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Ovarian dysfunction associated with zona pellucida-based immunocontraceptive vaccines

Carolynne J. Joonè[a,b,c], 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.

[c] Corresponding author. Email: carolynne.joone@jcu.edu.au. Address: School of Veterinary Sciences, College of Public Health, Medical and Veterinary Sciences, James Cook University, 1 James Cook Drive, Townsville, Queensland, 4811, Australia.

Abstract

Despite more than forty 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 anti-ovarian 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.

Keywords: porcine zona pellucida, contraception, anestrus, oophoritis, Freund's adjuvants, cytotoxic T-lymphocyte, glycosylation

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 aimed at humans, initial

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

This review re-visits initial studies describing ovarian tissue, oocytes or ZP as immunological agents, upon 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 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 fertilisation processes in the sea urchin [10] and frog [11] encouraged interest in the mammalian oocyte and ovary as putative anti-fertility antigens. Initial studies demonstrated the existence of organ-specific antigens in the guinea pig ovary and testis [12, 13]. Early immunofluorescence studies further localised 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 (FIA), 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. Super-ovulated hamster eggs exposed to rabbit anti-ovary 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].

Anti-ovarian antiserum, added to hamster ova prior to exposure to homologous spermatozoa *in vitro*, interfered with sperm-oocyte binding. None of 170 pre-treated oocytes was penetrated by spermatozoa, in comparison to nearly half of 58 control oocytes [17]. The use of homogenised 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 due to the specificity of anti-ZP antibodies [17].

Jilek et al. [3], using mice passively pre-immunized 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 anti-hamster ovary antiserum precipitated the ZP of pre-ovulatory 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, anti-oocyte 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 pre-incubation with anti-ZP antiserum had no effect on early embryonic development and zona hatching *in vitro*, nor implantation and further development of pre-treated embryos transferred to pseudopregnant recipients, despite visible precipitation of the zonae [22]. Similarly, mice passively immunized with anti-ZP antiserum two 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 peri-ovulatory oocyte. If so, oestrous cyclicity and reproductive behaviours should remain unaffected following vaccination. This feature of pZP immunocontraception has been an important, though 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

131 species may play a role in the species-specificity of sperm-zona binding [29]. Chemical de-
132 glycosylation of pZP3 was found to decrease its antigenicity and immunogenicity; precipitation of
133 ZP *in vitro* by the relevant antisera was more superficial than precipitation produced using
134 antisera to glycosylated pZP3 [30]. Consequently early workers in the field considered the de-
135 glycosylation of ZP antigens in order to negate pZP's oophoritogenic effects. In rabbits, the
136 degree of glycosylation of pZP was found to correlate directly with the degree of interference
137 with folliculogenesis, ovulation and oestrous cyclicity [31, 32]. Researchers hoped that qualitative,
138 rather than purely quantitative, differences in the immune response to de-glycosylated versus
139 glycosylated ZP antigens were responsible for the differences in oophoritogenic effect [32].
140 Subsequent trials in marmoset monkeys [33] and baboons [34] found that immunization with de-
141 glycosylated pZP3 did indeed result in lower levels of ovarian dysfunction compared to
142 glycosylated pZP3; a result that correlated to poorer antibody responses. In addition, antisera to
143 de-glycosylated ZP antigens provided poorer contraceptive efficacy *in vitro* [33]. Although now
144 abandoned as a possible means of preventing oophoritis, glycosylation remains a factor to
145 consider when designing synthetic vaccines. For example, Hardy et al. [35] investigated the use of
146 mammalian versus insect expression systems in the production of a recombinant murine ZP3
147 antigen. Recombinant ZP3 produced in a mammalian expression system caused a transient
148 antifertility effect. However, recombinant ZP3 produced in an insect expression system had no
149 anti-fertility effect despite evidence of an antibody response, possibly as a result of differences in
150 glycosylation of the protein product. Furthermore, differences in glycosylation may contribute to
151 the reduced immunogenicity associated with some synthetic vaccines, where multiple boosters
152 have been required to maintain adequate antibody titres [36-38].
153
154 **3.2. "Contamination with non-ZP ovarian proteins causes ovarian dysfunction"**
155

156 An early study investigating pZP immunocontraception in cynomolgus monkeys suggested that
157 contamination may have been responsible for the ovarian dysfunction detected, as a result of
158 immune responses directed towards extra-ZP ovarian epitopes [8]. This hypothesis currently
159 remains a commonly advanced argument [39].
160
161 Specific binding of anti-ZP antibodies within the developing ovarian follicle, primarily within the
162 ZP, occasionally involving adjacent oolemma or granulosa cells, has been demonstrated in the
163 rabbit [40] cat, dog, horse and African elephant [41, 42]. Similar findings have been demonstrated
164 using immunofluorescence in both primates [43, 44] and rabbits [45].
165
166 In addition, recombinant and synthetic peptide ZP antigens exclude the possibility of
167 contamination by non-ZP ovarian proteins [46]. A review of the literature describing the use of
168 these vaccines across a number of species includes multiple studies in which synthetic vaccines,
169 including antigens delivered within a virus or as a DNA plasmid vaccine, are nevertheless
170 associated with abnormalities on ovarian histology, including cellular inflammatory infiltration
171 and, or decreased follicle numbers [38, 40, 45, 47-57]. Although these findings do not completely
172 exclude the possibility of contamination of pZP vaccines as a cause of ovarian dysfunction, these
173 reports suggest that oophoritis or interference with folliculogenesis may be an inherent feature of
174 ZP-based vaccines.
175
176 In addition, attempts at purification of pZP to avert ovarian dysfunction has shown limited
177 success in primates [34, 58, 59]. In the bitch, purified pZP failed to prevent ovarian pathology,
178 although the observed pathology was milder and seen in association with lower antibody titres
179 than that induced by a crude pZP preparation [60, 61].
180
181 **3.3. "Freund's adjuvants are linked to ovarian dysfunction"**

