[Frontiers in Bioscience 7, d1979-1989, September 1, 2002]

IN VITRO OVARIAN TISSUE AND ORGAN CULTURE: A REVIEW

PJ Devine, KS Rajapaksa, and Patricia B Hoyer

Department of Physiology, The University of Arizona, 1501 N. Campbell Ave P.O. Box 245051, Tucson AZ 85724-5051

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1. ABSTRACT

Many investigations have utilized techniques for culturing ovarian tissue or isolated follicles *in vitro*. Whole ovaries from fetal or neonatal rodents have been incubated in organ culture systems. This has been utilized to understand the sequence of follicle formation and its hormonal requirements, activation of quiescent follicles, follicular growth and development, and acquisition of steroidogenic capabilities. Adaptations of this technique include incubation of ovaries in a chamber continuously perfused with medium or perfusion of medium through the intact vasculature. Late follicular development, ovulation, and steroidogenesis can also be examined in these systems. Another approach has been to culture individual follicles isolated by enzymatic or mechanical dissociation. The majority of this research has focused on improving follicular development *in vitro*. This review will discuss these various culture techniques and some of the results that have been acquired. Recent results from toxicological studies utilizing whole ovarian cultures performed will also be described. Future applications for ovarian and follicular cultures may include *in vitro* follicular development for eventual production of offspring from frozen ovarian tissue, mechanistic studies related to the impact of endocrine disruptors and ovotoxicants on ovarian function, and further investigations into follicle activation and development.

2. INTRODUCTION

2.1 Overall goals of *in vitro* culture

2.1.1. Review of ovarian physiology

The function of the mammalian ovary is two-fold: 1) to produce gametes for reproduction, and 2) to provide the proper environment for successful gamete production, embryo implantation, and growth of the embryo and fetus. The first function is an internal process, assisted by extra-ovarian hormones and growth factors, involving activation, growth, and development of ovarian follicles. The second function involves the synthesis and secretion of various ovarian hormones (estrogens, progestins, activin, inhibin) for regulating cyclic or pregnancy-related changes in other organs throughout the body, especially the uterus, hypothalamus, and pituitary.

Ovarian follicles consist of an oocyte surrounded by supporting granulosa cells, which in later stages of follicular development are in turn surrounded by theca cells. Depending upon the species, follicles are formed at the end of fetal development or shortly after birth. Germ cell proliferation ends and oocytes enter prophase of the first meiotic division. At that time, a very small, quiescent oocyte (approximately 20um, depending on the species) becomes surrounded by a layer of squamous granulosa cells, thus forming a primordial follicle. These quiescent follicles can remain dormant for years. Upon activation by an unknown signal, granulosa cells in a primordial follicle become cuboidal and begin proliferating. During the course of follicular development, the oocyte grows in size, and the follicle acquires theca cells from the surrounding tissue. Granulosa and theca cells work in concert to produce the steroids, progesterone, androstenedione, testosterone, and 17-beta-estradiol. Eventually, an antrum forms within the granulosa cell layers, which becomes filled with fluid. Within this compartment, the oocyte and some tightly opposed granulosa cells (called cumulus cells) are bathed in follicular fluid during the later stages of development. The end product of follicular development is a large antral follicle containing a fully competent oocyte (100um) which is expelled upon ovulation and can continue through meiosis, fertilization, and embryonic development. Most ovarian follicles do not develop to the pre-ovulatory stage, but rather undergo a process of degeneration called atresia. This process is characterized by apoptosis of granulosa cells and degradation of the oocyte, followed by hypertrophy of the theca cells. Of the follicles a female is born with, >99.9% die by atresia. In summary, the morphological alterations that reflect functional changes of follicular development include: 1) granulosa cells becoming cuboidal and proliferative upon activation, 2) growth of the oocyte to full size and meiotic competence, 3) formation of multiple layers of granulosa and theca cells, 4) formation of an antrum, or fluid-filled space, and 5) rupture of the follicle upon ovulation (1).

2.1.2. Goals of ovarian culture

This review will focus on the use of intact ovarian tissue (follicles or fragments) and organs for *in vitro* studies, describing the various experimental systems that have been developed and the questions such research is being used to answer. This chapter is intended to be an introduction to what has been accomplished thus far using these systems and what possibilities exist for the future. The use of primary ovarian cell cultures or cell lines will not be discussed, because that topic is deserving of its own review(s). The techniques for successful *in vitro* culture of intact organs and tissues were established early in this century (2), and have been utilized for many diverse research interests. The use of such systems has been to achieve two main goals: 1) a greater understanding of the physiological regulation of follicular development, ovulation, and atresia in the ovary, and 2) the development of ovarian follicles, possibly from cryopreserved ovarian tissue, to obtain mature oocytes for use in assisted reproduction technologies.

The use of ovarian tissue *in vitro* for experiments that cannot be accomplished easily or definitively *in vivo* can help identify extra-ovarian factors that affect regulation of ovarian function. Such studies might utilize expensive or toxic chemicals, metabolic substrates, nutrients, hormones, or specific agonists or antagonists of physiologically relevant signaling pathways. Further, with the advent of transgenic technologies, the role of individual gene products in ovarian development and function can be examined in tissue from genetically altered mice.

