

Wildlife population management: are contraceptive vaccines a feasible proposition?

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1. ABSTRACT

To minimize human-animal conflicts for habitation and burden of zoonotic diseases, it is imperative to develop new strategies for wildlife population management. In this direction, contraceptive vaccines eliciting immune response against hormones/proteins critical for reproduction have emerged as one of the promising options. Contraceptive vaccines based on neutralization of gonadotropin releasing hormone (GnRH) have been used for inhibition of fertility in various species such as wild horses, white-tailed deer, pigs, cats, dogs etc. It has been used for immunocastration of male pigs to improve meat quality. However, additional safety studies of GnRH vaccine will be needed in light of presence of its receptor at extra-pituitary sites. Native porcine zona pellucida (PZP)-based contraceptive vaccines have shown their utility in the management of the population of both captive and free-ranging wild horses and white-tailed deer. Long-term use of the PZP-based contraceptive vaccines has also demonstrated their safety. Ideally single injection of the contraceptive vaccine should elicit long lasting immune response and desired contraceptive efficacy, which will require development of novel vaccine delivery platforms and more potent adjuvants.

2. INTRODUCTION

The uncontrolled increase in population of free-ranging animal species such as elephants in Africa, kangaroos in Australia, feral horses and white-tailed deer in USA, street dogs and cats in several developing countries, and monkeys in India etc is leading to increasing conflicts for habitation between humans and wildlife species. Further, wild as well as domesticated animals may act as vectors for viruses, bacteria or parasites and thus pose a major risk to human health (1). There is an increase in the emerging infectious diseases in humans in last 3 decades and 70% of these are zoonotic in nature (2, 3). As an example, dogs are the main vectors that maintain rabies virus circulation within human communities. Human mortality from rabies has been estimated to be 55,000 deaths per year and 90% of deaths occur in the developing world (4). In India, approximately, 20,000 people die from rabies per year and 96% of these are infected by dogs (5, 6). Almost 1.8. million people annually receive post-exposure prophylaxis against rabies following bite or exposure to rabid or suspected rabid animal. The global threat posed by zoonoses (transmitted by bats and palm civets) is also illustrated by the 2003 outbreak of severe acute

respiratory syndrome coronavirus (SARS-CoV), which emerged in Hong Kong but affected 37 countries leading to over 8,000 cases of infection and approximately 800 deaths (7). Currently, there is concern about Middle East respiratory syndrome coronavirus (MERS-CoV), which emerged in Saudi Arabia in 2012 and is suspected to be zoonotic (initially transmitted by dromedary camels) in nature with over 1,100 cases of infection and 440 deaths, including travel related cases worldwide (8).

Wildlife managers have often used lethal means such as culling to control wildlife population; however, growing public concern over animal welfare issues and new legislative laws banning culling in several countries make such an approach increasingly unacceptable. Substantially reducing the number of abundant species may have indirect effects on the population of other species. For instance, in United Kingdom culling of Eurasian badgers (*Meles meles*) in an effort to reduce the incidence of bovine tuberculosis in cattle herd led to an increase in the population of red fox (*Vulpes vulpes*) (9). It has also been observed that repeated badger culling in the same area allow badgers from neighbouring area to re-colonize culled areas leading to increased prevalence of *M. bovis* infection in badger (10). Culling also leads to disturbance in the social group of animals leading to increased movement and thus instead of reducing transmission of zoonotic diseases it results in their increase (11). Thus, culling as a measure to control wildlife population and transmission of infectious disease has proven to be inefficient (12, 13). Translocation of animals from overpopulated areas to low population areas or in a confined environment has also been used as a measure for population control of wildlife in a particular territory, to minimise human-animal conflicts, and to reduce the burden of infection in the host wild species or its transmission to other animals including humans (14, 15). However, relocation of animals to new environment may lead to both physical and mental stress, disturbances in social culture, including adequate accessibility of food.

Surgical sterilization such as spaying of female dogs and castration of male dogs is used by few countries to control their population. Newly castrated male dogs may temporarily suffer from testosterone withdrawal leading to irritability and lethargy, a phenomenon known as the “irritable male syndrome” (16). Other potential complications with surgical castration include haemorrhage and excessive swelling, especially if the spermatic cords are cut but not crushed (17). Ovariohysterectomy of female dogs may lead to complications such as haemorrhage, ovarian remnant syndrome, stump pyometra, stump granuloma, fistula draining tracts and estrogen responsive urinary incontinence (17). To control monkey (*Rhesus macaque*) population in Himachal Pradesh, India, surgical sterilization of males by thermo-cauteric coagulative vasectomy and

of females by endoscopic thermo-cauteric tubectomy has been used (unpublished observations). However, long term side-effects of such a surgical sterilization program to control population of monkeys needs further investigation. Surgical sterilization by and large is more socially accepted than culling, though it is relatively expensive and required specialized facilities and staff. In addition to surgical sterilization, chemical sterilization by intra-testicular injection of zinc gluconate (Neutersol, Addison Biological Laboratory Inc.), which was approved in 2003 by the US Food and Drug Administration (FDA), that causes sclerosis of the testes and sterility has been used for controlling fertility of dogs and cats (18–20). In addition to zinc gluconate, intra-testicular injection of calcium chloride in dogs also led to dose-dependent decrease in sperm count (21). The low cost and ease of use of chemical sterilants as compared to surgical sterilization will be useful for their application at field level; however, additional field trials are needed to ascertain their safety and efficacy.

