Development of macaque secondary follicles exposed to neutral red prior to 3-dimensional culture

Daiane L. Bulgarelli¹ · Alison Y. Ting² · Brenda J. Gordon^{2,3} · Ana Carolina Japur de Sá Rosa-e-Silva¹ · Mary B. Zelinski^{2,4}

Received: 22 February 2017 / Accepted: 7 September 2017 / Published online: 21 September 2017
© Springer Science+Business Media, LLC 2017

Abstract

Purpose Neutral red (NR) may assist identification of preantral follicles in pieces of cortical tissue prior to cryopreservation in cancer patients requesting fertility preservation. This study is the first to analyze this effect by follicle growth rate after long-term culture in primates.

Methods Ovarian cortex was obtained from adult rhesus macaques, was cut into fragments, and was incubated with NR. Secondary follicles were readily visualized following NR staining and then were encapsulated into alginate beads and cultured individually for 4 weeks in α MEM media supplemented with 10 ng/ml FSH at 5% O₂.

Results The survival rates of secondary follicles during culture were similar between those derived from control tissue (71 $\pm$ 13%) and those treated with NR (68 $\pm$ 9%). The proportion of surviving follicles that formed an antrum were also similar in

both groups (70 $\pm$ 17% control; 48 $\pm$ 24% NR-treated). Follicle diameters were not different between control follicles (184 $\pm$ 5 μ m) and those stained with NR (181 $\pm$ 7 μ m) on the day of isolation. The percentages of surviving follicles within three cohorts based on their diameters at week 4 of culture were similar between the control group and NR-stained tissue group, fast-grow follicles (24 $\pm$ 6% vs. 13 $\pm$ 10%), slow-grow follicles (66 $\pm$ 5% vs. 60 $\pm$ 9%), or no-grow (10 $\pm$ 9% vs. 27 $\pm$ 6%), respectively. There were no differences in follicle diameters between groups during the culture period. Pre-exposure of secondary follicles to NR diminished their capacity to produce both estradiol and androstenedione by week 4 of culture, when follicles are exhibiting an antrum. Inhibitory effects of NR on steroid production by slow-grow follicles was less pronounced.

Conclusions NR does not affect secondary follicle survival, growth, and antrum formation during long-term culture, but steroid hormone production by fast-grow follicles is compromised. NR can be used as a non-invasive tool for in situ identification of viable secondary follicles in ovarian cortex before tissue cryopreservation without affecting follicle survival and growth in vitro. Whether maturation or developmental competence of oocytes derived from antral follicles in 3D culture that were previously isolated from NR-stained tissue is normal or compromised remains to be determined. Likewise, the functional consequences of pre-exposure to NR prior to ovarian cortical tissue cryopreservation and transplantation are unknown.

Supported by National Institute of Child Health and Human Development (NICHD)/National Institute of Health (NIH) Oncofertility Consortium UL1 RR024926, (RL1-HD058294, PL1-EB008542); ONPRC P51RR000163; NIH Fogarty International Center grant TW/HD-00668 to P. Michael Conn (DLB); M.J. Murdock Charitable Trust, Partners in Science 2010283 (BJG)

✉ Mary B. Zelinski
zelinski@ohsu.edu

¹ Department of Obstetrics and Gynecology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil

² Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Oregon Health & Sciences University, Beaverton, OR, USA

³ Cleveland High School, Portland, OR, USA

⁴ Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, OR, USA

Keywords Neutral red · Ovarian cortex · Primate · Secondary follicles · 3D culture · Vital staining

Introduction

Cryopreservation of ovarian tissue for fertility preservation in oncologic patients is a method currently under

investigation as a promising option for patients who are pre-pubertal or require immediate cancer therapy [1–11]. Due to gonadotoxic damage caused by cancer treatment through chemotherapy and/or radiotherapy in the ovaries, technologies for fertility preservation are required [12]. In 2004, the first documented human live birth following the transplantation of cryopreserved ovarian tissue was reported [4]; to date, there are 86 documented live births [13–15].

The limited progress for ovarian tissue vitrification is largely due to the complex physical and biological properties of the ovary as well as some practical challenges [16]. Ovarian tissue contains large number of preantral follicles, and there is different follicular density along the cortex and medullary regions. A crucial point before tissue cryopreservation and transplantation is in situ identification of viable follicles. Whereas stereomicroscopic localization is achievable, the density of the ovarian cortex can render preantral, particularly primordial, follicle identification difficult and unreliable [17, 18]. To assess follicular in situ localization and follicular viability in ovarian tissue, a new non-invasive and non-toxic method has been investigated using the supravital dye, neutral red (NR). The NR dye becomes a deep red color that, at slightly acid pH, enters cells/tissues readily through the plasma membrane and concentrates in the lysosomes of oocytes and granulosa/theca cells [19]. It is possible to distinguish preantral follicles within ovarian cortical tissue using standard light microscopy as well as determine their viability; follicles staining red are viable and unstained follicles are damaged or dead [20]. NR has been used to assess in situ localization and viability of follicles in ovarian tissue in a number of different species; ovine [21, 22], porcine [23, 24], bovine [25], rat [26], and human [27, 28].

