Effect of progesterone on the vaccination and immune response against *Chlamydia abortus* in sheep

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**ABSTRACT**

*Chlamydia abortus* produces ovine enzootic abortion (OEA). Symptoms are not observed until the organism colonises the placenta, eventually causing abortion. Infected animals become carriers and will shed the organism in the following oestruses. This process suggests that sex hormones might play an important role in the physiopathology of OEA, affecting the success of chlamydial clearance and also jeopardising the effectiveness of vaccination. However, the mechanisms through which sex hormones are involved in chlamydial pathogenicity remain unclear. The aim of this study, therefore, was to determine the effect of progesterone on the immune response against *C. abortus* and on the protection conferred by an experimental inactivated vaccine in sheep.

Eighteen sheep were ovariectomised and divided into four groups: vaccinated and progesterone-treated (V-PG), vaccinated and non-treated (V-NT), non-vaccinated and non-treated (NV-NT) and non-vaccinated and progesterone-treated sheep (NV-PG). Animals from both PG groups were treated with commercial medroxypregesterone acetate impregnated intravaginal sponges before and during the vaccination (V-PG) or just before challenge (NV-PG). The animals from both V groups were subcutaneously immunised with an experimental inactivated vaccine, which was seen to confer high protection in previous studies. All sheep were challenged intratracheally with *C. abortus* strain AB7 and were sacrificed on day 8 post-infection. Morbidity was measured as the variation in rectal temperature and samples of sera were collected for antibody and cytokine (IFN-γ and IL-10) analysis by commercial ELISA. In addition, lung and lymph node samples were collected for chlamydial detection by qPCR and for histopathological and immunohistochemical analyses.

Sheep from the V-PG group showed less severe or no lesions and lower morbidity than the other groups. They also had the highest abundance of regulatory T-cells. The sheep from V-NT also manifested high antibody levels against *C. abortus* and less severe lesions than those observed in non-vaccinated sheep, which showed high morbidity, low antibody levels and severe lesions, especially in NV-NT. These results confirm the effectiveness of the experimental vaccine employed and suggest that progesterone could enhance the effect.

1. **Introduction**

*Chlamydia abortus* is the causative agent of ovine enzootic abortion (OEA), a disease which mainly affects small ruminants worldwide but also other mammal species (including humans), causing abortion and the premature birth of stillborn or weak offspring (Essig and Longbottom, 2015) with economic implications for the farming industry. This pathogen has a predilection for the trophoblast cells of the placenta, where its unique intracellular biphasic developmental cycle takes place. The cycle is initiated when the infectious form of the organism, the elementary body (EB), enters the host cell to reside within a vacuole known as an inclusion. The EB undergoes conversion to the metabolically active reticulate body (RB), which replicates through binary fission. Towards the end of the cycle the RBs re-condense to EBs prior to lysis of both the inclusion and the host cell, allowing the release of infective organisms to infect neighbouring cells (Longbottom and Coulter, 2003). Some *in vitro* studies have reported that under stressful conditions, such as nutrient depletion (Raulston, 1997), coinfections with viruses (Deka et al., 2006; Borel et al., 2010) or protozoa (Romano et al., 2013), or exposure to antibiotics (Gieffers et al., 2004), cytokines (Beatty et al., 1993) or hormones (Amirshahi et al., 2011) chlamydial species develop enlarged pleomorphic RBs that neither undergo binary...
fission, nor differentiate back to EBs, called aberrant bodies (AB).

One especially intriguing feature of the pathology of OEA is that C. abortus is able to establish a latent or persistent, undetectable infection in non-pregnant sheep after oronasal infection, which, controversially, has been related with ABs in other chlamydia species (Bore et al., 2008; Pospischil et al., 2009; Rank et al., 2011; Phillips Campbell et al., 2012). The latent or persistent infection remains until the last few weeks of a subsequent pregnancy, when reactivation of the micro-organism causes abortion. After this, animals acquire protective immunity and do not experience further OEA abortive episodes, allowing successful rebreeding. However, they become carriers and can excrete the organism from their reproductive tracts in subsequent oestrus cycles, thereby providing an opportunity for transmission during breeding (Papp et al., 1994; Papp and Shewen, 1996). Animals may also shed infectious organisms at subsequent lambing, thus contributing to the spread of infection to naïve animals (Wilsmore et al., 1990).

