RESEARCH

Steroids and miRNAs in assessment of ovarian tissue damage following cryopreservation

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Abstract

Ovarian cortical tissue cryopreservation is a relatively novel approach to preserving fertility in women diagnosed with cancer. However, the effects of freezing-thawing are not fully understood, mainly due to the lack of suitable methods to assess tissue's survival after thawing. Disparities in steroid production have been associated with ovarian failure by disrupting folliculogenesis, ovulation and oocyte apoptosis. Moreover, specific miRNAs, identified in human ovarian follicles, are thought to play a fundamental role in folliculogenesis. In this study, we investigated the possible interplay between the ovarian steroidal production and miRNA expression patterns in spent culture media, as potential non-invasive markers for ovarian tissue damage after cryopreservation. Cryopreservation of ovarian cortical tissue decreased ($P < 0.05$) both steroid production (oestradiol and progesterone) and expression of miRNA-193b and 320A in spent culture media over 5 days; however, expression of miRNA-24 increased ($P < 0.05$). The number of primordial follicles was also reduced ($P < 0.05$) in fresh-cultured and cryopreserved-cultured cortical tissues when compared with fresh tissues. Downregulation of miRNA-193b and miRNA-320A together with upregulation of miRNA-24 could have a synergistic role in cell apoptosis, and consequently leading to reduced oestradiol and progesterone production. Thus, there appears to be an interplay between these miRNAs, ovarian steroid production and cell damage, which can be further explored as novel non-invasive markers of cell damage following cryopreservation.

Key Words

- ▶ ovarian cortical tissue
- ▶ cryopreservation
- ▶ steroids
- ▶ miRNA

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Introduction

Cryopreservation of ovarian cortical tissue is a novel approach to preserving fertility in women who are at risk of infertility, particularly in younger oncology patients (Abir *et al.* 2016). However, the effects of freeze-thawing are not fully understood, mainly due to the lack of suitable methods to assess tissue's survival after thawing. Among the cryopreservation techniques, vitrification of oocytes and embryos is an effective method resulting in high survival rate and pregnancy outcomes (Al-Azawi *et al.*

2013). However, for ovarian cortical tissue, slow freezing is the method that has provided best results. More than 130 live births have been reported following transplantation of slow-frozen cryopreserved ovarian cortical tissue, showing high primordial and primary follicle pool survival post transplant (Amorim *et al.* 2011, Donnez *et al.* 2015, Abir *et al.* 2017, Donnez & Dolmans 2017).

A succession of organisational and functional processes take place in ovary throughout the female's

life, indispensable for maintaining reproductive potential (Baerwald *et al.* 2012). For example, endocrine signals such as patterns of gonadotropins production and release (follicle-stimulating hormone (FSH), luteinising hormone (LH)) which modulate the production and release of sex steroidal hormones oestradiol, progesterone and androstenedione. These ovarian steroids are key local intra-ovarian factors involved in the regulation of folliculogenesis (Zumbado *et al.* 2010). Changes in the above endocrine network have been linked to premature ovarian failure (POF) due to disruption of folliculogenesis, ovulation failure and loss of oocytes via apoptosis (Yang *et al.* 2012).

miRNAs are evolutionarily conserved, single-stranded, non-coding RNA molecules, consist of 18–22 nucleotides. They are important intercellular signalling molecules, known to have major roles in post-transcriptional gene regulation (Weber *et al.* 2010, Cortez *et al.* 2011) and protein synthesis (Mukherji *et al.* 2011). In many cells, specific miRNAs are expressed differentially in disease states compared to normal tissue. Recent reports have identified miRNAs in human ovarian follicles that may be able to modulate reproduction (Houbaviy *et al.* 2003).

