite all its success, transplantation after OTC remains a matter of concern as ovarian tissue from certain cancer patients may contain cancer cells and transplantation of ovarian tissue could lead to the recurrence of cancer [18–26].

IVG of preantral follicles obtained from OTC would be a great advantage for female patients with cryopreserved ovarian tissue after cancer treatment and could reduce the risk of transplantation of malignant cells significantly [27–32]. It could be demonstrated that after isolation of follicles, separation from cancer cells can be obtained by the application of established laboratory protocols [33–35]. Promising results in mice show that IVG of follicles is a feasible way [36–39], while in humans, limited progress in IVG of ovarian tissue derived follicles may be due to tissue specific structural differences [40–44] and the limited access of researchers to cryopreserved human ovarian tissue [45]. The group of Telfer et al. showed that in vitro maturation from human unilaminar follicles grown in a multi-step culture system to Metaphase II oocytes is possible, but also implicating that further research is needed to focus on the optimization of a multi-step culture strategy [46], to obtain epigenetically normal oocytes [47, 48]. Proteomic based research approaches on HuFF identified over 800 components [49–62] contributing to development and maturation potential of the follicle and the oocyte [63–65], including hormones [66–68], growth factors and cytokines [69–73]. It has been shown by Molaeeghaleh et al. that cultivating mouse follicles supplemented with follicular fluid contributed to oocyte maturation and follicle growth [74]. Beside the composition of follicular fluid, it is necessary to highlight the oxygen distribution of the female reproductive tract that is considered mainly hypoxic, below atmospheric composition [75, 76]. Ovarian follicle environment is considered low oxygenic [77–79], the ovarian environment is regarded mainly as avascular [80]. Oocyte development under atmospheric oxygen composition has a harmful impact on maturation and development potential [81], and it could be demonstrated that follicle culture under hypoxic conditions contributes significantly to the follicular development potential and viability in comparison to atmospheric oxygen conditions [82].

In this prospective study, we analysed in vitro grown human follicles derived from frozen thawed ovarian cortex tissue after an 8 day period of tissue culture supplemented with human follicular fluid (HuFF) and FSH under hypoxic conditions. The experimental design of the study is indicated in Fig. 1.

Methods

Ethics

This study was approved by the ethics committees of the University Hospital of Bonn, Germany (007/09). Patients gave written, informed consent.

Collection of ovarian tissue

Ovarian tissue was obtained from 3 female cancer patients prior to gonadotoxic treatment via laparoscopy. Patients donated their tissue for research purposes and were at time of cryopreservation aged 26 (diagnosed with osteosarcoma), 22 (diagnosed with astrocytoma) and 19 (diagnosed with acute lymphocytic leukaemia [ALL]) years old.

Preparation of ovarian tissue

Cortex strips were cryopreserved and thawed according to established protocols [83–88] with minor modifications. Procedures were carried out under the European Union 2004a Directive 2004/23/EC providing maximum safety and quality of tissues [89, 90], including GMP (good manufacturing practice) procedures using certified materials and equipment under permanent hygienical and technical surveillance including particle and air germ measurement regarding room and sterile cabinet validation.

Cryopreservation of ovarian tissue

In brief, cortex strips were equilibrated in a precooled cryopreservation solution consisting of L-15 Leibovitz's medium (Life technologies, NY, USA) supplemented with 10% CryoSure DMSO (WAK Chemie, Steinbach, Germany), 11% human serum albumin (HSA) (Irvine Scientific, Santa Ana, CA, USA) and incubated for 35 min before slow freezing. Samples were stored in vapour phase of liquid nitrogen at −160 °C in automatically refilled storage tanks (MVE HEco Chart, Ball Ground, USA), monitored by an independent high end alarm system (Planer limited, Middlesex, GB).