Freund's complete adjuvant (FCA) consists of a water-in-oil emulsion, incorporating non-metabolizable oils (paraffin oil and mannide monooleate) and heat-killed *Mycobacterium tuberculosis* cells. Freund's incomplete adjuvant (FIA), generally preferred for booster vaccinations, consists of a similar water-in-oil emulsion without mycobacterial cells. Since its initial description nearly eighty 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*. While 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 titres 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 immunocontraceptive 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 titres that were lower, and, or diminished faster than antibody titres 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 recognise 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 recognised 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]. While 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 vacuolisation 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, characterised 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 titres have also been well documented. To illustrate this, Dall sheep maintained significant antibody titres for over three years following an initial pZP vaccination regime (primary plus booster) [81], whereas Muntjac deer required bi-annual boosters to maintain antibody levels [82].

A review of nine species groups (mice, rabbits, non-human primates, dogs, cats, sheep, deer, horses and African elephants), arguably the most studied species to date in terms of ZP-based

285 immunocontraception, revealed evidence of abnormal cyclicity in each one of these species
286 following treatment, with the notable exception of the cat and African elephant (Table 1). Cats
287 are refractory to pZP, showing neither cyclic disturbances nor any effect on fertility [83, 84]. One
288 study examining oestrous cyclicity during pZP immunocontraception (two to three years after the
289 start of treatment) in African elephants included evidence of anoestrus in a proportion of treated
290 cows [85]. Although this effect was possibly ascribable to seasonal effects, the lack of controls
291 complicates any definitive conclusions.
292
293 In horses, ovarian inactivity following pZP treatment, certainly in the short term, appears to have
294 been undetected for over twenty years. An initial study of pZP in horses found no significant
295 evidence of abnormal ovarian function following short-term treatment [86]. Similarly, Powell et
296 al. [87] found no differences in oestrous cycle characteristics between pZP-treated and untreated
297 mares. Although researchers noted depressed excretory steroid levels and slower reversal of
298 infertility following prolonged ($\geq$ 3 years) treatment [88, 89], the mechanism of action of pZP
299 immunocontraception in mares as an antibody-based interference with conception at the level of
300 the oocyte remained until recently the generally-accepted dogma in this species. Bechert et al.
301 [90] compared two long-acting pZP vaccine formulations in mares. Treated mares demonstrated
302 lower serum progesterone levels, smaller ovaries and fewer follicles than control mares; 93% of
303 treated mares ceased oestrous cyclicity within four months of treatment. Similarly, six of seven
304 mares treated with two doses of the conventional pZP vaccine demonstrated periods of
305 anoestrus post-treatment, characterised by baseline serum progesterone levels and a lack of
306 follicular development [39]. In part, the discrepancy between the latter two studies and other,
307 earlier studies might be explained by differences in antigen dose rates (100 µg [39] and 200 µg of
308 a single administered formulation [90], compared to 65 µg [91]). In addition, most trials observed
309 feral horses with associated constraints on clinical monitoring [27].
310

311 In white-tailed deer, ongoing oestrous behaviours during pZP immunocontraception has been
312 reported by a number of studies [92-94]. Yet, a study showing depressed progesterone levels
313 following pZP treatment suggests that ovarian inactivity may nevertheless be a feature of pZP
314 immunocontraception in this species [95]. The dose of pZP administered in the latter study (300
315 to 500 µg) was higher than that reported in other deer studies (65µg; [92, 93, 96]). This, however,
316 suggests that ovarian suppression is dose-dependent, rather than species-dependent. In a later
317 study, fewer normal secondary follicles were detected in recently re-vaccinated does in
318 comparison to controls and does vaccinated two years previously, although sample sizes were
319 low [97]. A third study anticipated observing increased mating behaviour in pZP-treated does but
320 found no differences in behaviours, including oestrus and dominance behaviours, between
321 treated and untreated groups of fallow deer [98]. Given the similar difficulties in oestrus
322 detection between feral horses and deer, further study of the ovarian effects of pZP in deer is
323 warranted.
324
325 In summary, ovarian suppression may be an inherent feature of effective ZP-based
326 immunocontraception, associated with the generation of elevated antibody titres over a
327 prolonged period of time and contributing to the vaccine's contraceptive effect, rather than a
328 species-specific response. However, further research to confirm this hypothesis is indicated.
329
330 **4. The link between antibody titres, contraceptive efficacy and ovarian dysfunction**
331
332 Although not an absolute rule, a recurring theme throughout the literature is the apparent link
333 between the immunogenicity of a ZP vaccine with the antifertility capabilities of the vaccine and
334 the presence of ovarian dysfunction. In the dog [60], sheep [99] and rabbit [7, 32, 67], ovarian
335 pathology and contraceptive efficacy showed a direct correlation when comparing two or more
336 alternative formulations. Similarly, in primates, significant antifertility efficacy is associated with

evidence of ovarian dysfunction [70, 71, 100]; with the converse also demonstrated (poor antifertility effect with no evidence of ovarian effects) [101, 102]. Finally, in the cat, the absence of contraceptive efficacy accompanies a consistent lack of ovarian effects [83, 84, 103].

