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Review

Ovarian dysfunction associated with zona pellucida–based immunocontraceptive vaccines

Carolynne J. Joonè^{a,b,*}, Martin L. Schulman^a, Henk J. Bertschinger^a^a Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa^b School of Veterinary Sciences, College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Queensland, Australia

ARTICLE INFO

Article history:

Received 1 June 2016

Received in revised form 9 September 2016

Accepted 9 September 2016

Keywords:

Porcine zona pellucida

Contraception

Anestrus

Oophoritis

Freund's adjuvant

Cytotoxic T-lymphocyte

ABSTRACT

Despite more than 40 years of research into zona pellucida (ZP)–based vaccines, relatively little is known about their mechanism of action. Early research demonstrated precipitation of ZP glycoproteins by antiovarian antiserum, rendering oocytes resistant to sperm binding *in vitro*. Subsequent work showed significantly decreased fertilization rates following passive immunization, sparking interest in anti-ZP immunocontraception for human and animal use. The primary mechanism of action of ZP vaccines is generally considered to be an antibody-mediated interference with sperm–oocyte binding and/or fertilization. However, this mechanism of action excludes the potential for ovarian dysfunction associated with anti-ZP treatment in some species. A review of relevant literature in pertinent model, domestic and wildlife species reveals a variety of previous and current hypotheses for ovarian effects following ZP-based immunization. Ovarian dysfunction has been suggested to be a species-specific response. In addition, cytotoxic T-lymphocytes and the use of Freund's adjuvants have been suggested to play a role. Finally, the type and extent of glycosylation of ZP antigens have been proposed to influence ovarian effects. The validity of these hypotheses is re-examined in the light of current knowledge. Further investigation of ovarian function in species believed to be resistant to the ovarian effects of anti-ZP vaccines is warranted. To this end, anti-Müllerian hormone may provide a novel tool for the assessment of ovarian function during ZP-based immunocontraception, particularly in wildlife species not amenable to frequent clinical examination.

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1. Introduction

The zona pellucida (ZP) is a complex glycoprotein matrix surrounding the mammalian oocyte and early conceptus. Comprised of either three or four glycoproteins, the ZP plays a pivotal role in the union of oocyte and spermatozoon during mammalian fertilization, arguably the most important joining event in biology. In addition, the ZP functions in the induction of the acrosome reaction, the prevention of polyspermy, and protection of the early

embryo [1]. Furthermore, the ZP is intimately involved in communication between the oocyte and its surrounding granulosa cells in the developing follicle [2]. These critical functions of the ZP in reproduction and its tissue-specific nature have encouraged research into its role as an immunocontraceptive for over 40 years [3].

Porcine zona pellucida (pZP) with added adjuvant remains the most common native form of the vaccine due both to the homology between the ZP proteins of many mammalian species and its availability in relatively large quantities [4,5]. Approximately 80 zoo and wildlife species have been successfully contracepted using pZP [6]. Despite this widespread application, relatively little is reported describing the vaccine's mechanism of action. In research

* Corresponding author. Tel.: (00) 61 747814398; fax: (00) 61 747791185.
E-mail address: carolynne.joone@jcu.edu.au (C.J. Joonè).

aimed at humans, initial enthusiasm for ZP-based immunocontraception waned sharply following reports of ovarian dysfunction in rabbits and nonhuman primates [7,8]. A number of hypotheses regarding the causes of ovarian dysfunction during ZP-based immunocontraception have since evolved.

This review revisits initial studies describing ovarian tissue, oocytes, or ZP as immunological agents, on which our current understanding of pZP's mechanism of action is based. In addition, several hypotheses advanced to explain ovarian dysfunction observed during ZP-based immunocontraception are re-evaluated based on relevant literature reporting on common laboratory and domestic animal species, as well as the feral horse, deer, and African elephant.

2. Early work on ZP antigens and antisera: a journey down the memory lane

Interest in immunological methods of fertility control dates back to the late 1890's with an initial focus aimed primarily at testes and spermatozoa as immunizing agents, reviewed by Tyler [9]. Reports of antisera to ovarian homogenates blocking fertilization processes in the sea urchin [10] and frog [11] encouraged interest in the mammalian oocyte and ovary as putative antifertility antigens. Initial studies demonstrated the existence of organ-specific antigens in the guinea pig ovary and testis [12,13]. Early immunofluorescence studies further localized common antigens to the ZP, atretic follicles, and the acrosome of spermatozoa [14].

