

Clinical Responses and Reproductive Pathological Changes Associated with *Brucella melitensis* and its Lipopolysaccharides in Female Mice

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Abstract: Brucellosis is a bacterial disease caused by the genus *Brucella*, in small ruminants it is caused by *Brucella melitensis* and is a Gram negative, facultative intracellular coccobacillus bacterium. It is characterized by significant reproductive problems and lead to massive economical loses. Hence, this study is designed to observed clinical responses and pathological changes in reproductive organs in female mice via intraperitoneal inoculation. Twenty four healthy female mice were divided into three groups. The treatment groups were inoculated with 1.0 mL of 10^9 wild type *Brucella melitensis* and 1.0 mL of 10^9 *Brucella* LPS intraperitoneally while; the control group was inoculated intraperitoneally with 1.0 mL of sterile phosphate buffer. The mice were observed for clinical signs for 10 days of post inoculation. Female reproductive organs were collected after 10 days for histopathological study. Mice in the *Brucella* group developed severe clinical signs compared to mice infected with LPS. The clinical signs observed were ruffled fur, movement, responsiveness and eye conditions. The pathological changes in the reproductive organs were moderate to severe in the *Brucella* group in relation to inflammatory cells, mild to moderate necrosis, degeneration; congestion and hemorrhage were also observed. The most affected reproductive organ post inoculation with the wild type *Brucella* was the ovary especially in relation to the infiltration of inflammatory cells and congestion/hemorrhage. LPS group developed similar lesions with the *Brucella* group except that the LPS group developed normal to mild necrosis and degeneration. The ovary of the mice infected with LPS developed moderate to severe lesions of inflammation with congestion/hemorrhage but lesser necrosis. In general, the oviducts were moderately inflamed with mild congestion. Some part of the oviduct also showed necrotic and degenerated lesions. Besides that, the vulva of mice in LPS group developed more necrotic lesions compared to the *Brucella* group. This finding suggests that, *Brucella immunogen* (LPS) is a good candidate for the development of Brucellosis vaccine.

Keywords: *B. melitensis*, clinical response, female mice, intraperitoneal inoculation, pathological changes, reproductive organs

INTRODUCTION

Brucella is gram negative, facultative intracellular bacteria that can infect many species of mammals and also human (Acha and Szyfres, 2003). There are at least six species of brucella recognized within the genus of the bacteria which comprises the *B. melitensis*, *B. abortus*, *B. ovis*, *B. suis*, *B. canis* and *B. Neotomae* (Alton *et al.*, 1988). The classification of brucella genus is mainly based on pathogenicity and hosts response (Corbel, 1997, 1989). Humans are also susceptible to *Brucella* species especially *B. melitensis* and *B. suis*, either through direct contact or consumption of affected milk (Gupta *et al.*, 2006; Seleem *et al.*, 2010; Joicy *et al.*, 2012).

B. melitensis is the main aetiological agent that causes Brucellosis in animals especially in small ruminants, goat and sheep. *B. melitensis* was the first in the genus *Brucella* described by Bruce (1887). Abortion in pregnant animals is the main clinical sign cause by *B. melitensis* (Lopes *et al.*, 2010). The infected animals shed the pathogen to various organs such as reproductive organ and milk (Hamd and Amin, 2002). It is usually being transmitted through direct contact with the placenta, aborted foetus and vaginal discharge of infected animals (Gupta *et al.*, 2006; Joicy *et al.*, 2012).

The pathogenesis of brucella is associated with their ability to pass the natural immunity barrier of the host and enter the cytoplasm of the variety of phagocytic cells especially macrophages (Lapaque

et al., 2005; Barquero-Calvo *et al.*, 2007). The virulence of these species and the establishment of chronic infections by brucella are thought to be essentially due to their ability to avoid the killing mechanisms within macrophages (López-Urrutia *et al.*, 2000).

