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Reproductive Toxicology

Lack of effects on female fertility or pre- and postnatal development of offspring in rats after exposure to AS03-adjuvanted recombinant plant-derived virus-like particle vaccine candidate for COVID-19



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ABSTRACT

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Keywords: SARS-CoV-2 COVID-19 vaccine Virus-like particles AS03 Developmental and reproductive toxicity study Pregnancy Fertility Immunogenicity Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection resulting in the coronavirus disease 2019 (COVID-19) has afflicted tens of millions of people in a worldwide pandemic. A recently developed recombinant Plant-Derived Virus-Like Particle Vaccine candidate for COVID-19 (CoVLP) formulated with AS03 has been shown to be well-tolerated and highly immunogenic in healthy adults. Since the target population for the vaccine includes women of childbearing potential, the objective of the study was to evaluate any untoward prenatal and postnatal effects of AS03-adjuvanted CoVLP administered intramuscularly to Sprague-Dawley female rats before cohabitation for mating (22 and 8 days prior) and during gestation (Gestation Days [GD] 6 and 19). The embryo-fetal development (EFD) cohort was subjected to cesarean on GD 21 and the pre/post-natal (PPN) cohort was allowed to naturally deliver. Effects of AS03-adjuvanted CoVLP was evaluated on pregnant rats, embryo-fetal development (EFD), during parturition, lactation and the development of the F1 offspring up to weaning Vaccination with AS03-adjuvanted CoVLP induced an antibody response in F0 females and anti-SARS-CoV-2 spike-specific maternal antibodies were detected in the offspring at the end of the gestation and lactation periods. Overall, there was no evidence of untoward effects of AS03-adjuvanted CoVLP on the fertility or reproductive performance of the vaccinated F0 females. There was no evidence of untoward effects on embryo-fetal development (including teratogenicity), or early (pre-weaning) development of the F1 offspring. These results support the acceptable safety profile of the AS03-adjuvanted CoVLP vaccine for administration to women of childbearing potential.

1. Introduction

In December 2019, a series of severe atypical respiratory disease cases occurred in Wuhan, China and a novel coronavirus named SARS-CoV-2 was rapidly identified as the causative agent of coronavirus disease 2019 (COVID-19) [1,2]. SARS-CoV-2 virions consist of a helical

nucleocapsid, formed by association of nucleocapsid phosphoproteins with viral genomic RNA that is surrounded by a lipid bilayer, into which three structural proteins are inserted: the spike (S), the membrane, and the envelope proteins. The new coronavirus rapidly spread around the globe resulting in the World Health Organization's (WHO) declaration of a pandemic on March 11, 2020. As of today, SARS-CoV-2 has caused

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Abbreviations: AS, adjuvant system; CI, confidence interval; COVID-19, coronavirus disease 2019; CoVLP, coronavirus-like particle; CT, cytoplasmic tail DART developmental and reproductive toxicity; EFD, embryofetal development; ELISA, enzyme-linked immunosorbent assay; GD, gestation day; GLP, good laboratory practice; GMT, geometric mean titer; HCD, historical control data; HRP, horse-radish peroxidase; IM, intramuscular; MRD, minimum required dilution; OECD, organisation for economic co-operation and development; PBS, phosphate buffered saline; PPN, pre and postnatal; PND, postnatal day; QA, quality assurance; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SOP, standard operating procedure; RBD, receptor binding domain; S, spike; SD, study day; TM, transmembrane domainVLP virus-like particle.

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over 260 million infections and more than 5.2 million deaths worldwide (WHO Coronavirus Disease (COVID-19) Dashboard, https://covid19.wh o.int/, 2021). Although therapeutics, including monoclonal antibodies and antivirals, are important to limit the burden of COVID-19, effective vaccines are essential to control the pandemic. Only a small number of vaccines against COVID-19 are currently approved on an emergency approval basis and, even together, they do not cover the global need for mass vaccination. Medicago has developed a SARS-CoV-2 vaccine candidate using a platform technology based on transient expression of recombinant proteins in the non-transgenic plant Nicotiana benthamiana and a disarmed Agrobacterium tumefaciens as a transfer vector to move targeted DNA constructs into the plant cells. The newly introduced DNA then directs the expression of the desired viral proteins. In this case, the newly synthesized full-length S proteins trimerize and move to lipid rafts where they spontaneously assemble into virus-like particles (VLPs) that 'bud' off the surface of the plant cell [3]. The S proteins in plant-derived coronavirus-like particles (CoVLP) are in a stabilized, prefusion conformation that resembles the native structure seen on SARS-CoV-2 virions. The prefusion form of S protein is preferred as a vaccine antigen since it contains several epitopes in the receptor binding domain (RBD) that are primary targets for neutralizing antibodies. Moreover, a study of the closely-related Middle-Eastern Respiratory Syndrome virus (MERS) suggests that the prefusion state of the S protein is a potent immunogen with dose-sparing properties [4].

In the pandemic context, CoVLP has been formulated with the immunostimulatory Adjuvant System AS03 to enhance the immune response [5-7] and as a consequence to reduce the amount of antigen needed per dose (AS03-adjuvanted CoVLP). The Adjuvant System AS03, an α -tocopherol-containing oil-in-water emulsion, has been used in the licensed pandemic A/H1N1pdm09 influenza vaccines Arepanrix H1N1 (in Canada) and Pandemrix (in Europe), as well as in other licensed (Q Pan H5N1, in the USA) or candidate vaccines [8]. Reproductive and developmental toxicity studies in rats revealed no evidence of toxicity of AS03 alone or in combination with influenza antigens [9]. The safety of AS03-adjuvanted CoVLP candidate vaccine has been evaluated in several nonclinical studies in mice (unpublished data), nonhuman primates [10] and clinical studies in adults (Phase 2/3 clinical trial (NCT04636697) ongoing) in which AS03-adjuvanted CoVLP was administered via intramuscular (IM) route. The results of the Phase 1 clinical trial showed that this vaccine has an acceptable safety profile and induces both humoral and cellular immune responses [11]. Since the target population for the vaccine includes women with childbearing potential, a nonclinical developmental and reproductive toxicity (DART) study was required to support the clinical development and licensure of the vaccine [12]. The results of the DART study are reported herein and demonstrate no untoward effects of AS03-adjuvanted CoVLP in F0 females, fetuses or F1 offspring. This is one of the first detailed descriptions of a DART assessment following the administration of an AS03-adjuvanted anti-COVID VLP vaccine. To our knowledge, animal DART data are currently available only for the Pfizer/BioNTech and AstraZeneca COVID-19 vaccine in rodents, which did not reveal any safety concerns [13,32].

2. Materials and methods

2.1. Regulatory guidelines and data quality

The study was conducted at Charles River Laboratories Montreal ULC (Senneville, Québec, Canada) and in accordance with the recommendations of international guidelines applicable on the development of vaccine and considerations for developmental toxicity study of the EMA [14], ICHS5 (R3) [15], OECD [16], FDA [17] and WHO [18].