Ownby et al. [15] injected golden hamster ovarian homogenates combined with Freund's complete adjuvant (FCA) into rabbits. Boosters, consisting of ovary homogenates with Freund's incomplete adjuvant, were followed by weekly serum sampling. The antisera produced included antibodies to at least one antigen unique to the ovary, demonstrated using agar-gel diffusion plates. Superovulated hamster eggs exposed to rabbit antiovary antisera formed a precipitate in the ZP that was visible under light- and phase-contrast microscopy. The precipitated ZP was found to be resistant to digestion by trypsin. Similar findings were reported by Sacco et al. [16].

Antiovarian antiserum, added to hamster ova before exposure to homologous spermatozoa *in vitro*, interfered with sperm–oocyte binding. None of 170 pretreated oocytes was penetrated by spermatozoa, in comparison to nearly half of 58 control oocytes [17]. The use of homogenized oocytes rather than the whole ovary as an immunogen produced similar results [18]. Despite ZP-based immunocontraception being in its infancy, researchers noted the potential advantages of this method of fertility control, including reversibility and the absence of somatic side effects because of the specificity of anti-ZP antibodies [17].

Jilek et al. [3], using mice passively preimmunized with rabbit anti-mouse ovary antisera, demonstrated via immunofluorescence the presence of anti-ZP antibodies bound to oocytes aspirated from antral follicles, as well as ovulated oocytes. This showed that anti-ZP antibodies were capable of reaching the ZP *in situ* within the follicle. In addition, passive immunization was found to decrease fertilization rates from over 91% to below 1%. The authors

concluded that the effect on fertility *in vivo* “seems to be a block to sperm penetration through the ZP, on which antibodies were actually detected within the follicles”.

Further study of aspirated oocytes and early embryos flushed from the uterus or uterine tubes of untreated hamsters showed that antihamster ovary antiserum precipitated the ZP of preovulatory oocytes as well as early embryonic stages *in vitro*. In addition, precipitation of the ZP following fertilization was thought to inhibit the attachment of transferred embryos to the endometrium [19], possibly as a result of interference with embryonic hatching [20]. In a similar study in mice, antioocyte and anti-ZP antisera had no effect on the development of early embryos to the blastocyst stage *in vitro*, although a small effect on zona shedding was noted [21]. A later study in the same species found that preincubation with anti-ZP antiserum had no effect on early embryonic development and zona hatching *in vitro*, nor implantation and further development of pretreated embryos transferred to pseudopregnant recipients, despite visible precipitation of the zonae [22]. Similarly, mice passively immunized with anti-ZP antiserum 2 days after mating showed no adverse effects on fertility or fecundity, and anti-ZP antisera had no effect on early embryonic development *in vitro* [23].

From these initial studies and those that followed, hypotheses regarding ZP vaccines' mechanism of action evolved as a primarily antibody-based interference with one or more of the following processes: sperm–oocyte binding, the acrosome reaction, sperm movement through the ZP, oocyte activation and/or the zona block; thus, at the level of the periovulatory oocyte. If so, estrous cyclicity and reproductive behaviors should remain unaffected following vaccination. This feature of pZP immunocontraception has been an important, although occasionally controversial, rationale supporting its application in species with complex social hierarchies, such as the African elephant and feral horse [24–28]. The detection, however, of ovarian dysfunction subsequent to treatment with ZP-based vaccines in some species has provided a challenge to researchers hoping to overcome this potentially undesirable outcome. During this process, a number of hypotheses regarding the cause of ovarian dysfunction have developed.

3. Hypotheses explaining ovarian dysfunction during ZP-based immunocontraception

3.1. Glycosylation causes oophoritis

Glycosylation refers to the pattern of binding of distinct carbohydrate moieties to amino acids, giving rise to the glycoprotein structure of the ZP. The diverse nature of ZP glycosylation across species may play a role in the species-specificity of sperm–zona binding [29]. Chemical deglycosylation of pZP3 was found to decrease its antigenicity and immunogenicity; precipitation of ZP *in vitro* by the relevant antisera was more superficial than precipitation produced using antisera to glycosylated pZP3 [30]. Consequently, early workers in the field considered the deglycosylation of ZP antigens to negate pZP's oophoritogenic effects. In rabbits, the degree of glycosylation of pZP was found to correlate directly with the degree of interference with