Administration of synthetic progestins such as megestrol acetate, melengestrol acetate, and levonorgestrel has been used as contraceptives for dogs and cats leading to inhibition of oestrous (For review see 22). This approach is suitable for confined companion animals or animals in zoo but unsuitable for free-roaming animals. Administration of melengestrol acetate as implants induced infertility for up to 2 years. Due to uterine pathology, long term administration of melengestrol acetate is not recommended (23). Levonorgestrel implant has also shown to be effective for prevention of fertility in tammar wallabies (*Macropus eugenii*), grey kangaroos (*Macropus giganteus*) and koala (*Phascolarctos cinereus*) females (24–26). In addition to synthetic progestins, gonadotropin releasing hormone (GnRH) agonists such as deslorelin (Suprelorin, Virbac) and azagly-nafarelin (Gonazon, Intervet International B.V.) have been used to inhibit fertility in dogs and tammar wallabies (22, 23, 27). For population management of free-ranging wildlife, hormonal contraceptive may not be a very good option due to high cost, difficulty in delivery, health risk in pregnant animals and changes in social behaviour. In addition, immunocontraception as one of the potential humane options for wildlife population management is being considered favourably by various agencies. In this article, various contraceptive vaccines that are being used for the wildlife population management will be reviewed. The efficacy and safety aspects of contraceptive vaccines will also be discussed. The current limitations for contraceptive vaccines and the plausible solutions will also be described.

3. CONTRACEPTIVE VACCINES

The basic principle of contraceptive vaccine is to generate either humoral and/or cell mediated immune response against hormones/proteins that are

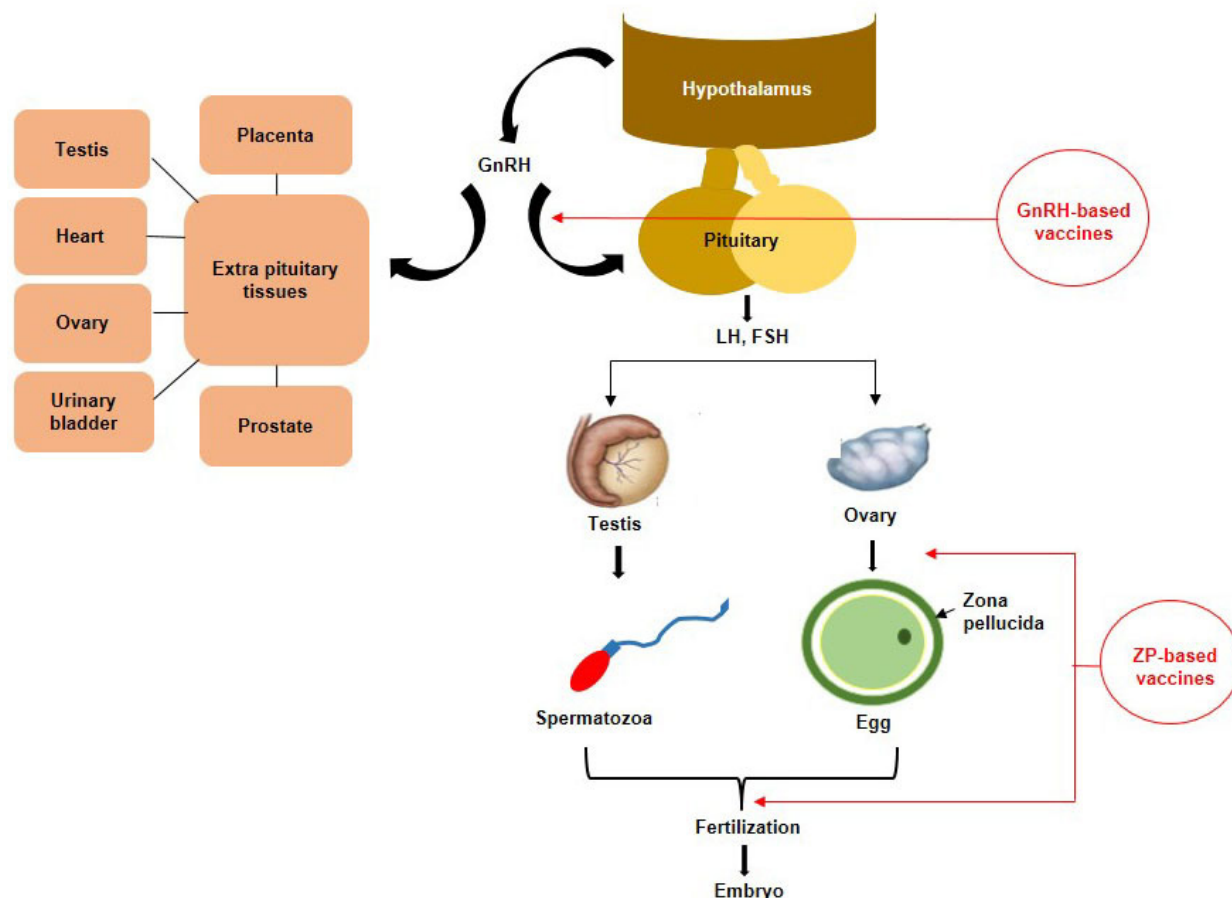


Figure 1. Schematic representation of the processes targeted by the contraceptive vaccines meant for wildlife population management. Possible sites have been shown for the action of GnRH- and ZP-based contraceptive vaccines. Antibodies against GnRH will inhibit production of LH and FSH from anterior pituitary thereby leading to inhibition of spermatogenesis in males and ovarian follicular development and ovulation in females. The anti-GnRH antibodies may also act on other extra-pituitary sites such as placenta, testis, ovary, heart, prostate, urinary bladder etc. Antibodies against ZP curtail fertility by either inhibiting folliculogenesis or fertilization or both.