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, 104-106]. 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 [107]. 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 anoestrous, 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

363 followed by superovulation and the flushing of oocytes from the oviducts. Antibody and
364 complement binding to oocytes was assessed using fluorescein-conjugated rabbit anti-mouse IgG,
365 and rabbit anti-mouse complement C3 followed by fluorescein conjugated goat anti-rabbit IgG,
366 respectively. In both tests, bright immunofluorescence was detected on oocytes from immunised
367 animals, with no immunofluorescence detected on control oocytes [108]. In contrast, no
368 complement binding was visualised in ovarian sections from immunocontracepted dogs, however
369 the small sample size and the lack of a positive control for the complement-binding assay were
370 major limitations to the study [61].
371
372 Alternatively, or additionally, the immune response might alter ZP structure and function in
373 developing follicles, affecting communication between the growing oocyte and its surrounding
374 granulosa cells [109]. This scenario is mimicked in mice lacking the functional gene for connexin
375 37, one of a family of proteins involved in intercellular communication between oocytes and
376 granulosa cells. Folliculogenesis is inhibited in connexion 37-deficient mice. In addition, ovaries
377 show abnormal accumulations of luteinised tissue [110], possibly resembling that described in ZP-
378 treated mice [111], rabbits [40, 45, 67] and primates [8, 36]. This mechanism may be particularly
379 plausible in the dog, where pZP treatment caused the formation of ovarian cysts associated with
380 prolonged oestrogen secretion [61].
381
382 **5. Concluding remarks**
383
384 The advantages of ZP-based immunocontraception include its efficacy as a contraceptive agent in
385 many species, safety during pregnancy, reversibility (at least in the short term) and freedom from
386 major side effects [112]. Apart from ovarian senescence, extensive macroscopic and microscopic
387 post mortem examinations of the major organ systems in pZP-treated mares failed to reveal any
388 pathology that could be linked solely to pZP [90]. The vaccine can be remotely delivered,

389 important for use in feral species [113]. Furthermore, the protein nature of the vaccine precludes
390 its entry into the food chain [29]. Permanent sterility, if indeed this could be induced by pZP
391 vaccination with a degree of reliability, may be a desirable side effect in certain so-called pest
392 species [46]. Importantly, pZP vaccination appears to show minimal adverse effects on animal
393 welfare, particularly when compared to alternative means of population control such as culling
394 [28]. No adverse behavioural or social effects were detected in a long-term study of pZP
395 immunocontraception in elephants [114]. In the feral horse, pZP immunocontraception has been
396 associated with both enhanced longevity and body condition [115]. Furthermore, the vaccine
397 showed few significant effects on social behaviours and time budgets in horses [116, 117]; one
398 study reported an increased frequency of reproductive behaviours and a lengthening of the
399 breeding season, which should be considered during the management of immunocontracepted
400 herds [118]. Further studies to confirm the lack of effects on behaviour and welfare are
401 warranted.
402
403 In most species studied, annual and sometimes biennial boosters are required if the
404 contraceptive effect is to be maintained. From a practical point of view, particularly in free-
405 ranging species like the African elephant, this requires considerable time and resource
406 investment. A long-acting pZP formulation which induces antibodies titres that are maintained for
407 two years or longer after a single treatment would be a major advantage. A series of studies
408 investigated the use of lactide-glycolide polymers to form pellets incorporating pZP. The release
409 delay, depending on the ratios of lactide and glycolide in the pellets, was either one, three or 12
410 months. Hand-injected pellets in combination with a conventional primary dose of pZP resulted in
411 antibody titres that were sustained at contraceptive levels for 21 to 22 months. The contraceptive
412 effect was evident for two years; a result which was promising [119]. An entirely different
413 approach to achieve long-lasting antibody titres and thus contraception has been the use of a
414 liposomal formulation consisting of lecithin and cholesterol and emulsified with FMCA, or

415 lyophilised and then reconstituted with FCMA [90]. Both formulations produced sustained
416 antibody titres in mares (monitored for 22 weeks) and induced cyclic changes alluded to earlier.
417 Moreover, the latter formulation maintained antibody titres for at least seven years in African
418 elephant cows [120]. The emulsified formulation has previously been tested in grey seals [121]
419 and deer [122], both producing infertility that was maintained over three years or longer. Bechert
420 et al. [90] proposed a number of possible mechanisms that may be responsible individually or,
421 more likely, collectively for the sustained antibody titres. These were sustained release of antigen
422 from the injection site, increased production of long-lived plasma cells in the bone marrow and
423 mobilisation of follicular dendritic cells within draining lymph nodes. Self-boosting by zona
424 capsules in developing follicles was also mentioned but, in the absence of local adjuvant, seemed
425 unlikely and would also apply to other pZP formulations. While these results are extremely
426 encouraging, the longer term effects on ovarian function have not been investigated
427 satisfactorily. Reversibility and thus lack of long-term ovarian effects in species like the African
428 elephant is an extremely important feature of this treatment.
429
430 The commercial availability of serum anti-Müllerian hormone (AMH) assays may provide access to
431 a novel method of monitoring the ovarian effects of immunocontraception in females. This
432 hormone, secreted by the granulosa cells of, primarily, preantral and early antral follicles, has
433 been correlated to antral follicle counts and ovarian reserve in mice [123], cattle [124] and
434 women [125]. Recently, AMH levels measured in mares during the course of one year of their
435 immunocontraception with either pZP or GnRH vaccines were compared. While the GnRH vaccine
436 had little effect on AMH levels, pZP suppressed serum AMH in the short term (Joonè et. al., *in*
437 *preparation*). Given the proposed link between low AMH levels and ovarian suppression in these
438 mares, AMH may prove useful in species not amenable to direct clinical examinations of their
439 reproductive organs. Likewise, pZP vaccination may provide an exciting tool for the study of AMH
440 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.

References

[1] Prasad S, Skinner S, Carino C, Wang N, Cartwright J, Dunbar B. Structure and function of the proteins of the mammalian Zona pellucida. Cells Tissues Organs 1999;166:148-64.

[2] Li R, Albertini DF. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. Nat Rev Mol Cell Bio 2013;14:141-52.

[3] Jilek F, Pavlok A. Antibodies against mouse ovaries and their effect on fertilization in vitro and in vivo in the mouse. J Reprod Fert 1975;42:377-80.

[4] Sacco AG. Antigenic cross-reactivity between human and pig zona pellucida. Biol Reprod 1977;16:164-73.