folliculogenesis, ovulation, and estrous cyclicity [31,32]. Researchers hoped that qualitative, rather than purely quantitative, differences in the immune response to deglycosylated versus glycosylated ZP antigens were responsible for the differences in oophoritis effect [32]. Subsequent trials in marmoset monkeys [33] and baboons [34] found that immunization with deglycosylated pZP3 did indeed result in lower levels of ovarian dysfunction compared with glycosylated pZP3; a result that correlated to poorer antibody responses. In addition, antisera to deglycosylated ZP antigens provided poorer contraceptive efficacy *in vitro* [33]. Although now abandoned as a possible means of preventing oophoritis, glycosylation remains a factor to consider when designing synthetic vaccines. For example, Hardy et al. [35] investigated the use of mammalian versus insect expression systems in the production of a recombinant murine ZP3 antigen. Recombinant ZP3 produced in a mammalian expression system caused a transient antifertility effect. However, recombinant ZP3 produced in an insect expression system had no antifertility effect despite evidence of an antibody response, possibly as a result of differences in glycosylation of the protein product. Furthermore, differences in glycosylation may contribute to the reduced immunogenicity associated with some synthetic vaccines, where multiple boosters have been required to maintain adequate antibody titers [36–38].

3.2. Contamination with non-ZP ovarian proteins causes ovarian dysfunction

An early study investigating pZP immunosuppression in cynomolgus monkeys suggested that contamination may have been responsible for the ovarian dysfunction detected, as a result of immune responses directed toward extra-ZP ovarian epitopes [8]. This hypothesis currently remains a commonly advanced argument [39].

Specific binding of anti-ZP antibodies within the developing ovarian follicle, primarily within the ZP, occasionally involving adjacent oolemma or granulosa cells, has been demonstrated in the rabbit [40], cat, dog, horse, and African elephant [41,42]. Similar findings have been demonstrated using immunofluorescence in both primates [43,44] and rabbits [45].

In addition, recombinant and synthetic peptide ZP antigens exclude the possibility of contamination by non-ZP ovarian proteins [46]. A review of the literature describing the use of these vaccines across a number of species includes multiple studies in which synthetic vaccines, including antigens delivered within a virus or as a DNA plasmid vaccine, are nevertheless associated with abnormalities on ovarian histology, including cellular inflammatory infiltration, and/or decreased follicle numbers [38,40,45,47–57]. Although these findings do not completely exclude the possibility of contamination of pZP vaccines as a cause of ovarian dysfunction, these reports suggest that oophoritis or interference with folliculogenesis may be an inherent feature of ZP-based vaccines.

In addition, attempts at purification of pZP to avert ovarian dysfunction have shown limited success in primates [34,58,59]. In the bitch, purified pZP failed to prevent

ovarian pathology, although the observed pathology was milder and seen in association with lower antibody titers than that induced by a crude pZP preparation [60,61].

3.3. Freund's adjuvants are linked to ovarian dysfunction

Freund's complete adjuvant consists of a water-in-oil emulsion, incorporating nonmetabolizable oils (paraffin oil and mannide monooleate) and heat-killed *Mycobacterium tuberculosis* cells. Freund's incomplete adjuvant, generally preferred for booster vaccinations, consists of a similar water-in-oil emulsion without mycobacterial cells. Since its initial description nearly 80 years ago, initially incorporating only paraffin oil and mycobacteria (reviewed in [62]), FCA has enjoyed widespread application in immunological research due to its marked efficacy as an adjuvant. However, side effects associated with FCA, particularly granulomatous injection site reactions, have discouraged its commercial use [63].

Concerns regarding false positive results to tuberculosis testing following the use of FCA led to the development of Freund's modified complete adjuvant (FMCA), incorporating *M. butyricum* instead of *M. tuberculosis*. Although most ZP-based research involving Freund's adjuvants has employed FCA, recent research in horses and African elephants has made use of FMCA. In horses, pZP with FMCA was found to induce antibody titers consistently higher than, although statistically no different to pZP with FCA [64]. Although most researchers likely refer to FCA when discussing Freund's adjuvants, the following discussion encompasses both FCA and FMCA.

A third hypothesis within ZP-based immunosuppressive research implicates the use of Freund's adjuvants in the pathogenesis of ovarian dysfunction. Early studies in primates detected disturbances of ovarian function in control groups administered Freund's adjuvant alone [59,65]. These findings were later contradicted by investigators who reported an absence of ovarian pathology in adjuvant control groups [33,66]. A similar lack of ovarian effects was found in rabbits administered Freund's adjuvant only, in comparison to saline-treated controls [31,32,67].