Ovarian tissue autotransplantation bears the risk of the reintroduction of cancer cells to the patients. So, to avoid the risk to re-seeding cancer cells an alternative option has been investigated the cryopreservation of isolated secondary follicles associated with in vitro maturation in 3-dimensional (3D) culture. In vitro maturation by 3D alginate matrix of follicles isolated has the function maintaining in vitro structural integrity, the capacity to antrum formation and yield mature oocytes that are capable of fertilization and embryo development, and have resulted in live offspring in mice [29]. Some studies showed that when encapsulated in alginate and cultured, macaque secondary follicles can to grow to the antral stage, produce steroids and growth factors, and yield healthy oocytes within 40 days [29, 30]. In the present study, we evaluated the influence of NR staining on non-human primate secondary follicle viability and development during long-term 3D culture in an alginate matrix in vitro.

Materials and methods

Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated.

Animals and ovary collection

The general care and housing of rhesus macaques (*Macaca mulatta*) at the Oregon National Primate Research Center (ONPRC) has been previously described [31]. The studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the ONPRC Animal Care and Use Committee. Adult female rhesus monkeys ($n = 4$, 8–11 years old) exhibiting normal menstrual cycles were used in this study. Ovaries were collected from anesthetized monkeys (on days 4–5 of the follicular phase, first day of menses is day 1) during necropsy procedures. Ovaries were placed into 3-(N-morpholino) propanesulfonic acid (MOPS)-buffered tissue holding media (HM; CooperSurgical Inc., Trumbull, CT, USA) and were immediately transported to the laboratory at 37 °C [32].

Ovarian tissue processing

Ovaries were processed in HM supplemented with 15% (v/v) serum protein substitute (SPS, CooperSurgical Inc.) and 29 mg/ml of the antioxidant L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (ascorbic acid phosphate, Sigma, St. Louis, MO, USA) [32]. Following the removal of fatty and non-ovarian tissue by trimming, 0.5-mm-thick outermost ovarian cortical pieces were collected using a Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ, USA). Using a tissue sectioner (McIlwain Tissue Chopper; Mickle Laboratory, Guildford, UK), the ovarian cortex slices were cut into $1 \times 1 \times 0.5 \text{ mm}^3$ fragments. Each fragment was examined under a dissecting scope for the presence of secondary follicles, and only tissues that showed visible secondary follicles were used in the current experiment. Due to the considerable heterogeneity of follicle distribution in the primate ovary [3], the inconsistency of follicular density among tissues was minimized by selecting only cortical pieces with secondary follicles. Ovarian tissue processing was handled and processed at 37 °C.

Incubation of ovarian tissue with neutral red

Ovarian cortex fragments that showed visible secondary follicles, as described above, were transferred to 35-mm culture dishes containing 6 ml of preheated McCoy's 5a culture medium (Core Cell Culture Facility) supplemented with 2.0 mM

L-glutamine, 100 µg/ml penicillin G, and 100 µg/ml streptomycin. The fragments were divided in two groups: (i) no-stained tissue (control) and (ii) NR-stained tissue. Ovarian fragments that were not stained were incubated in holding medium until follicle isolation. The ovarian cortex fragments were incubated with McCoy's culture medium supplemented with 50 µg/ml of neutral red (NR) solution (2-amino-3-methyl-7-dimethyl-aminophenazoniumchloride; cat# N2889, Sigma-Aldrich Saint Louis, MO, USA) at 37 °C in 5% CO₂ in atmospheric air for 5 min. The fragments were then transferred to plates containing McCoy's culture medium alone and were placed onto a shaker with a warming plate at 37 °C for 10 min. Fragments were rinsed in HM with SPS and ascorbic acid phosphate, as described above, on a orbital shaker for 10 min [32].

Secondary follicle isolation and encapsulation

The ovarian cortex fragments were assessed under a dissecting scope to identify follicles with red coloration and to classify the secondary follicles based on morphology (120–250 µm in diameter, partly surrounded by stromal tissue) with a visible oocyte (round and centrally located within the follicle), an intact basement membrane, and no antral cavity [32] (Fig. 1a–c). Secondary follicles with preserved morphology were isolated from tissue with/without NR incubation (as

described above) mechanically using 25-gauge needles and without collagenase treatment (Fig. 1d–g). Follicles with no red coloration were considered dead follicle and were not used for culture (Fig. 1d, e). Follicles from tissue incubated with NR were only considered to be viable when both the oocyte and more than 75% of the surrounding granulosa cells stained positive for NR (Fig. 1f, g). Isolated secondary follicles were then encapsulated in 0.25% alginate gel and cultured for 4 weeks as previously described [29, 33].