[5] Sacco A, Yurewicz E, Subramanian M, DeMayo F. Zona pellucida composition: species cross reactivity and contraceptive potential of antiserum to a purified pig zona antigen (PPZA). Biol Reprod 1981;25:997-1008.

[6] Kirkpatrick JF, Rowan A, Lamberski N, Wallace R, Frank K, Lyda R. The practical side of immunocontraception: zona proteins and wildlife. J Reprod Immunol 2009;83:151-7.

[7] Wood DM, Liu C, Dunbar BS. Effect of alloimmunization and heteroimmunization with zonae pellucidae on fertility in rabbits. Biol Reprod 1981;25:439-50.

[8] Gulyas BJ, Gwatkin RB, Yuan LC. Active immunization of cynomolgus monkeys (*Macaca fascicularis*) with porcine zonae pellucidae. Gamete Res 1983;7:299-307.

[9] Tyler A. The fourth Oliver Bird lecture: Approaches to the control of fertility based on immunological phenomena. J Reprod Fert 1961;2:473-506.

[10] Perlmann P. Immunochemical analysis of the surface of the sea urchin egg-an approach to the study of fertilization. Experientia 1959;15:41-52.

[11] Nace GW, Lavin LH. Heterosynthesis and autosynthesis in the early stages of anuran development. Am Zool 1963:193-207.

[12] Porter C. Ovarian antibodies in female guinea pigs. Int J Fertil 1965;10:257.

[13] Porter C, Highfill D, Winovich R. Guinea pig ovary and testis: demonstration of common gonad specific antigens in the ovary and testis. Int J Fertil 1969;15:171-6.

[14] Porter C, Highfill D, Winovich R. Guinea pig ovary and testis: localization of common gonad specific antigens. Int J Fertil 1970;15:177.

[15] Ownby CL, Shivers CA. Antigens of the hamster ovary and effects of anti-ovary serum on eggs. Biol Reprod 1972;6:310-8.

[16] Sacco AG, Shivers CA. Effects of reproductive tissue-specific antisera on rabbit eggs. Biol Reprod 1973;8:481-90.

[17] Shivers C, Dudkiewicz A, Franklin L, Fussell E. Inhibition of sperm-egg interaction by specific antibody. Science 1972;178:1211-3.

[18] Tsunoda Y. Inhibitory effect of anti-mouse egg serum on fertilization in vitro and in vivo in the mouse. J Reprod Fert 1977;50:353-5.

[19] Dudkiewicz A, Noske I, Shivers A. Inhibition of implantation in the golden hamster by zona-precipitating antibody. Fertil Steril 1975;26:686-94.

[20] Shivers CA. Immunological interference with fertilization. Acta Endocrinol 1975;80:S223-S44.

[21] Tsunoda Y, Chang M. Effect of antisera against eggs and zonae pellucidae on fertilization and development of mouse eggs in vivo and in culture. J Reprod Fert 1978;54:233-7.

[22] Tsunoda Y, Whittingham D. Lack of effect of zona antibody on the development of mouse embryos in vivo and in vitro. J Reprod Fert 1982;66:585-9.

493 [23] East IJ, Mattison DR, Dean J. Monoclonal antibodies to the major protein of the murine zona
494 pellucida: effects on fertilization and early development. Dev Biol 1984;104:49-56.
495 [24] Fayrer-Hosken RA, Grobler D, Van Altena JJ, Bertschinger HJ, Kirkpatrick JF.
496 Immunocontraception of African elephants. Nature 2000;407:149.
497 [25] Kerley GI, Shrader AM. Elephant contraception: silver bullet or a potentially bitter pill?:
498 commentary. S Afr J Sci 2007;103:181-2.
499 [26] Perdok AA, de Boer WF, Stout TAE. Prospects for managing African elephant population
500 growth by immunocontraception: a review. Pachyderm 2007:97-107.
501 [27] Nuñez C. Management of wild horses with porcine zona pellucida: history, consequences,
502 and future strategies. In: Leffhalm JE, editor. Horses: Biology, Domestication, and Human
503 Interactions. New York: Nova Science Publishers Inc; 2009.
504 [28] Swegen A, Aitken RJ. Prospects for immunocontraception in feral horse population control:
505 exploring novel targets for an equine fertility vaccine. Reprod Fert Develop 2014.
506 [29] Barber MR, Fayrer-Hosken RA. Possible mechanisms of mammalian immunocontraception. J
507 Reprod Immunol 2000;46:103-24.
508 [30] Sacco A, Yurewicz E, Subramanian M. Carbohydrate influences the immunogenic and
509 antigenic characteristics of the ZP3 macromolecule (Mr 55 000) of the pig zona pellucida. J Reprod
510 Fert 1986;76:575-85.
511 [31] Keenan J, Sacco A, Subramanian M, Kruger M, Yurewicz E, Moghissi K. Endocrine response in
512 rabbits immunized with native versus deglycosylated porcine zona pellucida antigens. Biol Reprod
513 1991;44:150-6.
514 [32] Jones GR, Sacco AG, Subramanian MG, Kruger M, Zhang S, Yurewicz EC, et al. Histology of
515 ovaries of female rabbits immunized with deglycosylated zona pellucida macromolecules of pigs. J
516 Reprod Fert 1992;95:513-25.