critical for reproduction, which will lead to interference in their biological activity resulting in inhibition of fertility. Basically, contraceptive vaccines can be broadly categorised as those i) inhibit production of gamete (spermatozoa, egg); ii) inhibit gamete functions such as fertilization; and iii) inhibit gamete outcome. GnRH primarily synthesized and secreted by the hypothalamus (though extra-hypothalamic sites for its secretion have been reported) acts on the anterior pituitary and in turn regulate the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Both, LH and FSH secreted by pituitary, in turn acts on testes and ovaries leading to production of sperm and oocytes, respectively. Thus neutralization of GnRH, LH and FSH or blocking LH/FSH receptor by generating specific antibodies may lead to inhibition of gamete production. Generation of immune response against spermatozoa- and/or egg-specific proteins will interfere in their respective functions thereby leading to inhibition of fertilization. Post-fertilization, the embryo synthesizes and secretes human chorionic gonadotropin (hCG), which has also

been used as target for development of contraceptive vaccine for women (28). In the context of wildlife population management, contraceptive vaccines based on GnRH and zona pellucida (ZP) have been tried at the field level. Figure 1 illustrates the basic principle and steps at which the GnRH and ZP-based contraceptive vaccines will act thereby leading to inhibition of fertility. In the present review, both GnRH- and ZP-based contraceptive vaccines will be briefly discussed with respect to their contraceptive efficacy and safety. In addition, the current limitations with respect to contraceptive vaccine delivery in free-ranging wildlife and plausible solutions will be discussed.

4. GnRH-BASED CONTRACEPTIVE VACCINES

GnRH, a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH₂) is primarily synthesized and secreted by the hypothalamus (29), though its presence and/or secretion in other tissues such as ovary, testis,

Table 1. List of commercially available GnRH-based contraceptive vaccines and their intended use

GnRH-based vaccines	Name of the company	Observations	References
GonaCon™	National Wildlife Research Centre, USA	<ul style="list-style-type: none"> • Provided multi-year contraception in female white-tailed deer • Single immunization achieved 80–100% contraception in female elk • Immunized prairie dogs showed high contraception • Showed inhibition of fertility in both male and female cats 	33, 35–38
Improvac®	Pfizer Animal Health, Australia	<ul style="list-style-type: none"> • Effective in reducing sexual and aggressive behaviour in pigs • Improvement of meat quality in male pigs • Suppressed testosterone concentration, number of sperm and sexual behaviour in boars 	39–42
Equity™	CSL, West Ryde, NSW, Australia	<ul style="list-style-type: none"> • Suppressed testicular function in elephant • Suppressed testicular function and sexual behaviour in stallion 	44, 45
Bopriva®	Pfizer Animal Health, Parkville, Australia	<ul style="list-style-type: none"> • Decreased testicular development, serum testosterone and physical activity in bulls • Suppressed testicular growth and testosterone secretion in male calf • Inhibit fertility in boars and dairy cattle 	46–49
Improvest®	Zoetis, Kalamazoo, MI, USA	<ul style="list-style-type: none"> • Castration of male pigs • Suppress heat in female gilts 	50, 51
Repro-BLOC™	Amplicon Vaccine LLC, Pullman, WA, USA	<ul style="list-style-type: none"> • Reduced uterine size, vascularity and suppressed ovarian steroidogenesis in elephant 	52

prostrate and placenta has also been reported (30, 31). To develop contraceptive vaccine, GnRH being a self-peptide hormone, its conjugation with various carrier proteins such as tetanus toxoid (TT), diphtheria toxoid (DT), keyhole limpet hemocyanin (KLH), blue protein from mollusc (*Concholepa concholepa*), ovalbumin etc have been made to provide T-cell help to enable generation of antibodies against GnRH. GnRH-based contraceptive vaccines work in both males and females as neutralization of its biological activity inhibit production of sperm in males and eggs in female. After establishing proof-of-concept of GnRH-based contraceptive vaccines in laboratory experimental animals including non-human primates, currently there are at least six commercial vaccines available (Table 1).

GonaCon™ developed by National Wildlife Research Center, USA is comprised of synthetic GnRH coupled to KLH (more recently with blue protein from mollusc) and supplied with AdjuVac™ (diluted version of Jones disease vaccine containing killed mycobacteria and oil adjuvant). GonaCon™ has been approved by the Environment Protection Agency (EPA), USA for fertility regulation in white-tailed deer in urban/suburban areas and subsequently for wild horses (*Equus caballus*) and burros (*Equus asinus*) (32). Single injection of GonaCon™ induced infertility ranging from 2 to 5 years in white-tailed deer as a measure to control their population (33). It also successfully blocked the cyclic activity and ovulation in captive wild mares (34). To reduce the potential of ecological damage and spread of brucellosis, the contraceptive efficacy of GonaCon™ has also been demonstrated in female elk (*Cervus elaphus*) (35). The contraceptive potential of GonaCon™ in black-tailed prairie dogs has also been

shown (36). The contraceptive efficacy ranging from 5 months to 5 years after single injection of GonaCon™ in female cats has also been reported, which depends on the immune response generated in the respective cat (37, 38). In general, the vaccine was more effective in female cats as compared to male cats (37).