Isolated secondary follicle 3D culture

Encapsulated follicles were transferred to individual wells of a 48-well plate containing 300 µl of alpha minimum essential medium (αMEM) culture media supplemented with 0.3% SPS, 10 ng/ml follicle-stimulating hormone, 0.5 mg/ml bovine fetuin, 5 µg/ml transferrin, 5 µg/ml insulin, and 5 ng/ml sodium selenite. A maximum of 12 encapsulated follicles were loaded per 48-well plate, while the remaining wells were loaded with 300 µl of sterile water to minimize media evaporation during long-term culture. Encapsulated follicles were cultured at 37 °C in 5% CO₂ in atmospheric air for 4 weeks. Every 2 days, half of the culture media (150 µl) was exchanged with fresh culture media (prepared weekly). All follicles stained with NR no longer exhibited orange-red color after 24 h of culture.

Fig. 1 Representative photomicrographs of macaque ovarian tissue and follicles not exposed or exposed to neutral red (NR). NR diffuses through the plasma membrane and concentrates in viable follicles making possible to distinguish follicles in a pieces of tissue (**a–c**); white arrowheads depict follicles stained with NR in situ. (**d**) Isolated

secondary follicles without NR staining from the no-stained tissue group (controls). (**e**) Secondary follicle (non-viable) that did not stain with NR isolated from ovarian tissue exposed to NR. (**f**) Isolated secondary follicle (viable) stained with NR. Isolated secondary follicles from tissue stained with NR (**g**). Scale bar = 200 µm (**a**), 100 µm (**b, d–g**), 50 µm (**c**)

Follicle survival and growth

Follicle health and diameter were assessed using an Olympus CK40 inverted microscope attached to an Olympus DP11 digital camera (Center Valley, PA, USA). Follicles were considered to be degenerating if (i) the oocyte was no longer surrounded by a layer of granulosa cells, (ii) the oocyte became dark, (iii) the granulosa cells became dark and lost connection with one another, or (iv) the diameter of the follicle decreased. For each follicle, weekly photographs were taken and growth was evaluated based on diameters measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). The mean of two measurements per follicle (perpendicular to each other) was then calculated and reported as the follicle diameter. Follicle growth was defined as a significant increase in follicle diameter in comparison to the day of isolation (week 0 or day 1). Similar to our previous reports [30] surviving follicles were divided into three distinct cohorts based on their size at week 5: (1) fast-grow (FG): diameters ≥ 500 μm , (2) slow-grow (SG): diameters between 250 and 500 μm , and (3) no-grow (NG): diameters < 250 μm .

Hormone assay

Concentrations of estradiol (E2) and androstenedione (A4) collected weekly (weeks 1, 2, and 4) in media of fast-grow, slow-grow, and no-grow follicles were measured by the Endocrine Technologies Support Core (ETSC) at the Oregon National Primate Research Center (ONPRC). Estradiol was analyzed using a Roche cobas e411 automated clinical platform (Roche Diagnostics, Indianapolis, IN, USA). The assay range for E2 was 5–4300 pg/ml. Intra- and inter-assay CVs for the Roche assays in the ETSC are consistently less than 7%. Androstenedione was measured by ELISA following the manufacturer's instructions (LDN, Nordhorn, Germany). The assay range was 0.1–10 ng/ml. LDN supplies two controls with the kit; the first control ranges from 0.29 to 0.77 ng/ml (control I), and the second 3.95–9.21 ng/ml (control II). Intra-assay CVs for control I ranged from 1.4 to 1.6% and inter-assay CV was 3.7% ($n = 2$). Intra-assay CVs for control II ranged from 0.6 to 1.0% and inter-assay CV was 1.4%. In addition, the ETSC includes an in-house non-human primate serum quality control pool with each assay. Intra-assay CVs ranged from 1.3 to 3.1% and inter-assay CV was 4.8% ($n = 2$).

Data analysis and statistics

Data are presented as mean $\pm$ SEM and analyzed by combining observations to make one single count per animal giving an equal contribution from each animal. Follicle survival and antrum formation is represented as the percentage rates (mean $\pm$ SEM) of four individual animals in each treatment group. The 4-week survival, antrum formation rate, as well as

hormone values (across timepoints within the same treatment group) were analyzed using one way analysis of variance (ANOVA: SigmaPlot 11.0, Systat Software, Inc., San Jose, CA, USA). *t* tests were performed for weekly follicle diameter as well as hormone values between treatment groups of the same culture timepoint. Differences were considered significant when $P \leq 0.05$.