The second objective of gaining the ability to produce mature oocytes from fresh or stored ovarian tissue is an important concern for humans. Specifically, storage of ovarian tissue for future use could potentially be worthwhile in patients being treated with chemotherapeutic agents or in women with active careers postponing child-bearing until later in life. Whereas, cryopreservation of sperm is currently possible, gaining the ability to collect and store ovarian tissue for future use would greatly enhance capabilities for dealing with fertility-related issues in a variety of species. Additionally, this technique could be useful for regulating fertility in economically, environmentally, or scientifically important animals, for example commercially important domestic animals, endangered species, or transgenic or knockout mouse lines.

3. WHOLE ORGAN CULTURE

3.1 Methodology

Techniques for successful ovarian tissue culture have been examined for more than 60 years (3). Studies have been conducted in mice, rats, hamsters, cows, baboons, and humans. Ovarian tissues have been successfully incubated for up to 50 days without visible signs of necrosis (4). Acquiring tissue for culture is fairly straightforward, excising the ovary and dissecting away the extraneous tissue. For studies involving older animals or larger species, it is possible to culture ovarian fragments of approximately $1mm^3$ in size (5-7). Most culture systems have involved placing ovarian tissue onto a physical support in culture wells, dishes, or watch glasses, minimally submerged, but with access to a sufficient quantity of medium below the support (Figure 1A). Incubation conditions of 37ºC and 5% CO_2 in air are typical, though few studies have reported actual

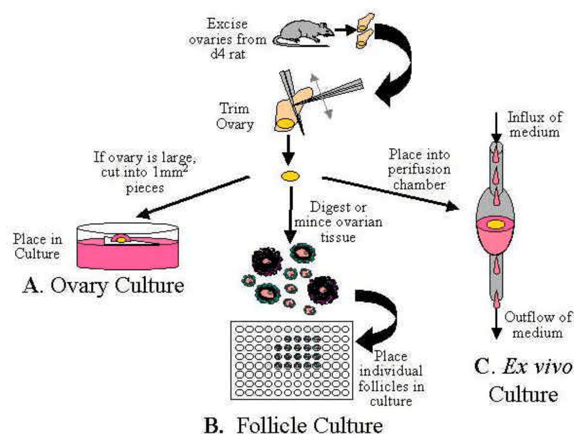

Figure 1. Diagram of three techniques involving *in vitro* culture of ovarian tissue. The techniques shown include A) whole ovarian culture or culture of ovarian fragments in a floating organ culture system, B) individual follicle culture of enzymatically digested or mechanically separated ovarian follicles in individual wells of a culture plate, and C) perifusion of an intact ovary in a flow-through system.

optimization of the culture conditions. Fainstat (8) examined the effects of various oxygen levels (20-95% O_2) on cultured ovarian tissue viability and follicular development. It was reported that insufficient O_2 caused centralized necrosis, whereas too much O_2 caused mitotic inhibition and damage to the periphery of tissues (8). Oxygen levels between 20 and 95% have been utilized (9, 10, respectively). Culture media tested have ranged from 100% serum or other biological extracts (3) to simple, commercially available salt solutions containing albumin as a protein source (11). More recent studies have favored the more defined media in an attempt to avoid exposure of the tissue to unrecognized or unquantified biological components. Required components of medium predominantly remain unknown, but may depend upon the species. All seem to require at least a protein source (BSA, serum). The physical support of ovaries has been achieved using gels, steel mesh, man-made membranes or filters, and even blood clots, all of which seem to perform the proper function. Studies have also been reported in which membranes coated with extracellular matrix proteins were used (laminin, collagen; 12). Characterization of the longest possible duration of tissue survival under various culture conditions has not been carried out, rather in most cases, time points have usually been chosen for expediency or based upon the time frame of expected results. The endpoints examined in such systems have typically included measurement of steroid secretion into the culture medium and histological assessment of stages of follicular development. Interestingly, normal ovulation *in vitro* has been observed, but only in ovarian tissue collected from PMSG-primed rats (13-15).

3.2 Applications

3.2.1. Developmental changes and acquisition of steroidogenic capacity

Many applications for culture systems of whole ovaries or ovarian pieces have involved a simple determination of steroidogenic synthetic capability in the cultured tissue. These types of experiments have led to a better understanding of the developmental timing of enzymatic capabilities and responses to gonadotropins in granulosa and theca cells in various species from fetal development through puberty and adulthood (9, 16, 17). For example, short-term *in vitro* culture of ovaries from postnatal day (pnd) 10-35 rats demonstrated that synthesis of 17-beta-estradiol was possible in pnd10 rat ovaries and was LH dependent (some FSH was present). This activity and responsiveness was lost between pnd12 and puberty, then was re-acquired (18). Monitoring follicular development or changes in steroid production has also helped identify when FSH and LH receptors are expressed during ovarian development. The effects of FSH, LH, and testosterone on steroidogenesis were examined in pnd5 rats by Funkenstein *et al.* (19), and all were found to stimulate both progesterone and estradiol production, supporting the presence of gonadotropin receptors.

