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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 medroxyprogesterone 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

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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 (Borel 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 microorganism 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 recrudescence 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 chlamydiae (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 (Lü et al., 2002). In the case of chlamydia infections, progesterone seems to promote the genital infection of *Chlamydia 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 pathogenesis, 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* infection as well as on the protection conferred by an experimental inactivated 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 antibodies. 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 intravaginal sponges impregnated with medroxyprogesterone acetate (Esponjavet®, 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 intravaginal 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 inactivated 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 immunopathological analysis, or frozen at −80 °C for chlamydial detection by qPCR.

2.2. Microorganism, vaccine and infection

C. abortus (AB7 strain) was propagated in the yolk sacs of developing 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 ethylenimine (BEI) inactivated *C. abortus* and 150 μg of QS21 adjuvant in 2 ml of sterile PBS.

For experimental infection, sheep were challenged by the intratracheal route with 5×10^7 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 immunohistochemical 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 (Dakocytomation, USA), Foxp3 (Abcam, USA) and CD163 (Bio-Rad, UK), using Avidin-Biotin-Peroxidase Complex (ABC), as previously described in

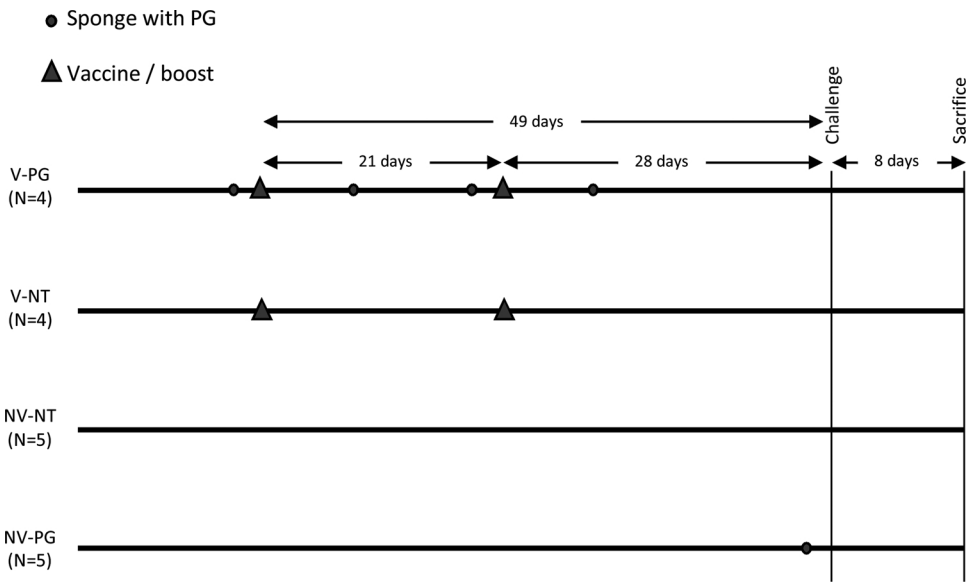

Fig. 1. Experimental design. Vaccinated and progesterone-treated sheep (V-PG) and Vaccinated and non-treated animals (V-NT) were vaccinated on day −49 and boosted on day −28, considering the day of challenge as day 0. V-PG animals were treated 2 days before their vaccination with medroxyprogesterone acetate impregnated intravaginal sponges. After vaccination, sponges were replaced every 10 days until 17 days before the challenge. Two days before challenge, intravaginal sponges were applied in Non-vaccinated and progesterone-treated sheep (NV-PG). Non-vaccinated and non-treated sheep (NV-NT) were used as a control group.

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 μm^2 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 *Chlamydophila 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:

(OD sample/OD positive control) × 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′-GCAACTGACA CTAAGTCGGTACA-3′), CpaOMP1-R (5′-ACAAGCATGTTCAATCGATAA GAGA-3′) and CpaOMP1-S FAM (5′-TAAATACCACGAATGGCAAGTTG GTTTAGCG-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 by 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 immunohistopathlogical 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 Sigmastat 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 2 pi. A second increase in temperature was observed in the NV-NT group at day 5 pi. 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

Table 1
PCR detection of *C. abortus* in organ samples at day 8 pi. No positive samples were identified in tracheobronchial and mediastinal samples.