Results

NR-stained follicle culture: survival and antrum formation rate

The survival rate after 4 weeks in culture (Table 1) of follicles from NR-stained tissue ($n = 26/39$; $68 \pm 9\%$) was similar to that of from non-stained tissue ($n = 36/51$; $71 \pm 13\%$). An antral cavity was evident within 2–4 weeks of culture for all follicles derived from no-stained tissue ($n = 23/33$) and NR-stained tissue follicles ($n = 12/25$). The proportion of surviving follicles that formed an antrum was also similar between no-stained tissue ($70 \pm 17\%$) and NR-stained tissue ($48 \pm 24\%$) (Table 1; Fig. 2).

NR stained follicle culture: growth

Follicle diameters were similar between follicles from no-stained tissue (184 ± 5 μm) and NR-stained tissue (181 ± 7 μm) on the day of isolation. During culture, three distinct cohorts of follicles were observed based on their growth rate. Weekly follicle diameters in the no-stained and NR-stained groups are presented in Fig. 3. No difference was observed between diameters of follicles in the no-stained tissue and NR-stained groups within each cohort at each week throughout culture. In addition, surviving follicles in the no-stained group ($n = 36$ total follicles) were composed of $11 \pm 6\%$ FG, $64 \pm 4\%$ SG, and $25 \pm 5\%$ NG follicles. No difference was found in the distribution of the 3 follicle growth cohorts in the NR-stained group ($n = 26$ total follicles; FG $31 \pm 3\%$, SG follicles $58 \pm 4\%$, and NG follicles $11 \pm 5\%$).

Table 1 Average number and total number (per animal, $n = 4$ animals) of secondary follicles and their survival rates (%) at 4 weeks and the ability to form antrum (%) among surviving follicles in no-stained and NR-stained follicles

Groups	% average (total number of follicles)	% survival rate (total number of follicles)	% antrum formation rate (total number of follicles)
No-stained	13 ± 3 (51)	71 ± 13 (36)	70 ± 17 (23)
NR-stained	10 ± 2 (39)	68 ± 9 (26)	48 ± 24 (12)

Data are presented as mean $\pm$ SEM ($n = 4$ animals per treatment group)

Fig. 2 Representative pictures of isolated secondary follicles throughout 4 weeks of culture in the FG cohort from control (top row) and NR-stained groups (middle row), and from the SG cohort in the NR-stained

group (bottom row). Antrum were formed by week (Wk) 3 in both treatment groups. The series of images represent the growth pattern of a single follicle from each group during culture. Scale bar = 100 μm

E2 and A4 concentrations

Estradiol (E2) levels produced by FG follicles in the no-stained tissue (control) group increased ($P < 0.05$) at week 2 and reached greater ($P < 0.05$) levels at week 4 (Table 2). E2 production by FG follicles in the NR-stained group were above baseline at week 1, increased at week 2, and remained similar at week 4. FG follicles of the no-stained group produced similar levels of E2 at weeks 1 and 2 relative to the NR-stained group. However, FG follicles from the no-stained group showed higher ($P < 0.05$) E2 levels at week 4. E2 levels of SG follicles in the no-stained group increased ($P < 0.05$) at week 4, and did not differ from SG follicles in the NR-stained group. E2 levels were at baseline and did not change in NG follicles throughout the culture period in both groups (data not shown).

Androstenedione (A4) concentrations for FG follicles in the no-stained group were similar between weeks 1 and 2, and increased ($P < 0.05$) at week 4, but did not change over the culture interval in FG follicles in the NR-stained group (Table 2). FG follicles in the no-stained group produced greater ($P < 0.05$) levels of A4 relative to the NR-stained group at week 4. A4 levels produced by SG follicles from the no-stained group decreased ($P < 0.05$) in week 2, compared to week 1, and returned back to week 1 levels in week 4. A4 levels produced by SG follicles from the NR-stained follicles also decreased ($P < 0.05$) in week 2, compared to week 1, but stayed low in week 4. A4 levels were at baseline and did not change in NG follicles throughout the culture period in both groups (data not shown).

Discussion

In this study, we evaluated the influence of NR staining on primate secondary follicle viability and development during long-term culture in 3D alginate matrix. Isolated secondary follicle survival, growth, antrum formation, E2, and A4 production in vitro were examined. The current experiment is the first to evaluate isolated secondary primate follicles cultured from ovarian tissue stained by NR. Results showed that NR tissue staining prior to secondary follicle isolation does not affect secondary follicle survival and the capacity to develop to advanced stages (antral stage). However, steroid hormone production by fast-grow follicles is compromised during long-term culture.

