MECHANISM OF OVARIAN DISRUPTION BY NEONATAL DES EXPOSURE: A FURTHER INVESTIGATION INTO OVARIAN FUNCTION IN THE HAMSTER CHEEK POUCH

A Thesis by

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MECHANISM OF OVARIAN DISRUPTION BY NEONATAL DES EXPOSURE: A FURTHER INVESTIGATION INTO OVARIAN FUNCTION IN THE HAMSTER CHEEK POUCH

I have examined the final copy of this Thesis for form and content and recommended that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Biological Sciences.

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DEDICATION

To my beloved thathi and ammi, Hemawansa and Kalyani Alwis, for your sacrifice, never-ending support and encouragement throughout all the years and to my wonderful wife, Melinda, who offered me love, constant support, and motivation that made all things possible
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ABSTRACT

Treatment of hamsters on the day of birth with the synthetic estrogen and prototypical endocrine disruptor, diethylstilbestrol (DES), induces severe anomalies throughout the female reproductive tract. The disruption phenomenon seen in the ovaries must be the result of either: 1) A direct mechanism that involves primary lesions in the neonatal ovary; and/or 2) An indirect mechanism that involves primary lesions in the hypothalamus and/or pituitary that then secondarily result in altered gonadotropin-regulated function of the mature hamster ovary. To test those alternative hypotheses, we used the convenient hamster cheek pouch system to perform homo-transplantation and cross-transplantation of ovaries among postnatal/prepubertal (day 21) control (CON) and neonatally DES-treated donor and host animals. Thereafter, host animals were monitored for estrous cycle activity and finally sacrificed at two months of age when blood was collected to determine endocrine status and both ectopic ovarian masses and in situ uteri were collected and fixed for histological and immunohistochemical analysis.

Evidence consistent with the indirect mechanism emerged early in the study from the monitoring of estrous cycle activity in hosts with viable ovarian transplants. Regardless of transplant treatment, all CON hosts exhibited regular, 4-day estrous cycles while all neonatally DES-treated hosts were non-cyclic. RIA analysis of sex steroids and gonadotropins in the serum of all treatment groups indicated maintenance of pituitary-ovarian axis function in terms of hormonal synthesis and secretion.

Histological analysis revealed that the viable, ectopic ovarian masses, either as homotransplants or as cross-transplants, did not replicate either the normal morphology seen in intact, CON animals or the characteristic disrupted morphology seen in intact, DES-exposed animals.
However, consistent with the equivalent serum estradiol levels measured in all four transplant groups, ovarian masses from all treatment groups were steroidogenically active as evidenced by immunohistochemical detection of aromatase. Despite the equivalent serum estradiol levels in all four transplant groups, severe endometrial dysplasia including evidence of inflammation was confined to the two neonatally DES-exposed host groups. We interpret the full spectrum of data reported here as being consistent with the alternative hypothesis that neonatal DES treatment disrupts morphogenesis and function of the adult hamster ovary via an indirect mechanism.
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CHAPTER 1
INTRODUCTION

1.1 **Estrogen Biosynthesis and Mechanism of Action**

The regulation, differentiation and maintenance of the female reproductive system as well as the promotion of secondary sexual characteristics are under the intricate control of the gonadotropin-releasing hormones (GnRH), the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH), and the steroid hormones estrogen and progestin (1). Produced and secreted in the hypothalamus, the decapeptide GnRH stimulates the secretion of FSH and LH. The secretory activity of GnRH neurons has a pulsatile nature, the frequency and amplitude of which is essential for the synthesis and cyclic release of LH and FSH (2). These gonadotrophins act upon the ovary to enable recruitment and maturation of follicles and must be released in a properly regulated manner in order for successful oocyte production (oogenesis), development, and release (ovulation) (3). For instance, LH stimulates ovulation and androgen production within the thecal cells. These androgens then diffuse into the granulosa cells and under the direction of FSH, are aromatized to produce estrogens (estradiol) which may have a localized effect related to ovum maturation (4). The steroid hormones estrogen and progesterone coordinate the actions of the reproductive tract and regulate LH and FSH secretion through a negative feedback mechanism in which the increased levels of estradiol at ovulation act upon the pituitary to suppress the secretion of GnRH which in turn leads to decreased levels of serum gonadotropins (1, 3).

Estrogens regulate the growth, differentiation, and function of many target tissues in the male and female reproductive tract including the uterus, mammary glands, testes, prostate, and
ovary. Naturally occurring estrogens are typically 19-carbon androgens aromatized to 18-carbon estrogens, all of which possess an aromatic A ring with phenolic hydroxyl. The three main estrogens are estradiol with a hydroxyl group at 3-beta- and 17-beta-positions, estrone with a 3-hydroxyl group and a 17-ketone, and estriol with a hydroxyl group at C3-beta, 16-alpha, and 17-beta positions. Estradiol is produced in ovarian follicular cells and is the main estrogen in non-pregnant females while the main estrogen in pregnant females is estriol and it is synthesized in the placenta from dehydroepiandosterone sulfate which is provided by the adrenal cortex of the fetus. The formation of estrogens is not limited to the gonads as smaller amounts may also be produced in the adrenal cortex. In men, the testes produce estradiol. Fat cells present in adipose tissue also have the ability to convert steroid precursors to estradiol, and are able to do so even after menopause (1, 2). Estradiol is more prevalent and potent than estriol and estrone, respectively (1, 4).

In plasma, the majority of sex steroids such as estradiol are bound to a β-globulin protein known as sex hormone binding globulin (SHBG) and another 10–40% is loosely bound to albumin, leaving only a small percentage free and biologically active. While the presence of estrogen and the state of pregnancy can increase SHBG levels in the blood, progestins, corticoids, and androgens decrease SHBG levels. Elimination of estradiol bound to SHBG or albumin involves its conversion to less active estrogens such as estrone and estriol. Estriol, a metabolite of both estrone and estradiol, is typically conjugated in the liver to sulfate and glucuronide groups and then excreted via the kidneys. Some of the water-soluble conjugates are also excreted via the bile duct and partly reabsorbed after hydrolysis in the intestinal tract. This process helps to maintain estradiol levels in the blood stream (5, 6).
Estrogens freely diffuse in and out of cells and are retained in target cells by high-affinity binding to proteins known as estrogen receptors (ERs). After definition of the receptor protein responsible for the specific binding of E2, two groups reported the cloning of the estrogen receptor gene in 1986. Then in 1995, a second ER (called ERβ, to distinguish it from the original receptor now called ERα) was cloned from a rat prostate cDNA library. Since then, ERβ has been cloned from several species and several isoforms of the receptor have also been identified. Since both receptors belong to the steroid/thyroid hormone superfamily of nuclear receptors, they display structural similarities. They are composed of an amino-terminal domain, a central DNA-binding domain, and a carboxy-terminal ligand-binding domain. The binding of ligand to ER causes the receptor to undergo conformational changes that allow the receptor to bind chromatin and modulate the transcription of estrogen-regulated genes. The conformational changes promote homodimerization and high binding affinity to specific estrogen response elements (EREs) which are located in the regulatory regions of target genes. ERα is expressed in the ovary, uterus, liver, kidney, and heart while ERβ is expressed in ovary, uterus, lung, neural tissue, as well as in male reproductive tissues. In the mouse ovary, ERα is expressed in the thecal and the interstitial regions while ERβ is expressed in the granulosa cells.

The human ERβ cDNA gene discovered in 1998 encodes a 530 amino acid protein which is longer than the original rat ERβ clone due to an additional 45 amino acid stretch in the amino-terminal domain. Since then, several isoforms and splice variants of both ERβ and ERα have been found. However, the biological and physiological roles of these isoforms and variants are still largely unclear. Differences in the amino-terminal regions of ERβ and ERα have been observed and are thought to be responsible for differences between the two receptors in their binding of different ligands. For instance, the antiestrogen tamoxifen can act as an agonist for
ERα and a pure antagonist for ERβ due to differences in activation factors (AFs) that mediate transcriptional activity (7, 8). It was previously thought that the only mechanism responsible for estrogen mediated transcription was by the direct binding of activated ER to EREs (Estrogen Receptor Elements). It is now known that both forms of ER can also modulate the expression of genes without being directly bound to DNA. For example, the interaction between ERα and the c-rel subunit of the NFκB complex prevents NFκB from binding to and stimulating expression from the interleukin-6 (IL-6) promoter thus leading to E2 inhibiting the expression of IL-6 (7).