	Lung		Tonsils		Retropharyngeal	
	PCR +/Sampled animals	Mean Cт [min-max]	PCR +/Sampled animals	Mean Cт [min-max]	PCR +/Sampled animals	Mean Cт [min-max]
V-PG	1/4	33.55 [27.86-35.69]	2/4	35.00 [34.78-35.34]	2/4	34.93 [34.44-35.39]
V-NT	3/4	31.56 [28.42-35.69]	3/4	34.93 [34.80-35.11]	2/4	34.98 [34.62-35.33]
NV-NT	4/5	30.40 [26.83-35.31]	1/5	35.18 [34.96-35.40]	1/5	35.24 [34.99-35.74]
NV-PG	3/5	31.28 [28.18-35.59]	2/5	35.08 [34.45-35.62]	3/5	35.08 [34.90-35.32]

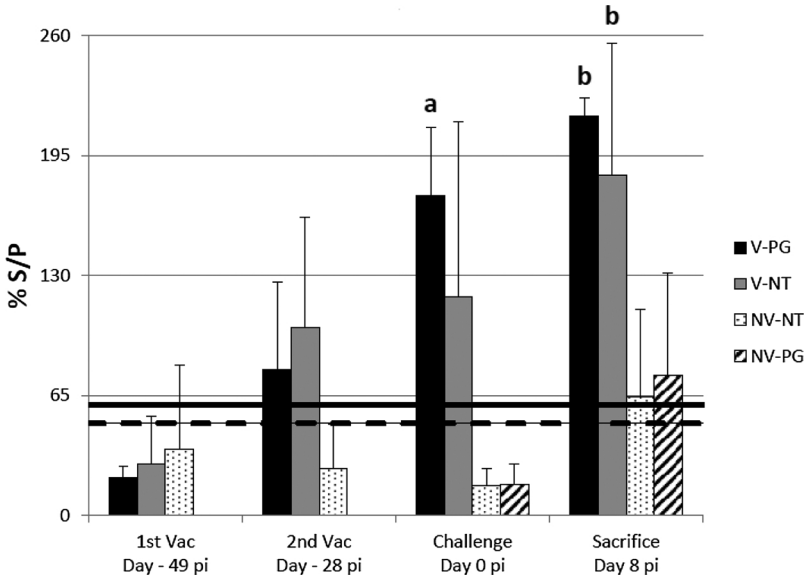

Fig. 2. Means (+SD) of antibody levels against *C. abortus*. Horizontal lines represent the threshold between negative and doubtful results (lower discontinuous line) and between doubtful and positive results (upper line). V: vaccinated; NV: non-vaccinated; PG: progesterone; NT: non-treated.
[a]: statistically significant differences between V-PG and NV-NT group on the day of challenge ($p < 0.05$).
[b]: statistically significant differences between V and NV-NT group on the day of sacrifice ($p < 0.05$).

retropharyngeal lymph node samples showed mixed results with high Cт values, as shown in Table 1. By contrast, most lung samples were positive in all the groups except V-PG, where only one sheep (of the 4 animals of the group) was positive. The positive samples in lung showed Cт values lower than 30.

3.3. Antibody response and cytokine production in sera

Sheep of the vaccinated groups manifested seroconversion after the first vaccination (day −28 pi), the antibody titre being higher in the V-NT group (Fig. 2). However, the antibody titre was higher in the V-PG group at day of the challenge (day 0 pi) with statistically significant differences ($p < 0.05$) between this group and non-vaccinated groups. In contrast, no statistical differences were found between V-NT and non-vaccinated groups. The challenge elicited a marked specific humoral response, which was determined at the day of sacrifice (day 8 pi) by a statistically significant ($p < 0.05$) increase in the antibody titre in the vaccinated groups compared with the non-vaccinated groups. Seroconversion of non-vaccinated animals was only observed at the end of the study.