In two studies reporting a direct comparison between Freund's and alternative adjuvants in combination with pZP, only the inclusion of Freund's adjuvant was associated with ovarian pathology in two monkey species [66,68]. However, the alternative adjuvants induced antibody titers that were lower, and/or diminished faster than antibody titers induced by Freund's adjuvants.

Furthermore, ovarian dysfunction or oophoritis is clearly not limited to the use of Freund's adjuvants, having been reported in association with a number of non-Freund's adjuvants, including alum [8,61]; muramyl dipeptide (MDP) [34,49,69]; MDP with Morris adjuvant [36,70]; squalene with Arlacel-A (and SPLS; primary vaccination) [38,71]; MDP with squalene and Arlacel-A [52]; and CP20,961 [60,72].

3.4. Cytotoxic T-cells cause oophoritis and ovarian dysfunction

The adaptive immune system is mediated largely by T-helper and T-cytotoxic lymphocytes. T-helper (CD4⁺)

cells recognize soluble and particulate antigens presented by professional antigen presenting cells, in association with major histocompatibility class (MHC) II molecules. In the classical endogenous pathway, intracellular-derived antigens presented in association with MHC class I molecules are recognized by cytotoxic T-lymphocytes (CD8⁺; CTL). An alternative pathway has been suggested, whereby dendritic cells present extracellular antigens, which could include ZP antigens, via MHC I [73]. Although T-helper cells play a role in the production of antibody by B-lymphocytes, CTL produce a direct cytotoxic effect. Other cells capable of cytotoxicity include members of the innate immune system such as macrophages and natural killer cells.

Various authors have suggested that a CTL response may be involved in the development of oophoritis during pZP immunocontraception [1,74–77], often citing Rhim et al. [47], Lou et al. [78], and Lou et al. [79] despite no reference to CTL within the cited studies. Few studies have actively attempted to identify CTL responses during ZP-based immunocontraception. In mice, the infiltration of CD4⁺ and CD8⁺ T-lymphocytes was demonstrated in ovarian sections using immunohistochemistry, following infection by a murine cytomegalovirus expressing mZP3 [56]. However, the presentation of the antigen by a virus provides a direct link to the classical MHC I T-cell pathway, likely contributing to the CD8⁺ response. Further work classifying the immune response to ZP vaccines, including the potential role of CTL in the pathogenesis of ovarian dysfunction, is warranted.

What does seem clear is the link between ovarian pathology, a helper (CD4⁺) T-cell-mediated immune response and an antibody response. Rhim et al. [47] showed that the adoptive transfer of CD4⁺ T-cells induced an oophoritis despite the absence of any detectable antibody response. This oophoritis was described as interstitial, excluding developing follicles, and did not appear to interfere with ovarian function in mice [50]. In a subsequent study, Lou et al. [51] reported that the presence of anti-ZP antibodies redirected the cell-mediated inflammatory response from the ovarian interstitium to the developing follicles, resulting in profound interference with ovarian function [51].

Furthermore, Lloyd et al. [57] found that immunoglobulin-deficient mice, incapable of mounting an antibody response but otherwise capable of normal adaptive immune responses, showed neither decreased fertility nor abnormalities on electron microscopy of ovarian sections following infection by a recombinant murine cytomegalovirus expressing murine ZP3 [57].

Immunocompetent mice infected with the same virus showed decreased fertility and fecundity, and evidence of abnormal ZP formation and vacuolization of oocytes on electron microscopy of ovarian sections. Although it could be argued that a recombinant ZP-expressing virus would be expected to show differences in immune response mechanisms to the conventional pZP vaccine, this study suggested a pivotal role for antibodies in the development of oophoritis during anti-ZP immunocontraception.

Taken together, these studies demonstrate the roles of both CD4⁺ T-lymphocytes and antibodies, in unison, in ZP vaccine-induced interference with ovarian function in

mice. Whether or not a similar dynamic plays a role in ovarian dysfunction in other species warrants further research.