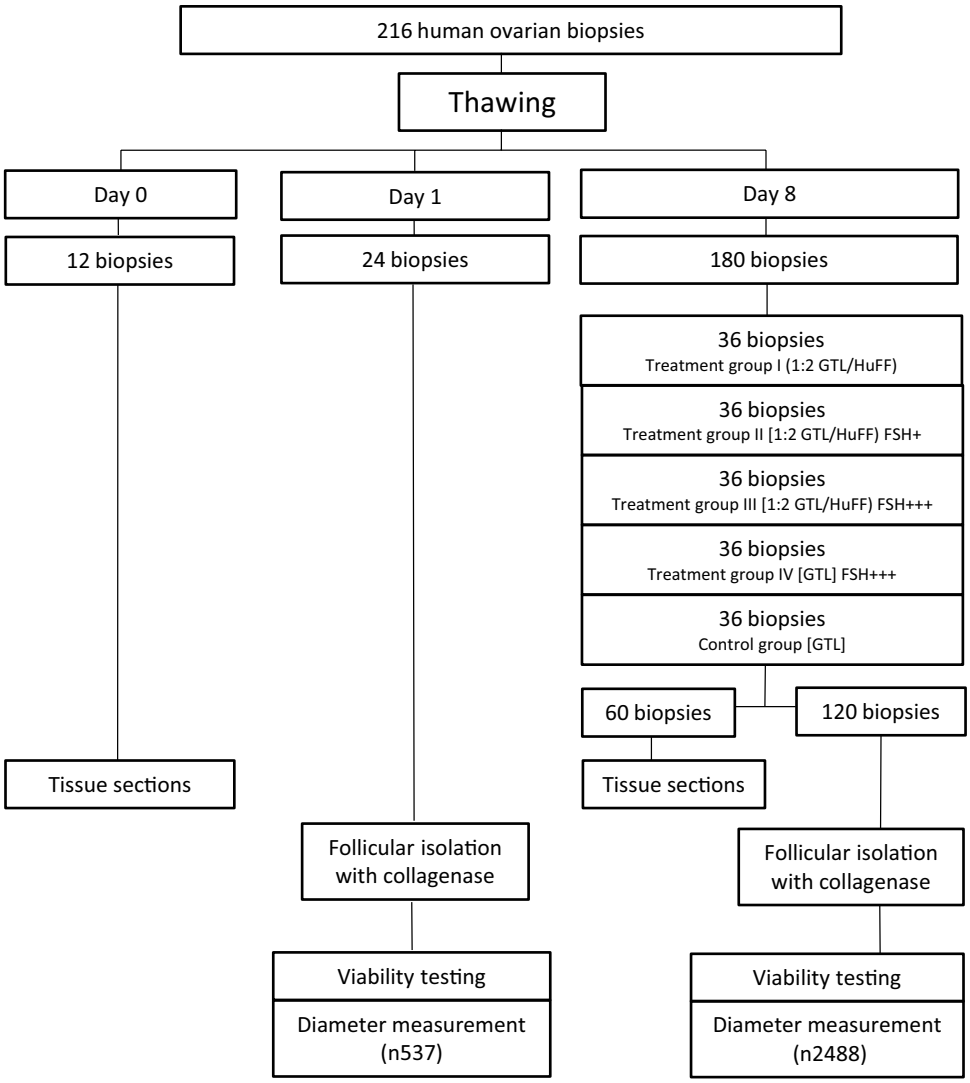

Fig. 1 Experimental design of the study

Thawing of ovarian tissue

In brief, slow frozen ovarian tissue pieces were exposed to room temperature for 40 s and submerged in a 37.5 °C pre-heated water bath for 130 s. Ovarian cortex pieces were subsequently transferred in 15 min. intervals to 3 thawing solutions consisting of DPBS CTS (Life technologies, NY, USA) supplemented with 11% human serum albumin (HSA) (Irvine Scientific, Santa Ana, CA, USA) with decreasing sucrose (Merck, Darmstadt, Germany) concentrations followed by 2 washing steps.

Collection and processing of HuFF

HuFF was collected and pooled from 3 pre-selected fertile patients aged 25–35 years with regular cycles without gynaecological abnormalities like endometriosis or polycystic ovarian syndrome (PCO) during ovum pick up (OPU) and processed under GMP conditions to produce a batch with constant quality in composition. After sterile filtration (Acrodisc Syringe Filter, Pall, Fribourg, Switzerland) and supplementation with 50 I.U/ml penicillin and 50 µg/ml streptomycin (Merck, Darmstadt, Germany), samples were stored in liquid nitrogen [91, 92]. Concentrations of LH (luteinizing hormone), FSH (follicle-stimulating hormone), E2 (Estradiol), Prog (Progesterone), TST (Testosterone), Dehydroepiandrosterone (DHEAS) and Anti-Müllerian hormone (AMH) were determined via ELISA after processing, freezing and thawing.

Tissue culture medium

Bicarbonate buffered, hyaluron and human serum albumin supplemented cell culture media (GTL, Vitrolife, Göteborg, Sweden) was used as a main carrier capable of maintaining a stable pH range between 7.2 and 7.4, even when supplemented 1:2 with frozen thawed HuFF. Prior to tissue culture, media were supplemented with ITS-G (Gibco, New York,

USA) containing insulin (1.0 g/l), transferrin (0.55 g/l) and selenium (0.00067 g/l). Performing preliminary pH measurements in 2 day intervals over a period of 8 days, tissue culture media was incubated at 37.1 °C, 5% O_2 and 6.8% CO_2. Calibrator solutions (WTW, Weilheim, Germany) were incubated at 37.1 °C as well. PH measurements were performed after 2-point calibration with inolab7110 (WTW, Weilheim, Germany) using a sentix 81 pH probe (WTW, Weilheim, Germany) on day 2, 4, 6 and 8.