This process of latency/active multiplication that leads to the re-

crudescence of C. abortus at certain moments of pregnancy and the reproductive cycle, suggests that female sex hormones might play an important role in the physiopathology of the OEA and also affect the immune system, reducing the success of chlamydia clearance and also jeopardizing the effectiveness of a vaccination. Despite the central role that this latency-to-recrudescence process plays in the pathogenesis of OEA, the underlying mechanisms that control it remain unclear.

Some lines of evidence suggest that fluctuations of hormones that control the reproductive cycles might act as triggering factors for the reactivation of dormant chlamydia (Guseva et al., 2003). Previous reports have shown that steroid hormones are able to modulate immune responses against bacteria and affect the interactions between intracellular pathogens and host cells (Beagley and Gockel, 2003; Amirshahi et al., 2011; Wan et al., 2014).

Progesterone is a steroid hormone widely employed to synchronise oestrus in sheep. This hormone is generally considered to have immunosuppressive effects, downregulating IFN-γ-associated genes (Dosiou et al., 2004) and also exerting inhibitory effects on antibody production by B cells (Li et al., 2002). In the case of chlamydia infections, progesterone seems to promote the genital infection of Chla-

mydia trachomatis and Chlamydia muridarum (Morrison et al., 2011) in mice by changing vaginal permeability to these bacteria.

Despite the role that progesterone plays in the chlamydia patho-
genesis, the underlying mechanisms by which this hormone is involved in chlamydial pathogenicity are not fully understood, and far less is known about the impact of sex hormones on responses to chlamydia vaccines. There are no studies that look deeply into the role of sex hormones in the C. abortus aetiopathology and any interaction with the vaccination process. The aim of this study was to determine the effect of progesterone on the kinetics and immune response in C. abortus infec-
tion as well as on the protection conferred by an experimental in-
activated vaccine against C. abortus previously tested in an intratracheal ovine infection model.

2. Materials and methods

2.1. Experimental design

All experiments were approved by the Bioethical Committee of the University of Murcia, Spain (approval number A13150203; date of approval 24 February 2015).

Eighteen conventionally raised Segureña sheep, aged 3 months, were serologically pre-screened by a commercial ELISA test (described in 2.4) to ensure they were seronegative for C. abortus-specific anti-

bodies. All animals were ovariectomised by the staff of the Veterinary Clinical Hospital of the University of Murcia before being randomly allocated into two groups of 4 animals and two groups of 5 animals according to the scheme represented in Fig. 1.

Sheep from the non-vaccinated and progesterone-treated group (NV-PG) were used to determine the effect of progesterone on C. abortus infection in relation to the non-vaccinated and non-treated control group (NV-NT). Animals of this group were treated with commercial intratracheal sponges impregnated with medroxyprogesterone acetate (Esponjaret®, Laboratorios Hipra, Spain) 2 days before their challenge.

Animals from the vaccinated and progesterone-treated (V-PG) and, vaccinated and non-treated (V-NT) groups, as well as the above mentioned NV-NT group, were used to determine the effect of progesterone on the effectiveness of vaccination against C. abortus. For this, sheep of group V-PG were also treated with commercial medroxyprogesterone acetate-impregnated intratracheal sponges that were periodically replaced every 10 days until the day of infection, in order to maintain progesterone level constant.

Two days after the hormonal treatment, animals from groups V-PG and V-NT were subcutaneously immunised with an experimental in-
activated vaccine (49 days before being challenged) and given a booster dose 21 days later under the same conditions. Animals of group NV-NT were left unvaccinated and untreated as an infection control group.

Blood samples were taken on the two vaccination days, on the day of the challenge and on the day of sacrifice, in order to obtain sera for the determination of C. abortus-specific antibodies and cytokines (IFN-γ and IL-10) by ELISA.

Sheep were challenged intratracheally as previously described in Álvarez et al. (2015). After C. abortus infection, rectal temperature was measured and recorded daily for each animal. On day 8 post-infection (pi) all animals were sacrificed using a captive bolt pistol, and lung, tonsils, mediastinal, tracheobronchial and retropharyngeal lymph nodes samples were collected in triplicate. Samples were fixed in 10% formalin or Zinc fixative agent (BD Biosciences, Pharmingen, USA) and embedded in paraffin for further histopathological and imm-

unohistochemical analysis, or frozen at –80°C for chlamydial detec-
tion by qPCR.

2.2. Microorganism, vaccine and infection

C. abortus (AB7 strain) was propagated in the yolk sacs of devel-
oping chick embryos and titrated by counting inclusion-forming units (IFUs) in McCoy cells as previously described (Buendía et al., 1999). Standardised aliquots were frozen at –80°C until use. The bacterial strain was used to prepare the inactivated vaccine and the inoculum for infection.