In the case of cytokines, no statistically significant differences were observed for IFN-γ between groups or days (data not shown). In contrast, statistically significant differences ($p < 0.05$) for the concentrations of IL-10 were found between NV-NT and NV-PG animals on the day of the challenge being higher in NV-PG at this time (data not shown). However, the values for IL-10 were low (around 12 pg/ml) and close to the detection limit of the commercial ELISA (5 pg/ml).

3.4. Gross lesions, histopathology and immunohistochemistry

Non-vaccinated animals developed lung lesions in the upper and middle lobes, consisting of irregular consolidation areas of a grey-red colour and catarrhal or purulent material (Fig. 3, A and B). Seven of the 10 non-vaccinated sheep, also showed enlarged tracheobronchial lymph nodes. In the vaccinated groups, all sheep of V-NT (Fig. 3, C and D) and only one of V-PG (the one which had positive qPCR) groups showed small consolidation areas in the cranial portion of the upper lobe with a firm texture and grey in colour. The remaining 3 animals of the V-PG group showed only small depressed areas with a firm texture and purple colour. Tracheobronchial lymph nodes of the vaccinated animals were enlarged, and had a tense capsule and the cut surface bulge.

The histopathological and immunohistochemical study of the lesions showed that animals from non-vaccinated groups (NV-NT and NV-PG) developed a suppurative bronchopneumonia with abundant inflammatory exudate in the lumen of the alveoli, bronchi and bronchioles (Figs. 4 and 5). This exudate was formed mainly of neutrophils and macrophages (CD163+). Moreover, a mononuclear infiltrate composed mainly of T-cells (CD3+) was located in the alveolar walls, septa and around the bronchi, bronchioles and vessels. Sheep of the NV-NT group (Figs. 4) also showed small necrotic areas with liquefaction of the pulmonary parenchyma. A high presence of chlamydial antigen was detected associated with the neutrophilic exudate, especially in necrosis areas, being lower in the NV-PG group (Figs. 4 and 5).

On the other hand, all sheep from V-NT (Fig. 6) and only one from the V-PG group developed an interstitial bronchopneumonia with abundant lymphoid infiltrate composed mainly of T-cells (CD3+), located in the alveolar walls, septa and around the bronchi, bronchioles and vessels. These animals also showed hyperplasia and the persistence of type II pneumocytes, fibrosis of the alveolar walls and macrophages (CD163+) in the lumen of bronchioles and alveoli, where they were associated with the scarce presence of chlamydia antigen. In addition, hyperplasia of bronchus-associated lymphoid tissue (BALT) was observed.

The remaining three animals of the V-PG group developed hyperplasia of BALT with prominent follicles associated with atelectasis corresponding to the depressed areas previously described in the macroscopic analysis. Chlamydial antigen was not observed in these animals (Fig. 7).

Regional draining lymph nodes of non-vaccinated animals showed follicular lymphoid hyperplasia with an active follicular centre, and an increase of the inner cortex, but low presence of Treg cells (Foxp3+), was observed. In contrast, in vaccinated animals, abundant Treg cells (Foxp3+) were found in follicles, interfollicular areas and inner cortex in regional draining lymph nodes, associated with a strong follicular hyperplasia and increase of the inner cortex. The increase in Treg cells observed was statistically significant ($p < 0.05$) in the V-PG group compared with the V-NT and NV-NT. In addition, statistically significant differences were observed between V-NT and NV-NT animals (Fig. 8).

4. Discussion

Chlamydia abortus, the causative agent of ovine enzootic abortion (OEA), is one of the widest ovine abortifacient agents worldwide.