The final stages of follicular development and meiotic competence have also been examined *in vitro* (13, 14). These studies determined *in vivo* and *in vitro* the optimal concentrations and time intervals of PMSG and hCG for inducing antral follicle development and ovulation in mouse ovarian fragments. The results showed that 5 IU (*in vivo*) or 3 IU/ml (*in vitro*) PMSG followed 40-48 hours later by 1-5 IU (*in vivo*) or 1 IU/ml hCG (*in vitro*) induced the maximal number of oocytes undergoing maturation, meiotic progression, and ovulation.

3.2.2. Ovarian hormonal and culture requirements

By mimicking the events that occur *in vivo* using an artificial system *in vitro*, researchers have been able to identify and analyze some of the complex factors involved in ovarian follicular development. Hormonal and particularly gonadotropin requirements have been a major focus of studies using ovarian culture. For example, co-culture of pnd5 rat ovaries with anterior pituitaries caused development of larger follicular stages than were obtained with ovaries alone (10), demonstrating the requirement for extra-ovarian hormones. Moreover, in hamster tissue, the addition of FSH and/or LH to the culture medium was beneficial to the development of ovarian follicles beyond the primary follicle stage (20).

Hovatta *et al.* (21), using biopsy material from human patients, demonstrated that ovarian tissue cultured on extracellular matrix-coated (Matrigel) membranes had improved viability of follicles compared to tissue on uncoated membranes. These results were similar to those described by Oktay et al (12), in which mouse ovaries cultured for 10 days on extracellular matrix-coated membranes (collagen laminin) displayed increased follicle density and development over poly-l-lysine coated membranes. These results suggest that follicular development is regulated by more than just the soluble, diffusable components within the ovarian environment. Further research into this topic is needed.

3.2.3. Initiation of Follicular Development

Only recently has the phenomenon of activation of primordial follicles to grow and develop been examined in detail. Though studies have involved examining culture-induced follicular changes in the baboon (7) and the fetal cow

(6), most work on this topic has been performed in rats and mice. Genetically modified or mutant mice have also been used to study these processes *in vivo* (GDF-9, 22; c-kit, 23). While this is a worthwhile approach, the results can be confounded by defects resulting from the effect of an altered gene on fetal development. Ovaries from neonatal rats or mice (pnd4) contain only primordial and small primary follicles, and may be an ideal system in which to examine the factors involved in both the maintenance of quiescence and the activation of primordial follicles. Such studies have identified activating effects of Kit-ligand (11), basic Fibroblast Growth Factor (24), Leukemia Inhibiting Factor (25), and insulin (26) in cultured rat ovaries.

3.2.4. Toxicologic assessment of endocrine-active compounds

Short-term culture of minced ovaries from pregnant (gestational day gd8-17) Sprague Dawley rats have been utilized for part of a Tier 1 screening assay to estimate the abilities of chemicals in both *in vivo* and *in vitro* exposures to inhibit steroid synthesis (5, 27). The greatest level of steroidogenesis was observed in ovaries from pregnant rats on gd8. Neither FSH nor 8-bromo-cAMP induced 17-beta-estradiol or progesterone production, and hCG increased steroid production by 2-3 fold. Ovaries from animals treated *in vivo* demonstrated an increased production of 17-beta-estradiol in response to ICI-182,780 (an estrogen receptor antagonist), whereas anastrozole (an aromatase inhibitor) caused a decreased production in both progesterone and estradiol. In ovaries exposed to chemicals *in vitro*, ICI-182,780 decreased progesterone and increased estradiol production, haloperidol (a D2 receptor antagonist) and anastrozole had no effects, and aminoglutethimide (an aromatase/C27-side chain cleavage mixed inhibitor) decreased estradiol and progesterone production. These results demonstrated that although alterations of steroidogenesis could be detected in the assays using ovarian tissue, false positives and negatives did occur. Such *in vivo* exposures followed by *in vitro* assessments may not be beneficial to most investigations, but are appropriate for the questions to which they were applied in this case.

3.3. Advantages/Disadvantages

Because of the intact structures present *in vitro*, there is a benefit to whole ovarian cultures or culture of ovarian pieces. Follicles in intact ovaries were found to behave differently than dispersed cells *in vitro* (28). Follicular development and interactions among populations of follicles and other cell types present in the ovary can be monitored in such cultured tissue, whereas cell dispersates and individual follicles do not have such complexity. Diffusion of nutrients and gases, however, can restrict the size of the ovary that can be maintained in organ culture. However, cutting larger ovaries into smaller fragments can improve this situation, even though some tissue damage occurs and larger follicles are lost during the processing.

