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Review article

Title. Researching immunocontraceptive vaccines with mares (*Equus caballus*) as both a target and model for African elephant (*Loxodonta africana*) cows: A review

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HIGHLIGHTS

- GnRH and ZP-based vaccines induced reversible suppression of ovarian cyclic activity in mares
- Domesticated mares provided an appropriate clinical and laboratory model for observations of the effects of immunocontraceptive vaccines for wildlife and feral horses
- The incorporation of non-Freund's adjuvants in recombinant and native zona pellucida vaccine formulations showed promising immunological and safety profile responses in treated mares

ABSTRACT

A sequence of studies is reviewed that reported the domestic horse (*Equus caballus*) mare as an appropriate and accessible research platform for recording clinical and laboratory data

post-immunisation with anti- GnRH and -zona pellucida (ZP) immunocontraceptive vaccines. Experience with a native porcine ZP (pZP) vaccine in African elephant (*Loxodonta africana*) cows highlighted needs for improving vaccine formulations and more clearly defining associated ovarian effects and safety profiles. Initially, the efficacy, reversibility and safety of the GnRH vaccine Improvac® in mares was demonstrated using reproductive tract ultrasonography and concurrently measuring serum antibody titres and progesterone concentrations. Results informed the study design and minimally invasive monitoring of post-treatment ovarian steroid responses of this vaccine in free-ranging African elephant cows. A subsequent sequence of studies reported reversible contraceptive and immunological efficacy in pony mares immunised with pZP formulated with Freund's adjuvants. By comparison, mares treated with a recombinant ZP3 and ZP4 (reZP) vaccine showed disappointing responses. Unexpectedly, most pZP-treated mares showed ovarian inactivity. In attempting to understand this response, results showed the involvement of cytotoxic (CD8+) T-cells negatively correlated to serum ovarian steroid and anti-Müllerian hormone (AMH) levels. Of concern was the prevalence of injection-site lesions ascribable to Freund's adjuvants. Following this, mares treated with both pZP and a novel reZP vaccine formulated with non-Freund's adjuvants showed comparable immunological responses and ovarian inactivity, notably without adverse treatment reactions. In addition, measuring AMH showed promise for monitoring ovarian function in anti-ZP-treated animals.

Keywords. Horse; African elephant; Immunocontraception; Vaccine formulation

1. Introduction

In most African countries, the African elephant (*Loxodonta africana*) is listed in the Convention on International Trade in Endangered Species (CITES) Appendix I as threatened with extinction. In contrast, populations in Botswana, Zimbabwe, Namibia and South Africa are burgeoning. The manipulation of these populations' growth rates is of critical importance to the future survival of populations and habitats. Lack of resources can result in elephant breakouts and human-wildlife conflict issues. Additionally, an over population of elephants has negative effects on other species through habitat degradation (Delsink et al., 2006; Kerley and Shrader, 2007; Valeix et al., 2008; Gandiwa et al., 2011).

Similarly, the presence of feral horses is associated with negative environmental effects, including soil compaction and erosion, damage to water bodies and disruption of local ecosystems (Nimmo and Miller, 2007). Yet, like the elephant, the horse attracts significant public empathy, with many populations now protected by law (Garrott and Oli, 2013).

Immunocontraceptive vaccines represent a promising means of population control for wildlife and feral species. Immunocontraceptives induce infertility via antibody production subsequent to stimulation by one or other antigenic component critical to reproductive pathways (Fayrer-Hosken, 2008). Vaccines have been successfully applied in many domestic and wildlife species as they offer a potentially cost-effective, minimally invasive and non-lethal alternative to traditional methods of population control such as culling and surgical sterilisation (Kirkpatrick et al., 2011).

The current focus of immunocontraceptive research and its management applications is with the anti-porcine zona pellucida (pZP) and -GnRH vaccines. The first reported application of immunocontraceptive vaccines in horses (*Equus caballus*) described the testing of a GnRH

vaccine (Garza et al., 1986) followed shortly thereafter by an investigation into pZP vaccination in this species (Liu et al., 1989). Subsequent reports most notably described administration of pZP vaccines in populations of feral horses in North America (Kirkpatrick et al., 1995; Turner et al., 2002; Turner et al., 2007; Killian et al., 2008; Bechert et al., 2013). The horse was reported as both a laboratory (Liu et al., 1989; Willis et al., 1994) and ecological model (Turner et al., 2002) within this body of research.