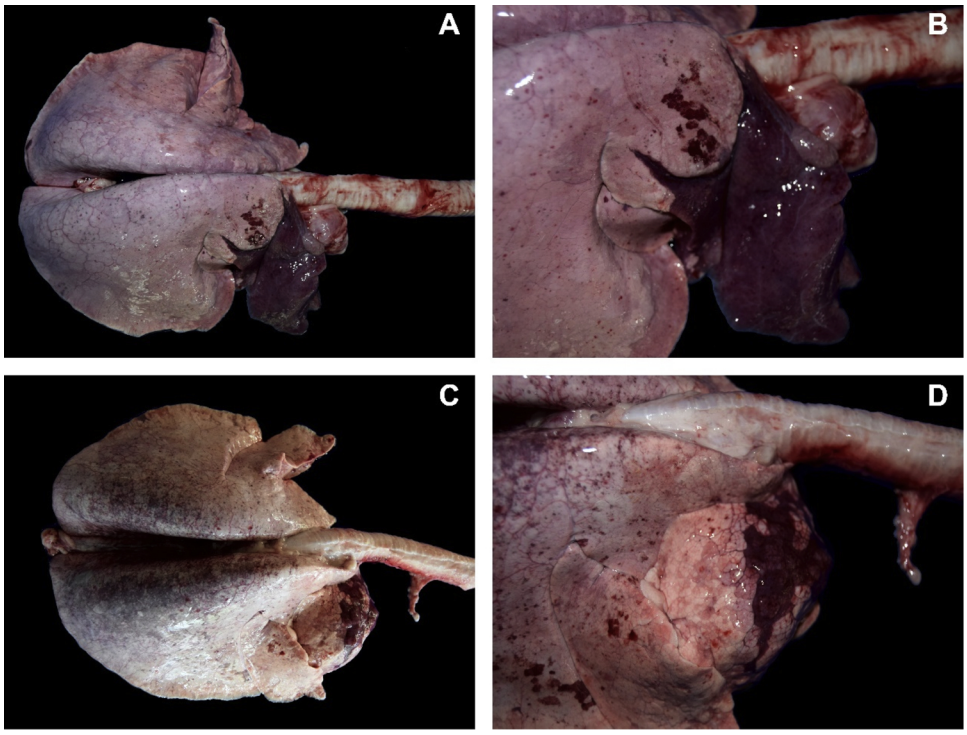

Fig. 3. Gross lesions. (A) Lung of a NV-PG sheep showing a grey-red consolidation of the upper lobe also in areas of the middle lobe. (B) Detail of the lesions shown in (A). (C) Lung of a V-NT sheep showing a small grey-red consolidation area in the cranial portion of the upper lobe. (D) Detail of the 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).

