DOI: 10.1111/rda.12862

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

New approaches to non-surgical sterilization for dogs and cats: Opportunities and challenges

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Over the last 40 years, researchers have explored methods to non-surgically suppress fertility in animals. Immunocontraception has been used to control wildlife populations but does not confer long-term immunity. The gonadotropin-releasing hormone (GnRH) agonist deslorelin, formulated as an implant to provide 6-month to 1-year suppression of fertility in male dogs, is available commercially in some countries. Neither of these approaches provide permanent sterility. A single-dose, permanent treatment would be a valuable tool in dog and cat population control. The Michelson Prize and Grants (MPG) programme was initiated "to eliminate shelter euthanasia of healthy, adoptable companion animals and reduce populations of feral and free-roaming cats and dogs" offering a $25 million US prize for a non-surgical sterilant that is effective as a single treatment in both male and female dogs and cats. Michelson Prize and Grants programme has offered US $50 million in grant money for research and has attracted scientists worldwide. Approaches under study include gene therapy, small interfering RNA to inhibit reproductive targets and delivery of cytotoxins to pituitary gonadotrophs or GnRH producing neurons in the hypothalamus. Research in implant technology that could deliver compounds over an animal's lifetime is also underway. Details of funded grants and results to date can be found at: http://www.michelsonprizeandgrants.org/michelson-grants/research-findings. The next steps are translating the most promising research into products. The Alliance for Contraception of Cats and Dogs (ACC&D) is helping to research practical methods of marking sterilized animals to avoid costly retreatment and population modelling that will help guide field workers in use of resources for sterilization programmes.

1 | BACKGROUND

Since the mid-1970s, researchers have been investigating alternatives to using surgery to sterilize dogs and cats. Early research investigated the novel idea that animals could be induced to mount an immune response to antigens that are important in reproduction, such as gonadotropins (Faulkner, Pineda, & Reimers, 1975) or zona pellucida proteins (Mahi-Brown, Huang, & Yamagimachi, 1982). Others began studying the use of gonadotropin-releasing hormone (GnRH) agonists that were then in development for the treatment of prostate cancer in men, for their potential to suppress fertility in dogs (Tremblay & Belanger, 1984).

Over the next few decades, many researchers published extensively on these non-surgical contraceptive approaches, yet this research has not resulted in products that are commercially available for dogs or cats, with one exception, a long-acting implant that releases the GnRH agonist deslorelin (Suprelorin®: Virbac) that is labelled in the EU, Australia and New Zealand for 6 or 12 month suppression of fertility in male dogs. A second implant of a GnRH agonist, azagly-nafarelin (Gonazon®: Intervet), was developed and approved in the EU for 1-year suppression of fertility in female dogs, but the product was never marketed.

No immunocontraceptives have been successfully developed for dogs or cats. An Australia company developed a GnRH vaccine for dogs that was acquired by Pfizer Animal Health and that vaccine

was approved by the United States Department of Agriculture and briefly marketed for "treatment of benign prostatic hypertrophy" in male dogs. Because of its mechanism of action of eliciting antibodies to GnRH which then suppressed testosterone production, not only did the vaccine decrease the prostate size of male dogs, but also suppressed fertility, although it never achieved regulatory approval for that use. A GnRH vaccine (GonaCon™, for deer and wild horses) and a porcine zona pellucida vaccine (Zona-Stat-H™, for wild horses) have been approved for use in the United States for suppression of fertility in these wildlife populations, but neither provides permanent sterility. A comprehensive review of approaches to non-surgical contraception describes other research that has been done (Alliance for Contraception in Cats & Dogs (ACC&D), 2013).

Developing a non-surgical contraceptive has proved to be a difficult problem, exacerbated by the lack of research funding support for reproductive research in dogs and cats. Pharmaceutical companies have been reluctant to invest in this area for a variety of reasons both technical and commercial. This lack of funding results in a lack of innovation and new approaches. Surgical sterilization has had an important role in reducing the number of dogs and cats euthanized in shelters, but it requires infrastructure and significant commitment of resources and it is widely acknowledged that a single treatment non-surgical alternative to spay/neuter would be an important tool in helping reduce the population of unwanted dogs and cats worldwide. (Miller et al., 2014).

