Current and proposed research in canine and feline non-surgical sterilization
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Introduction

Non-surgical contraception and sterilization of dogs and cats is a very active field of research. Techniques employed in this research are not those commonly used in small animal theriogenology and instead hail from oncology, molecular genetics, and other fields both in human and veterinary medicine. Much of the work that is being proposed is proprietary at this point and so cannot be discussed in detail. This review is a description of current research methodologies with some examples of specific research goals, to help practitioners and clinical theriogenologists better understand research as it is published. Few specific papers will be cited; the reader is referred to the website of the Alliance for Contraception in Cats and Dogs for proceedings of symposia with detailed information from specific researchers and descriptions of "think tanks" that have been held to address these methodologies and the larger issues of regulatory approval, manufacture, distribution, and marketing of non-surgical sterilants for dogs and cats.1

Contraception is reversible control of reproduction and usually is the goal in human medicine. Sterilization is permanent cessation of reproduction and usually is the goal in small animal veterinary medicine. Associated with that complete cessation of fertility is decrease in reproductive physiology and behaviors that we, as a society, have deemed unacceptable in pets, including exudation of serosanguinous vulvar discharge associated with heat in bitches, yowling and lordosis in queens, mounting in stud dogs, and urine spraying in tom cats. Current research is geared toward identification of a sterilant product that is 100% effective at decreasing fertility for the reproductive life of the animal, that is completely safe to that animal and to other species that may encounter the compound, and that can be administered as a single application in the animal's life. Ideally, the product also would be applicable for use in male and female dogs and cats, would have a clear path to regulatory approval in the United States and other countries, would be economical to mass produce and distribute, and would be appealing to shelter officials, veterinarians, and pet owners as an alternative to surgical sterilization.

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Surgical sterilization

The current standard of practice for sterilization of dogs and cats in the United States is surgical sterilization. Surgical techniques described in the literature for sterilization of females include ovariohysterectomy (OHE) via laparotomy or laparoscopy, and ovariectomy (OE) via laparotomy or transabdominal or natural orifice transluminal laparoscopy.2-6 The most common technique performed in the United States is ovariohysterectomy via laparotomy. The most common surgical sterilization technique for male dogs and cats is castration.7 Surgical sterilization is 100% effective in decreasing fertility and also in decreasing most reproductive physiologic events and behaviors that are of concern to pet owners. The reader is referred to current reviews of the literature documenting pros and cons of surgical sterilization.8-10 Surgical sterilization meets the primary criteria of efficacy, safety, and single application but is expensive and not readily available to help control owned and free-roaming populations in developing countries, where such populations negatively impact animal and human health.
Intratesticular injection

Another direct way to induce sterility is to destroy germ cells without removing the gonads. This is very difficult to achieve in females and will be discussed briefly under immunosterilization. In males, sterilizing agents have been described that are injected directly into the testes, epididymes, or vas deferens. The reader is referred to a good review of the material by Michelle Kutzler.11 The first product so approved, Neutersol™, was removed from the market. A product using the same compound, Esterisol™, is set to be approved this year. Zinc arginine is injected directly into the testes of puppies, with dose based on testicular size. The manufacturer of Esterisol™ recommends puppies be sedated before intratesticular injection is performed and cautions against extratesticular movement of the compound, which is associated with severe inflammation of scrotal tissue. An inflammatory and fibrous reaction within the testis impairs continuing spermatogenesis but does not completely inhibit testosterone production. Long-term effect on behavior and development of androgen-dependent disease is not clear. Intratesticular injection meets the primary criterion of efficacy regarding fertility. It is safe if administered properly and requires only one application. The drug is expensive and the need for sedation limits its use, especially for free-roaming populations that may require management without veterinary oversight.

More sophisticated methods of sterilization involve targeting of specific tissues or compounds involved in reproduction. The following is a brief review of relevant reproductive physiology.

Reproductive biology

The ovaries contain hundreds of thousands of primordial follicles at birth, each of which consists of a single ovum (queens) or one or two ova (bitches), surrounded by a single layer of flattened somatic cells called pregranulosa cells.12 The ova in these follicles are arrested in the diplotene stage of Meiosis I and bear connecting segments that permit interaction between the ovum and the surrounding somatic cells. A cohort of follicles is stimulated to begin development with each estrous cycle. Specific signals determining which follicles should begin development and how many follicles should be stimulated with each cycle are unclear but may include stimulatory factors, such as the phosphatidylinositol 3 kinase signaling network, which itself may be stimulated by insulin or growth factors, or cessation of release of inhibitory factors, such as members of the FOXO family of transcription factors or the tumor suppressor gene PTEN.13,14 Gonadotropin receptors are not expressed in primordial follicles.

