

Clinico-pathological Changes Associated with *Brucella melitensis* Infection and its Bacterial Lipopolysaccharides (LPS) in Male Mice

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Abstract: *Brucella melitensis* (*B. melitensis*) is gram negative, aerobic bacteria that cause Brucellosis in humans, sheep and goats. Brucellosis causes abortion in wild and domestic animals resulting in enormous financial losses. Therefore, the purpose of this study was to evaluate the clinico-pathological changes associated with *Brucella melitensis* infection and its bacterial Lipopolysaccharides (LPS) in male mice. Three groups of 24 Balb/c male mice consisting of 8 mice in each group were used as an animal model for the study. The control group were inoculated intraperitoneally with 1 mL of Phosphate Buffered Solution (PBS) pH 7 while, the treatment groups were inoculated intraperitoneally with 1 mL×10⁹ of *B. melitensis* colony and 1 mL×10⁹ of Lipopolysaccharides (LPS) extracted from *B. melitensis* respectively. Mice that showed severe clinical signs and those that survived were euthanized by cervical dislocation method after 5 days of post infection subsequently, post mortem was conducted and histopathological studies were carried out. *B. melitensis* group showed severe clinical signs between 6 to 17 h of post inoculation compared to the PBS and LPS groups. The LPS group became lethargic 2 h post inoculation but, they become active after 5 h post inoculation, while the control group (PBS) exhibited normal responses. Histopathology results showed severe tissue alterations in the reproductive organs of the *B. melitensis* group compared to LPS group. In conclusion, the atrophy of the spermatocytes in the testes and degenerative necrosis of the pseudo stratified epithelium of the vas deferens in the *B. melitensis* group were severe while, LPS group showed moderate atrophy of the spermatocyte of the testes and severe degenerative necrosis of the pseudo stratified epithelium of the vas deferens.

Keywords: Atrophy, *B. melitensis*, brucellosis, lipopolysaccharides, spermatocyte, vas deferens

INTRODUCTION

Brucella melitensis (*B. melitensis*) is gram negative, facultative intracellular coccobacillus or short rod (0.6-1.5 µm) aerobic bacteria that cause Brucellosis in humans, sheep and goats (Gupta *et al.*, 2006; Joicy *et al.*, 2012). The mucosal surface of the alimentary tract is the principal route of entry for *B. melitensis* (Carlos *et al.*, 2011). Unlike many other pathogens, *Brucella* spp. invade host cells without activating innate immune defence systems and then resist intracellular killing to persist in the host (Gorvel and Moreno, 2002; Barquero *et al.*, 2007). Brucellosis causes abortion in wild and domestic animals resulting in enormous financial losses especially in developing parts of the world (Andrew *et al.*, 2003).

The most current threat is on the possible use of *Brucella* species, principally *B. melitensis*, as an agent

of biological warfare because of the incapacitating ailment it causes. Extensive spreading of aerosolized *B. melitensis* would pose a biological, agricultural, as well as an economical threat to all countries engrossed (Gul and Khan, 2007). The disease is an emerging and re-emerging disease (Seleem *et al.*, 2010). The morbidity of the disease reported to be increase in number in central Asia and certain countries in the Middle East (Pappas *et al.*, 2006).

Brucella Lipopolysaccharides (LPS) is less endotoxic compared to enteric gram-negative bacteria, due to the presence of the unique components O-Polysaccharides (OPS) and Lipid A (Jarvis *et al.*, 2002; Cardosa *et al.*, 2006). The *Brucella* cell wall consists of a peptidoglycan layer which is strongly associated with the outer membrane. The outer membrane contains lipopolysaccharides, proteins and phospholipids (Galdiero *et al.*, 1995). *B. melitensis*, *B. abortus*, *B. Suis*

and *B. Neotomae* have smooth structure of LPS. The specific O-chain saccharides of the LPS structure determine the M (melitensis) type, or A (abortus) type antigenicity. Brucella antigenic characteristic of *Brucella melitensis* depends on LPS (Joicy *et al.*, 2012). There is limited information of host cell response towards immunogen of *B. melitensis* in mice. Therefore, the objectives of the present study are to compare the clinical signs and cellular changes of mice with reference to male reproductive organs following inoculation with *B. melitensis* and its bacterial Lipopolysaccharide (LPS).