1.1 Scope of review

Researchers from the Veterinary Population Management Laboratory of the University of Pretoria reported on a series of studies from 2008-2019 (Botha et al., 2008; Benavides Valades et al., 2012; Schulman et al., 2013; Joonè et al., 2017a; Joonè et al., 2017b; Bertschinger et al., 2018; Joonè et al., 2018; Nolan et al., 2018; Joonè et al., 2019; Nolan et al., 2019). These studies investigated the immunological and contraceptive efficacy, safety profiles and ovarian effects of administration of both a commercially available GnRH vaccine and various zona pellucida (ZP)-based vaccine formulations primarily in pony and horse mares. Our laboratory hypothesized that immunocontraceptive research in the domestic mare, while informing practical immunocontraceptive application to feral horses, would also be a promising model for the African elephant. Notably, domestic mares provided a more accessible research platform for recording clinical and laboratory data than would be possible in the African elephant cow and indeed in feral horse populations. Both mares and elephants are considered to be monovular and polyoestrous (Perry, 1953; Asa et al., 1979), although the oestrous cycle and gestation length of the elephant cow are markedly more protracted than in the mare (Ahlers et al., 2012). Both species are long-lived with complex social structures. The elephant social structure is matriarchal, with one cow presiding over a

herd of females and young (Gobush et al., 2009). In comparison, the free-roaming horse exists in a harem with a dominant stallion leading a herd of females and young, however the presence of dominant mares complements this system (Nuñez, 2009). Given the paucity of information related to reproductive physiology in the African elephant, further research to validate the mare as a model for this species is warranted. Interestingly, the mare is well established as an appropriate comparative animal model of reproductive dynamics in women (Ginther et al., 2004; Carnevale, 2008; Ginther, 2012). Similarly, the African elephant holds potential as a model for reproductive function in women, given the similarity in reproductive lifespan between the two species (Stansfield et al., 2011).

This review focuses on research objectives that investigated:

- Immunological, ovarian and contraceptive effects, reversibility and safety profile of a GnRH vaccine, Improvac® (Zoetis Animal Health, South Africa) (Botha et al., 2008; Schulman et al., 2013) and a native porcine zona pellucida (pZP) vaccine formulation derived from sow ovaries harvested post-slaughter (Joonè et al., 2017a; Nolan et al., 2018; Joonè et al., 2019; Nolan et al., 2019);
- Development and validation of minimally-invasive methods for measuring post-vaccination ovarian cyclical responses (Botha et al., 2008; Schulman et al., 2013; Joonè et al., 2018; Nolan et al., 2018);
- Application of this method to measure the response to Improvac® in free-ranging African elephant cows (Benavides Valades et al., 2012);
- Describing ovarian inactivity as an associated feature of ZP-based vaccination (Joonè et al., 2017a; Joonè et al., 2017b; Joonè et al., 2018; Nolan et al., 2018);

- Immunological, ovarian and contraceptive effects associated with vaccination using alternative ZP antigens, specifically recombinant ZP3 and ZP4 proteins (reZP) (Joonè et al., 2017a; Joonè et al., 2018; Joonè et al., 2019) and
- Alternative ZP vaccine formulations, specifically native pZP and reZP antigens formulated with non-Freunds adjuvants (Nolan et al., 2018; Nolan et al., 2019).