Secretion of pituitary gonadotropins and steroid hormones stimulate follicle development and the physical and behavioral manifestations of estrus. Kisspeptins, neuropeptides produced in the hypothalamus, control secretion of gonadotropin releasing hormone (GnRH), perhaps in concert with other neurotransmitters including neurokinin B and dynorphin.15 Other direct or indirect regulators of GnRH secretion include metabolic signals such as glucose, leptin, and ghrelin.15 Gonadotropin releasing hormone stimulates pituitary gonadotrophs to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH), which act on the primary and secondary follicles developed from the primordial follicles. As the follicles develop, the granulosa cells surrounding the ovum secrete estrogen, which causes the physical and behavioral signs of proestrus. In dogs, estrogen concentrations fall about nine days after the onset of proestrus; at this time, a surge of LH is released, causing ovulation, and the bitch will stand to be bred. In cats, ovulation does not occur until the queen is bred or otherwise induced to ovulate. Not all of the developing follicles in a given cohort will progress to be pre-ovulatory follicles, also called antral, Graafian, or tertiary follicles. Factors that determine which follicles will develop to this final stage include FSH, anti-Mullerian hormone, activin, inhibin, and insulin-like growth factors.16,17 The remaining
follies undergo atresia. At ovulation of the antral follicles, eggs are released from the follicles into the uterine tube, where fertilization occurs. At the time of ovulation, the egg is surrounded by the zona pellucida (ZP) and a layer of cumulus cells.

Spermatozoa are produced in the seminiferous tubules of the testes. The first stage of spermatogenesis is the proliferation phase, during which spermatogonia undergo mitotic division to replenish the stem cell population and produce a number of cells for further development. The second phase is the meiotic phase during which primary and secondary spermatocytes undergo meiosis to produce haploid spermatids. The final phase is the differentiation phase, a morphologic alteration to produce spermatozoa, each of which has a distinct head with an acrosomal cap, a midpiece, and a flagellum. Protamine packaging of the spermatozoal DNA also occurs during this phase, inactivating transcription. Further maturation of spermatozoa, including acquisition of capability for forward motion and ability to bind the outer layer of the ova, takes place as they traverse the epididymis. After ejaculation into the female reproductive tract, spermatozoa undergo capacitation, a complex interaction including loss of decapacitation factors, efflux of cholesterol with subsequent enhanced fluidity of the plasma membrane and transfer of a variety of proteins across the surface, activation of signal transduction pathways within the spermatozoon, and acquisition of hypermotility and ability to bind the ZP. Capacitated spermatozoa bind to the epithelium of the uterine tube until the ova are present; an undefined signal causes their release and permits binding of the spermatozoa to the ZP. A single spermatozoon binds to one of the ZP glycoproteins; this permits cross-linking with other ZP glycoproteins and enacts fusion of proteins on the head of the spermatozoon with a complex of receptors to form a multimeric zona recognition complex. This binding also prevents polyspermy.

**Targeted cytotoxins**

Targeted toxins work by binding to specific cells associated with reproductive function and destroying those cells only. This is analogous to chemotherapy. For this to work, a purified toxin must be attached to some sort of transport molecule for delivery to a specific target and that transport molecule must bind to the cell of interest and not to non-target cells. One example that has been published is conjugation of pokeweed antiviral protein to GnRH. As GnRH binds to gonadotrophs in the pituitary and is taken up, the toxin is introduced as well and function of those cells inhibited, decreasing release of FSH and LH. Another example is linking of a cytotoxic fragment of exotoxin A from *Pseudomonas aeruginosa* to a ligand that binds the G-protein-coupled receptor for FSH that is expressed specifically in testicular Sertoli cells and ovarian granulosa cells. With binding, cells that express receptors for FSH will be selectively destroyed. Problems lie in specificity of targeting to the cells of interest and ensuring non-reproductive tissues are not accidentally destroyed. For example, FSH receptors are not uncommonly expressed in tissues of the urinary tract in females of some species.

**Immunoc contraception**

Immunoc contraception relies on humoral and cell-mediated immune responses against specific proteins or tissues involved in reproduction. Humoral immunity is mediated by antibody production by B-cells exposed to extracellular antigens. Cell-mediated immunity is mediated by cytotoxic T cells, killer T cells, and macrophages, which often are activated by intracellular antigens including virus-infected cells. Antibodies may bind to regions of interest, blocking receptors and subsequent hormone responses such as stimulation of release of GnRH, or directly blocking reproductive events, such as fertilization. Immunosterilization requires cell-mediated destruction of reproductive proteins or tissues. Primary
concerns with immunocontraception are lack of antigenicity of many reproduction-specific proteins or tissues, desire not to block function of those proteins in non-target tissues, and inflammatory response associated with the immune reaction that may damage surrounding tissue.

