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# *IN VITRO* 17ß-OESTRADIOL RELEASE AS A MARKER FOR FOLLICULAR SURVIVAL IN CRYOPRESERVED INTACT BOVINE OVARIES

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## Abstract

Transplantation of cryopreserved intact ovaries from cancer patients is a technically challenging option for restoring fertility after sterilizing cancer therapy. In this paper we describe an assay based on 17ß-oestradiol (oestradiol) production, to monitor the functional damage sustained by the ovarian tissue during the freeze/thawing procedure. To this end, fresh bovine ovarian cortical biopsies were cultured *in vitro* for 7 days. As a control, the oestradiol release of biopsies that had sustained maximal cryodamage was analyzed. In addition the oestradiol release by cortical biopsies from two $ME_2SO$ perfused and cryopreserved intact ovaries was analyzed. Oestradiol production could be measured in culture supernatants, while oestradiol release of maximal cryo-damaged biopsies was at background levels. *In vitro* oestradiol release by cortical biopsies can be used as a functional marker for cryo-damage and indicates that our assay is suitable to optimize the cryopreservation procedure of intact ovaries.

**Keywords:** ovary, cryopreservation, oestradiol, in vitro assay, intact organ

## INTRODUCTION

New protocols in the early diagnosis and treatment of cancer have led to major improvements in the long-term survival of oncological patients. However treatment of cancer with aggressive chemotherapy, especially when using alkylating agents and/or radiotherapy of the pelvic region, often leads to infertility, due to the damage of the follicles and/or oocytes that are present in the ovaries (31). Safeguarding their reproductive potential is a very important issue for women that have not yet started or completed their family.

Several options, including ovarian tissue cryopreservation and transplantation, can now be offered to these women to (partially) preserve their fertility. New experimental approaches using *in vitro* growth of isolated human follicles are also explored (7, 8, 27).

Ovarian tissue can be frozen and grafted as cortical fragments, or as an intact ovary including its vascular pedicle (9). The cryobiological and surgical aspects of the preservation and transplantation of an organ *in toto*, is technically clearly more challenging than the cryopreservation and transfer of cortical fragments. The advantages of this approach are obvious; immediate revascularization of the transplanted ovary ensures that less ischemic damage will be inflicted to the ovarian tissue post-thawing, and that more follicles are likely to survive. In addition menses, normal reproductive functions, and normal hormonal status can be restored (4).

In the last few years, freezing and autologous grafting of whole ovaries has been attempted in several mammalian species, including rats (32, 34), rabbits (5), pigs (16, 17), and sheep (2, 4, 6, 16, 26), yielding promising results. In sheep, this procedure has even resulted in live offspring (16). However, it should be kept in mind that ovine ovaries are considerably smaller than human ovaries (11). Therefore, it remains to be seen whether cryopreservation of larger intact organs such as human ovaries can be performed equally successful, as volume has a considerable effect on parameters that affect successful cryopreservation. Although cryopreservation of an intact human ovary with its vascular pedicle has been described (3, 23), transplantation of a cryopreserved and thawed ovary has not yet been reported. Ideally, the optimal cryopreservation procedure, which obviously requires an assay that is able to detect cryo injury to preantral follicles, should be determined before cryopreservation and transplantation of human ovaries can be efficiently performed. In this paper, we used our previously described model system (11) employing bovine ovaries, to develop a suitable monitoring system for follicular survival after cryopreservation. Follicular survival in previous studies has been assessed predominantly by using cell based assays such as trypan blue exclusion, apoptosis assays, immunohistochemistry and conventional histology. The outcome of these assays has demonstrated a high percentage of viable follicles after cryopreservation (22). Whether these results represent functional follicular survival is not clear. In addition, follicular survival was analyzed directly after thawing of the tissue (22), whereas for instance apoptosis may take several hours to become manifest (15, 33). For this reason, we argued that an assay that specifically monitors functional follicular survival for several days after thawing of the ovarian tissue is a prerequisite for optimizing the cryopreservation process. Ideally, this assay should monitor the survival of primordial and primary follicles, as these are needed for replenishing the pool of developing follicles after retransplantation. On the other hand, secondary and antral follicles have been shown to be more prone to cryodamage, due to the fact that they are metabolically active, and contain fluid that may, by the formation of ice crystals, increase the induction of cryodamage (12). Monitoring the survival of the latter subsets of follicles may therefore be a more sensitive assay for determining cryodamage sustained during the cryopreservation process. We therefore studied the production of 17ß-oestradiol, as this is produced exclusively by viable secondary and antral follicles (1). Production of oestradiol by cortical biopsies *in vitro* was monitored during a culture period of 7 days. We show that oestradiol is produced *de novo* by cortical biopsies, and is related to the amount of cryo damage that is induced in an intact ovary by cryopreservation. Therefore, we consider this assay to be a valuable addition to the current array of techniques that are available to optimize the cryopreservation procedure of intact human ovaries.