Ovarian tissue culture

Thawed ovarian tissue was processed using 2 mm biopsy punches (pfm medical, Köln, Germany) to obtain 72 biopsy punches per patient. We defined 4 treatment groups and one control group per patient with different culture compositions for 8 days of tissue culture. Treatment group I (1:2 GTL/HuFF), Treatment group II (1:2 GTL/HuFF, FSH+) supplemented with 2.5 mIU/ml of recombinant FSH (Gonal-F® 200 IU, Merck-Serono, Darmstadt, Germany), Treatment group III (1:2 GTL/HuFF, FSH+++) supplemented with 15 mIU/ml of recombinant FSH (Gonal-F® 200 IU, Merck-Serono, Darmstadt, Germany), Treatment group IV [GTL, FSH+++] supplemented with 15 mIU/ml of recombinant FSH (Gonal-F® 200 IU, Merck-Serono, Darmstadt, Germany), and a control group [GTL only]. Groups of 4 punches were cultivated in 300 µl tissue culture media under oil [GM501 Mineral Oil, Gynemed, Lensahn, Germany] at 37.1 °C and 6.8% CO_2 respectively, to maintain a stable pH between 7.2 and 7.4 under hypoxic conditions [5% O_2]. 12 biopsy samples per patient and group were cultured, 8/12 samples per group were processed for vitality testing and size measurement, 4/12 samples per group were used for tissue sections and staining. For tissue culture incubation, G185 flatbed incubators [K-Systems, Cooper surgical, Berlin, Germany] were used. On day 2, 100 µl of medium was removed and 200 µl of pre-gassed medium was added, increasing the droplet volume to 400 µl. On day 4 and 6, 200 µl of the media and 50% of the cell culture oil was exchanged. After thawing, day 1 control was kept in AIMV (Thermo Fisher scientific, NY, USA) medium for 24 h prior enzymatic digestion and calcein staining.

Viability test and size measurement

On day 1 and day 8 of tissue culture, follicular viability was determined. In brief, tissue was incubated with 1 mg/ml Collagenase Type 1A (Merck, Darmstadt, Germany) supplemented with 2 µmol/l Calcein AM (Merck, Darmstadt, Germany) at 37.1 °C for 80 min shielded from light while promoting tissue resolution through cautious resuspension after 50 and 60 min. After 80 min, 120 overlapping pictures were taken via a preprogramed motorized microscope platform scanning pattern and processed for later follicle size calculation via NIS Elements software (Nikon, Düsseldorf) to determine follicle number and size of vital follicles with fluorescence microscopy (Nikon, Ti2, Düsseldorf). Vital follicles were defined as oocytes enclosed by a layer of granulosa cells (cubic or flat) with evenly distributed bright green colour. Vital cells convert the nonfluorescent calcein AM to fluorescent calcein induced by intracellular esterases after acetoxymethyl ester hydrolysis resulting in emission of green fluorescence at a wavelength of 415 nm when exposed to light of a wavelength of 495 nm [93]. Biopsy digest and viability determination procedure was carried out by two ovarian tissue experienced biologists to minimize bias through potential false identification or prolonged enzymatic exposition. Vital follicles were classified in size groups according to Kristensen et al. with minor modifications [94]. In brief, follicles were ranked in groups of >150 µm, >100–150 µm, 50–100 µm and <50 µm in relation to culture duration and media composition.

Tissue fixation and sectioning

To complement the viability tests and size measurements, tissue sections were conducted. Biopsies were fixed in 3.7% Formalin (Carl Roth; Germany) in PBS (PAN-Biotech; Germany) at 4 °C overnight. After incubation in 15% and 30% saccharin (Merck; Germany) in PBS for 10 min each, biopsies were embedded in CryoGlue (SLEE; Germany) and frozen. Tissue was cut transversely in a cryotom (SLEE; Germany) at 5 µm. 2 separate sections of the same block containing 4 biopsies per experimental group and patient were included per Hematoxylin-and eosin (H&E)-staining.

TUNEL staining

TUNEL Assay Kit was used following manufacturer's description. Tissue section demasking was performed using proteinase K., for staining, slides were incubated with Br-labelled nucleotides and TdT enzyme for 1 h at 37 °C. After washing, fluorescence labelled anti-BrdU antibody was added. Microscopic observation was performed at Ti2-E. Staining control (only anti-BrdU antibody incubation) was included. Staining intensity was assessed by NTS Elements software. Tissue was marked as region of interest. Mean intensity of TUNEL staining was measured. Value of negative staining control was set at 0.