Another important pathway of estrogen action involves the non-genomic effects of certain ligands. In endothelial cells, the membrane effects caused by E2 can lead to the activation of G-proteins, serine/threonine kinases, and mitogen-activated protein kinase (MAPK) pathways. This cascade effect has been postulated to result in the activation of endothelial nitric oxide synthase (eNOS) and release of nitric oxide (NO). Estrogen leads to the activation of tyrosine kinases, G-proteins, and MAPK at the membrane level in neurons resulting in neuroprotection, while in osteoblasts the membrane effects of estrogen are involved in apoptosis, cell differentiation and cell proliferation (7).

In the absence of E2, ERs may also be activated through other signaling pathways such as phosphorylation, which involves regulators such as protein kinase A (PKA), peptide growth factors, cytokines, or neurotransmitters and cell-cycle regulators. In the mouse uterus, insulin-like growth factor I (IGF-I) receptor signaling pathways are activated by estrogen. In E2-treated IGF-I knockout mice, uterine epithelium does not undergo mitosis which indicates the importance of the IGF-I pathway in estrogen responsiveness in the uterus. Exogenous doses of IGF-I can also induce estrogen-like responses such as increases in uterine mass and induction of estrogen target genes. This mechanism is now known as “cross-talk” since, in the absence of
estrogen, growth factor receptor activation may result in maintenance, activation, or induction of ER transcriptional activity (3, 7).

In ERα knockout mice (ERKO) ovaries, there is a lack of fully developed follicles and corpora lutea due to unsuccessful ovarian stimulation from pituitary gonadotrophins. Also present in ERKO ovaries are blood-filled cystic structures that have been attributed to increased concentrations of LH. Suppression of LH production by the administration of a GnRH antagonist prevented the incidence of these cystic structures. Conversely, ERKO mouse ovaries contained normal follicles at all stages of development surrounded by granulosa cells and corpora lutea. However, follicles that lacked oocytes and contained Sertoli-like cells appeared progressively with age. Also found were intermediate structures that appeared to possess degenerating oocytes as well as granulosa and Sertoli-like cells. This was evidence that follicles that appeared to be normal would begin to “trans-differentiate” as the degenerating oocyte underwent a transformation into seminiferous tubule-like follicles. The evidence gathered from these ER knockout models show that the lack of ERα and ERβ or the lack of ligand to activate ER gives rise to a unique phenotype and that both the combined effects of ERα and ERβ are required to ensure proper oocyte development in the ovary as well as successful folliculogenesis (3).

Comparisons of the levels of expression of ERα mRNA and protein with ERβ mRNA and protein in hamster ovarian somatic cells and oocytes prior to formation of primordial follicles showed that ERα maybe associated with the differentiation of interstitial cells, while ERβ expressing cells are involved in differentiation into granulosa cells. It has also been established that, during the formation of primordial follicles, ER mRNA levels increase while a decline in mRNA expression to more stable levels is brought about by the establishment of preantral folliculogenesis. Additionally, experiments conducted with pregnant female hamsters exposed to
FSH antiserum showed that periantral FSH action plays a vital part in ER expression and differentiation (9).

In recent years, several environmental chemicals that mimic the action of estrogen have been linked to an increased incidence of reproductive tract abnormalities and declining reproductive health among and men and women. In female rats, chemicals such as the pesticide atrazine and the plastics ingredient bisphenol A were shown to bind to ER and exert estrogenic effects. The most critical period for exposure to such chemicals seems to be at the fetal or neonatal stage when the reproductive organs are still under development. Studies are still underway to understand the interactions between ERα and ERβ and suspected environmental estrogenic chemicals (10).

1.2 Normal anatomy and physiology of the ovary

The main functions of the ovary are to produce and release eggs (oocytes) into the fallopian tubes (ovulation) and secrete steroid hormones such as estrogens, androgens, and progesterone that prepare the uterus for embryo implantation. The ovaries are covered externally by a layer of mesothelium. The body of the ovary is divided into an outer cortex and an inner medulla. The medulla consists of loose connective tissue with abundant blood vessels, lymphatic vessels, and nerve fibers. The cortex appears more dense and granular due to the presence of numerous ovarian follicles in various stages of development. Cortical fibroblasts are arranged in dense cellular whorls that make up the "ovarian stroma" (11).

At birth, the hamster ovary is still developmentally immature and undergoing organogenesis. Through the first few days of life, masses of oogonia and cortical mesothelieum are subdivided by somatic cells that originate from the hilus. The oogonia and mesothelial cells will eventually become the pregranulosa cells of the primordial follicles (12). Stroma-like theca
cells are recruited by oocyte-secreted signals. They surround the follicle's outermost layer, the basal lamina, and undergo cytodifferentiation to become the theca. An intricate network of capillary vessels forms and begins to deliver blood to and from the follicle (12, 13).

At day 9 of life, the periphery of the ovary is populated with oogonia associated with pre-granulosa cells forming primordial follicles. Below the zone of primordial follicles are rounded primary follicles containing one single layer of polyhedral-shaped granulosa cells. At this stage, granulosa and theca cells continue to undergo mitosis and the size of the oocyte increases. Directed by an oocyte-secreted morphogenic gradient, the tertiary follicle's granulosa cells begin to differentiate (12, 13). The prepubertal hamster ovary, as with most other rodents, contains a number of “polyovular follicles” (14).

At day 15, the hamster ovary contains numerous secondary follicles that are surrounded by multiple layers of granulosa cells. The oocytes of these follicles have a centrally located nucleus as well as a zona pelucida. While most of the stromal cells of the ovary have a well differentiated and organized appearance, the theca is still undergoing organization (13, 15). LH stimulates theca cells to produce the androgens androstenedione and, to a lesser extent, testosterone. Androstenedione and testosterone diffuse to the granulosa cells where they are converted to estrogens (estradiol or estrone) by the enzyme aromatase (12, 13).

The ovary undergoes general follicular differentiation until puberty that occurs around day 28 in the hamster. Then a surge of FSH from the anterior pituitary stimulates granulosa cell and follicle maturation. The onset of ovulation is then characterized by the release of LH which, in turn, stimulates formation of the corpus luteum. Immature ovarian follicles that do not respond to the action of LH undergo degeneration, known as atresia (16, 17).
1.3 Endocrine Disruption

The term “endocrine disrupting chemicals” (EDCs) refers to substances that may affect the endocrine system in a variety of ways. EDCs can interfere with the levels and action of normal circulating hormones by affecting their synthesis, secretion, transport, etc. They can also act by mimicking the action of hormones that are produced naturally in the body. While some EDCs can block a receptor site which leads to hormones being unable to carry out their function, others may act through a hormone-like process to initiate entirely new responses. EDCs can be naturally occurring phenolic plant compounds (phytoestrogens) or synthetic chemicals (xenoestrogens) from industrial, agricultural, and chemical sources.

Phytoestrogens, such as isoflavones that structurally resemble estrogen, can bind to ERs and thereby influence the transcriptional activity of specific genes. They can also act as antiestrogens by competing with the more potent endogenous estrogen for binding to ERs. Xenoestrogens may act as inappropriate estrogens that disrupt the function of endogenous estrogens such as estradiol (E2).

Endocrine disruptors have a variety of adverse effects on hormone-regulated processes such as immunity, thyroid function, and cancers of the uterus, breast, and prostate. However, most studies have focused on the effects of EDCs on the development and function of male and female reproductive organs in wildlife and in experimental animals that serve as models for their hypothesized effects on humans. In females, these chemicals may result in infertility, pregnancy loss, ovarian failure, and other reproductive defects.

The steroid hormones estrogen and progesterone play central roles in the regulation of several aspects of reproduction. Their effects are mediated through interaction with receptors such as ERα and ERβ (estrogen) and PR-A and PR-B (progesterone), which are members of the
nuclear superfamily of transcription factors (22). As mentioned above, endocrine disruptors can either induce or block the transcriptional regulatory activity of these hormones by binding to their receptors (23).

