Vesicle-associated protein 1: a novel ovarian immunocontraceptive target in the common brushtail possum, *Trichosurus vulpecula*

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Abstract

Ovarian-based immunological research is currently restricted to proteins of the zona pellucida. This study examined the immunocontraceptive potential of a novel vesicle-associated protein, VAP1, previously isolated from the vesicle-rich hemisphere of the brushtail possum oocyte. Seven female possums were immunized against recombinant glutathione *S*-transferase-VAP1 fusion protein. Control animals ($n=3$) received antigen-free vaccinations. Following immunization, regular blood sampling determined the level and duration of immune response. Animals were monitored daily, pre- and post-immunization, to determine estrous cycling activity and the percentage of reproductive cycles yielding viable young. The reproductive tracts and somatic organs of VAP1-immunized ($n=7$), control-immunized ($n=3$) and non-immunized ($n=5$) animals were collected and examined by histology and transmission electron microscopy. VAP1 immunization caused a strong and sustained immune response. Elevated levels of VAP1 antibody binding were detected in sera following initial injections, and immune titers rose as boosters were administered. Immunization had no adverse effect upon animal behavior or body condition. Immunized females demonstrated no major change in annual estrous cycling activity; however, the percentage of reproductive cycles resulting in pouch young decreased significantly ($P<0.05$) by 40%. Histological and ultrastructural analyses revealed an abundance of lipid-like degradation bodies within the ooplasm of developing oocytes and the cytoplasm of failing uterine zygotes. Active macrophage invasion of enlarged endometrial glands was observed in the uteri of two females. Reproductive tract changes are discussed in relation to observed fertility decline. The results of this study indicate that VAP1 has exciting potential as an immunocontraceptive target for possum control in New Zealand.

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Introduction

Manipulation of fertility, via immunocontraception, has been the subject of intense research for many years. Contraceptive vaccines may interfere with gamete production, fertilization, or embryogenesis by stimulating an immune reaction to key reproductive molecules. Common targets of interest include proteins of the zona pellucida (Barber & Fayrer-Hosken 2000), sperm surface antigens, and reproductive hormones (Naz *et al.* 2005, Cooper & Larsen 2006, Hardy & Braid 2007).

The zona pellucida (ZP) plays a critical role in mammalian fertilization, hence ZP glycoproteins are the most frequently proposed candidates for ovarian-based immunocontraception (Barber & Fayrer-Hosken 2000). Numerous trials have validated the contraceptive potential of various antigens (Frank *et al.* 2005); however, fertility reduction is frequently accompanied by ovarian pathology (Mahi-Brown *et al.* 1988, Curtis *et al.* 2007) and hormonal disturbance (Skinner *et al.*

1984, Stoops *et al.* 2005). An ideal ovarian immunological agent would inhibit fertility without causing ovarian dysfunction.

A major obstacle in developing contraceptive vaccines for the regulation of wildlife populations is the risk of inadvertently affecting non-target species. The situation in New Zealand is unusual in that the country's major mammalian pest is an introduced marsupial, the common brushtail possum (*Trichosurus vulpecula*). As New Zealand has no native marsupials, the development of a marsupial-specific contraceptive vaccine, derived from a unique feature of marsupial development, such as oocyte–conceptus polarity, would eliminate the threat to endemic species.

In marsupials, polarity is initially established in primary oocytes by the eccentric, peripheral location of the nucleus (Selwood 1992). Polarity is further enhanced in many species by the accumulation of conspicuous membrane-bound electron-lucent vesicles opposite the nucleus, either during oogenesis or following fertilization (Selwood 1994). Oocyte vesicles

originate via the fusion of multivesicular bodies, derived from endoplasmic, endocytotic, and Golgi vesicles in *Monodelphis domestica* (Falconnier & Kress 1992) as well as coated and heterogeneous vesicles in *Isodon macrourus* (Ullmann & Butcher 1996). Although the chemical composition of these vesicles remains largely unknown, there is some evidence they contain glycoproteins (Kress 1996) and saccharide residues (Breed 1996). Oocyte and zygote polarity is maintained by species-specific cytoskeletal architecture (Breed 1994, Merry *et al.* 1995, Frankenberg & Selwood 1998).