Ovarian tissue cryopreservation followed by in vitro follicle maturation has been highlighted as an alternative method for preserving fertility for patients with a high risk of malignant cell reoccurrence (i.e., for cancers originating in the ovary and for some hematological cancers). Follicles isolated from ovarian tissue can be cultured to antral stages wherein viable oocytes can be collected for in vitro fertilization [33, 34]. The inability of the conventional follicle culture system (two-dimensional (2D)) to sustain the in vivo-like follicle shape in mammals has limited the success of the follicle in vitro culture to generate mature oocytes. Currently, an alternative option for follicle culture has been developed wherein a calcium alginate-based matrix supports the three-dimensional (3D) architecture of follicles permitting the in vitro development of pre-antral follicles to the antral stage [29, 30, 35]. In the current study, secondary follicles were isolated from fresh tissue

Fig. 3 Follicle growth of three cohorts of surviving follicles during culture in alginate for 4 weeks: fast-grow follicles from tissue incubated with neutral red (NR) and tissue not stained (a), slow-grow follicles (b) and (c) no-grow follicles. Data are represented as the mean $\pm$ SEM ($n = 4$ animals). Follicle diameters in each growth cohort did not differ between groups through 4 weeks of culture. Different letters represent follicle diameters that are significantly different ($P < 0.05$) from week 0 within the control (lower case) and NR (upper case) groups

with or without prior staining with NR. Our data confirms that incubation of macaque ovarian cortical tissue in NR prior to culture does not compromise subsequent follicle survival in vitro, indicating the potential suitability of this approach in fertility preservation regimens. Even if NR is applied directly in isolated bovine ovarian follicles, it seems not to compromise viability [36].

Advanced technologies for cancer screening have been developed in the last years, with the goal of diagnosing patients at earlier stages. Thus, patients of a much younger age can be treated at younger ages. Currently, the survival rates (83%) of patients in reproductive age have been increasing [37]. Because cancer therapies destroy ovarian tissue by depleting

oocytes leading to infertility, fertility can be preserved by ovarian tissue cryopreservation as one option [12].

Since ovarian cortical tissue morphology, thickness, follicular density, and follicles at different stages of development vary with each piece of tissue prepared for cryopreservation, it can be difficult to identify and quantify the follicles in situ under light microscopy [17, 18]. Furthermore, methods that use fluorescent probes and staining ovarian cortical tissue with rhodamine 123 and calcein AM, which is pro-oxidative, have deleterious effects on enzymatic activity within cellular organelles and are cytotoxic under in vitro conditions [38–41]. These types of treatments do not support follicle development in vitro following visualization [18, 42]. The NR method is applicable as a simple, non-fluorescent staining method more suitable for in situ follicle density assessment and identification of viable follicles before and after ovarian tissue cryopreservation [26, 43]. After NR staining of macaque ovarian tissue, it was possible to confirm the presence of all classes of viable preantral follicles (NR staining follicles) under light microscopy. Similarly, Chambers and colleagues demonstrated that NR can be used to detect viable follicles within thin slices of fresh and cryopreserved ovine and human ovarian cortical fragments and found that follicle diameters after NR staining increased over 6 days of culture. NR can be used as a valuable tool in a number of species, now including non-human primates, to quantify follicle density in situ, without compromising follicle viability [22, 26, 27] and growth potential [22, 25–27].

While several studies indicate that NR is an efficient method to visualize viable preantral follicles in ovarian cortex [21–28], in the present study, NR does not improve secondary follicle isolation from macaque ovarian cortical tissue because they can be seen without NR if the tissue is prepared in thin pieces. However, the current study only used ovarian cortical tissues from young rhesus macaques. Therefore, for ovaries from older animals/patients with dense cortex, NR may be useful for identification of follicles in situ. Although a longer incubation period (4 h) in NR was required for follicles visualization in other studies [25], in this study, incubation of ovarian cortex fragments in NR for only 5 min under culture conditions was enough for NR to be stored in the lysosomes of viable follicles, resulting in red follicles. Thereby, NR allows easy and quick application in based media for identification of preantral follicles in ovarian cortex fragments.

During normal development, a secondary follicle grows in size by proliferation of granulosa cells and an increase in oocyte diameter. In the current study, no-grow follicles remained similar in size to the initial secondary follicle without significant change in diameters through 4 weeks of culture in NR group and control group. Slow-growth follicles (250–500 μ m) showed increase diameter along 4 weeks culture in

Table 2 Steroid production by fast- and slow-grow follicles collected from macaque ovarian cortical tissue not treated (no-stained) or treated with neutral red (NR-stained) during 3D encapsulated culture in vitro