Another commercial GnRH-based contraceptive vaccine, Improvac®, consists of synthetic incomplete analogue of gonadotropin-releasing factor (GnRF) linked to a carrier protein to make it immunogenic. Immunization of cross-bred Iberian female pigs with Improvac® led to reduction in the incidence of standing oestrous, serum progesterone levels, and development of uterus and ovaries (39). Immunization led to long-lasting immunity of at least 20 months after third injection (39). Immunization of male pigs with Improvac® led to a significant reduction in their sexual and aggressive behaviour as observed by reduction in mounting, fighting, pushing, head butting and tail manipulation (40). Pre- and post-pubertal rams and boars accumulates androgen derivatives namely androstenone and skatole in their adipose tissues, which gives an unpleasant odour to meat. To overcome this taint, surgical sterilization of male pigs is routinely done. Vaccination of boars with Improvac® contraceptive vaccine has shown to reduce the pig meat taint (41). Further, it has been shown that immunization of male pigs with Improvac® led to improvement in the lean meat content as compared to surgically castrated pigs (42). Immunization of adult ewes (White Alpine sheep) with Improvac® led to suppression of cyclicity concomitant with reduction in progesterone levels (43).

Immunization of a male Asian elephant with combination of two commercial GnRH-based contraceptive vaccines i.e. Improvac® and Equity™, led

to a decrease in serum testosterone concentrations, testicles diameter, penile atrophy and weight gain (44). After 1 year of initial treatment, no spermatozoa were observed in the semen suggesting that GnRH vaccine may be a useful non-invasive method of contraception for Asian elephants (44). Immunization of adult stallion with commercially available GnRH-based vaccine, Equity™, using three injections schedule led to suppression of testosterone levels, reduction in semen quality and changes in sexual behaviour, the extend of which was variable among the immunized animals (45).

Bopriva® is one of the commercially available GnRH-based vaccine, developed by Pfizer Animal Health, Parkville, Australia, which induces antibodies against GnRF. Active immunization of pubertal bulls with this vaccine led to decrease in testosterone levels in blood, testicular development and physical activity, leaving the body weight gain unaffected (46). Further, two injections of Bopriva® in the peripubertal bull also suppressed the testicular growth and blood testosterone concentration for at least 10 weeks after the booster injection (47). Bopriva® was also effective in suppression of testicular functions including sperm production in boars (48). Immunization of female dairy cattles (cyclic Swiss Fleckvieh cows) with Bopriva® showed decrease in progesterone levels without affecting estrogen levels, suppression of oestrus and impaired folliculogenesis (49).

Another commercial vaccine based on GnRH, Improvest® (Zoetis, Kalamazoo, MI, USA), has been used for castration of male pigs and improvement in the meat quality (50). Female gilts receiving two injections of Improvest® showed better growth performance and suppression of heat (51).

Repro-BLOC™ (Amplicon Vaccine, LLC, Pullman, WA, USA) is a recombinant ovalbumin-GnRH fusion protein vaccine developed for use in cattle, although there is limited data on its efficacy. Multiple injections (primary followed by 4 booster) of Repro-BLOC™ in a 59-year old Asian elephant (*Elephas maximus*) led to suppression of ovarian steroidogenesis for over a year and reduction in uterine size and vascularity (52).

4.1. Safety of GnRH-based contraceptive vaccine

GnRH-based vaccines have shown successful contraceptive efficacy in both male and female domestic horses, without any serious adverse effects (34, 45, 53–54). Suppression of oestrus and unwanted oestrus behaviour by GnRH-based vaccines (53, 54) may be desirable in domestic species including cats and dogs. However, inhibition in the production of reproductive steroid hormones (progesterone in females and testosterone in males) by GnRH-based vaccines may have impact on the complex evolutionary social organization of horses,

elephants and captive exotic species in zoos, which needs to be investigated more carefully (55).

There was no significant haematological or endocrinological changes in the two groups of mongrel dogs, when either immunized with Improvac® or surgically castrated, suggesting that immunological castration may provide a safer alternative (56). No adverse effects on body weight or blood chemistry were observed in black-tailed prairie dogs immunized with GonaCon™ (36). It has also been reported that immunization with Improvac® can improve the welfare of male pigs as it was able to inhibit sexual and aggressive behaviour (40). The vaccine was well tolerated in the pigs and no injection site reactions were observed (41). However, in few female cats immunized with GonaCon™, non-painful late-onset granulomas were observed at the injection site (38). A study demonstrated that simultaneous administration of anti-GnRH (GonaCon™) vaccine along with rabies vaccine had no adverse impact on the seroconversion against rabies virus antigen suggesting that GnRH vaccination can be used along with rabies immunization program (57).