During early cleavage, vesicular contents are generally shed in a polarized fashion into the perivitelline space (Breed & Leigh 1990, Taggart *et al.* 1993, Sathananthan *et al.* 1997), contributing to blastocoel formation (Frankenberg & Selwood 2001). Cell-zona attachment first occurs in the opposite hemisphere to vesicle discharge (Selwood 1992, 2001, Merry *et al.* 1995), where the pluriblast (future embryonic) cells develop. Trophoblast (future placental) cells line the opposite hemisphere and continue to secrete vesicular matter into the cleavage cavity. In a number of marsupials, *T. vulpecula* (Frankenberg & Selwood 1998), *Sminthopsis macroura*, and *Antechinus stuartii* (Selwood & Smith 1990), cytoplasmic vesicles are unevenly distributed in the first two cell lineages of the embryo, and the polarized discharge of extracellular material appears essential for normal development (Frankenberg & Selwood 1998, Kress & Selwood 2003, 2004).

Recently, a number of vesicle-associated molecules (VAMs) have been identified in the vesicle-rich hemisphere of the *T. vulpecula* oocyte (Selwood *et al.* 1999). VAMs are associated with oocyte polarity, cell lineage allocation, and are believed to be essential for normal oogenesis, cleavage, and blastocyst formation. Vesicle-associated protein 1 (VAP1), a previously characterized ovarian-specific protein, has been proposed as a potential immunocontraceptive target for possum control in New Zealand (Cui *et al.* 2005). This study was conducted to assess the ability of VAP1 immunization to elicit an immune response in *T. vulpecula* and to examine the effect of vaccination on female fertility.

Results

Animal health

Ten reproductively mature female *T. vulpecula* were treated in this study, conducted between 2003 and 2007. Seven animals were immunized with glutathione S-transferase (GST)-VAP1, and three received control vaccines. Animals were monitored daily and on the basis of body condition, weight, and behavior the treatments did not appear detrimental to animal health. Two experimental animals developed slight swellings at the primary injection sites; however, no animals developed

lesions, a common, undesirable side effect of Freund's complete adjuvant (FCA; Broderson 1989).

Estrous cycling activity and fertility

To determine whether female fertility was affected by VAP1 immunization, estrous cycling activity, mating, and birth data were collected from the same cohort of animals ($n=7$), pre- and post-VAP1 immunization. In general, most females cycled continuously between March and September. Following immunization, however, animals were more likely to continue cycling during the months of October and November, indicating that VAP1 immunization may prolong the breeding season in some females. Female fertility (the percentage of estrous cycles resulting in birth) showed a monthly decline following immunization, with later months of the breeding season (May to October–November) most affected (Fig. 1). Despite continuing to cycle during October–November, no immunized females produced young during this period. Following immunization, individual fertility levels dropped in six of the seven treated animals (Fig. 2), and mean female fertility ($\pm$ S.E.M.) declined significantly ($t=10.0$, $df=6$, $P\leq 0.01$). Changes in fertility were not significantly correlated with animal age or estrous cycle stage at immunization ($P>0.05$).

Immune response

An immune response to VAP1 was detected in the serum of five of seven VAP1-immunized females (Fig. 3). Unfortunately, a lack of protein prevented analysis of serum antibody titers for the final two experimental animals. An immune response was not detected in serum collected from any of the control-immunized females ($n=3$). Booster VAP1 injections, administered at intervals from the primary immunization, caused a rapid secondary response with marked elevation of serum

Figure 1 *T. vulpecula* annual estrous cycling activity before and after VAP1 immunization, collated from 2003 to 2007. The majority of females cycled continuously from March to September. The percentage of estrous cycles resulting in a birth declined after immunization, with later months of the breeding season (May to October–November) most affected. Despite continuing to cycle during October–November, no VAP1-immunized females produced young during this period. n =Number of cycles recorded.

Figure 2 The percentage of estrous cycles resulting in a birth for individual *T. vulpecula* females (numbers 1–7), prior to and following VAP1 immunization. After immunization, fertility was reduced in six of the seven animals. Mean female fertility ($\pm$ S.E.M.) fell from 96% ($\pm$ 3.6) pre-immunization to 56% ($\pm$ 8.1) post-immunization, a reduction of 40%. *Significant difference ($t=10.0$, $df=6$, $P\leq 0.01$).

VAP1-antibody levels. The highest immune titers were detected 1–4 months after the initial immunization and varied between individuals. Additional booster injections, given to three females between 6 and 18 months from the primary immunization, caused a subsequent increase in immune response.