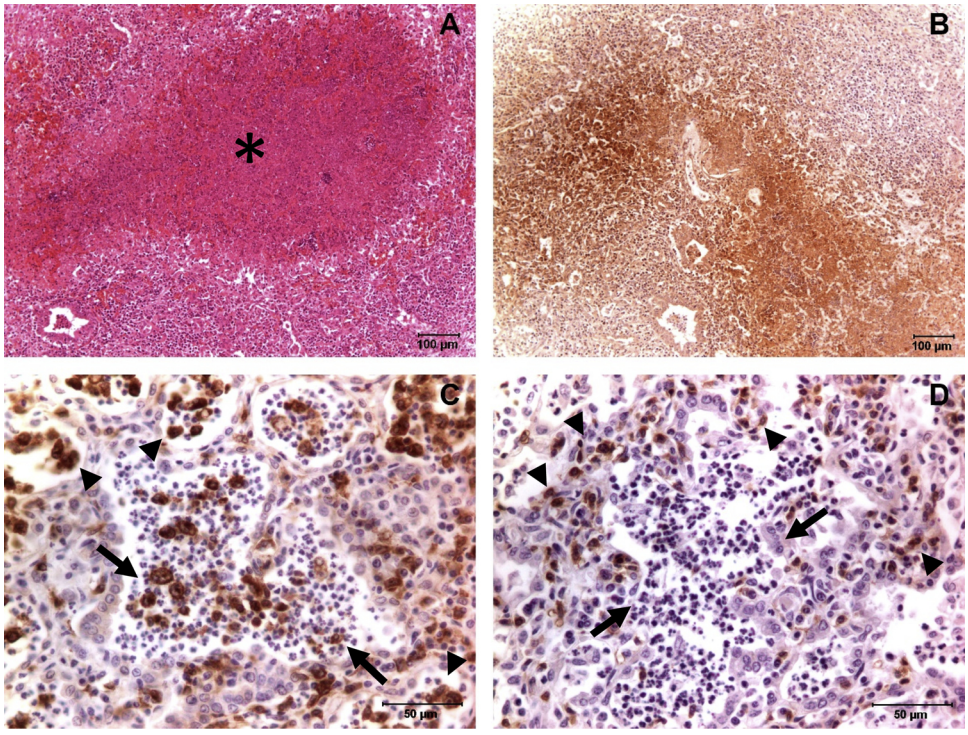

Fig. 4. Histopathological and immunohistochemical study of an NV-NT animal. (A) Section stained with haematoxylin and eosin (H&E) showing a necrosis area (*) with liquefaction of the pulmonary parenchyma. (B) Section immunostained by the ABC method contrasted with haematoxylin showing the association 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).

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

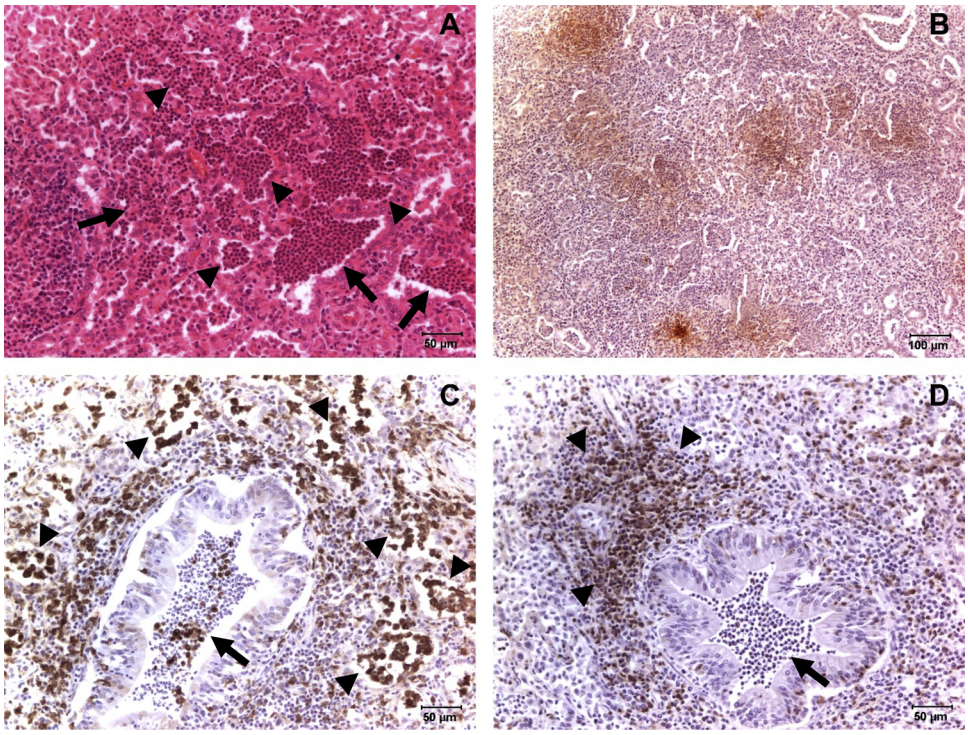

Fig. 5. Histopathological and immunohistochemical study of a NV-PG animal. (A) Section stained with H&E showing suppurative bronchopneumonia with abundant exudate composed of neutrophils and macrophages in the lumen of the alveoli (arrow heads), bronchi and bronchioles (arrows). (B) Section immunostained by the ABC method contrasted with haematoxylin showing the association of lesions with a positive chlamydial antigen immunoreaction (brown areas). The chlamydial antigen detection was lower than that observed in NV-NT animals (Fig. 4B). (C) Detail of a section immunostained for macrophage detection. Note the presence of these cells in the lumen of bronchioles (arrows) and alveoli (arrow heads) along with neutrophils. (D) Detail of a section immunostained for T cells detection. Note the presence of these cells in the alveolar walls, septa and around bronchioles (arrow heads) along with presence of neutrophils in the lumen of bronchioles and alveoli (arrow).