4. ISOLATED FOLLICLE CULTURE

4.1. Methodology

Incubation and *in vitro* development of individual ovarian follicles has been conducted by many laboratories (29-32). To date, the mouse has been the only species for which successful *in vitro* development of pre-antral (33, 34) and primordial (35) follicles to mature eggs and production of viable offspring has been achieved. There are more variables to address when experimenting with cultured follicles or cumulus-enclosed oocytes (CEO's, also called cumulus oocyte complexes or COC's) relative to whole ovarian organ culture. Not only are there obvious differences in ovaries from animals of different ages (neonatal, pubertal, adult), but there are also important differences in follicles placed in culture at different stages of development. The specific combination(s) of the donor's age and the follicle stage being cultured may all have different ideal culture conditions, growth factors, and hormonal milieus that are required. Thus, sequential changes in conditions over the course of culture may be necessary.

There are two principle techniques currently used to isolate ovarian follicles for *in vitro* culture: 1) enzymatic digestion with collagenase and DNase, or 2) mechanical dissection of ovaries using fine needles (Figure 1B). Both techniques can provide viable follicles, as demonstrated by their potential for fertilization and embryonic development at least to the blastocyst stage (35, 36). Many components of the medium have been examined for improving the development of isolated follicles. These have included ascorbic acid, growth factors (epidermal growth factor), and gonadotropins (FSH, LH, hCG). The duration of cultures has been from 24 hours (37) to 4 weeks (21). Culture of intact follicles has been carried out on man-made membranes, poly-l-lysine, extracellular matrix, and embedded in gels. These procedures are used for smaller, pre-antral follicles. Larger antral follicles can be cultured for short periods and are usually directly excised from ovarian tissue (38). Isolated cumulus-enclosed oocytes can be cultured, and are acquired by aspiration of mature antral follicles from gonadotropin-primed animals with fine needles (39). Such structures can be collected even from humans, by ultrasonography-directed aspiration of antral follicles (32). Maturation of CEO's is identified by an expansion or mucification of cumulus cells in response to FSH.

Denuded oocytes have also been cultured following removal of cumulus cells from aspirated CEO's, but the loss of contact between the oocyte and granulosa cells induces premature progression of meiosis (32, 40). Currently, chemical agents must be added to the culture medium of isolated CEO's or denuded oocytes to maintain the oocyte in meiotic arrest (e.g. hypoxanthine, isobutyl-methylxanthine). Spontaneous meiotic progression occurs in the absence of these agents upon release of CEO's or denuded oocytes from the follicle (41). Results suggest that disruption of pre-ovulatory follicles stimulates the oocyte to undergo germinal vesicle breakdown and progress through meiosis (41). Oocyte culture has been performed predominantly using oocytes of larger antral follicles, and endpoints examined predominantly have been germinal vesicle breakdown, although cAMP levels have also been measured.

4.2. Applications

4.2.1. Developmental changes of follicles from animals of various ages

As described above, the culture of isolated follicles is complicated by the donor animal's age and the stage of follicles being collected and cultured. Eppig and O'Brien (35) compared follicle development from animals of different ages, isolating and culturing pre-antral follicles from pnd6-12 mice. Though oocytes grew to similar sizes and fertilized at similar rates, oocytes from pnd6 mice had much poorer blastocyst formation. Few experiments have examined the effect of an animal's age on follicular development in culture, and this should be an area of focus for future research.

4.2.2. Ovarian hormone synthetic capabilities

Even with the small size of individually cultured follicles, some research has included assessment of steroidogenic capabilities of follicles cultured *in vitro*. This endpoint can be used to identify maturation of follicles and differentiation of granulosa cells (29, 42). Production of 17-beta-estradiol in cultured pre-antral follicles signals acquisition of aromatase and progression of granulosa cell differentiation. Addition of gonadotropin(s) to the medium is required for measurable levels of estradiol to be synthesized (29, 43).

4.2.3. Progression of Follicular Development *in vitro*

Most applications of isolated follicle cultures have been to induce and understand normal follicular development, and a thorough review of this research is beyond the scope of this chapter (29, 30, 44). Consistently acquiring mature oocytes from *in vitro* cultured follicles collected from a neonatal animal would be proof of a complete understanding of all requirements for full follicular development. This was accomplished once, though the success rate was low (35). In a two-step process, whole neonatal mouse ovaries were cultured for 8 days, digested enzymatically, and individual follicles were cultured for a further 14 days. Mature oocytes were then fertilized, implanted as 2-cell embryos into recipient females, and one live offspring was produced.

FSH and/or EGF improved development of cultured follicles in those experiments (35). FSH has also been demonstrated to be beneficial to large pre-antral hamster follicles (45) and to early mouse pre-antral follicles (36). Additionally, FSH was included in culture medium at the end of mouse follicle development before fertilization (35). The addition of LH to the medium improved antrum formation in cultured pre-antral mouse follicles, although overall survival in culture (12 days) was not affected (46). Cultured pre-antral mouse follicles were induced to ovulate following 5-6 days incubation *in vitro* by the addition of 5 IU/ml hCG (47) or LH (48). Inhibitors of prostaglandin synthesis or the progesterone receptor blocked ovulation both *in vivo* and *in vitro* (47). Growth of primary follicles was inhibited by Activin A secreted from co-cultured secondary follicles or added to the medium (49). These results suggest a role for Activins in establishing follicular dominance. Collectively, these results are helping to improve both our understanding of follicular development and tools that allow us to study developmental regulation.

