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Pathological Studies on Buffalo-Cows Naturally Infected with *Brucella melitensis*

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Abstract: The present study was carried out on a total number of 32 *Brucella melitensis* seropositive buffalo- cows. Specimens were taken from uterus, spleen and supramammary lymph nodes just after obligatory slaughter for pathological, immunohistochemistry as well as ultrastructural examinations. Histopathological findings showed different forms of endometritis {ulcerative (28.12%), granulomatous (6.25%), haemorrhagic (3.12%) and chronic (62.5%)}. Lymphoid depletion in spleen was evident. The immunohistochemical examination of uterine tissue revealed aggregations of extra cellular and periglandular moderate immunostaining *Brucella melitensis* antigen Meanwhile, in spleen, few and mild (weak positive) immunoreactive staining of brucella antigen was detected extracellularly among the lymphocytes of white pulp. Electron microscopical finding revealed presence of moderate aggregations (clusters) of dark bodies of intact cocobacilli within the cytoplasm of macrophages of medullary sinuses of supramammary lymph node. It could be concluded that brucella infection in buffalo cows caused pronounced histopathological changes in genital and lymphatic organs.

Key words: Buffaloes · *Brucella melitensis* · Histopathology · Immunohistochemistry

INTRODUCTION

Brucellosis is a bacterial zoonosis with economic and global importance [1, 2]. Late gestation abortion is the predominant clinical sign of *Brucella abortus* infection in cows, resulting in reproductive failure and consequently a decrease in milk production [3, 4]. The diversity of expression of pathological lesions in domestic animals is known to be influenced by species and strain of brucella, immune status of animal host, rout of exposure, sexual maturity, infection rate and virulence of organism [5].

Water buffaloes are an economically important livestock species in many Asian, Mediterranean and some European countries including Italy [1]. In Egypt since 1981, the General Organization of Veterinary Services (GOVS) has run a control program which is currently based on testing female ruminants older than 6 months of age and slaughtering of serologically positives. Also, voluntary vaccination of calves using *Brucella abortus* S19 vaccine and *Brucella melitensis* Rev 1 vaccine for lambs and kids was carried out [6].

Serological surveys indicated that brucella infections among buffaloes ranged from as low incidence as 0.13-1.49% [7-10] to as high incidence as 5.29-25.49 %. [11-13]. The diagnosis of brucellosis is based on direct or indirect laboratory methods. Direct methods such as bacterial isolation have high specificity but are time-consuming and require facilities with an appropriate degree of biosafety [14]. Serological methods are used more often, being quicker and less expensive. Alternative methods for the detection of *B. abortus* in tissues include immunofluorescence, which has high specificity but is of low sensitivity and, moreover, gives an inadequate picture of tissue morphology [15]. Immunohistochemical examination of paraffin wax-embedded tissues for *B. abortus* antigens is not only both sensitive and specific but also clearly shows tissue morphology; it is, therefore, capable of demonstrating the distribution of organisms in the tissues, a valuable attribute for the study of pathogenesis of *B. abortus* infection [16-18]. Polymerase chain reaction (PCR) has been explored for the rapid detection and confirmation of Brucella infection and considered very useful tools for differentiating Brucella spp., especially follow-up testing of unusual phenotypic results, all the collected samples of this work showed positive brucella infection using PCR techniques in previous studies [19]. Therefore the aim of the present work was to study the histopathological

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changes in the genital and lymphoid organs of buffalo cows infected with brucella in addition to detection of the organism in infected tissues by immunohistochemical and electron microscopical techniques.

MATERIALS AND METHODS

Animals: A total number of 32, *brucella melitensis* seropositive buffalo- cows was used in this study during the period extended from 2004-2005. These animals included 17 cases from buffalo -cows farm at Ismalia governorate, suffered from reproductive disorders as repeat breeding, decreased milk yield and retained placenta. Also, 15 obligatory slaughtered cases from Ossim slaughtered house in Giza governorate were included.

Tissue Samples: Tissue samples were taken from uterus and spleen for pathological examination and supramammary lymph nodes for ultrastructural examination.

