Antisperm response in rams experimentally infected with *Brucella ovis*

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Abstract

*Brucella ovis* infection causes chronic epididymitis in rams and may result in the formation of spermatic granulomas and a major reduction in fertility. To study the relationship between bacterial genital infection and antisperm response, 14 adult rams were experimentally inoculated via conjunctival (\(n = 7; \ G1\)) or preputial route (\(n = 7; \ G2\)) with a *B. ovis* strain. Serological response to *B. ovis* was evaluated by means of complement fixation (CF) and gel diffusion (GD). Autoimmunity was estimated by determination of antisperm antibodies (ASab) and the leukocyte migration inhibition test (MIT) using three different antigens: autologous sperm, ovine testicle, and *B. ovis* antigen. Immune responses were analyzed in relation to clinical signs, histopathological features, and bacterial isolations from semen and genital organs. Seven non-infected rams served as controls. Specific antibody titers were detected by CF during the second week post-inoculation (PI) in 100\% of the inoculated rams. The CF values reached a peak during the 8th and 6th week PI for groups G1 and G2, respectively. The percentage of seropositive animals decreased progressively in both groups until weeks 45 and 30 PI. Specific antisperm reaction was verified by detection of immobilizing ASab and by MIT. ASab were detected in the serum of 85.7\% and 71.4\% of rams in G1 and G2, respectively, from week 3 PI onwards. Genital alterations were clinically detected in 71.4\% of the inoculated rams. Gross epididymal lesions consisted of multiple caseous masses and cysts with purulent content. In those rams that presented clinical lesions, numerous spermatic granulomas were observed. 80\% of these granulomas were located in the epididymis tail. Epididymitis, seminal vesiculitis, and chronic ampullitis were histologically detected in 71.4\% of the animals of G1 and G2. *B. ovis* was isolated from 57\% of semen cultures and was recovered from genital tissues in 75\% of rams in both G1 and G2. At week 50 PI, four rams from each inoculated group (G1 and G2) received antibiotic treatment with long-acting oxytetracycline. In those animals treated with antibiotics, all semen and tissue cultures became negative to *B. ovis* at the time of the necropsy. However, cellular immunity was positive for autologous, testicular, and bacterial antigen and all rams demonstrated to be positive for ASab until week 80 PI. These data showed that rams with genital lesions caused by *B. ovis* developed a long-standing
antisperm immune reaction. This autoimmune process could be significant in the pathogenesis of reduced fertility observed in B. ovis infected rams. © 2000 Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Rams; Male infertility; Genital infections; Brucella ovis; Antisperm autoimmunity

1. **Introduction**

There is increasing evidence that genital infections in males are an important cause of infertility both in animals and men (Fowler, 1981; Foster, 1987; Megory et al., 1987; Eggert Kruse et al., 1996; Hinting et al., 1996). Venereal infectious agents are characteristic-ally protected or harboured along the genital tract (Alexander and Anderson, 1987). *Brucella ovis* is a facultative intracellular bacterium that causes epididymitis, seminal vesiculitis, and ampullitis in rams, followed by orchitis and a progressive loss of the animal’s reproductive performance (Foster, 1987; Ladds, 1993). Blasco (1990) described the serological response against *B. ovis* in spontaneous and experimental infections. Cell-mediated immune responses also have been evaluated in rams experimentally infected with *B. ovis* (Afzal et al., 1986) and the presence of several different types of immunoglobulin and immunocompetent cells was demonstrated in tissues and fluids of the male genital tract (Alexander and Anderson, 1987; Foster et al., 1988). Shott and Young (1971) described an autoimmune response in rams induced by injection of a testicular–epididymal homogenate. In humans, antisperm antibodies (ASab) are considered one of the important factors in male infertility (Yamamoto et al., 1996; Bates, 1997). In male dogs with chronic *B. canis* infection, an anti-sperm response with development of serum and seminal plasma antibodies was detected (George and Carmichael, 1984). However, the development of antisperm autoimmune phenomena in rams infected with *B. ovis* has not yet been described in detail. The formation of sperm granulomas has been used to indicate infertility in men with epididymal-orchitis of infectious or traumatic origin, or in vasectomized men (Hinting et al., 1996). The infectious agents associated with these granulomas can act as adjuvants in developing antisperm autoimmunity and consecutive sterility (Purvis and Christiansen, 1993).