4.3. Advantages/Disadvantages

The benefit of isolated follicle cultures over whole organ cultures stems from the ability to monitor the development of individual follicles over time. Studies can focus selectively on follicles of specific sizes, improving our understanding of developmental requirements for follicles at each stage. This system can also be advantageous by improving accessibility of follicles to specific reagents under conditions that could never be achieved *in vivo*. This approach can help identify specific requirements involved in follicular development. Unlike whole ovarian cultures, however, the physical process of acquiring isolated follicles may be somewhat detrimental to follicle integrity and viability. For example, some theca cells are lost from secondary to small antral-sized isolated follicles. Also, any agent added to follicle cultures must have a direct, rather than indirect, effect on those follicles.

5. PERFUSION AND PERIFUSION OF OVARIAN TISSUE

5.1. Methodology

Some researchers have maintained rat ovaries *in vitro* by perfusion of the organs through the vasculature which is dissected out intact with the gonadal tissue. Such a system has been successfully utilized for up to 24 hours. The requirements of proper oxygen and carbon dioxide levels are satisfied by bubbling gas (5% CO_2 in oxygen) through the medium that is perfused into the ovary, and modifications to conditions can be made by altering the components of the medium or the rate of perfusion. Abrahamsson *et al.* (50) perfused ovaries collected from women undergoing hysterectomy and oopherectomy for up to two hours to determine steroid production and responsiveness to hCG in post-menopausal ovaries. A similar technique, called perifusion, involves placing ovaries into incubation chambers in which fluid is pumped past the ovary rather than through the inherent blood vessels (51, 52, Figure 1**C**). Ovaries have been cultured by this method for as long as four days. These techniques sometimes are called *ex vivo* culture of ovaries. Regardless of which method is used, various agents or labeled compounds can also be administered to the ovary through the medium being passed through or around the tissue. Real-time monitoring of ovarian metabolism or hormone production can be accomplished in this system by evaluating the perfusate over the course of the culture period. Ovulations have been reported during incubations of PMSG-primed rat ovaries, as determined by the presence of CEO's in the medium (15, 52). Increases in follicle size and developmental stage can also be determined following culture, fixation of ovaries, and histological processing.

5.2. Applications

The literature suggests that this type of system has predominantly been used to examine the steroidogenic capabilities and the potential for final pre-ovulatory development within a relatively short time frame (24 hours). Perfusion experiments have been conducted using

ovaries collected from rabbits, mice, rats and humans. Using the recirculating perifusion system, hormone and gonadotropin levels have been varied over time, even in pulses as short as one hour to simulate the LH pulses. Morioka *et al.* (15) induced ovulations in ovaries collected from PMSG-primed rats both in the absence and presence of an aromatase inhibitor, suggesting that a pre-ovulatory surge in estradiol synthesis is not required for ovulation. Results suggest that responses to LH pulses are time and threshold dependent (53). The overall development of follicles in this system over a normal rat's estrous cycle, however, were still not identical to those developing *in vivo* (51, 52). In cultured ovaries, there were fewer growing follicles, and ovulating follicles had smaller diameters than those *in vivo*. These results suggest that the required *in vivo* conditions are not yet fully understood.

5.3. Advantages/Disadvantages

The perfusion/perifusion systems of ovarian culture have an advantage over the static ovarian culture system due to the dynamic and rapid changes that can be achieved during perfusion. Diffusion of gases and nutrient transfer may be improved by flow of the medium, though that hypothesis has not been tested. An added potential benefit of these systems may be the ability to use intact ovaries from older animals than can be successfully maintained under static culture conditions. The disadvantages of these systems are the complexity of the perfusion apparatus and the technical skill required for adequate dissection to retain the intact blood vessels.

6. SPECIAL CONSIDERATIONS: TRANSGENIC ANIMALS

As mentioned previously, ovarian cultures allow greater flexibility for adding exogenous agents to determine their effects on ovarian physiology and follicular development. The possibility of examining differences in follicle development and hormone synthesis in ovaries cultured *in vitro* between normal and transgenically modified animals has not yet been attempted. However, with knowledge of how normal ovaries develop *in vitro*, comparisons between wild-type and mutant/knockout ovaries may prove useful. Also, another powerful approach could be to perform gene complementation studies by adding a missing gene product to the culture medium. The potential effectiveness of this strategy has been demonstrated in ovaries from unmodified animals with the addition of growth factors, receptors, or ligands regulating primordial activation (c-kit/kit ligand, 11, GDF-9, 54).