Follicle growth Cohorts	Groups	Week 1	Week 2	Week 4
Estradiol (pg/ml)				
Fast grow follicles	No-stained	97 ± 9 ^a	185 ± 58 ^b	5613 ± 1929 ^{cA}
	NR-stained	87 [*]	177 ± 96 ^b	269 ± 107 ^{bB}
Slow grow follicles	No-stained	121 ± 38 ^a	152 ± 22 ^a	356 ± 99 ^b
	NR-stained	87 ± 4 ^a	145 ± 18 ^b	173 ± 34 ^b
Androstenedione (pg/ml)				
Fast grow follicles	No-stained	37 ± 16 ^a	24 ± 11 ^a	122 ± 5 ^{bA}
	NR-stained	8 ± 3 ^a	30 ± 18 ^a	10 ± 0 ^{aB}
Slow grow follicles	No-stained	33 ± 7 ^a	7 ± 2 ^b	20 ± 7 ^{ab}
	NR-stained	40 ± 9 ^a	9 ± 3 ^b	19 ± 9 ^b

Values represent the mean ± SEM from 4 animals per treatment, 3–5 follicles for fast-grow and 18–23 slow-grow follicles per treatment group per week. Lower case letters represent differences between weeks within a group. Upper case letters represent differences between groups within week. * SEM lacking due to $n = 1$ in the group

both groups, and large number of follicles was observed in this cohort. Fast-growth follicles were observed in both groups. Distribution of number of follicles along of three cohorts was similar to studies with rhesus macaque from Xu et al. 2009; 2010 [30, 35]. One might expect that NR-stained follicles exhibited higher developmental potentials in comparison to no-stained follicles, because only viable follicles are selected with NR staining, however this did not occur. There may be a slight impact on follicle development after NR staining that is compensated by the exclusion of no-viable follicles, in this way it optimizes the in vitro follicle culture avoiding unnecessary culture. In this study, similar follicle survival, antrum formation and growth were observed in no-stained and NR-stained follicles. It is possible that follicle selection by morphological observation is sufficient or there are few degenerating (non-NR stained) secondary follicles in the macaque cortex.

Steroid hormone production correlates with the growth and maturation of the secondary follicles cultured in this study [30, 33, 44]. Exposure of secondary follicles to NR within the ovarian cortex prior to encapsulated 3D culture allowed antrum formation and the correlated increase in estradiol, but not androstenedione, production in fast-grow follicles. In addition, pre-exposure of secondary follicles to NR diminished their capacity to produce both estradiol and androstenedione by week 4 of culture, when follicles are exhibiting an antrum. Inhibitory effects of NR on steroid production by slow-grow follicles was less pronounced. It has been consistently observed that both E2 and A4 production by fast-grow follicles from macaques and women increases during 4 weeks of culture [30, 33, 44]. Recently, it has been suggested that E2 levels produced by growing follicles during encapsulated 3D culture are associated with follicle survival, growth, antrum formation and oocyte health in primates [45]. Whether E2 acts directly on the oocyte or on the somatic cells to support oocyte health

and development remains to be determined. Although NR inhibited E2 production by fast-grow follicles in the present study, “optimal” levels of steroid production during encapsulated 3D culture of primate follicles are not defined for oocyte maturation and developmental competence. Thus, it is unknown whether this reduction, but not elimination, of E2 production would have detrimental effects on the developmental competence of oocytes derived from encapsulated 3D culture.

Finally, results of the present study showed that NR can be non-toxic with respect to survival, growth, and antrum formation in primate secondary follicles cultured in a 3D system. However, pre-exposure of secondary follicles to NR inhibited both estradiol and androstenedione production by antral follicles at week 4 of culture. NR can be used as a non-invasive tool for in situ identification of viable secondary follicles in ovarian cortex before tissue cryopreservation. Although other non-invasive methods have been described recently to identify follicles in situ [46], NR is inexpensive, practical, and does not demand specific equipment for its application. Whether maturation or developmental competence of oocytes derived from antral follicles in 3D culture that were previously isolated from NR-stained tissue is normal or compromised remains to be determined. Likewise, the functional consequences of pre-exposure to NR prior to ovarian cortical tissue cryopreservation and transplantation are unknown.

Acknowledgements We are grateful to the Division of Comparative Medicine for surgery and excellent animal care, and Maralee Lawson for her assistance.

Authors' contributions All authors contributed to the study design, execution, analysis, critical discussion, and drafting or revising of the manuscript. All authors have approved the final version and submission of this manuscript.