An inactivated vaccine with a purified derivate of saponin (QS21; Agenus) as adjuvant was used because of the good protection results obtained in previous experimental infections in pregnant mice (Caro et al., 2003) and in pregnant sheep (García de la Fuente et al., 2004). The vaccine was prepared according to the protocol described by García de la Fuente et al. (2004). Sheep received two doses of the experimental vaccine, each containing 180 μg of protein of binary ethyleneimine (BEI) inactivated C. abortus and 150 μg of QS21 adjuvant in 2 ml of sterile PBS.

For experimental infection, sheep were challenged by the in-

tratracheal route with 5 × 107 IFU of C. abortus in 0.5 ml of PBS with a sterile needle (23 G) as described by Álvarez et al. (2015).

2.3. Histopathological and immunohistochemical analyses

Formalin fixed lung, tonsils, mediastinal, tracheobronchial and retropharyngeal lymph node samples sections (4 microns) were stained with Haematoxylin-Eosin for histopathological study and im-

munohistochemical labelling, which was carried out to demonstrate the presence of chlamydia antigen, using an anti-chlamydia LPS antibody (Abcam, USA) as described in Buendía et al. (1998). Additionally, characterization of the inflammatory infiltrate was carried out in zinc-

fixed samples, using monoclonal antibodies against CD3 (Dakoocyto-
mation, USA), Foxp3 (Abcam, USA) and CD163 (Bio-Rad, UK), using Avidin-Biotin-Peroxidase Complex (ABC), as previously described in
Martínez et al. (2006). These markers are specific for T-cells, regulatory T-cells (Tregs cells) and macrophages, respectively. For quantification of the stained area, ten fields of 10,000 μm² per sample were evaluated under the microscope by counting the positive cells as described in Montes de Oca et al. (2000).

2.4. Antibody detection

Serum samples were analysed for the production of C. abortus-specific antibody levels by commercial ELISA (ID Screen Chlamydia abortus indirect multispecies, IDvet) following the instructions of the manufacturer. Optical densities (OD) were expressed as a percentage of the positive control using the following formula:

\[(\text{OD sample} / \text{OD positive control}) \times 100\].

Samples with values of ≥ 60% were considered positive, samples between 50% and 60% doubtful and samples of ≤ 50% negative.

2.5. IFN-γ and IL-10 detection

Serum from sheep was analysed to determine IFN-γ and IL-10 concentrations at each time point by a commercial ELISA kit (Sheep IFN-γ and Sheep IL-10 ELISA Kit, Cusabio, USA), following the manufacturer’s instructions.

2.6. Detection of C. abortus DNA in organ samples

DNA was extracted from organ samples for qPCR analysis using the FavorPrep Tissue Genomic DNA Extraction Mini kit (Favorgen Biotech Corporation, Taiwan). The presence of chlamydia DNA in the organ samples was determined by a Chlamydia abortus-specific qPCR (Pantchev et al., 2010), using primers CpaOMP1-F (5′-GCAACTGACCTAGTCGGTACA-3′), CpaOMP1-R (5′-ACAAGCATGTTCAATCGATAAGA-3′) and CpaOMP1-S FAM (5′-TAAATACCACAATGGAAGTTTAGCG-TAMRA-3′).

Samples with Cт ≤ 35 were considered positive and above this Cт were considered negative.

2.7. Statistical analysis

The data were compared using one-way ANOVA or the corresponding tests on ranks and were presented as the mean ± SEM. As a post-hoc test, Dunn’s method and Bonferroni t-test multiple comparisons of means with 95% confidence level were used. For immunohistopathological study, a Student’s t-test was used to compare vaccinated (V-PG versus V-NT or NV-NT) and non-vaccinated (NV-NT versus NV-PG) groups. Statistical analysis of the quantitative results was conducted using the Sigmasstat software package (SPSS). Minimal statistical significance was fixed at \(p < 0.05\).

3. Results

3.1. Clinical signs

The temperature of all the animals increased between day 1 and 2 pi, the animals from the NV-PG group showing the greatest increase (up to 2.24°C) at day 2pi. A second increase in temperature was observed in the NV-NT group at day 5pi. From day 5 onwards, the temperature of all the animals returned to normal values.