2. Investigating anti-GnRH vaccination in mares and elephant cows

In mares, pZP vaccination was reported as an effective contraceptive method that neither prevented oestrous cyclicity nor suppressed its related behaviours (Kirkpatrick et al., 2009). In contrast, GnRH vaccines induce production of antibodies to GnRH, preventing GnRH binding to the pituitary gonadotrophs with subsequent suppression of luteinising hormone (LH) and, to a lesser extent, follicle stimulating hormone (FSH) secretion. This withdrawal of gonadotrophic support for cyclical ovarian follicular activity ultimately results in anoestrus (Stout and Colenbrander, 2004; Hennessy, 2008). Several studies using GnRH vaccines reported variably successful but variable suppression of ovarian activity preventing oestrus behaviour in adult mares (Garza et al., 1986; Tshewang et al., 1997; Dalin et al., 2002; Stout and Colenbrander, 2004; Imboden et al., 2006). Additionally, age-associated effects on both ovarian response (Dalin et al., 2002; Stout and Colenbrander, 2004) and injection-site reactions (Stout and Colenbrander, 2004) to treatment were reported. Small study populations and brief monitoring periods that prevented observations of reversibility and the associated duration of vaccination-induced anoestrus limited these reports.

Our group's initial investigations assessed the potential of anti-GnRH vaccines as an effective, reversible and safe alternative to the well-established administration of pZP

vaccines in horse mares (Botha et al., 2008; Schulman et al., 2013). Following reports of the successful down-regulation of aggression and androgen-related behaviours via an anti-GnRH vaccine in African elephant bulls (De Nys et al., 2003; De Nys et al., 2010) these investigations additionally aimed at informing future application of these vaccines in free-ranging African elephant cows. The response to treatment was monitored via serial measurements of faecal androgen metabolite concentrations (Palme and Möstl, 1994), already validated in this species (Ganswindt et al., 2002).

Our study's primary aims were to observe the effects of active immunisation against GnRH on ovarian cyclic activity, reversibility, immunological responses and safety profile in a large group of horse mares (n= 65) during a 720 d observation period. Mares were age-categorised to observe age effects on treatment and a saline-treated control group was included. All treated mares received two gluteal intramuscular injections of 400 µg Improvac® (RnRF-protein conjugate, Zoetis Animal Health, South Africa) with a 35 d interval between treatments. Clinical data included findings from trans-rectal palpation and ultrasonographical examination of the internal reproductive tract, monitoring of general health parameters and injection sites. Laboratory data obtained by serial blood sampling measured serum progesterone concentration (SPC) via radioimmune assay (RIA) and anti-GnRH antibody titres by enzyme immunoassay (EIA). By 70 d post-treatment, all treated mares showed clinical and SPC measurements consistent with anoestrus. By 720 d, 92.2% resumed cyclic ovarian activity with a mean interval of 417.8 d (range: 232-488 d).

Significantly, an age effect on the interval to resumption, but not on antibody response, was observed between the youngest (≤ 4 y) with the longest interval, and the oldest (≥ 11 y), mare categories. Injection-site reactions were mild and transient in nature. Usefully, the close concordance in this large group between clinical variables and SPC served to validate

SPC as a minimally invasive measure of ovarian cyclical activity. This would potentially obviate (or at least minimise) more invasive clinical interventions when studying the cyclical effects of planned future vaccine treatment in free-ranging mares or wildlife species (Schulman et al., 2013).

Informed by the mare study and using a similar study design, Improvac[®] was administered for the first time in a group of free-ranging adult African elephant cows (Benavides Valades et al., 2012). This controlled study noninvasively monitored eight treated cows (two treatments of 600 µg Improvac[®] per 3 ml dart-delivered intramuscular dose) and four control cows over 12 months using faecal progesterone metabolite concentrations (FPMC) to assess the resultant cyclic ovarian activity. Vaccination failed to induce anoestrus in treated cows. Partially at least, this result was ascribed to an ineffectively low treatment dose. Subsequent investigations reported the efficacy of Improvac[®] at 1000 µg per dose in African elephant bulls with two initial treatments five weeks apart and subsequent booster intervals of 5-6 months (Bertschinger and Lueders, 2018), and ovarian suppression required more frequent administration at higher doses (Boedeker et al., 2012; Bertschinger and Lueders, 2018). Furthermore, the monitoring period proved too brief to distinguish the normal occurrence of an environmental dry season anoestrus from that due to the GnRH vaccine (Benavides Valades et al., 2012). However, the results indicated the occurrence of irregular oestrous cycles in free-ranging elephants similar to captive elephants and a probable relationship between ecological conditions and endocrine activity.