This autoimmune response in men is characterized by the presence of ASab, positive cellular immunity, altered seminal quality, high counts of cells from sperm progeny, and leukocytospermia in ejaculates (Mazzolli et al., 1988; González et al., 1992; Kamada et al., 1998).

In this study, we used an experimental model of genital infection with *B. ovis* in rams to evaluate the specific antibacterial immune response and to compare these results with the appearance of post-infection (PI) antisperm immune reaction.

2. **Materials and methods**

2.1. **Animals**

21 adult Corriedale rams from a Brucellosis-free flock were used. All animals were clinically normal, and serologically and bacteriologically negative to *B. ovis*, with normal seminal parameters determined two weeks before the beginning of the experiment. Basal level of ASab was determined in serum. Rams were fed on natural pasture, alfalfa hay, maize-corn concentrate negative to mycotoxins or zearalenone, and water ad-libitum.

2.2. **Inoculum and experimental inoculation**

The *B. ovis* strain 88E1 INTA, isolated from semen of a ram with epididymitis, was used as inoculum. It was cultivated on Columbia blood agar at 37°C under a 10% CO2 atmosphere for 6 days. The inoculum concentration was adjusted to 3.5 × 1010 colony forming units (CFU) ml⁻¹ using the technique described by Miles and Misra (1938). The average CFU was calculated from the arithmetic means of duplicate plates and the number of bacteria was expressed as log10. Seven adult male rams were allocated at random to any of three groups:

**Group 1 (G1): conjunctival.** The animals (n = 7) were each inoculated with 1 ml of inoculum conjunctivally.
Group 2 (G2): preputial. The rams (n = 7) were administered a 5 ml inoculum into the prepuce, according to Webb et al. (1980).

Group 3 (G3): non-infected controls. Control rams (n = 7) were given the equivalent volume of sterile PBS by both routes.

At week 50 four randomly selected animals from each group (G1, G2 y G3) were treated with long-acting oxytetracycline (Terramicina LA, Pfizer Lab., Argentina) at a dose of 20 mg/kg every 72 h during 30 days to eliminate the B. ovis infection in the genital tract. Each group was maintained in separated corrals during the experimental period. All rams were killed at week 80 PI for bacteriological and pathological studies.

2.3. Clinical examination

All animals were clinically examined on inoculation day and sampled for blood and/or semen. Genital organs were carefully observed and palpated.

2.4. Semen analysis

Semen samples were obtained through electroejaculation at weeks 4, 8, 12, 40, 60, 70, and 80 PI. Smears were stained with May Grunwald–Giemsa stain to evaluate sperm morphology, and also to detect spermatogenic progeny cells and white blood cells (WBC). The amount of WBC was determined in a 400× microscopic field; it was qualified from 1 to 5 according to Kott et al. (1988). The smears were stained with Gram and modified Ziehl Neelsen (ZN) strain (Stamp et al., 1950) to observe the presence of Brucella.

Aliquots of semen also were cultivated immediately after collection. Each sample was cultivated on Columbia blood agar, Skirrow agar, and modified Thayer Martin agar (Terzolo et al., 1991) at 37°C under a 10% CO₂ atmosphere for 10 days. B. ovis was identified by the morphology of colonies and by Gram and modified ZN stains. Suspicious colonies were referred to the Pan American Zoonosis Centre (CEPANZO) in Buenos Aires for further typing by standard procedures (Corbel and McHendry, 1985). Aliquots of semen were centrifuged at 500G for 10 min. The sperm pellets obtained were stored at −20°C; they were later employed as autologous spermatogenic antigen in the cell mediated immunity test (CMI).

2.5. Anti-Brucella serology

Blood samples were obtained at different PI periods: during the first four weeks samples were taken every 3 days, weekly from week 5 to 8, every 2 weeks until week 16, and once a month until week 40. Three additional blood samples were obtained from the animals at 60, 70 and 80 weeks. Sera were tested by complement fixation (CF) and gel diffusion (GD) tests for B. ovis. The antigen used was that described by Myers et al. (1972).

2.6. Autoimmunity determinations

Antisperm antibodies (ASab): One drop of hemolysis-free inactivated serum (56°C for 30 min) from each animal was tested against one drop of semen obtained from a normal donor ram [3 × 10⁶ sperm/ml] to identify antisperm immobilizing or agglutinating antibodies as described by Mazzolli et al. (1983). Those sera that agglutinated or immobilized 50% or more of the present sperms were considered as positive at a dilution higher than 1 : 8, considering this as basal ASab titer for all rams included in this study at the beginning of the experiment.

