EVIDENCE ON THE DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF

Progesterone

DRAFT

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AUTHORS AND REVIEWERS

The Office of Environmental Health Hazard Assessment's Reproductive and Cancer Hazard Assessment Section was responsible for the preparation of this document. Members of other technical sections within the Office of Environmental Health Hazard Assessment were drawn from to conduct internal peer review.

Primary Authors

Mari S. Golub, Ph.D.
Farla L. Kaufman, Ph.D.
Marlissa A. Campbell, Ph.D.
Ling-Hong Li, Ph.D.
Reproductive and Cancer Hazard Assessment Section

Internal OEHHA Reviewers

George V. Alexeeff, Ph.D., D.A.B.T.
Deputy Director for Scientific Affairs

Lauren Zeise, Ph.D.
Chief, Reproductive and Cancer Hazard Assessment Section

James M. Donald, Ph.D.
Chief, Reproductive Toxicology Unit
Reproductive and Cancer Hazard Assessment Section

Technical Support

Shirley Williams
Occupational and Environmental Health Library, OEHHA.

PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code 25249.5 *et seq.*) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The Act specifies that one of the mechanisms by which "a chemical is known to the state to cause cancer or reproductive toxicity [is] if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity" (Health and Safety Code Section 25249.8(b)). The "state's qualified experts" regarding findings of reproductive toxicity are identified as members of the Developmental and Reproductive Toxicant (DART) Identification Committee of the Office of Environmental Health Hazard Assessment's Science Advisory Board (Title 22, California Code of Regulations, § 12301). The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency.

This draft document provides the DART Identification Committee with information relevant to the reproductive toxicity of progesterone. While this hazard identification document does not provide dose-response evaluation, exposure assessment, or determination of allowable or safe exposure levels, the document does provide information which may be useful in such appraisals.

A public meeting of the Committee will be held on November 4, 2004, in Sacramento, California. Following discussion and Committee deliberation, the Committee will determine whether progesterone "has been clearly shown through scientifically valid testing according to generally accepted principles" to cause reproductive toxicity.

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A. Abstract

Progesterone is both a product and an early intermediate of steroid hormone synthesis in both males and females. Exposure to exogenous progesterone occurs due to therapeutic use (contraception, prevention of preterm labor, support of in-vitro fertilization (IVF) pregnancies, menstrual disorders), through cosmetic/supplement use, and through environmental media. Progesterone has a short half life and is less commonly used than progestagens (agents with progesterone-like action) with longer half lives in therapeutic applications. Recent development of micronized progesterone may lead to broader exposures.

Progestagens are used therapeutically as both male and female contraceptives, thus identifying a hazard to fertility when exposure is not intended or is intended for other purposes. Early studies showed suppressed menstrual cycling and ovulation in women and suppressed spermatogenesis in men treated with progesterone. Similar observations have been made in rabbits, monkeys and rats. Lower systemic gonadotropins (follicle-stimulating hormone, luteinizing hormone) are associated with suppression of ovulation and spermatogenesis, suggesting mediation by progesterone action at the hypothalamic-pituitary level. Other female reproductive endpoints identified in animals subsequent to progesterone exposure are prolonged gestation and impaired maternal behavior. Milk composition was altered in lactating women with progesterone-releasing IUDs. Other male reproductive effects demonstrated in animal studies include impaired mating and paternal behavior. Effects on the developing female reproductive system in mice exposed to progesterone as neonates are reflected in persistent vaginal estrous and altered sexual behavior. Effects on the developing male reproductive system from postnatal exposure in sheep and rats are reflected in delayed puberty and altered male mating behavior.

Effects on the developing embryo and fetus due to maternal progestagen exposure during pregnancy have long been a concern in human medicine. Endpoints that have been investigated in human and animal studies include altered genitalia in both the male and female fetus, cardiovascular malformations, and other birth defects. Some of these studies provide data specifically on progesterone (excluding other progestagens). Additional endpoints affected by gestational progesterone administration in animals are conceptus mortality and birthweight. Postnatal manifestations in animals include altered sexual behavior in males.

B. Introduction

Progesterone is an endogenous progestational hormone. Progesterone and other progestational agents are known collectively as progestagens or gestagens. Progestagens are well known for their therapeutic use as both male and female contraceptives, but full consideration of progesterone reproductive toxicity requires review of a variety of endpoints at all stages of life history. Since progesterone alone is being considered for listing under Proposition 65, it is necessary to examine evidence for progesterone specifically rather than for progestational agents as a group.

B.1. Chemical structure and sources

Progesterone (CAS# 57-83-0, MW 315, structure Figure 1) is a cholesterol-derived steroid hormone. It is both an intermediate and an end-product in the steroid hormone metabolizing pathways of both men and women (Figure 2). Commercially produced progesterone is synthesized using the plant steroid diosgenin as a precursor (Raber 1999). Progesterone is lipid soluble (octanol water coefficient 3.87), minimally water soluble (8.81 mg/L at 25^{o}C) and minimally volatile (1.3×10^{-6} mmHg).

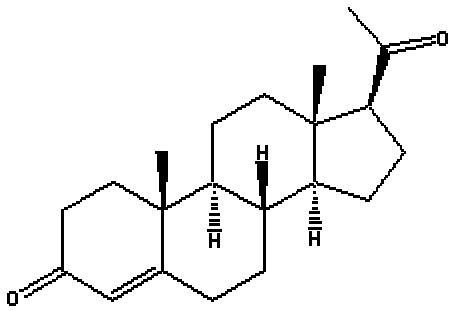

Figure 1. Structure of progesterone

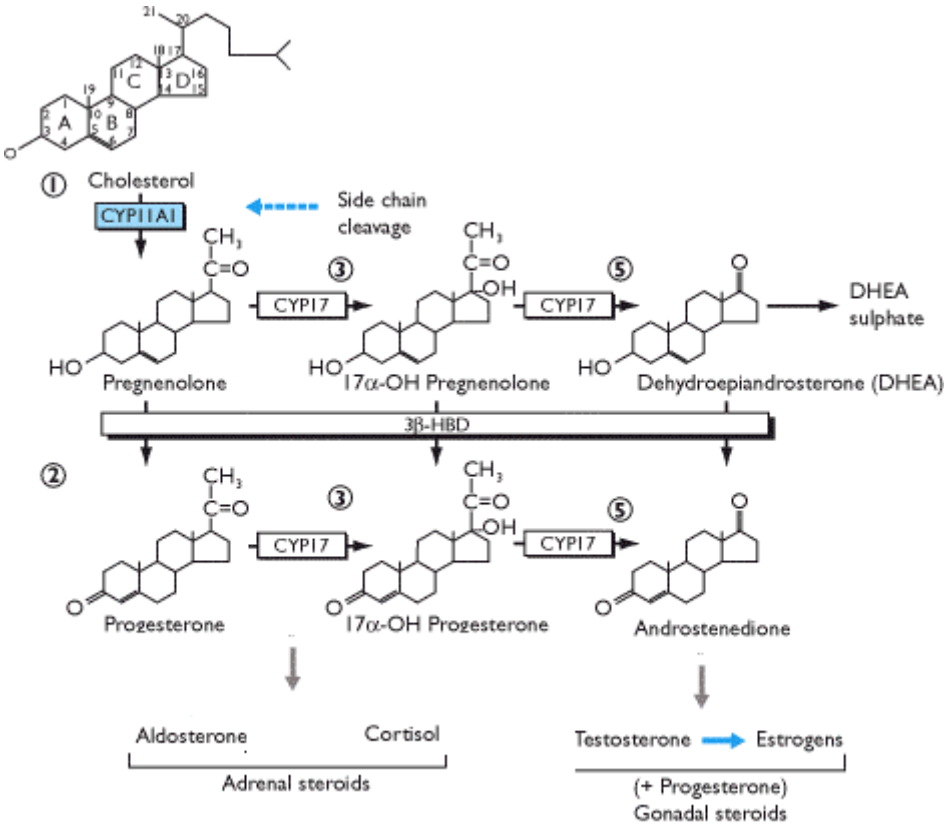

Figure 2. Steroid hormone metabolism. From (Nussey and Whitehead 2001)

Besides progesterone, a number of other agents are classified as "progestins", "progestagens" or "gestagens" based primarily on the activity of the agents in the Clauberg assay (Clauberg 1930), an early bioassay that measured the response of the estrogen-primed rabbit endometrium. Progestagens can be further divided by structural categories. As can be seen from Table 1, progestagens differ from progesterone in the range and potency of various endocrine actions, as summarized in a recent literature review (Schindler et al. 2003). The present review focuses specifically on progesterone; studies of other progestagens are mentioned as supportive material.

Table 1. Classification and endocrine actions of progestagens. From (Schindler et al. 2003)

Progestin	***Progesto-genic***	***Anti-Gonado-tropic***	***Anti-Estro-genic***	***Estro-genic***	***Andro-genic***	***Anti-Andro-genic***
Progesterone	+	+	+	-	-	±
Dydrogesterone	+	-	+	-	-	±
Medrogestone	+	+	+	-	-	±
17α-Hydroxy derivatives						
Chlormadinone acetate	+	+	+	-	-	+
Cyproterone acetate	+	+	+	-	-	++
Megestrol acetate	+	+	+	-	±	+
Medroxy-progesterone-acetate	+	+	+	-	±	-
19-Nor-progesterone-derivatives						
Nomegestrol acetate	+	+	+	-	-	±
Promegestone	+	+	+	-	-	-
Trimegestone	+	+	+	-	-	±
19-Nortestosterone derivatives						
Norethisterone	+	+	+	+	+	-
Lynestrenol	+	+	+	+	+	-
Norethinodrel	±	+	±	+	±	-
Levonorgestrel	+	+	+	-	+	-
Norgestimate	+	+	+	-	+	-
3-Keto-desogestrel	+	+	+	-	+	-
Gestodene	+	+	+	-	+	-
Dienogest	+	+	±	±	-	+

(++) highly effective (+) effective; (±) weakly effective; (-) not effective.

B.2. California use and exposure information

No exposure data specific to California were located. Exposures to progestagens occur in clinical medicine for male and female contraception, to prevent miscarriage, to support in-vitro fertilization (IVF) pregnancies, to extend postparturitional anovulation, to treat heavy menstrual bleeding, endometriosis and pelvic pain syndromes, for postmenopausal hormone replacement, and as an antilibidinal agent in sex offenders. While synthetic progestagens are most commonly used in these pharmaceutical products, micronized progesterone products have recently been developed for use, primarily as intravaginal contraceptives and to support IVF. The commercial availability via the internet and promotion of progesterone in the popular press (Lee 1999) has led to nonprescription use as a cosmetic/supplement. Suggested benefits (from internet advertisements) are

improved sexual libido, enhanced serenity, prostate support, opposition of "estrogen dominance," and production of healthy and more intelligent children.

Progesterone is given to production animals to speed weight gain. Federal law limits the allowable residues of progesterone in beef and lamb to between 3 and 15 parts per billion (US Food and Drug Administration 1977).

Progesterone is also an environmental contaminant. Progesterone is among the hormonally active agents found in the pulp mill effluent implicated in reproductive effects on wildlife in Florida (Jenkins et al. 2003). Screening of stream water in the U.S. found progesterone in 70 of 139 samples tested (Kolpin et al. 2002). A related progestagen, medroxyprogesterone, became a food contaminant for humans and livestock when waste water from a pharmaceutical company containing glucose, and contaminated with the progestagen, was recycled as a sugar source for soft drinks and animal feed (Graff 2002).

B.3. Pharmacokinetics

Endogenously produced progesterone is derived from cholesterol by side-chain cleavage and 3β-hydroxysteroid dehydrogenase (Figure 2). Progesterone production is classically associated with the corpus luteum and the placenta, with some contribution of adrenal steroids. Endogenous progesterone may be metabolized to other steroid hormones by appropriate enzyme systems in specialized cells or released into the circulation by endocrine organs (ovary, placenta, adrenals). The metabolism of progesterone in humans has been reviewed (Simon 1995; Aufrere and Benson 1976). In women, the rate of production of progesterone is about 2.3-5.4 mg/day during the follicular phase of the menstrual cycle and 22-43 mg/day during the luteal phase. During the later stages of pregnancy, when placental production is at its peak, the total rates of progesterone production can be well over 300 mg/day. The adrenals of young males produce progesterone at a mean rate of 0.75 mg/day. Approximately 20% of the total circulating progesterone is bound to corticosteroid binding globulin with most of the rest bound to albumin. As would be expected, plasma progesterone concentrations vary by sex and reproductive state. For males, levels average 0.03 μg/100 mL. In females during a normal menstrual cycle, levels rise from about 0.03-0.12 μg/100 mL during the follicular phase, to a maximum of 1-2 μg/100mL after ovulation. Plasma progesterone levels increase during pregnancy to a peak of 12-20 μg/100 mL near term.

Progesterone is rapidly metabolized and cleared in humans as well as in a variety of animal species studied (Simon 1995; Little et al. 1975). Clearance rates in L/day/kg have been reported to be 60-70 for humans, 40-50 for rhesus monkeys, 55-60 for rabbits and 120 for rats. In humans, the liver accounts for approximately two-thirds of progesterone metabolism, resulting in a strong first-pass effect for orally administered exogenous progesterone. The half-life of progesterone in serum is about 5 minutes. The primary metabolite (6-27%) is pregnanediol (5β-pregnane-3α, 20α-diol). Other metabolites include 20 α–hydroxypregn-4-en-3-one and 5 α-pregnan-3,20-dione. A variety of

hydroxylations at various carbons of the steroid structure can be accomplished by cytochrome P-450 (CYP) enzymes. The major circulating metabolites are 17 α-hydroxy-progesterone, 11-desoxycorticosterone, and 20-dihydroprogesterone. Pregnanediol and other metabolites are conjugated with glucuronic acid and excreted by the kidney.

Absorption of exogenous progesterone is rapid by all routes of exposure, including the vaginal mucosa (Archer et al. 1995). Low doses administered by the oral route, however, are almost completely metabolized in one pass through the gastrointestinal mucosa and the liver (Simon 1995). Progesterone that reaches the circulation from exogenous sources would be expected to follow the same metabolic pathways as progesterone released by endocrine organs.