The virulence of *B. melitensis* is associated with the smooth colony morphotype which contains the full Lipopolysaccharide (LPS). LPS is an immunogenic component which consists of expressed antigen on the surface of the Brucella (DeBagüés *et al.*, 2005). It is known as the main virulence factor of Brucella species. The virulence and intracellular survival potency depend mainly on the presence of the LPS (López-Urrutia *et al.*, 2000). LPS also contains a potent immunomodulator/stimulator on its inner segment known as lipid A (DeBagüés *et al.*, 2005). Brucella LPS is less endotoxic than enteric Gram-negative bacteria due to the presence of the unique components namely, OPS and Lipid A (DeBagüés *et al.*, 2005; Leornado *et al.*, 2004). There is inadequate knowledge on host cell response towards LPS immunogen of *B. melitensis*. Therefore, this study was designed to observed clinical responses and pathological changes in reproductive organs in female mice via intraperitoneal inoculation.

MATERIALS AND METHODS

Culture *B. melitensis* from stock: Wild type *B. melitensis* was isolated from previous outbreak of brucellosis in Malaysia and was obtained from Histopathology, Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The Brucella organisms were cultured on the selective Brucella agar which contains biotin, thiamin and nicotinamide. The culture was incubated for 4 days at 36-38°C with pH of 6.8. Brucella colonies are normally visible after 2 days of incubation period. Brucella colonies are round in shape with 1-2 mm in diameter, with smooth (S) margins, translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. The colonies will appear convex and pearly white when the plates are viewed from above where later the colonies will appear larger and darker.

Preparation 1 mL×10⁹ colony of *B. melitensis*: Several colonies of *B. melitensis* were chosen from the growing bacteria and mixed with distilled water. The concentration of the bacteria inoculums were determined using McFarland technique. Concentration of 10⁹ colony forming unit (cfu) was used in this study.

LPS extraction from 10⁹ colonies of *B. melitensis*: Lipopolysaccharide of *B. melitensis* was extracted by using LPS extraction kit (Intron Biotechnology). Brucella LPS was extracted from 10⁹ cfu *B. melitensis* colony. Five mL of 10⁹ *B. melitensis* was centrifuged at

13000 rpm at room temperature. Then, all the traces of the supernatant were removed and 1 mL of Lysis Buffer was added and was vortexed vigorously. Then 200 µL of chloroform were added and vortexed vigorously for 10-20 sec. The sample was incubated at room temperature for 5 min. After incubation, it was centrifuged at 13000 rpm for 10 min at 4°C. Then, 400 µL of supernatant were transferred to new 1.5 mL tube. Eight hundred µL of Purification Buffer was added and was mixed well. After that, the sample was incubated for 10 min at -20°C. After centrifuged at 13000 rpm for 15 min at 4°C, the upper layer was removed to obtain LPS pellet. 1mL of 70% ethanol was added to wash out the pellet in the tube. Finally, 30-50 µL of 10M Tris-HCL buffer pH 8 was added into the tube and it dissolved the LPS by boiling for 2 min.

Experiment design in mice: Twenty four healthy female ICR mice were divided into 3 equal groups. The groups consist of 8 female mice each. The first group of mice was inoculated with 1 mL 10⁹ of wild type of *B. melitensis* while group 2 was inoculated with 1 mL of LPS extracted from 10⁹ of *B. melitensis*. The third group (negative control group) was inoculated with 1.0 mL sterile phosphate buffered saline (PBS) pH 7. The inoculations were done via intraperitoneal route. The mice were observed for 10 days post-inoculation for clinical signs and responses. The mice showed severe clinical signs and survived mice after 10 days of post-inoculation were culled via cervical dislocation. Post mortem examination was conducted and the following reproductive organs consisting of ovary, oviduct, uterine body, vagina and vulva were collected for histopathology analyses.

Histopathology: Post mortem examination was conducted on all the mice that were culled after 10 days of the experiment. The survived mice were euthanized by using cervical dislocation. The female reproductive organs consisting of the ovary, oviduct, uterine body, vagina and vulva were collected for histopathology study. The organ samples were fixed with 10% formalin for 3 days prior to the processing. The tissues were processed in an automatic tissue processor. Tissue was processed into paraffin blocks and each section was routinely stained with standard hematoxylin and eosin (H&E) stain.