Over 200 miRNAs have been reported in human follicular fluid and other body fluids such as serum, urine and saliva (Rayner & Hennessy 2013, Xu *et al.* 2013). Due to the nature of these miRNAs and their ability to have an effect on multiple target sites, it is difficult to determine the specific role of a particular miRNA. Using bioinformatics analysis, Sang *et al.* (2013) studied the miRNA expression in granulosa cells and follicular fluid and its correlation with endocrine, reproductive and metabolic functions. They identified miRNA-320 and miRNA-24 as regulators of oestradiol production, whereas miRNA-24 and miRNA-193b regulate progesterone. MiRNA-24 was found to decrease oestradiol secretion while miRNA-320 was found to increase it (Sang *et al.* 2013). The lack of tight regulation of these processes by miRNAs can cause disruptions and prevent certain stages of development occurring within the correct time frame during folliculogenesis, and it can lead to abnormalities in oocyte development. It is also thought to have an effect on oocyte–granulosa cell relationships and in turn could have detrimental effects on normal recruitment and development of follicles (Toloubeydokhti *et al.* 2008).

Rotatory cell culture system (RCCS) has been used in cancer cell lines and stem cell culture because it allows cell co-culture and high nutrient transfer with low shear force (Morabito *et al.* 2015, Snyder *et al.* 2016). This vessel simulates the 3D environment in which tissue normally

develops and functions (Barrila *et al.* 2010). Previous work from our laboratory involving ovine ovarian cortical tissue, cultured in the RCCS for 7 days showed significant decrease in the primordial follicles and increase in the number of primary and secondary follicles. Telfer and colleagues in 2008 reported successful antrum formation and oocyte growth within ovarian cortical tissues using a two-step culture system of 6 days with the addition of activin. However, there was significant reduction in the number of primordial and primary follicles between at the time of cortical tissue collection and after 6 days of culture (Telfer *et al.* 2008).

In this experiment, we investigated the possible interplay between steroid production and miRNAs' expression pattern that could help in the assessment of tissue damage post freeze-thaw cycles. We studied steroid production and miRNAs' expression to evaluate the tissue functionality following cryopreservation of ovarian cortical tissue using a controlled rate cryopreservation protocol. Expression of miRNA-193b, 24, 320A and oestradiol, progesterone and androstenedione production was evaluated in ovarian cortical tissue subjected or not to cryopreservation followed by 5 days culture in a RCCS system. In this study, sheep was used as model species due to their well described pre-antral follicular dynamics and similarities to human reproductive tissues (Campbell *et al.* 2012)

Materials and methods

All materials and media were obtained from Sigma-Aldrich Ltd, unless stated otherwise.

Collection of ovaries and preparation of ovarian cortical tissue

Sheep ovaries were collected from a local abattoir, transported to the laboratory and kept in pre-warmed dissection media (20mM HEPES in Medium 199 supplemented with 1% penicillin/streptomycin, 1% fungizone (amphotericin B-250µg/mL)) at 37°C until use.

Ovarian cortical tissues of approximately 5mm² in area and 1mm in thickness were collected using a sterile Rosenberg skin grafting knife (4 Med Ltd., Sderot, Israel). After collection, cortical tissues were randomly assigned into three groups: fresh (*n*=20), fresh-cultured (*n*=20) and cryopreserved-cultured (*n*=20). Fresh cortical tissues (*n*=20) were immediately fixed in 4% paraformaldehyde (PFA) for histological evaluation using haematoxylin & eosin (H&E) staining. The remaining cortical tissues were

either cryopreserved ($n=20$) or not ($n=20$), followed by a 5-day RCCS culture, after which tissues were fixed and stained for histological evaluation. A detailed schematic diagram describing the experimental units has been shown in Fig. 1.

Ovarian cortical tissue culture

Ovarian cortical tissues were placed into RCCS vessel (fresh-cultured and cryopreserved-cultured groups) through the main lock port using forceps. The vessel was tilted at 45° to ensure the ovarian tissues remain immersed in culture media. Vessels containing the tissues were filled with 10 mL of granulosa cell (GC) culture media (McCoy's 5A with sodium bicarbonate PLUS containing 1 mg/mL bovine serum albumin (BSA), 1% penicillin/streptomycin, 1.5% L-glutamine, transferrin (10 μ g/mL), Selenium (4 ng/mL), IGF-1 LR3 (10 ng/mL), insulin (10 ng/mL) and FSH (1 ng/mL, NIAMDD-FSH-20)), mentioned by Campbell *et al.* (1996). The rotational speed was set to 10–14 rpm according to the size of the tissue. Tissues were incubated at 37°C and 5% CO_2 for 5 days. Culture media (6 mL) was replaced with fresh media daily. Collected spent culture media was stored at -20°C until used for steroid determination (oestradiol,

progesterone and androstenedione) and miRNA (24, 193b and 320A) extraction.