It is imperative to take into consideration that use of GnRH-based vaccine in pregnant animals may lead to abortion, if LH is critical to maintain the corpus luteum throughout during gestation such as in bison. GnRH receptors have been described at several extra-pituitary sites such as ovary, testes, placenta, prostate including urinary bladder and heart (30, 31, 58, 59). These observations suggest that more carefully monitored field trials of GnRH-based contraceptives vaccines should be undertaken to rule out any adverse effect on other physiological processes than reproduction.

5. CONTRACEPTIVE VACCINES BASED ON ZONA PELLUCIDA GLYCOPROTEINS

In mammals, egg is surrounded by an extracellular translucent glycoproteinaceous matrix known as zona pellucida (ZP). It acts as a relatively species-specific 'docking site' for binding of the spermatozoa to the oocyte, induces acrosomal exocytosis in zona-bound spermatozoa, prevents polyspermy, and plays an important role in the protection of a pre-implanted blastocyst. Due to their critical role in reproduction, ZP glycoproteins have been used as candidate antigens for contraception via immunological intervention. Zona matrix is composed of either 3 or 4 glycoproteins. In mice, it is composed of 3 glycoproteins namely ZP glycoprotein -1 (ZP1), -2 (ZP2), and -3 (ZP3), whereas pig and canine ZP matrix is also composed of 3 glycoproteins but instead of ZP1, ZP glycoprotein-4 (ZP4) is present. In non-human primates and humans, ZP matrix is composed of 4 glycoproteins that are ZP1, ZP2, ZP3,

Table 2. Sequence homology of porcine ZP2, ZP3 and ZP4 with other species at amino acid level

Animal Species	Percentage sequence homology with porcine		
	ZP2	ZP3	ZP4
Mouse (<i>Mus musculus</i>)	55	66	50 ¹
Dog (<i>Canis lupus</i>)	70	75	72
Horse (<i>Equus caballus</i>)	72	77	75
Monkey (<i>Macaca radiata</i>)	63	72	67 ¹
Bison (<i>Bison bison</i>)	78	88	77 ¹
Rat (<i>Rattus norvegicus</i>)	54	65	60
African Bush Elephant (<i>Loxodonta africana</i>)	65	73	65
Brush-tail Possum (<i>Trichosurus vulpecula</i>)	51	50	56 ¹

¹Instead of ZP4, ZP1 is present

and ZP4. Structure and functions of all the four ZP glycoproteins during fertilization from various species has been investigated by various groups, which has been reviewed elsewhere (60). Characterization and sequencing of ZP glycoproteins from various species revealed that their basic structure is evolutionary conserved but have variable degree of amino acid sequence identity. For example porcine ZP3 has 66% identity at amino acid level with mouse ZP3 and 75% with canine ZP3. The extent of amino acid sequence identity of various porcine ZP glycoproteins with their respective homologues from other species is listed in Table 2. This property of ZP proteins has made heterologous immunization as a feasible proposition. It is further strengthened by the observations that polyclonal antibodies generated against porcine heat solubilised isolated zona pellucida (SiZP) cross-react immunologically with ZP of human, squirrel monkey, rabbit, rat, and mouse (61). Due to easy accessibility of porcine ovaries from abattoirs, contraceptive vaccine based on porcine ZP preparations became the antigen of choice.

5.1. Native porcine zona pellucida (PZP)-based contraceptive vaccine

Contraceptive potential of either native porcine SiZP (PZP) or its purified component (ZP3) has been demonstrated in female rabbits (62), bitches (63), non-human primates (64, 65), domestic and captive wild horses (66), captive white-tailed deer (67) and a variety of zoo animals (68). The field trial of native PZP-based contraceptive vaccine for the management of wild horses population on Assateague Island National Seashore, a barrier island off the coast of Maryland, USA demonstrated its contraceptive efficacy (69). The vaccine was delivered remotely using dart gun. Inhibition of fertility in wild horses could be maintained by giving annual booster of PZP vaccine. It was shown that third consecutive annual booster of porcine ZP led to 79% efficacy in preventing pregnancies in mares (70).

Smithsonian Institute's Conservation and Research Center at Front Royal, VA, USA initiated the first field trials of PZP-based contraceptive vaccine in white-tailed deer inhabiting Fire Island National Seashore, NY, USA (71). Deer were captured, tagged, given primary injection of PZP vaccine, and then released. Subsequent booster injections were given remotely by using dart gun. Between 1993 and 1999, twinning rates among individually known vaccine treated adult female deer decreased by 78.9% from pre-treatment rates (72). The contraceptive efficacy of PZP vaccine was also demonstrated in free-ranging African elephants (*Loxodonta africana*) in the Kruger Park, South Africa (73). Thus PZP-based contraceptive vaccine has been successfully used for the management of the population of wild horses (34, 66, 69, 70, 74, 75, 76), urban deer (71, 72, 77, 78), wapti (79), and African elephants (73, 80). Keeping in view the above, currently three commercial vaccines based on PZP are available as listed in Table 3.