3.5. Ovarian dysfunction is species-specific

Finally, ovarian dysfunction during ZP-based immunocontraception, characterized as a cellular oophoritis and/or interference with folliculogenesis has been described as a species-specific complication. Antibody responses to ZP-based vaccines show some variation between individuals within a species, and likely represent variation in the overall immune response to vaccination. Reasons for individual variation in immune response include factors influencing the physiological status of the individual during or after vaccine administration, including physiological stress, nutritional status, and the presence of concurrent systemic conditions. In addition, genetic differences in immune response to immunocontraceptive vaccines have been suggested to play a role [80].

Significant species differences in terms of the endurance of antibody titers have also been well documented. To illustrate this, Dall sheep maintained significant antibody titers for over 3 years following an initial pZP vaccination regime (primary plus booster) [81], whereas Muntjac deer required biannual boosters to maintain antibody levels [82].

A review of nine species groups (mice, rabbits, nonhuman primates, dogs, cats, sheep, deer, horses, and African elephants), arguably the most studied species to date in terms of ZP-based immunocontraception, revealed evidence of abnormal cyclicity in each one of these species following treatment, with the notable exception of the cat and African elephant (Table 1). Cats are refractory to pZP, showing neither cyclic disturbances nor any effect on fertility [92,93]. One study examining estrous cyclicity during pZP immunocontraception (2–3 years after the start of treatment) in African elephants included evidence of anestrus in a proportion of treated cows [106]. Although this effect was possibly ascribable to seasonal effects, the lack of controls complicates any definitive conclusions.

In horses, ovarian inactivity following pZP treatment, certainly in the short term, appears to have been undetected for over 20 years. An initial study of pZP in horses found no significant evidence of abnormal ovarian function following short-term treatment [101]. Similarly, Powell et al. [102] found no differences in estrous cycle characteristics between pZP-treated and untreated mares. Although researchers noted depressed excretory steroid levels and slower reversal of infertility following prolonged (≥ 3 years) treatment [103,104], the mechanism of action of pZP immunocontraception in mares as an antibody-based interference with conception at the level of the oocyte remained until recently the generally accepted dogma in this species. Bechert et al. [105] compared two long-acting pZP vaccine formulations in mares. Treated mares demonstrated lower serum progesterone levels, smaller ovaries, and fewer follicles than control mares; 93% of treated mares ceased estrous cyclicity within 4 months of treatment. Similarly, six of seven mares treated with two doses of the conventional pZP vaccine demonstrated

Table 1

Overview of the literature reporting on ovarian dysfunction during zona pellucida-based immunocontraception.

Species	No evidence of ovarian dysfunction detected ^a	Evidence of ovarian dysfunction detected ^a	Inconclusive evidence of ovarian dysfunction detected	Fertility rates ^b reported for the relevant vaccine formulation, within the cited studies
Mice	[74,83–85]	[48,50,53,55–57,86,87]		9%–100% 0%–100%
Rabbits	[7,54]	[7,31,32,40,45,54,88]		100% 0%–30%
Nonhuman primates	[36,37,49,68,70,89–91]	[8,33,34,36,49,52,58,59,68–71,89]		25%–100% 0%–50%
Cats	[92–94]			16% ^c –100%
Dogs	[38,60,72]	[60,61,72]		100% 0%–34%
Sheep	[95]	[95]		100% 0%
White-tailed deer	[96–99]		[100]	0%–95% 11%
Horses	[39,101,102]	[39,103–105]		0%–57% 0%–8%
African elephants			[106]	0%

^a Evidence of ovarian dysfunction includes histological evidence of oophoritis or decreased follicle numbers, or behavioral or hormonal evidence of abnormal estrous or menstrual cyclicity.

^b Expressed as a proportion of the control group, where control groups are available.

^c Not statistically different to control group.

periods of anestrus posttreatment, characterized by baseline serum progesterone levels and a lack of follicular development [39]. In part, the discrepancy between the latter two studies and other, earlier studies might be explained by differences in antigen dose rates (100 µg [39] and 200 µg of a single administered formulation [105], compared with 65 µg [107]). In addition, most trials observed feral horses with associated constraints on clinical monitoring [27].