517 [33] Paterson M, Koothan PT, Morris KD, O'Byrne KT, Braude P, Williams A, et al. Analysis of the
518 contraceptive potential of antibodies against native and deglycosylated porcine ZP3 in vivo and in
519 vitro. Biol Reprod 1992;46:523-34.
520 [34] Dunbar B, Lo C, Powell J, Stevens V. Use of a synthetic peptide adjuvant for the immunization
521 of baboons with denatured and deglycosylated pig zona pellucida glycoproteins. Fertil Steril
522 1989;52:311-8.
523 [35] Hardy CM, Ten Have JF, Pekin J, Beaton S, Jackson RJ, Clydesdale G. Contraceptive responses
524 of mice immunized with purified recombinant mouse zona pellucida subunit 3 (mZP3) proteins.
525 Reproduction 2003;126:49-59.
526 [36] Paterson M, Wilson MR, Morris KD, van Duin M, Aitken RJ. Evaluation of the contraceptive
527 potential of recombinant human ZP3 and human ZP3 peptides in a primate model: their safety
528 and efficacy. Am J Reprod Immunol 1998;40:198-209.
529 [37] Govind CK, Gupta SK. Failure of female baboons (Papio anubis) to conceive following
530 immunization with recombinant non-human primate zona pellucida glycoprotein-B expressed in
531 Escherichia coli. Vaccine 2000;18:2970-8.
532 [38] Srivastava N, Santhanam R, Sheela P, Mukund S, Thakral S, Malik B, et al. Evaluation of the
533 immunocontraceptive potential of Escherichia coli-expressed recombinant dog ZP2 and ZP3 in a
534 homologous animal model. Reproduction 2002;123:847-57.
535 [39] Joonè CJ, Bertschinger HJ, Gupta SK, Fosgate GT, Arukha AP, Minhas V, et al. Ovarian function
536 and pregnancy outcome in pony mares following immunocontraception with native and
537 recombinant porcine zona pellucida vaccines. Equine Vet J 2015.
538 [40] Kerr PJ, Jackson RJ, Robinson AJ, Swan J, Silvers L, French N, et al. Infertility in female rabbits
539 (Oryctolagus cuniculus) alloimmunized with the rabbit zona pellucida protein ZPB either as a
540 purified recombinant protein or expressed by recombinant myxoma virus. Biol Reprod
541 1999;61:606-13.

542 [41] Fayrer-Hosken RA, Bertschinger HJ, Kirkpatrick JF, Grobler D, Lamberski N, Honneyman G, et
543 al. Contraceptive potential of the porcine zona pellucida vaccine in the African elephant
544 (Loxodonta africana). Theriogenology 1999;52:835-46.
545 [42] Barber M, Lee S, Steffens W, Ard M, Fayrer-Hosken R. Immunolocalization of zona pellucida
546 antigens in the ovarian follicle of dogs, cats, horses and elephants. Theriogenology 2001;55:1705-
547 17.
548 [43] Rath A, Choudhury S, Hasegawa A, Koyama K, Gupta SK. Antibodies generated in response to
549 plasmid DNA encoding zona pellucida glycoprotein-B inhibit in vitro human sperm-egg binding.
550 Mol Reprod Dev 2002;62:525-33.
551 [44] Afzalpurkar A, Shibahara H, Hasegawa A, Koyama K, Gupta SK. Immunoreactivity and in-vitro
552 effect on human sperm-egg binding of antibodies against peptides corresponding to bonnet
553 monkey zona pellucida-3 glycoprotein. Hum Reprod 1997;12:2664-70.
554 [45] Gu W, Holland M, Janssens P, Seamark R, Kerr P. Immune response in rabbit ovaries
555 following infection of a recombinant myxoma virus expressing rabbit zona pellucida protein B.
556 Virology 2004;318:516-23.
557 [46] Gupta SK, Gupta N, Suman P, Choudhury S, Prakash K, Gupta T, et al. Zona pellucida-based
558 contraceptive vaccines for human and animal utility. J Reprod Immunol 2011;88:240-6.
559 [47] Rhim SH, Millar SE, Robey F, Luo AM, Lou YH, Yule T, et al. Autoimmune disease of the ovary
560 induced by a ZP3 peptide from the mouse zona pellucida. J Clin Invest 1992;89:28.
561 [48] Lou Y, Ang J, Thai H, McElveen F, Tung KSK. A zona pellucida 3 peptide vaccine induces
562 antibodies and reversible infertility without ovarian pathology. J Immunol 1995;155:2715-20.
563 [49] VandeVoort CA, Schwoebel ED, Dunbar BS. Immunization of monkeys with recombinant
564 complimentary deoxyribonucleic acid expressed zona pellucida proteins. Fertil Steril 1995;64:838-
565 47.

566 [50] Bagavant H, Adams S, Terranova P, Chang A, Kraemer FW, Lou Y, et al. Autoimmune ovarian
567 inflammation triggered by proinflammatory (Th1) T cells is compatible with normal ovarian
568 function in mice. Biol Reprod 1999;61:635-42.
569 [51] Lou Y, Park K, Agersborg S, Alard P, Tung KSK. Retargeting T cell-mediated inflammation: a
570 new perspective on autoantibody action. J Immunol 2000;164:5251-7.
571 [52] Bagavant H, Sharp C, Kurth B, Tung KS. Induction and immunohistology of autoimmune
572 ovarian disease in cynomolgus macaques (Macaca fascicularis). Am J Pathol 2002;160:141-9.
573 [53] Clydesdale G, Pekin J, Beaton S, Jackson RJ, Vignarajan S, Hardy CM. Contraception in mice
574 immunized with recombinant zona pellucida subunit 3 proteins correlates with Th2 responses and
575 the levels of interleukin 4 expressed by CD4+ cells. Reproduction 2004;128:737-45.
576 [54] Mackenzie S, McLaughlin E, Perkins H, French N, Sutherland T, Jackson RJ, et al.
577 Immunocontraceptive effects on female rabbits infected with recombinant myxoma virus
578 expressing rabbit ZP2 or ZP3. Biol Reprod 2006;74:511-21.
579 [55] Li J, Jin H, Zhang A, Li Y, Wang B, Zhang F. Enhanced contraceptive response by co-
580 immunization of DNA and protein vaccines encoding the mouse zona pellucida 3 with minimal
581 oophoritis in mouse ovary. J Gene Med 2007;9:1095-103.
582 [56] O'Leary S, Lloyd ML, Shellam GR, Robertson SA. Immunization with recombinant murine
583 cytomegalovirus expressing murine zona pellucida 3 causes permanent infertility in BALB/c mice
584 due to follicle depletion and ovulation failure. Biol Reprod 2008;79:849-60.
585 [57] Lloyd ML, Papadimitriou JM, O'Leary S, Robertson SA, Shellam GR. Immunoglobulin to zona
586 pellucida 3 mediates ovarian damage and infertility after contraceptive vaccination in mice. J
587 Autoimmun 2010;35:77-85.
588 [58] Sacco AG, Subramanian MG, Yurewicz EC, DeMayo FJ, Dukelow WR. Heteroimmunization of
589 squirrel monkeys (Saimiri sciureus) with a purified porcine zona antigen (PPZA): immune response
590 and biologic activity of antiserum. Fertil Steril 1983;39:350-8.