Table 1 Determination of hormonal key factors in pooled HuFF used for tissue culture

	LH [U/l]	FSH [mIU/ml]	E2 [pg/ml]	Prog [ng/ml]	TST [ng/ml]	DHEAS [µg/ml]	AMH [ng/ml]
Value	0.7	4.8	>30,000	>600	3.63	2.89	1.01

Table 2 Follicle size of study groups with different media composition and culture duration in relation to the control group on day 1

Patients 1–3 *n* 3025	Treatment group I GTL/Huff [day 8] *n* 337	Treatment group II GTL/Huff/FSH+ [day 8] *n* 487	Treatment group III GTL/Huff/FSH+++ [day 8] *n* 641	Treatment group IV GTL/FSH+++ [day 8] *n* 680	Control group GTL [day 8] *n* 343
Control group [day 1] *n* 537	$P < 0.01$	$P < 0.01$	$P < 0.01$	P 0.095	$P < 0.01$

Mann–Whitney *U* test

Results

Determination of basic hormone levels in Huff

After batch processing of pooled Huff and one cycle of cryopreservation and thawing we detected the following concentrations of LH [0.7 IU/l], FSH [4.8 mlU/ml], E2 [> 30,000 pg/ml], Prog [> 600 ng/ml], TST [3.63 ng/ml], DHEAS [2.89 µg/ml] and AMH [1.01 ng/ml], as shown in Table 1.

Viability test and size measurements

Analysing the follicular viability on day 1 after thawing we classified 537 follicles as vital in the day 1 control group as indicated in Fig. 1. On day 8 after thawing, we rated 337 follicles in treatment group I, 487 follicles in treatment group II, 641 follicles in treatment group III, 680 follicles in treatment group IV and 343 follicles in the control group as vital.

Analysing 3025 vital follicles, our analysis showed a significant difference in vital follicle size between the control group on day 1 and treatment groups I–III on day 8 as indicated in Table 2 and Figs. 2, 3, 4, 5. In treatment groups I–III a considerable rise in numbers of vital follicles sized 50–100 µm and vitality sustainment of individual follicles sized > 100 µm were detected, results indicated in Fig. 3. In treatment group IV, vital follicle size showed a borderline significance while in the control group on day 8 the proportion of vital follicle size was significant smaller compared to the basis control on day 1. Interestingly, no vital follicles > 100 µm were detected in treatment group IV and the control group on day 8.

Tissue sections

Early follicle growth requires tissue integrity. To assess the effect of in vitro culture on apoptosis, tissue was stained for nicked DNA (TUNEL). After thawing, the tissue barely showed sign of apoptosis. After 8 days of culture, the tissue was still viable as seen in the calcein staining. However, the culture in GTL only caused a significant increase in apoptosis. The supplementation of hormones or HuFF showed different patterns in all three patients as indicated in Figs. 6 and 7. Complementary HE staining of tissue sections revealed the presence of follicles with different sizes prior and posterior tissue culture, shown in Fig. 8.

Discussion

In this study, we first describe the effect of HuFF in a tissue culture system of frozen thawed human ovarian tissue cortex, supplemented with different FSH concentrations under hypoxic conditions.

Analysing the control group on day 1, we found 537 vital follicles with an average size of 39.16 µm—this finding is in agreement with Vanacker et al., observing similar follicle diameters after performing enzymatic isolation ahead of in vitro culture of 7 days—reporting significant growth of follicles but also a substantial loss in follicle numbers [95].

As expected, the majority of vital follicles in the day 1 control group was sized < 50 µm and reached a 87.15% ratio, this also accords with the morphologic properties observed in stained tissue sections, most follicles were primordial and primary stages next to a small proportion of secondary follicles sized > 100 µm. These results are partially consistent with those of Telfer et al. classifying 90% of healthy follicles as primordial or transitory stages when analysing follicular composition prior a tissue culture period of 6 days [96].

In our study, the control group on day 8 (GTL media only) showed significantly smaller vital follicles compared to the day 1 control. As vital follicular stages sized > 100 µm were present in the day 1 control group but not in the day 8 control, these might have become atretic during the culture period. This implicates a certain role of hormones not