The study was conducted in accordance with the OECD Principles of Good Laboratory Practices (GLP) [19] and as accepted by Regulatory Authorities throughout the European Union, USA (FDA), Japan (MHLW), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement; and in accordance with Charles River Laboratories Montreal ULC (Senneville, Québec, Canada) standard operating procedures (SOPs). The study was monitored by Charles River Quality Assurance (QA) personnel to ensure the facilities, equipment, personnel, methods, practices, records, and controls used were in conformance with GLP standards for the conduct of nonclinical studies. The QA personnel also reviewed the study protocols and amendments, conducted inspections at intervals considered adequate to assure the integrity of the study, and audited the final report to assure that it accurately described the methods and SOPs followed and that reported results were an accurate reflection of the raw data.

2.2. AS03-adjuvanted CoVLP vaccine candidate

The full-length S glycoprotein of SARS-CoV-2, strain hCoV-19/USA/ CA2/2020, corresponding in sequence to nucleotides 21563–25384 from EPI_ISL_406036 in GISAID database (https://www.gisaid.org/) was expressed in *Nicotiana benthamiana* plants as previously described [3]. The S protein was modified with R667G, R668S and R670S substitutions at the S1/S2 cleavage site to increase stability, and K971P and V972P substitutions to stabilize the protein in prefusion conformation. The signal peptide was replaced with a plant gene signal peptide and the transmembrane domain (TM) and cytoplasmic tail (CT) of S protein was also replaced with TM/CT from Influenza H5 A/Indonesia/5/2005 to increase VLP assembly and budding. The self-assembled VLPs bearing S protein trimers were isolated from the plant matrix and subsequently purified using a process similar to that described for Medicago's plant-derived influenza VLP vaccine candidates [20].

The AS03 Adjuvant System, an oil-in-water emulsion containing 11.86 mg DL- α -tocopherol, 10.69 mg squalene and 4.86 mg Polysorbate 80 per human dose of 0.25 mL, was supplied by GSK, (Rixensart, Belgium) and was used as recommended by the manufacturer. The control article was phosphate buffered saline (PBS) solution with Polysorbate 80. On each dosing day, CoVLP was diluted with PBS to achieve the appropriate concentration and then mixed in a 1:1 (volume:volume) ratio with adjuvant prior to administration.

2.3. Animals and husbandry

Sprague-Dawley Crl:CD rats were supplied by Charles River (Raleigh, North Carolina, USA). Female rats were approximately 7 weeks of age at the start of the study. In summary, animals were maintained under standard laboratory conditions (lighting: 12 / 12 h, temperature: 22 \pm 3 °C, relative humidity: 30-70 %) with certified rodents pellet feed (Lab Diet Certified Rodent Diet 5002) and had ad libitum access to water treated by reverse osmosis. Animals were acclimated for at least 15 days to the laboratory environment prior to initiation of dosing. F0 females were group-housed, except during cohabitation for mating, in polycarbonate cages with bedding. After a 22-day pre-mating dosing period, each F0 female was cohabited on a 1:1 basis with a untreated proven breeder male of the same strain for up to 14 days. At the end of the mating period, females failing to show signs of mating were newly paired with a new proven breeder male for up to 7 additional days. Females with confirmed presence of spermatozoa observed in a smear of the vaginal lavage and/or a copulatory plug observed in situ were considered to have mated. On the day mating was confirmed (referred as GD 0), the female was transferred back to group housing. On GD 20, PPN Phase F0 females were single housed until parturition at which time dams were housed with their litter until termination.

2.4. Study design

All procedures involving the care and use of animals were performed in accordance with the Canadian Council on Animal Care, the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Academies Press, Washington, DC) 8th edition and Animal Research Reporting *in vivo* Experiments guidance and approved by Charles River Institutional Animal Care and Use Committee. The overall design of the DART study is illustrated in Fig. 1.

Animals assigned to the study were selected based on a screening for the presence of full-length trimeric S protein to minimize bias during immunogenicity analyses. Sprague-Dawley female rats (n = 92) were randomly assigned either to a placebo group (n = 46) or to AS03adjuvanted CoVLP group (n = 46) using a computer-based randomization program balanced by body weight. Once mated, females were allocated to either a cesarean delivery cohort (embryofetal development phase [EFD], n = 22/group) euthanized on gestation day (GD) 21 or to a natural delivery cohort (pre-and postnatal phase [PPN], n = 24/group) and allowed to rear offspring until postnatal day (PND) 21. Animals were administered AS03-adjuvanted CoVLP or placebo 22 days prior to cohabitation for mating (study day [SD] 1), 7 days prior cohabitation (SD 15), on GD 6 and GD 19. The chosen administration regimen allowed induction of peak immune response at mating and during the critical phases of pregnancy (i.e., the period of organogenesis) and maximized exposure to maternal antibodies throughout the embryonic, fetal, and early postnatal periods. On each administration day, each female rat received 0.2 mL of either AS03-adjuvanted CoVLP (CoVLP dose of 3 µg) or phosphate buffered saline by IM injection at one injection site either in the lateral compartment of the right or left thigh muscles. The F1 offspring did not receive any injections. As per FDA Guidance to the industry [17], a 3 µg dose of CoVLP was deemed sufficient to assess its safety when administered with AS03 since the antigen dose exceeded the human dose on a body weight basis by 160-fold (based on 3 µg CoVLP for a 0.250 kg rat and 3.75 µg CoVLP for a 50 kg woman), while the dose of AS03 exceeded the human dose by 80-fold (based on 0.1 mL AS03 for a 0.250 kg rat and 0.25 mL AS03 for a 50 kg woman). The dose administered to animals was confirmed by dose formulation analyses at the time of the first (SD 0) and last (GD 19) administrations.

2.5. In-life evaluations

Observations for moribundity and mortality were performed twice daily during pre-mating, gestation, and lactation. Clinical observations were performed at least once daily. Each injection site was observed for erythema (redness) and edema (swelling) prior to dosing and daily for 7 days following each dosing. Erythema was scored as 1 for the presence of skin redness and edema was scored as 1 for area around of exposure and 2 for area beyond area of exposure. Body weights and food consumption were recorded throughout the study. Estrous cycles were determined for at least 14 consecutive days before initiation of dosing and continuing until the day of positive identification of mating. From GD 20, natural delivery cohort females were checked at least three times daily for signs of parturition. The day of completion of littering was defined as PND 0. Dams were evaluated for length of gestation, litter size and pup viability at birth. Each pup was examined daily for clinical observations (including any external developmental abnormalities), viability, and sex. On PND 4, litters were reduced to a maximum of 8 pups per litter (4 pups/sex when possible) using a computerized randomization procedure based on random number generator. The pups selected to be culled were terminated. Live pups were weighed on PND 0, 4, 7, 14 and 21. Each pup was evaluated once daily from PND 1 for achievement of pinna unfolding, from PND 12 for achievement of eye opening and auricular startle response and on PND 21 to test pupillary closure and visual placing responses.