7. FUTURE APPLICATIONS: TOXICITY STUDIES

Few toxicological studies have utilized ovarian follicles or tissue in culture. Baker and Neal (55) made comparisons of multiple species' ovarian sensitivities to gamma radiation *in vitro*. Reproductive failure in women undergoing irradiation or chemotherapy suggests the need for such research in the future (56). The screening assay to detect endocrine disruptors capable of inhibiting steroid synthesis reported by Powlin *et al.* (27) described in section 3.2.4 also utilized minced and cultured ovarian tissue from pregnant rats, but only for short-term assessments. Inhibition or induction of steroidogenesis could be detected in response to chemical exposures *in vitro* in ovaries cultured for 3 hours, however results did not completely concur with effects observed *in vivo*. Whereas, this application of *in vitro* technology is important, it has not taken full advantage of the potential flexibility of ovarian culture for use in ovarian toxicological studies. Ovotoxicants known to be effective *in vivo* include cyclophosphamide, epoxide-containing chemicals, products of cigarette smoke including, 9,10-dimethylbenzanthracene, benzo-a-pyrene, and 3-methylcholanthrene, and other alkylating agents used for chemotherapy (57). The mechanisms involved in oocyte loss due to exposures to these compounds remain unknown, and such questions may benefit from investigations *in vitro*. Initial characterization of ovotoxicity caused by one particular chemical, 4-vinylcyclohexene diepoxide (VCD), has recently been investigated in an *in vitro* system, and the results will be summarized below.

7.1. Overview of VCD-induced ovotoxicity

The occupational chemical 4-vinylcyclohexene (VCH) was reported to cause an increased incidence of benign tumors and rare neoplasms in ovaries of female mice treated for two years by oral gavage (58), whereas rats were not affected. This chemical had been determined previously to be mutagenic in bacteria (59, 60). It is a byproduct formed during the manufacture of rubber tires, flame retardants, anti-oxidants, pesticides, and plasticizers. Subsequent studies in mice have determined that the diepoxide metabolite of VCH, VCD, is responsible for the toxicity which results in depletion of small pre-antral ovarian follicles in mice and rats (61, 62). Dosing with VCH for 30 days (800 mg/kg, ip) led to premature ovarian failure in mice within one year (63). This chemical, though not widely distributed in the environment, provides a model ovotoxicant for further investigation into possible general mechanisms of ovarian follicle loss.

7.2. Species specificity

The species specificity of VCH and VCD was examined through assessment of ovarian follicle counts and measurement of metabolites in exposed rats and mice. Administration of VCH (800 mg/kg, ip) caused loss of pre-antral follicles in female mice, but not rats, following 30 days of daily intraperitoneal dosing. In contrast, daily dosing with VCD (80 mg/kg, ip) induced follicular loss in both species (62). As with the year-long study in which mice were treated with VCH, dosing of rats with VCD for 30 days (80mg/kg, ip) also caused reproductive failure within one year (Mayer et al., unpublished). The monoepoxide intermediates were detected in serum of mice at higher levels than in rats dosed with VCH, suggesting that more of the ovotoxic form can be produced in mice. Furthermore, excretion of these compounds was slower in mice than in rats (64). Taken together, these results suggested that the difference in sensitivity to VCH between mice and rats was due in part to differences in bioactivation, detoxification, and excretion between the species.

7.3. Characterization of follicular loss

The time course and specific follicular targets of VCD were also examined. The smallest ovarian follicles

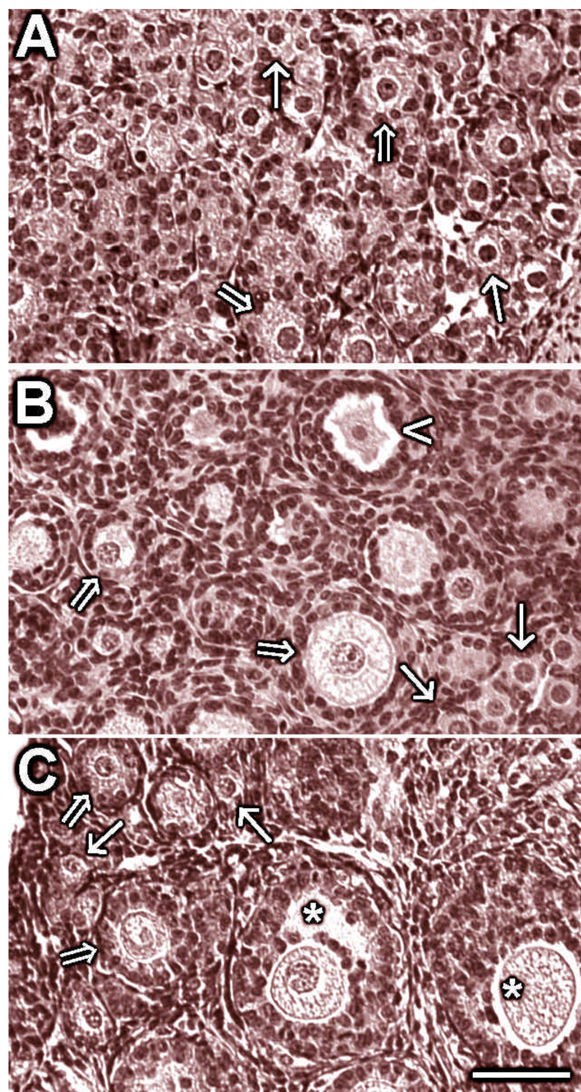

Figure 2. Comparison of morphological appearance of rat ovaries developing *in vivo* or *in vitro*. Ovarian micrographs are A) pnd4 rat, B) pnd4 rat; cultured 15 days *in vitro*, or C) pnd19 rat. Scale bar in C) represents 50um. "←" = primordial follicles; "⇐" = primary follicles; "*" = secondary and small antral follicles; "<" = eosinophilic, unhealthy follicle.