1.4 The DES Syndrome

DES is the classic example of an endocrine disruptor and exemplifies the damage done by xenoestrogens. DES is a non-steroidal, synthetic estrogen that was developed to supplement a woman's natural estrogen production. DES was first prescribed by physicians in 1938 for women who experienced miscarriages or premature deliveries that were believed due to insufficient levels of estrogen. However, in 1971 Herbst et al. reported the occurrence of vaginal clear cell adenocarcinoma in a group of women in their twenties who were exposed to DES in utero (24). Previously, this form of cancer was rare and reported only in post-menopausal women (25). Many women who were exposed to DES in utero also had a range of reproductive tract abnormalities which included structural changes in the cervix and/or upper vagina as well as vaginal epithelial changes consisting of vaginal adenosis (26). As a result of such structural abnormalities, DES-exposed daughters are prone to infertility and adverse pregnancy outcome (27). Since then, numerous studies in both humans and animal models on the effects of perinatal DES exposure showed neoplastic effects in both the male and female reproductive tract (19). While experiments conducted in male rats showed that high DES doses induced adverse changes in the developing reproductive system, low doses had an inhibitory effect on the plasma concentration of testosterone and irreversibly stimulated thyroid function (28).

In male offspring of women who were exposed to DES in utero, genital structural abnormalities, sperm and semen abnormalities, and increased risk of prostate cancer and
testicular cancer have been reported (27). This wide spectrum of clinical phenomena linked to perinatal DES exposure became known as the DES syndrome (25).

Figure 1: The chemical structure of estradiol and diethylstilbestrol (DES). The structure of the natural estrogen estradiol is shown on the left and that of the synthetic estrogen DES is shown on the right.

Our lab uses Syrian golden hamsters (*Mesocricetus auratus*) to investigate the phenomenon of neonatal DES-induced endocrine disruption. A single injection of DES on the day of birth resulted in a high incidence of endometrial adenocarcinoma following severe hyperplasia and altered apoptosis dynamics in the uterus of adult hamsters (29, 30).

In other studies, morphological disruption was also seen in the uteri of neonatally DES-exposed hamsters after they were ovariectomized prepubertally (day-21) and then chronically stimulated with estradiol. The observed abnormalities included endometrial hyperplasia, leukocytic infiltration, and adenocarcinoma (19, 30) Because these abnormalities were not seen in the uteri of similarly ovariectomized and estradiol-stimulated control animals, the interpretation was that DES had a direct disruptive effect on the developing, neonatal hamster uterus.
Evaluation of ovaries in 3-week old rat pups from DES-exposed moms showed that the percentage of primary and secondary ovarian follicles increased while the percentage of primordial ovarian follicles decreased (28). Plasma levels of FSH were also increased suggesting that DES promotes maturation of rat ovarian follicles perhaps by an ER-dependent mechanism (28).

Preliminary studies conducted in our lab showed that neonatal exposure to DES induced morphological and functional abnormalities in the hamster ovary (31). Newborn Syrian golden hamsters were injected with a single dose of either corn oil alone (Control) or corn oil plus either 100µg of DES or estradiol-17β. Ovaries were then removed at different time points and the tissues were processed for histological analyses. We observed an increase in the usually low levels of polyovular follicle (POF) formation in the DES-exposed ovaries after about 9 days of life. We also observed altered stromal/interstitial morphology, large antral and preovulatory follicles but no corpa lutea, and a thickened theca layer after 28 days of life in DES-exposed animals. These effects were not seen to the same extent in the the neonatally estradiol- treated animals. The results from this study showed that DES was a more potent endocrine disruptor than estradiol in the hamster (Hendry WJ, unpublished data).

An increased incidence of POFs was also seen in the ovaries of neonatally DES-exposed mice. The DES-exposed ovaries responded to eCG and hCG with ovulation and a reduction in POF incidence (32). Experiments involving ovaries of newborn mice that were cultured in serum-free medium containing DES and then transplanted under the renal capsules of ovariectomized hosts resulted in POF formation in the ovarian grafts. However, ovaries that were cultured in serum-free medium without DES before transplantation showed an absence in POFs suggesting that DES acts directly on the ovary of neonatal mice to induce POF formation (32).
Evaluations of the long-term effects of prenatal exposure to DES in mice revealed altered gross morphology of the ovarian interstitial compartment (33). DES-exposed ovaries had a larger interstitial compartment compared to controls as well as a higher number of vacuolated cells and a dramatic increase in lipid content in their interstitial compartment. Comparisons of ovarian steroid production in ovarian tissue harvested from 3 and 4 month control and DES-exposed mice showed that the latter expressed a significant increase in the levels of estrogen, progestrone, and elevated levels of testosterone. The increase in testosterone was attributed to the increase in the interstitial compartment (33).

Permanent disturbances in reproductive performance also occurred in mice who were treated with 5µg of DES daily for the first 5 days of birth (34). Observations included the presence of hypertrophy and hyperplasia in the interstitial tissue and follicles at different stages of development but no corpora lutea. Adult females that were neonatally exposed to DES also had a larger number of ova compared to controls, possibly due to the occurrence of POFs (34).

Tenenbaum and Forsberg showed that the ovaries of adult mice treated with daily doses of DES for the first five days of birth lacked corpora lutea, and possessed large interstitial cells with clear cytoplasm (35). Grafting of ovaries from 4 week old control mice under the kidney capsule of a DES-treated ovariectomized host and vice versa resulted in DES grafts with normal histomorphology, follicles, corpora lutea, and interstitial cells while the control ovarian grafts showed evidence of normal follicle structure but lacked any visible corpora lutea. That study suggested that the DES-induced ovarian effects were reversible and secondary to an altered hormonal environment (35).
1.5 DES Mechanism

The primary attribute of DES is its ability to avidly bind to ERs and mimic the actions of E2 in target organs. In mice, DES is an ERα agonist in the uterus and vagina of neonatal and ovariectomized adult mice (36). Experiments conducted on adult ERα knock out (ERKO mice) and wild-type females that were neonatally administered with vehicle or DES from day 1 through day 5 showed a decrease in the uterine expression of Wnt7a, Hoxa10 and Hoxa11 in the DES treated wild-type mice but not in the ERKO mice. However, the DES-effect on uterine expression of Wnt4 and Wnt5a was conserved in both types of mice suggesting a developmental role for DES mediated by ERβ while the lack of DES effects on gene expression and resulting tissue differentiation in ERKO mice suggest that ERα plays a key role in mediating the effects of neonatal DES exposure in the murine reproductive tract (37).

In utero exposure to DES affects the expression of Hox genes thought to be involved in development of the anterior-posterior axis of embryonic Müllerian duct segments (38). In mice and humans, Hox genes that are involved in the patterning of the reproductive tract include Hoxa9 (oviducts), Hoxa10 (uterus), Hoxa11 (cervix) and Hoxa13 (upper vagina) (39).

Alterations in the expression of Hox genes can lead to abnormalities in the reproductive tract (39). In situ hybridization and Northern blot analyses in prenatally DES-exposed mice showed that DES altered the expression of a number of Hox genes. While Hox9 expression is normally expressed in the oviduct region of mice, in neonatally DES-exposed mice it was absent from the oviduct and dramatically increased in the uterus. Also in neonatally DES-exposed mice, Hoxa10 expression was absent from both glands and stroma in the anterior part of the uterus and instead was expressed more caudally in the uterus. Hoxa11 expression was low and normally distributed along the tract. The expression levels of Hoxa13 were unaltered and normally
concentrated in the vagina (39). The DES-elicited posterior shift in *Hoxa-10* gene expression is thought to be responsible for an anterior transformation in the pattern of uterine development in mice, a pattern also seen in women who were exposed to DES *in utero* (39). These results provide a possible molecular explanation for the tetratogenic effects seen in the developing reproductive tract (39, 40).