Compliance with ethical standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

- Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *New Eng J Med*. 2000;342:1919.
- Radford JA, Lieberman BA, Brison DR, Smith ARB, Critchlow JD, Russell SA, et al. Orthotopic reimplantation of cryopreserved ovarian cortical strips after high-dose chemotherapy for Hodgkin's lymphoma. *Lancet*. 2001;357:1172–5.
- Schmidt KL, Byskov AG, Nyboe Andersen A, Muller J, Yding AC. Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries. *Hum Reprod*. 2003;6:1158–64.
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet*. 2004;364:1405–10.
- Andersen CY, Rosendahl M, Byskov AG, Loft A, Ottosen C, Dueholm M, et al. Two successful pregnancies following autotransplantation of frozen/thawed ovarian tissue. *Hum Reprod*. 2008;23:2266–72.
- Silber SJ, DeRosa M, Pineda J, Lenahan K, Grenia D, Gorman K, et al. A series of monozygotic twins discordant for ovarian failure: ovary transplantation (cortical versus microvascular) and cryopreservation. *Hum Reprod*. 2008;23:1531–7.
- Kim SS, Lee WS, Chung MK, Lee HC, Lee HH, Hill D. Long-term ovarian function and fertility after heterotopic autotransplantation of cryobanked human ovarian tissue: 8-year experience in cancer patients. *Fertil Steril*. 2009;91:2349–54.
- Donnez J, Kim SS, Albertini DF. Proceedings of the first world congress on fertility preservation: executive summary. *J Assist Reprod Genet*. 2010;27:191–5.
- Rosendahl M, Schmidt KT, Ernst E, Rasmussen PE, Loft A, Byskov AG, et al. Cryopreservation of ovarian tissue for a decade in Denmark: a view of the technique. *Reprod BioMed Online*. 2011;2:162–71.
- Silber SJ. Ovary cryopreservation and transplantation for fertility preservation. *Mol Hum Reprod*. 2012;2:59–67.
- Poirot C, Abirached F, Prades M, Coussieu C, Bernaudin F, Piver P. Induction of puberty by autograft of cryopreserved ovarian tissue. *Lancet*. 2012;9815:588.
- Schmidt KT, Larsen EC, Andersen CY, Andersen AN. Risk of ovarian failure and fertility preserving methods in girls and adolescents with a malignant disease. *BJOG*. 2010;2:163–74.
- Donnez J, Jadoul P, Pirard C, Hutchings G, Demylle D, Squifflet J, et al. Live birth after transplantation of frozen-thawed ovarian tissue after bilateral oophorectomy for benign disease. *Fertil Steril*. 2012;3:720–5.
- Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. *Fertil Steril*. 2013;6:1503–13.
- Jensen AK, Macklon KT, Fedder J, Ernst E, Humaidan P, Andersen CY. 86 successful births and 9 ongoing pregnancies worldwide in women transplanted with frozen-thawed ovarian tissue: focus on birth and perinatal outcome in 40 of these children. *J Assist Reprod Genet*. 2017;34(3):325–36.
- Ting AY, Yeoman RR, Campos JR, Lawson MS, Mullen SF, Fahy GM, et al. Morphological and functional preservation of pre-antral follicles after vitrification of macaque ovarian tissue in a closed system. *Hum Reprod*. 2013;28:1267–79.
- Hovatta O, Wright C, Krausz T, Hardy K, Winston RML. Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. *Hum Reprod*. 1999;14:2519–24.
- Soleimani R, De Vos W, Van Oostveldt P, Lierman S, Van Den Broecke R, De Sutter P, et al. Two novel techniques to detect follicles in human ovarian cortical tissue. *Hum Reprod*. 2006;21:1720–4.
- Allison AC, Young MR. Uptake of dyes and drugs by living cells in culture. *Life Sci* 1964;1407–14.
- Borenfreund E, Puerner JA. Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol Letter*. 1985;24:119–24.
- Campbell BK, Scaramuzzi RJ, Webb R. Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. *J Reprod Fertil*. 1996;106:7–16.
- Chambers EL, Gosden RG, Yap C, Picton HM. In situ identification of follicles in ovarian cortex as a tool for quantifying follicle density, viability and developmental potential in strategies to preserve female fertility. *Hum Reprod*. 2010;25:2559–68.
- Shores EM, Picton HM, Hunter MG. Differential regulation of pig theca cell steroidogenesis by LH, insulin-like growth factor I and granulosa cells in serum-free culture. *J Reprod Fertil*. 2000;118:211–9.
- Brankin V, Mitchell MR, Webb B, Hunter MG. Paracrine effects of oocyte secreted factors and stem cell factor on porcine granulosa and theca cells in vitro. *Reprod Biol Endocrinol*. 2003;1:55.
- Jorssen EPA, Langbeen A, Fransen E, Martinez EL, Leroy JLMR, Bols PEJ. Monitoring preantral follicle survival and growth in bovine ovarian biopsies by repeated use of neutral red and cultured in vitro under low and high oxygen tension. *Theriogenology*. 2014; 1–9.
- Milenkovic M, Diaz-Garcia C, Wallin A, Brännström M. Viability and function of the cryopreserved whole rat ovary: comparison between slow-freezing and vitrification. *Fertil Steril*. 2012;97:1176–82.
- Kristensen SG, Rasmussen A, Byskov AG, Andersen CY. Isolation of pre-antral follicles from human ovarian medulla tissue. *Hum Reprod*. 2011;26:157–66.
- Khosravi F, Reid RL, Moini A, Abolhassani F, Valojerdi MR, Kan FWK. In vitro development of human primordial follicles to pre-antral stage after vitrification. *J Assist Reprod Genet*. 2013;30:1397–06.
- Xu M, Kreeger PK, Shea LD, Woodruff TK. Tissue-engineered follicles produce live, fertile offspring. *Tissue Eng*. 2006;10:2739–46.
- Xu J, Bernuci MP, Lawson MS, Yeoman RR, Fisher TE, Zelinski MB, et al. Survival, growth, and maturation of secondary follicles from prepubertal, young, and older adult rhesus monkeys during encapsulated three-dimensional culture: effects of gonadotropins and insulin. *Reproduction*. 2010;5:685–97.
- Wolf DP, Thomson JA, Zelinski-Wooten MB, Stouffer RL. In vitro fertilization-embryo transfer in nonhuman primates: the technique and its applications. *Mol Reprod Dev*. 1990;3:261–80.
- Ting AY, Yeoman RR, Lawson MS, Zelinski MB. Synthetic polymers improve vitrification outcomes of macaque ovarian tissue as assessed by histological integrity and the in vitro development of secondary follicles. *Cryobiology*. 2012;1:1–11.
- Xu J, Lawson MS, Yeoman RR, Pau KY, Barrett SL, Zelinski MB, et al. Secondary follicle growth and oocyte maturation during encapsulated three-dimensional culture in rhesus monkeys: effects of gonadotrophins, oxygen and fetuin. *Hum Reprod*. 2011;5:1061–72.
- Xiao, et al. In vitro follicle growth supports human oocyte meiotic maturation. *Sci Rep*. 2015;5:17323.