Because many of the proposed targets are recognized as "self" and therefore do not elicit an immune response, investigators have tried several things to enhance immune response. These include conjugation to other large proteins and packaging of antigen to create a repeat of antigens every 50-100 angstroms, as is a common presentation on bacteria and viruses that stimulate a strong immune response. Many reproductive targets are immunologically privileged because they are sequestered from the immune response early in embryologic development. While these may be antigenic when administered systemically, the tissues may still be protected from the immune response. For example, spermatozoa are protected from the immune system by the blood-testis barrier and neurons producing GnRH are protected by the blood-brain barrier.

Potential immunogens include GnRH, LH, FSH, receptors for those hormones, ZP, and specific proteins associated with germ cells or reproductive organs. Gonadotropin releasing hormone is a small peptide hormone and is highly conserved, raising concerns about inadvertent immunization of non-target species in any compound distributed as an oral bait or otherwise introduced into the environment. Increased antigenicity requires conjugation of GnRH with a larger protein; those that have been employed include tetanus toxoid and keyhole limpet hemocyanin. There have been many studies completed evaluating use of homologous or heterologous ZP proteins for immunocontraception. One example of a specific protein that could be used as an immunocontraceptive agent is a protein called "maternal antigen that embryos require" or MATER, that is expressed solely by oocytes.

Research in immunocontraception has mostly involved ZP or GnRH-based vaccines. Vaccines using porcine ZP in dogs cause erratic estrous cycling and do not consistently prevent pregnancy long-term. Vaccines using recombinant canine ZP proteins conjugated to diphtheria toxin in dogs caused a rise in titers and subsequent inhibition of ovarian follicular development but did not prevent estrous cycling and pregnancy in all cases. Most ZP vaccine studies in dogs were associated with at least short-term infertility in more than 75% of cases but were associated with prolonged progesterone bleeding and estrous behavior and with ovarian cystic disease. In cats, vaccines developed using ZP proteins from dogs, cats, mink, and ferrets all were demonstrated to induce a significant, measurable antibody response but did not protect against pregnancy as the antibodies did not bind to the queen’s own ZP in vitro. It may be that variation in sperm binding sites on the ZP vary enough among species to minimize the effect of antibodies raised against ZP proteins.

Another reported problem with immunocontraceptive vaccines evaluated to date is the adjuvant used. In one study in cats, using Freund’s complete adjuvant, seven of 10 cats developed granulomatous reactions at the injection site and in distant tissues including lymph nodes and brain. One of the 10 cats died of a vaccine-associated sarcoma at the injection site, and three of 10 suffered from hypercalcemia and compromised renal function. Granulomatous reactions also have been reported at the injection site in dogs. A commercial ZP vaccine with Freund’s adjuvant (SpayVac®) was available from 2002-2005 through a Canadian company. As of this writing, no ZP vaccine is commercially available for use in companion animal species.

In a study in male dogs using GnRH conjugated to tetanus toxoid, rises in antibody titers against the tetanus toxoid but not against the GnRH were demonstrated. A study in bitches using GnRH conjugated to canine distemper virus proteins demonstrated a rise in titers but no inhibition to conception and pregnancy. A recent study in cats using multiple tandem repeats of GnRH conjugated to proteins
from *Pasteurella* sp. showed high titers against GnRH, lack of follicular development, and no estrous cycling or pregnancy for up to 20 months after vaccination. Finally, GnRH conjugated to hemocyanin from the keyhole limpet and adjuvanted with a commercial preparation using *Mycobacterium avium* (AdjuVac®) has been demonstrated to decrease testosterone and sperm count in male dogs and cats; work in bitches and queens is ongoing. A commercial GnRH vaccine using AdjuVac® (GonaCon®) is reported to be undergoing registration for use in hoofstock by the USDA. There are no reports of a commercial vaccine for companion animals as of this writing.