The percentage reduction in fertility per individual was positively correlated with the maximum immune titer reached ($R=0.769$, $n=5$, $P=0.037$), indicating that the level of immune response determined the degree to which fertility was reduced (Fig. 4).

Tissue response

Histological examination of the lymphatic system and somatic tissue (heart, kidney, liver, lung, spleen, lymph nodes, and oviduct) of VAP1-immunized animals ($n=7$)

Figure 3 The immune titer ($\log_2$) of five VAP1-immunized *T. vulpecula* females. Primary immunizations (Month 0) were followed by two booster injections (Months 1 and 2). The highest immune titers were recorded between 1 and 4 months from the initial immunization. Three females received an additional booster as indicated by the arrows. The final data point on each line indicates euthanasia of the animal.

Figure 4 Following VAP1 immunization, the percentage reduction in fertility per *T. vulpecula* individual was positively correlated with the maximum immune titer reached ($R=0.769$, $n=5$, $P=0.037$).

revealed no abnormalities when compared with tissue from control-immunized ($n=3$) or non-immunized ($n=5$) females. Normal uterine morphology, typical of early gestation, was observed in five of the seven VAP1-immunized females (examined 0–8 days from ovulation). The uteri of the remaining two females (examined 7 days after removal of pouch young (RPY) and during anoestrus) showed greatly enlarged endometrial glands projecting into the uterine lumen (Fig. 5A). Large clusters of actively phagocytotic macrophages were present within the glandular lumen (Fig. 5B). Fertility reduction was greater than 40% in both these animals. All control-immunized ($n=3$) and non-immunized ($n=5$) animals demonstrated normal uterine morphology with endometrial gland development typical of the reproductive stage examined.

In contrast to the ovaries of control-immunized ($n=3$) and non-immunized ($n=5$) animals, an abundance of oocyte inclusions, termed degradation bodies, were observed in the ovaries of four of the seven VAP1-immunized females. Transmission electron micrography (TEM) revealed that the degradation bodies were lipid like in that they were bordered by an osmiophilic ring rather than a true, limiting trilaminar membrane. They contained defined electron-dense and electron-lucent regions, which presumably correlate with the saturated and unsaturated fatty acid content of the triglyceride and the degree of unsaturation of fatty acids present (Mehta & Ghadially 1973). The degradation bodies were roughly spherical in shape, showed evidence of fusion with cytoplasmic vesicles, and were generally accompanied by regions of amorphous, electron-dense cytoplasm. *T. vulpecula* oocytes typically contain a few cytoplasmic lipid droplets of homogeneous appearance (Frankenberg

Figure 5 Light micrographs of uterine tissue from a VAP1-immunized *T. vulpecula* female (8 µm, H&E). (A) Transverse section through the uterus showing the uterine lumen (L), glandular endometrium (E), and muscular myometrium (M). Within the endometrial layer, several enlarged glands are visible and a cluster of macrophages (MC) can be seen in the glandular lumen. Normal glands (G), stromal tissue (S). Scale=300 µm. (B) Higher magnification of clustered macrophages with pseudopodia (P) within the lumen of an enlarged endometrial gland. Scale=50 µm.

& Selwood 2001). Normal lipid droplets were observed in the oocytes of all animals examined; however, degradation bodies (determined by the characteristics described above) were only observed in the oocytes of VAP1-immunized animals. Gross deposits of lipid of this nature imply fatty degeneration.

Degradation bodies first became apparent in primary oocytes after the transition of primordial to the primary follicle stage (Fig. 6A and B). As follicles developed, the inclusions increased in size and accumulated to various extents within the ooplasm (Fig. 6C–F). Analysis of primary oocyte cross-sections (from VAP1-immunized