2.6. Blood collection

Blood samples were collected from all F0 female rats by jugular venipuncture prior to the first dose administration for prescreening, baseline antibody levels (SD -1), and on SD 14, and from the abdominal aorta (terminal procedure) on GD 21 (EFD cohort only) and PND 21 (PPN cohort only). On GD 21, the EFD F0 dams were deeply anesthetized using isoflurane, the abdomen and uterus were opened to expose the fetuses and blood samples were collected by cardiac puncture from approximately 50 % of the live fetuses in each litter using capillary tubes, pooled per litter into serum separator tubes and processed. Following completion of the fetal blood collection, blood samples were collected by abdominal aorta puncture from the anesthetized dams. Blood was also collected from 1 male and 1 female pups from each litter at terminal euthanasia on PND 21. All samples were centrifuged, and the resultant sera were separated and frozen immediately on dry ice and transferred in a -80° C freezer until analyzed.



Fig. 1. Female rats from Placebo group injected with 0.2 mL of PBS; female rats from test group injected with 0.2 mL of CoVLP adjuvanted with AS03 (CoVLP dose of 3 µg). EFD: Embryofetal Development, GD: Gestation Day, PND: Postnatal Day, PPN: Pre and Postnatal, SD: Study Day. N, total number of rats; n, number of rats within the group. Syringe indicates immunization day.

2.7. Necropsy and terminal procedures

EFD F0 female rats and the PPN F0 females and their offspring were euthanized and necropsied on GD 21 and PND 21, respectively. F0 females and F1 offspring were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. Fetuses selected for immunogenicity analysis were euthanized by cardiac puncture and the remaining animals were euthanized by subcutaneous administration of an euthanyl/lidocaine mixture. All F0 and F1 animals retained on the study were subjected to a complete necropsy at termination, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. The gravid uteri of the EFD F0 female rats were examined for number and distribution of corpora lutea, implantation sites, live and dead fetuses and early/late resorptions as well as any abnormalities in placentae (size, color and shape). At cesarean, fetuses were removed from the uterus and retained for examination (body weight, sex, external, visceral and skeletal abnormalities). Approximately half of the fetuses were selected for visceral examination using a modification of the microdissection techniques of Staples [21]. The heads of these fetuses were removed and fixed in Harrison's solution for examination by the technique of Wilson [22]. The remaining fetuses were selected immunogenicity blood collection and for skeletal examination after staining with alizarin red S [23,24]. Fetal observations were classified as either malformations (rare structural defects thought to be life threatening or of major physiological consequence) or variation (commonly minor abnormalities, defects or alternative forms that are of no known major physiological consequence). For PPN F0 dams, the number of implantation site scars were recorded at euthanasia.

2.8. Immunogenicity

Specific-IgG antibodies to full-length trimeric S protein were measured in the serum samples using a qualified semi-quantitative ELISA method at Charles River Facility. Sera were diluted 1/100 (minimum required dilution or MRD) with EZ Block and loaded on a microtiter plate coated with recombinant full-length trimeric S protein from SARS-CoV-2/Wuhan/2019 (Immune Technology, New York, NY). Rat anti-SARS-CoV-2 S protein IgG antibodies present in the rat serum samples bound to the immobilized antigen. The detection of bound anti-SARS-CoV-2 S protein IgG was performed using a goat anti-rat IgG horseradish peroxidase (HRP) conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). The bound HRP metabolized tetramethylbenzidine, subsequently added to each well, to generate a chromophore, for which the absorbance at 450 nm was determined using a spectrophotometer. The absorbance of the chromophore correlated with the level of rat anti-S protein IgG antibodies present in the samples.

2.9. Statistical analyses

The descriptive statistics and statistical comparisons were performed where appropriate. Numerical data were analyzed according to occasion and, as applicable, sex and/or by litter. Levene's test was used to assess the homogeneity of group variances. For in-life evaluations and postmortem observations, pairwise comparisons were conducted using two sided tests followed by a Dunnett's (equal variance) or Dunn's (unequal variance) post-hoc test. The non-parametric variables were evaluated using an overall Kruskal-Wallis test followed by a Dunn's test. For incidence, Fisher's exact test or Chi² test were used to conduct pairwise group comparisons of interest. For immunogenicity analyses, data were Log₂-transformed and pairwise comparisons were performed for each study day using the Student *t*-test. All statistical analyses were performed using Provantis and/or SRS (testing facility in-house application built with SAS) and/or in-house reporting software Nevis 2012 (using SAS) and/or GraphPad Prism software (Version 9.2.0; GraphPad Software, La Jolla, CA) and statistical significance was set at p < 0.05 and reported at the 1 % and 5 % levels (unless otherwise noted).

3. Results

3.1. Antibody response

The presence of anti-S IgG antibodies was assessed in serum from maternal (SD 14, GD 21 and PND 21), fetal (GD 21) and pup (PND 21) blood samples collected at different occasions. The AS03-adjuvanted CoVLP induced a significant antibody response in F0 females when measured on SD 14 and on GD 21 and this response was maintained throughout lactation as illustrated by results obtained on PND 21 (p < 0.01 in comparison with placebo group) (Fig. 2). The IgG antibodies produced by the vaccinated F0 females were shown to cross the placenta as illustrated by the antibody level measured in fetuses on GD 21 (Fig. 2). The IgG antibodies were also shown to be transferred equally to male and female pups, as shown by the levels measured at PND 21 (Fig. 2).

3.2. Mortality, clinical and injection site observations

All animals remained in good health during the study and no mortality was reported. Daily clinical observations were limited to swollen hindlimb and inguinal area observed with a high incidence for F0 females treated with AS03-adjuvanted CoVLP immediately after the second administration (SD 15) and at lower incidence after the subsequent administrations (GD 6 and GD 19). Incidence of animals with swollen hindlimb and inguinal area was statistically higher (p < 0.0001) in this group confirming that these observations were related to the administrations of AS03-adjuvanted CoVLP. These findings generally disappeared within a week.

Injection site edema, around and beyond the injection site (grade 1 or 2), was observed 24 h post injection in almost all F0 females in the AS03-adjuvanted CoVLP group after the second (SD 15), third (GD 6) and fourth (GD 19) administrations. The incidence of detectable edema was very low or absent in the control placebo group. Statistical analyses showed that the incidence of edema was significantly higher in animals treated with AS03-adjuvanted CoVLP (p < 0.0001). The severity of edema decreased over time (i.e., after third and fourth injections) and



Fig. 2. Serum SARS-CoV-2 Spike protein specific IgG antibody response in F0 females (SD 14, GD 21 and PND 21), fetuses (GD 21) and pups (PND 21). Results are presented as GMT with 95 % CI for the pre-mating period (SD14; n = 46), EFD phase (GD 21; n = 22 for placebo and n = 21 for CoVLP + AS03) and PPN Phase (PND 21; n = 22 for placebo and n = 21 for CoVLP + AS03, except for male pups n = 20). Half of the minimum required dilution (MRD) of the method was assigned to non-responders (i.e., 50 for IgG titers) for GMT calculation purposes. In each panel, statistical comparisons were performed using Student's *t*-test between Log₂-transformed IgG titers of the control and the vaccine groups for each study day, ** p < 0.01.

was generally gone within 7 days post-injection. These observations were consistent with gross pathology findings at the injection sites for the AS03-adjuvanted CoVLP group and consisted of thickness, pale or raised foci, firm abnormal consistency, swelling and adhesion of the skin to lower tissues. The incidence of these observations at the injection sites was lower in animals of the PPN phase as they were euthanized approximately 3 weeks following the last vaccine administration. No erythema was observed in any animals during this study.