Growth factors from the WNT family play roles in embryogenesis and cancer (41). The *Wnt7a* gene participates in “cross-talk” with *hox* gene expression and thus is involved in the appropriate expression of genes such as *Hoxa-10* and *Hoxa-11* in the female reproductive tract (42). Many of the abnormalities seen in the female reproductive tract of neonatally DES-treated mice are also seen in *Wnt7a* mutants. Neonatal DES exposure in mice disrupted *Wnt7a* expression and resulted in an altered response to estrogenic stimulation later in life (41, 43).

Early DES exposure also appears to influence gene expression by an epigenetic mechanism. For instance, neonatal but not mature (day 30) DES exposure results in demethylation of specific CpG sites in the estrogen response element of the lactoferrin gene. The site remained methylated in the neonatally DES-exposed, ovariectomized mice, suggesting that DES-induced methylation was under ovarian hormonal control (36, 44). Further investigation of DES-elicited methylation patterns showed that down-regulation of *Hoxa10* and *Hoxa11* as a result of neonatal DES-exposure was not due to methylation of their promoter CpGs (45). Neonatally DES-exposed mouse reproductive tracts also express high levels of *c-fos* and *c-jun* mRNAs which are linked to observations involving high mitotic activity in the vagina and uterus (36).

Numerous DES metabolites have been identified in the urine of Syrian golden hamsters. The variety of metabolites indicate extensive DES biotransformation in the hamster. These
metabolites may be chemically active themselves or be transformed in vivo into reactive metabolites that play a role in tumor formation (46). Using Syrian hamster embryo (SHE) cells to study DNA damage possibly involved in cell transformation, DES induced the formation of DNA adducts as well as numerical chromosome changes (aneuploidy) (47). Also, Syrian hamsters treated with a single injection of DES (200 mg/kg body weight) developed distinct DNA adduct patterns in the kidney, liver, uterus, and testes and adduct concentrations were 4-6-fold higher in females than in males (48). In addition, studies examining the characteristics of the DES-DNA adduct formation in the liver and kidney of hamsters showed that the phenomenon was dose dependent and more pronounced in older compared to younger animals (49). Neonatal DES exposure has been known to alter gene expression in the reproductive tract of rodents perintally exposed to DES. In the hamster uterus, we showed that neonatal DES exposure altered the expression of a number of proto-oncogenes (c-jun, c-fos, c-myc) involved in cell proliferation and another group of genes (bax, bcl-x, bcl-2) involved in the regulation of apoptosis (50).

1.6 The Hamster Cheek Pouch

The hamster cheek pouch is a convenient and well established site for tissue transplantation studies. It is a diverticular, bilateral, symmetric, and double-walled epithelial structure that is derived from the oral cavity and is used to store and transport food. The pouches expand dorsally from the mouth to the shoulder region located ventral to the ears (12, 17).

The wall of the cheek pouch consists of four layers: an outer later that contains keratinized and stratified epithelium, the lamina propria that is made up of dense fibrous connective tissue, the tunica muscularis that is composed of striated skeletal muscle with varied thickness invested by loose fibrous connective tissue, and the fibrous connective tissue layer that makes up the inter-membranous space in the pouch (51).
The hamster cheek pouch has been used extensively due to its ability to accept a variety of grafts (allogenic and xenogenic) and to sustain them (52) because of its “immunologically privileged” nature (53). No lymphatic tissue exists in the wall of the cheek pouch (51) so the reduced number of Langerhans cells and a lack of lymphatic drainage results in blockage of the immune response and thus a local immunological tolerance. The areolar connective tissue barrier also aids in cheek pouch immunity (12, 53).

The hamster pouch has been exploited in numerous ways including transplantation and microcirculatory studies, cancer chemotherapy, tissue and cell grafting, vascular physiology, and drug testing (12). In addition to the immunologically privileged nature of the hamster check pouch, other advantages include: 1) The ability to frequently evert the check pouch in order to observe, measure, or photograph transplants at different stages without causing any undue trauma to the animal, 2) the membrane is almost transparent, a feature that facilitates the observation of the implant as well as neovascularization around it, and 3) implantation of grafts as well as preparation of the cheek pouch is relatively easy and sophisticated or sterilized equipment is not necessary (52).

The hamster cheek pouch was first tested in our lab using uterine tissue transplants from 7-day old untreated hamsters (donors) which were then transplanted into the pouches of 1-month old female, hamsters (hosts). At the time of implantation, the hosts were either ovariectomized only or were ovariectomized and an estrogen-releasing pellet was also implanted. Periodic inspection of the pouches of both groups showed sustained growth of the uterine tissues, especially in those hamsters that were ovariectomized and treated with the estrogen-releasing pellet. The transplants also thrived without the host’s health being compromised, exhibiting sepsis, or any other complications. Histological analysis of successful uterine transplants in
ovariectomized and estrogen-stimulated hosts showed an endometrium that was hypertrophic/hyperplastic while those in ovariectomized-only hosts were atrophic (52). The results from this experiment showed that uterine transplants in cheek pouches could in fact respond to endocrine hormones, grow, and differentiate (52).

In order to further investigate whether DES has a direct effect on the response of uterine tissue to estradiol stimulation, a different trial was conducted. Control and neonatally DES-treated hamsters were ovariectomized and received an estradiol-releasing implant at 21 days of life (prepubertal). Also at that time, uteri from 7-day old control and DES treated neonatal donors were transplanted into the contralateral cheek pouches of each host animal. After 2 months, transplant tissues were harvested and histological analysis showed that uterine disruption was confined to DES-exposed donor uteri in both the control and DES-exposed group of host animals. This proved that the differences in uterine disruption were not due to differences in the neuroendocrine function of the two treatment groups. These results also concurred with previous evidence that neonatal DES exposure permanently disrupts the estrogen responsiveness in the hamster uterus via a direct mechanism (30).

We had mixed results in previous attempts to transplant hamster ovaries into hamster cheek pouches. In one trial run, prepubertal animals (day 21) were ovariectomized and the ovaries implanted into another hamster’s pouch (cross transplantation). In that run, the ovaries were dissected out of the bursa that surrounds it and then bisected in the belief that such a procedure would better expose it to the blood vessels in the hamster cheek pouch after transplantation. This method was tested because previous attempts to transplant ovaries still within their bursae proved unsuccessful. Inspections over subsequent weeks of the everted cheek pouch containing the bursa-free and bisected ovarian transplants revealed extensive
vascularization. Tissue harvesting occurred two months later and uteri in the ovariectomized and successfully-transplanted host hamsters had dimensions similar to those of normal uteri in intact animals. The uteri of the hamsters with unsuccessful ovarian transplants were atrophic due to the absence of ovarian estrogens. This experiment supported the feasibility of the transplantation procedure and showed that the immature ovary can reach mature functionality once transplanted into the cheek pouch (54).

1.7 Ovarian Transplantation

Current advances in cancer treatment have led to more cancer patients experiencing recovery through the action of potentially sterilizing radiotherapy and/or chemotherapy. Cryopreservation of ovarian tissues has recently been considered as an alternative to assisted conception technologies (e.g., IVF) for storage of embryos or oocytes in order to help deal with the later effects of cancer treatment such as gonadal failure and infertility (55, 56). Studies have shown that cryopreserved whole rat ovaries can restore follicular activity and secretion after successful transplantation. Supporting evidence included a relatively uninterrupted estrus cycle, normal uterine weight, serum FSH and estradiol-17β levels, a normal ovarian morphology as well as no significant differences in the numbers of primordial and growing follicles. The possibility of pregnancy after these transplants suggest that there is a potential to conserve fertility in animal models as well as in humans. However, allotransplants were rejected in the rat model which appeared to rule out donor ovarian transplants unless the donor and host were close tissue matches (56).

Additionally, allotransplant experiments conducted with ovarian tissue taken from a mouse donor with lymphoma showed that malignancy was transferred to recipient mice, while ovarian tissue taken from human lymphoma patients did not display malignancy when
transplanted into mouse recipients. As a result, xenotransplantation of ovarian tissue was shown to be a more successful model/research system and a safer clinical approach as the risk of malignancy transfer was reduced (55).

