

Diagnostic Approaches to Reproductive Toxicology in Animals

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As a profession, veterinarians are often focused on a definition of reproductive toxicology that refers primarily to the teratogenic effects of compounds on the developing fetus. These proceedings discuss instead the types of exposures that can negatively impact reproductive performance of the male and female animal. Specifically, poor or abnormal sperm production or disrupted estrus cycle or ovarian function which causes suboptimal performance for the breeding animal. An overall approach to reproductive failure is provided, including assessment of potential reproductive toxicant exposure.

Reproductive Toxicology of the Male

Recently the critical role of the male gamete (sperm) in predicting final reproductive outcome has become of interest. Specifically, it is now recognized that toxins which effect the reproductive system need not cause total sterility of the male to have a deleterious effect on a population. Rather, compounds that damage sperm function including the DNA (chromatin), can lead to fertilization failure, embryo loss, spontaneous abortion of the fetus, and birth defects or even disease in the offspring (e.g. cancer).

Toxin damage to the male can result in abnormal reproductive outcomes including:

- Fertilization failure
- Early embryonic losses
- Spontaneous abortions
- Birth defects in the offspring
- Diseases of the offspring (such as cancer)

As elsewhere in medicine, a *problem oriented approach* is warranted for cases presenting with a male reproductive disorder. In order to rule out a toxin exposure as a cause for the disorder the clinician must understand the mechanisms by which compounds can exert a deleterious effect on the male animal. This understanding optimizes the practitioner's ability to identify and treat clinical problems, and to discuss prognosis with the client.

Specifically, fertility of mammalian males, can be altered by disruption of either the:

A) Endocrine system (hypothalamic-pituitary-gonadal axis). Disruption of the endocrine system can effect sexual differentiation of the fetus, sexual maturation at puberty, libido or male behavior in the adult animal (e.g. delivery of sperm to the female), and actual sperm production.

B) Sperm production (number, quality and function of gametes). Abnormalities of sperm production (including numbers of sperm, sperm shape and motility), and damage to sperm chromatin (DNA) prior to fertilization, can occur by separate mechanisms and are not necessarily correlated. Both poor sperm quality and abnormal sperm DNA can deleteriously effect

reproductive outcome. Recent studies into the mechanisms of DNA damage of sperm have shown classes of sperm in males where normal fertilization can occur, (e.g. sperm quality is sufficient to support fertilization); however, abnormal DNA is passed on to the offspring with resulting fetal losses and potential pathology.

Normal and Abnormal Endocrine Function of the Male

Hormonal control of the mammalian male involves a continuous feedback loop between the hypothalamus, the pituitary and the gonads (testicles). This hypothalamic-pituitary-gonadal (HPG) axis controls testosterone secretion from the Leydig cells in the testicle. Adequate testosterone levels *in the testicle* are requisite for normal sperm production. The level of testosterone in the *blood stream* impacts libido and secondary sex characteristics, and controls the hypothalamic secretion of GnRH leading to the production of *testicular testosterone*. Many toxicants that disrupt male reproduction act by mimicking steroidal activity. By doing so they disrupt the HPG axis, causing a decrease in hypothalamic activity and lower testosterone levels in the testicle, thus negatively impacting sperm production, even though libido and other peripheral sex characteristics may not be affected.

The level of exposure to toxic compounds with steroid-like activity required to interfere with fertility is highly variable among individuals. Some of this variability may be due to individual animal responses after exposure to compounds with steroid activity. However, breed and species differences may exist. For example, metabolism of phytoestrogens (from dietary soyabean products) may be less efficient in cats than other species, possibly leading to infertility in some animals. Additionally, dogs appear to be very sensitive to glucocorticoid disruption of the HPG with concomitant decreases in sperm production.