3.2. C. abortus DNA detection

The organ samples were used to detect C. abortus DNA on the day of sacrifice. The qPCR was negative in all of mediastinal and tracheobronchial lymph node samples from all groups. Tonsil and...
Chlamydia abortus, the causative agent of ovine enzootic abortion (OEA), is one of the widest ovine abortifacient agents worldwide.
Increasing our knowledge of the mechanism whereby progesterone influences the immune response and kinetics of C. abortus infection may provide important insights into the possible control of infection and the development of a suitable protective immune response induced by vaccination. For this reason, the present study assesses the effect of progesterone on the kinetics and immune response against C. abortus infection, as well as on the protection conferred by an experimental inactivated vaccine previously tested in the natural host (García de la Fuente et al., 2004).

Our results regarding the lesions and effectiveness of vaccination are similar to those obtained by Álvarez et al. (2015), who validated the intratracheal inoculation route for testing vaccines against OEA in sheep. In addition, the QS21 adjuvated experimental vaccine conferred protection against the pathogen, providing an adequate immune response.

In our study, vaccinated sheep showed the highest antibody titre against chlamydia, which was significantly different from that of non-vaccinated sheep. However, no differences between V-PG and V-NT were found. The effects of progesterone on antibody production are still not clear. Some authors describe that the administration of this

Fig. 3. Gross lesions. (A) Lung of a NV-PG sheep showing a grey-red consolidation of the upper lobe also in areas of the middle lobe. (B) Detail of the lesions shown in (A). (C) Lung of a V-NT sheep showing a small grey-red consolidation area in the cranial portion of the upper lobe. (D) Detail of the lesion shown in (C) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 4. Histopathological and immunohistochemical study of an NV-NT animal. (A) Section stained with haematoxylin and eosin (H&E) showing a necrosis area (*) with liquefaction of the pulmonary parenchyma. (B) Section immunostained by the ABC method contrasted with haematoxylin showing the association between necrosis areas and chlamydial antigen positive immunoreaction (brown areas). (C) Detail of a section immunostained to detect macrophages. Note the presence of these cells in the lumen of bronchioles (arrows) and alveoli (arrow heads) along with presence of neutrophils. (D) Detail of a section immunostained for T cell detection. Note the scarce presence of these cells in the alveolar walls (arrow heads), septa and around bronchioles and vessels (arrows) and the high presence of neutrophils in the lumen of bronchioles (arrows) and alveoli (arrow heads).
hormone to animals reduces (Lü et al., 2002) or enhances (Vermeulen et al., 2001) antibody production, whereas other authors found that progesterone had no effect (Nikolaevich et al., 1991) on the humoral response. Our results tend to support the last mentioned study, suggesting that progesterone has no critical effect on the production of specific anti-\textit{C. abortus} antibodies. As chlamydia is an intracellular pathogen, antibodies do not appear to be the major protective component of the adaptive host immune response against OEA (Entrican et al., 2012). However, the humoral response against \textit{C. abortus} is important for the establishment of an adequate and complete protective immune response after vaccination in experimental models (de Sa et al., 1995; Buendia et al., 2009).

Other important components of the memory immune responses generated by vaccines are the T-cells. We found that sheep from the vaccinated groups (V-PG and V-NT) showed the highest number of T-cells (CD3+) in lungs compared to the other groups. T-cells have a pivotal role on the establishment of a protective memory immune response against \textit{C. abortus} infection (del Río et al., 2000; Martínez et al., 2006). This cellular immunity seems to be mediated by interferon (IFN-\(\gamma\)) in all chlamydiaceae species. Thus, Chlamydia-specific T-cells wield...
their protective effects through the secretion of this cytokine (del Río et al., 2001; Rottenberg et al., 2002). However, no differences were observed for IFN-γ levels among groups at any time of our study. It is possible that vaccinated animals displayed an early IFN-γ increase after infection, which had already decreased by the day of sacrifice, as has been reported in mouse models (Caro et al., 2003).

Some authors have reported that low concentrations of IFN-γ may lead to persistent chlamydia infections (Hogan et al., 2004), while others suggest that chronic chlamydial infections could be generated by an increase in IL-10 (del Río et al., 2018). It has been shown that IL-10 is able to moderate immunopathological events associated with the overproduction of IFN-γ linked to its regulatory role as an anti-inflammatory cytokine (Jakobshagen et al., 2015; Kulcsar and Griffin, 2016). The only differences found in the IL-10 levels in our study were those observed between NV-NT and NV-PG animals on the day of challenge. Non-vaccinated sheep displayed a great innate-like immune response and the severity of lesions was lower in NV-PG ewes. These results could be associated with the fact that progesterone may prevent severe tissue damage by promoting IL-10 production in these animals as described in Trautman et al. (1997), who found that progesterone induced a modest increase of IL-10 implicated in the anti-inflammatory response.