3.3. Maternal data

No effects related to AS03-adjuvanted CoVLP were reported as evidenced by the lack of differences in the many parameters evaluated. Indeed, maternal body weights (Figs. 3A and 3B) and food consumption (not shown) for the AS03-adjuvanted CoVLP treated females were comparable to the placebo group during the pre-mating, gestation (A) and lactation (B) periods.

Estrous cyclicity (the number of days in estrus, the number of cycles or the mean cycle length of the observed cycles) was evaluated between SD 1 to SD 23 for all F0 females, i.e. in females vaccinated twice (SD 1 and SD 15) during the pre-mating period. Results (Table 1) showed that the administration of AS03-adjuvanted CoVLP did not affect estrous cyclicity of the vaccinated F0 females. The reproductive parameters (Table 2) and the ovarian uterine data (Table 3) for the AS03-adjuvanted CoVLP treated females were comparable to the placebo group.

Slightly higher group pre-implantation loss was observed in animals treated with AS03-adjuvanted CoVLP (4.60 % for placebo vs 10.76 % for AS03-adjuvanted CoVLP, not statistically significant) and was mainly due to one female with a preimplantation loss value of 57.1 %. However, the mean pre-implantation loss value including this animal (10.76 %)



Fig. 3. Mean Body Weight in F0 Females during pre-mating and gestation (A) and lactation (B). Results are reported as mean body weight and standard deviation (\pm SD) per group. Statistical comparisons between AS03-adjuvanted CoVLP and placebo groups were performed using Dunnett's (equal variance) or Dunn's (unequal variance) test. No statistically significant differences were detected between both groups (p > 0.05).

Table 1

| Estrous | Cyclic | city Dat | a (EFD | and | PPN). |
|---------|--------|----------|--------|-----|-------|
| | ~ | 2 | | | |

| | Treatment G | roups | Historical Control |
|-------------------------------------|---------------------------------|----------------------|--------------------|
| Parameters | Placebo | CoVLP 3 µg + AS03 | Data |
| Total Females (EFD and PPN) | 46 | 46 | N/Ap |
| Number of Cycles (\pm SD) | 4.0 ± 0.7 | 4.0 ± 1.1 | 2.6 - 4.0 |
| Mean Cycle Length (Days | 4.67 \pm | 4.91 ± 1.99 | 3.8 - 4.6 |
| \pm SD) | 1.28 | | |
| Number of Days in Estrus $(\pm SD)$ | $\textbf{6.0} \pm \textbf{1.2}$ | 5.7 ± 1.5 | N/A |

EFD: Embryofetal development, PPN: Pre-and postnatal, SD: Standard deviation, N/A: Not available, N/Ap: Not applicable.

|--|

| | Treatment O | TTista via al | | |
|--|---------------------------------|---------------------------------|----------------|--|
| Parameters | Placebo | CoVLP 3 µg + AS03 | Control Data | |
| Paired Females | 46 | 46 | N/Ap | |
| Mated Females (confirmed) | 44 | 43 | N/Ap | |
| EFD Phase (Caesarean | 22 | 21 | | |
| Subgroup) | | | | |
| PPN Phase (Littering | 22 | 22 | | |
| Subgroup) | | | | |
| Pre-Coital Interval (Days \pm SD) | $\textbf{3.2} \pm \textbf{3.2}$ | $\textbf{4.6} \pm \textbf{5.2}$ | 1.6 – 4.6 | |
| Pregnant Females | 46 | 45 | N/Ap | |
| Pregnant Females with no | 2 | 3 | N/Ap | |
| Confirmed Mating | | | | |
| Mating Index ¹ | 100 % | 100 % | 73.9 % - 100 % | |
| Fertility Index ² | 100 % | 97.8 % | 62.5 % - 100 % | |
| Pregnancy Index ³ | 100 % | 97.8 % | 75.0 % - 100 % | |
| Females with all Nonviable (EFD Phase Only) | 0 (0.0 %) | 0 (0.0 %) | 0-1 | |
| Females with Resorptions (EFD | 10 (45.5 | 7 (33.3 %) | N/Ap | |
| Phase Only) | %) | | • | |
| Normal Placenta Exam (EFD | 22 (100 | 21 (100 %) | N/Ap | |
| Phase Only) | %) | | - | |

EFD: Embryofetal development, PPN: Pre-and postnatal, SD: Standard deviation, N/Ap: Not applicable. No significant differences detected between AS03-adjuvanted CoVLP and placebo groups (p > 0.05) using either Dunn's test or Fisher's test; ¹ Mating index = (No. of females mating/No. of females placed for mating) x 100; ² Fertility index = (No. of pregnant females/No. of mated females) x 100; ³ Pregnancy index = (No. of pregnant females/No. of mated females) x 100.

still remained within the historical control range of the testing facility for this parameter (from 3.3 to 19.2%). All observations on reproductive parameters and ovarian and uterine data remained within the historical control ranges of the Test Facility established on 78 studies from 2007 to 2020 and were not considered related to the administration of AS03adjuvanted CoVLP. The absence of effects on and fertility and reproductive endpoints was supported by macroscopic examination of the F0 females at scheduled termination (GD 21 for the EFD cohort or PND 21 for the PPN phase) and did not indicate any AS03-adjuvanted CoVLP effect on reproductive performance. Macroscopic changes other than those observed at the injection site in animals that received AS03adjuvanted CoVLP were limited to enlargement of the iliac lymph node (the lymph node draining the injection site) and swelling in the hindlimb adjacent to the injection site in one EFD female, and to a nodule adjacent to the injection site in one PPN female (not shown).

3.4. Fetal data

No significant differences in number of fetuses per litter (Table 3), sex ratio (Fig. 4A) or mean female and male fetal body weights (Fig. 4B) were noted between the AS03-adjuvanted CoVLP treated females and

Table 3

Ovarian and Uterine Data (EFD).

| | Treatment | Groups | Historical Control | |
|--|------------------------------|-----------------|--------------------|--|
| Parameters | Placebo CoVLP 3 µg + AS03 | | Data | |
| Number of Females Examined (Pregnant) | 22 | 21 | N/Ap | |
| Number of Corpora Lutea/ | 16.9 \pm | 16.5 ± 1.8 | 13.7 – 20.1 | |
| Female (\pm SD) | 2.0 | | | |
| Pre-Implantation Loss (% \pm | 4.60 \pm | 10.76 ± 13.40 | 3.3 – 19.2 | |
| SD) ¹ | 5.65 | | | |
| Number of Implantations/ | 16.0 \pm | 14.8 ± 2.8 | 11.6 – 17.6 | |
| Female (\pm SD) | 1.7 | | | |
| Post Implantation Loss (% \pm | 3.65 \pm | 3.62 ± 5.60 | 1.6 – 12.8 | |
| SD) ² | 4.80 | | | |
| Examined Live Litter | 22 | 21 | N/Ap | |
| Number of Live Fetuses/Litter | 15.5 \pm | 14.2 ± 2.7 | 11.1 – 16.6 | |
| (+ SD) | 1.8 | | | |

SD: Standard deviation, N/Ap: Not applicable. No significant differences detected between AS03-adjuvanted CoVLP and placebo groups (p > 0.05) using Dunn's test; ¹ Pre-implantation loss = [(No. of corpora lutea – No. of implants)/ No. of corpora lutea] x 100; ² Post-implantation loss = [(No. of implants – No. of live fetuses)/No. of implants] x 100.

the placebo group.