Clinical scoring: The clinical signs in this study consist of 4 parameters which are ruffled fur, movement, eye condition (eye discharge, closed eye) and responsiveness. The scoring for ruffled fur in mice was evaluated based on scores of, 0; normal (normal fur):

- Mild (ruffled fur by 30% of the body)
- Moderate (ruffled fur by 60% of the body)
- Severe (ruffled fur more than 60% of the body)

Table 1: Histopathology lesion scoring

Lesion	Score	Justification
Inflammatory cells	0	Normal (no inflammatory cells)
	1	Mild (inflammatory cells on 30% of the field)
	2	Moderate (inflammatory cells on 60% of the field)
	3	Severe (inflammatory cells on more than 60% of the field)
Necrosis/degeneration	0	Normal (no necrosis/degeneration)
	1	Mild (necrosis/degeneration on 30% of the field)
	2	Moderate (necrosis/degeneration on 60% of the field)
	3	Severe (necrosis/degeneration on more than 60% of the field)
Congestion/haemorrhage	0	Normal (no congestion/haemorrhage)
	1	Mild (congestion and haemorrhage on 30% of the field)
	2	Moderate (congestion/haemorrhage on 60% of the field)
	3	Severe (congestion/ haemorrhage on more than 60% of the field)

Table 2: Comparison between clinical signs in the various groups of mice using mean rank

Group	Ruffled fur	Movement	Eye condition	Responsiveness
Brucella	20.50 ^a	20.50 ^b	20.50 ^a	20.50 ^a
LPS	12.50 ^b	9.50 ^b	9.50 ^b	9.50 ^b
Control	4.50 ^{bc}	7.50 ^b	7.50 ^b	7.50 ^b

^{a,b,c}: Mean ranks in the same column differ at p<0.05

Table 3: Mean score values and severity of clinical signs in mice inoculated with *B. melitensis*, LPS and PBS

Group	Parameter	Mean scores
Brucella	Ruffled fur	1.86±0.12*
	Movement	1.15±0.05
	Eye condition	0.33±0.05
	Responsiveness	1.08±0.05
LPS	Ruffled fur	0.65±0.11*
	Movement	0.01±0.0
	Eye condition	0.01±0.0
	Responsiveness	0.01±0.0
Control	Ruffled fur	0±0
	Movement	0±0
	Eye condition	0±0
	Responsiveness	0±0

*: Significant value p<0.05 (Mann Whitney t-test to evaluate differences between experimental groups and control group)

Table 4: Comparison between scores of pathological lesions in the various groups of mice using mean rank

Group	Inflammatory cells	Necrosis/ degeneration	Congestion/ hemorrhage
Brucella	83.29 ^a	79.11 ^a	79.43 ^a
LPS	74.90 ^b	64.88 ^b	72.56 ^a
Control	21.50 ^{bc}	35.50 ^{bc}	27.50 ^b

^{a,b,c}: Mean ranks in the same column differ at p<0.05

Clinical scoring for movement in mice was divided into 4 scores, which were score 0; normal (normal movement and activity), score 1; mild (reduced movement and activity by 30%), 2; moderate (reduced movement and activity by 60%) and score 3; severe (reduced movement and activity more than 60%).

Clinical sign for eye condition in mice were divided into 4 scores. Score 0 was for normal eye condition (normal eye with no discharge and eye closed), score 1; mild (eye discharge and eye closed 30%), score 2; moderate (eye discharge and eye closed 60%) and score 3; severe (eye discharge and eye closed more than 60%). Lastly, for clinical sign of responsiveness, 4 scores were used. Score of 0; normal (normal responsiveness), score 1; mild (reduced responsiveness by 30%), score 2; moderate (reduced

responsiveness by 60%) and score 3; severe (reduced in responsiveness more than 60%).

Histopathology lesion scoring: Cellular changes were scored by the examination of 6 microscopic fields for each slide at 100 and 200 x magnification. Lesion scoring was divided into 4 scores namely: score of 0; normal (normal), score 1; mild (less than 30% of the field involved), score 2; moderate (30-60% of field involved) and score 3; severe (more than 60% of field involved). The histopathological lesions examined were the presence of inflammatory cells, necrosis/degeneration and congestion/haemorrhage (Table 1 to 4).