B.4. Biological actions and mechanisms

Progesterone (P4) is a phylogenetically old and versatile hormone (Li and O'Malley 2003; Mahesh et al. 1996; Gemmell 1995). It has an extensive suite of cellular targets including specific nuclear and membrane receptors, neurotransmitter receptors and signal transduction pathway components. Many effects of progesterone are mediated by the progesterone receptor (PR), a member of the nuclear receptor superfamily (Schumacher et al. 1999). Two isoforms, PRA and PRB, have been cloned and their functions are currently being studied with specific knock out mice (Conneely et al. 2003b; Gruber and Huber 2003). It has been proposed that PRB is the major mediator of gene transcription activation, whereas PRA provides an inhibitory effect on transcription at PRB as well as the estrogen and glucocorticoid receptor (Gruber and Huber 2003). PR is known to be expressed in uterus, mammary gland, ovary, fallopian tube (Christow et al. 2002) and placenta (Shanker et al. 1997). In male reproductive tissues less work has been done, but PR mRNA was identified in nonhuman primate testes, prostate, and epididymis (Heikinheimo et al. 1995). In nonreproductive tissues, PR is found in the thymus, vascular smooth muscle, bone, the central nervous system (Conneely et al. 2003a), the peripheral nervous system (Martini et al. 2003; Melcangi et al. 2003), the immature bladder (Celayir et al. 2002), the lung (Gonzalez-Arenas et al. 2003), and the islet cells of the pancreas (Doglioni et al. 1990). Progesterone actions in regulating gonadotropin-releasing hormone (GnRH) production and release at the hypothalamic and pituitary level are critical to its effects on ovarian cycling and spermatogenesis, as discussed in section D3.

There is also recent documentation of a membrane progesterone receptor (mPR) that activates signal transduction pathways in selected cell types (granulosa cell, thymocyte, fish oocyte) (Zhu et al. 2003a; Zhu et al. 2003b). The mPR is particularly notable as a promotor of oocyte meiosis. There are three mPR isoforms (alpha, beta and gamma) They are expressed in neural, kidney and intestinal tissues in addition to the reproductive tract. Membrane effects of progesterone are also implicated in induction of the acrosome reaction in sperm (Ambhaikar and Puri 1998). In addition to PR and mPR, progesterone also has some ability to bind the glucocorticoid receptor. Binding and activation of the androgen receptor has been considered negligible but extensive studies have not been

conducted (Pollow et al. 1989). Progesterone metabolites (3 alpha hydroxyprogesterones) are modulators of the GABAa receptor and act as "neurosteroids" (Mahesh et al. 1996; Lambert et al. 2003). Progesterone also has modifying effects at the nicotinic acetylcholine receptor and the sigma receptor (Schumacher et al. 1999). In addition to receptor mediated and modulating effects, progesterone can play a role in signal transduction pathways (Schumacher et al. 1999).

Limited work has been done on PR expression in the embryo. PR mRNA and protein were expressed in preimplantation pig embryo prior to the fifth cell division but not at later stages through blastocyst formation (Ying et al. 2000). No studies of PR expression during early organogenesis were found. Human fetuses at 11-21 weeks of gestation (late embryonic, early fetal period) expressed PR (primarily PRB) in all tissues examined (heart, liver, kidney, spleen, pancreas, intestine, thyroid, adrenal, ovary and uterus) but expression was limited later in gestation to reproductive organs, pancreas and intestinal cells and (Inoue et al. 2001). PR mRNA and protein are expressed at increasing amounts in the female reproductive tract of the rat after organogenesis (Okada et al. 2002).

Because progesterone is an early intermediate in synthesis of all steroid hormones (androgens, estrogens, glucocorticoids), exogenous progesterone can presumably alter production of these hormones, although this has not been studied in detail. Progesterone is not known to be an inhibitor of cholesterol side chain cleavage enzyme, a rate limiting enzyme in the steroid synthesis pathway, but induces expression of StAR mRNA and protein in Leydig cells (Schwarzenbach et al. 2003). Progesterone has been shown to inhibit 5 α-reductase, another important enzyme in steroid hormone metabolism, in cultured fibroblasts from human newborn genital skin (Dean and Winter 1984).

B.5. Non-DART toxicities

B.5.1. Acute Toxicity

A lethal dose of progesterone for mice by the i.v. route is 100 mg/kg (RTECS 2004). An LD50 for rats by the intraperitoneal (i.p.) route is 327 mg/kg (RTECS 2004). An acute anesthetic effect has been documented in the mouse at 16 mg/kg i.p. (RTECS 2004).

B.5.2. Nonreproductive tract target tissues

Apart from the reproductive system, the nervous system is a major target organ of progesterone (Gruber and Huber 2003; Lambert et al. 2003). Hypothalamic PRs are involved in regulating the hypothalamic-pituitary-gonadal axis, but PRs in the peripheral nervous system and the cortex subserve other functions. Neurosteroid effects on GABAa receptors are thought to mediate anxiolytic, sedative, and anticonvulsant effects of progesterone metabolites (primarily 3α-hydroxy-5 α-pregnan-20-one); progesterone itself also has limited activity. Progesterone is also known to promote normal sleep cycles. Progesterone can alleviate migraine headache syndromes via altered release of mediators from the meninges. An adverse effect associated with meningeal progesterone actions is

enhancement of growth of menangiomas. In the peripheral nervous system, progesterone produced by Schwann cells promotes myelination. No literature was found concerning the adverse actions of exogenous progesterone or altered circulating progesterone levels on myelination.

Progestagens have been hypothesized to affect bone loss through action at glucocorticoid receptors (Ishida et al. 2002). No data on progesterone administration in vivo were identified in literature searches, but progesterone receptors have been documented on human osteoblasts and progesterone activates osteoblasts in primary cultures derived from human females. When given as part of hormone replacement therapy, progesterone has been shown to lower high density lipoprotein (HDL) (Ottoson et al. 1984).

Other nonreproductive tissues expressing progesterone receptors are lung (Gonzalez-Arenas et al. 2003), bladder (Celayir et al. 2002), coronary artery smooth muscle cells (Dubey et al. 2004), and immune cells. Progesterone toxicity associated with these tissues has not been explored.

B.5.3. Mutagenicity and cancer

The National Toxicology Program (NTP) has listed progesterone since 1985 as "*reasonably anticipated to be a human carcinogen,"* based on sufficient evidence of carcinogenicity in experimental animals (NTP 2002). Similiarly the International Agency for Research on Cancer (IARC) has classified progesterone in Group 2B, possibly carcinogenic to humans, based on sufficient evidence in experimental animals (IARC International Agency for Research on Cancer 1982). Progesterone has been listed as a carcinogen under Proposition 65 since 1988 (Title 22, Cal. Code of Regs. § 12000). The carcinogenicity data for progesterone have been summarized by NTP (NTP National Toxicology Program 2002) as follows:

> "When progesterone was implanted subcutaneously, mammary carcinomas were induced at a significantly earlier age and at a higher incidence in female mice. Long-term subcutaneous implants induced ovarian granulosa cell tumors or endometrial stromal sarcomas in female mice (IARC 1974, 1979). Subcutaneous injections of progesterone induced increased incidences of mammary tumors in adult female mice and lesions of the vaginal or cervical epithelia and genital tract lesions in newborn female mice. Hyperplastic alveolar-like nodules and other dysplasias were also induced in female neonatal mice (IARC 1979). Long-term subcutaneous injections in female dogs induced endometrial hyperplasia, inhibition of ovarian development, marked mammaryhyperplasia, and some fibroadenomatous nodules of the mammary gland (IARC 1979, 1982).
>
> "Female mice injected subcutaneously with progesterone showed decreased latent periods for the induction of mammary tumors by 3-methylcholanthrene. Ovariectomized female mice receiving injections of progesterone developed sarcomas of the uterine horn when given an intrauterine implant of 3-

> methylcholanthrene and developed increased incidences of squamouscell carcinomas of the cervix or vagina when treated intravaginally with 7,12dimethylbenz[*a*]anthracene (IARC 1974, 1979). Local applications of 3-methylcholanthrene and subcutaneous implantations of progesterone induced increased incidences of vaginal-cervical invasive squamous cell carcinomas in female mice (IARC 1979). Rats receiving subcutaneous or intramuscular injections of progesterone had decreased latent periods and/or increased incidences of mammary tumors induced by oral administration of 3-methylcholanthrene or 7,12dimethylbenz[*a*]anthracene, but only when the known carcinogens were administered first. An increased incidence of mammary tumors was induced in female rats fed 2-acetylaminofluorene in the diet and injected intramuscularly with progesterone. Newborn female rats receiving a subcutaneous injection of progesterone and a subsequent intragastric instillation of 7,12dimethylbenz[*a*]anthracene developed increased incidences of mammary adenocarcinomas (IARC 1979)."

In 1999 IARC reviewed "progestogen only contraceptives," and designated them "possibly carcinogenic to human (Group 2B)" based on sufficient evidence in experimental animals (IARC International Agency for Research on Cancer 1999).

Genotoxicity data on progesterone have been summarized as follows (IARC International Agency for Research on Cancer 1987):

> "Progesterone did not induce dominant lethal mutations in mice or chromosomal aberrations in rats treated in vivo. It did not induce chromosomal aberrations or sister chromatid exchanges in cultured human cells, nor chromosomal aberrations or DNA strand breaks in rodent cells. Studies on transformation of rodent cells in vitro were inconclusive: a clearly positive result was obtained for rat embryo cells, a weakly positive result for mouse cells and a negative result for Syrian hamster embryo cells. Progesterone was not mutagenic to bacteria."

C. Developmental Toxicity

Concern about the adverse developmental effects of progesterone has arisen from progestagen use as a therapeutic agent in human pregnancy. The majority of the literature concerns progestagens other than progesterone. Endpoints that have received attention are female virilization, male hypospadias, cardiovascular malformation, birth defect incidence, parturition, and postnatal brain and behavior. Data on standard pregnancy outcome variables (prematurity, birthweight, stillbirth) are also available from some studies.

C.1. Human studies

The large literature evaluating clinically used progestagens in pregnancy was screened to identify studies that contained information specifically on progesterone. These studies are reviewed below with some information on the broader progestagen-related literature.

C.1.1. Malformation

Six studies were identified which examined the association of exposure to progesterone and the risk of congenital malformations, three prospective studies (Michaelis et al. 1983; Heinonen et al. 1977; Harlap et al. 1975) two a retrospective review of medical records (Check et al. 1986; Rock et al. 1985), and two retrospective studies (Resseguie et al. 1985). These are described below. Other studies which have examined maternal hormone use during pregnancy in relation to the occurrence of congenital abnormalities were not considered for this review as exposure to progesterone alone, exclusive of other sex hormones, was not considered in the analysis (Gal et al. 1967; Nora and Nora 1975; Ericson and Kallen 2001; Levy et al. 1973; Ferencz et al. 1980)

A prospective study was conducted in pregnant women attending prenatal clinics in West Jerusalem between 1966 and 1968 (Harlap et al. 1975). Interviews with the women included a question about drugs taken early in pregnancy. Of the 11,468 babies in the cohort a total of 432 babies were born after exposure to hormones, which included stilbestrol (2), gestanin (27), medroxyprogesterone (259), progesterone (15), abortifacients (29), and drugs to guard against miscarriage (100). In the 15 babies exposed to progesterone, there were two anomalies, hip dislocation and melanoma (no statistical analysis reported). Forty-seven of the babies exposed to hormones prenatally, including estrogens or progesterones, were born with a malformation giving a rate of 108.8 per 1,000 compared with 77.6 per 1,000 in babies born with no exposure to hormones ($p<0.02$). A significantly elevated rate of malformations was found in babies exposed to either medroxyprogesterone or progesterone during pregnancy, 127.7 per 1,000 births, including genetic defects, compared to babies with no exposure 77.6 per 1,000 ($p<0.002$). There was a wide range of malformations in babies exposed to medroxyprogesterone including structural anomalies, (such as hypospadias, heart defects, and cleft palate), as well as an enzyme deficiency. It was noted that some of the use of hormones might be for therapeutic purposes and that an excess of malformations would be expected among infants born to mothers with threatened or previous abortions. Control for potential covariates or confounders was not conducted in this study.

Heinonen et al. (1977) conducted a prospective cohort study, the Collaborative Perinatal Project, which included 50,282 mother child pairs from 12 hospitals throughout the United States. Women were interviewed before the birth of the child. Information on drug use was recorded at each antenatal visit and confirmed by the attending physician or by chart review. Diagnoses of cardiovascular defects were ascertained from one or more of the following sources: daily examinations during the first week of life, physical examinations at one year and autopsy data. Cardiovascular defects were observed in 404 children, 41 per cent of whom died. Of the 1042 women who used sex hormones during

the first four lunar months of pregnancy, 428 (41%) used progestagens exclusively. However, only three women were exposed solely to progesterone and, due to the small number in this group, progesterone was not analyzed separately.

In a prospective study conducted in West Germany, Michaelis et al. (1983) examined exposure to various drugs and the risk of congenital malformations. Women were enrolled within the first trimester of pregnancy from 1964-1976 at clinics in participating hospitals. Detailed information was kept by the physicians on drug intake.The women recorded information in diaries on drug intake, exposure to chemical agents, (e.g. detergents, insecticides, fertilizers), illnesses, daily work load, and other factors. Infants were routinely examined up to 36 months of age. Congenital malformations were reported by the clinics, evaluated by an expert committee and classified as major or minor malformations and other abnormalities. Drug use in this sample pertained to specific drugs taken during the first three months past the last menstrual period (LMP). A control group consisted of births from the cohort unexposed to any sex hormones and matched by maternal age, number of previous pregnancies and abortions, and marital status.

Women had taken progesterone (Proluton) (n = 186) and Proluton-Depot (hydroxyprogesterone caproate) (n = 462) mainly to prevent abortion; however, about half of these cases has also taken one or more other sex hormones. No major malformations were observed following the intake of Proluton. Four malformations were reported in women taking Prolution-Depot only within the first 12 weeks past the LMP. Malformations were listed as: chromosomal aberration, central nervous system, musculoskeletal, and not specified. Restricting the analysis to women only taking Proluton-Depot, no increased risk of malformations was observed in 320 matched pairs (number of major malformations = 4 cases and 6 controls, odds ratio = 0.66, 90% CI, 0.17 – 2.30). No information concerning doses was provided.

Rock et al. (1985) conducted a review of medical records to examine the risk of fetal malformations following progesterone therapy during pregnancy. The records of all women treated with progesterone from 1949 to 1977 at Johns Hopkins Hospital were examined. Progesterone was the sole medication for the treatment of threatened abortion, luteal phase deficiency, and/or habitual abortion. Newborn status was determined from review of records or phone conversations with parents, pediatricians, and/or obstetricians. Mean period of follow-up was 6.8 years, with a range from birth to 30 years of age. However, many patients (41%) had information available only at birth. Progesterone was initially administered on the third day of temperature rise, or at the beginning of the fifth week of pregnancy.

Ninety-three women conceived while taking progesterone suppositories or progesterone in oil intramuscularly. Among 42 pregnancies reported in women taking suppositories, 29 resulted in term births, 12 in spontaneous abortions, and one in a preterm delivery. All these women were treated with progesterone during the first trimester with the average dose being 2236 mg (range, 75 mg - 4500 mg). The average total dose

administered to each patient was 11,530 mg (range, 6000 – 13,5000 mg). Among 51 pregnancies reported in women taking progesterone in oil intramuscularly 45 were term births, three ended in spontaneous abortion, one in neonatal death, one in preterm delivery, and one was stillborn. Two malformations occurred in the term births (2/45=4.4%), one with a unilateral undescended testis, and one with a meningomyelocele. The average dose in the first trimester was 1009 mg (range, 75-3800 mg). The spontaneous abortion rate in women receiving progesterone suppositories was 28.6% versus 16.1% in women receiving progesterone in oil. No statistical analysis was reported. The authors concluded that there was no increased risk of congenital malformations associated with administration of progesterone. However, they did acknowledge the limitations of the study including the small sample size and the lack of a control group.