591 [59] Sacco A, Pierce D, Subramanian M, Yurewicz E, Dukelow W. Ovaries remain functional in
592 squirrel monkeys (Saimiri sciureus) immunized with porcine zona pellucida 55,000
593 macromolecule. Biol Reprod 1987;36:481-90.
594 [60] Mahi-Brown C, Yanagimachi R, Hoffman J, Huang T. Fertility control in the bitch by active
595 immunization with porcine zonae pellucidae: use of different adjuvants and patterns of estradiol
596 and progesterone levels in estrous cycles. Biol Reprod 1985;32:761-72.
597 [61] Mahi-Brown CA, Yanagimachi R, Nelson ML, Yanagimachi H, Palumbo N. Ovarian
598 histopathology of bitches immunized with porcine zonae pellucidae. Am J Reprod Im Mic
599 1988;18:94-103.
600 [62] Freund J. The Effect of Paraffin Oil and Myco-bacteria on Antibody Formation and
601 Sensitlzation. A Review. Am J Clin Pathol 1951;21:645-56.
602 [63] Stils HF. Adjuvants and antibody production: dispelling the myths associated with Freund's
603 complete and other adjuvants. ILAR J 2005;46:280-93.
604 [64] Lyda RO, Hall JR, Kirkpatrick JF. A comparison of Freund's Complete and Freund's Modified
605 adjuvants used with a contraceptive vaccine in wild horses (*Equus caballus*). J Zoo Wildl Med
606 2005;36:610-6.
607 [65] Aitken RJR, D.W.; Hulme, M. Immunological interference with the properties of the zona
608 pellucida. In: Crighton DB, editor. Immunological aspects of reproduction in mammals. London:
609 Butterworth; 1984. p. 305 - 25.
610 [66] Sacco A, Yurewicz E, Subramanian M, Lian Y, Dukelow W. Immunological response and
611 ovarian histology of squirrel monkeys (Saimiri sciureus) immunized with porcine zona pellucida
612 ZP3 (Mr= 55,000) macromolecules. Am J Primatol 1991;24:15-28.
613 [67] Sehgal S, Gupta SK, Bhatnagar P. Long-Term Effects of Immunization with Porcine Zona
614 Pellucida on Rabbit Ovaries. Pathology 1989;21:105-10.

615 [68] Upadhyay SN, Thillaikoothan P, Bamezai A, Jayaraman S, Talwar GP. Role of adjuvants in
616 inhibitory influence of immunization with porcine zona pellucida antigen (ZP-3) on ovarian
617 folliculogenesis in bonnet monkeys: a morphological study. Biol Reprod 1989;41:665-73.
618 [69] Mahi-Brown CA, McGuinness RP, Moran F. The cellular immune response to immunization
619 with zona pellucida antigens. J Reprod Immunol 1992;21:29-46.
620 [70] Paterson M, Wilson M, Jennings Z, Van Duin M, Aitken R. Design and evaluation of a ZP3
621 peptide vaccine in a homologous primate model. Mol Hum Reprod 1999;5:342-52.
622 [71] Govind CK, Srivastava N, Gupta SK. Evaluation of the immunocontraceptive potential of
623 Escherichia coli expressed recombinant non-human primate zona pellucida glycoproteins in
624 homologous animal model. Vaccine 2002;21:78-88.
625 [72] Mahi-Brown CA, Huang TT, Yanagimachi R. Infertility in bitches induced by active
626 immunization with porcine zonae pellucidae. J Exp Zool 1982;222:89-95.
627 [73] Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu Y-J, et al. Immunobiology of
628 dendritic cells. Annu Rev Immunol 2000;18:767-811.
629 [74] Millar SE, Chamow SM, Baur AW, Oliver C, Robey F, Dean J. Vaccination with a synthetic zona
630 pellucida peptide produces long-term contraception in female mice. Science 1989;246:935-8.
631 [75] Bradley MP, Eade J, Penhale J, Bird P. Vaccines for fertility regulation of wild and domestic
632 species. J Biotechnol 1999;73:91-101.
633 [76] Hinds LA, Hardy CM, Lawson MA, Singleton GR. Developments in fertility control for pest
634 animal management. Aciar Mg S 2003;96:31-6.
635 [77] Barfield JP, Nieschlag E, Cooper TG. Fertility control in wildlife: humans as a model.
636 Contraception 2006;73:6-22.
637 [78] Lou Y, Tung KS. T cell peptide of a self-protein elicits autoantibody to the protein antigen.
638 Implications for specificity and pathogenetic role of antibody in autoimmunity. J Immunol
639 1993;151:5790-9.