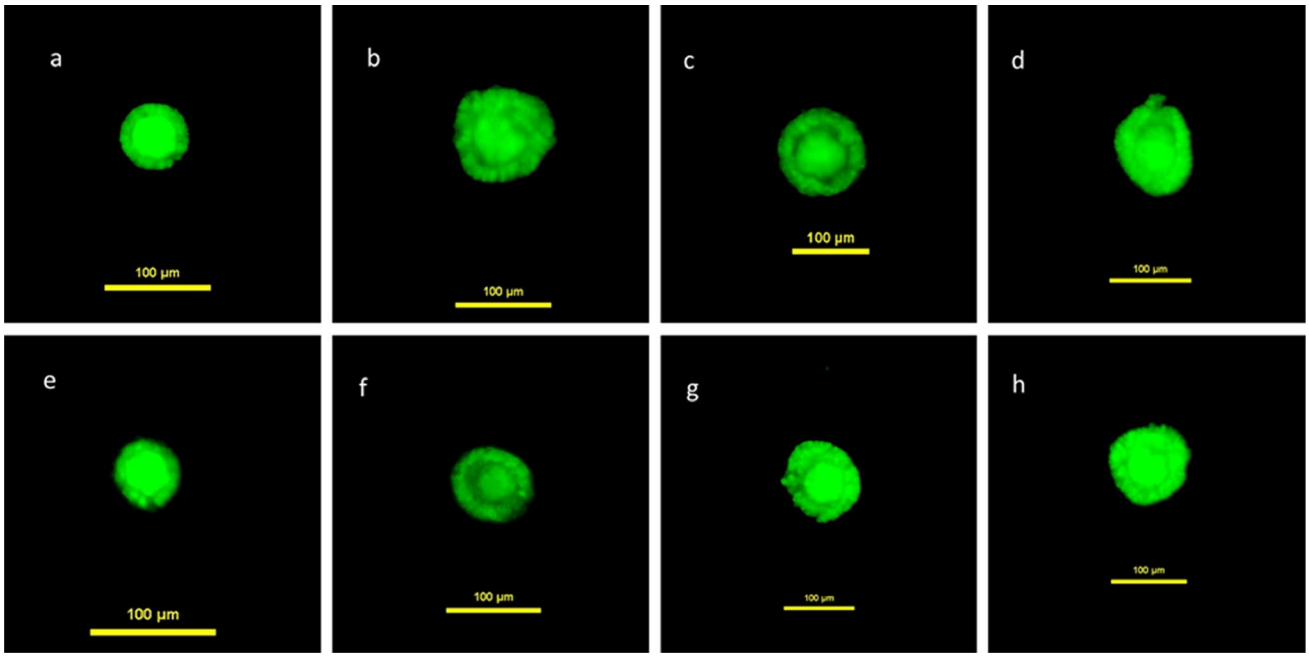

Fig. 2 Calcein staining (green fluorescence light [495 nm]) of follicles **a**–**h**. Scalebar 100 µm

only for follicular growth but also in maintaining viability of grown follicles.

Treatment group IV (GTL with FSH+++) showed a borderline significance [*P* 0.095] comparing overall vital follicle size with the basis control on day 1. We detected a proportion of follicles sized 50–100 µm of 9.41% while no vital follicles sized > 100 µm were observed. This implicates a non-optimal initiation of follicle activation and a non-sustainment of follicular viability of advanced stages under the used culture conditions. An overall lower FSH concentration and a lack of additional key factors present in HuFF, e.g. activin could have caused this difference to the HuFF-supplemented treatment groups.

Comparing the treatment groups I–III on day 8 with the control on day 1, we observed similar compositions of vital follicles sized > 100 µm but detected significant differences of overall vital follicle size in all 3 groups. On closer examination we detected a considerable increase of vital follicles sized 50–100 µm to 32.64–34.63%, compared to a 11.9% ratio on day 1.

These results partially match those observed in earlier studies of Telfer et al., cultivating ovarian tissue for a period of 6 days with flattened sheets of ovarian cortex [97], while our approach included tissue culture supplementation with HuFF under hypoxic conditions, extensive numeric follicle evaluation via follicular viability tests and large-scale size measurement prior and posterior tissue culture for 8 days.

Furthermore, standardized 2 mm cortex biopsy samples in our culture system offer several advantages: size and numbers of biopsy punches providing a substantial contribution to minimize the effect of unevenly follicle distribution in the human ovary [98–101]. Enhanced nutrition uptake of small cortex samples as in vitro engineered tissues show size dependant constraints due to limited nutrition supply [102, 103]. Additionally, a minimization of the effect of potential follicular interactions in an early or later stage of growth. An extensive appraisal of follicle evaluation increasing the information value regarding follicular size composition of cultured groups as well as conducting viability tests combined with semi-automated size measurements. Finally, 2 mm biopsy punches are well suited for processing in oil overlaid culture drops under hypoxic conditions providing stable and comparable culture conditions similar to IVF embryo culture.

The addition of HuFF (with or without FSH supplementation) did not only hinder the supposed follicular atresia of the day 8 control group, but also promoted follicle growth significantly and sustained follicular viability of larger stages sized > 100 µm of the treatment groups I–III. Given the fact that this size group reached similar proportions as the control group on day 1, it can be assumed that beside viability sustainment a potential growth inhibition of advanced stages occurred that has been described by other groups [104–106].