A study by Check et al. (1986) examined the risk of fetal anomalies as a result of progesterone therapy. Questionnaires on the results of fetal outcome were sent to 475 patients who had been part of a study to determine whether progesterone given prophylactically could reduce the rate of spontaneous abortions in high risk patients. The questionnaires were sent a minimum of one year after the birth of the infant in order to detect any problems that developed during the first year. Responses were received from 382 women (80% response rate). Women received 50 mg/day of progesterone, as suppositories, 3 or 4 days following release of the ovum as confirmed by sonographic examination. If there was clinical or laboratory evidence for a need for increased progesterone therapy, 17-α-hydroxyprogesterone, given intramuscularly, was added to the vaginal progesterone. Progesterone was increased to 100 mg/day following a confirmed pregnancy diagnosis by beta-subunit human chorionic gonadotropin test 18 days after forming a mature follicle. For the majority of patients, progesterone therapy was stopped by 14 weeks; however, some extended the therapy for a few weeks while other remained on it until 34 weeks, depending on clinical circumstances.

Of five women who reported birth defects, four were taking progesterone and one was taking 17-OHP. The defects of the infants of women taking the progesterone included: club foot, cleft palate, hydrocephaly; transposition of great vessels, Sprengle's deformity, congenital elevation of the scapula**,** and omphalocele. The defect reported by one woman taking 17-OHP was ventricular septal defect and pulmonic stenosis. The duration of treatment with either hormone was 16 weeks. The authors stated that the 1.3% incidence of anomalies in women treated with hormones was low and concluded that there was no increased risk associated with progesterone therapy. No control group was included in this study. No statistical analyses were conducted.

Resseguie et al. (1985) conducted a retrospective cohort study to examine the relationship of exposure in utero to progestins and the risk of congenital malformations. The medical records of 24,000 women who had received prenatal care at the Mayo Clinic were reviewed to identify children who had been exposed to sex hormones before birth. The medical records of all singleton live births or stillbirths delivered between 1936-1974 were eligible for the study. The exposed cohort included all children exposed in utero to

any exogenous progestin but not to any other sex hormone or gonadotropin. Two control subjects were matched to each exposed subject by sex, age of mother, and number of previous liveborn children. Medical records of all the children were manually searched for diagnosis of anomalies by one investigator. Cox proportional hazards model was used to compare the exposed and unexposed groups while adjusting for potential covariates. These covariates considered for inclusion into the models included occurrence of bleeding during gestation, death or malformation in a previous pregnancy of the mother, record of the mother's examination or treatment for infertility, and age of the mother. However, it was not specified in the article which of these were actually included in the analyses. Age of the mother was one of the variables used for matching of the subjects however, perfect matching did not appear to have been achieved.

In the exposed group, 649 were exposed to 17 α-hydroxyprogesterone caproate, 244 to progesterone, with 141 exposed to other progestins. The study found no tendency for an excess of cardiovascular, central nervous system or limb reduction anomalies, or hypospadias in the exposed group. Due to the rarity of specific anomalies all major anomalies were combined for comparison between groups. Statistical analyses were reported not to support an association between exposure to the specific progestins and the occurrence of congenital malformations. Although data were presented separately for those exposed to 17 α-hydroxyprogesterone caproate only, the multivariate analyses appeared to include subjects exposed to any of the progestins. No detailed results of the multivariate analyses were presented.

C.1.2. Other pregnancy outcome variables

Data on the effects of progestagens, including progesterone, on various pregnancy outcome parameters are scattered throughout the literature. The Cochrane Library produces literature reviews and meta-analyses of clinical trials and clinical studies which are available by subscription to promote "evidence-based medicine." A recent Cochrane review (Oates-Whitehead et al. 2004) conducted a meta-analysis of all randomized controlled trials of progestagen therapy to prevent spontaneous abortion during the first 20 weeks of pregnancy. Both efficacy and toxicity were considered. The adverse outcomes relevant to this reproductive hazard identification document were: miscarriage, preterm birth, stillbirth, neonatal death, low birth weight, fetal genital abnormalities, teratogenic effects and admission to special care unit. Fourteen studies were included in a meta-analysis; an additional sixteen studies were reviewed but could not be included in the meta-analysis due to deficiencies. The meta-analysis reported an odds ratio (OR) of 1.05 (95% CI, 0.83 – 1.34) for birth rates between progestagen and placebo groups. In examining the six studies that reported incidence of preterm birth, the meta analysis reported an OR of 1.15 (95% CI, 0.68 – 1.93). For three studies that reported neonatal death as an outcome, no significant differences were seen between the treatment groups.

The Cochrane review identified three studies, described below, that used progesterone as the progestagen (Nyboe Andersen et al. 2002; Gerhard et al. 1987; Swyer and Daley 1953).

In a study of IVF pregnancies, half the women (n=153) received the usual regimen of 200 mg progesterone vaginally per day for five weeks after embryo transfer, while another group (n=150) received progesterone for a shorter period of time (two weeks) (Nyboe Andersen et al. 2002). No differences were seen between the two groups in the number of abortions, the number of deliveries, the length of gestation or birthweight. A similar study (Smitz et al. 1992) administered progesterone either by intra-muscular (i.m). injection (50 mg/day) or intra-vaginally (600 mg/day) for the first 12 weeks of pregnancy to support IVF pregnancies. The number of resulting implantations did not differ between the two groups. Studies of progesterone in IVF pregnancies do not have control (no progesterone) groups since progestagens are needed to induce uterine preparation for implantation.

Other investigators studied clinical populations of women at risk for abortion. Swyer (1953) randomly assigned "habitual aborters" (women with two or more consecutive previous spontaneous abortions) to receive 150 progesterone i.m. (n=53) or no treatment (n=60). No differences were found in the number of abortions or of living children. Gerhard et al. (Gerhard et al. 1987) studied 56 women with vaginal bleeding (threatened abortion), 27 of whom were randomly assigned to receive 25 mg vaginal progesterone twice daily. Four women were later excluded for unrelated pregnancy complications. About 25% of each group had induced ovulation. The treatment continued until an abortion occurred or bleeding ceased. Progesterone had no apparent effect on the vaginal bleeding. There were five abortions in the 26 women in the placebo group and three abortions in the 26 treated women. Pregnancy length, birthweight, birth length and biparietal diameter were provided in tables only for a subgroup of patients who began bleeding prior to the seventh week of gestation; no differences were apparent between progesterone and placebo treated patients in this subgroup. The authors noted lack of any indication of virilization in the newborns.

C.1.3. Virilization

Certain abnormalities of the external genitalia (e.g., clitoral hypertrophy) of newborn girls indicative of virilization are seen primarily in connection with the congenital adrenal hyperplasia syndrome. Shortly after introduction of nor-testosterone agents as progestagens in clinical practice in the 1950's, virilization began to be reported in newborns without associated congenital adrenal hyperplasia and was soon attributed to the use of progestagens (Kupperman 1961; Hayles and Nolan 1957; Jones and Wilkins 1960; Wilkins 1959; Wilkins et al. 1958; Burstein and Wasserman 1964; Grumbach et al. 1959; Hillman 1959; Shirkey 1972). More that 400 girls are estimated to have been virilized in this manner. The case reports eventually led to discontinuation of use of nor-testosterone progestagens and no formal case-control or epidemiological studies were conducted. A number of papers, mostly case reports and case series, examined whether

progesterone and nor-progesterone derivatives had the same effect as the nor-testosterone derivatives. Studies in animal models, reviewed below, were also undertaken. Review of human and animal studies concluded that virilization was a property of the testosterone derivatives (Scialli 1988).

Following review of the literature pertaining to virilization, and screening for studies in which only progesterone were administered prenatally, three case reports were identified as appropriate for inclusion in this document. These reports were of abnormalities of the external genitalia associated with virilization in three female infants (Hayles and Nolan 1958, Reilly et al. 1958). In addition, a report of five male infants described hypertrophy of penis and scrotal hyperplasia (Russell 1969, in Shirkey 1972)

Two cases of virilization of female fetuses were reported by Hayles and Nolan (1958). In the first case an infant 6 weeks of age was brought to the Mayo Clinic because of abnormality of the genitalia, an enlarged clitoris and fused labia. The infant was born to a 27-year-old woman who had previously given birth to two normal children, one male and one female. During the third pregnancy, three injections per day of progesterone (lutocyclin) 10 mg each, were administered on March 4, 5, and 8 because of a small amount of uterine bleeding. The date of the LMP was December 17 and the date of conception was about December 28. No bleeding occurred after the injection on March 4. The mother reported that the injections were followed by an increase in libido and a growth of long hairs under the chin and about the breast. The delivery of the infant was normal. The sex chromatin pattern was female. The authors reported a suggested diagnosis of female pseudohermaphroditism without evidence of adrenogenital syndrome.

In the second case a three-year-old girl was brought to the Clinic due to enlargement of the clitoris, which had been observed at birth. The 29-year-old mother had had two miscarriages and so when a diagnosis of pregnancy was confirmed the woman was started on a treatment of progesterone linguets (lutocylol) of 10 mg twice daily. The date of the LMP was September 22 and the progesterone treatment began on November 2. On December 31 treatment began with daily oral doses of 2.5 mg of estrogen. In late March following hospitalization for threatened abortion the dose of progesterone was increased to 20 mg every eight hours. This dose was continued until June 1 when it was reduced to 10 mg four times daily. The progesterone treatment was discontinued on June 14. A normal delivery occurred on June 27. No abnormalities were noted except that of the external genitalia. The sex chromatin pattern was that of a female.

In another case report by Reilly et al. (1958), a mother had five previous miscarriages and two live births. During the 18th week of the mother's eighth pregnancy, daily doses of 120 mg daily of progesterone (lutocylol) were begun. This dose was increased to 240 mg at the 28th week until delivery and occasionally 320 mg daily may have been taken. An infant girl, weighing 1815 gm, was delivered during the 34th week of pregnancy. The only abnormalities detected were an enlarged clitoris without a urethra and enlarged

labia. The clitoris began to decrease in size after the third day of life and continued to do so until the last examination at two years of age.

A report by Russell (1969), as described by Shirkey (1972), described enhanced virilization in five male fetuses whose mothers received 100 mg/week to 300 mg/week of progesterone by i.m. injection from the end of the 2nd to the 9th week of gestation. It was noted that no persistent or progressive alteration of urinary steroid occurred despite what the author believed were wide-ranging and long-lasting physical and behavioral effects. The following abnormalities were seen: hypertrophy of penis and scrotal hyperplasia persisting for two years or more in all five boys; pubic hair was noted in three shortly after birth; exceptional muscularity was noted in all; walking occurred as early as 7 months. Hyperkinesia and aggressiveness was characteristic for the first year of life, with resistance to sleep.

C.1.4. Hypospadias

Hypospadias are a relatively common congenital anomaly with a birth prevalence ranging from 0.3% to 0.8% of Caucasian male live births in the United States. Studies have reported different risk factors for hypospadias including genetic polymorphisms and mutations; infants with lower birth weights, shortened length of gestation, and/or evidence of growth retardation in utero; parental subfertility and maternal age (Kallen and Winberg 1982; Sweet et al. 1974; Manson and Carr 2003; Fisch et al. 2001). Environmental exposures have also been associated with hypospadias incidence (Manson and Carr 2003; Baskin et al. 2001; Pierik et al. 2004).

Early case reports of hypospadias in progestagen treated pregnancies (Kupperman 1961) have led to a number of studies examining the relationship between progestagens administered in pregnancy and the risk of hypospadias (Czeizel and Toth 1990; Kallen et al. 1992; Kallen et al. 1991; Kallen et al. 1991; Kallen 1988; Kallen et al. 1986; Kallen and Winberg 1982; Sweet et al. 1974; Stoll et al. 1990; Aarskog 1979; Mau 1981; Katz et al. 1985; Czeizel et al. 1979; Polednak and Janerich 1983; Macnab and Zouves 1991; Silver et al. 1999). Most of these studies have case-control designs with hypospadias cases selected from birth registries or birth defect registries. None control for the major risk factors known to be associated with hypospadias such as genetic defects in steroid metabolism, small for gestation age, subfertility in parents, paternal smoking, and paternal genital anomalies. Some have reported a positive association (Kallen 1988; Macnab and Zouves 1991; Aarskog 1979; Calzolari et al. 1986; Czeizel et al. 1979), while others, sometimes from the same research group, are negative or inconclusive (Czeizel and Toth 1990; Kallen et al. 1991; Kallen et al. 1986; Kallen and Winberg 1982; Sweet et al. 1974; Stoll et al. 1990; Mau 1981; Katz et al. 1985). A major barrier in reaching a weight of evidence conclusion regarding the association between this endpoint and exposure to progesterone is that the studies use varied exposure categories such as "progestins", "maternal hormone administration" or "sex hormones" which include a spectrum of agents with different pharmacological properties. Meta-analyses and reviews have not been able to confirm an association between progestins in pregnancy

and hypospadias (Scialli 1988; Raman-Wilms et al. 1995; Oates-Whitehead et al. 2004; Manson and Carr 2003).

Because initial therapeutic use of progesterone was rapidly superseded by more bioavailable synthetic progestagens, there are very few papers concerning hypospadias which include information on exposure to progesterone. These papers are described below.

Two studies were identified in which progesterone was administered during pregnancy (Kupperman 1961; Macnab and Zouves 1991). One of these studies was in a population of women who had undergone IVF (McNab and Zouves 1991). The second study involved treatment for the prevention of abortion (Kupperman 1961).

One additional study of IVF is discussed (Silver et al., 1999). Although the form of "gestational progestins" used to support the IVF pregnancies was not specified in this study, the standard procedure is to administer progesterone (Yoshida 1999). This was also implied in the discussion section of the study.

Kupperman (1961) examined 62 newborn infants whose mothers had received a daily dose of 1000 mg of progesterone and 14 whose mothers had received 200 mg of acetoxyprogesterone. The drugs were given to prevent abortion and the major concern when these agents were used was partial masculinization of the females. Although no abnormality in the genitalia of the females was found, two of the 38 males, one in each treatment group, had hypospadias. No information was presented concerning potential covariates or confounders.