Figure 6 Light micrographs of oocytes from VAP1 immunized *T. vulpecula* females illustrating varying degrees of cytoplasmic lipid accumulation (normal lipid droplets and degradation bodies) during oogenesis (1 µm, Toluidine blue). (A) A normal primary oocyte (primary follicle) showing few cytoplasmic lipid droplets (arrow). Granulosa cells (G), zona pellucida (arrow head), nucleus/germinal vesicle (N). Scale=15 µm. (B) An affected primary oocyte (primary follicle) showing elevated cytoplasmic lipid levels. Degradation bodies (arrow) are present throughout the oocyte. The largest degradation bodies are located in an area of amorphous, electron-dense cytoplasm that appears almost devoid of vesicles (V). Granulosa cells (G), zona pellucida (arrow head). Scale=15 µm. (C) A badly affected primary oocyte (tertiary follicle) containing numerous degradation bodies (arrow), patches of amorphous, electron-dense cytoplasm, and an abnormally low level of vesicles (V). Granulosa cells (G), zona pellucida (arrow head), nucleus/germinal vesicle (N). Scale=15 µm. (D) A normal primary oocyte (early antral follicle) containing no degradation bodies, very few lipid droplets (arrow), an abundance of vesicles (V), and no areas of amorphous, electron-dense cytoplasm. Granulosa cells (G), zona pellucida (arrow head), nucleus/germinal vesicle (N). Scale=15 µm. (E) A badly affected primary oocyte (antral follicle) in which the cytoplasm has segregated into two distinct areas, one containing degradation bodies (arrow) and amorphous, electron-dense cytoplasm, and the other coalesced vesicles (V). Granulosa cells (G), zona pellucida (arrow head). Scale=26 µm. (F) A mildly affected antral follicle oocyte containing an abundance of vesicles interspersed with lipid and degradation bodies (arrow). No areas of amorphous, electron-dense cytoplasm are evident. Granulosa cells (G), zona pellucida (arrow head), nucleus/germinal vesicle (N). Scale=30 µm.

and non-immunized females) revealed that the number of lipid inclusions (degradation bodies and normal lipid droplets) per μm^2 oocyte cytoplasm was significantly greater in primary ($t=3.37$, $\text{df}=44$, $P=0.02$) and antral ($t=4.28$, $\text{df}=15$, $P=0.02$) follicle oocytes of VAP1-immunized animals. The number of vesicles (greater or equal in size to the largest observed lipid inclusion) per μm^2 oocyte cytoplasm was significantly reduced in primary follicle oocytes ($t=4.45$, $\text{df}=44$, $P=0.12$) of VAP1-immunized females but not in antral follicle oocytes ($P<0.05$). Degradation bodies remained prominent in secondary oocytes and appeared largest in the most mature oocytes.

An unfertilized oocyte, two zygotes, and a late bilaminar blastocyst were obtained from the uteri of VAP1-immunized females. Both zygotes showed an intact, well-developed shell coat, with numerous spermatozoa embedded in the mucoid layer (Fig. 7A). The embryonic hemisphere was closely adhered to the ZP, whereas cytoplasm in the opposite, ab-embryonic hemisphere had become detached in places. Although cytoplasmic polarization was pronounced and yolk extrusion had begun, cytoplasmic fragmentation was evident and neither zygote appeared viable. In addition to normal cytoplasmic contents, such as filamentous, elongated mitochondria, electron-lucent vesicles and homogeneous, saturated lipid droplets, both zygotes contained a profusion of degradation bodies (Fig. 7B). Under TEM, the degradation bodies (Fig. 7C and D) appeared equivalent to those observed in the oocytes of VAP1-immunized animals.

Discussion

The immunization of *T. vulpecula* with GST-VAP1 elicited an oocyte-specific systemic antibody response and a cell-mediated uterine response, both novel reactions to an ovarian immunocontraceptive. In addition to a significant reduction in female fertility, VAP1 immunization caused embryonic failure and generated oocyte and uterine changes, suggesting that multiple mechanisms may contribute to fertility decline.

VAP1 is encoded by a unique gene with no significant homologies (Cui *et al.* 2005). Some regions of the protein, however, show structural similarity to the active regions of cystatin C, an endogenous cysteine proteinase inhibitor (Barrett *et al.* 1984, Turk & Bode 1991, Dieckmann *et al.* 1993). Cystatin C, a member of the type 2 cystatin superfamily (Abrahamson *et al.* 2003), regulates cysteine proteinase activity in intra- and extracellular biological reactions (Barrett 1981). In addition to similar spatial form, the conserved presence of all five cystatin C functional motifs (disulfide-paired cysteine residues) within the VAP1 sequence implies a homologous role. If so, VAP1 may contribute to extracellular matrix (ECM) stability during oogenesis and early development by allowing the accumulation