Observations of fetal external malformations (Table 4) showed edema of the entire subcutis, malrotation of the hindlimb, hyperflexion of the forepaw, omphalocele of the trunk in a total of 3 fetuses, out of 153 evaluated, from 3 different litters (out of a total of 21 litters) from the AS03-adjuvanted CoVLP treated females.

The incidences of each of these observations were within the historical control ranges of the Test Facility when considering the mean number of litters affected and mean number of fetuses per litter affected. Visceral malformations (Table 5) consisting of *Situs Inversus* of the thorax was noted in the AS03-adjuvanted CoVLP group at a very low incidence. A small eye lens was noted in the placebo group.

Skeletal malformations (Table 6) consisting in fused rib occurred on average in less than 1 % of the fetuses per litter of the AS03-adjuvanted CoVLP group and this finding was within the normal historical control range and thus was not considered treatment-related. Visceral variations consisting of small renal papilla and convolution/dilatation of the ureter were observed in AS03-adjuvanted CoVLP and/or placebo groups at low incidence. Some skeletal variations (Table 6) also occurred either in the AS03-adjuvanted CoVLP or placebo groups. The incidence of each observation was within historical control range of the testing facility, and these findings were considered to be of a spontaneous nature and were therefore not considered related to the administration of the

A. Sex Ratio



B. Fetal Weight



vaccine candidate [25].

3.5. Offspring data

The administration of AS03-adjuvanted CoVLP had no effect on F0 generation dams' parturition or gestation length. There were no effects on the live birth index or on pup mortality as illustrated by the comparable PND 4 viability index between the litters from the AS03-adjuvanted CoVLP and placebo groups. The high variability observed for the live birth index for the AS03-adjuvanted CoVLP group was due to one female with a live birth index of 20.0 %. Nonetheless, the mean viability at birth value including this animal remained within the historical control range of the testing facility for this parameter (from 84.8 to 95.5 %). All other females that received AS03-adjuvanted CoVLP had a live birth index between 80.0 % and 100.0 %. By way of comparison, viability at birth for the placebo females ranged from 81.3 %–100.0 %. Moreover, the survival index up to PND 21 was 100 % in both groups (Table 7).

The male and female pup body weights measured during the preweaning period and the clinical condition of these pups during the corresponding period were unaffected by the administration of AS03adjuvanted CoVLP to the F0 generation dams (Fig. 5). The few minor clinical observations noted during the pre-weaning period for the male and female pups in this group were also observed in the placebo group or occurred at a low incidence and, therefore, were not considered to be treatment related.

The mean number of development days to observe eye opening, pinna unfolding, righting reflex and the auricular startle response was unaffected by the administration of AS03-adjuvanted CoVLP (Fig. 6). All pups tested positive for pupillary closure and visual placing at PND21. There were no AS03-adjuvanted CoVLP treatment-related findings at macroscopic examination of the F1 pups between PND 0 and 4 and at scheduled termination (PND 21) (not shown).

4. Discussion

Several health authorities such as the WHO have advocated for the use of COVID-19 vaccines during pregnancy since pregnant women are at elevated risk of developing severe illness and SARS-CoV-2 infection has been associated with adverse pregnancy outcomes [26,27]. Therefore, to support the use of the AS03-adjuvanted CoVLP vaccine candidate in women of childbearing potential and pregnant women, the potential untoward effects of AS03-adjuvanted CoVLP on reproduction and development (embryo-fetal and pre-weaning) were evaluated in female rats following repeated administrations prior to mating and during gestation.

Fig. 4. Sex ratio (% male fetuses) is presented (A). Statistical comparisons between adjuvanted CoVLP and placebo groups were performed using Dunn's test. No significant differences were detected between both groups (p > 0.05). Mean fetal body weigh results (B) are reported as mean body weight and standard deviation (\pm SD) per group. Statistical comparisons between AS03-adjuvanted CoVLP and placebo groups were performed using Dunnett's test. No significant differences were detected (p > 0.05) and annotated on the graphs as ns.

Table 4

Fetal External Examination (Gross).

| | | Treatment | Groups | Historical | |
|--|--------------------------------------|----------------------|---------------------------|---------------|--|
| Type of Malformati | Placebo | CoVLP 3 µg + AS03 | Control Data ¹ | | |
| Number of Fetuses | [Litter | 170 | 153 [21] | N/Ap | |
| Examined] | | [22] | | | |
| Total Number of Fe | etuses with | 0 | 3 | N/Ap | |
| Abnormalities | | | | | |
| Total Litter % of Fetuses with Abnormalities ² | | 0.00 | 1.72 | N/Ap | |
| Total Number of Li | tter Affected | 0 | 3 | N/Ap | |
| Fetuses Edema: Entire Affected (%) ³ | | 0 (0.00) | 1 (0.60) | N/Ap | |
| Subcutis | Litter Affected (%) | 0 (0.0) | 1 (4.8) | N/Ap | |
| Malrotation; | Fetuses Affected (%) ³ | 0 (0.00) | 1 (0.53) | 0.00 - 0.64 % | |
| Hindlimb | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-8.00 % | |
| Hyperflexion; | Fetuses Affected (%) ³ | 0 (0.00) | 1 (0.60) | 0.00 - 0.78 % | |
| Forepaw | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-5.00 % | |
| Omphalocele; | Fetuses Affected (%) ³ | 0 (0.00) | 1 (0.60) | 0.00 - 0.68 % | |
| Trunk | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00–9.09 % | |

N/Ap: Not applicable. No significant differences detected between AS03- adjuvanted CoVLP and placebo groups using Dunn's test (p > 0.05); ¹Historical control ranges of the Test Facility for the % of fetuses/litters affected; ²Litter % of fetuses with abnormalities = (No. of fetuses in litter with a given findings/No. of fetuses in litter examined) x 100; ³Mean % fetuses with abnormalities per litter.