Results of large-scale viability and size measurements were supplemented by tissue sections, highlighting that enzymatic digestion ahead calcein staining offers a comprehensive approach while tissue sections illuminate solely a sub section of the follicular group composition and may not be representative for the entire cohort, based on that,

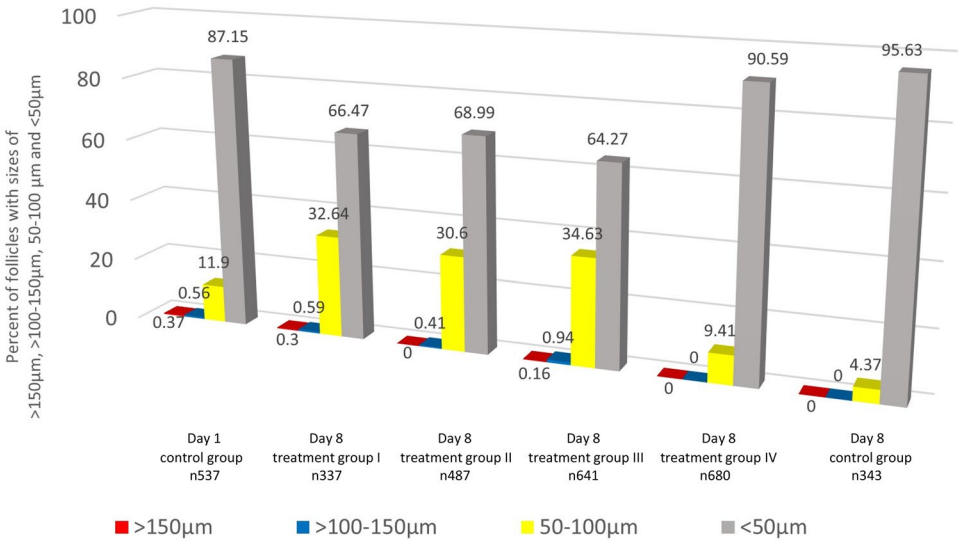

Fig. 3 Proportion of vital follicle size [%] in relation to culture duration and media composition [*n* 3025]

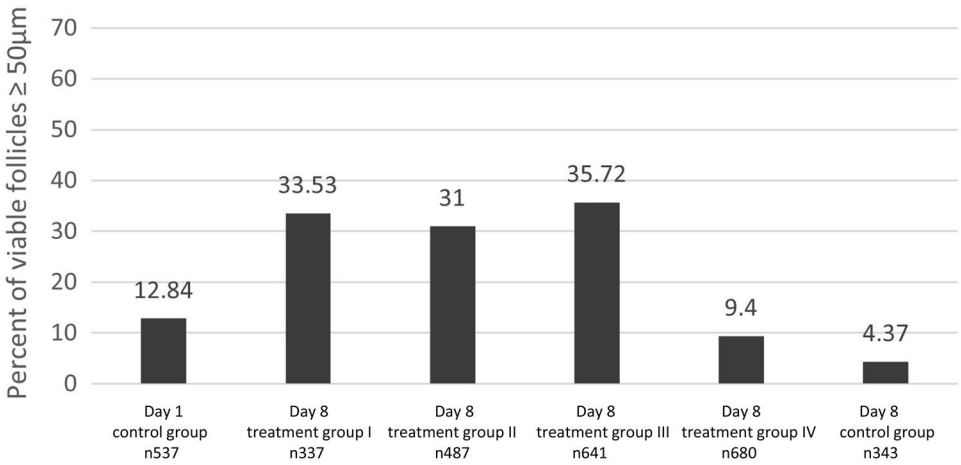

Fig. 4 Proportion of vital follicles with size of ≥ 50 µm in relation to media composition and culture duration [*n* 3025]

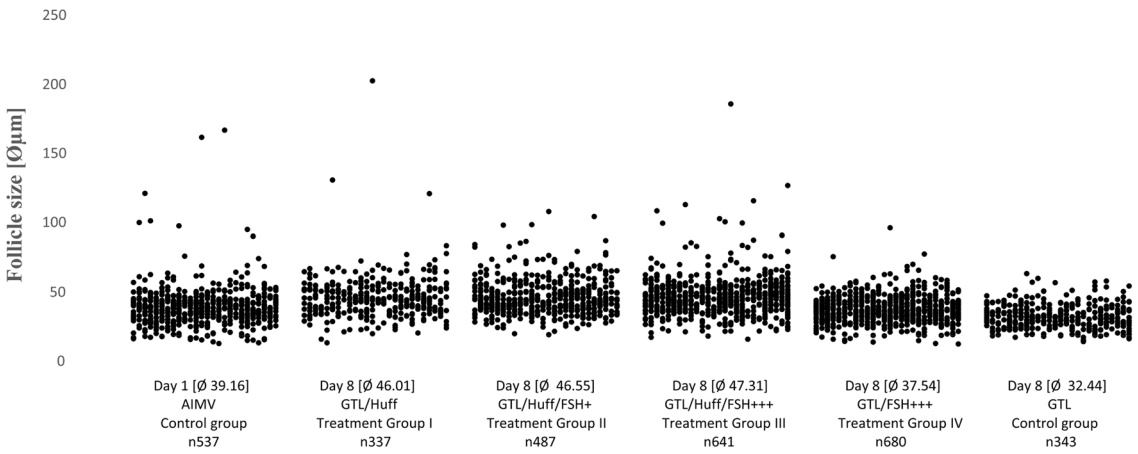

Fig. 5 Size of vital follicles in relation to culture duration and media composition [*n* 3025]