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-*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 *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; Buendía 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 *C. abortus* infection (del Río et al., 2000; Martínez et al., 2006). This cellular immunity seems to be mediated by interferon (IFN-γ) in all chlamydiaceae species. Thus, Chlamydia-specific T-cells wield

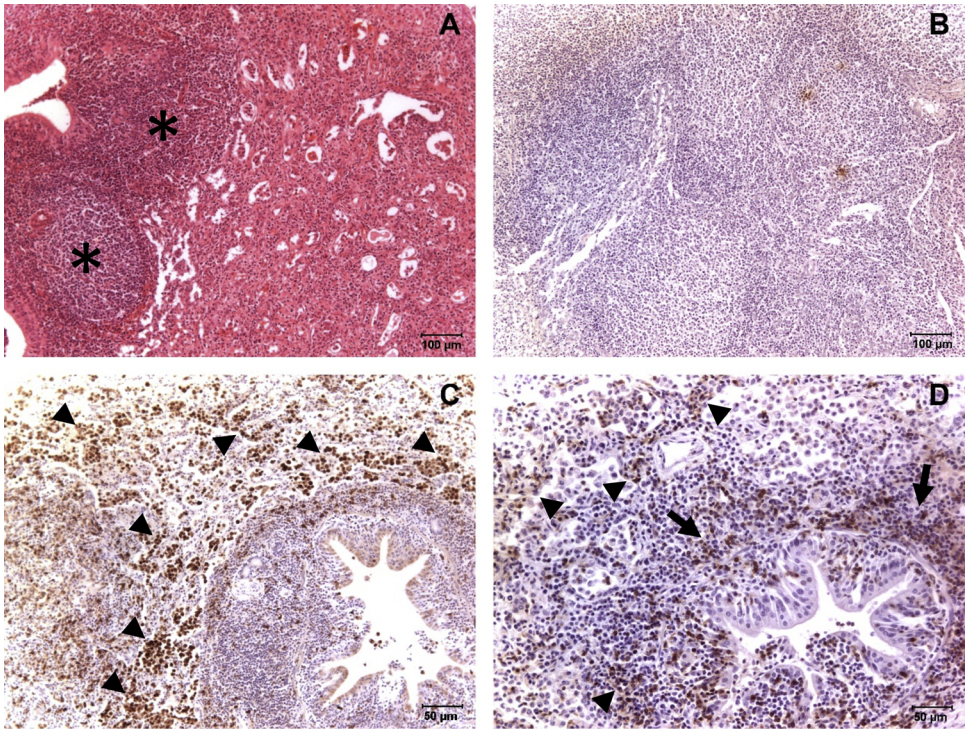

Fig. 6. Histopathological and Immunohistochemical study of a V-NT animal. (A) Section stained with H&E showing interstitial bronchopneumonia and hyperplasia of BALT (*). (B) Section immunostained by the ABC method contrasted with haematoxylin, showing small areas with a positive chlamydial antigen immunoreaction (brown areas). Chlamydial antigen detection was weaker in these animals than in the non-vaccinated groups (Fig. 4 B and 5 B). (C) Detail of a section immunostained for macrophage detection. Note the presence of these cells in the lumen of alveoli (arrow heads) with no presence of neutrophils. (D) Detail of an immunostained section for T cells detection. Note the abundance of these cells in the alveolar walls (arrow heads), septa and around bronchioles and vessels (arrows).