Overall, the results of the current study showed no systemic toxicity in AS03-adjuvanted CoVLP treated females and only transient local signs of inflammation (edema) with associated macroscopic findings were observed at the injection sites of most animals. Local reactogenicity is frequently observed following the intramuscular administration of vaccines adjuvanted with AS03 [9,28-30], and is indicative of a local inflammatory process and the initiation of an innate and adaptive immune response [31]. The AS03-adjuvanted CoVLP vaccine candidate induced a robust anti-SARS-CoV-2 S protein IgG antibody response in vaccinated female rats, and did not result in any maternal, reproductive or developmental toxicity. The lack of adverse effect on maternal and reproductive performances correlated with the absence of macroscopic findings on female reproductive organs. Other safety parameters, such as organ weights have been assessed in several safety studies in rats during the development of this vaccine at doses up to 10 μ g of AS03-adjuvanted CoVLP and no relevant changes were reported, with the exception of increased relative and absolute spleen weight in some animals (unpublished data). Exposure of the fetuses and F1 offspring up to weaning to maternal antibodies was not associated with embryofetal (including teratogenicity) or early postnatal development toxicity. Although the current study design for the PPN phase focused on pre-weaning development, as recommended by FDA Guidance to Industry for the development of vaccines, another recent DART study performed with the COVID vaccine ChAdOx1 nCoV-19, suggests that the absence of adverse findings during pre-weaning development might correlate with a normal post-weaning development [32].

In pregnant women, it has been reported that the developing fetus can sometimes be infected by SARS-CoV-2 *in utero* (ie: vertical transmission) as demonstrated by positive PCR results from amniotic fluid, umbilical cord blood, or neonatal blood shortly after birth [33,34]. Although the short- and long-term impact of fetal/neonatal SARS-CoV-2 infection are currently unknown, elevated levels of inflammatory cytokines were detected in the serum of neonates born of SARS-CoV-2 infected women [35].

Table 5Fetal Visceral Examination.

| | | Treatment | Groups | TTista di sal | |
|--|---|----------------------|---|---------------|--|
| Type of Malformation | Placebo | CoVLP 3 µg + AS03 | Historical Control Data ¹ | | |
| Head Razor Examinat | ion (Malformatio | on) | | | |
| Number of Fetuses [L Examined] | itter | 170 [22] | 152 [21] | N/Ap | |
| Total Number of Fetu Abnormalities | ses with | 1 | 0 | N/Ap | |
| Total Litter % of Fetu Abnormalities ² | ses with | 0.57 | 0 | N/Ap | |
| Total Number of Litte | r Affected | 1 | 0 | N/Ap | |
| Small Lens: Eve | Affected | 1 (0.57) | 0 (0.00) | 0.00 - 0.66 % | |
| onian Lenis, Lyc | Litter | 1 (4.5) | 0 (0.0) | 0.00-4.76 % | |
| Visceral Examination | - Malformation | | | | |
| Number of Fetuses [L Examined] | itter | 170 [22] | 152 [21] | N/Ap | |
| Total Number of Fetu Abnormalities | ses with | 0 | 1 | N/Ap | |
| Total Litter % of Fetu Abnormalities ² | ses with | 0.00 | 0.60 | N/Ap | |
| Total Number of Litte | r Affected Fetuses | 0 | 1 | N/Ap | |
| Situs Inversus; | Affected (%) ³ | 0 (0.00) | 1 (0.60) | 0.00-1.64 % | |
| THOTAX | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-10.53% | |
| Fresh Visceral Fetal Abnormalities (Va | | riation) | | | |
| Number of Fetuses [Litter Examined] | | 170 [22] | 152 [21] | N/Ap | |
| Total Number of Fetuses with Abnormalities | | 2 | 2 | N/Ap | |
| Total Litter % of Fetuses with Abnormalities ² | | 1.22 | 1.48 | N/Ap | |
| Total Number of Litte | r Affected | 2 | 2 | N/Ap | |
| Small (Moderate); | Fetuses Affected | 0 (0.00) | 1 (0.95) | 0.00–1.94 % | |
| Renal Papilla | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00–9.52 % | |
| Convolution; | Fetuses Affected (%) ³ | 0 (0.00) | 2 (1.48) | 0.00-2.58 % | |
| Ureter | Litter Affected (%) | 0 (0.0) | 2 (9.5) | 0.00–14.29 % | |
| Dilatation (Moderate); | Fetuses Affected (%) ³ | 2 (1.22) | 1 (0.53) | 0.00-4.80 % | |
| Ureter | Litter Affected (%) | 2 (9.1) | 1 (4.8) | 0.00-26.32 % | |
| Dilatation | Affected (%) ³ | 1 (0.57) | 1 (0.95) | 0.00-3.70 % | |
| (Severe); Ureter | Litter Affected (%) | 1 (4.5) | 1 (4.8) | 0.00-20.00 % | |

N/Ap: Not applicable. No significant differences detected between AS03adjuvanted CoVLP and placebo groups using Dunn'test (p > 0.05); ¹Historical control ranges of the Test Facility for the % of fetuses/litters affected; ²Litter % of fetuses with abnormalities = (No. of fetuses in litter with a given findings/No. of fetuses in litter examined) x 100; ³Mean % fetuses with abnormalities per litter.

Because many of the physiologic changes associated with pregnancy make pregnant women more susceptible to severe illness with respiratory viruses such as influenza [36–38] and COVID-19 [27], the benefit/risk of the vaccination generally far exceeds the potential consequences of developing such infections during pregnancy. Although the clinical manifestations of COVID-19 in pregnant women are generally similar to those in non-pregnant adults [39], pregnant women are at increased risk of severe illness [27] associated with adverse pregnancy events such as preterm birth, fetal vascular malperfusion, and premature

Table 6

Fetal Skeletal Examination.

Table 6 (continued)