5.2. Safety of PZP-based contraceptive vaccine

Ovarian specific expression of zona proteins and lack of cross-reactivity of antibodies generated against zona proteins with other tissues and protein hormones (81, 82) is of an advantage of PZP-based vaccines as compared to GnRH-based contraceptive vaccine. Zona pellucida-based contraceptive vaccine primarily works by either inhibiting fertilization and/or folliculogenesis (Figure 1). Potential changes in ovarian pathology represented by follicular atresia along with sometime depletion of primordial follicles pool in rabbits (62), dogs (63), sheep (83) immunized with porcine zona proteins was one of the main concern for the application of PZP-based contraceptive vaccine for wildlife population management. However, no changes in ovarian histology were found in PZP-based contraceptive vaccine immunized horses and deer (66, 71). Inhibition of fertility was reversible in the captive deer immunized with PZP-vaccine after one to four years of immunization (67). In another study,

Table 3. List of commercially available porcine ZP-based vaccines and their applications

Commercial PZP-based vaccines	Name of the company	Applications	References
Spayvac®	ImmunoVaccineTechnologies™, Halifax, Nova Scotia, Canada	<ul style="list-style-type: none"> • Showed 100% contraceptive efficacy the first year then 83% the next 3 years in wild horses • Showed 85–90% infertility in female deer 	34, 78
ZonaStat-H	Humane Society of the United States, Washington, DC, USA	<ul style="list-style-type: none"> • Only PZP vaccine approved by the Environmental Protection Agency (EPA) for use in female wild horses and burros • Suppressed fertility by 86% in domestic and wild horses 	76
PZP-22	University of Iowa, School of Pharmacy, Iowa City, USA	<ul style="list-style-type: none"> • Provided two years of infertility in feral horses 	74, 76

no changes in ovarian histology of PZP immunized deer were reported. However, breeding season was extended by 1–2 months (71). PZP-vaccinated deer were more active and gained significantly more weight by summer following immunization than untreated deer, presumably because of avoidance of lactation, but by the following fall, all weight differences disappeared (84). Ovarian eosinophilic oophoritis was reported in PZP immunized deer, which; however, was not statistically different as compared to unimmunized group (85). Injection site abscesses were reported only in 0.5% of PZP immunized deer, but all of them revealed the formation of granuloma at the injection site without any complication (72, 85). In elephants immunized with PZP-based contraceptive vaccine, examination of the ovaries and uteri by ultrasound revealed no adverse changes (73, 80). However, there may be species-specific differences in the ovarian histopathology subsequent to PZP-based contraceptive vaccine immunization, which should be investigated in detail in the respective target species.

Long-term treatment of wild horses with PZP-based contraceptive vaccine did not lead to any permanent or significant changes in ovarian endocrine parameters and oestrous cyclicity as monitored by urinary steroid metabolites (70). It had no adverse outcome when used in pregnant mares. The contraceptive effect was reversible (86). Increased body conditions and longevity were observed in PZP-immunized horses (87). There was no significant change in the social organization or behaviour of PZP-immunized wild horses (88). However, there is some concern over injection site reactions (55). It is likely that the remote delivery of PZP-based contraceptive vaccine by dart may result in surface bacteria and debris being pushed into the injection site resulting in injection site reactions (55).

Few significant changes in blood chemistry such as levels of urea and creatine were observed in PZP immunized deer but none of these were associated with any physiological abnormalities (85, 89). PZP immunized Dall sheep (*Ovis dalli*) and domestic goats (*Capra hircus*) also did not reveal any significant changes in blood chemistry (90).

5.3. Recombinant zona proteins as candidates for contraceptive vaccine

Due to limited availability of native porcine zona proteins from pig oocytes and to circumvent the apprehension of their probable contamination by other ovarian-associated proteins, recombinant zona proteins have also been used as candidate immunogens. Immunization of female baboons (*Papio anubis*) with *E. coli*-expressed recombinant bonnet monkey (*Macaca radiata*) ZP4 (previously designated as ZPB) coupled with diphtheria toxoid (DT) led to reversible block of fertility (91). However, immunization of female bonnet monkeys with the above immunogen led to inhibition of fertility which was not reversible. Histology of the ovaries from immunized monkeys revealed follicular atresia (92). Active immunization of female marmosets (*Callithrix jacchus*) with mammalian-expressed recombinant human ZP3 also led to inhibition of fertility associated with ovarian pathology characterized by depletion of primordial follicle pool (93). Immunization of cynomolgus monkeys (*Macaca fascicularis*) and baboons (*Papio cynocephalus*) with mammalian expressed recombinant human ZP2, ZP3 and ZP4 respectively showed higher contraceptive efficacy in animals immunized with ZP4 (94). These studies in non-human primates showed the potential of recombinant zona proteins as candidate immunogens for the development of contraceptive vaccine.

In order to control the population of street dogs and thereby reduce the burden of rabies infection, it was demonstrated that non-descript female dogs immunized with *E. coli*-expressed recombinant dog ZP3 conjugated to DT failed to conceive (95). Ovarian histology of the immunized dogs revealed degenerative changes in the ZP matrix and follicular atresia. Subsequently, to avoid chemical conjugation of recombinant dog ZP3 with DT and to obtain recombinant protein without His₆-tag, it has been expressed in *E. coli* as a fusion protein with promiscuous T cell epitope of tetanus toxoid (TT, amino acid residues 830–844) with dilysine linker (TT-KK-ZP3) (96). Immunization of female mice with recombinant TT-KK-ZP3 led to significant reduction in fertility, which was associated with antibody titres (96). In Australia and

New Zealand, the potential of recombinant brushtail possum (*Trichosurus vulpecula*) ZP3 protein to control the fertility of Koalas (*Phascolarctos cinereus*) and Eastern Grey Kangaroos (*Marcopus giganteus*) has also been explored (97, 98).