2 | THE MICHELSON PRIZE AND GRANTS PROGRAMME

In 2008, Gary Michelson, MD, established the Found Animals Foundation to reduce or eliminate shelter euthanasia of cats and dogs in the United States. Previously, he had been active in funding spay/neuter clinics in the area near Los Angeles, California, United States. He hypothesized that a non-surgical sterilant could have significant positive impact on decreasing shelter populations and therefore he committed $25 million US Michelson Prize in Reproductive Biology as well as an additional US $50 million for the funding of Research Grants in Reproductive Biology (www.michelsonprizeandgrants.org).

The prize and grant programmes have the goal of discovering a safe, low-cost, single-dose non-surgical sterilant for male and female cats and dogs that lasts for their 10–20 year lifespan. To win the prize, the sterilant also must ablate the presence or action of sex steroids (thereby preventing sexual behaviours as well as mating), have a pathway to FDA regulatory approval as a veterinary prescription product and be able to be delivered in a field setting, preferably a subcutaneous or intramuscular injection. The Foundation's mission is to reduce euthanasia of shelter pets in the United States, but also anticipates that a successful product will be made available internationally to groups managing feral and community owned populations of cats and dogs.

This level of resources for research with the goal of better understanding reproductive control in cats and dogs has caught the attention of scientists in the global biomedical research world. Grant applications have been received from around the world in disciplines such as reproductive biology, gene therapy, neuroendocrinology, immunology, oncology, bioengineering, materials science, medicinal chemistry and many more. Found Animals Foundation has received 351 letters of intent and 132 full applications for research funding since 2008, of which 35 applications have been funded. Proposals funded to date encumber approximately US $15 million of the US $50 million available. Research approaches in these funded projects can be grouped into four main approaches: immunocontraception, targeted delivery of cytotoxins, high-dose/long-term GnRH agonists and gene silencing/gene therapy.

2.1 | Immunocontraception

Immunocontraception is active immunization against an antigen important to reproductive function, whose immune response will suppress fertility. Gonadotropin-releasing hormone, luteinizing hormone (LH), follicle stimulating hormone (FSH) or zona pellucida proteins are possible antigens, but they are "self"-antigens that exist in animals, rather than foreign proteins such as bacteria or viruses. For a robust immune response against a self-antigen, various adjuvants and conjugates are required.

The creation of an immune response which induces antibodies that bind GnRH is a promising target because GnRH is a decapeptide which is conserved in male and female dogs and cats, and if effectively suppressed, will result in suppression of all downstream hormones including LH, FSH, estradiol and testosterone, suppressing reproduction in both males and females. Vaccines against GnRH have been reported to suppress reproduction safely in cats (Enright & Swift, 1995) and dogs (Jung et al., 2005), but booster vaccinations are necessary at intervals to maintain a high enough anti-GnRH antibody titres to result in suppression of fertility. New approaches are needed to develop methods to prolong the immune response to GnRH in such a way that booster injections are not needed and anti-GnRH antibodies are maintained for years. Three new areas are under investigation in immunocontraception are the use of novel antigens, novel delivery of antigens and new ways of increasing the immune response.

2.1.1 | Novel antigens

Researchers are investigating the GnRH receptor as a potential target for inactivation by anti-GnRH receptor antibodies. If the GnRH receptor is complexed with antibody, it will inhibit the binding of GnRH to its receptor and result in infertility. Another approach is to refine the zona pellucida antigen, using specific recombinant zona pellucida proteins instead of the extract of porcine ovaries that is the source of the porcine zona pellucida antigens used in the current wildlife vaccine (Zona-Stat-H). It is important to understand the species specificity of these proteins; research has shown that the zona pellucida proteins from one species may elicit an immune response, but not interfere with fertility. For example, cats vaccinated with porcine zona pellucida developed anti-pig zona pellucida antibodies, but these did not interfere with fertility, likely because the feline zona pellucida has structural differences great enough that these antibodies did not bind the zona pellucida of cats (Levy, Mansour, Crawford, Pohajdak, & Brown, 2005).