640 [79] Lou YH, Bagavant H, Ang J, McElveen MF, Thai H, Tung KS. Influence of autoimmune ovarian
641 disease pathogenesis on ZP3 contraceptive vaccine design. J Reprod Fertil Suppl 1996;50:159-63.
642 [80] Gray ME, Cameron EZ. Does contraceptive treatment in wildlife result in side effects? A
643 review of quantitative and anecdotal evidence. Reproduction 2010;139:45-55.
644 [81] Lyda RO, Frank KM, Wallace R, Lamberski N, Kirkpatrick JF. Immunocontraception of captive
645 exotic species: V. Prolonged antibody titers in Dall sheep (Ovis dalli dalli) and domestic goats
646 (Capra hircus) immunized with porcine zona pellucida. J Zoo Wildl Med 2013;44:S21-5.
647 [82] Frank KM, Lyda RO, Kirkpatrick JF. Immunocontraception of captive exotic species - IV.
648 Species differences in response to the porcine zona pellucida vaccine, timing of booster
649 inoculations, and procedural failures. Zoo Biol 2005;24:349-58.
650 [83] Gorman SP, Levy JK, Hampton AL, Collante WR, Harris AL, Brown RG. Evaluation of a porcine
651 zona pellucida vaccine for the immunocontraception of domestic kittens (Felis catus).
652 Theriogenology 2002;58:135-49.
653 [84] Eade JA, Roberston ID, James CM. Contraceptive potential of porcine and feline zona
654 pellucida A, B and C subunits in domestic cats. Reproduction 2009;137:913-22.
655 [85] Ahlers MJ, Ganswindt A, Munscher S, Bertschinger HJ. Fecal 20-oxo-pregnane concentrations
656 in free-ranging African elephants (Loxodonta africana) treated with porcine zona pellucida
657 vaccine. Theriogenology 2012;78:77-85.
658 [86] Liu IK, Bernoco M, Feldman M. Contraception in mares heteroimmunized with pig zonae
659 pellucidae. J Reprod Fert 1989;85:19-29.
660 [87] Powell DM, Monfort SL. Assessment: effects of porcine zona pellucida immunocontraception
661 on estrous cyclicity in feral horses. J Appl Anim Welf Sci 2001;4:271-84.
662 [88] Kirkpatrick J, Liu I, Turner J, Naugle R, Keiper R. Long-term effects of porcine zonae pellucidae
663 immunocontraception on ovarian function in feral horses (*Equus caballus*). J Reprod Fert
664 1992;94:437-44.

665 [89] Kirkpatrick J, Naugle R, Liu I, Bernoco M, Turner Jr J. Effects of seven consecutive years of
666 porcine zona pellucida contraception on ovarian function in feral mares. Biol Reprod Mg
667 1995;1:411 - 8.
668 [90] Bechert U, Bartell J, Kutzler M, Menino A, Bildfell R, Anderson M, et al. Effects of two porcine
669 zona pellucida immunocontraceptive vaccines on ovarian activity in horses. J Wildlife Manage
670 2013;77:1386-400.
671 [91] Henderson C, Hulme M, Aitken R. Contraceptive potential of antibodies to the zona pellucida.
672 J Reprod Fert 1988;83:325-43.
673 [92] McShea WJ, Monfort SL, Hakim S, Kirkpatrick J, Liu I, Turner Jr JW, et al. The effect of
674 immunocontraception on the behavior and reproduction of white-tailed deer. J Wildlife Manage
675 1997:560-9.
676 [93] Curtis PD, Pooler RL, Richmond ME, Miller LA, Mattfeld GF, Quimby FW. Comparative effects
677 of GnRH and porcine zona pellucida (PZP) immunocontraceptive vaccines for controlling
678 reproduction in white-tailed deer (Odocoileus virginianus). Reproduction Suppl 2002;60:131-41.
679 [94] Miller LA, Fagerstone KA, Wagner DC, Killian GJ. Factors contributing to the success of a
680 single-shot, multiyear PZP immunocontraceptive vaccine for white-tailed deer. Human-Wildlife
681 Interactions 2009:34.
682 [95] Miller LA, Johns BE, Killian GJ. Long-term effects of PZP immunization on reproduction in
683 white-tailed deer. Vaccine 1999;18:568-74.
684 [96] Turner Jr JW, Kirkpatrick JF, Liu IK. Effectiveness, reversibility, and serum antibody titers
685 associated with immunocontraception in captive white-tailed deer. J Wildlife Manage 1996:45-51.
686 [97] Curtis PD, Richmond ME, Miller LA, Quimby FW. Pathophysiology of white-tailed deer
687 vaccinated with porcine zona pellucida immunocontraceptive. Vaccine 2007;25:4623-30.
688 [98] Darhower SE, Maher CR. Effects of immunocontraception on behavior in fallow deer (Dama
689 dama). Zoo Biol 2008;27:49-61.

690 [99] Stoops MA, Liu IKM, Shideler SE, Lasley BL, Fayrer-Hosken RA, Benirschke K, et al. Effect of
691 porcine zonae pellucidae immunisation on ovarian follicular development and endocrine function
692 in domestic ewes (*Ovis aries*). Reprod Fert Develop 2006;18:667-76.
693 [100] Martinez ML, Harris JD. Effectiveness of zona pellucida protein ZPB as an
694 immunocontraceptive antigen. J Reprod Fert 2000;120:19-32.
695 [101] Bagavant H, Thillai-Koothan P, Sharma M, Talwar G, Gupta S. Antifertility effects of porcine
696 zona pellucida-3 immunization using permissible adjuvants in female bonnet monkeys (*Macaca*
697 *radiata*): reversibility, effect on follicular development and hormonal profiles. J Reprod Fert
698 1994;102:17-25.
699 [102] Kaul R, Sivapurapu N, Afzalpurkar A, Srikanth V, Govind CK, Gupta SK. Immunocontraceptive
700 potential of recombinant bonnet monkey (Macaca radiata) zona pellucida glycoprotein-C
701 expressed in Escherichia coli and its corresponding synthetic peptide. Reprod Biomed Online
702 2001;2:33-9.
703 [103] Harrenstien LA, Munson L, Chassy LM, Liu IK, Kirkpatrick JF. Effects of porcine zona pellucida
704 immunocontraceptives in zoo felids. J Zoo Wildl Med 2004;35:271-9.
705 [104] Sun W, Lou YH, Dean J, Tung KS. A contraceptive peptide vaccine targeting sulfated
706 glycoprotein ZP2 of the mouse zona pellucida. Biol Reprod 1999;60:900-7.
707 [105] Lea IA, Widgren EE, O'Rand MG. Analysis of recombinant mouse zona pellucida protein 2
708 (ZP2) constructs for immunocontraception. Vaccine 2002;20:1515-23.
709 [106] Borillo J, Coonrod SA, Wu J, Zhou C, Lou Y. Antibodies to two ZP3 B cell epitopes affect zona
710 pellucida assembly. J Reprod Immunol 2008;78:149-57.
711 [107] Lloyd ML, Shellam GR, Papadimitriou JM, Lawson MA. Immunocontraception is induced in
712 BALB/c mice inoculated with murine cytomegalovirus expressing mouse zona pellucida 3. Biol
713 Reprod 2003;68:2024-32.
714 [108] Gwatkin R, Williams D, Carlo D. Immunization of mice with heat-solubilized hamster zonae:
715 production of anti-zona antibody and inhibition of fertility. Fertil Steril 1977;28:871-7.