were determined to be selectively depleted by VCD. This was shown by an initial depletion of only primordial and primary follicles (15 days, 65), whereas a reduction in numbers of secondary follicles was seen only after significant loss of the smaller pre-antral follicles had occurred (30d, 66). It was determined that daily dosing of rats with VCD (80 mg/kg/day; ip; 15 days) induces a 50% loss of the smallest ovarian follicles (primordial and primary, 67). This dose was used to determine that significant primordial follicle loss can first be measured after 12 days of daily dosing (65).

7.4. Mechanisms of follicular loss

In order to elucidate the mechanism of VCD-induced follicular loss, evidence for apoptosis was investigated following 10 daily doses of VCD in rats. Small ovarian follicles were isolated after the final dose by enzymatic digestion of ovaries followed by manual sorting according to follicle size. Levels of mRNA encoding *bax* were significantly increased over controls specifically in the ovarian fraction containing small pre-antral follicles that are selectively targeted by VCD (25-100um, 67) following 10 days of daily dosing. Additionally, there was a time-dependent increase at the 10d time point in DNA fragmentation in isolated small ovarian follicles in response to VCD treatment (67). Levels of the apoptosis-related proteins, Bad, Bcl-x_L, and Bax were measured in cytosolic and mitochondrial fractions of small ovarian follicles in rats following 15 days of dosing. Bad protein levels were increased in small pre-antral follicles, whereas Bcl-x_L and Cytochrome C proteins were redistributed from the mitochondrial fraction to the cytosolic fraction, events known to be associated with apoptosis (68). Furthermore, repeated dosing with VCD increased the activity and protein levels of Caspase-3, one of the executioner proteases involved in the final apoptotic signaling pathway. This effect was again specific for isolated small ovarian follicles targeted by VCD (69). The entire specific triggering event(s), however, have still not been elucidated for VCD-induced apoptosis.

8. *IN VITRO* ASSESSMENT OF VCD-INDUCED OVARIAN FOLLICULAR LOSS

8.1. Effects of VCD on Ovarian Follicles in Cultured Rat Ovaries

The potential usefulness of a neonatal rat ovarian culture system has been evaluated for investigations into the ovotoxicity induced by environmental chemicals, such as VCD. Once proven to be effective *in vitro*, the mechanism(s) underlying follicular depletion can be examined in more mechanistic detail. The sensitivity of neonatal rats to VCD-induced follicle loss was established by daily dosing of pnd4 female rats (80 mg/kg, ip, 15 days). VCD dosing caused a selective depletion of primordial and small primary follicles, whereas follicles in later stages of development were not significantly affected (data not shown). This pattern of follicle loss mimicked that seen in older pre-pubertal and adult rats (66). Thus, the sensitivity of small pre-antral follicles to VCD *in vitro* was investigated.

The neonatal rat ovarian culture system was established (11). Ovaries collected from pnd4 rats were cultured in Ham's F12/DMEM medium containing 1mg/ml bovine serum albumin, 1mg/ml Albumax (Gibco, Grand Island, NY), 250uM vitamin C, 5 U/ml penicillin, 5 ug/ml streptomycin, and 27.5 ug/ml transferrin. Following 15 days of incubations, ovaries remain healthy, and follicle development progresses over time (Figure 2A, B). Follicle populations in ovaries collected from pnd4 rats and fixed immediately consist solely of primordial and small primary follicles (Figure 2A). Following 15 days of culture, larger pre-antral follicles containing a single layer of granulosa cells (primary follicles) have developed (Figure 2B). Maximal follicular development under these conditions was, however, limited to small secondary, pre-antral

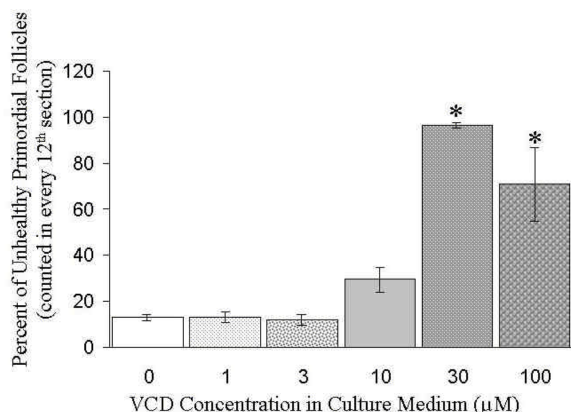

Figure 3. Effect of VCD exposure *in vitro* for 15 days on the number of unhealthy ovarian primordial follicles in cultured rat ovaries. Ovaries were collected from pnd4 rats, placed into floating organ cultures with control medium or medium containing VCD (1-100uM). Ovaries were collected for histologic assessment and follicle counts 15 days following the start of culture. Columns represent percent unhealthy ovarian follicles (eosinophilic oocytes) counted in every 12th section (5μm) of ovaries relative to the total number of follicles observed. Results demonstrate a concentration-dependent increase in unhealthy follicles. Values are mean±standard error; * = $p<0.05$, different from controls, n=3-4 ovaries per treatment.

follicles containing two layers of granulosa cells, and only a few were observed per ovary. This was in contrast to ovaries developing *in vivo*, in which the majority of follicles on pnd19 are at the secondary or small antral stages (Figure 2C).