Statistical analysis: The clinical signs and lesion scoring data obtained were analyzed using Kruskal-Wallis H test as the data were not normally distributed. Then, all the significant parameters (p<0.05) were further analyzed using Mann-Whitney U test. JMP9 was used to analyse the data collected.

RESULTS

Clinical signs: After 240 h (10 days) of post inoculation, mice from all the treated and control groups showed significant differences (p<0.05) for clinical signs of ruffled fur, reduced movement, eye condition and reduced responsiveness. Brucella group was significantly different (p<0.05) of clinical signs compared to the other groups. LPS group was significantly different (p<0.05) from Brucella group for all the clinical signs but, there were no significant differences (p<0.05) between LPS and control groups for clinical signs of movement, eye condition and responsiveness.

Brucella group started to develop clinical signs as early as 7 h of post inoculation where most of the mice

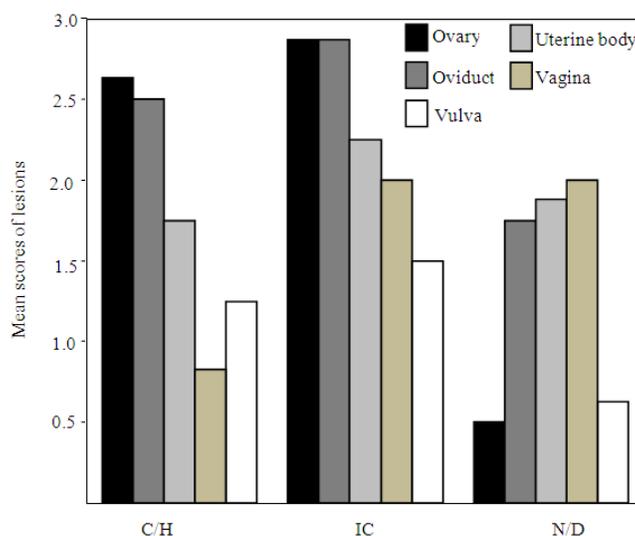


Fig. 1: Mean scores of lesions in organs inoculated with brucella bacteria; C/H = Congestion/Hemorrhage, I/C = Inflammatory cells; N/D = Necrosis/Degeneration

in Brucella group showed reduction in movement and responsiveness between 7-9 h of post inoculation. For the clinical sign of ruffled fur, mice in Brucella group showed progressive development of ruffled fur at 30 h of post inoculation. After 35 h of post inoculation, mice in Brucella group showed improvement in clinical signs. They seem to become more active and responsive towards the environment. After 65 h of experiment, the mice in Brucella group started to become sick and lethargy. At the end of experiment, the mice in Brucella group were dull and inactive with severe ruffled fur with sme developed eye discharged. In LPS group, only 3 mice developed clinical signs within 24 h of post inoculation. The mice in LPS group developed mild ruffled fur throughout the experiment with no evidence of dullness and unresponsive. After 48 h of post inoculation, the mice had mild ruffled fur, reduce in movement, eye discharge and reduction in responsiveness. The mice become more active and alert after the third day of post inoculation. For control group, all the mice were healthy and active throughout the experiment period.

Histopathological lesion (Fig. 3-10): The reproductive organs were examined for presence of inflammatory cells, necrosis, degeneration, congestion and haemorrhage. There were significant ($p < 0.05$) pathological changes in the reproductive organs namely ovaries, oviducts, uterine body, vagina and vulva from Brucella and LPS groups.

There were significant differences ($p < 0.05$) between in the groups regarding cellular changes such as the presence of inflammatory cells, necrosis and degeneration. There were no significant differences ($p < 0.05$) between Brucella and LPS groups for lesions such as congestion and haemorrhage.