| Fetal Skeletal Examir | nation. | | | | | | Treatment | Groups | |
|--|---|---------------|----------------------|---------------------------|----------------------------|---|---------------|----------------|---|
| | | Treatment | t Groups | Historical | Type of Malformation/ | Variation | Placebo | CoVLP 3 | Historical Control Data ¹ |
| Type of Malformation | /Variation | Placebo | CoVLP 3 µg + AS03 | Control Data ¹ | | Fetuses | | $\mu g + AS03$ | control Data |
| Fetal skeletal Abnorm | alities (Malformat | ion) | | | | Affected | | | |
| Number of Fetuses [L | itter Examined] | 170 [22] | 147 [21] | N/Ap | | (%) ³ Litter | 2 (9.1) | 1 (4.8) | 0.00-5.26 % |
| Total Number of Fetu Abnormalities | ses with | 0 | 1 | N/Ap | | Affected (%) | | | |
| Total Litter % of Fetu Abnormalities ² | ses with | 0.00 | 0.68 | N/Ap | | Fetuses Affected | 3 (1.83) | 1 (0.79) | 0.00–18.57 % |
| Total Number of Litte | r Affected Fetuses | 0 | 0 | N/Ap | IO; Parietal Bone | (%) ³ Litter | | | |
| Fused; Rib | Affected (%) ³ Litter | 0 (0.00) | 1 (0.68) | 0.00 – 0.76 % | | Affected (%) Fetuses | 3 (13.6) | 1 (4.8) | 0.00-52.38 % |
| Fetal cheletal Abnorm | Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-4.55 % | IO; Supraoccipital Bone | Affected (%) ³ | 5 (2.71) | 3 (2.00) | 0.00-6.76 % |
| Number of Fetuses []. | itter Framined] | 170 | 147 [21] | N/An | Done | Affected | 3 (13.6) | 3 (14.3) | 0.00-28.5 7% |
| Total Number of Fetu | ses with | [22] | | 10,11p | | (%) Fetuses | 0.0 | | |
| Abnormalities Total Litter % of Fetu | ses with | 65.40 | 96 | N/Ap | IO: Sternehra | Affected (%) ³ | 23 (13.79) | 25 (17.97) | 0.00-20.83 % |
| Abnormalities ² | | 03.40 | 04.47 | млар | io, stemesta | Litter | 14 | 10 ((1.0) | 0.00 (1.11.0) |
| Total Number of Litte | r Affected Fetuses | 22 | 21 | N/Ap | | Affected (%) | (63.6) | 13 (61.9) | 0.00-61.11 % |
| IO; Phalanges Forenaw | Affected (%) ³ Litter | 0 (0.00) | 2 (1.47) | 0.00-4.17 % | | Fetuses Affected (%) ³ | 67 (39.76) | 62 (41.92) | 0.00–57.97 % |
| rorepan | Affected | 0 (0.0) | 2 (9.5) | 0.00-22.22 % | IO; Thoracic Centrum | Litter | 21 | 10 (00 5) | 0.00.100.00.0/ |
| | (%) Fetuses Affected | 4 (2 50) | 1 (0.68) | 0.00-18.57.% | | Affected (%) Fetuses | (95.5) | 19 (90.5) | 0.00-100.00 % |
| IO; Femur | (%) ³ Litter | 4 (2.39) | 1 (0.00) | 0.00-18.37 % | Isolated Ossification | Affected (%) ³ | 0 (0.00) | 1 (0.68) | 0.00 - 0.81 % |
| | Affected (%) Fetuses | 4 (18.2) | 1 (4.8) | 0.00–57.14 % | Site; Cervical Arch | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-5.88 % |
| IO; Ischium | Affected (%) ³ | 1 (0.51) | 0 (0.00) | 0.00-1.65 % | Isolated Ossification | Fetuses Affected | 7 (3.90) | 16 (10.73) | 0.00–16.98 % |
| | Affected (%) | 1 (4.5) | 0 (0.0) | 0.00-5.26 % | Site; Lumbar Arch | Litter Affected | 5 (22.7) | 9 (42.9) | 0.00-62.50 % |
| IO: Pubis | Fetuses Affected (%) ³ | 1 (0.76) | 0 (0.00) | 0.00-2.48 % | | Fetuses Affected | 8 (4.60) | 2 (1.59) | 0.00–6.71 % |
| 10,1 4010 | Litter Affected (%) | 1 (4.5)) | 0 (0.0) | 0.00-5.26 % | Unossified; Hyoid Body | (%) ³ Litter Affected | 5 (22.7) | 1 (4.8) | 0.00-31.82 % |
| | Fetuses Affected (%) ³ | 1 (0.65) | 1 (0.60) | 0.00–7.44 % | | (%) Fetuses Affected | 1 (0.57) | 2 (1.28) | 0.00–3.64 % |
| IO; Rib | Litter Affected | 1 (4.5) | 1 (4.8) | 0.00-23.81 % | Unossified; Sternebra | (%) ³ Litter | 1 (4 5) | 0.(0.5) | |
| | (%) Fetuses Affected | 1 (0.51) | 2 (1.39) | 0.00-2.68 % | | (%) Fetuses | 1 (4.5) | 2 (9.5) | 0.00-22.22 % |
| IO; Frontal Bone | (%) ³ Litter | 1 (4 5) | 0 (0 5) | | Unossified; Thoracic | Affected (%) ³ | 0 (0.00) | 1 (0.68) | 0.00 - 0.80 % |
| | Affected (%) Fetuses | 1 (4.5) | 2 (9.5) | 0.00–16.67 % | Centrum | Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-5.26 % |
| IO; Hyoid Body | Affected (%) ³ Litter | 24 (14.62) | 14 (8.61) | 0.00-32.14 % | Misaligned; Thoracic | Fetuses Affected (%) ³ | 0 (0.00) | 1 (0.68) | 0.00 - 0.72 % |
| | Affected (%) | 13 (59.1) | 8 (38.1) | 4.00-86.36 % | Centrum | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-4.76 % |
| IO; Interparietal Bone | Affected (%) ³ | 20 (12.14) | 19 (12.03) | 2.27-44.29 % | | Fetuses Affected | 5 (3.24) | 3 (1.98) | 0.00-3.66 % |
| | Affected (%) | 13 (59.1) | 7 (33.3) | 9.09-80.95 % | Misshapen; Sternebra | Litter Affected | 5 (22.7) | 2 (9.5) | 0.00–27.27 % |
| IO; Nasal Bone | | 2 (1.33) | 2 (1.59) | 0.00–1.45 % | | (%) | 0 (0.00) | 1 (0.79) | 0.00-1.36 % |
| | | | | | | | 0 (0.00) | (conti | nued on next page) |

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Table 6 (continued)

| Type of Malformation/Variation | | Treatment | Groups | Historical Control Data ¹ | |
|--------------------------------|---|-----------|--|---|--|
| | | Placebo | $\begin{array}{c} \text{CoVLP 3} \\ \mu\text{g} + \text{AS03} \end{array}$ | | |
| Full; Thoracolumbar, Rib | Fetuses Affected (%) ³ Litter Affected | 0 (0.0) | 1 (4.8) | 0.00–8.70 % | |
| Short; Thoracolumbar, | (%) Fetuses Affected (%) ³ | 0 (0.00) | 1 (0.68) | 0.00–7.80 % | |
| Rib | Litter Affected (%) | 0 (0.0) | 1 (4.8) | 0.00-36.36 % | |

IO: Incomplete Ossification, N/Ap: Not applicable. No significant differences detected between AS03 adjuvanted CoVLP and placebo groups using Dunn's test (p > 0.05); ¹Historical control ranges of the Test Facility for the % of fetuses/litters affected; ²Litter % of fetuses with abnormalities = (No. of fetuses in litter with a given finding/No. of fetuses in litter examined) x 100; ³Mean % fetuses with abnormalities per litter.

Table 7

Viability Data of Offspring.