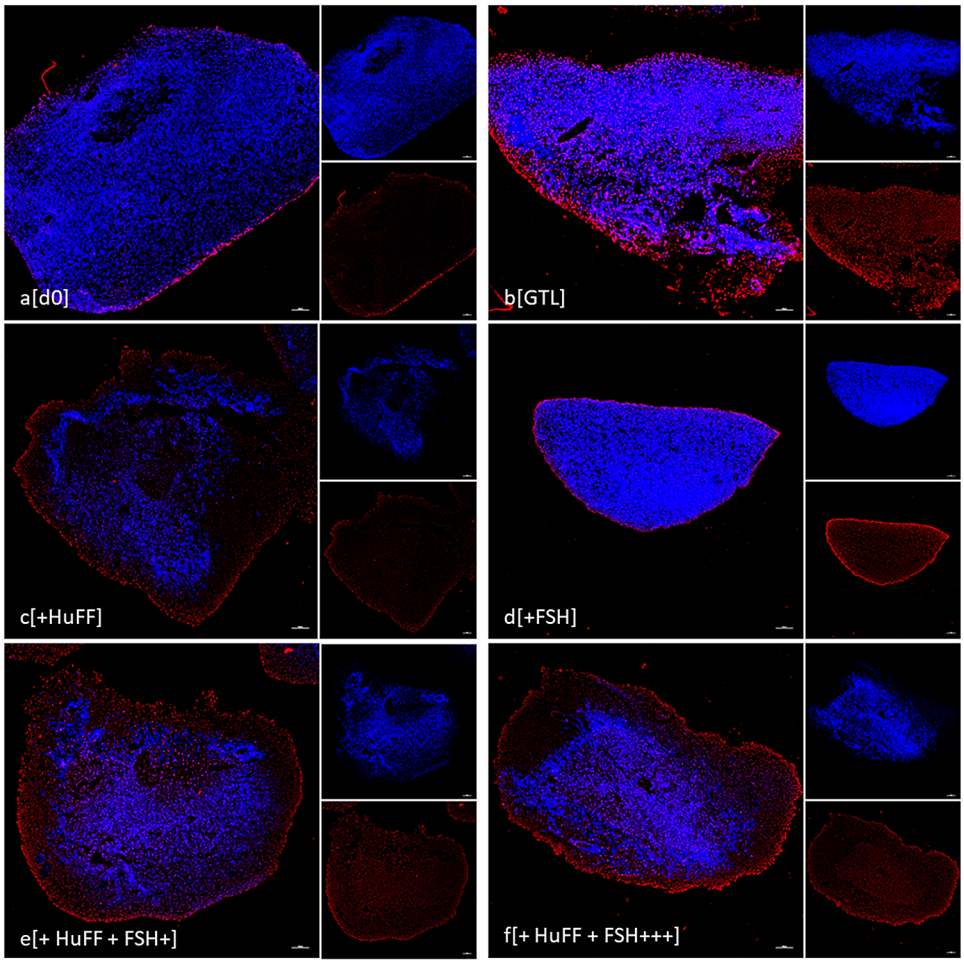

Fig. 6 **a–f** TUNEL staining of ovarian cortex biopsies. Ovarian cortical tissue was thawed and cultured with the mentioned supplements. After thawing and tissue culture, biopsies were fixed and stained for nicked DNA as a marker of apoptosis induction. Mean staining intensity of whole sections were assessed

the majority of biopsy samples were processed for viability testing and calcein staining.

Tissue sections stained for nicked DNA (TUNEL) implicated that the chosen culture conditions predominantly ensured the integrity and viability of the tissue.

As proposed by different working groups, successful IVG of ovarian tissue requires a multi-step [107–110] culture approach, involving follicle activation as a key event in the first step [111, 112]. The mechanisms of follicle activation still need to be elucidated while a combination of maintenance, inhibitory and stimulatory factors is assumed [113], among them activin [114], that is present in follicular fluid [115, 116].

In mammals, the potential of oocyte development [117, 118] is significantly influenced by the follicular environment [119]. According to De Vos et al., when maturating germinal vesicle (GV) oocytes to metaphase II cells (MII) using in vitro maturation (IVM) techniques, fertilization competence depends on the development potential gained under in vivo conditions [120], casting a spotlight on follicular tissue culture mimicking in vivo environment of the early oocyte as best as possible.