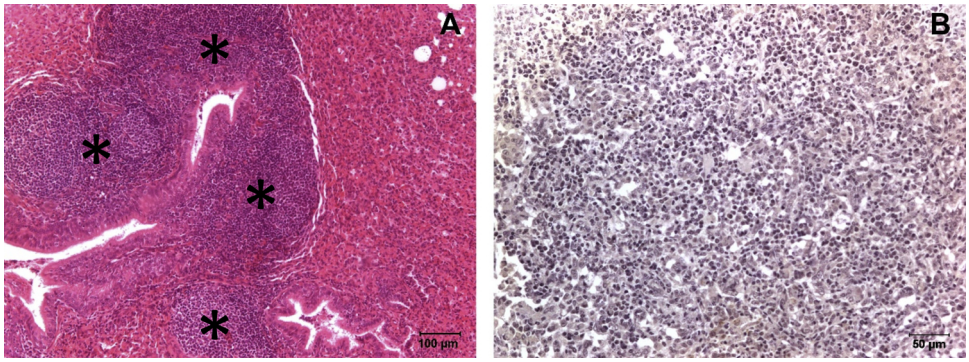

Fig. 7. Histopathological and Immunohistochemical study of a V-PG animal. (A) Section stained with H&E showing hyperplasia of BALT with prominent follicles (*). (B) Section immunostained by the ABC method contrasted with haematoxylin for detection of chlamydial antigen. No chlamydial antigen was detected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

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

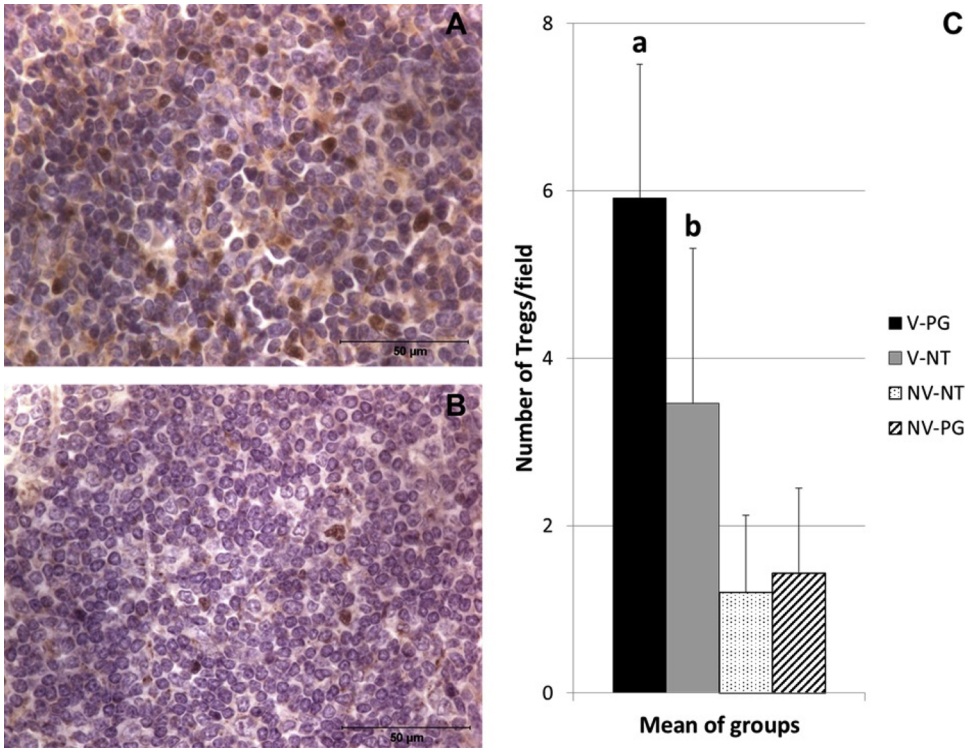

Fig. 8. Detail of an immunostained section for Foxp3+ antigen. Note the large amount of positive lymphocytes detected in the germinal centre follicle in the tracheobronchial lymph node of a vaccinated sheep (A) compared with a non-vaccinated sheep (B) (the immunostaining of the antigen is intranuclear). (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-PG 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-PG 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-PG 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. arbortus* infection (Montes de Oca et al., 2000; del Río 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 minimum 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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