| | Treatment Gr | TTL-4 | |
|--|---|-------------------------------------|----------------------------|
| Parameters | Placebo CoVLP 3 AS03 | | Historical Control Data |
| Number of Females Examined (Pregnant) | 22 | 21 | N/Ap |
| Number of Pregnant Females with no Live Pups | 0 | 0 | N/Ap |
| Length of Gestation (Days \pm SD) | 22.0 ± 0.4 | 22.3 ± 0.5 | 21.3 - 22.0 |
| Sex Ratio (% of Males) ¹ | 45.93 ± 12.45 | $\textbf{46.41} \pm \textbf{16.45}$ | 42.5 - 60.6 |
| Number of Implant Scars (\pm SD) | 15.3 ± 2.6 | 15.2 ± 3.1 | 12.6 - 17.2 |
| Litter Size (Live Pups) on PND 0 (\pm SD) | 14.1 ± 2.9 | 13.9 ± 3.5 | N/Ap |
| Live Birth Index (% \pm SD) ² | $\begin{array}{c} 91.68 \pm \\ 6.96 \end{array}$ | $\textbf{89.44} \pm \textbf{16.89}$ | 84.8 – 95.5 |
| Viability Index PND 0–4 (% \pm SD) ³ | $\begin{array}{c} 97.17 \pm \\ 7.03 \end{array}$ | $\textbf{99.10} \pm \textbf{2.94}$ | 91.1 - 100.0 |
| Survival Index PND 4–7 (% \pm SD) ⁴ | $\begin{array}{c} 100.00 \pm \\ 0.00 \end{array}$ | 100.00 ± 0.00 | 95.5 - 100.0 |
| Survival Index PND 4–14 (% \pm SD) ⁴ | $\begin{array}{c} 100.00 \pm \\ 0.00 \end{array}$ | 100.00 ± 0.00 | 94.9 - 100.0 |
| Lactation Index PND 4–21 (% \pm SD) ⁴ | $\begin{array}{c} 100.00 \pm \\ 0.00 \end{array}$ | 100.00 ± 0.00 | 94.1 - 100.0 |
| Dead Pups at Birth (Mean per litter \pm SD) | $\textbf{0.0} \pm \textbf{0.2}$ | 0.1 ± 0.3 | N/Ap |
| Number of Litter Dead Affected | 1 | 2 | N/Ap |
| Pups Number of Pups Affected | 1 | 2 | 0 – 1 |
| Pups with Malformations at Birth (Mean per litter \pm SD) | $\textbf{0.0} \pm \textbf{0.0}$ | $\textbf{0.0} \pm \textbf{0.0}$ | N/Ap |

SD: Standard deviation, N/Ap: Not applicable. No significant differences detected between AS03-adjuvanted CoVLP and placebo groups (p > 0.05) using Dunn's or Fisher's test; ¹Sex ratio = (No. of male pups/Total No. of pups) x 100; ²Live birth index = (No. of live pups at birth/No. of implantation scars) x 100; ³Viability index = (No. of live pups on PND 4 (pre-cull)/No. of live pups on PND 0) x 100; ⁴Survival index = (No. of live pups on PND7, 14 or 21/No. of live pups on PND4 (post cull)) x 100.

fetal membrane rupture [26]. These women are also more susceptible to COVID-related thromboembolic complications due to higher levels of circulating coagulation factors during pregnancy [40]. Pre-eclampsia and COVID-19 also have overlapping features including high blood pressure, thrombocytopenia (i.e. low platelet count), and immune



Fig. 5. Mean Body Weight in F1 pups. Results are reported as mean body weight and standard deviation (\pm SD) per group. Statistical comparisons between AS03-adjuvanted CoVLP and placebo groups were performed using Dunnett's (equal variance) or Dunn's (unequal variance) test. No statistically significant differences were detected between both groups (p > 0.05).

dysregulation which can complicate timely diagnosis of the former condition [41]

In the context of COVID-19 pandemic and the elevated risk of serious outcomes in pregnancy [42], it is crucial for pregnant women to be adequately protected against viral infection. Interestingly, data available suggest that antibodies induced by natural SARS-CoV-2 infection during pregnancy may not confer the same neutralizing potential [43] and be less efficiently transferred to the fetus compared to those generated by vaccination [44]. In the current study, we reported placental transfer of SARS-CoV-2-specific IgG to the fetuses at ~ 15 % of the levels seen in the dams. Even though no direct correlation between the placental transfer rate of rat and human can be made, this observation is reassuring since the placenta is the major route by which maternal antibodies are transferred to the fetus/neonate in humans [45] in contrast to rats in which lactation is the principal mechanism of such transfer [46]. This observation suggests that significant transfer of maternal antibodies to the fetus is likely to occur following vaccination of pregnant women.

While the acquisition of maternal antibodies by human infants via breastmilk is more limited than placental transfer, anti-SARS-CoV-2 antibodies are present in women who have natural infection [47-50] and would be expected to be even higher following administration of vaccines that elicit strong neutralizing antibody responses like AS03-adjuvanted CoVLP [51]. The general benefits of breastfeeding are well understood [48,48,49,50] and would be further enhanced by the presence of antibodies in the breastmilk that may have a protective effect for the recipient infant against SARS-CoV-2 infection [52]. In the current study, the level of serum IgG antibodies in the pups on PND 21 was \sim 70 % of the level measured in the dams at the same time. Even though antibody levels were not measured directly in the breastmilk, these results strongly suggest successful antibody transfer during lactation, which is the expected route for rodents. It is noteworthy that the exposure of the fetus and pups to the very high levels of maternal anti-SARS-CoV-2 IgG antibodies directed against the S protein induced by AS03-adjuvanted CoVLP was not associated with any sign of toxicity during development.

Based on the evidence outlined above, most authorities agree that pregnant women [42] should be included in the priority populations for SARS-CoV-2 vaccination [53]. Although safety data are rapidly accumulating in pregnancy registries for a small number of widely deployed

A. Physiological Development



B. Auricular Startle

ns

C. Visual Function



vaccines [54] and that several studies of SARS-CoV-2 vaccine use in pregnancy are on-going, we are not aware of any human trials that have demonstrated fetal and neonatal safety with any COVID-19 vaccines. The data currently available from animal developmental and reproductive toxicity studies are also very limited but have not suggested any safety concerns with regards to female fertility, fetal, embryonal, or postnatal development [13,32].

5. Conclusions

The current study was conducted according to regulatory guidelines to support the use of AS03-adjuvanted CoVLP vaccine in women of childbearing potential and during pregnancy. Results presented shows that the administration of the AS03-adjuvanted CoVLP to female Sprague-Dawley rats twice before mating and twice during gestation did not result in any measurable developmental or reproductive toxicity. There was no effect on mating, fertility, pregnancy, parturition,

Fig. 6. Developmental landmark achievements in F1 pups are presented for Physiological Development (A), Auricular Startle (B), and Visual Function (C). Results are reported as litter mean and standard deviation (\pm SD) per group. Visual placing score is defined as: 0 =negative response and 2 = positive response. Pupillary closure score is defined as: 0 = negative response, 1 =positive response for one eye and 2 = positive response for both eyes. Statistical comparisons between AS03adjuvanted CoVLP and placebo groups were performed using Dunn's (physiological development and auricular startle) or Fisher's (visual function) test. No statistically significant differences were detected between both groups (p > 0.05) and annotated on the graphs as ns.

maternal care of offspring, or prenatal or postnatal offspring viability, survival, growth, or development. A robust immune response was confirmed in F0 female rat prior to mating and at the end of gestation and lactation. High levels of antibodies were also detected in fetuses and F1 offspring. Overall, these results suggest an acceptable safety profile of AS03-adjuvanted CoVLP vaccine for administration in women of childbearing potential.

Trademark statement

Arepanrix and Pandemrix are trademarks of the GSK group of companies.

Declaration of Competing Interest

CD, SPR, IP, BJW, NL and ST are either employees of Medicago Inc or receive salary support from Medicago Inc. Charles River received payments or financial compensations from Medicago Inc for the services provided in this study. ED is an employee of the GSK group of companies and reports ownership of GSK shares.

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