35. Xu M, West-Farrell ER, Stouffer RL, Shea LD, Woodruff TK, Zelinski MB. Encapsulated three-dimensional culture supports development of nonhuman primate secondary follicles. *Biol Reprod*. 2009;81:587–94.
36. Langbeen A, Jorssen EP, Granata N, Fransen E, Leroy JL, Bols PE. Effects of neutral red assisted viability assessment on the cryotolerance of isolated bovine preantral follicles. *J Assist Reprod Genet*. 2014;12:1727–36.
37. Howlader N, Noone A, Krapcho M, Neyman N, Aminou R, Altekruse S, Kosary C, Ruhl J, Tatalovich Z, Cho H. SEER Cancer Statistics. Review, 1975–2009 (Vintage 2009 Populations). National Cancer Institute.
38. Sheen VL, Macklis JD. Apoptotic mechanisms in targeted neuronal cell death by chromophore-activated photolysis. *Exp Neurol*. 1994;130:67–81.
39. Hockberger PE, Skimina TA, Centonze VE, Lavin C, Chu S, Dadras S, et al. Activation of flavin-containing oxidases underlies light-induced production of H₂O₂ in mammalian cells. *Proc Natl Acad Sci U S A*. 1999;11:6255–60.
40. Jover R, Ponsoda X, Castell JV, Gómez-Lechón MJ. Evaluation of the cytotoxicity of ten chemicals on human cultured hepatocytes: predictability of human toxicity and comparison with rodent cell culture systems. *Toxicol in Vitro*. 1992;1:47–52.
41. Avery B, Greve T. Effects of ethanol and dimethylsulphoxide on nuclear and cytoplasmic maturation of bovine cumulus-oocyte complexes. *Mol Reprod Dev*. 2000;55:438–45.
42. Cortvrindt RG, Smitz JE. Fluorescent probes allow rapid and precise recording of follicle density and staging in human ovarian cortical biopsy samples. *Fertil Steril*. 2001;75:588–93.
43. Nemes Z, Dietz R, Lüth JB, Gomba S, Hackenthal E, Gross F. The pharmacological relevance of vital staining with neutral red. *Experientia*. 1979;15;35(11):1475–6.
44. Xu M, Barrett SL, West-Farrell E, Kondapalli LA, Kieseewetter SE, Shea LD, et al. In vitro grown human ovarian follicles from cancer patients support oocyte growth. *Hum Reprod*. 2009;24:2531–40.
45. Ting AY, Xu J, Stouffer RL. Differential effects of estrogen and progesterone on development of primate secondary follicles in a steroid-depleted milieu in vitro. *Hum Reprod*. 2015;30:1907–17.
46. Peters IT, Stegehuis PL, Peek R, Boer FL, van Zwet EW, Eggermont J, Westphal JR, Kuppen PJ, Trimboos JB, Hilders CG, Lelieveldt BP, van de Velde CJ, Bosse T, Dijkstra J, Vahrmeijer AL. Noninvasive detection of metastases and follicle density in ovarian tissue using full-field optical coherence tomography. *Clin Cancer Res*. 2016;16.