Slow freezing of ovarian cortical tissue

Ovarian cortical tissues were cryopreserved following a controlled rate or slow freezing protocol, previously described by Gosden *et al.* (1994). Briefly, cortical tissues were first equilibrated on a roller mixer in 10 mL cryoprotectant (CPA) solution (1.5 M DMSO, 0.1 M sucrose and 10% v/v foetal calf serum (FCS) in Leibovitz-L15 medium (L-15)) at 4°C for 30 min to allow maximum penetration of CPA. Equilibrated tissues were then transferred to a cryovial containing 1.8 mL CPA. Cryovials were transferred to a programmable freezer (Planer, Kryo 10 Series model 10-20, Planer Products Ltd, Sanbury-on-Thames, UK) held at 0°C (starting temperature). The freezing programme was then cooled down to -9°C at a rate of $-2^\circ\text{C}/\text{min}$ and held at this temperature for manual seeding. Following this, the chamber temperature was brought down to -40°C at a rate of $-0.3^\circ\text{C}/\text{min}$ and then to -140°C at a rate of $-10^\circ\text{C}/\text{min}$. At the end of the programme (-140°C), the samples were removed from the freezer and stored in liquid nitrogen at -196°C (Gosden *et al.* 1994).

Thawing of ovarian cortical tissue

The cryovials were kept at a room temperature for 1 min and rapidly thawed at 37°C in a water bath for a maximum of 10 min. After this, tissues were incubated in three different thawing media (TM) with decreasing concentrations of DMSO and sucrose in L15 medium: TM₁ (1.0 M DMSO, 0.1 M sucrose, 10% FCS), TM₂ (1 M DMSO, 10% FCS) and TM₃ (10% FCS only). Tissues were incubated for 10 min in each pre-warmed thawing solution (37°C) on a roller mixer. After thawing, cortical tissue was cultured in RCCS for 5 days.

Determination of oestradiol, progesterone and androstenedione concentration

The concentration of oestradiol, progesterone and androstenedione produced by tissues in the spent culture media (fresh-cultured and cryopreserved-cultured groups) during culture in RCCS was determined using a competitive ELISA method, which has been optimised and validated in-house. To account for a possible effect of size differences in cortical sections incubated in the RCCS, steroid concentrations are presented as steroid production (ng) per volume of tissue (mm^3).

Figure 1

Detailed schematic diagram of materials and methods describing the experimental units.

MiRNA extraction, cDNA synthesis and real-time PCR

Total RNA in the spent culture media for cortical tissues (fresh-cultured and cryopreserved-cultured groups) were extracted using a Qiagen miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions.

Total RNA concentration was determined using a NanoDrop spectrometer measuring the absorbance at 260 nm. Purity was measured by calculating the 260/280 ratio. The TaqMan™ advanced miRNA cDNA Synthesis Kit (AB Applied Biosystems) was used for the preparation of cDNA templates from the entire pool of mature miRNAs in each sample according to the manufacturer's instructions. cDNA template (miRNA reaction product) was diluted (1:10) and PCR reaction mix was prepared using PCR master mix and human miRNA-specific primers (Table 1) according to the number of samples. The diluted cDNA template for each sample was added to a 96-well PCR plate containing the PCR mix (Applied Biosystems) and Real-time PCR reactions were performed using 7500 Fast Real-Time PCR System (Applied Biosystems) with following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in triplicate. The level of miRNA expression was measured using the threshold cycle (CT), miRNA PCR results (CT values) were normalised against the internal control miR-26b using $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

Follicle determination in ovarian cortical tissue

PFA-fixed cortical tissues from all groups (fresh, fresh-cultured and cryopreserved-cultured) were processed, embedded in paraffin and sectioned into 5 µm thin serial sections using a microtome (Leica). Approximately 100–105 sections were made from each ovarian cortical tissue and stained using H&E.