3. Investigating ZP-based vaccination

3.1 Native pZP vaccine in mares

Traditionally, the mechanism of action of anti-pZP vaccines was considered to be an antibody-based block to sperm-oocyte binding and subsequent fertilisation (Barber and Fayer-Hosken, 2000). In theory, therefore, pZP should exert little influence on the ovary and reproductive cyclicity. This factor has been an important determinant in its selection as a preferred immunological method of population control in species with complex social structures including horses and African elephants (Fayer-Hosken et al., 2000; Kirkpatrick et al., 2011). However, reported ovarian dysfunction associated with pZP vaccination in rabbits (Wood et al., 1981), non-human primates (Gulyas et al., 1983), dogs (Mahi-Brown et al., 1988) and sheep (Stoops et al., 2006) supported further research defining the vaccine's mechanism of action. The few reports describing ovarian effects in the mare included an early study reporting no affect one year after treatment (Liu et al., 1989) and conflicting results from studies involving repeated contraceptive treatments in feral horse populations (Kirkpatrick et al., 1992; Kirkpatrick et al., 1995; Powell and Monfort, 2001). More recently, a long-acting pZP vaccine formulation resulted in ovarian inactivity in nearly all treated mares (Bechert et al., 2013).

Experience with pZP vaccination for fertility control in African elephant cows highlighted both the needs for improvement of both the available vaccine formulations and understanding of their effects on targeted animals (Fayer-Hosken et al., 2000; Bertschinger et al., 2008; Ahlers et al., 2012; Bertschinger et al., 2012; Delsink et al., 2013; Bertschinger and Caldwell, 2016; Bertschinger et al., 2017). The current vaccine, produced and supplied solely by the Veterinary Population Management Laboratory (University of Pretoria) in terms of current national legislation (Bertschinger et al., 2018), consists of solubilised pZP proteins produced from abattoir-derived pig ovaries and formulated with Freund's modified

complete adjuvant (FMCA) as a primary and Freund's incomplete adjuvant (FIA) as a booster dose, respectively.

Together with an increased awareness of potentially deleterious ovarian and vaccine reactions reported in the mare, these considerations prompted us to identify several further research priorities. These included investigating immunological, ovarian, contraception and safety effects associated with alternative ZP antigens, specifically recombinant ZP3 and ZP4, and investigations into non-Freund's adjuvants. Study designs, informed by the GnRH vaccine studies, again employed domestic horse mares as a research platform to obtain clinical and laboratory data.

A randomly allocated group of pony mares (n=7) was treated with native pZP incorporating FMCA and FIA into primary and booster vaccinations respectively. Mares were monitored via transrectal palpation and ultrasonography of the reproductive tract and serum sampling for antibody titres and ovarian steroid measurements. Post-treatment, any cyclic mares were artificially inseminated using fresh semen at an optimal breeding time. One mare showed continued oestrous cyclicity characterised with apparently normal, but infertile, ovulations. Notably, however, the remaining six mares showed aberrant oestrous cyclicity, characterised by intermittent to persistent anoestrus. These results suggested that infertility during pZP immunocontraception was, at least, as much a result of ovarian inactivity as it was to an antibody-based block to sperm-oocyte binding (Joonè et al., 2017a).

This study incorporated the novel application of measuring anti-Müllerian hormone (AMH) levels following pZP treatment. Mares treated with pZP showed significantly lower levels of AMH than control mares (Joonè et al., 2018). As AMH is expressed primarily by granulosa cells of small to medium sized follicles (Claes et al., 2014), these results suggested

either profound inhibition of follicular development or abnormal granulosa cell function during effective pZP immunocontraception.

In attempting to understand the observed ovarian response, these studies additionally investigated the cell-mediated immune response to ZP-based vaccines in domestic mares. Previously it was hypothesized that cytotoxic (CD8+) T-cells were responsible for the ovarian effects of pZP vaccines in other species (Millar et al., 1989; Bradley et al., 1999; Prasad et al., 1999; Hinds et al., 2003; Barfield et al., 2006). Interestingly, our results confirmed that both helper (CD4+) and cytotoxic T-cells were involved in the immune response to pZP in mares. Moreover, significant negative correlations between cytotoxic T-cell responses and serum AMH, oestradiol and progesterone levels were detected (Joonè et al., 2019). Clarifying the role of cytotoxic T-cells during pZP immunocontraception warrants further research.