Several different types of vaccines can be used to induce immunocontraception. Protein vaccines are those that have been described, with a purified protein of interest associated with larger proteins and adjuvants to enhance immune response. Another type of vaccine is DNA vaccine, which introduces DNA that encodes the protein of interest into cells where antigen is then produced and available to the immune system. Either system may be incorporated into virus-like particles or other carriers to enhance delivery of antigen and immune response. Virus carriers that have been used include canary pox, which expresses antigen well and cannot reproduce in mammalian tissues, non-replicating adenovirus, lentivirus, and alpha-virus. One group has demonstrated a non-infectious *Salmonella* sp. vector that colonizes lymphoid tissue, expresses associated antigen, and then self-destructs. True immunosterilization requires complete destruction of specific cell populations required for fertility. Some work suggests irreversible fertility in females after inoculation with ZP proteins destroyed the population of primordial germ cells, including the rabbit doe, bitch, and female monkey. This has not been demonstrated consistently in any species, perhaps because the ZP vaccines employed were adulterated with other ovarian proteins inducing significant oophoritis. In women, the number of primordial follicles must fall to less than 1000 before onset of menopause, suggesting a threshold number that must be maintained for estrus cycling to continue. This limit has not been identified in other species.

The final consideration is need for boostering of immunocontraceptives. Research currently underway is looking at packaging of antigenic molecules in some sort of encapsulated form that would gradually degrade and be released as a self-booster.

**Gene silencing**

Gene silencing most commonly is accomplished with a class of double-stranded RNA molecules. These molecules, when introduced into a cell containing genes with homologous DNA, block transcription and effectively abolish expression of that gene. Specific types of RNA used in this way, small interfering RNAs (siRNAs) and small temporal RNAs (stRNAs), are themselves regulated by the enzyme Dicer. As a group, these are referred to as interfering RNAs (iRNAs). Introduction of iRNAs to cells of interest decreases gene transcription for a variable amount of time. Specificity of the iRNA is vital, to ensure mRNA of a desirable gene is not blocked unintentionally. Similarly, targeting of the iRNA to specific tissues is vital to ensure that production of the protein of interest is abolished as completely as possible but that transcription is not blocked in non-target tissues requiring that gene product.

Because gene silencing does not permanently shut down cell function, the challenge is to create long-term infertility from a transient silencing event. Examples of how this might be achieved include by silencing inhibitory factors that control apoptosis (controlled cell death) or otherwise altering secretion of gene products required for maintenance of the germ cell population. Another question that has not yet been answered is what percentage of active cells must be silenced to effect a change in reproduction.
example, if a system could be created that silences 50% of kisspeptin secretion, would that alter GnRH secretion or is there a much lower threshold needed, such that a much higher percentage of cells must be silenced to effect change?

Besides siRNA, another group of compounds that have been described for gene silencing are chemically modified oligonucleotides with a nuclease resistant backbone. These bind to DNA and RNA and interrupt transcription and translation. Because these are not broken down by nucleases, they may be able to exert a long-term effect.

One example of gene silencing would be decreased function of neurons secreting kisspeptin or other factors controlling release of GnRH. In this particular example, a second challenge is accessing kisspeptin neurons within the blood brain barrier. Other examples include silencing of genes in the piwi protein family, which are expressed in male germ cells have some function in male fertility, and silencing of the gene for the MSY2, a protein expressed in male and female germ cells and again, somehow supporting fertility. Mice with homozygous knockout of these gene products are infertile, suggesting that long-term silencing could be a method of inducing sterility.

As with immunosterilants and specific cell toxins, delivery of the siRNAs or these other silencing agents only to the appropriate cells is a challenge. Ideas for how to introduce these compounds into cells include packaging siRNAs into particles that bind to the tissue of interest and activate surface receptor mechanisms; antibodies that bind to an siRNA and a target cell, such that the siRNA is taken into the cell by endocytosis; and aptamers, chimeric RNA molecules that bind protein targets on cells, are taken in by endocytosis, and then are activated by Dicer. Viruses also may be used, as described for immunosterilization.

The Red Queen Hypothesis in evolutionary biology states that continuing adaptation is needed in order for a species to maintain its relative fitness amongst the systems with which it is co-evolving. Reproduction is a biologic imperative for animals, and is a complex system with built-in safeguards and fail-safes we have not yet identified. For example, kisspeptin is produced primarily in the arcuate nucleus and anteroventral periventricular area of the hypothalamus but that kisspeptin neurons also are scattered elsewhere in the brain. There is evidence of stem cells permitting follicular renewal in mammalian species. Movement of proteins across the plasma membrane of spermatozoa during capacitation is fluid and involves formation of lipid rafts that permit construction of complex binding platforms to ensure binding of that spermatozoon to the egg in varying conditions. Some crocodile species can alter the environment of the nest to ensure population balance by gender as eggs hatch, and some social insects, like bees, can evaluate available resources and determine how many offspring to produce in a given season. Current research continues to deepen our understanding of animal reproduction to lead us toward the stated goal of complete control of animal reproduction.

References