Considering the presence of inflammatory cells, both Brucella and LPS groups showed moderate to severe lesions with average mean scores of 2.32 ± 0.87 and 2.00 ± 0.78 , respectively. While, Brucella group developed mild to moderate lesions of necrosis and degeneration with an average mean score of 1.32 ± 1.09 . Furthermore, the LPS group had a mean score of 0.80 ± 0.94 which was within normal to mild. Finally, both groups developed mild to moderate pathological changes in relation to congestion and hemorrhage of the reproductive organs especially the ovaries. In conclusion, Brucella group showed more severe and extensive lesions of cellular changes compared to LPS group.

The most affected reproductive organ in the Brucella group was the ovary where infiltration of inflammatory cells and congestion/hemorrhage were observed. The second most affected organ was oviduct where severe infiltration of inflammatory cells and congestion were observed. Most of the blood vessels in oviduct were severely congested with red blood cells and inflammatory cells especially neutrophils. For the uterine body, the endometrium was necrotized with the presence of inflammatory cells. The lumen of the uterine body was also filled with inflammatory cells mainly neutrophils and mononuclear cells. Vagina and vulva were the organs had least pathological changes with moderate inflammation and necrosis in the Brucella group (Fig. 1).

The ovary from LPS inoculated group showed moderate to severe lesion of the presence of inflammatory cells and congestion/hemorrhage. The oviducts of LPS group had moderate inflammatory cells and mild congestion. The oviduct and vulva of the LPS inoculated group showed lesions of necrosis and degeneration and the severity of the lesions was intense compared to the Brucella group (Fig. 2).

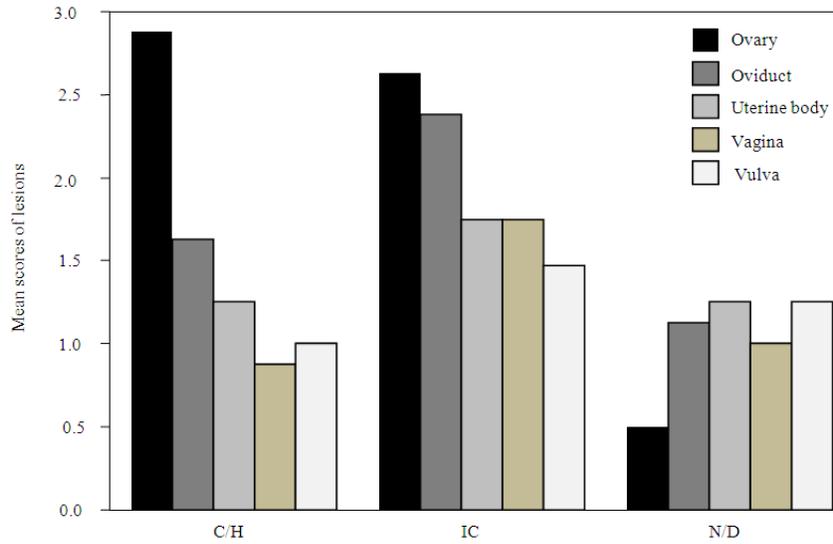


Fig. 2: Mean scores of lesions in organs inoculated with Brucella Lps; C/H = Congestion/Hemorrhage, I/C = Inflammatory cells; N/D = Necrosis/Degeneration

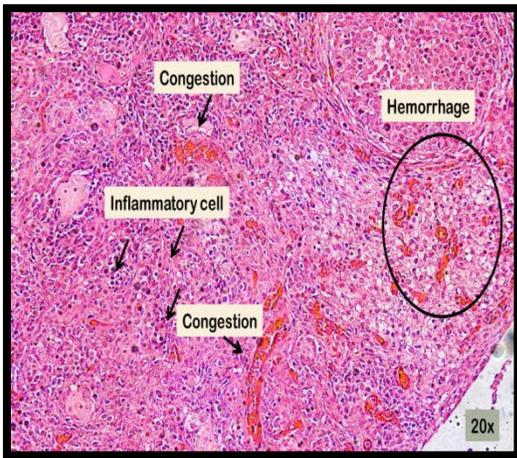


Fig. 3: Necrosis of the tunica mucosa of the oviduct of mice in Brucella group with presence of inflammatory cells and moderate congestion of blood vessels