2.1.2 | Novel delivery of antigens

Biomaterials that release antigen slowly over long periods of time or that could provide a "self-boost" after a single injection are being investigated. Some type of pulsatile release of antigen is preferable than slow release, as slow release exposure to antigen may cause immune anergy. Another approach to delivering antigen long term is the use of a viral vector that expresses GnRH, zona pellucida proteins or other reproductive antigens and can remain active within the host over the animal's lifetime, thereby providing continuous boosters (Munks, 2012).

2.1.3 | Novel ways of augmenting the immune response

If antigen is presented in novel ways to the immune system, there is the potential to increase the immune response in both robustness and duration. Given the extensive research into the immune response, and the advances in cancer immunotherapy, refining understanding of the feline and canine immune response could result in developing an approach for long-term fertility suppression. One interesting possibility is to present the antigen using a DNA vaccine, where DNA with the gene coding for the reproductive antigen is administered, often intradermally, to elicit a robust immune response that could be long term, as DNA is incorporated into cells and the antigen produced from expression of that DNA over time. Unlike a viral vector delivered DNA, where the virus is usually administered intravenously, and has a tropism for specific tissues such as liver or muscle, DNA vaccines incorporate the DNA at the site of injection.

2.2 | Targeted Delivery of Cytotoxins

In human biotechnology companies, scientists are researching the use of cytotoxins that can be targeted specifically to tumour cells, by conjugating them to monoclonal antibodies that bind tumour cell antigens. For example, a prostate cancer therapy has been developed that uses antibodies to a protein specific to many prostate cancer cells and attaches (conjugates) this antibody to a potent toxin. Once the antibody has bound the prostate cancer cell surface, the toxin is delivered and specifically kills the cancer cells (Olson & Israel, 2014).

It may be possible to create a cytotoxin conjugate that could directly target a subset of cells critical for reproduction, such as the GnRH neurons in the brain, pituitary gonadotrophs or primordial follicle cells in the ovary, and kill those cells without damaging other tissues. Three things are required for this approach to be effective: a method for targeting a particular subtype of cell important in reproduction, a potent toxin that can kill cells when delivered by the targeting mechanism and, finally, a way to get the drug–toxin conjugate into only the desired cells, avoiding other cells to reduce toxicity.

Several grants using this approach have been funded by the Michelson Prize and Grants (MPG) programme. Researchers are targeting neurons in the brain that secrete GnRH; the challenge with this approach is getting treatments through the blood/brain barrier. Despite this obstacle, these neurons are an appealing target, as they are the master reproductive regulatory cells. Another target is the population of gonadotrophs in the anterior pituitary since the gonadotrophs are located outside of the blood/brain barrier. If gonadotroph populations in dogs and cats can be killed with toxins, it is not known whether or not these cells can be repopulated with stem cells in the pituitary (Struthers, 2012) which could be a limitation of this approach. Preliminary evidence using a GnRH small molecule antagonist conjugated to a potent toxin shows that the compound can bind receptors in the gonadotrophs, causing a suppression of LH in male rats, but that the cells are not killed (Betz, Struthers, Zhu, & Kusnetzow, 2016; Struthers, 2012). Further research is needed to understand how better to deliver the toxin to the gonadotrophs, which will then allow evaluation of the potential for pituitary stem cells to regenerate these populations, and over what time frame such regeneration might take place.

Cells in the gonads, that is the cells that differentiate into ovarian follicles (primordial follicles) and sperm (spermatogonia), are another potential target for causing sterility. Targeting these cells may be easier in females born with a finite population of follicles than in male animals that continuously make sperm. Targeting of the exact cell population to be killed is important to this approach as well, and male and female gonadal cells will likely present different targets. Scientists targeting gonadal stem cells are searching for homing peptides that target gonadal stem cells and can deliver a toxin to those specific cells. Work is also underway to develop toxins that can be internalized into primordial follicles or spermatogonia and destroy them.