MATERIALS AND METHODS

Bacteria: Stock of *B. melitensis* was obtained from the Histopathology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). The organism was re-cultured onto media in which biotin, thiamine and nicotimide were used as a supplement for bacterial growth. The incubation period of this bacterium is 3-4 days and the optimum temperature for the growth is 36-38°C. All procedures and experiments illustrated were undertaken under a project license approved by Animal Utilization Protocol Committee with reference number: UPM/FPV/PS/3.2.1.551/AUP-R120.

Preparation of 1 mLx10⁹ colony of *Brucella melitensis*: Mac Farland technique was used to determine the concentration of *B. Melitensis* (10⁹ colonies).

LPS extraction from 10⁹ of colony *Brucella melitensis*: The LPS extraction kit (Intron biotechnology®) was used to prepare the LPS from *B. melitensis* bacteria. In the present study, 10⁹ cfu of organism (*B. melitensis*) was extracted. The bacteria were harvested by centrifugation in room temperature at 13,000 rpm. Then, 1 mL lysis buffer was added and it was vortex vigorously. Thereafter, an amount of 200 µL of chloroform was added and it was vortexed vigorously for 10-20 sec after which it was incubated at room temperature for 5 min. Following this, it was centrifuged again at 13,000 rpm for 10 min at 4°C. The supernatant of 400 µL was transferred to a 1.5 mL tube. 800 µL Purification buffer was added into the tube and it was mixed well. Subsequently, it was incubated in -20°C for 10 min. Then it was centrifuge at 13,000 rpm for 15 min at 4°C. The upper layer was removed to obtain the LPS pellet. One millilitre of 70% ethanol was added to the pellet to wash the pellet. The 30 µL of 10 M Tris-HCL buffer pH 8 was added to LPS and was dissolved by boiling it for 2 min.

Experimental design: Balb/c mice were obtained from the Institute of Cancer Research (ICR). Twenty four Balb/c mice were divided into 3 groups, with 8 mice in

each group. Mice in group 1 were inoculated intraperitoneally with 1.0 mLx10⁹ of Phosphate Buffer Solution (PBS), pH 7, mice in group 2 were inoculated with 1.0 mLx10⁹ of *B. melitensis* and group 3 were similarly inoculated with 1.0 mLx10⁹ of Lipopolysaccharides (LPS) of *B. melitensis* colony. All the groups were observed for 120 h (5 days) for clinical signs such as ruffled hair, movement, ocular discharge, closed eyed and responsiveness. Mice showed severe clinical signs and those that survived were euthanized after 5 days via cervical dislocation method and the reproductive organs namely testes, seminal vesicle and vas deferens were obtained for histopathological study.

Clinical scoring: The clinical signs of all the three groups of mice namely, control, whole bacterium (*B. melitensis*) and LPS were scored in scale of 0-3 based on the presence of ruffled fur, ability to move and discharges from the eye as well as the degree of responsiveness following infection of *B. melitensis* and its LPS. The score 0 represented no abnormality of clinical signs observed, 1 for mild, 2 for moderate and 3 for severe.

Lesion scoring: Cellular changes were scored following the evaluation of 10 slides. 3 organs were examined. Lesion scoring was divided into 4 namely; 0 = normal; 1 = mild lesion less than 1/3 of the field was involved; 2 = moderate lesion between 1/3 and 2/3 of the field were involved; 3 = severe more than 2/3 of the field was involved.

Statistical analysis: Data obtained were analyzed using the statistical software package JMP 9 (SAS, QSAS Institute Inc, Cary, NC, USA). Analyses were considered as significant at p<0.05.

RESULTS

Clinical observation: All challenged groups showed significant clinical changes following infection with *B. melitensis* and its LPS. In Brucella group, the clinical signs did appear earlier and were severe in comparison to the control and LPS inoculated group. Lethargic and laboured breathing signs were exclusively noted at 11 h post-inoculation. The severity of the clinical sign reached its peak at 15 h when animals were humanly euthanized at this point. Brucella group showed significant changes in all clinical observations compared to the control and LPS group (Table 1). In contrast, animals treated with LPS extracted from *B. melitensis* exhibited the clinical signs at 12 h post-inoculation. The severity of the clinical signs in this group ranged from mild to moderate with significant differences (Table 1).