## MATERIALS AND METHODS

*Tissue collection*. Ovaries were obtained from freshly slaughtered adult cows at a local abattoir, as described previously (11). Ovaries that were used for direct culturing of biopsies were collected on ice and transferred to the laboratory for further preparation. Ovaries that

were used for cryopreservation *in toto* were perfused with 15 ml of a PBS/heparin (50 IE/ml, Leopharm, Breda, The Netherlands) solution via the *vena ovarica* that was transected approximately 10 cm under the ovarian hilus. Vessels branching from this vein were occluded using sutures. After perfusion ovaries were placed on ice and transferred to the laboratory within 30 min.

*Cortical biopsy preparation and culturing*. Ovarian biopsies were collected from 15 different ovaries, derived from 15 individual animals. Each ovary corresponds to a separate experiment. Only areas free from visible follicles were used to obtain biopsies. Using a biopsy punch and scissors, biopsies with a diameter of 6 mm and an approximate depth of 2 mm were obtained. Biopsies were transferred to 6-well plates (Falcon, Heidelberg, Germany) for culturing. For each ovary duplicate wells were prepared, each of which containing 3 fresh biopsies in 5 ml of culture medium (DMEM supplemented with 10% FCS, 100IU per ml of penicillin and 0.1 mg per ml of streptomycin). After 4 days, culture medium was collected and fresh medium was added to the wells. In some experiments, biopsies were cultured in the presence of 2 U/ml urinary human FSH and 2 U/ml LH (Menopur, Ferring, Hoofddorp, the Netherlands). In addition, fresh biopsies were collected in a cryovial (Nunc, Roskilde, Denmark), and subjected to maximal cryo injury by 3 cycles of freezing and thawing by alternate incubation in liquid nitrogen and 37 $^{o}$C for 5 minutes each. Next, biopsies were cultured in 5 ml of medium as described for fresh biopsies. Culture supernatants were analysed for oestradiol at day 4 and day 7.

*Oestradiol levels in culture supernatants.* Oestradiol was measured by radioimmunoassay after extraction and Sephadex-20 chromatography (30). The sensitivity of the assay was 10 pmol/l. The within-assay coefficient of variation was 4.9% at a level of 510 pmol/l. The between-assay coefficient of variation of the mean of duplicate measurements was 5.6%.

*Cryopreservation and thawing of intact ovaries.* Ovaries were immersed in a bath containing 25 ml of the cryoprotective solution (10% dimethylsulphoxide ($ME_2SO$) in DMEM supplemented with 2% FCS) and perfused with this same solution using a peristaltic STC-521 Syringe pump (Terufusion, Tokyo, Japan) at a flow rate of 2.5 ml/min for 5 min (23). After perfusion ovaries were transferred to a 5100 Cryo Freezing Container (Nalgene, VWR, Belgium) containing 2-propanol, precooled at 4 $^{o}$C . The container was stored at -80 $^{o}$C overnight, allowing for a gradual decrease in temperature. Subsequently, the cryovial was stored in liquid nitrogen for at least 1 week. Ovaries were thawed at 60 $^{o}$C for 10 min, followed by an additional incubation at 37 $^{o}$C for 20 min. After thawing, the ovaries were removed from the cryovial, and submerged in 25 ml of DMEM. The cryoprotectant was gradually removed by 3 consecutive perfusion steps for 10 min at room temperature at a flow rate of 2.5 ml/min using DMEM with 0.25 M sucrose, DMEM with 0.1 M sucrose and DMEM without sucrose, respectively. Biopsies from cryopreserved/thawed ovaries were prepared and cultured as described for fresh ovaries.