Cell mediated immunity (CMI): This was evaluated through the leukocyte migration inhibition test (MIT) using heparinized peripheral blood samples (Mazzolli, 1971; Mancini et al., 1972). Three different antigens were used: (a) autologous sperm (obtained from the semen of each ram at the beginning of the experiment), (b) ovine testicular homogenate, and (c) bacterial antigen of the B. ovis 88E1 INTA strain. Inhibition of migration was expressed as a percentage; it was the mean value of several determinations per animal (eight tubes). Inhibition levels of 25% or more were considered significant in our experimental conditions and indicated the presence of positive cellular immunity.

2.7. Post-mortem examination

Samples for bacteriological determinations were taken from testes, epididymides, seminal vesicles, ampullae, bulbourethral, and prostatic glands. Each
tissue sample was ground separately and cultivated as described above. Specimens from the same tissues were fixed in Bouin’s, solution embedded in paraffin, cut at 5 µm, and stained using haematoxylin and eosin.

2.8. Statistical evaluation

The geometric means of CF titers were calculated for each group of rams. The simple linear correlation was calculated at different moments of the experimental period between the percentage of rams with CF and with ASab positive titers for each sampling time.

3. Results

3.1. Bacteriological and serological findings

The results corresponding to G1 and G2 are shown in Tables 1 and 2. Control rams (G3) were clinically normal, serologically negative for GD and CF anti-Brucella, ASab and CMI. They had neither macroscopic nor histological lesions in their genital organs. All the bacteriological cultures of semen and tissues were negative.

3.2. Anti-Brucella serology

All inoculated rams were positive to B. ovis for both tests, i.e., GD and CF (1/10 or higher). All G1 rams presented positive titers from week 3 PI onwards. The maximum percentage of rams positive to CF was reached from week 8 PI onwards, then descended and became negative in some rams, or retained low titers in others until week 80 PI.

57% of G2 animals had positive CF titers from week 3 PI onwards and all (100%) the G2 rams from week 6 PI onwards. The maximum percentage of positive CF was reached at week 12 PI onwards. The percentage of seropositive animals in G1 and G2 groups decreased progressively until week 40 and 30 PI, respectively. Disappearance of antibody titers coincided with remission of epididymal lesions in 4 out of 10 rams from both groups. After antibiotic treatments all rams in G1 (n = 4) and G2 (n = 4) were CF negative, (Fig. 1a and b).

3.3. ASab and cell mediated immunity

Positive immobilizing ASab were detected in 85.7% and 71.4% of ram serum from groups G1

| Table 1 |

<table>
<thead>
<tr>
<th>Serologic anti-Brucella titers (CF), presence of ASab, and isolates of B. ovis of genital tissues in rams inoculated via conjunctival (G1) or preputial route (G2)</th>
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<tbody>
<tr>
<td>Inoculation route</td>
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<td>Conjunctival</td>
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a Results at the time of necropsy, 80 weeks post-inoculation.

b Complement fixation reciprocal titers (>1/10).

c Rams treated with oxytetracycline (20 mg/kg) during weeks 50–60 post-inoculation.
and G2 respectively, starting from week 3 PI onwards. In G1 rams the positive ASab decreased to 42.8% from week 6 to 15 PI, whereas in animals from G2 ASab diminished in week 30 PI and kept their positive titers until week 80 PI. Simultaneously, the percentage of rams with positive anti-Brucella antibodies decreased until all were negative. In all rams from both groups G1 and G2 a negative correlation between number of rams with positive CF titers and positive ASab titers was determined at the beginning (10–20 weeks post-inoculation) and at the end of the experimental time (60–80 weeks post-inoculation). In 7/8 of the inoculated animals treated with antibiotics, the cellular immunity was positive (25% or more CMI) for spermatogenic, testicular, and bacterial B. ovis antigens until week 80 PI. These rams showed ASab positive, but negative anti-Brucella titers and negative isolation of B. ovis.

### 3.4. Semen examination

Inflammatory WBC were detected in the semen of 71.4% of the rams in both G1 and G2 and coincided with clinical detection of testicular–epididymal lesions. The score reached 4 and 5 (5–10 and >10 cells values for microscopic field respectively) in all cases and remained unchanged during the assay. The semen presented oligospermy and spermatogenic cells increased in 71.4% and 66%, in G1 and G2 rams respectively, at week 9 PI. B. ovis was initially recovered from semen of two G2 rams (29%) at week 4 PI, but from week 8 PI onwards it was recovered from 57% of the animals in each group. In both groups, B. ovis was recovered from the genital tissues of 43% and 29% in G1 and G2 rams, respectively. Occasionally, the ZN technique applied to seminal smears showed B. ovis in phagocytic cells or around them. Neither WBC nor bacteria were detected in the semen of antibiotic treated rams.