3.2 Recombinant ZP vaccines in mares

Despite its recognised successful administration for population management in various species, important limitations are associated with native pZP vaccine. These include restriction of registration as a commercial product due to its biological origin and associated risks of disease transfer and the uneconomical and cumbersome production methods (Nolan et al., 2019). A vaccine incorporating recombinant ZP proteins has the potential to be produced more economically and efficiently, providing a purer and more standardised product with the potential for international distribution (Gupta and Bansal, 2010). The use in mice of a reZP vaccine derived from an *Escherichia coli* platform expressing porcine ZP glycoproteins 3 and 4 was reported (Gupta et al., 2013). This vaccine was included with native pZP vaccine in a comparative study in mares to assess its contraceptive efficacy, ovarian, clinical and immunological effects (Joonè et al., 2017a). Freund's modified

complete and FIA adjuvants were incorporated into this reZP vaccine formulation for administration in seven mares. The results in these mares suggested that reZP exerted a partial, but not statistically significant, contraceptive effect. Furthermore, anti-ZP antibody titres measured using enzyme-linked immunosorbent assay (EIA) following reZP treatment were lower than the titres induced by native pZP (Joonè et al., 2017a) (Figure 1).

Subsequently, in collaboration with Biosciences, Council for Scientific and Industrial Research, South Africa, the reZP was modified and incorporated in a novel reZP vaccine and was observed to induce the development of anti-ZP antibody titres comparable to those in native pZP-treated horses (Nolan et al., 2019) (Figure 1). In addition to observing this immunological response, notably both the native pZP and the reZP vaccines in this study were formulated with non-Freund's adjuvants and treated mares showed varying levels of ovarian suppression (Nolan et al., 2018).

3.3 Zona pellucida-based vaccines formulated with non-Freund's adjuvants in mares

Our initial studies observed an unacceptable prevalence of adverse injection site reactions in both vaccine-treated (pZP and reZP) and adjuvant-treated control mares. These ranged from ultrasonographically visible intramuscular lesions to overt sterile granuloma formation (Joonè et al., 2017a). These findings were supported by reports describing similar findings in mares (Bechert et al., 2013) and treated elephant cows (Bertschinger et al., 2018). Freund's adjuvants were common to these studies. Consequently, an important focus in our research group has been investigating suitable alternatives to Freund's adjuvants. A pilot study in geldings compared various alternative adjuvants and established that the commercially available adjuvants PetGel A (Montanide™ PetGel A, Seppic, France) and Poly (I:C) (HMW VacciGrade™, Invivogen, USA) were promising alternatives for incorporation in

ZP vaccines (Nolan et al., 2019). Subsequently, these two adjuvants replaced Freund's adjuvants for incorporation into vaccine formulations with both native pZP and the novel reZP for investigation in a comparative mare study. Neither administration of pZP nor the reZP vaccines formulated with PetGel A and Poly (I:C) (nor indeed the adjuvant-treated controls) were associated with any appreciable adverse post-injection effects in treated mares (Nolan et al., 2018).

3.4. Zona pellucida-based vaccination in African elephants

The first field trial with pZP vaccine administration in free-ranging African elephants occurred from 1996 to 2000 in the Kruger National Park of South Africa (Fayrer-Hosken et al., 2000). Animals were immobilised to facilitate ultrasonographical pregnancy detection and hand injected with native pZP vaccine and subsequently boosted via remotely delivered treatments. An initial contraceptive efficacy of approximately 58% later improved to 80% by decreasing the intervals between the three administered treatments to two weeks. Since then, the vaccine has proved to be a practical and non-lethal method of manipulating the growth rate of elephant populations particularly in small to medium-sized fenced reserves in South Africa (Bertschinger et al., 2018). In smaller reserves, enabling individual cow identification, a contraceptive efficacy of 100% was achieved whereas in larger reserves, where this is impossible, a blanket treatment approach was shown to be $\geq 90\%$ effective (Bertschinger et al., 2018). By 2018, ≥ 800 cows were undergoing treatment across 27 South African reserves (Nolan, 2019).