*Histochemical staining of follicles*. Sections of ovaries were fixed O/N in phosphate buffered 4% formaldehyde, and embedded in paraffin. Five μm sections were stained with Azan trichrome histological staining (13), a staining method that highlights connective tissue in darkblue, whereas cellular nuclei are stained bright red. Tissue sections were analysed by conventional light microscopy (x100) and photographed.

*Ovarian biopsy lysates.* Per ovary, 3 snap frozen fresh (non-cultured) biopsies were pulverized by using either a mortar and pestle (5 ovaries) or by using a Mikro-Dismembrator II (4 ovaries) (Braun Biotech, Melsungen, Germany). Each ovary was derived from a separate animal. To allow for direct comparison with the results from cultures with intact tissue biopsies, 5 ml of medium was added to the pulverized samples, and oestradiol was allowed to diffuse into the culture medium overnight. Subsequently, supernatants were analyzed for oestradiol content.

*Statistical analysis.* Data were tested for normality using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com, and found to be normal. Two-sided Student's t-test was performed using the same software package.

## RESULTS

*Oestradiol release by cultured ovarian biopsies.* We evaluated the usefulness of oestradiol release as a marker for follicular viability before and after sustained cryo-injury. Duplicate sets of three cortical biopsies were cultured in vitro for 4 or 7 days, after which oestradiol levels in the culture supernatant were assessed. To induce maximal cryo-damage (MCD), additional sets of biopsies from the same ovary were subjected to three freeze/thaw cycles before culturing. A representative experiment is shown in figure 1. After 4 days of culture,

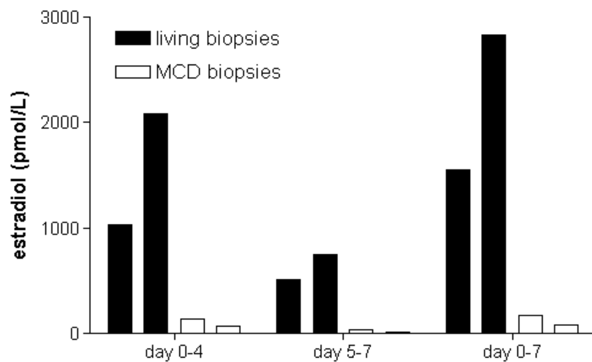


**Figure 1**. *In vitro* oestradiol release (duplicate measurements) by bovine cortical ovarian biopsies. Solid bars: viable biopsies; open bars: biopsies frozen and thawed 3 times to induce maximally cryo-damage. Oestradiol levels in culture medium were assessed after 0-4 days and 5-7 days of culture. Total release (day 0-7) is represented in the right hand set of columns.

oestradiol levels derived from vital biopsies were well above background levels (duplicate measurements of 1030 and 2080 pmol/L versus a mean level of 18 pmol/L in 5 separate unconditioned medium samples). Oestradiol release decreased considerably during the subsequent culture period (day 5-7). In a separate experiment, biopsies were cultured in medium containing a mixture of human FSH/LH. This FSH/LH treatment did not affect oestradiol production by the cortical biopsies (data not shown).

MCD samples released considerably less oestradiol (136 and 75 pmol/L for a duplicate measurement), but still higher than the medium control level. Cumulative oestradiol release during the entire 7 day culture period was approximately 20 times higher for vital biopsies compared to MCD-biopsies, indicating that cryo-damage of ovarian tissue significantly impairs oestradiol release.