Incubation of pnd4 rat ovaries for 15 days with VCD (1-30uM) did not demonstrate visible signs of necrosis, nor was follicle morphology or development grossly altered. Follicular atresia (apoptosis) was demonstrated by pyknosis of granulosa cells in hematoxylin and eosin-stained sections in the largest follicles observed in the cultured rat ovaries (Figure 2B). Additionally, degenerating follicles could be identified by intense eosinophilic staining of the oocytes in both primordial and atretic larger growing follicles of untreated and VCD-exposed cultured ovaries. Therefore, follicles cultured for 15 days were evaluated for healthy versus unhealthy appearances. The percent of follicles that were unhealthy was 13% in untreated ovaries, and VCD exposures (30 and 100μM) for 15 days increased this percent to nearly 100% ($p<0.05$, Figure 3). Additionally, in ovaries incubated with VCD, some primary follicles exhibited oocytes that had a small amount of cytoplasm split off into one or two polar body-like fragments. This was observed almost exclusively in ovaries continuously exposed to 10 or 30uM. This may be a symptom of VCD-induced molecular changes in the oocyte, and requires further investigation.

VCD (30uM) exposures significantly reduced primordial and small primary follicle numbers by 8d, and caused nearly complete depletion of those follicles within 15 days of *in vitro* exposure (Devine *et al.*, unpublished). The numbers of more developed follicles were unaltered by 1-30uM VCD exposure, although secondary follicles were not observed in ovaries treated with VCD at 100uM. There may not have been sufficient small pre-antral follicles for recruitment to the secondary stages in ovaries exposed 100μM VCD for 15 days. Concentrations below 30uM had no significant effects on follicle numbers during exposures for 15 days *in vitro*, demonstrating that there was a very narrow range of VCD concentrations at which specific ovotoxic effects were observed in cultured ovaries.

In summary, experiments involving VCD-exposures *in vitro* have confirmed that this chemical is a direct ovotoxicant and has similar effects *in vivo* and *in vitro*. Overall, these results demonstrate the utility of the ovarian culture system for assessing the effects of ovarian toxicants and the mechanisms involved.

8.2. Future Investigations

Future investigations into the mechanism of VCD-induced follicle loss will utilize the neonatal rat ovary culture system to identify specific molecular changes. These investigations will involve identification of the specific cellular target (somatic versus germ cell) by assessment of oocyte- and granulosa cell-specific growth factor and receptor levels. Additionally, VCD-induced oxidative stress will be evaluated by measurement of the intracellular antioxidant, glutathione, as was described following *in vivo* exposures (70). Ovarian-specific metabolism of VCD and expression of cytochrome P450's in both mouse and rat ovarian tissues will also be examined.

9. PERSPECTIVES

In conclusion, investigations into ovarian physiology and follicular development have benefited from the use of ovarian tissue cultured *in vitro*. Our demonstration that *in vitro* culture of pnd4 rat ovaries with VCD mimics the response to *in vivo* dosing, in terms of the follicle types targeted, provides support that this will be a valid model on which to base more extensive mechanistic studies. With such validation in hand, the culture system can be used to design more simplified and specific experiments. Our lack of understanding of all factors that are required for proper follicle and oocyte development lends itself to investigation of large numbers of variables that can be examined in order to gain a full understanding of the various elements that regulate follicular development and function. Future research will benefit from expanding and exploiting the various *in vitro* systems that have been developed. For example, such systems would lend themselves well to exploring the effects of endocrine disruptors and ovarian toxicants.

10. ACKNOWLEDGEMENTS

This research was funded by NIH grant RO1ES08979, RO1ES09246 and the NIEHS Center grant 06694.

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Abbreviations: PMSG: pregnant mare serum gonadotropin; LH: luteinizing hormone; FSH: follicle stimulating hormone; hCG: human chorionic gonadotropin; um: micrometer; IU: international units; ip: intraperitoneal; BSA: bovine serum albumin; pnd4: postnatal day 4; uM: micromolar; CEO: cumulus-enclosed oocytes; cAMP: cyclic adenosine monophosphate; VCD: 4-vinylcyclohexene diepoxide; VCH: 4-vinylcyclohexene; ug: micrograms

Key words: Culture, Ovary, Pre-antral Follicle, Oocyte, *In vitro*, VCD, 4-Vinylcyclohexene Diepoxide, Review

Send correspondence to: Dr Patricia B. Hoyer, Physiology Dept., The University of Arizona, 1501 N. Campbell Ave P.O. Box 245051, Tucson AZ 85724-5051, Tel: 520- 626-6688, Fax: 520-626-2382, E-mail: hoyer@u.arizona.edu