4. Measuring the ovarian response to treatment in mares and elephants

The mare studies reviewed here used clinical examination of the ovaries and reproductive tracts corroborated by laboratory measurements of ovarian steroids to define post-treatment ovarian inactivity. Anoestrus was defined by bilaterally small ovaries (both $<25\text{ cm}^3$) lacking a corpus luteum or any follicle $>15\text{ mm}$ in diameter and basal SPC ($<1\text{ ng/mL}$) (Botha et al., 2008; Schulman et al., 2013; Joonè et al., 2017a; Nolan et al., 2018; Nolan et al., 2019). This enabled relatively precise determination of both the onset of ovarian suppression and reversibility over extended post-treatment monitoring periods. Interestingly, in the final study reviewed in this series, both anti -pZP and -reZP antibody titres were significant predictors of ovarian shutdown ($P= 0.001$ 95% CI [0.644, 2.537], $P= 0.001$, 95% CI [1.321, 4.758], respectively (Nolan et al., 2019). This model was used to monitor luteal profiles as an indication of post-treatment ovarian response to GnRH vaccine in free-ranging elephant cows (Benavides Valades et al., 2012). The study circumvented serial serum sampling by non-invasive serial sampling for faecal progestagen metabolite (FPM) evaluation via an EIA detecting faecal 5α -reduced pregnanes.

Anti-Müllerian hormone concentration was measured using a commercially available EIA in mares treated with both anti-GnRH and-ZP-based vaccines and was compared with other established clinical and laboratory variables including age, ovarian volumes, follicular dynamics and SPC (Joonè et al., 2018; Nolan et al., 2018). In these studies, AMH levels in GnRH-treated mares were unchanged, whereas pZP-treated mares showed profound and reversible AMH suppression (Joonè et al., 2018), supporting AMH as a marker for ovarian function during ZP-based immunocontraception. A single sampling intervention for AMH analysis, unlike sampling for ovarian steroids or their metabolites would suffice to define ovarian and follicular status. This may prove useful in situations where serial gynaecological exams are unfeasible, such as in free-ranging African elephant cows.

5. Conclusions and future directions

Fayrer-Hosken (2008) stated “The ultimate goal of fertility control of a population is an agent that it is non-toxic, completely reversible, has multi-year efficacy, is easily administered, is devoid of behavioural side-effects, cost-effective and is efficacious in multiple species and both sexes”. The administration of contraceptive agents and the subsequent monitoring of their efficacy and safety profiles in free-ranging domestic and wildlife populations is associated with several unique challenges. Previously, most reported mare studies described vaccine application in feral or free-roaming horse populations (Kirkpatrick et al., 1997; Turner Jr et al., 1997) with obvious limitations in these populations on the frequency and scope of sampling interventions for clinical data, hormonal and immunological assays. Safety profiles, including injection site reactions, were difficult to assess without compliance and continuous access. Further research-associated limitations in wildlife species have included lack of controls, identification of individual study animals and relatively brief monitoring durations, impeding detection of reversibility (Kirkpatrick et al., 1990; Turner Jr et al., 1997; Turner Jr et al., 2001; Turner et al., 2002).

This sequence of reported studies has shown that both GnRH and ZP-based vaccines induce reversible suppression of ovarian cyclic activity in mares. Using domestic mares facilitated serial, in-depth clinical and laboratory observations of individual animals over extended observation periods. Results improved understanding of these vaccines’ mechanism of action and validated the use of serum AMH concentrations for the monitoring of ovarian activity in horses not amenable to serial gynaecological examinations.