Normal and Abnormal Sperm Production

The production of sperm (spermatogenesis) involves a complex process of cellular divisions leading from germ cells (the “parent” cells in the tubules of the testicles) to functionally mature sperm cells that can fertilize an egg. This process allows the germ cells containing both chromosomes from the father (2 n) to rapidly divide and provide a continual source of sperm with one set of chromosomes (1 n) for ejaculation and the production of offspring.

The high rate of division of testicular germ cells makes them uniquely sensitive to toxic agents. If the germ cells are damaged several outcomes can occur. Sperm production may be decreased or cease, abnormal sperm can be produced (e.g. shape, motility), or the chromosomal material (DNA) of the sperm can become abnormal. Whether or not these changes are reversible depends on whether or not the insult was sufficient to cause permanent damage to the parent germ cell in the testicle. There is a great deal of individual variation with regards to when the intensity of exposure to a compound is sufficient to irreversibly effect the germ cells in the testicle. The more primitive the germ cell effected (in the continuum of spermatogenesis) by an exposure, the more long lasting and potentially detrimental the results on future reproduction. In general, the process of spermatogenesis from germ cell to mature sperm cell, takes about 60 days. Therefore, to determine if observed abnormalities in sperm production are permanent or temporary requires clinical observation through several 60 day cycles. In fact, recovery of normal sperm production up to a year after insult has been reported.

Abnormal sperm production can occur following: cellular insult to the germ cell with disruptions in spermatogenesis; decreases in testicular testosterone (as above) which disrupts spermatogenesis; or damage to the DNA of germ cell or sperm chromosomes at any stage of

development. With several toxins, levels of exposure which damage sperm DNA, occur before any observable loss of sperm quality or fertilization is seen. Such sperm can lead to abnormal reproductive outcomes such as early embryonic death, spontaneous abortions, and possible pathology in any resulting offspring.

Potential Problems in Male Fertility Disorders Which Require Toxin Exposure Rule Out

The following checklist (Table 1) can be used to identify potential reproductive disorders in the male which should include toxin exposure as a differential diagnosis. For each problem identified determine, as appropriate, if the duration has been lifelong, chronic (6 months or more) or acute (< 6 months). It is important to note that by the time a clinical problem in reproduction is observed, one time acute exposures to a toxin may have occurred weeks to months in the past. Therefore, recollection of past possible exposures are warranted.

- **A problem oriented approach for evaluating disruptions in male fertility should include a detailed history of any and all *possible* pharmaceutical, environmental (including in the home) or dietary exposures for the animal.**

In conclusion, poor reproductive function of the male animal may be due to exposure of known or unknown toxins. In order to determine this, first identify specific problems with the reproductive system which are present. Second, through a detailed history, determine if possible toxin exposure(s) have occurred, keeping in mind that one time exposures in the past may have occurred. Be sure to take into account any additional conditions which may exacerbate reproductive disorders such as an older male animal or an obese animal (both of which results in decreased testicular function). Further diagnostic assays to evaluate the endocrine axis (via hormone stimulation tests) and detailed semen analysis will provide more specific answers with regards to the severity of the insult.

With regards to long-term prognosis, a diagnosis of total sterility is not possible without either a testicular biopsy showing loss of germ cells, or sequential ejaculations over a year with no motile sperm. However, the prognosis for recovery of normal sperm function is poor for males with no increase in serum testosterone after a hormone stimulation test of injecting GNRH or HCG (LH activity), suggesting that testicular function is severely disrupted. Successful treatment options in these cases are currently limited in animals.