Pathological changes: Table 2 showed the mean score of cellular changes of the reproductive organs of male

Table 1: Summary of clinical signs observed in mice inoculated with PBS, *Brucella melitensis* and LPS

Time	Group 1 (PBS)	Group 2 (<i>Brucella</i>)	Group 3 (LPS)
2 h	Absent	Absent	-Mild movement, responsiveness
3 h	-	-	-Mild movement
5 h	-	1/8 (laboured breathing, ruffled fur, closed eye, responsiveness). Others were in good shape	-
6 h	-	1-died	-
7-10 h	-	2/7 (ruffled fur, laboured breathing), 7/7 (responsiveness)	-
11-12 h	-	7/7 (severe movement, laboured breathing, responsiveness) at 12 h: diarrhea observed in all the mice	All look very active
13-15 h	-	Severe clinical signs in all parameter, also diarrhea and seizure. At 15 h 1-died	Still active and exhibit normal clinical signs
15-17 h	-	Mice euthanized (due to severe clinical signs)	-
120 h	Euthanized	-	Euthanized

Table 2: Mean score of cellular changes of reproductive organs observed in mice 5 days post inoculation with immunogen

Organs	Parameter	Group 1 (PBS)	Group 2 (<i>Brucella</i>)	Group 3 (LPS)
Testes	Atrophy of spermatocyte	0.0000±0.0000	*2.3333±0.33333	*1.6667±0.33333
Vas deferens	Degenerative necrosis	0.0000±0.0000	*2.8333±0.16667	*2.6667±0.16667

*: Significant value $p < 0.05$; Comparison between immunogens group and control group

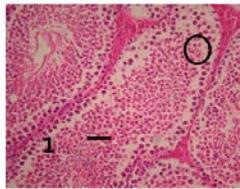


Fig. 1: Photomicrograph of a section of testes of mouse inoculated with *B. melitensis*. Part of atrophy of spermatocyte (circle) (HE, bar = 50 µm)

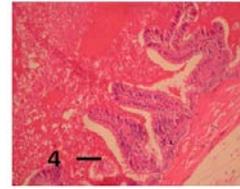


Fig. 4: Normal photomicrograph of a section of seminal vesicle of mouse inoculated with LPS extracted from *B. Melitensis* (HE, bar = 50 µm)



Fig. 2: Photomicrograph of a section of testes of mouse inoculated with LPS extracted from *B. melitensis*. Part of atrophy of spermatocyte (circle) (HE, bar = 20 µm)

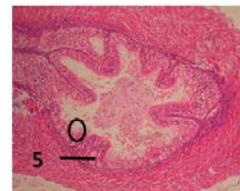


Fig. 5: Photomicrograph of a section of vas deferens of mouse inoculated with *B. melitensis*. Part of degeneration necrosis (circle) (HE, bar = 20 µm)

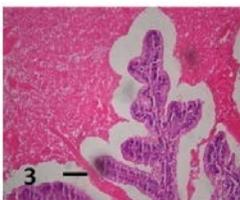


Fig. 3: Normal photomicrograph of a section of seminal vesicle of mouse inoculated with *B. Melitensis* (HE, bar = 50 µm)



Fig. 6: Photomicrograph of a section of vas deferens of mouse inoculated with LPS extracted from *B. melitensis*. Part of degeneration necrosis (circle) (HE, bar = 20 µm)

mice in the present study after 5 days of post inoculation with immunogen while, Fig. 1 to 6 showed the histopathological changes in the male reproductive organs of mice after 5 days of post inoculation with immunogen. There were significant differences in the

pathological changes in the testes between *B. melitensis* group, LPS group and the PBS group. There were also significant differences in the cellular changes of the spermatocyte between *B. melitensis* and LPS groups compared to the PBS group. The mice in *B. melitensis*

inoculated group showed severe atrophy of the spermatocyte with the mean score of 2.3333 ± 0.3333 compared to the PBS group. Mice in LPS group showed moderate atrophy of the spermatocyte with mean score of 1.6667 ± 0.3333 . *B. melitensis* group showed no significant difference in cellular changes of seminal vesicle compared to PBS group. *B. melitensis* and LPS group showed significant differences in cellular changes of vas deferens whereas the *B. melitensis* group showed severe degenerative necrosis with mean score of 2.8333 ± 0.16667 whereas the LPS group showed severe degenerative necrosis with mean score of 2.6667 ± 0.16667 .