716 [109] Skinner S, Mills T, Kirchick H, Dunbar B. Immunization with zona pellucida proteins results in
717 abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid
718 secretion. Endocrinology 1984;115:2418-32.
719 [110] Simon AM, Goodenough DA, Li E, Paul DL. Female infertility in mice lacking connexin 37.
720 Nature 1997;6616:525 - 9.
721 [111] Jackson RJ, Maguire DJ, Hinds L, Ramshaw IA. Infertility in mice induced by a recombinant
722 ectromelia virus expressing mouse zona pellucida glycoprotein 3. Biol Reprod 1998;58:152-9.
723 [112] Kirkpatrick JF, Lyda RO, Frank KM. Contraceptive vaccines for wildlife: a review. Am J Reprod
724 Immunol 2011;66:40-50.
725 [113] Kirkpatrick JF, Liu IK, Turner Jr JW. Remotely-delivered immunocontraception in feral
726 horses. Wildlife Soc B 1990;18:326-30.
727 [114] Delsink AK, Kirkpatrick J, van Altena JJ, Bertschinger HJ, Ferreira SM, Slotow R. Lack of
728 spatial and behavioral responses to immunocontraception application in African elephants
729 (Loxodonta africana). J Zoo Wildl Med 2013;44:S52-74.
730 [115] Turner A, Kirkpatrick JF. Effects of immunocontraception on population, longevity and body
731 condition in wild mares (*Equus caballus*). Reproduction Suppl 2002;60:187-95.
732 [116] Powell DM. Preliminary evaluation of porcine zona pellucida (PZP) immunocontraception
733 for behavioral effects in feral horses (Equus caballus). J Appl Anim Welf Sci 1999;2:321-35.
734 [117] Ransom JI, Cade BS, Hobbs NT. Influences of immunocontraception on time budgets, social
735 behavior, and body condition in feral horses. Appl Anim Behav Sci 2010;124:51-60.
736 [118] Nuñez C, Adelman JS, Rubenstein DI. Immunocontraception in wild horses (*Equus caballus*)
737 extends reproductive cycling beyond the normal breeding season. Plos One 2010;5:e13635.
738 [119] Turner JW, Rutberg AT, Naugle RE, Kaur MA, Flanagan DR, Bertschinger HJ, et al. Controlled-
739 release components of PZP contraceptive vaccine extend duration of infertility. Wildlife Res
740 2008;35:555-62.

741 [120] Berchert U, Fraker MA. The response of African elephants to a single dose of SpayVac®, a
742 pZP contraceptive vaccine, over a seven-year period. Pachyderm 2016:97-108.
743 [121] Brown R, Bowen W, Eddington J, Kimmins W, Mezei M, Parsons J, et al. Evidence for a long-
744 lasting single administration contraceptive vaccine in wild grey seals. J Reprod Immunol
745 1997;35:43-51.
746 [122] Fraker MA, Brown RG, Gaunt GE, Kerr JA, Pohajdak B. Long-lasting, single-dose
747 immunocontraception of feral fallow deer in British Columbia. J Wildlife Manage 2002:1141-7.
748 [123] Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groome NP, et
749 al. Serum anti-mullerian hormone levels reflect the size of the primordial follicle pool in mice.
750 Endocrinology 2006;147:3228-34.
751 [124] Ireland J, Scheetz D, Jimenez-Krassel F, Themmen A, Ward F, Lonergan P, et al. Antral follicle
752 count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of
753 young adult cattle. Biol Reprod 2008;79:1219-25.
754 [125] Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with
755 histologically determined primordial follicle number. Fertil Steril 2011;95:170-5.

756

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, 104-106]			9% to 100%
		[48, 50, 53, 55-57, 107, 111]		0% to 100%
Rabbits	[7, 54]			100%
		[7, 31, 32, 40, 45, 54, 109]		0 – 30%
Non-human primates	[36, 37, 49, 68, 70, 100-102]			25 – 100%
		[8, 33, 34, 36, 49, 52, 58, 59, 68-71, 100]		0 – 50%
Cats	[83, 84, 103]			16[c] - 100%
Dogs	[38, 60, 72]			100%
		[60, 61, 72]		0 – 34%
Sheep	[99]			100%

		[99]		0%
White-tailed deer	[92-94, 97]			0 – 95%
			[95]	11%
Horses	[39, 86, 87]			0 – 57%
		[39, 88-90]		0 – 8%
African elephants			[85]	0%

[a]Evidence of ovarian dysfunction includes histological evidence of oophoritis or decreased follicle numbers, or behavioural or hormonal evidence of abnormal oestrous or menstrual cyclicity

[b]Expressed as a proportion of the control group, where control groups are available

[c]Not statistically significant