Extensive growth of adherent fibroblast-like cells was observed during the culture of the cortical biopsies. Generally, 50 % of the surface of the culture well was covered with these cells after 7 days of culture. This cell growth was never observed in wells containing MCD biopsies, confirming that presumably no living cells were present in these biopsies anymore. To assess the possible contributions of these adherent cells to the measured oestradiol levels, we removed the cortical biopsies from a number of wells, and added fresh culture medium to these wells (without biopsies). After an additional 4 day culture period, oestradiol levels were determined in the medium conditioned by the adherent cells, and found to be below the detection level of the oestradiol assay (comparable to fresh medium, data not shown).

As can be observed in figure 1, the difference between the duplicate oestradiol measurements was quite substantial. In the next set of experiments, we therefore analysed the intra- and inter-ovarian variation in oestradiol release in more detail.

*Intra- and inter-ovarian variation in oestradiol release.* In a set of 15 ovaries derived from 15 individual animals, we compared the differences between duplicate oestradiol

measurements to assess intra-ovary variation (figure 2a). Results are expressed as the fold difference between the duplicates. When both measurements are equal, this results in a fold difference of 1.0. Oestradiol levels were determined after culture periods of 0-4 days and 5-7 days, for both vital biopsies and MCD-biopsies.

The intra-ovary variation in oestradiol release between days 0-4 ranged between 1.1 and 3.5, with a mean of about 1.8. The variation appeared to diminish during the 5-7 days culture period, with a range of 1.0 and 2.3 and a mean of 1.5, but was not statistically different from the variation observed at the day 0-4 period. Obviously, the variation observed in the cumulative oestradiol release (day 0-7) was intermediate (ranges 1.0-2.7, mean 1.7). Variation in oestradiol release from MCD samples was generally lower than the variation found with vital biopsies, but no significant differences were observed. No influence of the phase of the ovary on the level of variation was observed.

Inter-ovary variation was analysed by comparing the mean of duplicate measurements derived from biopsies obtained from the same 15 ovaries, expressed as absolute oestradiol levels (figure 2b). The same parameters as mentioned in the previous section were analysed. A similar pattern in variability was observed as in the intra-ovary variation; variation was clearly lower in the 5-7 day culture period (range 200-905, mean 601 pmol/L) compared to the 0-4 day period (range 305-1750, mean 1015 pmol/L), and variation in MCD samples was lower compared to vital samples (range 135-338 with mean 235, and range 505-2655 with mean 1755 pmol/L, respectively, in cumulative day 0-7 samples). Based on morphological criteria