Xenotransplantations of ovarian tissue grafts were most successful in nude or severely combined immunodeficient (SCID) mice. In mice that are homozygous for the SCID mutation, the humoral as well as cellular immunity systems fail to mature. The inability of SCID mice to make T and B lymphocytes results in them being unable to reject ovarian tissue explants from other mammalian species (43). However, the major drawback of SCID mice is the fact that they are extremely vulnerable to pathogenic and opportunistic microorganisms and as a result, cannot be housed in traditional barrier rooms without risking infections (57). The use of SCID mouse model is thus a labor-intensive and expensive process as their use depends on the construction of specialized microisolation systems to protect the animals for long periods of time and to ensure that the integrity of their biomedical research potential is not compromised. Xenografts have been reported in several mammalian species such as mice (58), hamsters (17), rats (59), and cows (43).

Autotransplantations of human ovarian tissue can be done to the human pelvic side wall (60), forearm, and beneath the skin of the abdomen to preserve endocrine function in women undergoing sterilizing cancer therapy or surgery (61). In 2004, the first live birth as a result of orthotopic transplantation of cryopreserved ovarian tissue in humans was reported (62).

Significant advances in the field of ovarian preservation and transplantation have provided several advantages. The ability to provide additional oocytes for livestock production (43), preserving reproductive capacity in humans (61), as well as boosting biodiversity through
the conservation of DNA from rare, valuable or endangered species (59) can all be attributed to the recent advances in ovarian preservation and transplantation techniques.
1.8 Research Plan

Alternative working hypotheses:

Information collected from our lab indicates that neonatal treatment with DES disrupts early ovarian morphogenesis resulting in altered histology that could be accompanied by a loss of regulated function in the mature animal. Two alternative hypotheses were proposed to explain this phenomenon.

Neonatal exposure to DES disrupts the morphogenesis and function of the neonatal hamster ovary by either: 1) A direct mechanism that involves immediate DES-induced alterations in the neonatal ovarian tissue or 2) An indirect mechanism that involves alteration of the development and function of the hypothalamic/pituitary axis which in turn results in altered gonadotropin-regulated function of the mature hamster ovary.

In order to test these hypotheses, we decided to modify our previous cheek pouch system to include dissection of prepubertal hamster ovaries out of the encapsulating ovarian bursa and accompanying oviduct. This experimental system provides a convenient site for homotransplantation and cross-transplantation of ovaries among control and neonatally DES-treated donor and host animals. Prepubertal (post partum) ovaries (from control and DES-exposed donor hamsters) were transplanted into the cheek pouches of control vs. DES-exposed host hamsters thereby exposing the ovaries to potentially different adult endocrine environments.
CHAPTER 2
MATERIALS AND METHODS

2.1 Animals

All experimental procedures were performed in accordance with the Wichita State University Institutional Animal Care and Use Committee (IACUC). Pregnant Syrian golden hamsters (*Mesocricetus auratus*) were maintained under a 14-h light/10-h dark schedule at 23-25°C and fed laboratory chow and tap water *ad libitum*. New-born hamsters were sexed within 6-8 hours of birth (day 0) and litter size adjusted to eight neonates per litter by eliminating extra males. All animals in a litter were treated with a single subcutaneous injection of 50µl of vehicle corn oil (Control hamsters, CON) or vehicle containing 100µg DES (~33 mg/kg body weight).

2.2 Cross-transplantation of Ovaries into the Cheek Pouch

Prior to puberty (Day 21), CON and DES-treated animals were bilaterally ovariectomized through two dorsal incisions. For this surgical procedure, animals were anesthetized with an intraperitoneal injection of Nembutal sodium solution at a dose of 0.075mg/100mg body weight. Sterile tissue culture media was prepared by filtering 50ml DMEM/F-12 with 0.39g HEPES and antibiotics in a 60cc syringe using a 45µm filter tip. The excised ovarian masses were placed in the sterile media prior to implantation into the cheek pouches. With the aid of a dissecting scope, each ovary was dissected out of its bursa and accompanying oviduct.
Table 1: Ovarian transplantation groups.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Host neonatal treatment</th>
<th>Type of transplantation</th>
<th>Group Name</th>
<th>n^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CON</td>
<td>Intact animals</td>
<td>CON Intact</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(No transplantation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DES</td>
<td>Intact animals</td>
<td>DES Intact</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(No transplantation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CON</td>
<td>Homo-transplantation</td>
<td>CON Homo</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(One ovary per cheek pouch)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>DES</td>
<td>Homo-transplantation</td>
<td>DES Homo</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(One ovary per cheek pouch)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CON</td>
<td>Cross-Transplantation</td>
<td>CON Cross</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(One ovary per cheek pouch)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>DES</td>
<td>Cross-Transplantation</td>
<td>DES Cross</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(One ovary per cheek pouch)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Number of animals in each group.

The ovarian transplant procedure was performed using groups of two animals and the ovaries were implanted randomly into either the right or the left cheek pouch. The entire transplantation procedure is done while the animal is still under anesthesia. The cheek pouch is everted using forceps, spread on a paraffin plate, and held with T-pins. With the aid of a dissection microscope, an incision is made in the epithelium of the cheek pouch. The opening of the incision is widened slightly and a pocket between the two epithelial layers is made using a pair of forceps. The ovary is then inserted into the pocket using a specialized microforcep and the incision is then sealed using a liquid suturing product (Vetbond Tissue Adhesive, 3M Animal Care Products). The cheek pouches were inspected weekly and the size and vascularization of the transplants was evaluated. At the same time, the animal’s estrus cycle stage was also observed. This was done by the observation of the distinctive preovulatory discharge that denotes day 1 of the estrus cycle and subsequent discharge.
2.3 Tissue Harvesting and Processing

At two months of age, the specific day of estrus was determined for animals that underwent control treatments. Animals from all groups were anesthetized with CO₂, terminated by decapitation, and trunk blood was collected. This time point was chosen because dramatic morphological abnormalities throughout the female reproductive tract were observed then in DES-exposed hamsters (29). Transplant tissue masses as well as reproductive tracts were harvested, fixed in two changes (24 hours each) of freshly made fixative (4% Paraformaldehyde, pH 7.2), and finally transferred to 70% ethanol. Samples were sent to HSRL laboratories for histological processing and paraffin embedded sections were analyzed for changes at the histomorphological (standard hematoxylin and eosin staining) and the biochemical/molecular level (immunohistochemistry). To assess uterotropin activity, 0.5 cm long segments of fixed uterine horns were cut, lightly blotted between paper towels, and weighed three times.

2.4 Steroid Hormone and Gonadotrophin Radioimmunoassays

To assess one aspect of ovarian activity at the time of animal termination, blood levels of the serum steroid hormones estradiol and progesterone and the gonadotrophins, leutinizing hormone and follicle stimulating hormone were measured by radioimmunoassay (RIA) as described previously (63). The sensitivities of the E₂ assays were 50 pg/ml, P₄ assays were 19ng/ml, and LH and FSH assays were 0.78ng/ml and 15.6ng/ml respectively. Assays were performed by Dr. Shyamal K Roy at the University of Nebraska Medical College.

2.5 Immunohistochemistry

Slides containing paraffin-embedded tissue sections were incubated at 70°C for 15 minutes and de-paraffinized in fresh xylenes thrice for 10 minute each time. Next, tissues were
re-hydrated in decreasing concentrations of ethanol (100%, 95%, 85% and 30% [v/v] in deionized/distilled water) and washed briefly in deionized/distilled water. Endogenous peroxidase activity was blocked by quenching in 3% [v/v] hydrogen peroxide in methanol for 30 minutes at room temperature (RT). After washing once in water and three times in PBS/Tween-20 (0.05% [v/v] Tween in Dulbecco phosphate buffered saline, p.H 7.5), slides were incubated in PBS/Tween for 15 minutes at 37°C to block non-specific protein binding. Antigen retrieval was performed by incubating slides in tri-sodium citrate buffer (10mM tri-sodium citrate, p.H 6.0) in a plastic coplin jar immersed in boiling water for 30 minutes. Next, slides were incubated in a secondary antibody-specific 10% serum dilution (1:10 in PBS/Tween) for 30 minutes at RT. Slides were then incubated with primary antibody diluted in PBS/Tween overnight at 4°C in a humidified chamber, followed by a 20 minute incubation at 37°C with appropriate species-specific biotinylated antibody (Vector Laboratories, Burlingame, CA) diluted 1:100 in PBS/Tween. Next, slides were incubated for 20 minutes at 37°C in an avidin-biotin-peroxidase complex reagent (Vectastain® ABC or Elite® ABC). The ABC reagent was prepared by mixing 12µl of Elite® ABC in 1200µl PBS/Tween (dilution 1:50). Finally, slides were reacted with a commercial diaminobenzidide substrate solution (SigmaFast™ from Sigma, Saint Louis, MO) at RT until reaction was visualised. After rinsing with deionized/distilled water, slides were counterstained with 0.2% (w/v) methyl-green (in 0.1M ammonium acetate, pH 4.6), rinsed in deionized/distilled water, and covered with glass coverslips using Permount mounting medium.