### 3.5. Clinical and pathological findings

Genital alterations were detected in 71.4% of the inoculated rams. Two rams belonging to G1 showed bilateral epididymal lesions and another two animals presented unilateral lesions from the 6th week PI onwards. The other rams manifested epididymitis from week 8 onwards. Two animals from group G2 with clinical lesions from week 3 onwards were detected while the others showed lesions from week 6 onwards. The lesions comprised testicular adherences, deformation and increase in epididymal tone.

### Table 2

Serologic anti-Brucella titers (CF), presence of ASab, and isolates of B. ovis of semen (BoS) or genital tissues (BoGT) in rams inoculated via conjunctival (G1) or preputial route (G2).

<table>
<thead>
<tr>
<th>Inoculation route</th>
<th>Ram No.</th>
<th>During experimental period</th>
<th>At necropsy</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CF (&lt;1/8)</td>
<td>ASab (&gt;1/8)</td>
</tr>
<tr>
<td>Conjunctival</td>
<td>1</td>
<td>1/320</td>
<td>+</td>
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<tr>
<td></td>
<td>2</td>
<td>1/320</td>
<td>–</td>
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<tr>
<td></td>
<td>3</td>
<td>1/320</td>
<td>+</td>
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<tr>
<td></td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/80</td>
<td>+</td>
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<td></td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/80</td>
<td>+</td>
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<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/40</td>
<td>+</td>
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<tr>
<td></td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/320</td>
<td>+</td>
</tr>
<tr>
<td>Preputial</td>
<td>8</td>
<td>1/320</td>
<td>–</td>
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<tr>
<td></td>
<td>9</td>
<td>1/320</td>
<td>+</td>
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<tr>
<td></td>
<td>10</td>
<td>1/10</td>
<td>–</td>
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<tr>
<td></td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/80</td>
<td>–</td>
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<td></td>
<td>12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/40</td>
<td>+</td>
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<tr>
<td></td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/160</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/320</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Complement fixation, maximal reciprocal titers.
<sup>b</sup> Rams treated with oxytetracycline (20 mg/kg) during weeks 50–60 post-inoculation.

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Fig. 1. Percentage of seropositive rams for anti-Brucella ovis (CF ≥ 1/10) and antispermatic antibodies (ASab ≥ 1/8) in eight rams after: (a) conjunctival (G1) and (b) preputial (G2) experimental inoculation with *B. ovis* during 80 weeks. Antibiotic treatment was administered on week 50 in four rams in both G1 and G2.
Multiple caseous masses and cysts with purulent contents were observed in the sectioned epididymis. Epididymitis, seminal vesiculitis, and chronic ampullitis were observed histologically in 71.4% of the animals of G1 and G2 in coincidence with the presence of macroscopic lesions. In those rams that presented clinical lesions, numerous spermatic granulomas, 80% of them in the tail of the epididymis, were observed as a mass of neutrophils, macrophages with phagocyted spermatozoa, and giant cells in direct contact with mononuclear cells. Degeneration, atrophy, and mineralization were frequently found in testicles. The histological findings were similar to those presented by other authors in cases of epididymitis due to *B. ovis* (Biberstein et al., 1964; Shott and Young, 1971; Searson, 1987; Foster, 1987; Blasco, 1990; Ladds, 1993).

4. Discussion

The results of the present study indicate that genital brucellosis in rams is associated with a long-standing antispermatic response that could be confirmed by the presence of ASab and CMI against autologous sperm and testicular tissue. The inoculations via mucosal routes was very effective in establishing the disease. This was confirmed by a high percentage of rams with specific positive titers to *B. ovis*, the isolation of the agent from semen and genital tissues, as well as by the development of typical lesions. Similar findings were reported by other authors in both naturally and experimentally *B. ovis* infected rams (Shott and Young, 1971; Afzal et al., 1986; Foster et al., 1988).

Allergic orchiepididymitis has been induced in bulls and guinea pigs (Parsonson et al., 1971; Mazzolli, 1971) after inoculation of testicular homogenates, with lesions similar to those of our study. The exposure of the immune system to spermatic antigens, as a result of trauma or infection in the genital tract, stimulates a high titer of ASab production that could persist for many years (Alexander and Anderson, 1987; Purvis and Christiansen, 1993; Hinting et al., 1996).