Figure 7 Light and transmission electron micrographs of a zygote retrieved from the active uterus of a VAP1 immunized *T. vulpecula* female. (A) The shell coat (arrow head) surrounding the zygote appears well-developed and numerous spermatozoa are embedded in the mucoid layer (M). The embryonic hemisphere (E) of the zygote is closely adhered to the zona pellucida (arrow), whereas ooplasm in the opposite, ab-embryonic (AB) hemisphere is detached in places. Polar bodies (not shown) were located in the ab-embryonic hemisphere. No cleavage furrow was observed. Scale = 100 μm . (B) A failed zygote fragment containing filamentous mitochondria (M) and numerous degradation bodies (arrow) of varying size. ($\times 2200$). (C) Smaller degradation bodies generally contained an electron-dense centre. Irregularly shaped, electron-lucent regions were located toward the periphery and bordered by a thin, osmiophilic ring. Evidence of fusion with cytoplasmic vesicles was regularly observed. ($\times 21\,000$). (D) Larger degradation bodies contained central electron-lucent regions, contrasted with greater electron density in the surrounds ($\times 11\,500$).

and stable storage of proteins within the oocyte cytoplasm, and/or stabilizing proteins as they are secreted into the blastocoel during cleavage (Cui *et al.* 2005).

Sparsely distributed lipid droplets have been described in the ooplasm of *T. vulpecula* (Frankenberg & Selwood 2001), *I. macrourus*, *I. obesulus*, and *Perameles nasuta* (Ullmann 1981, Lyne & Hollis 1983) and various eutherian species (Fleming & Saacke 1972, Tesoriero 1981, Homa *et al.* 1986, Kikuchi *et al.* 2002). Although rare, degradation bodies have been observed in *T. vulpecula* zygote cytoplasm (Frankenberg & Selwood 1998) but not during normal oogenesis (Frankenberg & Selwood 2001). Degradation bodies of the size and magnitude observed here are previously unreported.

Despite the unusual oocyte changes induced by VAP1 immunization, local inflammatory cell levels were not

elevated, therefore the ovarian immune response appears systemic. In contrast to ZP-based immunocontraceptives (Kitchener *et al.* 2002), VAP1 immunization affected only the germ cells of the ovary. The preservation of surrounding somatic tissue and normal ovarian morphology strengthens previous speculation that *Vap1* expression may be oocyte specific (Cui *et al.* 2005). Following immunization, degradation bodies first became apparent in primary oocytes after the transition of primordial follicles to the primary follicle stage, correlating with a period of high *Vap1* expression (Cui *et al.* 2005), and the initiation of oocyte vesicle formation (Frankenberg *et al.* 1996, Frankenberg & Selwood 2001). The accumulation of degradation bodies in affected oocytes was associated with decreased vesicle number, suggesting that immunization compromises normal vesicular accumulation during oogenesis.

If VAP1 plays a role akin to cystatin C, then the transfer of VAP1 antibodies into the developing oocyte via serum would disrupt the normal cysteine proteinase-inhibitor balance, effectively allowing uncontrolled proteolytic activity of this type within the oocyte. In humans, imbalances between cathepsins of the cysteine proteinase family and their endogenous inhibitors are related to a variety of pathological conditions (Lenarcic *et al.* 1988, Henskens *et al.* 1996, Cimerman *et al.* 2001, Kos *et al.* 2001). We propose that the degradation bodies observed in this study are a direct result of increased proteolysis of oocyte storage proteins and/or ECM molecules due to VAP1 inhibition. During early development in *T. vulpecula*, the polarized discharge of ECM material appears important for embryonic–abembryonic patterning (Frankenberg & Selwood 1998, Kress & Selwood 2003, 2004) and vesicles are preferentially distributed in the first two cell lineages (Frankenberg & Selwood 1998). Decreased ECM stability, change in vesicular secretion and disruption of cell lineage allocation may all contribute to reducing embryonic success following immunization.

In addition to the antibody-mediated ovarian response, a small number of VAP1-immunized females demonstrated uterine changes consistent with endometrial cystic hyperplasia (De Bosschere *et al.* 2001), raising the possibility that a uterine immune response may contribute to this condition. The presence of macrophage clusters within enlarged endometrial glands suggests a cell-mediated reaction, possibly in response to the presence of a failed conceptus. All conceptuses obtained from the uteri of VAP1-immunized females showed cytoplasmic fragmentation; therefore, it appears probable that the macrophages observed were attacking the remains of a disintegrated oocyte or conceptus. There was no evidence to suggest that macrophages attack the intact conceptus, implying that the shell coat may have immunoprotectant properties. The absence of T lymphocytes within VAP1-immunized uteri is not unusual as experiments were generally scheduled during

early pregnancy (prior to shell coat loss) in the hope of obtaining a conceptus. Due to the limited number of animals available, we were unable to examine all stages of pregnancy.