In a study conducted by McNab and Zouves (1991) at a University Hospital in Vancouver, British Columbia between 1985-1988, two cases of hypospadias were reported that occurred in 53 male infants conceived as a result of Assisted Reproductive Technology. The first case involved IVF in which one hour after retrieval of the oocytes the woman, a 28 year old, was given 50 mg progesterone in oil by i.m. injection. Forty-eight hours later a second dose was administered by the same route and the embryos were replaced. Three days after embryo replacement 25 mg of natural progesterone was administered twice a day as an intravaginal suppository. This was continued until eight weeks gestation. The male infant born with hypospadias was one of a set of dizygotic twins. No other medications apart from prenatal vitamins were taken during pregnancy.

In the second case reported the mother, a 32 year old, had IVF and Gamete Intrafallopian Transfer performed. One hour before embryo replacement, the patient was given 50 mg of progesterone by intramuscular injection. Three days later, 25 mg of progesterone was administered two times a day as a vaginal suppository. Progesterone was discontinued at eight weeks of gestation. There was no family history of congenital anomalies and no other medications were taken during pregnancy.

Silver et al. (1999) conducted a retrospective study which included "chart reviews" of all live male births conceived by IVF at the Greater Baltimore Medical Center from 1988-1992 to identify those born with hypospadias. Also included in the study were all patients with hypospadias after IVF who were referred to Johns Hopkins Hospital between 1988 and 1995. Control data were taken from patients with hypospadias seen at Johns Hopkins Hospital without a history of IVF as well as from statistics from the Maryland Birth Defects Registry from 1988 to 1994. A total of 14 patients conceived by IVF and born with hypospadias were identified. The control group consisted of 14 contemporaneous patients with hypospadias but without a history of IVF. Controls were selected from patients without an abnormal karyotype. The distribution of hypospadias severity was similar for both groups.

The incidence of hypospadias in the IVF group was 1.46%, compared with 0.27% in the control group, representing a 5-fold increase in risk in infants conceived by IVF ($p<0.001$). Although there was no difference between the two groups with respect to mean age at study, family history of hypospadias, cryptorchidism, or male twin, there was a statistically significant difference in exposure to gestational progestins (100% in the IVF group vs. 14% in the control group). There was also a difference in family history of infertility (IVF group = 33%, control group = 7%); however, this was not statistically significant ($p<0.26$). Complete data on maternal age was not available for the control group.

Some information is also available from a large international case-control study of hypospadias (846 cases) drawn from birth defect registries in eight countries (Kallen et al. 1992; Kallen et al. 1991; Kallen et al. 1986). Among the risk factors considered were "oral contraceptives" and "hormones." One report broke down hormone use by period of gestation and specific agent. Twenty-nine cases were found to have been exposed to hormones during the period of urethral fold closure (weeks 8-16 of gestation), thought to be the critical period for induction of hypospadias. There were 15 controls matched to these cases (next nonmalformed singleton male born in the hospital) for whom hormone exposure data were available. The odds ratio was 2.3 (95% CI, 1.2-4.4) for hormone exposure during the critical period. The individual hormonal agents were also described, most of which were progestagens. The odds ratio for progestagens was 2.3 (95% CI, 1.01-5.15). Eight of 29 cases and four of 15 controls were exposed only to progesterone, and an additional two cases and one control were exposed to progesterone and other hormones. The odds ratio for progesterone only exposure was not calculated. but the group proportions are very similar (0.28 for cases and 0.27 for controls).

C.2. Animal studies.

A few studies have examined pregnancy outcome in animals with designs similar to contemporary developmental toxicology studies. Because of the potential association of progestagens with female virilization and male hypospadias in the human literature, fetal genital development has been studied in animal models. Conceptus mortality due to progesterone has also been the topic of some research.

C.2.1. Fetal/newborn parameters

Piotrowski published a series of papers looking at adverse developmental effects of prenatal progesterone administration on chicks, rabbits and rats. In the rat study (Piotrowski 1968b), 0.1, 1, 5, or 20 mg/kg were administered i.m. to pregnant Wistar rats. The two intermediate doses (1 and 5 mg/kg) were given during each of 3 gestational period (gd 1-6, gd 6-14 or gd 14-20), while the low dose was given only on gd 6-14 and the high dose only on gd 1-6. Group size varied from 7-13 but most groups contained 10 dams. This study was remarkable in demonstrating several developmentally toxic effects of progesterone. The 20 mg/kg dose was stated to "bring about the intrauterine death" of all fetuses. Birthweights were significantly reduced with the 5 mg/kg dose given on gd 1-6 or 14-20. Placental weights were also lower in the 5 mg/kg group treated from gd 14-20. An increased incidence of resorptions and "foetuses with developmental defects" was also reported for the 5 mg/kg treatment in the tabular data presentation, although no statistics were provided for these endpoints. At the lower doses (0.1 and 1 mg/kg) there was no progesterone effect on fetal or placental weights, although early resorptions were elevated in incidence for the 1 mg/kg dose given from gd 1-6. As regards morphological defects, the authors described an incidence of 4/163 (2.5%) in the 1 mg/kg group and 11/362 (3.0%) in the 5 mg/kg group. Cranial hematoma was the most common defect (10 of 11 defective fetuses in the 5 mg/kg group). No occurrence of this defect was reported in controls.

A similar study using only one dose of progesterone was conducted in rabbits (chinchilla breed) (Piotrowski 1968a). A 30 mg/kg dose was administered i.m. from gd 8 to 16 (n=10). The progesterone treated group was compared to vehicle controls (n=9). Pregnancy was established by housing females with males and fetal exams were conducted on gd 28 (term is gd 31). The progesterone group had a significantly higher male/female ratio (sex confirmed by dissection) and a statistically significantly greater anogenital distance (both males and females). Four resorptions occurred in the ten progesterone-treated pregnancies as compared to none in the nine control pregnancies. Fetal weights were comparable in the two groups. As regards the higher sex ratio, Piotrowski reported a similar result in the rat study (Piotrowski 1968a). A significantly higher sex ratio was also reported in another rat study in which progesterone (10 mg/day) was administered on gd 12-20 by s.c. injection (Hahn and Hays 1963). In this study the progesterone treated rats also received estrone 0.5 µg/day on gd 8-20. The sex ratio in that study was 1.22, compared to 1.82 in the Piotrowski rabbit study.

A study in pigs documented pregnancy outcome in sows (n=34) given 200 mg progesterone/day i.m. on gd 2 and 3 (Vallet 2002). This study was reported as an abstract only. The progesterone was intended as a treatment to reduce stillbirth. No differences relative to control (n=78) were found in litter size, stillbirth, birthweight or weaning weight.

A preclinical study of intravaginal progesterone was conducted in rabbits in connection with development of the progesterone-releasing intrauterine device Progestasert (Hudson et al. 1978). In this study, the progesterone-releasing device was inserted on gd 6, after induction of ovulation and artificial insemination, by a surgical procedure that included laparatomy. The intrauterine device released 1 μg progesterone/day. The progesterone-treated group (n=25) was compared to two control groups (n=20) which received either sham surgery or sham surgery and a device that did not release progesterone. Less than half of the does in each group completed pregnancy and 6/65 does died. In the remaining does, there were no group differences in implantations, resorptions, internal or external malformations, skeletal defects, live fetuses or fetal weight and length as determined on gd 29/30. In a subgroup allowed to deliver, gestation length and live birth index were similar in the three groups; however, most of the pups died within 4 days of birth. In a second experiment, the intrauterine progesterone device was inserted surgically on gd 9 rather than gd 6 (n=10-11/group) and all does were allowed to deliver. Thirteen of the 32 does did not deliver due to maternal death or abortion. In the remaining does, there was a significantly lower litter size and a significantly greater number of resorptions in the progesterone group than in controls. The designation of the control group for statistical purposes was not clear from the report. This is the only study located for the present review that provided a complete teratological exam (gross, dissection, skeletal) in connection with progesterone administration during organogenesis. Although the authors concluded that progesterone was not detrimental to the developing rabbit embryo, the high mortality and abortion rates, possibly related to the surgery to implant the device, detract from a conclusion regarding developmental toxicity.

Other studies looking primarily at virilizing and anti-androgenic effects on the fetus (Sections C.2.2 and C.2.2), contained data on standard pregnancy outcome parameters (gestation length, litter size, live fetuses per litter, fetal/birth weight were sometimes reported). In rats injected with 50 or 200 mg progesterone daily from gestation day (gd) 15-21, mean birthweight was reported as higher than in controls, although no statistics were presented (Scholer and de Wachter 1961). Litter sizes were similar to controls. Normal birthweights and gestation length were reported in 6 monkeys (Wharton and Scott 1964) treated with 50 mg/day intramuscular (i.m.), five days per week from gd 26 to term. No controls were provided in this study. In guinea pigs treated with 1 mg/day progesterone by subcutaneous (s.c.) injection on gd 18-60, no statistically significant effects on birthweight or gestation length were seen relative to untreated controls (Foote et al. 1964). In their studies of Wistar rats, described below, Kawashima et al. (Kawashima et al. 1977) provided only data on urovaginal septum length, with no information on birthweight or litter size.

C.2.2. Female sexual differentiation-prenatal exposure

In response to the reports of virilization of female fetuses from pregnancies treated with synthetic progestagens (Wilkins 1959; Wilkins et al. 1958), several animal studies examined the masculinizing effects on female genitalia after gestational hormone administration, usually using anogenital distance as a measure. Studies including progesterone are summarized in Table 3. Several studies confirmed the virilizing effects of the 19-nor-testosterone agents, the first of the synthetic progesterone analogues to be used clinically. Effects of progesterone on anogenital distance were also reported in two of the studies, described in detail below. One of these studies also included sex-differentiated behavioral measures.

A mouse study found effects of progesterone on female virilization reflected in both genitalia and postnatal behavior (Wagner et al. 1986). Pregnant mice were injected s.c. with 0.25 or 0.5 mg progesterone on gd 12-16. At birth, pups were killed and anogenital distance was measured under a dissecting microscope to the nearest 0.05 mm. In addition to the anogenital distance, the relative anogenital distance was determined by dividing by the body weight. Data are shown in Table 2. Statistically significant differences from control groups were found for the relative anogenital distance measure.

Table 2. Body weights and absolute and relative anogenital distances of female Rockland-Swiss albino mice that were prenatally treated with s.c. progesterone. From Wagner et al. (1986).

Group	N	Body weight(g)	Anogenital distance (mm)	Relative anogenital distance[a]
Not injected	29	1.51±0.03[b]	0.84±0.01	55.62±0.97
Vehicle (oil)	33	1.51±0.02	0.85±0.01	56.29±0.88
0.25 mg Prog	29	1.47±0.03	0.89±0.02	60.54±1.32*
0.50 mg Prog	36	1.48±0.03	0.86±0.01	58.12±1.06**

[a] anogenital distance/body weight x 100[b] mean ± SEM
*significantly different from not injected and vehicle control groups ($p<.05$)
** significantly different from not injected control group ($p<.05$)

For the behavioral portion of the study, offspring were fostered and reared by untreated dams. No progesterone effects on fertility and pregnancy outcome were found when the adult offspring were mated. The adult female offspring were then tested for aggressive behavior on postpartum days 6-8, the period when maternal aggression toward an unfamiliar male peaks. Over 80% of the lactating mice exhibited aggression. The composite aggression score (combined attacks and lunges) was over 50% higher in the progesterone-treated mice than in the controls, with no difference between the two progesterone doses. ANOVA analysis of the composite aggression score demonstrated a

significant treatment effect ($p<0.01$), and significant post-hoc comparisons between each of the progesterone groups and both control groups.

Another study in chinchilla rabbits (Piotrowski 1968a) also recorded an effect on anogenital distance. Notably, this is the only study in Table 3 that used exposure during organogenesis. The early fetal period was selected for most of the studies as the time when differentiation of genitalia occurs. The rhesus monkey study also included exposure during organogenesis but did not measure anogenital distance, looking instead for frank virilization.

An interesting characteristic of the chinchilla rabbit study is the presentation of all the individual data for anogenital distance, along with the fetal weights, the sex and the litter membership. This made it possible for RCHAS to confirm the findings of a significant progesterone effect on anogenital distance in an analysis that included body weight as a covariate and used litter as the basis of analysis.

Table 3. Assessment of virilization of female fetuses by progesterone and progestagens in animal studies.

Species/timing Group size	Progesterone dose	Endpoint	Progestagen effect* (Yes, No)	Reference
Mice gd 12-16 n=29-36	0.25, 0.5 mg, s.c.	Anogenital distance	Yes, progesterone	Wagner et al. 1986
Rabbits gd 8-16 n=84, 77	30 mg/kg, i.m.	Anogenital distance	Yes, progesterone	Piotrowski et al. 1968a
Rats, Long-Evans gd 15-20 n=13-39	5, 50, 100, 200 mg, s.c.	Anogenital distance, visual inspection	No, progesterone; Yes, nor-testosterone and acetoxy progesterone agents	Revesz et al 1960
Rats gd 15-21 n=4 dams	50, 200 mg s.c.	Anogenital distance Reproductive tract histology Gonad examination	No, progesterone or retroprogesterone; Yes, nor-testosterone agents	Scholer and deWachter 1961
Rats gd 14-19	2.5, 5, 10 mg, s.c.	Anogenital distance	No, progesterone Yes, nor-testosterone agents	Lerner et al. 1962

Table 3. Assessment of virilization of female fetuses by progesterone and progestagens in animal studies (continued).

Species/timing Group size	Progesterone dose	Endpoint	Progestagen effect* (Yes, No)	Reference
Rats, gd 16-19 group size not stated	10, 30, 100 mg, s.c.	Urovaginal septum length; prostate buds	No, progesterone, but quantitative effects	Suchowsky et al. 1967
Rats, Wistar gd 1-6, 6-14, 14-20 n=10 dams	0.1 mg/kg i.m. gd 6-14 1 mg/kg, gd 1-6, 6-14, or 14-20	Anogenital distance	No, progesterone	Piotrowski et al. 1968a
Rats, Wistar gd 17-20 Group size not stated, statistics presented	50, 200 mg, oral	Urovaginal septum length from histological sections	No, progesterone; Yes, testosterone, testosterone derivatives, medroxyprogesterone and megestrol acetate	Kawashima et al. 1977
Rhesus monkeys gd 26-term n=6 progesterone	50 mg/day, 5 days/week i.m. .	Necropsy with gross and microscopic examination of reproductive tract	No, progesterone; Yes, norethindrone; Endometrial hyperplasia in 3 female infants treated with progesterone	Wharton and Scott 1964
Guinea pigs gd 18-60 n=7 dams n=8 newborns	1 mg/day s.c. progesterone	Dissection and histology of genitals	No, progesterone; Yes, testosterone, medroxyprogesterone, norlutin	Foote et al. 1964

*see text for complete description of the effect

Other studies (Piotrowski 1968b; Scholer and de Wachter 1961; Wharton and Scott 1964; Kawashima et al. 1977; Suchowsky et al. 1967; Foote et al. 1964; Revesz et al. 1960; Lerner et al. 1962) failed to find a virilizing effect of progesterone using either anogenital distance or other measures. These studies, summarized in Table 3 and described below, generally had smaller group sizes and less precise measures of anogenital distance than the two studies demonstrating an effect. Species/strain differences might also have been a factor. Also potentially relevant to virilizing actions of progestagens, some studies commented on maternal genital changes with gestational progesterone treatments.