The presence of severe macroscopic and histological lesions in the genital tissues of rams infected with *B. ovis* indicates a possible break in the immune tolerance and a response to spermatic autosensitization. Infection does not necessarily initiate an autoimmune phenomenon, but it is known that infections in the genital tract are often associated with positive cellular immunity, and ASab in animals as well as in men (Afzal et al., 1986; Foster, 1987; Ladds, 1993; Hinting et al., 1996). Dogs chronically infected with *B. canis* with a bacteremia lasting for more than four months had cutaneous delayed-type hypersensitivity reactions when tested with soluble canine testicular extracts. Those dogs with testicular atrophy had the most severe skin test responses. Seemingly, isoimmune responses to sperm antigens are involved in infertility caused by *B. canis* infection of male dogs (George and Carmichael, 1984).

Although antibodies do not necessarily mean infertility, many cases showed a close correlation between reproductive tract infection and autosensitization with positive cellular immunity and ASab in serum, seminal plasma or both (Mazzolli and Barrera, 1989). Moreover, ASab in infertile men were inversely correlated with gestation percentage (Rumke et al., 1974), conception rate (Fuchs and Alexander, 1983), poor initial spermatic motility, and decreased cervical mucus penetration (Mathur et al., 1986). The ASab were demonstrated in men with prostatitis, positive seminal cultures, and testicular obstruction (Hendry, 1989). Obstruction of the male genital tract, whatever the cause, is often associated with antisperm immune reaction and infertility (Hinting et al., 1996; Yamamoto et al., 1996). González et al., 1992 found that 50% of men with ASab in the seminal plasma showed astenozoospermia, teratozoospermia, leukocytospermia, and hypo-function of seminal vesicles with high prevalence of IgA in the seminal plasma. Also, chronic genital infection could have an adjuvant role in spermatic autoimmunity (Purvis and Christiansen, 1993). Shott and Young (1971) attempted to demonstrate the contribution made to the pathological process by addition of testicular–epididymal homogenates in rams inoculated with *B. ovis*, but they did not find a correlation between the pathological and the immunological processes. However, other authors communicate that *B. canis* infection in dogs induced an immune response of variable intensity against spermatozoa due to absorption of sperm antigens (Serikawa et al., 1984).
Male Beagles infected with *B. canis* for 3 months or more developed serum antibodies that agglutinated normal canine spermaotzoa, and antibodies were observed in seminal plasma of chronically infected dogs (George and Carmichael, 1984). In our work, the antispermatic response in rams had a similar behaviour in both groups (G1 and G2) and coincided with the presentation of epididymal lesions. Moreover, with the antibiotic treatment a gradual decrease of titers against *B. ovis* as well as the elimination of the bacterium in semen and genital tissues allowed the identification of the rams with positive ASab and cellular immune responses. The ASab were detected early after infection, remaining higher than the basal titer during a long period of time, as could be observed for 80 weeks PI. In men presenting obstruction and/or infection of genital organs, ASab have been considered the cause of prolonged sterility and the period of infertility was estimated to be of 5 years in average (Hinting et al., 1996).

The relevant leukocytospermia together with the alterations in seminal quality observed in our study coincides with the findings of other authors in similar experimental studies (Kott et al., 1988; Kortebani et al., 1992). It was demonstrated that leukocytes produce cytokines, tumor necrosis factor-α and free radicals, and also reduce motility and the fertilizing potential of spermatozoa (Tomlinson et al., 1992). In fact, polymorphonuclear leukocytes are a major source of harmful oxygen-derived free radicals, especially after interaction with bacterial products and cytokines (Wang et al., 1997). Additionally, *B. ovis* infection in rams modified the carbohydrate composition of organs participating in maturation, transport, and storage of spermatozoa. Glycoconjugates are instrumental in receptor–ligand interactions. These alterations have been postulated to be involved in the impaired fertility observed in *B. ovis* infected rams (Paolicchi et al., 1995).

Today, ASab detection is one of the most important steps in the evaluation of infertility in men (Dondero et al., 1997; Lenzi et al., 1997), and ASab could become an important tool in the diagnosis of animal infertility. Continued investigation of infertility in rams will lead to novel treatment regimens as well as to the development of new prophylactic measures. Finally, this ovine disease could be a useful model in comparative pathology and biomedical research.

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