Chlamydial DNA and antigen were found in lung of all non-vaccinated animals, V-NT animals and one animal from the V-PG group. Mixed results were obtained in tonsils and retropharyngeal lymph nodes probably because some sheep coughed after challenge, spreading bacteria over the mucosal tissue near to the lymph nodes. The C. abortus DNA and chlamydial antigen detected in the lung of only one animal from the V-PG group might be related with the fact that C. abortus remains in the lymphoid organs during a latency phase of infection (Longbottom et al., 2013; Álvarez et al., 2015).

One intriguing result obtained was the significantly higher number of Treg cells (Foxp3+) in the regional draining lymph nodes of V-PG animals compared with the animals of the V-NT and NV-NT groups. Treg cells have been reported to dampen function on inflammation and to have a suppressive role on innate immunity, preventing an excessive immune responses, which would result in serious tissue damage (Antunes and Kassiotis, 2010; Hou et al., 2015). Weinberg et al. (2011) reported that the increase in progesterone levels during the menstrual cycle in women was associated with higher Treg cell frequencies and lower cell-mediated immunity. Our results show that V-PG animals developed less severe morbidity and lesions than the other groups and

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**Fig. 7.** Histopathological and Immunohistochemical study of a V-PG animal. (A) Section stained with H&E showing hyperplasia of BALT with prominent follicles (*). (B) Section immunostained by the ABC method contrasted with haematoxylin for detection of chlamydial antigen. No chlamydial antigen was detected in this group.

**Fig. 8.** Detail of an immunostained section for Foxp3+ antigen. Note the large amount of positive lymphocytes detected in the germinal centre follicle in the tracheobronchial lymph node of a vaccinated sheep (A) compared with a non-vaccinated sheep (B) (the immunostaining of the antigen is intranuclear). (C) Number of Treg cells per field (63x). a: statistically significant differences between V-PG, V-NT and NV-NT groups (p < 0.05). b: statistically significant differences between V-NT and NV-NT groups (p < 0.05).
scarce presence of neutrophils and macrophages was observed. In addition, chlamydial DNA and antigen were only detected in 1 out of 4 animals of this group. This suggests that, in our model, progesterone enhances the presence of Treg cells, whose presence prevents an excessive innate immunity reaction in lung that might lead to the formation of lesions but, they do not block the development of an effective memory immune response induced by vaccination. The results displayed by V-NT animals seem to support this hypothesis. The number of Treg cells in the regional draining lymph nodes found in V-NT sheep was significantly lower than the number observed in V-PC animals. Nonetheless, in spite of the great protection conferred by the vaccine, we found that V-NT animals showed lesions with more abundant macrophages than V-PC sheep. We also detected the presence of chlamydial DNA and antigen in lungs of V-NT ewes, which suggests that a lower presence of Treg cells could be related with a less efficient chlamydial clearance.

Of interest was the lack of neutrophils found in lesions of vaccinated animals (V-PC and V-NT). Previous studies reported that neutrophils act to prevent the uncontrolled multiplication of bacteria in the early stages of primary C. abortus infection (Buendía et al., 1999) and that they are necessary for the recruitment of T-cells to the inflammatory foci, a key event for the clearance of C. abortus infection (Montes de Oca et al., 2005; del Rio et al., 2000; Martínez et al., 2006). Other authors support the idea that inactivated vaccines, such as the one used in our study, are able to control the infection by producing antibodies (Buendía et al., 2009), so the presence of neutrophils would not be so important for a vaccine efficient immune response against C. abortus (Ortega et al., 2007). It is possible that the lack of neutrophils in vaccinated animals was due to the early specific memory immune response generated by the vaccine used. Thus, the high levels of antibodies against chlamydia and the high number of Treg cells allow the chlamydia clearance with minimal involvement of the components of the innate immune response.

In conclusion, it was found that progesterone treatment of sheep together with vaccination prevents or reduces the severity of chlamydial lesions. The results support the effectiveness of the experimental vaccine used and suggest that progesterone does not interfere in the immune response elicited by it. Moreover, the results suggest that progesterone may enhance its protective effect. However, further studies are required to deepen the knowledge of the mechanisms involved in the effect of progesterone on the immune response to C. abortus.

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