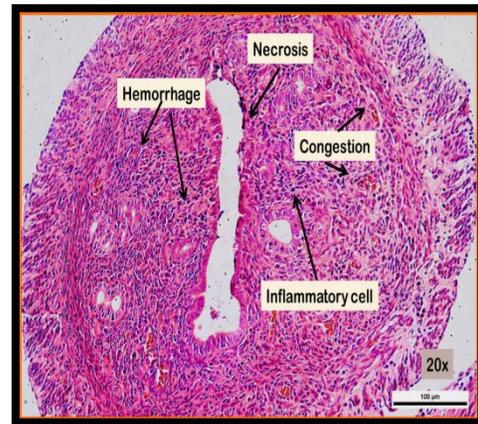


Fig. 4: Necrosis of the tunica mucosa of the oviduct of mice in Brucella group with presence of inflammatory cells and moderate congestion of blood vessels

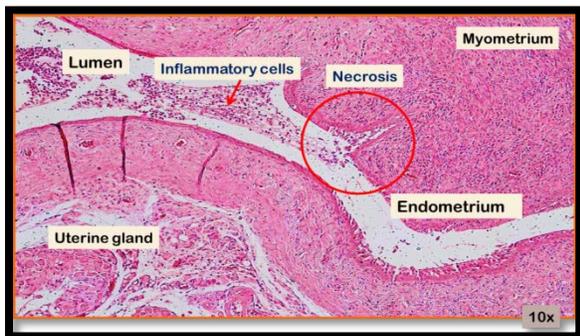


Fig.5: Severe necrosis and thinning of the endometrium and infiltration of inflammatory cells in the lumen of the uterine body of mice in Brucella group

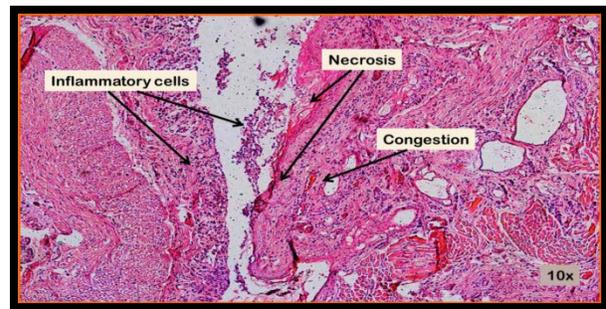


Fig.6: Severe necrosis and thinning of the mucosa layer, severe inflammation of the mucosa layer and infiltration of inflammatory cells (neutrophils and mononuclear cells) in the lumen of the vagina of mice in Brucella group

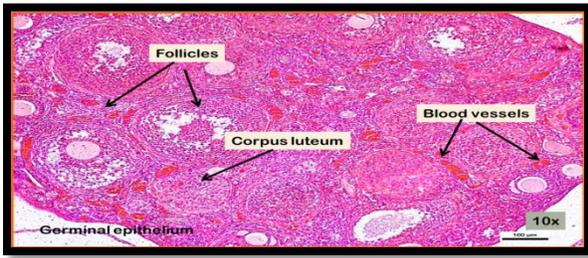


Fig. 7: Severe congestion of blood vessels in ovary of LPS group of mice

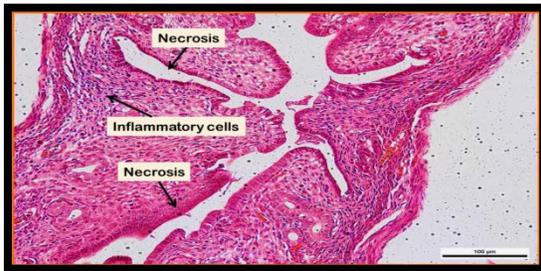


Fig. 8: Severe necrosis of the tunica mucosa and severe inflammation of the tunica muscularis of the oviduct of mice in LPS group