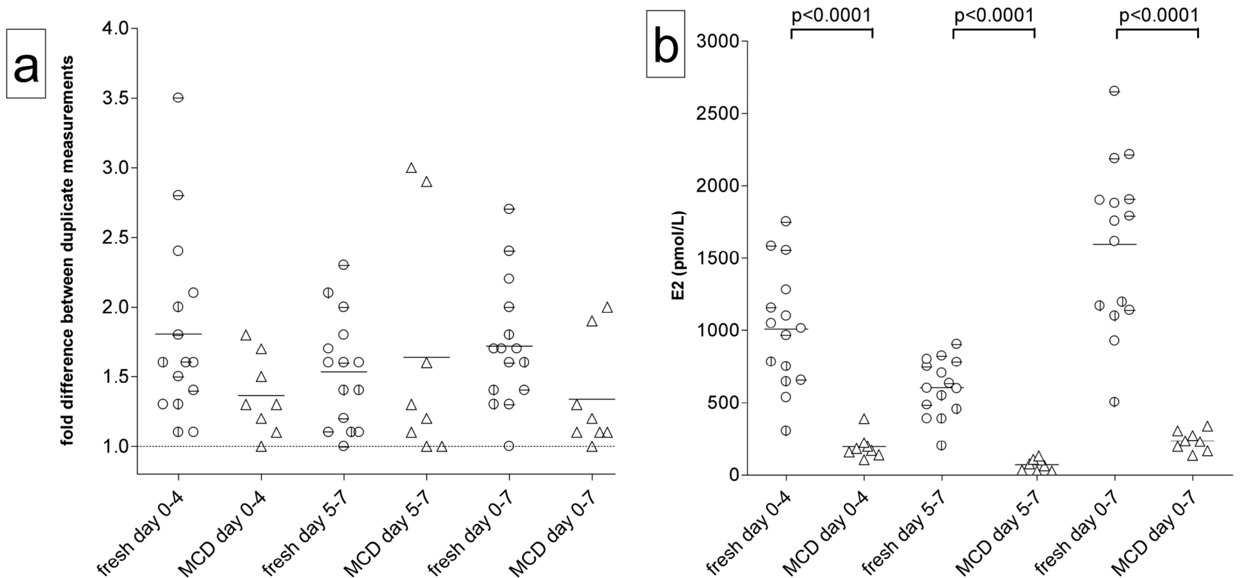


**Figure 2**. Assessment of intra- and inter- ovary variation of *in vitro* oestradiol release by ovarian biopsies, derived from fifteen ovaries, each from a separate animal. Each circle (fresh tissue) or triangle (tissue with maximal cryo damage, MCD) represents a single ovary. Circles with a horizontal bar represent ovaries in luteal phase, with vertical bar ovaries in follicular phase, open circles represent ovaries of unknown phase. Mean values are indicated by horizontal lines. (a) Intra-ovary variation is expressed as fold-difference between duplicate oestradiol levels in culture medium of biopsies derived from an individual ovary. (b) Inter-ovary variation is represented by the absolute levels (pmol/L) of oestradiol release by different ovaries, expressed as mean of a duplicate measurement for each ovary.

(clear absence or presence of a corpus luteum), the phase of the ovaries could be assigned unambiguously in 10 out of 15 ovaries. In general, ovaries in the luteal phase produced more E2 than ovaries in the follicular phase. This difference was actually statistically different ($p=0.02$, 0.01 and 0.01 at culture periods 0-4, 5-7 and 0-7, respectively). Statistical analysis of the effect of the maximum cryo-damage inducing procedure on the level of oestradiol release (figure 2b), showed a highly significant decrease compared to fresh biopsies. This decrease was apparent in the day 0-4 samples ($p<0.001$) , the day 5-7 samples ($p<0.0001$), as well as in the 0-7 samples ($p<0.0001$). This finding, together with the lack of outgrowth of fibroblast-like cells from MCD-biopsies, indicates that the MCD procedure indeed results in non-viable tissue.

*Distribution of follicles in bovine ovarian tissue.* To explain the observed intra- and inter-ovary variation in in vitro oestradiol release by ovarian biopsies, we investigated the distribution of follicles in histological sections of four separate ovaries. Sections were stained in order to highlight follicular structures (figure 3). As highlighted by the intense blue staining, the sub-epidermal part of the cortex is rich in extracellular matrix components. Follicles (oocytes stained red, surrounded by granulosa cells; indicated with white arrowheads in figure 3a and b, and shown in more detail in figure 3c), are oriented beneath this layer. As can be seen in figure 3 a and b (representing different areas of the same ovary) the distribution of follicles in the ovarian cortex was not random, but clustered. As a consequence, biopsies that are taken randomly may contain different numbers of follicles, ranging from several dozens to none at all. This will obviously affect subsequent oestradiol release by the biopsies.