In light of limited availability of native PZP-based contraceptive vaccine, recombinant porcine ZP3 and ZP4 have also been expressed in *E. coli* and their contraceptive efficacy was evaluated (99). Immunization of female mice with recombinant porcine ZP3 and ZP4 led to significant inhibition of fertility and decrease in litter size as compared to the adjuvanted control (99). Interestingly, *E. coli*-expressed recombinant porcine ZP3 and ZP4 boosted the antibody response in female mice primed with porcine native SI-ZP, suggesting that recombinant proteins can be used as booster doses thereby enabling wider coverage of immunization program in light of limited supply of native PZP-based contraceptive vaccine (99). Contraceptive efficacy of recombinant porcine ZP3 and ZP4 vis-à-vis PZP vaccine has also been evaluated in pony mares. Extended anoestrus was observed in 86% of the PZP immunized mares as compared to 14% in recombinant porcine ZP3/ZP4 immunized group, which correlated with basal serum oestradiol and progesterone levels (100). All mares resumed cyclicity by 10 months post-treatment. None of the PZP-immunized mare conceived whereas all the unimmunized mares became pregnant. Out of 7 pony mares immunized with recombinant porcine ZP3/ZP4, 3 failed to conceive. These experiments suggest the potential of recombinant proteins for controlling fertility in mares, which; however, need further investigations (100).

6. VACCINE DELIVERY: MAJOR CHALLENGE IN THE APPLICATION OF CONTRACEPTIVE VACCINES FOR WILDLIFE POPULATION MANAGEMENT

In the context of free-ranging wildlife population management, it will be ideal, if single injection of contraceptive vaccine generates adequate immune response leading to inhibition of fertility for several years or even permanent sterility. Though it has been shown that single injection of GnRH-based contraceptive vaccine may induce infertility ranging from 2–5 year in white-tailed deer (33), by and large booster doses of the contraceptive vaccine are required to achieve desirable contraceptive efficacy and duration of infertility. It will necessitate development of sensitive tracking technologies to distinguish unvaccinated animals from those that have been vaccinated and among vaccinated animals to distinguish how many prior booster dose(s) have been delivered. Additionally, it will be imperative to develop more potent adjuvants and novel vaccine delivery platforms.

6.1. Remote vaccine delivery by either dart gun or oral bait

It is an expensive and cumbersome exercise to capture the free-ranging animals to deliver the contraceptive vaccine by intramuscular route. Immunization of free-ranging large animals such as horses and white-tailed deer by remote delivery of contraceptive vaccine by dart-gun has been very useful (69, 72). However, vaccine delivery by dart gun may not be a feasible proposition in small free-ranging animals such as monkeys etc, as vaccine dart may hit some sensitive part of the body and thereby leading to injury. Another strategy to deliver vaccines may be to use oral baits. In the second half of the 20th Century, oral rabies vaccines in the form of different types of baits incorporating different strains of live replication-competent rabies virus strains were developed with an aim to eliminate rabies infection circulating in European wildlife in particular foxes (101). The oral baits were dropped by helicopters in the forests inhabited by wild foxes. Within the past 30 years, the overall incidence of rabies in Europe has decreased by approximately 80% and it has been completely eliminated from Western and Central Europe (www.who-rabies-bulletin.org). However, delivery of contraceptive vaccine as oral baits for the management of free-ranging wildlife population may be a difficult proposition as it will have adverse consequences on the fertility of other animal species, who may also consume these baits beside the target species. Contraceptive vaccines as oral baits may still be a feasible proposition for captive wildlife, where the individual animal is fed under supervision.

6.2. Edible vaccine

Plants can be engineered to produce foreign proteins in large amount which can be fed to the animals thereby eliciting appropriate immune response. As a proof-of-principle, it was shown that genetically modified potatoes (by inserting gene encoding the heat labile enterotoxin unit B from enterotoxigenic *E. coli* bacteria) when fed to mice as well as humans led to generation of immune response against the heat labile enterotoxin unit B antigen (102, 103). In an effort to control the population of possums in New Zealand, attempts are being made to develop genetically modified carrots expressing possum ZP3 (104). However, the outcome of these efforts is not as yet published.

6.3. Virus-like particles and bacterial ghosts

Virus-like particles (VLPs) are essentially non-infective viruses, which comprised of self-assembled viral envelop proteins without the genetic material. VLPs have unique property to display multiple copies of small foreign peptides on its surface, without losing the self assembly property of

the envelop protein, thereby leading to potent immune response against the foreign peptide (105). Their size typically falls in nanoparticles range and is stable and versatile in nature (106). VLPs offer excellent adjuvant characteristics which can induce innate and adaptive immune responses. As a matter of fact, VLPs have shown to enhance immunogenicity of peptides and proteins which otherwise are weakly immunogenic. There are various VLP vaccines that have been evaluated such as HPV L1 VLP, norovirus VLP vaccine and chikungunya (106). Johnson grass mosaic virus (JGMV) coat protein self-assemble to form rod-shaped VLPs and can be engineered to express on its surface foreign peptides (107). Immunization of mice with JGMV coat protein-based VLPs expressing a fusion peptide comprising of mouse ZP3 and spermatozoa-specific YLP₁₂ peptide separated by diglycine spacer led to sub-fertility in the immunized animals (108). The basic problem with VLPs as a platform to deliver contraceptive vaccine is that in addition to antibodies generated against the foreign peptide/protein, a more potent antibody response is also generated against the envelop/coat protein of the virus.