Table 1. Presenting Problem Checklist

Problem Observed	Duration Lifelong, Chronic, Acute		
☐ Abnormal external genitalia or sexual differentiation (such as): ☐ Cryptorchidism ☐ Hypospadia ☐ Short preputial opening to anal distance ☐ Agenesis of vas or epididymis ☐ Other	Not Applicable		
☐ Abnormal Libido	_____	_____	_____
☒ observe exposure to several different estrus females for accurate assessment in a variety of settings			
☐ Abnormal Copulation/Intromission	_____	_____	_____
☒ best if can be observed by clinician in quiet distraction free environment			
☐ Failure to produce any offspring	_____	_____	_____
☒ detailed history of management and females			
☐ Production of aborted, small or abnormal litters (circle all applicable)	_____	_____	_____
☒ detailed history of any work up done on abnormal outcomes			
☐ Small Testicles (for body weight)	_____	_____	_____
☒ references for small and large animals suggest normal testicular size for species and size			
☐ Abnormal Endocrine Response on Stimulation Assays	_____	_____	_____
☒ Use hcg or GNRH stimulation assays to confirm if Leydig cells remain functional (their degeneration occurs after spermatogonial function is lost).			
☐ Abnormal Ejaculate Quality	_____	_____	_____
☐ Abnormal Semen Analysis	_____	_____	_____
☒ Confirm that full ejaculate is being evaluated (e.g. run alkaline phos assay in the canine)			
☐ Abnormal Sperm Chromatin Assay	_____	_____	_____

Reproductive Toxicology of the Female

All periods of reproductive development and function are potential targets for disruption of normal female reproduction. Female reproduction is unique to other areas of organ function in that it is intermittently expressed, and reproductive function can only be fully evaluated in a mating pair and their offspring.

Target Sites of Reproductive Toxicants in the Female

Endocrine Effects. The Hypothalamic-Pituitary-Gonadal-Axis (HPG) is very vulnerable to disruption by toxicant injury or pharmaceutical exposure. It is regulated in a complex manner that requires the appropriate interaction of the central nervous system, ovaries, and reproductive tract. To achieve the successful outcome of live, normal, healthy offspring the individual components of the HPG must each function properly and this involves *critical timing and integration of signaling* between the sites involved.

The hypothalamus

The hypothalamus has permissive control of the cyclicity of the female through the pulsatile release of gonadotropin-releasing hormone (GnRH). The timing, frequency, and amount of GnRH released are critical to the female reproductive cycle including the initiation of ovulation. The hypothalamus is modulated by both positive and negative feedback from factors released by the CNS, ovaries, and uterus (e.g. neurotransmitters, androgens, estrogens, and progestogens). The release of GnRH signal into the hypophyseal portal system causes the anterior pituitary to secrete the gonadotropins, follicle stimulation hormone (FSH) and luteinizing hormone (LH). Catecholamines, dopamine, serotonin, GABA, and endorphins all have the potential for altering the release of GnRH. Therefore, agents that are agonist or antagonists of these compounds have the potential to modify GnRH release and thereby interfere with pituitary function.

The anterior pituitary

The anterior pituitary secretes three hormones (FSH, LH, and prolactin) that are essential for female fecundity. Their critical roles are in maintaining ovarian cyclicity, governing follicular recruitment and maturation, ovulation, and luteinization. The anterior pituitary is also a site of positive and negative feedback from the ovary. The appropriate pattern of release of FSH and LH during the cycle controls the events of normal follicular development, while the alteration in the secretion pattern may result in abnormal follicular development, acyclicity, and ovarian atrophy. Toxicant-induced alterations in the synthesis, storage, or secretion of gonadotropins can seriously disrupt female reproductive function. Steroid agonist and antagonists can initiate an inappropriate release of gonadotropins from the pituitary, thereby disrupting the ovarian cycle.

Direct effects on the oocyte

Several hundred thousand follicles exist in the mammalian ovary at birth. Only about half the number of oocytes present at birth remain at puberty. This number continues to decline in adulthood. Only several hundred follicles will result in mature ova during the reproductive lifespan of the female. Therefore, anything that decreases this available oocyte pool can lead to reduced fertility. The developing ovarian follicle secretes estrogens which act to thicken the uterine endometrium (“proliferative stage”). Ovulation is triggered by an LH peak which then promotes formation of a corpus luteum (CL) which secretes progesterone (“secretory phase”).