Our results demonstrate that tissue culture for 8 days prior follicle isolation offers the advantage of exploiting a high yield of the ovarian follicle reserve through promoting growth initiation while sustaining viability of larger stages simultaneously. This culture approach could facilitate a higher follicular yield while isolating ovarian follicles from unstimulated ovarian tissue bears the risk of obtaining only a small proportion of follicles suited for further single follicle culture. In summary our findings indicate a significant shift from the quiescent to the growing follicle pool while sustaining viability of advanced stages, providing a promising platform for further research in a multi-step culture approach tailored for individual ovarian tissue culture for female cancer patients. We propose that hypoxic ovarian

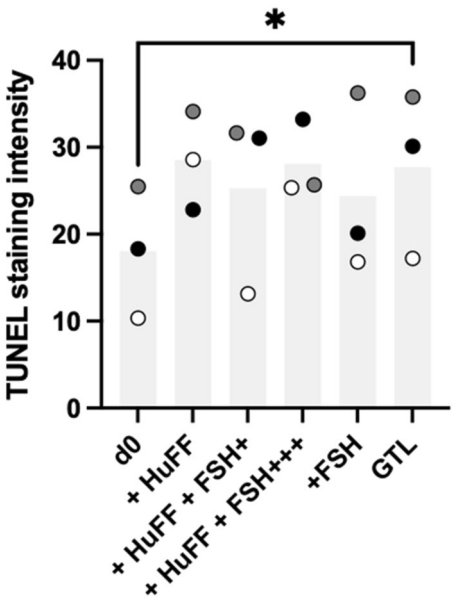

Fig. 7 TUNEL staining of ovarian cortex biopsies. Mean TUNEL staining intensity of the tissue section was assessed. Staining control value was subtracted. Each colour represents one patient. Every dot represents the mean of 2 sections of 4 distinct biopsies per patient. Representative fluorescence images from the patient marked filled dots show the TUNEL stain in red and the staining of DNA with DAPI in blue

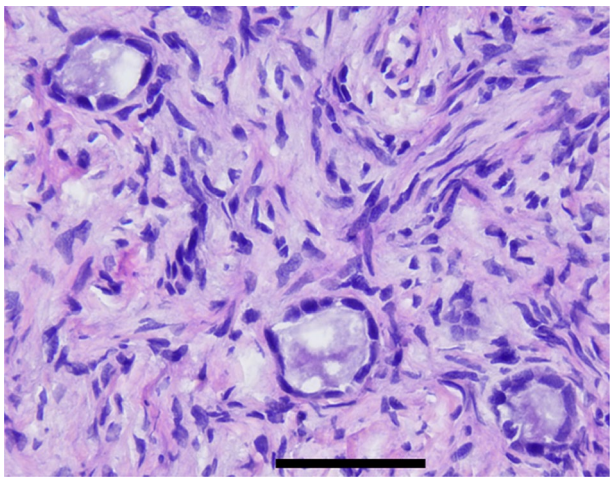

Fig. 8 Hematoxylin–Eosin (HE) staining of tissue sections. Complementary histological evaluation of tissue showing the presence of follicles with different sizes. Scalebar 50 µm

tissue culture supplemented with HuFF and FSH instead of applying single factors like activin or FSH only might benefit IVG of ovarian tissue prior single follicle culture and posterior IVM of GV oocytes as HuFF serves as a rich source of diverse oocyte-nurturing and imprinting factors mimicking in vivo conditions to a large extent.

Limitations

To maintain protein activity and function, an undamaged sustainment of the 3-D protein structure is obligatory. Batch processing of HuFF included sterile filtration that can have a negative impact on protein quality and quantity [121, 122]. To minimize further negative effects, HuFF samples were frozen in 1 ml aliquots to avoid repeated freezing thawing cycles and stored at − 196 °C in liquid nitrogen to keep protein degradation at a low level. Keeping in mind that HuFF contains more than 800 factors potentially contributing to oocyte growth and development we focussed on the determination of hormonal key factors of the processed batch.

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Author contributions AS and RE designed the study and were involved in thawing, processing of the samples, viability tests, writing and revision of the manuscript and interpretation of the results. AS obtained the approval of the ethics committee, designed and conducted the tissue culture system, HuFF collection/processing, statistical evaluation and delivered a first draft of the manuscript. RE designed and conducted TUNEL assay and tissue sections/staining. CF contributed to the design of the study, manuscript writing and revision. NS funded the study, supervised the project, contributed to the design of the study, manuscript writing, revision and final approvement.

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Data availability The data underlying this article will be shared on reasonable request to the corresponding author.

Declaration

Conflict of interest The authors declare that they have no conflict of interest.

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