Enlargement of the dam clitoris was reported in one dam each in two studies (Scholer and de Wachter 1961; Revesz et al. 1960).

Piotrowski (Piotrowski 1968b) found no effects of progesterone on anogenital distance in rats. This study is described in detail in section C.2.5. The authors also note in their discussion the absence of clitoral enlargement or imperforate vagina in the female fetuses.

Kawashima et al. (Kawashima et al. 1977) studied 12 progestagens, including progesterone and nor-testosterone derivatives, as well as 7 testosterone derivatives in a standardized assay of female virilization. The endpoint used was the fetal urovaginal septum length expressed in mm/g body weight. The urovaginal septum length was obtained from stained sagittal sections of the pelvic region of the gd 21 fetal rat examined microscopically. The distance from the tip of the septum separating the urinary and vaginal cavities to the cranial tip of the pubic symphysis was measured. The urovaginal septum is shorter ("abridged") in virilized female fetuses and is not seen in male fetuses. The agents were administered to pregnant Wistar rats (group size not stated) by the oral route from gd 17 to gd 20 at two-four different doses. Compared to vehicle control, all but one of the testosterone and nor-testosterone agents were found to produce shorter urovaginal septum lengths by statistical analysis (test not stated). Two progesterone derivatives, medroxyprogesterone and megestrol, were also effective. Progesterone, at 50 and 200 mg/day, and retroprogesterone did not alter the urovaginal septum length.

Further studies on progestagen structure-activity relationships relative to genital virilization of female rat fetuses has been conducted by Kawashima and co-authors (Kasahara et al. 2001; Furukawa et al. 2001; Kiguchi et al. 2001). These experiments are available only in non-English language journals or as abstracts. When available, English abstracts state that progesterone had no virilizing effects.

A very weak androgenic effect of progesterone was suggested in a detailed quantitative study (Suchowsky et al. 1967). In this study, female rat fetuses were examined histologically for the length of urovaginal septum and also for the presence of prostatic buds. The authors report that 42-80% of untreated controls demonstrated small prostatic buds, considered grade 1. Prostatic buds equivalent to male newborns were considered grade 3, and an intermediate category was also defined (grade 2). Twelve steroid drugs, including estrogens, androgens and progestagens, were examined in this system. The female fetuses whose dams received 100 mg progesterone s.c. on gd 16-19 had a higher incidence of grade 1 prostate buds than controls, but did not demonstrate grade 2 or 3 buds, and were not considered to be virilized. The authors did not evaluate group differences statistically but provided the following description "the frequency of the appearance of prostatic buds scarcely exceeded the normal rate." A second measure obtained by Suchowsky et al was urovaginal septum length, which was used to compute a percent inhibition measure relative to untreated controls. A linear dose-response relationship for 100 and 30 mg progesterone was plotted. The authors describe the

results for this measure as follows: "the urethrovaginal septum was only slightly impeded in its development." Statistical group mean comparisons were not presented.

Other studies from this research group (Suchowsky et al. 1967; Junkmann and Neumann 1964) compared 12 progestagens with progesterone (as a negative control) and testosterone (as a positive control) for effects on vaginal development, as examined microscopically and histologically. Treatments were administered during several stages of gestation (gd 5-8, gd 10-13, gd 16-19) as well as throughout gestation (gd 5-20) by injection. The 19 nor-testosterone agents were found to be virilizing when given on gd 16-19 or throughout gestation. Since progesterone was considered a negative control, and there were no untreated controls in the study, the effects of progesterone relative to controls could not be evaluated in these studies.

Recently, the question of progestagen induction of female genital virilization has been taken up by Baskin and colleagues using tools of modern biology such as computer reconstruction of fetal anatomical structures from histological sections (Kim et al. 2004). Among progestagens, only medroxyprogesterone has been studied (De Souza et al. 2004). Doses of 50, 100 or 200 mg/kg were administered s.c. on gd 12-18 to C57BL/6 mice and genitalia of fetuses were examined on gd 19. These doses elicited virilization in terms of increased urethral length and hypertrophied periurethral spongiosum in 53, 64 and 74% of fetuses respectively at the three dose levels. The authors mention hypothalamic-pituitary effects as well as regulation of HOXa genes as possible mechanisms.

In addition to studies in mice, rats and rabbits, a few studies have been conducted in guinea pigs and nonhuman primates. These studies are particularly relevant because the long post-embryonic period of development in these species resembles that of humans. However, the studies are very limited. The single guinea pig study examined only eight progesterone-exposed fetuses (Foote et al. 1964), and one of the monkey studies contained only three progesterone-treated female fetuses (Wharton and Scott 1964). Neither of these studies reported virilization. A second study in monkeys used progesterone combined with estradiol to simulate birth control pills at several doses that were multiples of human oral contraceptive doses (Hendrickx et al. 1987). Seven to ten monkeys were treated per group. Fifty to 100% embryolethality was observed at doses 10 or 25 times the human contraceptive dose. At 25 times the human dose, growth retardation was observed in the four of ten surviving fetuses. A limb reduction defect was seen in one fetal death (25× dose) and one fetus (of ten pregnancies treated at the 10× dose) died at 96 days and had "masculinized external genitalia." Masculinized external genitalia also occurred in the same study in four of eight monkeys treated with 300 and 1000 times the human contraceptive dose of norethisterone plus ethinylestradiol. However, the authors suggested that the virilized progesterone + estradiol fetus "may have been a spontaneous occurrence" because the drug was given prior to the time of sensitivity for androgen-induced virilization.

C.2.3. Male sexual differentiation-prenatal exposure

Progestagen induction of hypospadias has not been extensively studied in animal models because of the difficulty of readily detecting this abnormal morphology in a routine fetal exam of rats and mice. Anogenital distance and retained areolas are more common indices of aberrant male reproductive tract development of rodents as determined in the perinatal period (Clark 1998). Examinations for hypospadias are more often conducted in adults after preputial separation.

Several of the studies of female virilization (Table 3) also contained information on male fetuses, usually measurements of anogenital distance. Hypospadias and reduced anogenital distance can be considered part of a syndrome of failure of differentiation of the male genitalia associated with closure of the urethral fold and are sometimes referred to as "feminization."

- Piotrowski (1968b) found an *increased* anogenital distance in male rabbit fetuses whose dams were exposed to progesterone during organogenesis.
- Revesz et al (Revesz et al. 1960) present tabular data showing smaller average anogenital distances in progesterone exposed male fetuses, but no statistics were reported. Variability data is given but the indices (sd's or sem's) are not stated and it is not possible to independently assess statistical significance.
- Suchowsky et al. (1967), Kawashima et al.(1977) and Wagner et al. (1986) examined only female fetuses.
- Scholer and de Wachter (1961) found male fetal anogenital distances to be in the normal range, but did not present group means and variability.
- Foote et al. (1964) reported normal reproductive tracts in treated guinea pigs, although examination of external genitalia was not specifically mentioned.
- Wharton and Scott (1964) reported no abnormalities in when external genitalia were examined in three male monkey infants exposed to progesterone.

In an additional study not included in Table 3 (because female newborns were not included), Pointis et al. (Pointis et al. 1987) reported no effect on anogenital distances in newborn male mice after maternal progesterone administration (2 mg/kg) on gd 14-16.

The most extensive data on male fetuses is from an article published in German (Junkmann and Neumann 1964). Progesterone as well as several experimental chlorinated progestagens were administered to pregnant rats on gd 16-19 by s.c. injection. The authors report that progesterone at the high dose of 30 mg had no antiandrogenic activity. However, the data reported in tabular form lists 3% hypospadias in male fetuses examined as compared to 0% in controls. The fetuses from the groups receiving the chlorinated progestagens had up to 100% hypospadias. Interpretation of this information is complicated by a question mark following the data on progesterone in the table ("% Tiere mit hypospadie –3?"). In addition, the group size stated in the table is presumably treated dams and the number of examined fetuses is not clear. However this

paper has been cited as demonstrating that progesterone can cause hypospadias in animals (Goldman and Bongiovanni 1967; Briggs 1982).

Only one study was found which examined reproductive function of males after gestational exposure to exogenous progesterone. Pointis et al. (Pointis et al. 1987) administered 2 mg/day progesterone to pregnant mice on gd 14, 15 and 16 via subcutaneous injections. Plasma obtained from fetuses on gd 17 showed significantly lower testosterone, and higher LH with no change in FSH. Other pregnancies went to term and anogenital distance and body weight were measured at 21, 40, 64, and 90 days of age. No progesterone effects were detected. At 90 days, males were exposed to females in estrous once weekly for three weeks to evaluate mating behavior. The number of animals exhibiting mounting, intromission or ejaculation was lower in the prenatally treated males (n=15) than in controls (n=14). About half the treated males achieved mounting and intromission, compared to all of the controls. None of the treated males ejaculated, as compared to 5/14 controls. At necropsy the testes and seminal vesicle weights of the two groups were not significantly different.

A small unpublished study was described in a recent review (Silver 2004). A progesterone pellet (dose not stated) was implanted subcutaneously in female rats on gd 10. The authors state that three of 82 gd 21 male fetuses thus exposed had hypospadias; no hypospadias were reported in controls, but the number of control fetuses was not stated and no statistics were provided. Hypospadias was apparently determined by inspection of the genitals along with dissection to determine sex.

Baskin and colleagues have recently undertaken studies of steroid hormone induced hypospadias in a mouse model (Baskin et al. 2001). Medroxyprogesterone has been studied in this regard, as reported in a recent abstract (De Souza et al. 2004). Doses of 50, 100 or 200 mg/kg were administered on gd 12-18 to C57BL/6 mice and genitalia of fetuses were examined on gd 19. Hypospadias as determined by microscopic examination of histological sections were found in 50.6, 53.3 and 58.2% of male fetuses respectively in the three dose groups. The hypospadias incidence in controls was not stated.

C.2.4. Conceptus Mortality

Some studies have investigated whether, in addition to effects on ovulation and implantation, progesterone can impair fertility by a direct influence on the viability of the embryo.

To examine this issue in the preimplantation embryo (gd 1-6), Ball et al. (Ball et al. 1992) treated pregnant pony mares with progesterone (450 mg/day i.m.) on gd 0-6 after artificial insemination. Serum sampling confirmed elevation of progesterone by the treatment; ovulation was confirmed by ultrasound. On gd 7, embryos recovered from the oviduct of treated and control mares (n=13/group) did not differ in recovery rate, diameter, developmental stage, quality or number of cell nuclei. A similar study

administered progesterone (1 mg) to rabbits on gd 1-4 and found no effect on blastocysts (Adams et al. 1961, described in IARC 1987). However, progesterone administered to rabbits prior to mating (0.5 mg 2 days prior, 1 mg 1 day prior, 1 mg day of mating) led to increased embryonic death by gd 4 (McCarthy et al. 1977).

Embryotoxic effects of progesterone later in development (gd 10) were studied in cell culture (Bowden et al. 1993). CD rat embryos were obtained at gd 10 and cultured with estradiol, DES, 17 α ethynylestradiol, or progesterone for 48 h. Two progesterone concentrations, 30 and 100 μg/mL, were used. In contrast to the estrogens, progesterone did not influence survival, embryo size or somite number relative to controls.

Conceptus mortality at the fetal stage was addressed in a study that compared conceptus mortality when progesterone was injected into the pregnant Brown Belt mouse dam vs. into the amniotic sac of the fetus (2 fetuses/pregnant dam were injected) (Petrelli and Forbes 1964). The dose and timing were based on a number of previous studies cited by the authors demonstrating the embryotoxic effects of progesterone in pregnant mice. Fetal mortality (gd 18) was 65% when mouse dams (n=20) were injected subcutaneously with progesterone (3.5 mg) on gd 15, 16 and 17. Fetuses in this group also received a sham intra-amniotic injection. A similar fetal mortality (61%) was observed when progesterone (3.5 mg) was injected directly into the amniotic sac of mouse fetuses on gd 15 (n=49 dams/group). The mortality was 13% in procedural controls (laparatomy and injection vehicle into amniotic sacs, n=49). Unmanipulated controls had a mortality rate of 2%. No statistics were calculated. The authors conclude that the progesterone was directly toxic to the gd 15 mouse fetus, possibly due to CNS narcotic effects, since no damage was seen grossly when embryos were examined. They pointed out that endogenous progesterone production declines rapidly after gd 15 in mice. Progesterone administered within 16 h after birth has also been shown to increase mortality in mice (Jones and Bern 1977).

C.3. Developmental toxicity: Other relevant data

C.3.1. Distribution and metabolism in pregnant females and conceptuses

There is some information on endogenous hormone levels after administration of exogenous progesterone during gestation to humans and laboratory animals. One study (Ferre et al. 1984) administered 400 mg micronized progesterone orally immediately before c-section to 20 women. Progesterone, estradiol and estrone were measured in maternal plasma, placenta and myometrial samples, which were taken at the time of c-section, ranging from 100-250 min after progesterone administration. Progesterone increased to about 120% of baseline in plasma and myometrium, but not placenta, peaking at about 150 min after administration and declining within an hour. Estradiol increased in the placenta. No fetal measurements were taken.

In laboratory animals, studies have been done in rats and mice. Endogenous progesterone levels declined from 8 to 5 ng/mL from gd 15 to 21 in rat fetuses (gender

not stated). At the same time maternal levels declined from 63 to 8 ng/mL (Tapanainen et al. 1979). Postnatally, endogenous progesterone levels were low in pups during lactation (< 4 ng/mL) but could be elevated by administration by subcutaneous injection of progesterone to the pups (Tapanainen et al. 1979), or to the lactating rat dams (Hull 1981). In mice, a single 2 mg progesterone injection led to elevated fetal plasma progesterone one and three hours after the injection (Pointis et al. 1984).

C.3.2. Clinical implications of fetal virilization/masculinization

Follow-up studies of offspring virilized/masculinized in utero by progesterone are not available for either humans or animals. Fertility would be a major concern. Women with genital virilization at birth due to congenital adrenal hyperplasia have low fertility potential, although pregnancies have occurred with appropriate treatment and assistance (Lo and Grumbach 2001). Fertility has been reported as normal in followup of boys who received surgery for hypospadias as well as in rats with drug induced hypospadias (Kojima et al. 2002), although mating performance was impaired in the rats.

C.3.3. Mechanism(s) of developmental toxicity.