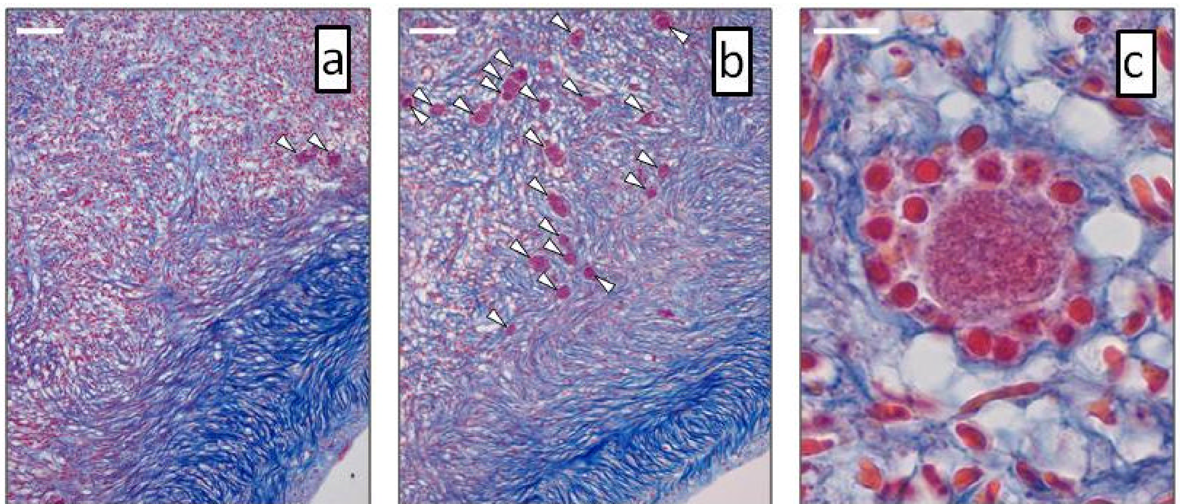


**Figure 3**. Tissue sections of bovine ovaries were stained using the Azan protocol which stains extracellular matrix components to highlight follicles. Note heterogeneous distributions of follicles (white arrowheads), with hardly any follicles present in figure a, while clustering of follicles can be observed in figure b . Figure c shows a magnified pre-antral follicle with an oocyte surrounded by a single layer of granulosa cells. Original magnification: 100x. Scale bar in figure a and b represents 250 µm, and in figure c 50 µm.

*Contribution of resident oestradiol content of ovarian cortical biopsy to total oestradiol release.* Our results with MCD-samples shown in figures 1 and 2, strongly suggest production de novo rather than release by diffusion of oestradiol already present in the ovarian biopsies. We decided to assess the possible contribution of resident oestradiol to the observed total oestradiol release levels in more detail. To this end, we prepared lysates of fresh (non-cultured) ovarian cortical biopsies via two different protocols (mortar or dismembrator), and

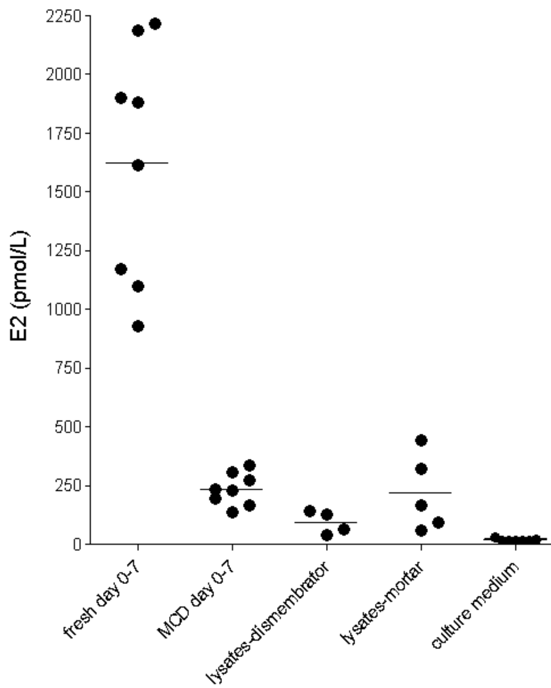


**Figure 4**. Distinction between resident and *de novo* synthesized oestradiol. Oestradiol levels were determined in conditioned medium from fresh tissue, tissue with maximal cryodamage (MCD), and in tissue lysates. Lysates were prepared using two different protocols; dismembrator method and mortar method. Ovaries were derived from separate animals. Levels of oestradiol present in culture medium is shown for comparison.

measured their oestradiol content. As shown in figure 4, the oestradiol present in the two types of lysates generally did not exceed oestradiol levels released by the MCD-samples, indicating that oestradiol released by vital biopsies is indeed constituted almost entirely by de novo production, subsequent diffusion, and release into the medium during the culture period.