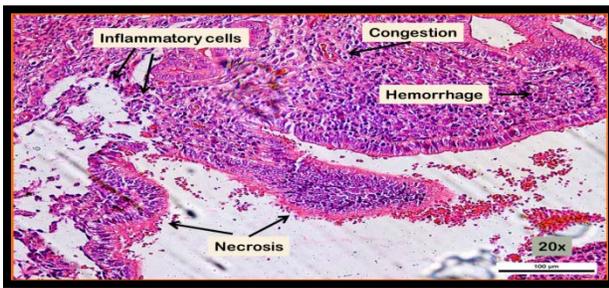


Fig. 9: Severe necrosis and inflammation of the endometrium, mildly congested blood vessels, and infiltration of inflammatory cells inside the lumen of uterine body of mice in LPS group

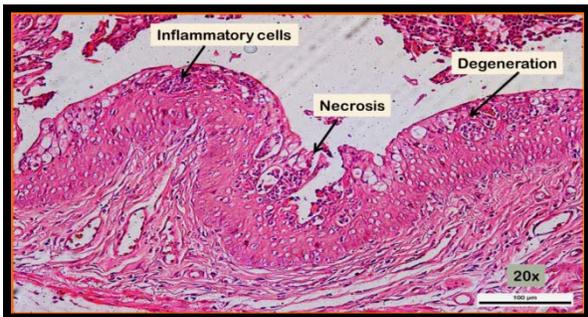


Fig.10: Severe necrosis and inflammation of the mucosa layer, degeneration of mucosa layer, and infiltration of inflammatory cells inside the lumen of vagina of mice in LPS group

DISCUSSION

Brucellosis is a bacterial disease that is caused by many genus of *Brucella*. *B. melitensis* is gram negative, facultative intracellular, coccobacillus, non-spore-forming and non-capsulated bacteria. *Brucella* belongs to the alpha 2 subdivision of the Proteobacteria, along with *ochrobactrum*, *rhizobium*, *rhodobacter*, *agrobacterium*, *bartonella* and *rickettsia* (Yanagi and Yamasato, 1993; Scholz *et al.*, 2008a). It is an important zoonotic disease and a significant cause of reproductive losses in animals and humans (Robinson, 2003). The *Brucella* cell envelope consists of an outer layer of lipopolysaccharide-protein about 9 nm thick. *B. melitensis* infection in sheep and goats is similar to *B. abortus* infection in cattle. Nevertheless, differences are significant and each species of *Brucella* causes a different disease (OIE, 1997). Depending on species, strain and size of infecting inoculum, the virulence of *Brucella* varies accordingly (Foster *et al.*, 2007). Other than that, the host susceptibility to the infection is also variable and mostly associated with the reproductive status of the animals. The infection in females follows a route very similar to *B. abortus* infection in cattle. The major route of infection appears to be through the mucous membranes of the oropharynx and upper respiratory tract or the conjunctiva. Other potential routes of infection are through the mucous membranes of the male or female genital tract (European Commission, 2001). When the bacteria successfully survive over the body defenses, a bacteraemia is generally established. In many cases the bacteraemia is detectable after 10 to 20 days and may persists from 30 days to more than 2 months. On the other hand, if the animal is pregnant, bacteraemia often leads to the invasion of the uterus. At the same time, infection may occur in various lymph nodes and organs, often in the udder and sometimes in the spleen (European Commission, 2001).

Generally, transmission occurs in the same way in sheep and goats as in cattle, materials excreted from the female genital tract forming the main supply of organisms for transmission to other animals and man. Therefore, in most circumstances, the primary route of dissemination of *Brucella* is the placenta, fetal fluids and vaginal discharges expelled by infected ewes after abortion or full-term parturition.