C.3.3.1. Active agent

The active agent for various progesterone effects on development has not been studied in detail. The well known and widespread actions of progesterone at its specific receptors provides support for direct action of the parent compound. In addition, the progesterone metabolite 17 α hydroxy progesterone has been widely used as a contraceptive and is known to bind to the progesterone receptor and exert progestagenic effects. The progesterone metabolites 3 α, 5 α –THP and 5 α DH have been identified for their effects at GABAa receptors. Progesterone narcotic effects may have played a role in conceptus death after maternal progesterone administration, as suggested in discussion of one study by the author (Petrelli and Forbes 1964).

C.3.3.2. Biological mechanisms of action

Progesterone actions during development could be mediated by a number of mechanisms:

- Progesterone binding and activation of PR or mPR in fetal or maternal tissues
- Progesterone inhibition of enzymes in the steroid hormone synthesis pathways
- Progesterone suppression of gonadotropin release in the fetal or maternal hypothalamus/pituitary
- Direct androgenic effects of progesterone due to binding to androgen receptors in the mother/fetus

Very little is known about the role of embryonic and fetal PR in development, and no discussion of a direct PR-mediated effect on the fetus was identified in the literature on progesterone developmental toxicity.

Progesterone feedback inhibition of steroid hormone synthesizing enzymes is a possible factor in female virilization and male feminization. In a widely cited paper (Goldman and Bongiovanni 1967), an attempt was made to integrate the virilization and feminization effects of progestagens under a single mechanism, inhibition of 3 β hydroxysteroid hydrogenase. This enzyme is required in the early steps of steroid hormone synthesis from cholesterol. The authors describe a peak in activity of this enzyme in the testes of the human and rat male fetus at the time of urethral fold fusion and in the adrenals of the female fetus at the time of urethrovaginal differentiation. Testosterone action on the developing male urogenital system requires activity of this enzyme, which is highly expressed in the testes of the rat beginning on gd 15. The associated accumulation of dehydroepiandrosterone due to lack of 3 β hydroxysteroid hydrogenase activity in the adrenal of the female fetus was hypothesized to lead to female genital virilization. Dehydroepiandrosterone (DHEA) was identified as the steroid hormone responsible for female virilization in adrenal hyperplasia syndromes. In support of their hypothesis the authors point out the high incidence of hypospadias in human males with genetic defect in 3 β hydroxysteroid hydrogenase activity, and virilization of females seen in adrenal hyperplasia. The authors conducted *in vitro* studies that suggested progesterone inhibition of a bacterial 3 β hydroxysteroid hydrogenase enzyme, although progesterone was much less potent than estradiol.

This hypothesis was tested in an experiment in rats (Briggs 1982). A known 3 β hydroxysteroid hydrogenase inhibitor was compared to norethisterone, ethynylestradiol and medroxyprogesterone for its effects on DHEA metabolism in the fetal (gd 20) wolffian ducts after administration to the pregnant dam. While the known enzyme inhibitor reduced DHEA metabolism, the other agents were without effect at doses 100 times an estimated human clinical dose.

Another hypothesis involves progesterone inhibition of 5 α-reductase, the enzyme that converts testosterone to dihydrotestosterone. Dihydrotesterone is known to be the local mediator of male penile development. Genetic 5 α-reductase deficiency in humans (Silver 2000; Wilson et al. 1993; Imperato-McGinley et al. 1991) and administration of the 5 α-reductase inhibitor finasteride to rats (Clark et al. 1990) are associated with hypospadias. One study demonstrated potent progesterone inhibition of 5 α-reductase in cultured fibroblasts from human newborn genital skin (Dean and Winter 1984).

Direct androgenic effects of progesterone, identified in three early studies, are potentially relevant to female fetal virilization.

1. A direct androgenic effect of progesterone was tested in castrated male rats (Suchowsky et al. 1967). The weight range indicated that the rats were immature (35-45 g). The endpoint was the dose (ED100) that produced a 100% increase in the wet weight of the accessory organs (ventral prostate, seminal vesicles, levator ani muscle). For progesterone, this dose was 6 mg/d for seven days by s.c. injection. The corresponding dose for testosterone propionate was 0.016 mg/day. This indicates that progesterone has

1/360th the potency of testosterone *in vivo* for these endpoints when endogenous testosterone production is eliminated in rats.

2. A similar assessment was conducted in castrated immature male guinea pigs (Clausen 1942) using a single dose of progesterone (1 mg/day for 12 days). The mean prostate and seminal vesicle weights (n=10/group) increased approximately 4-fold. No variability measures or statistical analysis were provided. The histology of these tissues was similar for progesterone and testosterone treated groups, and failed to demonstrate the atrophy seen in controls.

3**.** A third study (Greene et al. 1939) used both castrated and intact immature (19 day old) male rats. They received a daily dose of 2 (castrated) or 3 (intact) mg progesterone for 5 days (route not stated). Only the castrated rats showed greater prostate and seminal vesicle weights than controls. These organ weights were similar in castrated, progesterone-treated rats to those in intact controls. A dose-response study was also conducted in adult castrated rats with 2, 5, 9 , 12, 17 and 35 mg/day for 7 days. Increased prostate and seminal vesicle weights were seen at 17 and 35 mg doses with indication of a dose-response curve. Group sizes were one to three in this report and no statistical analysis was conducted.

Receptor-binding studies have compared the relative binding affinities of progesterone and other progestagens (Phillips et al. 1990), although no contemporary studies of receptor activation could be located (Pollow et al. 1989). These relative binding affinities are potentially relevant to the androgenic action of progesterone vs. other progestagens that have been more widely studied for their prenatal effects on sexual differentiation in humans.

Table 4. Relative receptor binding affinities of progesterone and other progestagens. Data are from in vitro incubations of human uterus (progesterone) and rat prostate (androgen) with appropriate labeled reference steroids. From Pollow et al. (1989).

Progestagens	Relative receptor binding affinity	
	Progesterone receptor	Androgen receptor
Progesterone	40	<.01
Medroxyprogesterone	115	5
Desogesterol	1	<.01
Levonorgestrel	120	45
Cyproterone acetate	90	6
Gestodene	85	100

Table 5. Relative receptor binding affinities of progesterone and other progestagens. Data are from in vitro incubations of rabbit uterus (progesterone) and rat prostate (androgen) with appropriate labeled reference steroids. From Phillips et al. (1990).

Progestagens	Relative receptor binding affinity	
	Progesterone receptor	Androgen receptor
Progesterone	1.00	.0005
Norgestimate	1.24	.0003
Desogesterol	8.49	.118
Levonorgestrel	5.41	.220
Gestodene	9.21	.154
Dihydrotestosterone	0.03	1.00

Anti-androgenic effects of progesterone are relevant to male feminization during development (shorter anogenital distance, hypospadias). Progesterone has been found to influence testosterone production in mouse fetuses (Pointis et al. 1984). When progesterone (2 mg) was given as a single s.c. injection on gd 18, testosterone in fetal plasma was higher 1 h after injection but depressed 3 h after injection in male fetuses; however, in female fetuses, testosterone was elevated at both time points. Antiandrogenic effects in the fetus could be mediated by suppression of gonadotropin release as has been demonstrated in adult men (Brady et al. 2003).

C.4. Integrative evaluation

Developmental exposure to progesterone could occur as a consequence of progesterone therapeutic use during pregnancy, use as a supplement, or unintended exposures through environmental or food source contamination.

Epidemiologic studies of exposure to progesterone and risk of adverse developmental outcomes are difficult to evaluate due to the limitations of the studies which include the nature of the chemical being administered, the administration of progesterone alone, the dosage and timing of exposure, small sample sizes and lack of control for potential confounding variables.

Six human studies were identified which examined exposure to progesterone and the risk of congenital malformations: three large prospective studies (Michaelis et al. 1983; Heinonen et al. 1977; Harlap et al. 1975); two retrospective studies, one using medical records (Rock et al. 1985) and one using questionnaires (Check et al. 1986); and one retrospective cohort study (Resseguie et al. 1985). Only one study observed an increase in risk of malformations associated with exposure to progesterone (Harlap et al. 1975).

There are no systematic studies of pregnancy outcome (miscarriage, gestation length, birthweight, stillbirth) in progesterone-treated vs untreated pregnant women. Studies comparing longer and shorter progesterone therapeutic regimens (Nyboe Andersen et al. 2002) and different routes of progesterone administration (Smitz et al. 1993) found no difference on limited pregnancy outcome assessments. Two studies found no difference in spontaneous abortion rates inwomen at risk for abortion who were untreated or treated with progesterone (Swyer and Daley 1953).

Although many studies have examined the risk of hypospadias associated with exposure to progestagens during pregnancy, very few have been conducted on exposure to progesterone. Two studies have reported cases of hypospadias with exposure to progesterone, one for prevention of spontaneous abortion (Kupperman 1961), one associated with assisted reproductive technology including IVF and GIFT (Macnab and Zouves 1991), while a third study, a retrospective study, examined births conceived by IVF (Silver et al. 1999). Another large, international study (Kallen et al. 1986) of exposure to progestagens identified a subset of subjects exposed to progesterone only. Although no analysis was conducted on these individuals, no difference was evident between the proportion of cases and controls exposed.

Virilization of female genitalia of newborns was reported in a number of case reports in association with use of nor-testosterone based progestagens in pregnancy (Kupperman 1961). This literature contains three case reports of girls exposed to progesterone as the only administered progestagen (Reilly et al. 1958, Hayles and Nolan 1957). Another study reported on five boys with apparently enhanced virilization whose mothers had been treated with progesterone during pregnancy (Shirkey 1972)..

Although contemporary developmental toxicity studies are not available for progesterone, effects of injection with progesterone during gestation have been studied in animal models. Approximate doses can be calculated using data from the experiments and assumptions concerning body weights when necessary. Effects have been identified on fetal mortality at 116 mg/kg/day in mice (Petrelli and Forbes 1964), on altered sexual behavior of adult male offspring at 66 mg/kg/d in mice (Pointis et al. 1987), on

anogenital distance and sex ratio at 30 mg/kg/d in rabbits (Piotrowski 1968a), on anogenital distance and postpartum aggression at 8 mg/kg in mice (Wagner et al. 1986) and on birthweight and resorptions at 5 mg/kg/d in rats (Piotrowski et al. 1968b). Maximal endogenous progesterone production during pregnancy can be estimated at 300 mg/day, or 5 mg/kg/d, in women assuming a 60 kg body weight; however, progesterone titers vary widely depending on the stage of pregnancy. Animal models have not successfully identified progesterone-induced male hypospadias.

D. Female Reproductive Toxicity

Progesterone was the first steroid hormone to be examined as a potential contraceptive agent. However, it was rapidly replaced by synthetic progestagens with longer half-lives, and the earlier literature establishing its antifertility action is limited. In addition to fertility, other female reproductive endpoints of interest are effects on parturition, lactation and maternal behavior. Effects at nonfertile lifestages (prior to puberty, menopause) are largely unstudied, although there is some animal literature on progesterone administration in the early postnatal period.

D.1. Human female reproductive toxicity studies

The initial studies demonstrating the antifertility effects of daily progesterone were conducted in the late 1950's in Boston. Later controlled clinical trials were undertaken with synthetic progestins in Puerto Rico. After the combination birth control pill became widely used, many studies of adverse effects of contraceptives on reproductive organs and processes have reached the literature. In general, these studies were conducted with combination birth control pills using synthetic progestagens, and more recently, with subcutaneous progestagen (depoprovera, medroxyprogesterone). Some studies are available for an intrauterine device containing progesterone (Progestasert), the only progesterone-based contraceptive that has been marketed.

The main concerns for side effects of contraceptives have been reproductive tract cancer, cardiovascular thrombosis, and bone mineral density. Concerns related to reproductive toxicity include effects on lactation, on fertility after discontinuation of contraceptives, and on pregnancy outcome endpoints when contraceptives are inadvertently taken after conception. Only information on fertility and lactation are available specifically for progesterone-based contraception.

D.1.1. Individual studies

D.1.1.1 Fertility

Informal clinical trials with progesterone in the early 1950's were described jointly with later experiments with synthetic progestagens (Rock et al. 1957; Pincus 1956; Pincus 1956). In the first preliminary trial, 80 women with "conceptive failure" were given oral diethylstilbestrol and progesterone to simulate the hormonal changes of pregnancy for 3

cycles. The intent was to potentially induce a later normal pregnancy by these "pseudopregnancy" hormone regimens. The authors (Rock et al. 1957) reported that menses was suppressed in all cases, but resumed immediately after treatment in all but one patient. This suggested an antiovulatory effect of the hormone treatments.

The major study (Pincus 1956) administered progesterone only and used three indices of ovulation (vaginal smears, daily temperature, endometrial biopsy). The subjects were women described as "regular ovulators." A maximum of 32 subjects is suggested by the data presentation, although not all measures were taken on all women. The three indices of ovulation were ascertained during an initial cycle with no treatment and during 1-3 subsequent cycles in which 300 mg progesterone was administered orally daily from day 5 to 25 of the cycle.

Urinary pregnanediol, assessed during the luteal phase, was elevated in progesterone treated cycles (13.8 ± 1.5 mg/24 h) compared to control cycles (3.2 ± 0.4 mg/24h), indicating bioavailability of the administered progesterone.

Because progesterone was discontinued in the late luteal phase (cycle day 25) menstruation occurred during treated cycles. Cycle length was on the average 2.4 days shorter for treated than control cycles, a difference which the authors attributed to "breakthrough" bleeding in 18% of the treated cycles.

Ovulation was suppressed in the progesterone treated cycles. Table 4 shows data for the individual indices and Table 5 for combination of two or three indices. Ovulation was completely suppressed by the third cycle of treatment in the five women who completed three cycles and were assessed with all three ovulation indices (vaginal smears, daily body temperature, endometrial biopsy).

Table 4. Analysis of temperature curves, endometrial biopsies and vaginal smears during control and experimental cycles. Progesterone (300 mg, oral) was administered daily from day 5 to 25 of the experimental cycles. From Pincus (1956).

Parameters	Control Cycles							During Progesterone Medication					
	No. Exam-ined	Positive for ovulation		Anomalous		Negative For ovulation		No. Exam-ined	Positive for ovulation		Anomalous		Negative for ovulation	
		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%
Temperature Curves	30	30	100	0	0	0	0	69	27	39	5	7	37	54
Endometrial Biopsies	27	27	100	0	0	0	0	53	18	34	24	43	11	21
Vaginal smears	17	17	100	0	0	0	0	50	6	12	14	28	30	60

Table 5. Analysis of occurrence of "Ovulation-time" in control and successive cycles of treatment with progesterone. From Pincus (1956).

"Ovulation-time" by	Control Cycles %		1st Experimental Cycle		2nd Experimental Cycle		3rd Experimental Cycle	
	No.	% Positive	No.	% Positive	No.	% Positive	No.	% Positive
Two criteria	28	100	32	25	20	25.0	8	12.5
Three criteria	14	100	20	20	17	0.0	5	0.0

Additionally, in nine progesterone-treated subjects, laparotomies were conducted during the luteal phase of a treated cycle to visually assess the presence of corpora lutea. Five subjects had no corpora lutea, two had mature corpora lutea and two had regressed corpora lutea, which could have resulted from ovulation in the previous cycle. No control data were presented for comparison. Subsequent studies using a similar protocol with synthetic progestagens produced similar data on suppression of ovulation (Pincus et al. 1956).