*Oestradiol release by biopsies derived from cryopreserved intact bovine ovaries.* To assess the relevance of our assay for the assessment of follicular survival after the cryopreservation of an intact ovary, we perfused two ovaries (independent experiments) with a standard 10% $ME_2SO$ solution, followed by cryopreservation, storage in liquid nitrogen, and subsequent thawing. From these ovaries cortical biopsies were taken, cultured for 7 days, and oestradiol

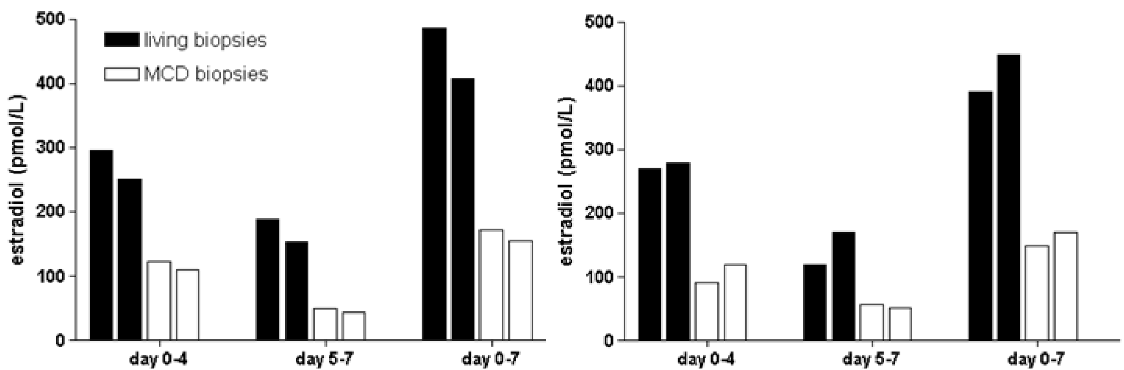


**Figure 5**. Oestradiol release by cultured cortical ovarian biopsies from two $ME_2SO$ perfused and liquid nitrogen preserved intact bovine ovaries, derived from two animals. Oestradiol release by biopsies with MCD is shown for comparison.

levels were determined. For reference values, cortical biopsies from the same ovaries were subjected to the maximal cryo-damage inducing procedure and cultured as well. As shown in figure 5, results obtained from the 2 ovaries were very similar. In both instances, we were able to detect oestradiol levels that were 2-3 times higher in vital biopsies compared to MCD biopsies, indicating that viable 17ß-oestradiol-producing follicles were still present in the

cryopreserved tissue. Compared to the mean oestradiol level released by fresh biopsies from 8 separate ovaries presented in figure 2b, oestradiol release by biopsies from the two cryopreserved ovaries had dropped by approximately 70%, indicating that the standard method of cryopreservation as presented in literature may not yield optimal functional follicular survival. Oestradiol release in the day 5-7 period in the two cryopreserved ovaries was decreased with 56 and 61% respectively, compared to the 0-4 day period. This decrease is in the same range as the mean decrease observed for fresh ovaries (45%, figure 2b).

## DISCUSSION

Of the several options to preserve fertility of women undergoing fertility-threatening therapies, cryopreservation of an intact ovary may in a number of instances have additional value compared to the currently existing options. Considering the difficulties that will be encountered while freezing intact organs such as ovaries, it is clear that selection of the optimal cryopreservation procedure is a prerequisite before this option can be offered to human subjects. This procedure should be aimed specifically at preserving primordial and primary follicles, as these constitute the follicular reserve after retransplantation of the intact ovary.

As oestradiol is only produced by theca-containing follicles (more specifically, secondary and antral follicles), the outcome of our assay is in theory an indication for the number of secondary and antral follicles that is present in the biopsies. We are fully aware of the fact that, as a consequence, our assay does not directly measure the survival of the target population of primordial and primary follicles. Theoretically, the optimal cryopreservation conditions may differ for primordial and primary, versus secondary and antral follicles. This may be due to the fact that cells in different stages of development show different susceptibility to cryodamaging events, and/or react differently to the protective effects of cryoprotectants (10, 29). Data from literature, indeed suggest that secondary and antral follicles do not tolerate freezing and thawing as well as primordial and primary follicles (12). As our assay monitors the most vulnerable subpopulation of follicles, it may therefore be considered as a sensitive assay for the survival of total follicle population.