In conclusion, VAP1 immunization induces a novel oocyte-specific systemic immune response, which decreases female fertility without detrimental ovarian pathology. A secondary cell-mediated uterine response may also contribute to embryonic failure. Passive immune transfer (Adamski & Demmer 2000), from immunized mothers to female young during lactation, may impart a generational effect. The ovary has a finite reserve of oocytes. As VAP1 targets all stages of oogenesis, immunization has the potential to impair fertility throughout an animal's lifespan. With the marsupial-specific VAP1 protein identified and shown to have an antibody mediated response to oocytes *in vivo*, VAP1 antibodies can now be used to carry fertility control agents into developing oocytes of the marsupial ovary.

Materials and Methods

Animals

Female *T. vulpecula* used in this study were housed in an outdoor colony maintained by L Selwood at The University of Melbourne. Colony maintenance and experiments followed Australian National Health and Medical Research Council Guidelines for the Care and Use of Animals for Scientific Purposes. A female-biased sex ratio (~2:1) was preserved throughout the experimental period, and the colony contained an average of 18 individuals at any given time.

Reproductive monitoring

Adult females were monitored from March to November to establish the reproductive status. Estrous cycle stage was determined by cytology and mating success by the presence of spermatozoa in daily urine samples (Duckworth *et al.* 1999). *T. vulpecula* are monovular, polyoestrous seasonal breeders (Pilton & Sharman 1962). Following RPY females return to estrus within 7–15 days (Duckworth *et al.* 1998). Estrus occurs 1–3 days prior to ovulation (Shorey & Hughes 1973), and young are born 17.3 (± 0.2) days post-coitus (Duckworth *et al.* 1998). Experimental females were monitored for 6–18 months before VAP1 immunization. Data obtained during this period served as respective (pre-immunized) controls for each individual, enabling the comparison of pre- and post-immunization activity. Immunizations were undertaken during the breeding season and post-immunization data were collected during the remainder of that season and the subsequent breeding season. The length of time animals were monitored post-immunization varied between individuals.

Immunization

Animals were randomly assigned to VAP1-immunized ($n=7$) or control-immunized ($n=3$) groups. The immunization

antigen, recombinant GST-VAP1 fusion protein, was prepared according to a previous paper (Cui *et al.* 2005). The immunization protocol comprised an initial dose of 300 µg GST-VAP1 in 500 µl magnesium- and calcium-free PBS[−] emulsified with 500 µl Freund's Complete Adjuvant (Sigma–Aldrich). Two booster vaccines followed at 28-day intervals, each containing 300 µg GST-VAP1 in 500 µl PBS[−] in the presence of 500 µl Freund's Incomplete Adjuvant (Sigma–Aldrich). Four animals received a further booster between 6 and 18 months from the initial immunization. Control-immunized animals were treated over the same period with equivalent volumes of antigen-free PBS[−] ($n=2$) or GST in PBS[−] ($n=1$) using the same adjuvants. Immunizations were delivered to multiple sites by s.c. injection. Prior to immunization and/or blood collection animals were sedated via i.m. injection with 10 mg/kg Zoletil (Virbac Animal Health, Peakhurst, NSW, Australia). Blood samples were collected from the lateral tail vein or the basilic forearm vein, prior to the initial injection (pre-immune control), 7–10 days after booster injections (Cooper & Paterson 2008), and at 3 monthly intervals throughout the remaining experimental period. Prior to autopsy, animals were anesthetized with 15 mg/kg Zoletil delivered intramuscularly. The blood was collected via heart puncture and animals euthanized by intracardial injection of 150 mg/kg sodium pentobarbitone (Lethabarb; Virbac Animal Health). The blood samples were centrifuged at 2130 g for 10 min. Sera were removed and stored at -20°C for analysis.