To estimate the number of ovarian follicles of the categories described below, slides that contained sections 50 µm apart where selected, to avoid double-counting of follicles. Follicles were categorised according to the

morphological appearance of GCs based on (Telfer *et al.* 2008) as follows: (1) primordial follicles, an oocyte surrounded by a few squamous GCs; (2) transitional follicles, an oocyte surrounded by squamous and at least one cuboidal GC; (3) primary follicle, an oocyte surrounded by a single layer of cuboidal GCs; (4) secondary follicle, an oocyte surrounded by two or more layers of cuboidal GCs and (5) early antral follicle (Telfer *et al.* 2008).

Statistical analyses

Data in this study were reported as means ± s.e.m. unless otherwise specified. Obtained data were checked for normality using Kolmogorov–Smirnov/Shapiro–Wilk test and log transformed when necessary. Data were analysed using GenStat statistical software (v19; VSNi, UK). Repeated-measures ANOVA was used to compare miRNA expression, oestradiol, progesterone and androstenedione concentrations between fresh-cultured and cryopreserved-cultured groups. Follicle populations were not normally distributed and therefore transformed, compared between fresh, fresh-cultured and cryopreserved-cultured groups using two-way ANOVA and Tukey test. A *P* value of <0.05 was considered statistically significant.

Results

Ovarian steroidal production

Throughout culture, as shown in Fig. 2, oestradiol production was significantly lower in cryopreserved-cultured tissues compared to fresh-cultured tissues ($P < 0.001$; Fig. 2). Despite, an increased observed in oestradiol in the spent culture media produced by fresh-cultured cortical tissue and an apparent decrease in cryopreserved-cultured tissue from Day 1 to Day 5, day of culture was not significantly different ($P = 0.894$; Fig. 2). There was statistically significant interaction between treatment and day of culture ($P = 0.025$), indicating that in fresh-cultured tissue, oestradiol concentrations increased in Days 4 and 5, contrary to what occurred in cryopreserved-culture tissue.

Progesterone production was similar between fresh-cultured and cryopreserved-cultured groups during the initial 3 days in culture (Fig. 3). However, from Day 4, progesterone production decreased abruptly in cryopreserved-cultured tissues but increased in fresh-cultured tissue ($P = 0.02$; Fig. 3). Moreover, while progesterone concentrations seemed to increase throughout culture, those from cryopreserved-cultured

Table 1 TaqMan probe choices and their corresponding mature miRNA sequence in humans (Griffiths-Jones *et al.* 2006).

TaqMan probe	Mature human miRNA sequence
Human: has-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG
Human: has-miR-193b-3p	AACUGGCCCUCAAAGUCCCGCU
Human: has-miR-320A	AAAAGCUGGGUUGAGAGGGCGA
Human: has-miR-26b-3p	CCUGUUCUCCAUAUACUUGGCUC

Figure 2

Oestradiol concentration (ng) per volume of tissue (mm³) in the spent culture media for both fresh and cryopreserved cortical tissue, over 5 days of culture. Significant differences were noted in oestradiol concentrations between groups (fresh-cultured and cryopreserved-cultured) ($P < 0.001$) and its interaction with culture day ($P = 0.025$; indicated by *), but not in culture day within treatments ($P = 0.894$).

tissue decreased in Days 4 and 5 of culture ($P = 0.013$ treatment $\times$ culture day; Fig. 3).

Concentrations of androstenedione in spent culture media between fresh-cultured and cryopreserved-cultured groups followed a similar pattern to those of progesterone (Fig. 4). Production of androstenedione was comparable between both groups during the initial 3 days of culture, but from Day 4 onward, while concentrations decrease in cryopreserved-cultured tissues, they reach maximal

Figure 3

Progesterone concentration (ng) per volume of tissue (mm³) in the spent culture media for both fresh and cryopreserved ovarian cortical tissue, over 5 days of culture. A significant difference was observed between treatments (fresh-cultured and cryopreserved-cultured; $P = 0.02$), culture day ($P = 0.02$) and there was a significant interaction between these ($P = 0.013$). Different letters indicate these interaction ($P = 0.013$).

Figure 4

Androstenedione concentration (ng) per volume of tissue (mm³) in the spent culture media for both fresh and cryopreserved cortical tissue, over 5 days of culture. A significant difference was observed between treatments (fresh-cultured and cryopreserved-cultured; $P < 0.001$) but not between culture days ($P = 0.646$) nor was there an interaction between terms ($P = 0.11$).

production in fresh-cultured tissues ($P < 0.001$; Fig. 4). However, within groups no differences were observed in androstenedione concentrations between different days of culture ($P = 0.646$).