The only contraceptive based on progesterone to be marketed commercially was the Progestasert IUD, which contained progesterone imbedded in silicon and released 65 µg progesterone/day for approximately one year. An early review (Murad 1977) cited pregnancy rates of 2.5% in nulliparous women and 1.9% in parous women using the Progestasert IUD; however, the data on which this estimate was based are not cited. Only one controlled study of fertility with Progestasert vs. no contraception was located. A study conducted in Egypt (Badraoui et al. 1982) compared 80 women receiving the Progestasert IUD after delivery with a control group of lactating women not using contraception who had previously been studied with an identical protocol (group size not stated). Significantly more women in the control than in the Progestasert group menstruated during each of the first 12 months postpartum. The authors reported a return to ovulation in the postpartum period of 42.7% in controls using no contraception compared to 7.5% in women using Progestasert. Ovulation was determined by "examination of endometrial shedding during menstrual bleeding." In a smaller study comparing Progestasert (n=6), and controls (n=11), blood samples were collected daily during the luteal phase of the menstrual cycle in lactating women. Assays demonstrated that circulating LH, FSH and progesterone were decreased relative to women using no contraception (Abdalla et al. 1981). Only the LH differences were statistically significant. With anovulation defined as serum progesterone less than 4.5 nmol/L, all of the Progestasert group and 59% of the control group were considered anovulatory. These data suggested that the intravaginally administered progesterone was acting centrally on the hypothalamus/pituitary through feedback to inhibit gonadotropin release and ovulation.

Progesterone has been administered during the luteal phase of the menstrual cycle to promote fertility. Clifford et al. (1996) randomly assigned women with recurrent miscarriage (n=106) to receive progesterone (n=25) or no progesterone during the luteal phase of six menstrual cycles. The author reported no effects on conception or the number of live births (Clifford et al. 1996).

D.1.1.2 Lactation

Progesterone, in the form of the Progestasert IUD, was studied for its effect on lactation in Egyptian women (Badraoui et al. 1982). The Progestasert IUD was commonly introduced for contraception immediately after delivery, so effects on lactation were of concern. A group of 80 young, parous Progestasert-treated women were compared to

controls not using contraception. Milk samples were obtained monthly using a standardized procedure. The amount of breast milk was higher in the Progestasert group each of the first 12 months postpartum. The difference in milk yield was as much as 50% early in lactation. No differences were found in average serum prolactin. Progestasert users showed a significant rise in prolactin at the onset of menses, whereas the rise was not significant in the group using no contraception (Badraoui et al. 1981). As regards milk composition, the protein concentration of milk was higher in the Progestasert group throughout lactation; however, the lactose concentration was lower from the 4th to the 10th month postpartum and the total lipid concentration was lower from the 7th to the 12th month postpartum. Statistical significance was reported in the figures, but the statistical technique was not described. The authors concluded that the intravaginal progesterone had no adverse effects on lactation.

D.2. Animal female reproductive toxicity studies

D.2.1. Fertility

Early studies showing antifertility effects of progesterone were conducted by Pincus (Pincus 1956; Pincus and Chang 1953; Slechta et al. 1954) in rabbits and rats based on even earlier observations that progesterone blocked ovulation in laboratory animals.

In rats, single injections or oral administration of 5, 10, 25, or 50 mg progesterone (n=8-20 per group) were given to females one day prior to caging with proven male breeders (Slechta et al. 1954). Treated groups were compared to vehicle controls (n=59) for time to mating (sperm in vaginal smear), an index of both estrus and ovulation in rats. Time to mating increased from 4.7 to 27.5 days in a dose-dependent manner for the subcutaneous injection administration. This effect was not evaluated statistically. When a 5 mg dose was given twice weekly by injection for 9 weeks, 3 of 10 treated females became pregnant (no control data were presented). Time to mating also increased in the oral group, but a dose-response pattern was not seen. Females that became pregnant were examined and found to be similar to injection controls in number of corpora lutea, embryos, and resorptions.

A later study looked at a lower dose range during chronic progesterone administration (Peterson and Edgren 1965). Ninety-day old females rats received daily s.c. injections of 0.1, 0.3, 1 or 3 mg progesterone for 30 days. Of the eight females in each dose group, only one rat at the 3 mg group failed to mate (sperm in vaginal smear). However, five of seven rats who mated in this group did not become pregnant. Tabular data indicated a mating delay of 2-8 days in rats of the 1 and 3 mg groups. One rat in the 1 mg group also failed to become pregnant. After discontinuation of progesterone all rats mated and became pregnant.

In rabbits, which ovulate reflexly in response to coitus, progesterone was administered orally, by subcutaneous injection, or intra-vaginally at various times prior to mating in doses of 1-30 mg (Pincus and Chang 1953). The animals were killed for examination of

ovulation the day after mating. In eight rabbits injected s.c. with 30 mg progesterone, none of the rabbits had ovulated when mating occurred between 1 and 24 days after injection; at 10 mg dose, three of eight rabbits ovulated with post-injection periods of 1-14 days. Five of the eight rabbits at the 30 mg dose demonstrated sperm in the tubes or uterus, indicating that mating had occurred. Progesterone at doses of 0.1 or 0.5 mg administered intravaginally was not effective in blocking ovulation in a small number of tests with one or two rabbits per group, but a 1 mg dose prevented ovulation in two rabbits by this route.

Similar studies in other species are not reviewed here. The Registry of Toxic Effects of Chemical Substances (RTECS 1995) listed 33 studies for progesterone under the category "effects on fertility" in species including dogs, sheep, hamsters, mice, monkeys and pigs, in addition to rats and rabbits.

D.2.2. Female reproductive tract development-postnatal exposure

One issue that was addressed in early studies was interference with female reproductive tract development due to early postnatal exposure to progesterone. Studies using prenatal exposure are discussed under Developmental Toxicity (Section C). Other studies administered progesterone in the early postnatal period. Under current Proposition 65 guidance limiting developmental toxicity to prenatal exposure, these studies with postnatal administration are relevant to female reproductive toxicity.

In response to the demonstration of diethylstilbestrol effects on the neonatal mouse vagina, experiments were undertaken with neonatal progesterone administration (Jones et al. 1984; Jones and Bern 1979; Nagasawa et al. 1978; Kawashima et al. 1978; Jones and Bern 1977). Jones and Bern (1979) used injection of 100 μg progesterone during the first 5 days of life to female neonates of BALB/C mice fostered to C3H mice. (This strain of mice was used as a model of spontaneous mammary tumors, which were one of the endpoints of the study.) The progesterone treatment led to persistent vaginal cornification in adult females that affected 100% of the group (n=32) when vaginal smears were evaluated for 25 successive days beginning at 40-50 days of age. None of 17 injection controls showed persistent vaginal cornification. If the ovaries were removed at postnatal day (pnd) 40, the vaginal cornification failed to develop in 23 of 26 progesterone-treated mice, suggesting mediation by the ovary. In contrast, neonatal estradiol treatment led to persistent vaginal cornification that was not ovary-dependent. Either persistent estrous or diestrous vaginal smears indicate impaired ovulatory cycling. Other experiments demonstrated hypertrophied ovarian interstitial tissue and an increase in gonadotropes in the pituitary, further implicating indirect mechanisms of neonatal progesterone action. Mammary tumor incidence was elevated and age of appearance of tumors was earlier in the mice treated with progesterone as neonates compared tovehicle controls and these differences were statistically significant (Jones and Bern 1979)

A review by Takasugi and Tomooka (Takasugi and Tomooka 1976) describes a 1964 study which demonstrated a similar phenomenon in a different strain of mice (A/Ms).

Progesterone (20 μg) was injected the first 5 days after birth. Some of these mice were ovariectomized in the neonatal period (pnd 6-8) and others as adults (pnd 107). The mice ovariectomized as adults had persistent vaginal cornification which ceased after ovariectomy. Those ovariectomized in the neonatal period had persistent diestrous vaginal smears as adults.

Interestingly, neonatal estrogen and testosterone led to a similar pattern of adult persistent vaginal cornification (Takasugi and Tomooka 1976). The critical period for these effects was determined to be within the first five days of birth in the mouse models used.

In a small study that was part of an experiment on androgenic properties of progesterone, four newborn female rats were injected with progesterone from birth to 30 days of life in increasing doses to a total dose of 45 or 73 mg. The authors reported that the clitoris was increased 3 fold in size as compared to littermates (Greene et al. 1939).

One study examined sexual behavior of females whose mothers were treated with progesterone in the postpartum period (Hull 1981). In this study, rat dams received 3.3 mg/kg progesterone per day by injection after giving birth and presumably continuing to weaning at pnd 28. The authors state that these injections increased progesterone levels in nursing pups to 2-3 standard deviations above controls. Female offspring were ovariectomized as adults and tested for sexual behavior in response to hormone injections. No group differences were found, although the authors note that more of the progesterone treated females showed mounting in response to testosterone than control females. Masculine behavior of the male offspring was influenced by the maternal progesterone treatment, as described in section E.

D.2.3 Parturition

Progestagens are known to inhibit uterine muscle contractility. Recently, a clinical trial for the use of 17-α-hydroxy progesterone in preterm labor was conducted (Meis et al. 2003). 17-α-hydroxy progesterone is a synthetic progestagen which is also a major metabolite of endogenously produced progesterone.

In an early study (Buhrdel et al. 1974), progesterone (0.5, 1 or 1.5 mg/day) was administered via s.c. injection to pregnant rats from gd 15 until parturition (maximum gd 25). The rats normal gestation length was reported as 23 days. There were either 8 or 9 pregnant rats/group. All rats in the unhandled and vehicle injected groups and the 0.1 mg progesterone group delivered with normal gestation length, while all of the 1.0 or 1.5 mg progesterone group had longer gestations. For the 1.0 mg, group four rats had 24 day gestations and four had 25 day gestations while for the 1.5 mg progesterone group, two had 24-day gestations and six had 25-day gestations. Shorter gestation length was found in a study with progesterone administration to pigs, described previously in section C.2.5 (Vallet 2002). The length of gestation was slightly but significantly shortened by progesterone (115.47± 0.21 days vs. 115.97 ± 0.14 days in controls

D.2.4 Maternal Behavior

Because endogenous progesterone decreases prior to parturition, studies have been conducted to determine whether exogenous progesterone administration could interfere with postpartum lactation and maternal behavior. However, most of these studies (see review by Numan (Numan 1978)) used animals that had their ovaries and uteri removed in order to eliminate endogenous hormone production and thus are not appropriate for toxicology evaluations that generalize to the normal condition. One early study (Moltz et al. 1969) demonstrated a blockade of maternal behavior by progesterone in rat dams that had not previously reared litters. Wistar rats were bred, but fetuses were removed by c-section on gd 21 (this procedure eliminated the hormonal changes immediately associated with the process of parturition). The rats were injected s.c. with 3 mg progesterone on gd 19-23, and were offered litters from other dams 24 h after the cesarean section. Maternal behavior was blocked in a subgroup of rats that had not previously reared litters, but not in a multiparous group that had raised litters. If the progesterone injections were delay to gd 24, after the dams had experience with litters, maternal behavior was not depressed in the primiparous dams.

The most recent work in this area suggests that progesterone inhibits maternal behavior by antagonizing estrogen effects in the hypothalamus (Sheehan and Numan 2002). A number of studies have also demonstrated that progesterone *antagonism* during pregnancy has adverse effects on maternal behavior in rodents (e.g. Crombie et al. (Crombie et al. 1995)).

D.3. Female reproductive toxicity: other relevant data

Progesterone receptors (PRs) are widely distributed in the female reproductive tract, but central actions of progesterone in suppressing release of gonadotropins are most commonly discussed in connection with progesterone effects on female fertility, female reproductive development, lactation and maternal behavior. PRs are expressed in the hypothalamus and pituitary, where they are regulated by circulating estrogen and progesterone (Camacho-Arroyo et al. 1998; Camacho-Arroyo et al. 1996; Szabo et al. 2000). Progesterone regulation of ovulatory cycling has been attributed to PR in specific cell groups in the preoptic area of the hypothalamus, rather than direct effects on gonadotrophin releasing hormone (GnRH) synthesizing neurons (Lauber et al. 1991). However, the proximal regulation of GnRH synthesis, storage, and release is still incompletely defined (Moenter et al. 2003). In the human female, the antigonadotrophic effects of synthetic progestagens have been investigated (Couzinet et al. 1996) and shown to be mediated through the hypothalamus and/or pituitary. Androgen receptor effects are apparently not related to progesterone suppression of gonadotropin. The suppressive effects of synthetic progestagens on LH and FSH were not reversed by selectively blocking the androgen receptor with flutamide and their antigonadotrophic action was similar in both healthy subjects and those with complete androgen insensitivity. Effects of progesterone on maternal behavior are thought to be mediated by

PR in the medial preoptic area, as well as areas of the forebrain and amygdala (Sheehan and Numan 2002).

D.4. Integrative evaluation

Progestagens, including progesterone as the Progestasert IUD, are used therapeutically as female contraceptives. Early studies showed suppressed menstrual cycling and ovulation in women treated with progesterone. Similar observations have been made in rabbits, monkeys and rats. Progesterone administration also prolonged gestation in pregnant rats and impaired maternal behavior in inexperienced rat dams. Milk composition was altered in lactating women with Progestasert IUDs. Effects on the developing female reproductive system are reflected in persistent vaginal estrus and altered sexual behavior in mice exposed to progesterone as neonates. Lower systemic gonadotropins (FSH, LH) are associated with suppression of ovulation, suggesting mediation by progesterone receptors at the hypothalamic-pituitary level.

E. Male Reproductive Toxicity

E.1. Human male reproductive toxicity studies

Antifertility effects of progesterone in men were evaluated during the 1950's, at about the same time that progesterone contraceptive effects were being investigated in women. As was the case in women, progesterone was compared to the newly introduced synthetic progestagens. More recently, medroxyprogesterone, administered as an intramuscular depot preparation, has been entered into clinical trials as a male contraceptive (Brady and Anderson 2002) and progesterone mechanisms of suppression of spermatogenesis have been further investigated in humans.

E.1.1. Individual studies

Heller et al. (1958, 1959) studied effects of progestational compounds including progesterone on the reproductive processes in men among volunteer adult men from the inmate population of the Oregon State Penitentiary, Salem, Oregon. The majority of the data in the 1958 report was also presented in summary format in the 1959 report. However, there are some differences in the data on progesterone between these two reports. These differences will be pointed out below.