ELISA

The titer of polyclonal antibodies (against GST-VAP1) in the sera of VAP1-immunized ($n=7$) and control-immunized ($n=3$) animals was determined by ELISA, with pre-immune samples serving as negative controls for each individual. Assays were performed in 96-well ELISA plates (Immunlon 4 HBX; Thermo Scientific, Noble Park, VIC, Australia) using recombinant GST-VAP1 as the antigen. Non-specific binding was blocked with 3% albumin bovine serum (Sigma–Aldrich) and plates were washed between incubations with a buffering solution of 0.05% Tween-20 (Selby Scientific, Notting Hill, VIC, Australia) in PBS[−]. Serial dilutions of the primary antibody, *T. vulpecula*-raised polyclonal antisera, were detected by application of the secondary antibody, horseradish peroxidase (HRP)-conjugated protein A (GE Health Care, Castle Hill, NSW, Australia), diluted 1:5000 in PBS[−]. Incubation with tetramethyl benzidine substrate (Sigma–Aldrich), which reacts with HRP, produced an insoluble compound. Reactions were terminated after 30 min by the addition of 0.5 M sulfuric acid. Color absorbance was measured at 450 nm using an ELISA reader (Multiscan EX; Thermo Electron Corporation, Vantaa, Finland). The three negative controls included on all plates comprised no primary antibody incubation, no secondary antibody incubation, and no primary or secondary antibody incubation.

Endpoint antibody titers were obtained from the lowest dilution factor at which the absorbance of post-immunization sera remained greater than the absorbance of pre-immunization sera (1:200 dilution). For analysis, endpoint dilution factors were expressed as log₂ values (Doolin *et al.* 2002).

Histology

Tissue segments (heart, kidney, liver, lung, spleen, lymph node, ovary, oviduct, and uterus) were collected from all immunized animals and fixed by immersion in 4% paraformaldehyde (PFA; Merck) in PBS[−] for 24 h, dehydrated in ethanol, embedded in paraffin, serially sectioned at 6–8 µm, mounted, and stained with Harris' Hematoxylin and Putt's Eosin. Tissue morphology was compared between VAP1-immunized ($n=7$) and control-immunized ($n=3$) animals. The tissue from healthy, non-immunized colony animals ($n=5$) was also collected, processed in the same manner, and used as a reference for normal morphology.

Transmission electron microscopy

Ovarian follicles, oocytes, and conceptuses were immersed in Superfix (2.5% glutaraldehyde (ProSciTech, Thuringowa, QLD, Australia), 3% PFA, 0.2 M sodium cacodylate buffer) at RT for 2 h (pH 7.4). After three rinses in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixation took place in 1% osmium tetroxide (ProSciTech) diluted with 0.2 M sodium cacodylate buffer for 2 h at RT. Following rinsing in 0.1 M sodium cacodylate buffer, the tissue was dehydrated in an ethanol series, infiltrated with a propylene oxide–Epon araldite mixture, embedded in pure Epon araldite, and polymerized at 60 °C for 48 h. The resin consisted of a mixture of 25 ml Procure 812, 15 ml Araldite 502, 55 ml dodecenyl succinic anhydride, and 1.25 ml benzyldimethylamine (ProSciTech).

Semi-thin sections (1 µm) were cut with glass knives on an ultramicrotome (Ultracut E; Reichert-Jung, Heidelberg, Germany) and stained with 1% toluidine blue and 1% sodium borate in distilled water at 90 °C for 1–2 min. At least three resin-embedded ovarian segments were serially sectioned, for each VAP1-immunized ($n=7$) and non-immunized ($n=5$) animal. Primary oocyte diameters, in primary and antral follicles, were measured at their largest cross-section, and counts made of the number of lipid inclusions (degradation bodies and normal lipid droplets) and the number of vesicles (greater or equal in size to the largest lipid inclusion) within the oocyte cytoplasm.

Thin sections (70 nm), cut with a diamond knife, were contrasted with 3% uranyl acetate and 0.6% lead citrate, mounted on coated grids and examined with a transmission electron microscope (CM 10; Philips, Eindhoven, The Netherlands).

Statistical analysis

Paired-samples *t*-tests were used to compare female fertility (i.e., the percentage of estrous cycles resulting in birth), pre- and post-VAP1 immunization. The correlation between individual fertility reduction and maximum immune titer reached was determined by linear regression. Independent-samples *t*-tests compared the number of lipid inclusions and the number of vesicles per µm² oocyte cytoplasm between VAP1-immunized and non-immunized animals.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research.

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