Expression of miRNAs

Relative expression of miRNA-24 was higher in cryopreserved-cultured tissues throughout culture compared to fresh-cultured tissues ($P < 0.001$; Fig. 5). However, relative expression of miRNA-24 in cryopreserved-cultured tissue increased from Day 1 to Day 2, but decrease on Days 3 and 4, and suddenly increased on Day 5 ($P = 0.031$) (Fig. 5). For fresh-cultured tissues, relative expression of miRNA-24, decreased from Day 1 to Day 4 but increased on Day 5 ($P = 0.031$) (Fig. 5).

Relative expression of miRNA-193b in cryopreserved-cultured tissue was detected on Day 1 (Fig. 6) but appeared to be downregulated (no detectable expression) in subsequent days ($P = 0.043$). Concentrations of miRNA-193b were higher in fresh-cultured compared to cryopreserved-cultured tissues on Day 1 (Day 1 = 58.4, $P = 0.004$). However, expression in fresh culture decreased from 58.4 to 3.4 on Day 1 to Day 3, respectively, to then steadily increased to 7.6 on Day 5 ($P = 0.043$) (Fig. 6).

Relative expression of miR-320A showed higher expression on the first day in culture and then a decline on Day 2 with concentrations remaining constant there on ($P = 0.001$; Fig. 7). However, expression levels of this

Figure 5

Log concentration of miRNA-24 relative to miRNA-26b, in the spent culture media of both fresh and cryopreserved cortical tissue, over 5 days of culture. A significant difference was noted in the expression of miRNA-24 between treatments (fresh-cultured and cryopreserved-cultured) ($P < 0.001$) and culture day ($P = 0.031$), with no significant interaction between these factors observed ($P = 0.089$).

miRNA were lower in cryopreserved-cultured tissue ($P = 0.049$) compared to tissues without freezing-thawing throughout culture (Fig. 7).

Proportion of follicles in ovarian cortical tissue

The proportion (percentage) of follicles present in a total of 24 ovarian cortical tissues section was histologically estimated: fresh ($n = 8$ sections); cryopreserved-cultured

Figure 6

Log concentration of miRNA-193b relative to miRNA-26b, in spent media of both fresh and cryopreserved ovarian cortical tissue, over 5 days of culture. A significant difference was noted in the expression of miRNA-193b between treatments (fresh-cultured and cryopreserved-cultured) ($P = 0.004$) and culture day ($P = 0.043$), with no significant interaction between these factors observed ($P > 0.05$).

Figure 7

Log concentration of miRNA-320A relative to miRNA-26b, in spent media of both fresh and cryopreserved ovarian cortical tissue, over 5 days of culture. A significant difference was noted in the expression of miRNA-320A between treatments (fresh-cultured and cryopreserved-cultured) ($P = 0.049$) and culture day ($P = 0.001$), with no significant interaction between these factors observed ($P = 0.431$).

($n = 8$ sections) and fresh-cultured ($n = 8$ sections). The number of primordial follicles decreased ($P < 0.05$) and late-stage follicles increased in fresh-cultured and cryopreserved-cultured groups compared to fresh cortical tissues (Table 2). Two-way ANOVA and Tukey test showed a significant difference between fresh and fresh-cultured cortical tissue for primordial ($P = 0.0014$), transitional ($P = 0.0019$) and secondary ($P = 0.0027$) follicles, fresh and cryopreserved-cultured cortical tissue of primordial ($P < 0.001$), transitional ($P = 0.0018$) and primary follicles ($P = 0.0393$), as well as fresh-cultured and cryopreserved-cultured cortical tissue in case of primary ($P = 0.0131$) follicles (Table 2).

Discussion

Ovarian tissue cryopreservation was associated with a differential expression of the studied miRNAs and a reduction in steroidal hormone production. Furthermore, primordial follicle number decreased nearly 50 and 25% in cryopreserved-cultured and fresh-cultured tissues, respectively, compared to fresh tissues. To the best of the authors' knowledge, this is the first study to identify a possible correlation between cryopreservation, hormones (oestradiol, progesterone and androstenedione) and miRNAs' in spent culture media. The protocol used was successful at obtaining and measuring miRNAs' expression and oestradiol, progesterone and androstenedione in spent culture media, and thus, provides a novel non-invasive way to estimate tissue quality in culture.