2.6 Microscopy

Analysis of immunohistochemistry and hematoxylin/eosin-stained tissue sections was conducted using a Nikon Eclipse E800 using Infinity Optics and Plan Apo objectives. All images were taken using a MagnaFire digital camera (Optronics, Goleta, CA) and AnalySIS® digital
image analysis software (Soft Imaging System Corp., Lakewood, CO). Images for the purposes of uterine cross-section measurements were taken using a Nikon SMZ-10 microscope and a 7.1 megapixel Kodak digital camera.

2.7 **Statistical analysis**

All statistics were determined using the Statistical Analysis System (SAS®) PC version 9.1.2 SAS Institute Inc. Cary, North Carolina 2004. For circulating steroid hormone and gonadotropin levels an Analysis of Variance (ANOVA) (64) and the Duncan-Waller test (65) was performed by the MEANS and the WALLER option respectively.
CHAPTER 3
RESULTS

3.1 Circulating Steroid Hormone and Gonadotropin Levels

Serum levels of steroid hormones were determined by RIA and are shown in Figure 2. Mean estradiol levels in the intact DES-treated animals were significantly higher than those in the intact CON-treated animals and those in all four cheek pouch transplant groups. Although striking differences in estrus cycle activity and uterine histomorphology were observed (see below), mean serum estradiol levels were equivalent in all four transplant groups (CON Homo, DES Homo, CON Cross and DES Cross) and lower (significantly so except for the CON Cross group) than in the intact control group. Mean serum progesterone levels were highest in the intact CON-treated animals and equivalent to that in the CON Homo group. Progesterone levels were lowest and least variable in the DES Homo group, and intermediate in both magnitude and variability in the DES Cross and CON Cross groups.

Figure 3 displays RIA results for the gonadotropins LH and FSH. Mean LH levels were lowest in the intact CON group, intermediate in the intact DES-treated group, and highest in all four transplant groups. Similarly, the highest mean FSH levels were seen in all four transplant groups when compared to the intact CON and DES-treated groups. The degree of enhanced gonadotropin levels in the four transplant groups compared with the two intact groups was statistically significant with the exception of LH values in the DES Cross groups.

3.2 Estrus cycle activity

Estrus cycle activity for all animals in all experimental groups was monitored in the weeks prior to harvest. Evidence of a regular 4-day estrus cycle was seen in all of the 16 control
host animals (CON Homo and CON Cross groups) but was absent in all of the 14 DES-exposed host animals (DES Homo and DES Cross groups) regardless of whether the cheek pouch transplants came from control or DES-exposed donors. Furthermore, regularity of the 4-day estrus cycle in control hosts appeared the same regardless of whether they harbored one or two viable transplants or whether follicles in the transplant appeared healthy or not (see below).

3.4 Uterotropic Activity

As one means to assess ovarian transplant function, reproductive tracts in homo-transplant, cross-transplant, as well as intact groups of control and DES-treated animals were harvested and fixed at 2-months of age. Figure 4 depicts mean weights of 0.5 cm-long uterine horn segments harvested from CON and DES-treated cheek pouch implant and intact groups. The mean uterine segment weights from the DES-treated groups were significantly heavier than those from the CON treatment groups. Those from the CON-treated intact group were lighter but not significantly different than those from the CON Homo-transplant group. Additionally, those from the CON Cross and CON Homo groups were not significantly different.

Figure 5 depicts mean cross sectional area of 0.5cm sections of host uterine horn segments from CON and DES-treated cheek pouch implant and intact groups. The cross-sectional area of uteri that underwent DES treatments were significantly larger than those that underwent CON treatments. Cross-sectional area from the DES-treated intact and DES-Homotransplant group were not significantly different while the DES Homo-transplant group uterine cross-sectional area was larger but not significantly different from the CON-treated intact group. CON and DES-treated ovariectomized animals had the lowest cross-sectional area in comparison to the rest of the treatment groups and were not significantly different from each other and the CON Cross- and CON Homo-transplantation groups.
Figure 6 shows representative control host tracts and tracts from control intact and ovariectomized groups. Those from the CON Homo group and CON Cross group animals (Figure 4, B and C) were comparable in overall dimensions to those from CON-treated intact animals (Figure 6, A). Due to estrogen withdrawal, harvested uteri from ovariectomized 2-month CON-treated animals were clearly atrophic (Figure 6, D) compared to uteri from the CON-treated intact and transplantation group animals.

Figure 7 shows representative host tracts from DES-treated cheek pouch transplants animals and from intact and ovariectomized DES-treated animals. Uteri from all four groups of the DES-treated animals were more massive than those from the CON-treated animals. Furthermore, uteri from the DES Homo group and DES Cross group animals (Figure 7, B and C) were comparable in size to those from the DES-exposed intact animals (Figure 7, A). Uteri from the DES-exposed, ovariectomized animals were relatively atrophic but still more massive than those from the control, ovariectomized animals (compare Figs. 6D and 7D).

3.5 Histomorphology of the Cheek Pouch Transplants

Histomorphological analysis of hemotoxylin/eosin-stained tissues representing the viable transplant masses from all the treatment groups revealed the presence of ovarian tissue containing follicles at various stages of development. However, none of the analyzed transplant tissues exhibited normal ovarian histomorphology. In addition, stromal compartments within the ovarian transplants were disorganized and most of the follicles appeared distorted. Transplants representing the CON Homo-transplant group (Figure 8, A through D) displayed clear evidence of antral, primary, and tertiary follicles. Transplant tissue from a DES Homo-transplant group animal (Figure 9, A through D) contained primordial, primary, and numerous secondary follicles. Transplant tissue from DES-exposed ovaries that were placed in CON hosts (Figure 10, A
through D) contained primary follicles, some secondary follicles, as well as oocytes without distinct granulosa or thecal compartments (Figure 10 C). Transplant tissue from CON ovaries that were placed in DES-exposed hosts (Figure 11, A through D) contained cystic structures as well as primary follicles and numerous secondary follicles.

The histomorphological profile of the transplant ovarian masses depended on neonatal treatment history of the host animal rather than neonatal treatment history of the donor animal. Within control hosts, ovarian transplants from both control (Figure 8) and neontally DES-exposed (Figure 10) donors displayed evidence of a relatively normal morphology. However, within neontally DES-exposed hosts, ovarian transplants from both control (Figure 9) and neontally DES-exposed (Figure 11) donors displayed evidence of cystic structures that were indicative of DES-exposure.

### 3.6 Histology of Host Uteri

Histomorphological analysis was performed on hematoxylin/eosin-stained host uteri from all four transplant groups as well as control and DES-exposed groups of 2-month old intact and ovariectomized animals. Figures 12 and 13 show representative uterine cross-sections from some of the animals in CON and DES-treated transplantation groups, all at low magnification. These figures illustrate the general histomorphology and uterotropic state characteristic of each group.

In the uteri of intact CON-treated animals, observations at high magnification showed that the epithelium lining the lumen consisted of simple columnar cells with centrally located oval nuclei (Figure 14, A). A similar luminal epithelial organization was observed in the CON-Homo and CON-Cross group uterine compartments (Figure 14 B and C). In contrast, uteri from
the 2-month old CON-treated ovariectomized animals were atrophic with low cuboidal epithelial cells (Figure 14, D).