Histopathological Examination: Parts from the taken specimens, were fixed in formal saline 10% then washed, dehydrated, embedded in paraffin, sectioned at 4-5 micron thickness with hematoxylin and eosin as a routine work for histopathological studies [20]. Special stains are used for detection of specific lesions as Prussian blue stain for haemosidrosis [21] and Von Kossa stain for calcification [22].

Immunohistochemistry: Avidin- Biotin complex peroxidase technique was applied for detection of Brucella in formalin-fixed, paraffin -embedded tissue sections from uterus and spleen [23] using peroxidase detection kit purchased from Novocastra Co.UK.

Ultra Structural Examination: Small tissue specimen was taken from supramammary lymph nodes for ultra structure examination. The specimens were fixed in 5% cold cocodylate buffer glutraldehyde (4°C 0.1N, pH 7.2) then kept at 4°C until processing for electron microscopic examination [24]. This work was done at the electron microscope unit, National Research Centre, the model of apparatus is Zeiss E.M.10 -West Germany.

RESULTS

Pathological Results

Macroscopical Findings: The gross examination of uterus revealed pathological lesions in eight out of thirty two collected cases. Erosion and ulceration of the endometrial mucosa with presence of moderate amount of mucous exudates, dark reddish brown coloration in a focal manner and focal areas of marked subendometrial hemorrhages were seen. There were multiple white necrotic foci in the endometrium. The supramammary lymph nodes of most examined cases were relatively small in size and firm in consistency. However, in few cases, supramammary lymph nodes appeared enlarged and edematous. On cut section watery exudates oozes. In one case, the spleen was firm in texture with presence of whitish necrotic foci.

Histopathological Findings

Uterus: The microscopical examination of uterus of 9 out of 32 cases showed ulcerative endometritis which is characterized by multifocal desquamation of surface epithelium and its basement membrane, the lamina propria subepithelialis showed diffuse and heavy infiltration of mononuclear inflammatory cells. Endometrial stroma was diffusely edematous separating the uterine elements from each others. The uterine glands were atrophied. The lumen of some glands was heavily infiltrated with mononuclear inflammatory cells. The epithelial lining of some uterine glands was completely destroyed, necrosed and sloughed in the lumen. The blood vessels appeared markedly dilated and congested. Two out of thirty two cases revealed granulamatous endometritis (Fig. 1) which was characterized by presence of multiple granulamatous structures leading to loss of the architecture details of uterine tissue. The granuloma consists of a central area of caseous necrosis associated with massive calcification surrounded with zone of mononuclear inflammatory cells and finally encircled with thick fibrous tissue capsule (Fig. 2). The myometrium was edematous, in addition to the smooth muscle fibers were also vacuolated and the interstitial tissue was infiltrated with macrophages and plasma cells either in focal or diffuse manner. Sections stained with Von Kossa stain revealed brownish black coloration in the center of granuloma (Fig. 3). In one case, severe haemorrhagic endometritis was noticed, meanwhile chronic endometritis was seen in the remaining cases. The covering epithelium was multifocal desquamated. The endometrial stroma showed periglandular fibrosis associated with atrophied uterine gland. Perivascular fibrosis associated with thickening of the wall and stenosis of the lumen were seen, in addition to chronic perimetritis was observed.

Spleen: Displayed marked thickening of connective tissue capsule and trabecullae, in addition to lymphoid depletion of white pulp. In some cases, necrosis of the lymphocytic cell was evident. Moderate diffuse neutrophilic infiltration, especially around lymphoid