In white-tailed deer, ongoing estrous behaviors during pZP immunocontraception have been reported by a number of studies [96–98]. Yet, a study showing depressed progesterone levels following pZP treatment suggests that ovarian inactivity may nevertheless be a feature of pZP immunocontraception in this species [100]. The dose of pZP administered in the latter study (300–500 µg) was higher than that reported in other deer studies (65 µg; [96,97,108]). This, however, suggests that ovarian suppression is dose-dependent, rather than species-dependent. In a later study, fewer normal secondary follicles were detected in recently revaccinated does in comparison to controls and does vaccinated 2 years previously, although sample sizes were low [99]. A third study anticipated observing increased mating behavior in pZP-treated does but found no differences in behaviors, including estrus and dominance behaviors, between treated and untreated groups of fallow deer [109]. Given the similar difficulties in estrus detection between feral horses and deer, further study of the ovarian effects of pZP in deer is warranted.

In summary, ovarian suppression may be an inherent feature of effective ZP-based immunocontraception, associated with the generation of elevated antibody titers over a prolonged period of time and contributing to the vaccine's contraceptive effect, rather than a species-specific response. However, further research to confirm this hypothesis is indicated.

4. The link between antibody titers, contraceptive efficacy, and ovarian dysfunction

Although not an absolute rule, a recurring theme throughout the literature is the apparent link between the immunogenicity of a ZP vaccine with the antifertility capabilities of the vaccine and the presence of ovarian dysfunction. In the dog [60], sheep [95], and rabbit [7,32,67], ovarian pathology and contraceptive efficacy showed a direct correlation when comparing two or more alternative formulations. Similarly, in primates, significant antifertility efficacy is associated with evidence of ovarian dysfunction [70,71,89]; with the converse also demonstrated (poor antifertility effect with no evidence of ovarian effects) [90,91]. Finally, in the cat, the absence of contraceptive efficacy accompanies a consistent lack of ovarian effects [92–94].

These observations support the hypothesis that ovarian dysfunction is an inherent feature, or at least a component, of the mechanism of action of ZP-based vaccines [46,60]. To the best of the authors' knowledge, no vaccine formulation, whether native or synthetic, has yet achieved near complete contraceptive efficacy without some evidence of ovarian effects following further study. Attempts at vaccine design, aimed at the inclusion of B-cell epitopes while excluding putative oophoritogenic T-cell epitopes, have indeed shown decreased ovarian effects but, again, limited contraceptive success [70,79,83–85]. To further complicate matters, ovarian effects may inadvertently be missed. Periods of ovarian dysfunction may be transient and their detection consequently dependant on the timing of sampling interventions for ovarian histology in relation to vaccine administration [61]. In addition, the difficulty associated with the detection of cellular inflammatory infiltrates in ovarian tissue sections under light microscopy has been demonstrated. In an initial study, no

inflammatory infiltrate was detected in ovaries observed under light microscopy [86]. However, in a follow-up study using the same population and methodology, lymphocytic infiltration of the ovary was detected using immunohistochemistry [56]. The value of immunohistochemistry over conventional histopathology in detecting oophoritis was confirmed by Bagavant et al. [52].

Possible causes of interrupted folliculogenesis, leading to ovarian atrophy and anestrus, include immune-mediated follicular destruction and interference with normal follicle development [60]. Destruction of oocytes and follicles could occur as a result of antibody-mediated complement activation. Few studies have investigated the role of complement in ZP-based immunocontraception. In an early study, mice were treated with solubilized hamster ZP followed by superovulation and the flushing of oocytes from the oviducts. Antibody and complement binding to oocytes was assessed using fluorescein-conjugated rabbit anti-mouse IgG, and rabbit anti-mouse complement C3 followed by fluorescein-conjugated goat anti-rabbit IgG, respectively. In both tests, bright immunofluorescence was detected on oocytes from immunized animals, with no immunofluorescence detected on control oocytes [110]. In contrast, no complement binding was visualized in ovarian sections from immunocontracepted dogs; however, the small sample size and the lack of a positive control for the complement-binding assay were major limitations to the study [61].

Alternatively, or additionally, the immune response might alter ZP structure and function in developing follicles, affecting communication between the growing oocyte and its surrounding granulosa cells [88]. This scenario is mimicked in mice lacking the functional gene for connexin 37, one of a family of proteins involved in intercellular communication between oocytes and granulosa cells. Folliculogenesis is inhibited in connexin 37-deficient mice. In addition, ovaries show abnormal accumulations of luteinized tissue [111], possibly resembling that described in ZP-treated mice [87], rabbits [40,45,67], and primates [8,36]. This mechanism may be particularly plausible in the dog, where pZP treatment caused the formation of ovarian cysts associated with prolonged estrogen secretion [61].