Table 2 Proportion (percentage) of follicles in fresh, fresh-cultured and cryopreserved-cultured cortical tissues.

Cortical tissues	Fresh	Fresh-cultured	Cryopreserved-cultured
Total follicles	884	779	526
Primordial follicles	76 ± 4.2% (a)	45 ± 4.6% (b)	27.3 ± 3.4% (b)
Transitional follicles	22.8 ± 2.1% (d)	23.8 ± 4.3% (c)	35.8 ± 2.7% (c)
Primary follicles	7 ± 1.3% (f)	8 ± 1.4% (f)	6 ± 1.5% (e)
Secondary follicles	2.8 ± 1.2% (h)	4 ± 0.9% (g)	6.8 ± 1.1% (g)
Early antral follicles	2.4 ± 0.3%	3 ± 0.8%	4.9 ± 0.9

Different letters indicate significant differences between fresh, fresh-cultured and cryopreserved-cultured groups within same follicle category ($P < 0.05$).

The elevated expression of miRNA-24 in spent culture media for cryopreserved-cultured tissues may be due to solution effect toxicity of CPA during slow freezing. On the other hand, for cortical tissues cultured without freezing and thawing, cells recover and more follicles have activated during the culture process, resulting in decreased expression of miRNA-24. However, toward the end of culture, cells and tissue damage may have caused elevated expression of miRNA-24. Interestingly, miRNA-24 has also been described as a regulator of oestradiol concentration; increased miRNA-24 expression resulting in decreased transforming growth factor (TGF) signalling, and ultimately leading to inhibition of oestradiol secretion (Sang *et al.* 2013). Another recent study has shown that transfection with miRNA-24 mimics in the steroidogenic human granulosa-like tumour cell line, KGN, resulting in decreased oestradiol secretion (Sorensen *et al.* 2014). MiRNA-24 also regulates the genes of cellular survival pathway and is associated with reduced cellular viability (Manvati *et al.* 2015), also induce apoptosis and DNA damage in breast (Srivastava *et al.* 2011) and ovarian cancers (Sorensen *et al.* 2014, Manvati *et al.* 2015). Our findings support this inverse relationship between miRNA-24 expression and oestradiol concentration measured over the 5 days of culture for fresh-cultured and cryopreserved-cultured cortical tissue.

For fresh-cultured ovarian cortical tissues, relative expression of miRNA-193b in the spent culture media was detected throughout the culture period (Fig. 6), but expression showed a downward progression on days 1–3 followed by an increase in days 4–5. This may be linked to initial adaptation to culture and to follicular survival, as shown by increased oestradiol production, followed by tissue damage during later culture stages. Contrary to the previous, expression of miR-193b was only detected on Day 1 in cryopreserved-cultured ovarian cortical tissue (Fig. 6). Abnormal downregulation of this miRNA is frequently observed in human disease (Sorensen *et al.* 2014) such as pancreatic cancers (Jin *et al.* 2015) and systemic sclerosis (Iwamoto *et al.* 2016). Reduced

expression of miRNA-193b is also linked to increased expression of caspase-3, which is an important marker for cellular apoptosis (Wu *et al.* 2017). Therefore, changes in expression observed in this study support the role of miRNA-193b as apoptosis regulator.

The relative expression of miRNA-320A in spent culture media was detected on Day 1 in fresh-cultured and cryopreserved-cultured tissues, but higher in the former group (Fig. 7). As observed in Fig. 7 concentrations decreased in a similar pattern in both groups as culture progressed. MiRNA-320 expression has been previously reported in human follicular fluid (Feng *et al.* 2015) and along with miRNA-24, it is also involved in oestradiol regulation (Sang *et al.* 2013), with increased expression levels of miRNA-320A is linked to decrease in the level of oestradiol (Yin *et al.* 2014). Interestingly, a similar relationship is also observed in this study with oestradiol concentrations in spent media increasing in fresh-cultured tissue as expression of miRNA-320 decreased. Nonetheless, oestradiol levels in spent culture media remained low in spite of continuous low expression of miRNA-320A from cortical tissue undergoing cryopreservation and culture. Continuous low expression of miRNA-320 in case of cryopreserved-cultured tissues may be related to adverse cryopreservation effects resulting in increased cellular loss.