The epithelium lining the uterine lumen in intact DES-exposed animals was characteristically dysplastic (Figure 15, A). For instance, there was no distinct demarcation between the epithelial cells and the uterine stroma and the epithelial compartments contained abnormally tall (hypertrophic) and pseudostratified (hyperplastic) columnar cells that had a “foamy” appearance to it as a result of infiltrating leukocytes and cavities that harbored apoptotic bodies. Interestingly, both of the DES-exposed transplants from DES Homo and DES Cross groups exhibited dysplastic states that were very similar to that seen in the intact DES-exposed animals (Figure 15, B and C). Analysis of the DES-exposed, 2-month old uteri from ovariectomized animals showed a less cellular stroma and a more pseudostratified epithelium (Figure 15, D) than in control, 2-month old uteri from ovariectomized animals (Figure 15D).

3.7 Immunohistochemistry of cheek pouch tissue sections

Paraffin-embedded tissue sections representing all four experimental groups were analyzed to determine if the ovarian tissue transplanted in the cheek pouch expressed steroidogenic activity. For this analysis, we targeted the enzyme aromatase on tissue sections from groups 1 through 6 as well as ovarian tissue sections from intact CON and DES-exposed adult animals which served as our positive controls. A clear, positive signal was detected in sections from both CON and DES-intact animals (Figure 16, A and B) and it was localized within granulosa cells from antral follicles as well as tertiary follicles in both groups. A similar pattern was detected in tissue sections from cheek pouch transplants within all four transplant groups (Figure 16 C, D, E and F). Granulosa cells in antral follicles as well as tertiary follicles
showed a positive signal, while the stromal compartment and primary and secondary follicles did not.
4.5 Transplantation of Ovaries into the Cheek Pouch

The effects of neonatal DES exposure, at critical stages of reproductive system development, result in profound morphological and functional abnormalities in ovarian tissue. In the present study, we tested two alternative hypotheses that focused on whether the morphological and functional disruption of the mature hamster ovary as a result of neonatal DES-exposure are due to 1) a direct effect mechanism involving immediate DES-induced changes in the developing ovarian cells/tissue and/or 2) an indirect mechanism involving an immediate alteration in the neonatal development/function of the hypothalamus-pituitary axis that then leads to an altered gonadotropin-regulated function of the mature hamster ovary.

Our transplantation procedure involved a modified approach based on a prior attempt at performing homo and cross-transplantations of CON and DES-exposed ovaries into the hamster cheek pouches of CON and DES-exposed hosts. We hypothesized that the prior approach of transplanting the ovary encapsulated in the ovarian bursa led to inhibition of a necessary vascularization process that thus led to unsuccessful transplants. Our main procedural modification involved dissecting the ovary out of the ovarian bursa and accompanying oviduct and transferring it into the cheek pouch in hopes that this would not hinder the vascularization process. Additional changes to the procedure included prepping the cheek pouch with phosphate buffered saline to reduce bacterial contamination, tissue culture media supplemented with fetal calf serum containing antibiotics was used to store the excised and dissected ovaries to prevent bacterial infection prior to transplantation, and specialized non-serrated micro forceps were
utilized to prevent damage to the ovarian surface and to keep the opening to the cheek pouch implant site as small as possible. Using this procedure, we went on to implant CON and DES-treated pre-pubertal ovaries in the cheek pouches of CON and DES-treated hosts. The development of any abnormalities in ovarian histomorphology characteristic of DES-exposure in only the DES-treated transplants in a CON-treated host would indicate that neonatal DES treatment induces ovarian disruption by a direct mechanism. Conversely, the development of such abnormalities in CON-treated transplants placed in DES-treated hosts would indicate that neonatal DES treatment induces ovarian disruption by an indirect mechanism.

Preliminary transplantation efforts involved the transplantation of bisected ovaries in an attempt to expose the blood vessels in the hilum to the blood vessels in the intra-membranous space of the cheek pouch in order to hasten the process of angiogenesis. However those attempts were unsuccessful since no significant vascularization was observed during the cheek pouch inspections and transplants typically degraded within the first week following implantation. As a result, subsequent transplantations involved the entire ovary being transplanted into the cheek pouch. Thereafter, weekly inspections of the cheek pouch revealed that the transplants underwent extensive vascularization and that this procedural modification resulted in a greater number of viable transplants within the cheek pouch.

Histomorphological analysis of the cheek pouch transplants revealed that characteristic ovarian morphology was not maintained regardless of the neonatal treatments of the transplanted ovaries or the host. Nevertheless, in all treatment groups, ovarian tissue was present in most of the transplant masses. Transplants from different treatment groups appeared to have a number of primordial, primary, secondary, tertiary and antral follicles present within the ovarian stroma. While we were able to determine the developmental stage of these follicles, it was clear that they
were not healthy, with most follicles being deformed and some oocytes undergoing degradation. In addition to the presence of follicles, we also observed the presence of cystic structures, an indicator of DES exposure, which appeared to be present only in transplants that were placed in DES-treated hosts, regardless of the neonatal exposure of the ovaries. Overall however, we must acknowledge that in none of the four transplant groups did we observe a histomorphology profile consistent either with a normal ovary in a CON-treated intact animal or with the characteristic disrupted ovary in a DES-treated intact animal. In other words, the histomorphological data does not definitively support or rule out either the direct or indirect disruption mechanism hypothesis.

Taking into consideration the modifications made to our transplantation procedure, we expected our ovarian transplants to mature and maintain their characteristic morphology within the hamster cheek pouch. However, we found this not to be the case with transplants containing various follicles and being steroidogenically active but not maintaining normal CON or DES morphology. A possible reason for this would probably be due to the lack of possible beneficial biochemical factors (e.g. immune responses) and physical stresses that are exerted on the ovary within the ectopic site.

4.2 Gross uterine Morphology and Histological Analysis

It was observed that host uteri in all treatment groups that possessed viable transplants were estrogically stimulated regardless of the condition of the overall ovarian tissue or the follicles they contained. Gross morphological analysis of the host reproductive tracts showed that CON treated hosts that were ovariectomized at 21-days of age and that had CON or DES-treated transplants exhibited morphology that was comparable to intact CON animals. Similarly, DES-treated hosts that were ovariectomized at 21-days of age exhibited uterine morphology that was characteristic of DES-exposure. The histomorphological analysis of hemotoxlin/eosion stained
tissues confirmed our findings with host uteri exhibiting histomorphology that was characteristic of their neonatal treatment. In contrast, reproductive tracts from both CON and DES-treated hosts that did not have any viable cheek pouch transplants resulted in severely atrophic uteri, which was an indication of estrogen withdrawal. These observations provided us with initial evidence that the cheek pouch transplants were indeed estrogenically active.

4.3 Immunohistochemical Analysis

The steroidogenic activity of the transplants from all treatment groups was further evidenced by the expression of the enzyme aromatase. Aromatase was expressed in the granulosa cells of pre-ovulatory as well as tertiary follicles in both CON and DES-treated intact animals. Similarly, there was clear aromatase expression in the granulosa cells of pre-ovulatory and tertiary follicles in the ovarian transplant tissue regardless of neonatal exposure while the ovarian stroma as well as primary and secondary follicles remained relatively unstained.

4.4 Estrus Cycle Observations

Our observations of the 4-day hamster estrus cycle gave evidence of the indirect rather than the direct mechanism of neonatal DES-exposure since only hosts that were administered CON-treatments exhibited typical estrus cycle activity regardless of the neonatal exposure of their ovarian transplants. Neonatal DES-exposure has been known to cause hamsters to become acyclic. As a result, we expected the DES-homotransplant group to be acyclic. It was also found that neonatally exposed DES hosts remained acyclic even though they had CON-treated ovaries placed within their cheek pouch.

4.5 Steroid Hormone and Gonadotropin Analysis

Our results showed that the DES-treated intact and cheek transplant animals had characteristically low levels of P4 when compared to CON-treated intact and cheek- pouch
transplant animals. Keeping with the “persistent estrus” nature of DES-treated ovaries, elevated levels of E2 were seen in all of the DES-treated intact animals when compared to CON-treated intact animals (66). However, E2 levels among all four check-pouch transplant groups were similarly low (67). This could be attributed to the fact that the precise day of estrus for some of the CON-treated animals on the day of serum collection was unknown. Thus, it was possible that these animals were in stages in their estrus cycle that exhibited low levels of E2. It is also possible for hamsters to cycle with low levels of steroids provided that follicular development and their subsequent demise followed regular patterns. Subfertile animals have been known to exhibit an estrus cycle similar to that of normal animals despite a decreased number of mature follicles and consequently low levels of steroid hormones.