DISCUSSION

B. melitensis is a gram negative, coccobacillus or short rod bacteria (OIE, 2009; Joicy *et al.*, 2012) which cause brucellosis in humans, sheep and goats. This bacterium is usually transmitted via contact with the placenta, fetus, fetal fluid and vaginal discharge. Goats are shedding this bacteria in vaginal discharge for about 2-3 months, while sheep for about 3 weeks. Sheep and goats were infected with these bacteria through the mucous membrane of oropharynx, upper respiratory tract and conjunctiva. *B. Melitensis* also can be transmitted through the broken skin. Male goats infected with these bacteria, resulted in the development of orchitis and epididymitis leading to infertility (Lilenbaum *et al.*, 2007). Arthritis was also observed but occasionally. Goats were the source of the infection for human population through the consumption of goats' products (Wallach *et al.*, 1997; De Massis *et al.*, 2005; Kahler, 2000; Acha and Szyfres, 2003; Sriranganathan *et al.*, 2009).

In the present study, mice inoculated with *B. melitensis* exhibited severe clinical signs and all the mice died gradually after 6 h and up to 15 h of post inoculation. In contrast, mice that were inoculated with LPS became lethargic 2 to 3 h post inoculation, but after that, they exhibited normal clinical signs. Similarly, in a study conducted by Alton (1990) in goats the infection lasted for a duration of short period of slight infection to persistence for years while sheep were resistance to the infection probably due to breed disposition. LPS group become more active at 12 h of post inoculation and all mice survived until the day they were euthanized. A study conducted by Edmond *et al.* (2002) proved that Brucella species lacking major outer membrane protein Omp 25 were attenuated in mice and protected them against *B. melitensis*. Furthermore, a study by Mina *et al.* (2004) stated that during infections by gram-negative bacteria, the presence of Lipopolysaccharide (LPS) stimulated the innate immune system whereby the inflammatory response played a critical role in helping to clear the bacteria and prevent infection. The mice in PBS group showed normal clinical signs throughout the 5 days of the experiment.

In the current study, mice in groups and LPS group showed atrophy of spermatocytes in the testes and also degenerative necrosis in the pseudo stratified epithelium of the vas deferens. Atrophy of the spermatocyte was also seen to increase the space between the spermatocytes as shown in (Fig. 1 and 2). In the degenerative necrotic cells there were ballooning appearances in the cytoplasm of the pseudo stratified epithelium cells as indicated in (Fig. 5 and 6). However, there were no lesions in the seminal vesicles as observed in the *B. Melitensis* group, LPS and PBS. In a previous study conducted by Izadjoo *et al.* (2008) stated that mice inoculated with *B. melitensis* ($5 \text{ mL} \times 10^{11}$) via oral route, showed inflammation of testes on day 57, but, recovered on day 88. In the present study, inoculation with $1 \text{ mL} \times 10^9$ of *B. melitensis* intraperitoneally indicated the development of clinical signs and histopathologic lesion similar to the findings of Izadjoo *et al.* (2008). Additionally, Study by Takele *et al.* (2009) indicated that mice inoculated with 5×10^8 cfu *B. melitensis* colony showed clinical signs at the early stages such as extreme shivering, erection of hair coat, anorexia and dullness. These clinical signs virtually disappeared at the later stages after one month of post inoculation.

In another study conducted by Jinkyung *et al.* (2002) stated that mice inoculated with 5×10^5 of *B. abortus* intraperitoneally were unable to resolve the infection and died between 10-20 days post inoculation. In the present study, the mice were inoculated with $1 \text{ mL} \times 10^9$ *B. melitensis* intraperitoneally and they died within 15 h of post inoculation.

Another study conducted by Apurba *et al.* (2002) stated that the intranasal immunization of *B. melitensis* LPS with *Neisseria meningitides* Group B Outer Membrane Protein (GOMP) significantly protected the mice against the virulent *B. melitensis* (strain 16 M). It reduced the dissemination of spleen and liver injury but not lung infection. LPS is indispensable for the viability of most Gram-negative bacteria. Brucella LPS is less endotoxic than that of enteric Gram-negative bacteria, a feature attributed to unique components in the OPS and lipid A (Jianwu and Thomas, 2011).

In conclusion, this experiment prove that mice infected intraperitoneally with *B. melitensis* developed severe clinical signs whereas mice inoculated with *B. melitensis* LPS developed normal to mild clinical signs. Furthermore, both *B. melitensis* immunogen inoculated in male mice developed pathological changes in the testes and vas deferens. The *B. melitensis* LPS could be a promising candidate for the development of vaccine against *B. Melitensis* in mice, sheep, goats and humans.

Conflict of interest: The authors declare that they have no conflict of interest.

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