We have shown that bovine cortical ovarian biopsies produce and release considerable amounts of oestradiol in the culture medium. The oestradiol release was highest during the first 4 days of culture, but also in the consecutive 3 days considerable oestradiol levels could be measured, indicating follicular survival beyond the 4 day period. The observed decrease in oestradiol release after 4 days can most probably be attributed to tissue degeneration in the course of time, as, obviously, our culture system is not designed to sustain the survival of large tissue fragments for prolonged (> 1 week) periods of time. It should be noted that our assay provides information on cryo-damage and follicular survival for up to one week after completion of the cryopreservation procedure, whereas the currently used cellular assays (apoptosis detection etc.) only provide information on cryo-damage that is present at one time point shortly after the procedure. At this time point, the effects of the cryo-damage may not yet have become apparent.

An important consideration for the usefulness of our assay is the origin of the oestradiol we measured in the culture supernatants. In vitro oestradiol release in medium from cultured ovarian tissue has been shown before (14, 18, 19, 21). In general, oestradiol secretion by cortical biopsies *in vitro* could be readily measured up to two weeks of culture (20, 24). However, differences in the species of which the ovarian tissue originated and differences in the amount of cultured cortical tissue, culture volumes and time points of sampling make direct comparison to the results presented in this study very difficult. In theory, oestradiol

found in culture supernatants may represent only resident (meaning already present at the start of the culture) oestradiol that slowly diffuses out of the cortical biopsies during the culture period, rather than oestradiol produced *de novo*. In this study, we actually provide evidence that the observed estradiol levels represent for the larger part estradiol that is produced *de novo*. More specifically, the contribution of resident oestradiol to our measurements was approximately 15% of the amount produced by fresh biopsies, indicating that 85% was newly synthesized oestradiol. The fact that some oestradiol was already present in the cortical biopsies before start of the culture, strongly suggests that oestradiol production is not an artifact induced by the *in vitro* culture process, but rather is a naturally occurring phenomenon.

We observed a considerable intra- and inter-ovary variation in oestradiol production. Several factors may contribute to this variation. One obvious explanation for the observed intra-follicular variation would be a non-random distribution of follicles within the ovarian cortex, resulting in different numbers of follicles (and consequently different levels of oestradiol production) in each cortical biopsy. This was confirmed by performing an extracellular matrix staining that highlighted follicular structures, demonstrating a highly clustered follicular distribution that was also observed in human ovaries (12, 25, 28).

The inter-ovary variation in oestradiol production may be affected by age of the donor, and/or the phase of the cycle at the moment of ovary removal. We have not been able to link the age of the donor to oestradiol production levels. Evidence for an influence of the phase of the cycle was provided by our finding that biopsies derived from ovaries in the luteal phase produced significantly higher levels of 17ß-oestradiol than biopsies derived from ovaries in the follicular phase. Further studies to elucidate this matter are obviously required.

To further assess the usefulness of our assay to evaluate different cryopreservation protocols, we performed preliminary experiments in which we perfused intact ovaries with the $ME_2SO$ containing cryopreservation solution employed by Martinez Madrid et al (23). After freezing of the ovary, storage in liquid nitrogen for one week, thawing and reperfusion to remove the $ME_2SO$, cortical biopsies were cultured and oestradiol was determined in the culture supernatants. Oestradiol production could be readily measured in these supernatants. Compared to the mean oestradiol production by biopsies derived from eight fresh ovaries, the oestradiol production had decreased by about 70%, indicating considerable cryo-damage induced by this particular cryopreservation protocol. Our results thereby clearly demonstrate the necessity for further optimization of the cryopreservation procedure. Furthermore, we show that our assay may provide a valuable addition to the current array of tests and techniques that are available for the assessment of damage induced in cryopreserved ovarian tissue.

The observed variation of oestradiol production levels implies that several ovaries will have to be tested for each different protocol. In future studies we will attempt to reduce this variation by taking the phase of the cycle of the donor into account. In addition, other soluble factors that are produced by subsets of follicles will be assessed for their capacity to act as an indicator of follicular survival after cryopreservation. Other interesting candidates may be locally produced factors such as anti-Müllerian hormone and growth differentiation factor 9, that play an important role in follicular development.

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