4.1. Conclusions

The advantages of ZP-based immunocontraception include its efficacy as a contraceptive agent in many species, safety during pregnancy, reversibility (at least in the short term), and freedom from major side effects [112]. Apart from ovarian senescence, extensive macroscopic and microscopic postmortem examinations of the major organ systems in pZP-treated mares failed to reveal any pathology that could be linked solely to pZP [105]. The vaccine can be remotely delivered, important for use in feral species [113]. Furthermore, the protein nature of the vaccine precludes its entry into the food chain [29]. Permanent sterility, if indeed this could be induced by pZP vaccination with a degree of reliability, may be a desirable side effect in certain so-called pest species [46]. Importantly, pZP vaccination appears to show minimal adverse effects on animal

welfare, particularly when compared to alternative means of population control such as culling [28]. No adverse behavioral or social effects were detected in a long-term study of pZP immunocontraception in elephants [114]. In the feral horse, pZP immunocontraception has been associated with both enhanced longevity and body condition [115]. Furthermore, the vaccine showed few significant effects on social behaviors and time budgets in horses [116,117]; one study reported an increased frequency of reproductive behaviors and a lengthening of the breeding season, which should be considered during the management of immunocontracepted herds [118]. Furthermore, studies to confirm the lack of effects on behavior and welfare are warranted.

In most species studied, annual and sometimes biennial boosters are required if the contraceptive effect is to be maintained. From a practical point of view, particularly in free-ranging species like the African elephant, this requires considerable time and resource investment. A long-acting pZP formulation which induces antibodies titers that are maintained for 2 years or longer after a single treatment would be a major advantage. A series of studies investigated the use of lactide-glycolide polymers to form pellets incorporating pZP. The release delay, depending on the ratios of lactide and glycolide in the pellets, was either 1, 3 or 12 months. Hand-injected pellets in combination with a conventional primary dose of pZP resulted in antibody titers that were sustained at contraceptive levels for 21 to 22 months. The contraceptive effect was evident for 2 years; a result which was promising [119]. An entirely different approach to achieve long-lasting antibody titers and thus contraception has been the use of a liposomal formulation consisting of lecithin and cholesterol and emulsified with FMCA or lyophilized and then reconstituted with FMCA [105]. Both formulations produced sustained antibody titers in mares (monitored for 22 weeks) and induced cyclic changes alluded to earlier. Moreover, the latter formulation maintained antibody titers for at least 7 years in African elephant cows [120]. The emulsified formulation has previously been tested in gray seals [121] and deer [122], both producing infertility that was maintained over 3 years or longer. Bechert et al. [105] proposed a number of possible mechanisms that may be responsible individually or, more likely, collectively for the sustained antibody titers. These were the sustained release of antigen from the injection site, increased production of long-lived plasma cells in the bone marrow, and mobilization of follicular dendritic cells within draining lymph nodes. Self-boosting by zona capsules in developing follicles was also mentioned but, in the absence of local adjuvant, seemed unlikely and would also apply to other pZP formulations. Although these results are extremely encouraging, the longer term effects on ovarian function have not been investigated satisfactorily. Reversibility and thus lack of long-term ovarian effects in species like the African elephant are an extremely important feature of this treatment.

The commercial availability of serum anti-Müllerian hormone (AMH) assays may provide access to a novel method of monitoring the ovarian effects of immunocontraception in females. This hormone, secreted by the

granulosa cells of, primarily, preantral, and early antral follicles, has been correlated to antral follicle counts and ovarian reserve in mice [123], cattle [124], and women [125]. Recently, AMH levels measured in mares during the course of 1 year of their immunocontraception with either pZP or GnRH vaccines were compared. Although the GnRH vaccine had little effect on AMH levels, pZP suppressed serum AMH in the short term (Joonè et. al., in preparation). Given the proposed link between low AMH levels and ovarian suppression in these mares, AMH may prove useful in species not amenable to direct clinical examinations of their reproductive organs. Likewise, pZP vaccination may provide an exciting tool for the study of AMH and its relationship to follicular dynamics and the ovarian reserve.

In conclusion, this review suggests a re-evaluation of dogmas that have emerged within the field of ZP-based immunocontraception, regarding these vaccines' ovarian effects. The suggestion that ovarian suppression could be an inherent feature of effective ZP-based immunocontraception, across all species, requires further investigation. Nevertheless, pZP remains a valuable, practical, and humane means of population management with application in a number of mammalian species.

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