3 | HIGH-DOSE/LONG-TERM GNRH AGONISTS

Gonadotropin-releasing hormone agonists are a group of small peptide compounds with a sequence similar to GnRH but with substitutions of artificial amino acids designed to increase serum half-life. These compounds bind to GnRH receptors on gonadotroph cells in the anterior pituitary, initially causing a release of LH and FSH which results in an increase in testosterone in males, and in females may induce an oestrous cycle in anoestrous females. After approximately 2 weeks of continuous treatment, GnRH receptors are downregulated, resulting in lack of release of LH and FSH from the pituitary, which then reduces testosterone levels in males and inhibits oestrous cyclicity in females.

These compounds were originally developed for humans in the 1970s to suppress secretion of reproductive hormones. Gonadotropin-releasing hormone agonists such as leuprolide, historelin, deslorelin and nafarelin are used in human medicine to treat prostate cancer, endometriosis, precocious puberty and other conditions. Gonadotropin-releasing hormone agonists have been formulated as subcutaneous implants that can release drug for as long as a year. In veterinary medicine, the GnRH agonist deslorelin has been formulated in implants that release the drug for either 6 or 12 months (Suprelorin™: Virbac). These implants are approved for use in Australia, New Zealand and Europe and labelled for suppression of fertility for 6 or 12 months in male dogs.

Gonadotropin-releasing hormone agonists have been shown to suppress fertility in dogs and cats, but the duration of the effect is

limited by the ability of the implant to release the compound over time. If an implant could deliver a treatment for >5 years, this duration of extended release might be long enough in some populations (e.g. feral cats) to cause lifetime sterility. A novel implant formulation or perhaps a medical device that could hold sufficient drug for lifetime administration might prove effective. A company (Microchips Biotech, Lexington, MA, USA) is developing a medical device for human contraception, and it is possible that this device could hold sufficient GnRH agonist for lifetime release in dogs and cats. Because only very low continuous levels of GnRH agonists are needed to maintain reproduction suppression, such long-term delivery, using advanced formulation technologies, might be possible.

Researchers have suggested that giving high doses of GnRH agonists to very young kittens or puppies might delay or prevent puberty. Carranza et al. (2014) demonstrated significant delay of onset of puberty (to 42–91 weeks of age) in male and female kittens given a 1.6 mg deslorelin acetate implant within 24 hr of birth; control kittens demonstrated onset of puberty at 15.5 ± 1.7 weeks. Similar puberty delay was seen in deslorelin-treated puppies (Faya, Priotto, Marchetti, Dela Sota, & Gobello, 2016). Although puberty delay was achieved, puberty proceeded normally, limiting the promise of this approach for eliciting permanent sterility.

4 | GENE SILENCING/GENE THERAPY

The discovery of small interfering RNA (siRNA) or micro-RNA molecules, and the potential of these molecules to bind specific genes and inhibit their expression, has presented novel targets for the development of therapeutics (Soutschek et al., 2004). In order to deliver siRNA to their target, and to ensure that the RNA is expressed over time to prolong its effect, viral vectors are used. It has been known for many years that it is possible to genetically engineer viruses to be vectors to deliver genes to cells. Although first investigated over 10 years ago, there are still high hurdles to manufacturing the RNA molecules and delivering them to the appropriate cells. The first therapeutics in human medicine using this approach are in clinical testing now. Companies such as Alnylam are developing small RNA drugs for amyloidosis and porphyria using this mechanism of action. In veterinary medicine applications, gene therapy for haemophilia in dogs has resulted in restoration of normal clotting function for the dogs' entire lifetime (Herzog et al., 2001; Niemeyer et al., 2009).

But can this approach be used for suppression of reproductive genes? Three things are needed for the gene silencing approach to be effective: definition of a specific gene to "turn off," identification of a viral vector to deliver the siRNA required to suppress that gene and, finally, the siRNA needs to get to the cells that express the gene.

One approach under investigation is construction of a viral vector that targets kisspeptin, a peptide that is required to elicit GnRH secretion (Albers-Wolthers et al., 2014; Dissen et al., 2012). The viral vector must deliver the siRNA to the hypothalamic neurons secreting kisspeptin, a tall order, since penetrating the blood/brain barrier is difficult. However, if the right hypothalamic neurons secreting GnRH could be targeted, that viral vector could cause the cell to produce continuous siRNA to shut down reproduction. If this could be achieved, a single product could theoretically work in both male and female cats and dogs for a lifetime of fertility suppression.