Each developmental stage in follicular development has a varying degree of sensitivity to toxicant exposure. For example, a toxicant which targets development of primordial follicles will not be readily identifiable, rather a shortened reproductive life span (b/c of fewer oocytes) will be noticed years later. Conversely, toxicity to preovulatory follicles will result in infertility during attempts to conceive.

Oocytes are vulnerable to damage or destruction by toxic agents. Potential sites of injury include oocyte maturation and meiotic cell division. Alkylating agents have been shown to destroy oocytes. Lead has also been observed to produce ovarian toxicity characterized by follicular atresia in rodents and nonhuman primates. Other metals, including mercury and cadmium, have also been shown to produce ovarian damage that may be mediated through oocyte toxicity.

Pathophysiology of Female Reproductive Toxicology

The exposure, by an effective means, to a reproductive toxicant in a mature female can have a *variety* of outcomes. The initial sign of a problem is often observed as a disruption in normal cyclicity. Based on this individual symptom no conclusion regarding the sight of disruption of the reproductive system can be made. A chemical that interferes with the HPG could block the release of regulatory gonadotropins that are required for oocyte development and ovulation. Another outcome may be a chemical that interferes with ovarian function and directly inhibits ovulation by a variety of mechanisms, including a reduction in numbers of oocytes that develop and mature or disruption of the critical pattern of ovarian hormone release and levels. Chemicals that disrupted reproductive function can also prevent successful fertilization. This can be due to altered oocyte development, or a direct effect on the reproductive tract such as defective tubular transport of oocyte and sperm, or improper preparation for implantation within the uterus.

Fertility effects can range from temporary infertility to an irreversible inability to reproduce (sterility). Other outcomes include premature reproductive senescence and increases in ovarian and uterine cancer.

Diagnostic Approach:

A careful analysis of retrospective or prospective reproductive history is required to identify most cases of domestic animal toxin exposure in the female.

Historical

1. Detailed description/record of estrus and diestrus activity (days), including observational and clinical (cytologies, ultrasound of follicles, blood or urine tests).
2. Dates of mating and history of stud. Any unusual conditions?
3. Evaluation or testing for infectious causes of poor fertility?
4. Pregnancy diagnosis? Numbers of offspring present early and at later evaluation.
5. Description of parturition.
6. Number of live and dead neonates.

Prospectively

1. Monitor induced or naturally occurring estrus. Include careful evaluation (tracking) of follicular development (cytology in companion animals; ultrasound in large animals).
2. Carefully evaluate semen quality, timing of insemination (natural or artificial) and quality of breeding.
3. Rule out infectious causes of reproductive failures with appropriate cultures, serology.
4. Confirm luteal function with measurement of blood progesterone one week after estrus. Continue to monitor luteal function at intervals (at least weekly) throughout pregnancy.
5. Confirm early pregnancy by ultrasound.
6. If early pregnancy can not be confirmed by ultrasound, embryo recovery to determine if conception is occurring can be done in species where this is routine (cattle/horses).
7. Monitor fetal health throughout pregnancy and parturition.

Most common environmental toxins that effect the female's reproduction will manifest as abnormal cycle length (failure to cycle), luteal failure or in spontaneous early embryo death or abortion. Specific teratogens and their effect on the fetus are not discussed in these proceedings.

Conclusions. Numerous references cite appropriate methods for semen evaluation in the male or reproductive cycle analysis in the female. Key to ruling out of a potential role for toxicant exposure in abnormal reproduction is determining if exposure to a compound that either: disrupts the HPG axis or damages the gamete has occurred. By far, most cases of reproductive toxicology affecting male or female reproduction in animals occur through exposure to environmental toxins, chemicals or pharmaceuticals that negatively impact the hormonal axis. In the male, this can lead to abnormal libido, poor sperm production, or production of sperm with abnormal quality including DNA damage. In the female, this can lead to abnormal follicular development, luteal failures, and loss of the conceptus at various stages.

References available upon request.