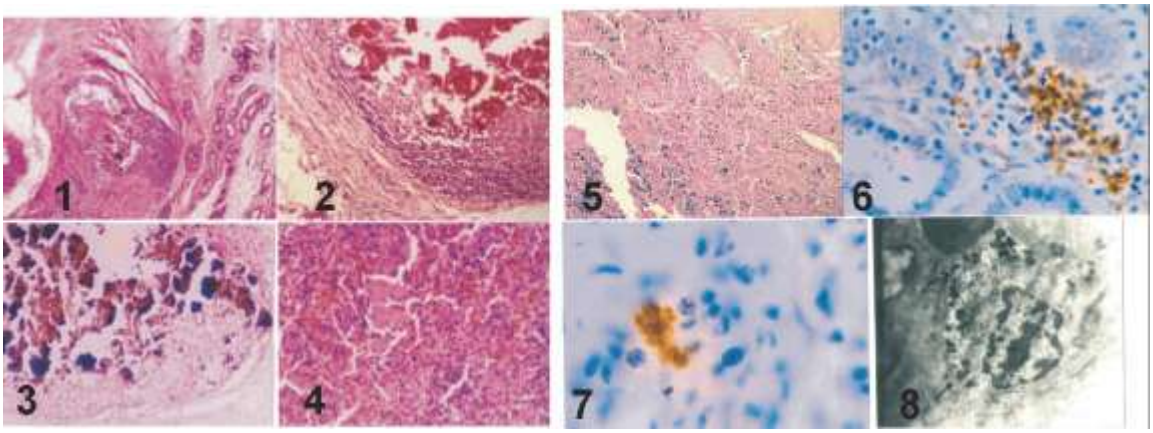

Fig. 1: Uterus of infected buffalo cows, showing Granulomatous structure. (H&E, X40)
Fig. 2: High magnification of Fig. 1 (H&E, X100)
Fig. 3: Uterus of infected buffalo cows, showing brownish to black calcium deposition in the granulomatous structure. (Von Kossa stain, X400).
Fig. 4: Spleen of infected buffalo cows, showing multiple patches of golden brown pigmentation in the red pulp. (H&E, X 200)
Fig. 5: Spleen of infected buffalo cows, showing haemosidrosis, (Prussian blue, X40).
Fig. 6: Uterus of infected buffalo cows, showing extra cellular and periglandular golden brown deposition of brucella melitensis antigen (moderate positive immunoperioxidase). Indirect peroxidase technique. (DAB, X 200).
Fig. 7: Spleen of infected buffalo cows, showing golden brown deposition of *Br. Melitensis antigen* among lymphocytes and plasma cells (mild positive immunoperioxidase). Indirect peroxidase technique. (DAB, X400).
Fig. 8: Electron micrograph revealed the presence of moderate aggregates of dark bodies of intact coco bacilli within the cytoplasm of macrophage in the medullarysinuses of supramammary lymph node. (Uranylacetate, X10000)

follicles was seen. Spleenic artery was constricted with protrusion of the endothelial cell lining toward the lumen. Golden brown patches of irregular shapes of haemosidrosis were occasionally observed (Fig. 4). These patches were stained positively with Prussian blue stain and appeared as diffuse bluish precipitations of iron pigments in white pulp of spleen (Fig. 5).

Immunohistochemistry Results: In uterine tissue, aggregations of extra cellular and periglandular moderate immunostaining *brucella melitensis* antigen was seen. (Fig. 6) Meanwhile, in spleen, few and mild (weak positive) immunoreactive staining of brucella antigen was detected extracellularly among the lymphocytes of white pulp (Fig. 7).

In all positive cases deposition of golden brown chromogen pigment at the site of antigen-antibody reaction was seen within the cytoplasm of mononuclear cells and detected extracellularly among the lymphocytes.

Electron Microscopical Finding: Electron microscopy revealed presence of moderate aggregations (clusters) of dark bodies of intact cocobacilli within the cytoplasm of macrophages of medullary sinuses of supramammary lymph node (Fig. 8).