Previous work from our laboratory involving ovine ovarian cortical tissue culture in the RCCS showed improved survivability of the follicles compared to conventional methods. This vessel simulates the 3D environment in which tissue normally develops and functions (Barrila *et al.* 2010). Another advantage is waste materials around tissue are diluted with media due to periodic circulation of media flow (Morabito *et al.* 2015). This enabled the ovarian cortical tissue to secrete their miRNAs, steroid hormones and other substances into the culture media as close to what they normally would do *in vivo*. The ovarian cortical tissue cultured in a 3D culture vessel demonstrated a decrease in the number of primordial follicles and increase in the number of

more developed follicle stages in fresh-cultured and cryopreserved-cultured tissue compared to fresh cortical tissue. Surgical sectioning of the cortical tissue, as well as tissue processing, may induce ischaemia which leads to the loss of primordial follicles (Campos *et al.* 2011). On the other hand, culture of ovarian cortical tissues in RCCS have increased activation of follicles which in turn have increased their number than the fresh cortical tissues.

Higher concentrations of steroid production were detected in cortical tissue during RCCS culture in later stages of fresh-cultured compared to cryopreserved-cultured tissues (Figs 2, 3 and 4). Detection of these steroids in the spent culture media for both fresh-cultured and cryopreserved-cultured cortical tissue is an indication of steroidogenic function of the cortical tissue (Xu *et al.* 2011). Previous studies have also suggested that steroidogenic activity is maintained in the ovarian cortical tissue after cryopreservation by slow freezing (Campos *et al.* 2011). In this study, oestradiol and androstenedione were higher in fresh-cultured tissues which is consistent with aromatisation of androstenedione into oestradiol at these stages. Furthermore, progesterone concentrations were higher toward the end of culture in fresh-cultured tissues, which also indicates active steroidogenic pathways. Bastings *et al.* in 2014 reported a decline in the level of oestradiol and progesterone in the spent culture media from the cortical tissue undergoing freezing-thawing and culture for 7 days (Bastings *et al.* 2014). Loss of follicles of about 30–60% have been reported in various studies where ovarian cortical tissue has been frozen using slow freezing technique (Maltaris *et al.* 2006, Silber *et al.* 2010, Campos *et al.* 2011). Taking together, steroidal production indicates that fresh-cultured tissues were able to adapt better to the culture conditions and survive up to 5 days, with higher oestradiol, androstenedione and progesterone production compared to cryopreserved-cultured tissues.

For androstenedione production, there was a continuous downward progression in case of ovarian cortical tissue undergoing culture following freezing-thawing apart from Day 3 where the concentration spiked suddenly, while cortical tissue cultured without freezing showed a steady increase. It is expected that androstenedione production would parallel the oestradiol production because it is largely a result of aromatisation of androgens by the enzyme aromatase (George & Wilson 1978).

There were significant differences noted between miRNA expression pattern in spent culture media and follicle number for ovarian cortical tissues cultured with or without freezing. We therefore plan to investigate

further the relationship between these miRNAs and follicle survivability, as well as elucidate into which specific cell types are expressing those miRNAs in our next study

In conclusion, downregulation of miRNA-193b and miRNA-320A along with upregulation of miRNA-24 for cryopreserved ovarian cortical tissue cultured in RCCS might have a synergistic role in cell apoptosis and consequently reduced oestradiol and progesterone production. As those miRNA markers paralleled oestradiol and progesterone concentration production by the follicular GCs, it appears they are good indicators of apoptosis occurring within the granulosa compartment of follicles when cultured in RCCS. Thus, there appears to be an interplay between these miRNAs and oestradiol and progesterone production and these could be further explored as novel markers of cell injuries and/or damage following cryopreservation in RCCS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The original experiment was designed by N I and W E M. N I and U P S performed all the laboratory procedures. Data collection, analysis and interpretation were done by N I, whereas W E M, J H M and P M made the critical evaluation and final data analysis approval. N I and W E M prepared the manuscript which all authors have read and approved.

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