In the 1958 report (Heller et al. 1958), one group of four subject received daily intramuscular injection of one dose of 50 mg progesterone for 10 weeks. Each of three other groups (5 men per group) was given two daily oral doses of nilevar (norethandralone), norlutin (norethindrone), or enovid (a combination birth control agent containing norethynodrel and mestranol). Testicular biopsies, semen samples, and urine samples for determination of levels of gonadotrophins, estrogens, pregnanediol, and 17-ketosteroids were obtained from each subject at pretreatment and immediately before the

end of treatment. During the treatment, sperm counts and motility were evaluated at 1- or 2-week intervals.

The authors found that progesterone and the three other progestagen treatments caused azoospermia in all subjects. Decreases in sperm motility and sperm counts in progesterone-treated men started after the second week of treatment and azoospermia was reached in Week 10. All subjects also lost libido and had difficulty in producing semen samples by masturbation after three to four weeks of treatment. At the end of treatment, testicular size and absolute number of mature sperm forms per tubule in testicular biopsies from progesterone-treated men was decreased. Gynecomastia occurred in two of four treated men. As determined at the end of treatment, progesterone led to decreases in 17-ketosteroid excretion in urine, but there was no change in urinary excretion of gonadotrophins (measured in a whole animal bioassay), estrogens, or pregnanediol.

Following the withdrawal of treatment, the mean sperm count remained depressed for about 12 weeks and then abruptly rose to a level that far exceeded the pretreatment level.

The majority of the observations described above were presented in brief summary format in the 1959 paper (Heller et al. 1959). However, OEHHA noticed two major differences between the two reports regarding data on progesterone.

1. In the 1958 report, the authors stated that four subjects were included in the progesterone group, all of who developed azoospermia. In the 1959 report, the authors stated that five subjects were included in the progesterone group, one of whom did not develop complete azoospermia. Although the data on sperm count, sperm motility, histology of testicular biopsies were almost identical, it is not clear to OEHHA staff if the subjects reported in the 1958 paper were the same as those in the 1959 paper.

2. In the 1959 paper, the authors reported three subjects (in addition to the five subjects) were treated daily by intramuscular injection of 50 mg progesterone for 90-110 days, but no significant reduction in sperm counts or change in spermatocytes in testicular biopsies was observed. The authors stated that the dosage of progesterone used (50 mg) might be at a threshold level, so that some subjects responded more completely than others and some not at all.

Brady et al. (2003) investigated the effect of progesterone on gonadotrophin secretion in normal healthy men and compared it to that produced by desogestrel, a synthetic progestagen. Desogestrel is a synthetic third-generation 19-nor-testosterone derivative. It has relative low affinity for the androgen receptor and high progesterone receptor selectivity in comparison to other synthetic progestagens such as gestodene and levonorgestrel.

Twenty normal healthy men, aged 18-38, were randomly allocated to the two groups, one for progesterone and the other for desogestrel treatment. The number of subjects per

group was not reported; since the author stated the study design "was a single-center open parallel-group study," it is most likely that 10 men per group was used.

Subjects in the two groups were treated daily either with 50 mg progesterone i.m. or 300 μg desogestrel orally for seven days. Frequent blood sampling at 15-min intervals over 12 hours was undertaken before and after treatment. Each subject was administered 100 μg GnRH by i.v. injection two hour before the end of the frequent blood sampling period.

Mean hormone levels, mean pulse frequency, and pulse amplitude from the peak to nadir difference were calculated over 10 h of frequent blood sampling prior to the GnRH administration. The response to GnRH was calculated from the mean hormone values following the administration of GnRH over the final two hours of frequent blood sampling. Hormonal data from the two groups were log-transformed, paired when appropriate, and compared between the groups using Student's *t*-test.

Treatment with progesterone for seven days caused significant decreases in mean concentrations of LH, FSH, and testosterone. There was no effect on the mean concentration of inhibin B. The frequency and amplitude of LH secretion pulses were significantly reduced. Mean concentrations of LH and FSH during the 2-hr post-GnRH period were significantly lower after 7-day progesterone treatment than before the treatment, suggesting a significant attenuation of the response to GnRH in men treated with progesterone. The effects of desogestrel on gonadotrophin secretion were similar to that caused by progesterone. The authors concluded that progesterone suppressed gonadotrophin secretion via a progesterone receptor-mediated mechanism and that synthetic gestogens exert their antigonadotrophic effect through their progestogenic properties.

E.2. Animal male reproductive toxicity studies

E.2.1. Mating and paternal behavior

The majority of experiments evaluating progesterone effects on male reproduction involved investigation of spermatogenesis; studies involving mating and pregnancy outcome are rare. As regards mating behavior, adult male guinea pigs (n=14) were observed for mating behavior during 10-min sessions with estrous females (Diamond 1966). A baseline period was followed by 50 mg i.m. injection on the first day and 10 mg injections on subsequent days for ten weeks. Progesterone injection significantly reduced the composite score of mating behavior; the authors stated that ejaculation frequency was the most impacted individual index. Significant recovery occurred during a 10-week recovery period, but scores did not return to pretreatment levels. Similar results were obtained with progesterone administration to castrated guinea pigs and castrated guinea pigs treated with testosterone. At the conclusion of treatment, testes of the progesterone treated group were smaller but did not demonstrate histological abnormalities relative to a control group. This suggested to the authors that the inhibition of sexual behavior was mediated by effects on the brain.

A recent study (Schneider et al. 2003) examined the effects of PR deletion and progesterone administration on paternal behavior of mice. Parental behavior (pup retrieval and nurturance) and aggression toward pups were recorded in adult PRKO male mice and wildtype C57BL/6 mice with progesterone implants designed to release physiological levels of progesterone. Progesterone-treated males (n=15) demonstrated aggression toward pups more often ($p<0.037$) than vehicle controls (n=22), but no differences in parental behavior scores were recorded. PRKO mice showed statistically significantly higher parental behavior scores and lower incidence of pup aggression than isogenic controls. PRKO mice were also found to exhibit more parental behavior in several other experiments.

E.2.2. Effects on spermatogenesis in various species

E.2.2.1. Rabbits

Ericsson et al. ((Ericsson et al. 1964)) treated two mature male rabbits (strain, age, and body weights not reported) with 10 mg progesterone in propylene glycol solution by subcutaneous injection every other day for 14 days. Semen quality from ejaculates was evaluated. The only change in semen characteristics during an 18-week collection period was an increase in the frequency of spermatozoa with protoplasmic droplets. The appearance of protoplasmic droplets in ejaculated sperms indicates an arresting effect on maturation of the sperm in the epididymis.

In another experiment, in the same report, two additional male rabbits were injected with 10 mg progesterone every other day for 28 days. Two control males were included but the authors did not report if the control males were treated with vehicle only or untreated. One of the progesterone-treated rabbits showed reduced libido shortly after beginning of the treatment. Average total sperm counts and semen volume were significantly reduced from the 15th through 27th week. Both treated males were azoospermic from the 19th through 21st week of treatment. Decrease in semen volume was partially due to a lack of gelatinous material, produced by glandulae vesiculaes (seminal vesicules).

Testicular biopsies were not taken from the four rabbits injected with 10 mg progesterone in order to avoid possible interference with semen collection. In a separate experiment, a male was injected with 20 mg of progesterone every other day for 60 days. Testes from the treated male and a control male were removed 10 days after the last injection and histological sections of the testis were prepared and evaluated. Seminiferous epithelia in the tubules from the treated male were disorganized. There were sloughed germ cells and debris in the lumen. The authors stated that spermatogenic activity in the treated male was disrupted. The authors concluded that progesterone interferes with spermatogenesis and accessory gland function in the male rabbit.

E.2.2.2. Nonhuman Primates

A study in rhesus monkeys (Anand Kumar et al. 1980) investigated the effects of progesterone, estradiol, and norethisterone (a 19-nor-testosterone-derivative synthetic progestin) administered by nasal spray on serum testosterone levels and spermatogenesis.

In experiments involving progesterone, three adult male rhesus monkeys weighing 7.5-11.0 kg were treated by intranasal spraying of solvent (as control, solvent type not reported; one animal) or progesterone (30 μg/day, two animals) for 60 days from October to December. The authors measured serum testosterone levels in treated animals and compared them to that in the control animal. Blood samples obtained at intervals of 10 min over a period of two hours from the control and treated animals on the day prior to the start of the treatment, 30 days after treatment, and on the day following the end of the 60-day treatment were analyzed. Mean values of serum testosterone levels in the blood samples from the control and treated animals were statistically compared using Cochran's modified t-test. Testicular biopsies containing a few seminiferous tubules were evaluated.

The authors found that progesterone treatment reduced the diameter of the seminiferous tubules, though not as markedly as the reduction observed in animals treated with estradiol or norethisterone. Seminiferous tubules in progesterone treated animals also had fewer spermatids than those in the control animal. Mean values of serum testosterone in blood samples from progesterone-treated monkeys were progressively reduced as compared to that in the control monkey ($p<0.01$). The authors concluded that intranasal administration of progesterone to adult male rhesus monkeys caused an impairment of spermatogenesis and a significant reduction in levels of circulating serum testosterone.

A similar study in bonnet macaques also used 30 μg/day nasal spray administration of progesterone in 3 males (Moudgal et al. 1985). After 90 days, sperm count was reduced 71%. Serum testosterone was lower in two of the three males at this time, although only a marginal decrease was seen, and this was only apparent in the night time sample (when testosterone is typically elevated), but not in the daytime sample.

E.2.2.3. Rats

A study demonstrating progesterone suppression of spermatogenesis in rats was conducted by the dermal route (Setty and Kar 1967). Progesterone was administered in ethanol daily for 30 days at a dose of 2 mg to 8 adult albino rats. The dose was applied by dropper to a shaved 2x2 area of skin in nape area. Weights of the testes, prostate, and seminal vesicles were statistically significantly lower than those of controls at the end of the treatment ($p<0.01$). The mean reduction in size was greater than 50%. The weight of the pituitary, however, was increased relative to controls ($p<0.05$). The gonadotropin content of the pituitary, as determined by an in vivo bioassay, was not altered by progesterone. All rats were azoospermic as determined from examination of the vas deferens. The description of testicular histology noted "disorganization of the

seminiferous epithelium" but no change in tubule diameter and "mostly normal" Leydig cells. Spermatogenesis was described as arrested at the secondary spermatocyte stage.

E.2.3. Male reproductive tract development-postnatal exposure

A thorough examination of the effect of prepubertal progesterone on male reproductive development was conducted in rams implanted with progesterone implants at 2 weeks of age and continuing for either 4, 8 or 12 weeks (puberty at 18-24 weeks) (Echternkamp and Lunstra 1984). The amount of progesterone in the implants was not stated, but plasma progesterone was about 4-fold higher during the implant period compared to measurements made after implant removal (plasma progesterone was not measured in controls.). Scrotal circumference measured during the implant period was smaller in treated rams than controls, but recovered by 14 weeks of age. Testes weights at the conclusion of the experiment (22 weeks) were not different in the groups. The 12-week implants led to decreased testosterone in blood samples taken biweekly from 4 to 14 weeks and decreased LH from 4 to 12 weeks. Average age at puberty, as assessed from testicular biopsy, was later in the treated than control rams. The percent of rams with elongated sperm in the seminiferous tubules at 18 weeks was 37% in control, 34% after 4-week progesterone exposure and 0% after 8 or 12 weeks exposures. By 22 weeks of age, similar number of rams in each group had reached puberty.

When progesterone was injected s.c. in rat pups (0, 10, 30 or 100 μg/kg) daily for 1, 2 or 3 weeks, testicular weights at one and two weeks of age were somewhat greater in the two higher dose groups than controls and in addition the density of Leydig cells in histological sections was enhanced at two weeks of age (Tapanainen et al. 1979). Although circulating testosterone was not affected by the progesterone treatment, testicular testosterone was lower, suggesting to the authors feedback enhancement of Leydig cell proliferation and testicular size due to suppression of testosterone production.

E.2.4. Male behavioral development-postnatal exposure

The sexual behavior of males exposed to progesterone in the postpartum period has also been studied (Hull 1981). Dams received 3.3 mg/kg progesterone per day by injection beginning at birth and presumably continuing to weaning at pnd 28. The authors state that these injections increased progesterone levels in nursing pups to 2-3 standard deviations above controls. No information was provided about maternal behavior, offspring growth or genital development. Male offspring were castrated as adults and tested for sexual behavior (reported as a composite score) in response to hormone injections. The progesterone treated group had lower masculine behavior scores in response to both testosterone and estradiol injection. In addition, significantly fewer males in the progesterone group demonstrated intromission during the testing. There were no group differences in demonstration of lordosis in response to estradiol. In another study, neonatal male rats were injected with three doses of progesterone (1.25, 2.5 or 5 mg s.c.) on pnd 3 and tested for mating behavior on pnd 110 (Diamond et al.

1973). Significantly fewer intromissions and ejaculations were recorded in the males injected with 5 mg.

E.3. Male reproductive toxicity: Other relevant data

E.3.1. Mechanistic considerations

Potential sites of action for progesterone effects on male reproduction are similar to those discussed above in section C.3.3.2. Progesterone could act through binding and activation of nuclear or membrane PR in peripheral organs or in the central nervous system. Additionally, progesterone could interfere indirectly with steroid hormone metabolism, bind directly to androgen receptors, or suppress gonadotrophin production or release in the brain. This last mechanism has been most explored in the male.

As described in detail above, progesterone has been shown to reduce both circulating testosterone and circulating gonadotropins in men (Brady et al. 2003). It has been difficult to ascertain at what level gonadal steroids and their synthetic derivatives exert their inhibitory effect on gonadotrophin secretion, as this is dependent on the integrated response of both the hypothalamus and pituitary. One possible mechanism whereby progesterone and synthetic gestogens may alter gonadotroph function is through decreasing the number of GnRH receptors in the pituitary. In the orchidectomized sheep, progesterone stimulation reduces the concentrations of the GnRH receptor and GnRH receptor mRNA in pituitary tissue (Sakurai et al. 1997). Progesterone may regulate gonadotrophin synthesis at both transcriptional and post-transcriptional levels. Progesterone has been reported to shorten the length of the poly(A) tail of mRNA encoding gonadotrophin subunits (Wu et al. 1994). Therefore, progesterone may affect the steady-rate of gene transcription and the degree of mRNA stability. Other mechanisms at the hypothalamic/pituitary level remain to be explored.

E.4. Integrative evaluation

Progestagens have been used therapeutically as male contraceptives. Studies show reduced testosterone and sperm production in men treated with progesterone. Similar observations have been made in rabbits, monkeys and rats. Progesterone also impaired mating behavior after administration to adult male guinea pigs. Effects on the developing male reproductive system are reflected in altered male mating behavior in rats exposed to progesterone as neonates. One study found delayed puberty in rams exposed postnatally to progesterone. Lower systemic gonadotropins (FSH, LH) are associated with suppression of testosterone and sperm production, suggesting mediation by progesterone receptors at the hypothalamic-pituitary level.

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