The development of a synthetic pZP vaccine showed significant progress. These initial studies support its further investigation as a potentially effective alternative to the native

pZP vaccine without the associated limitations particularly with its production and supply. Moreover, alternatives to Freund's adjuvants for incorporation into ZP-based vaccine formulations have undergone preliminary investigation with promising results that supported immunological efficacy with an enhanced safety profile in horses. Future investigations should aim at further clarifying the ovarian, immunological and importantly, the contraceptive effects of reZP vaccines formulated with non-Freund's adjuvants. Fulfilling these aims supports the important goal of investigating the administration of the novel reZP vaccine in free-ranging elephant cows as a population control tool with the abovementioned potential benefits. Furthermore, management circumstances may indicate selection of alternatives to ZP-based vaccines in elephant cows, warranting further investigation of GnRH vaccines.

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Declaration of interests

None.

ECAR INVITED LECTURE: CONFLICT OF INTEREST STATEMENT

On behalf of my co-authors, I would like to submit this revised manuscript for consideration for publication by your journal. This manuscript is a review linked with an ECAR Invited Lecture. The manuscript title is entitled: **Researching immunocontraceptive vaccines with mares (*Equus caballus*) as both a target and model for African elephant (*Loxodonta africana*) cows: A review**

There are no conflicts of interest or requirements for permission to include any of the material.

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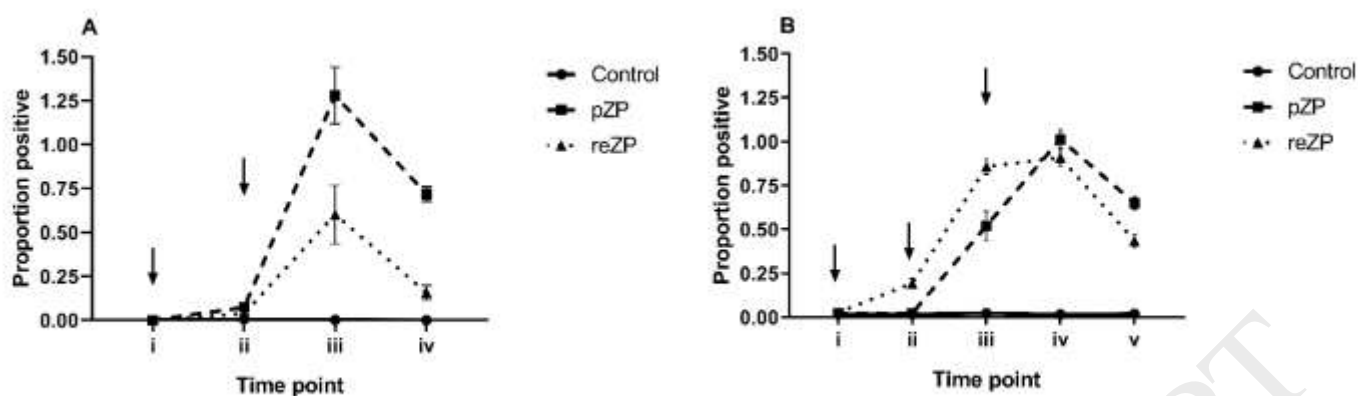


Figure 1

Mean anti-pZP antibody response expressed as a proportion of a positive standard (+ s.e.)

A: pZP and reZP* vaccines formulated with Freund's modified complete (FMCA) and incomplete (FIA) adjuvants for primary and booster vaccinations, respectively

Control; pZP & FMCA/FIA; reZP & FMCA/FIA at four time points: i (pre-vaccination); ii (28 d post primary vaccination); iii (28 d post booster vaccination), iv (70 d post booster vaccination) (Joonè et al., 2017a)

B: pZP and reZP** vaccines formulated with PetGelA and Poly (I:C) adjuvants for primary and booster (pZP single booster; reZP double booster) vaccinations

Control; pZP & PetGel A & Poly (I:C); reZP** & PetGel A & Poly (I:C) at five time points: i (pre-vaccination (and primary vaccination for reZP)); ii (35 d post primary vaccination for reZP and pre-vaccination pZP and control); iii (35 d post primary/booster vaccination); iv (35 d post booster/second booster vaccination); v (70 d post booster/second booster vaccination) (Nolan et al., 2019)

Arrows depict approximate treatment time

*Gupta et al., 2013; **CSIR Biosciences, South Africa