DISCUSSION

In the present study, the histopathological findings were characterized by granulamatous endometritis. This finding comes in accordance with the observations reported in goats [25]. Also, infection with *Br. abortus* biovar 2 caused mild and diffuse lymphocytic infiltrate of the endometrium with several, large focal, endometrial accumulation of lymphocytes in Bison [26]. In this respect, the occurrence of granulamatous lesions indicates the chronicity of the condition and reflects the nature of the persisting infection [5], wherever the organisms are localized and a granuloma develops whereas the phagocytes that are attracted by the bacilli engulf them. The bacteria multiply within the cytoplasm of the phagocytes which are transformed into epithelioid cells. Around these cells lymphocytes, Langhans giant cells and plasma cells accumulate [27]. The organisms seek out cells that are capable of providing the nutrient

erythritol, hence their predilection site to live within cells of the genital tracts of animals and development of a granulamatous cellular response to chronic brucellosis is a classic hallmark [28]. *B. abortus* intracellular survival is dependent upon its ability to resist the acidified intraphagosomal environment and to inhibit phagosome–lysosome fusion [2].

Previous studies revealed that *Br. abortus* can induce the release of pro inflammatory cytokines in a variety of cell types such as interleukin(IL)-12 and tumour necrosis factor alpha (TNFα) [29]. It has been shown that TNF α is required for the influx of phagocytes to the site infection for granuloma formation [30] and for macrophage activation. Granuloma formation localizes pathogens and exposes them to phagocytosis [28] thus, potent cytokine-stimulatory properties possessed by *Br. abortus* may explain the correlation between tissue invasion and localized inflammation. On the other hand, endometritis associated with multifocal ulceration of superficial endometrium together with periglandular and perivascular fibrosis was also seen in the present work. Similar observations were recorded in *Brucella abortus* infected goats and cattle [3, 4].

The present work revealed presence of positive immunostaining brucella antigen in formalin-fixed, paraffin embedded tissue sections of uterus and spleen by using avidin -biotin complex peroxidase technique. Similar results were reported in cows, goats and mice inoculated with *Br. abortus* [3, 4, 16], in tissues of naturally aborted bovine fetuses infected with *Br. abortus* [18] and in adult female buffaloes naturally infected with *Br. Melitensis* [31]. The last authors concluded that this technique is sufficiently sensitive for detecting brucella antigens in formalin fixed, paraffin embedded tissues and could be a complementary tool to serological and bacteriological examination for diagnosis of brucellosis. Moreover, it was reported that immunoperoxidase technique may enhance diagnosis capabilities of brucellosis particularly in chronic infection and is an efficient mean for detecting brucella organisms when are inherently slow or difficult to diagnose by isolation or culture from tissues obtained from field cases due to contamination [32]. In addition to, this technique is relatively rapid and enables detection of dead and or low numbers of bacteria [33] although, cross reaction of the polyclonal antibodies with other microorganisms such as *Yersinia enterocolotica* and *E. coli* cannot rule out. Also, immunohistochemical staining has been used to study and assist in the understanding of the pathogenesis of infectious agents as the quantity, tissue and cellular locations of agent can be visualized [4, 17]. This technique could be a complementary tool to serology and bacteriology for the diagnosis of brucellosis [18].

The ultra structural finding of the current study showed presence of aggregates of intact electron dense cocci or cocobacilli within the macrophages which means the ability of the organism to survive intracellularly [3, 34, 35]. It has been suggested that lymph nodes draining areas of infections or lesions have the highest chance of being positive for brucellae [1]. Since, it was found that *B. abortus* is incorporated into phagosomes and remains in membrane-bound compartment until the host cell dies. The ability of brucella to survive in the intracellular environment is apparently due to inhibition of phagosome-lysosome fusion [34].

Transmission electron microscope (TEM) studies of rough strain brucella infection showed that in addition to necrosis, brucella -infected macrophages underwent oncosis, which is a prelethal pathway leading to cell death characterized by cell organelle swelling, cell blebbing and increased membrane permeability [36, 37]. These findings were confirmed by previous reports indicating that infected cells were not killed via apoptosis [38]. The outcomes of infection can be explained as the organism can reach replication niches and survive and the host cells will be killed. Otherwise the bacteria will be cleared by the host cells [35].

Finally, it could be concluded that brucella infection in buffalo cows caused pronounced histopathological changes in genital and lymphatic organs which were necrosis and granulomatous formation and fibrosis. Immunoperoxidase and electron microscopical technique enhance the diagnosis capabilities of brucellosis particularly in chronic infection.

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