Dissen et al. (2014, 2016) have developed a method to deliver interfering siRNA to the hypothalamus of cats using an adeno-associated virus (AAV) to silence genes involved in the central control of reproduction, such as Kiss1 and Tac2. They target coding regions common to both dogs and cats. Roesl et al. (2016) described delivery of micro-RNA that will suppress androgen receptor gene expression in Sertoli cells. They have demonstrated that male mice given this construct are infertile for up to 5 months (when the experiment was terminated). Further experiments to evaluate longer duration suppression are in progress.

Scientists are also studying the feasibility of inserting genes that cause the overexpression of gonadotropin-inhibiting hormone (GnIH) or Mullerian inhibiting substance (MIS). GnIH could potentially decrease the synthesis and release of GnRH (Tsutsui et al., 2010). MIS regulates primordial follicle recruitment in adult females and testosterone production in adult males in both cats and dogs (Pepin et al., 2016). It is hypothesized that delivering a gene that could express high enough levels of MIS over the lifetime of dogs and cats could suppress follicular recruitment in females and potentially decease testosterone in males resulting in both female and male infertility.

Gene therapy can also be used to deliver a gene that could result in the continuous secretion of anti-GnRH monoclonal antibodies. This passive immune response would require the production of an antibody of high enough titre and affinity to inactivate GnRH, resulting in infertility. The DNA would need to be designed to elicit canine- or feline-specific antibodies to be effective. Early work has proved promising: genes have been delivered to male and female mice using AAV vectors that cause the overexpression of monoclonal antibodies to GnRH and zona pellucida proteins causing suppression of fertility for over 6 months (Hay, 2015).

Another approach is to use micro-RNA technology to inhibit androgen receptor expression in the testes, as androgen stimulation is required for normal testicular function including sperm production. Androgen receptor gene silencing constructs could be delivered with viral vectors to "turn off" male reproduction. Roesl et al. (2016) describe delivery of micro-RNA that will suppress androgen receptor gene expression in Sertoli cells.

Gametes can also be targeted using gene therapy; there are small RNA molecules identified as vital for ova and sperm to undergo maturation. Interfering with these RNA molecules, which are unique to gametes, is another interesting target (Fu & Wang, 2014; He et al., 2009). Once the right cat and dog small RNAs that regulate, for example, spermatogenesis have been identified, then antagonists to those RNAs can be designed and potentially delivered using viral vectors.

5 | SAFETY AND ANIMAL WELFARE

The Found Animals Foundation has committed to assuring that any product that will be developed using the various approaches outlined

above is both effective and safe for the treated dogs and cats. The goal is to suppress reproduction without disrupting other organs and systems.

The Foundation and MPG programme are committed to the highest welfare of all research animals used in grant-supported research. Grantees are required to follow the Foundation and MPG Program's Guidelines for Use of Animals in Research (www.michelsonprizeandgrants.org/resources/animal-welfare-policy) and to rigorously justify numbers of animals (usually rodents, occasionally cats and dogs) used in projects and must, prior to approval of any project, demonstrate that behavioural enrichment will be provided to research animals. Research dogs and cats must be placed in adoptive homes at the end of the study where they can enjoy living as a pet.

6 | SUMMARY

The development of a single-dose non-surgical sterilant is a long-term goal, and although an effective solution has not yet been found, the work supported by the Found Animals Foundation has resulted in new findings that are available to the research community and help shed light on basic reproductive mechanisms in cats and dogs. As these findings are shared via publication in peer-reviewed journals, other researchers benefit and the knowledge of reproductive biology is enriched. This, in turn, should encourage new approaches and collaborations.

ACKNOWLEDGEMENTS

The author would like to thank Dr. Shirley Johnston for discussion of the MPG programme and for historical context. In addition, perspective and historical context was provided by discussions with the late Dr. Wolfgang Jöchle.

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