In the present study, female mice were inoculated with wild type 10^9 *B. melitensis* and they begin to develop clinical sign as early as 7 h of post inoculation. Most of the mice in *Brucella* group exhibited mild reduction in movement and responsiveness between the first 7 to 9 h of experiment. They also showed mild ruffled fur during that period. No death has been reported in the present study except one of the mice that died on day 6 of the experiment may be due to other factors. As compared to a previous study conducted by

Jesse *et al.* (2013), male mice that were inoculated with wild type of 10^9 *B. melitensis* developed severe clinical signs and begin to show mortality after 6 to 15 h of post inoculation. A similar Study conducted by Cannat and Serre (1984), mentioned that with the exception of the Brucella resistance character of C57BL mice, which seem to be partially dominant with polygenic control in females, no comparative studies have been performed between sexes of mice (Grilló *et al.*, 2012). After 35 h of post inoculation, mice in Brucella group showed slight improvement in clinical signs. They seem to become more active and more responsive toward environment. Then, in later time they gradually become weak until the end of experiment. This study was similar to the study conducted by Takele *et al.* (2009) which indicated that mice inoculated with $1 \text{ mL} \times 10^8$ cfu *B. melitensis* colony showed clinical signs at early stages such as extreme shivering, erection of hair coat, anorexia and dullness. After one month of post inoculation, the clinical signs almost disappeared. On the other hand, a comparison with a previous study conducted on male mice, the clinical signs showed by the mice progressively become worse and finally lead to death.

In contrast, female mice which were inoculated with *B. melitensis* Lipopolysaccharides (LPS), the mice exhibited mild clinical signs such as ruffled fur after 24 h of post inoculation. Mice in LPS group had mild clinical signs such as ruffled fur; reduced movement, eye discharge and also reduction in responsiveness only for the first 2 days of the experiment period. After 2 days of post inoculation period, the mice become more active and alert. No severe clinical signs were observed in LPS group. This might be due to development of antibody towards LPS component of Brucella and this finding was in agreement with a study conducted by Kshash *et al.* (2009) in which he stated that LPS is a potent antigen which will stimulate immune response and LPS is able to initiate inflammatory reaction and clear the LPS activity in the animals.

In the present study, mice in Brucella and LPS group developed significant histopathological changes in all reproductive organs including ovaries, oviduct, uterine body, vagina and also vulva. There were infiltration of inflammatory cells, necrosis, degeneration, congestion and haemorrhage for all the three organs. Severe congestion of ovaries of Brucella and LPS groups can be observed which was similar with the study conducted by Enright *et al.* (1990). Inflammatory cells mainly neutrophils and mononuclear cells were massively infiltrated in tunica mucosa and tunica submucosa of the oviduct. Uterine body of mice which are from Brucella and LPS groups showed severe necrosis and myositis of myometrium layer with thinning of the wall of endometrium. The endometrium of the uterine body was also necrotized and thinning. Report by Scientific Committee in

Animal Health and Animal Welfare in Brucellosis in Sheep and goat, (2001) stated that Brucella-infected animals generally develop granulomatous inflammatory lesions which frequently are found in lymphoid tissues and organs such as reproductive organs, udder and supramammary lymph nodes and sometimes joints and synovial membranes and this findings is in accord with the present study.

The difference between Brucella and LPS groups was the severity of the lesion. Cellular changes in reproductive organs of mice inoculated with wild type 10^9 Brucella showed more severe histopathological lesions compared to 10^9 LPS group. According to Silva *et al.* (2011), female pregnant mice infected with 10^6 cfu of *B. abortus* virulent strain 2308 developed a moderate multifocal necrotic placentitis with severe neutrophilic infiltration and intra lesion of bacteria in trophoblastic cells. The lesions described in the female pregnant mice were similar to those observed in cows, which suggests that this model may be useful to study Brucella-induced placental disease, although mice and cattle have different morphological types of placenta.

As compared to a previous study using male mice conducted by Jesse *et al.* (2013), mice in groups Brucella and LPS showed atrophy of spermatocytes in the testes and also degenerative necrosis in the pseudostratified epithelium of the vas deferens. Therefore, the findings of the cellular changes in the female organs of the mice in the present study are in agreement with other work (Silva *et al.*, 2011; Jesse *et al.*, 2013).

CONCLUSION

In conclusion, *B. melitensis* causes progressive clinical signs in mice where the clinical signs deteriorated with time. Compared to the previous study in male mice, female mice seem to have more resistant towards Brucella infection. Both groups of female mice inoculated with Brucella and its immunogen developed significant pathological changes in all reproductive organs. Hence, LPS could be the likely candidate for the development of vaccine against *B. melitensis*.

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