Bacterial ghost, made-up of non-living bacterial cell without genetic component, has also been effectively used as platform to deliver antigens for eliciting immune response. Immunization of female brushtail possum with bacterial ghost expressing either N-terminal (41–316 amino acid residues) or C-terminal (308–636 amino acid residues) fragments of possum ZP2 fused to maltose-binding protein in *E. coli* NM522 strain led to the generation of both humoral and cell-mediated immune responses (109). Animals immunized with C-terminal fragment of ZP2 showed significant reduction in fertility (109). Bacterial ghost expressing brushtail possum ZP3 has also been prepared by using bacteriophage phiX174 lysis gene E in *E. coli* NM522 and their immunogenicity demonstrated in homologous animal model (110).

6.4. Live-vector based contraceptive vaccines

By using reverse genetic approach, attenuated *Salmonella typhimurium*-expressing mouse ZP3 was developed and delivered in mice through oral route. Immunized animals showed reduction in fertility (111). Mimivirus expressing B cell epitope of spermatozoa specific protein, eppin, has also been developed (112). Intranasal immunization of male mice with the above mimivirus led to generation of anti-eppin IgA antibodies in the genital tract. Mating of immunized males with unvaccinated females showed reduction in the fertility. Interestingly, immunization with the above antigen did not lead to any histopathological changes in the testes or their function (112).

Host-specific live vectors expressing various zona proteins have also been evaluated

for their contraceptive potential. For example, mice infected with recombinant ectromelia virus (a natural pathogen that causes mouse pox) expressing mouse ZP3 showed infertility ranging from 5 to 9 months that was associated with disruption of ovarian folliculogenesis (113). Immunization of female rabbits with recombinant myxoma virus expressing either rabbit ZP3 or ZP4 also showed infertility accompanied with ovarian pathology (114, 115). Infection of mice with recombinant cytomegalovirus (mouse-specific β herpes virus) expressing mouse ZP3 led to permanent infertility, primarily due to induction of the ovarian autoimmune pathology leading to progressive depletion of follicles (116). Basic concept of developing host-specific live vector-based contraceptive vaccine was to release these in the environment so that the recombinant virus gets transmitted from one animal to another thereby leading to effective management of pests such as rats and rabbits. It will save the task of individually capturing the animal, followed by vaccination and releasing the immunized animals in its natural habitat. The overall concept was good; however, it was observed that recombinant viruses have lower infectivity as compared with the wild type virus, which will eventually reduce the contraceptive efficacy of live vector-based contraceptive vaccine (117, 118). Another major concern was about the consequences on the population of other species, if by chance the recombinant virus loses its host specificity (117). Keeping in view the above, it is less likely that host specific live-vector-based contraceptive vaccine will be used in near future for population management of free-ranging pests.

6.5. Microparticles for contraceptive vaccine delivery

One of the exciting option to deliver the contraceptive vaccine is to use inert nanoparticles or microparticles which themselves may not be immunogenic but provide sustained immune response against an entrapped antigen for a long period. It is primarily due to the slow release of the antigen from such particles. Liposomes comprising of phospholipids and cholesterol is one such option. Grey seals (*halichoerus grypus*) immunized with porcine ZP entrapped in liposomes, decreased fertility in 90% of the immunized animals over a 5-year period (119). Antigen entrapped in biodegradable and biocompatible polymeric particles of poly-lactide (PLA) and poly(lactic-co-glycolic acid) (PLGA) have been used to reduce the number of injections and to generate long-lasting immune response. Immunogenicity of PLA based microparticles have been shown to improve by the use of permissible adjuvant like alum (120). Incorporation of CpG (small stretches of synthetic oligodeoxy nucleotides corresponding to unmethylated DNA of bacterial origin) motif can also enhance immune response of microparticles (121).

In general, nanoparticles favour generation of cell mediated immune response whereas microparticles favour humoral immune response (122). It has been shown that polymer particle-entrapped vaccines can elicit memory antibody response from single-point immunization (123). Therefore, a microsphere particles-based formulation supplemented with suitable adjuvant(s) is expected to reduce the number of doses of the vaccine required to achieve the desired immune response and efficacy. In addition to PLA/PLGA polymer, microparticles of water-soluble polyphosphazenes have also been used successfully as antigen delivery platform. Indeed, it was shown that microparticles of poly(di(sodiumcarboxylatoethylphen oxy) phosphazene) entrapping pertussis toxoid, CpG motif and synthetic cationic innate defense regulator peptide 1002 elicited effective immune response against *Bordetella pertussis* in mice (124). It has been shown that hCG entrapped in PLA/PLGA microparticles showed antibody response following one injection which was comparable with four injections of free hCG in rats (125). In the microparticle immunized group, antibody titres reached at peak on day 56 and maintained a steady state till day 84. Hence, it seems that contraceptive candidate proteins entrapped in PLA/PLGA based microparticles supplemented with appropriate adjuvant may lead to long-lasting immune response with lesser number of injections and thereby making single dose contraceptive vaccine a reality in future.

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