Low levels of FSH and LH corresponded to appropriate levels of sex steroid hormones (13). In the four transplant groups, high levels of LH and FSH corresponded to the low levels of serum steroid hormones indicating a functional pituitary-ovarian axis (68, 69), although there seemed to be a lesser negative feedback mechanism acting to lower the gonadotropin levels.

In order to make accurate conclusions in future trials involving RIA steroid and gonadotropin assays, it would be beneficial to monitor the estrus cycle of the hamsters up to 4 weeks prior to the harvest date in order to accurately predict their stage in the cycle. Performing cohort studies using all the different intact and treatment groups are an option to obtain accurate circulating steroid and gonadotropin levels. Serum from animals that are in the same stage of their estrus cycle can then be pooled to form an accurate picture of the gonadotropin and steroid levels, including androgens, for the cheek pouch-transplant animals. One consideration is that numerous animals will need to be sacrificed in order to get sufficient amounts of serum for each stage of the estrus cycle (diestrus1, diestrus2, proestrus and estrus). The same number of animals
will then have to be repeated for each treatment group. Power analysis can be performed prior to data collection which can be used to calculate an appropriate sample size to achieve adequate power. Serial reproductive hormone measurements can also be conducted by performing cardiac punctures (69, 70) close to or on the 2-month harvest date.
A modified ectopic approach was performed involving ovarian cross-transplantations between neonatal CON-treated and DES-treated hamster ovaries to answer the question as to whether DES-induced disruption of the postpubertal ovary was due to an indirect mechanism or a direct mechanism. However, the utility of that transplantation strategy was less than hoped for because ectopic ovarian masses failed to replicate either the normal ovarian morphology seen in intact, control animals or the characteristic disrupted morphology seen in intact, neonatally DES-exposed animals.

On the other hand, observations of their gross morphology showed that host uteri in all treatment groups that possessed viable transplants were estrogenically stimulated regardless of the overall condition of the ovarian transplant. Transplants also were steroidogenically active and analysis of serum sex steroid and gonadotropin levels indicated maintenance of pituitary-ovarian axis function.

Lastly, estrous cycle activity was observed only in CON-treated hosts regardless of the treatment of their viable donor ovarian transplants. Together, these findings suggest that neonatal DES treatment disrupts morphogenesis and function of the adult hamster ovary via an indirect mechanism.
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APPENDIX
Figure 2. Mean ± standard error of circulating steroid hormones Progesterone (P4) and Estradiol (E2) concentrations as measured by RIA. Mean values with the same letter are not significantly different as determined by Waller-Duncan k-ratio t-test.
Figure 3: Mean ± standard error of circulating gonadotropins Leutinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) concentrations as measured by RIA. Mean values with the same letter are not significantly different as determined by Waller-Duncan k-ratio t-test.
Figure 4. Mean ± standard error of 0.5 cm-long uterine horn segment weights harvested from CON and DES-treated cheek pouch implant and intact groups. Mean values with the same letter are not significantly different as determined by Waller-Duncan k-ratio t-test.
Figure 5. Mean ± standard error of 0.5 cm-long uterine horn segments cross-sectional area harvested from CON and DES-treated cheek pouch implant, intact, and ovariectomized groups. Mean values with the same letter are not significantly different as determined by Waller-Duncan k-ratio t-test.
Figure 6. Gross morphological reproductive tracts representing 2-month old CON-treated animals. Figure A represents intact CON-animals. Figure B represents the CON-Homo (CON host with CON ovaries transplanted) group, figure C represents the CON-Cross (CON host with DES ovaries implanted) group, and figure D represents CON-treated 2-month old ovariectomized animals.
Figure 7. Gross morphological reproductive tracts representing 2-month old DES-treated animals. Figure A represents intact DES-animals. Figure B represents the DES-Homo (DES host with DES ovaries implanted) group, figure C represents the DES-Cross (DES host with CON ovaries implanted) group, and figure D represents DES-treated 2-month old ovariectomized animals.
Figure 8. Histomorphology of hematoxylin and eosin-stained cheek pouch sections from the CON-Homo (CON host with CON ovaries implanted) group. Panel A shows ovarian implant tissue contained within the cheek pouch. Panels B through D show follicles in various stages of development within the implant tissue. AF indicates antral follicles, PF primary follicles and TF tertiary follicles. Panel A is at 20X magnification, other panels are at 200X magnification.
Figure 9. Histomorphology of hematoxylin and eosin-stained cheek pouch sections from the DES-Homo (DES host with DES ovaries implanted) group. Panel A shows ovarian implant tissue contained within the cheek pouch. Panels B through D show follicles in various stages of development within the implant tissue. Cy indicates cystic structures, PF primary follicles, SF secondary follicles and Pr primordial follicles. Panel A is at 20X magnification, other panels are at 200X magnification.
Figure 10. Histomorphology of hematoxylin and eosin-stained cheek pouch sections from the CON-Cross (CON host with DES ovaries implanted) group. Panel A shows ovarian implant tissue contained within the cheek pouch. Panels B through D show follicles in various stages of development within the implant tissue. PF indicates primary follicles and TF tertiary follicles. Panel A is at 20X magnification, other panels are at 200X magnification.
Figure 11. Histomorphology of hematoxylin and eosin-stained cheek pouch sections from the DES-Cross (DES host with CON ovaries implanted) group. Panel A shows ovarian implant tissue contained within the cheek pouch. Panels B through D show follicles in various stages of development within the implant tissue. Cy indicates cystic structures, PF primary follicles and SF secondary follicles. Panel A is at 20X magnification, other panels are at 200X magnification.
Figure 12. Histology representing uterine cross-sections from 2-month old CON-treated animals. Figure A represents uterine cross sections from intact CON-animals. Figure B represents CON-Homo (CON host with CON ovaries implanted) group, figure C represents the CON-Cross (CON host with DES ovaries implanted) group and figure D represents CON-treated 2-month old ovariectomized animals. All photos are taken at 20X magnification.
Figure 13. Histology representing uterine cross-sections from 2-month old DES-treated animals. Figure A represents uterine cross sections from intact DES-animals. Figure B represents the DES-Homo (DES host with DES ovaries implanted) group, figure C represents the DES-Cross (DES host with CON ovaries implanted) group and figure D represents DES-treated 2-month old ovariectomized animals. All photos are taken at 20X magnification.
Figure 14. Uterine histology of neonatally CON-treated animals. Figure A represents hemotoxylin-eosin uterine cross sections from intact CON-animals. Figure B represents the CON-Homo (CON host with CON ovaries implanted) group, figure C represents CON-Cross (CON host with DES ovaries implanted) group and figure D represents CON-treated 2-month old ovariectomized animals. In all panels E indicates epithelial tissue, S indicates stromal tissue, EG indicates endometrial glands and L indicates uterine lumen All photos are taken at 400X magnification.
Figure 15. Histology representing uterine cross-sections from 2-month old DES-treated animals. Figure A represents uterine cross sections from intact DES-animals. Figure B represents the DES-Homo (DES host with DES ovaries implanted) group, figure C represents the DES-Cross (DES host with CON ovaries implanted) group and figure D represents DES-treated 2-month old ovariectomized animals. All photos are taken at 400X magnification.
Figure 16. Immunohistochemistry results for an aromatase probe in intact ovaries and cheek pouch implants. Upper panels represent ovarian cross sections from intact CON-treated (left) and intact DES-exposed (right) hamsters. Middle panels represent ovarian transplant tissue from groups 1 (left) and 3 (right) while the lower panels represent ovarian transplant tissue from groups 2 (left) and 4 (right). S indicates ovarian stroma, SF indicates secondary follicle, TF indicates tertiary follicle and AF indicates antral follicles. All panels are shown at 200x magnification.