A Study on Some Serological and Immunological Parameters in *Brucella* Infected Bovines

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**ABSTRACT**

This study was aimed to determine the possible effect of brucellosis on the oxidative/antioxidative status and some immunological parameters. A total of 1432 sera from infected as well as brucellosis negative private bovine farms located in Giza governorate were collected. These serum samples were tested serologically for bovine brucellosis using Rose Bengal Plate test (RBPT), serum agglutination test (SAT), rivanol test (Riv.T) and complement fixation test (CFT). Based on serological results, four groups were created as follows; 24 selected positive serum samples of infected cows (Group A), 18 selected positive serum samples of infected buffaloes (Group B), 20 and 13 selected negative serum samples of *Brucella* negative cow and buffalo herds into (Group C) and (Group D) respectively. To support serological findings, bacteriological examination was done and resulted in the recovery of 3 isolates recognized as *Brucella melitensis* biovar 3. Evaluation of oxidative stress using malondialdehyde and the activity of anti-oxidant enzymes (catalase and superoxide dismutase) was investigated in these groups and revealed insignificant differences between cattle and buffaloes and a significant elevation of these parameters in infected bovine groups. Serum interferon gamma (IFN-γ) levels displayed a significant decrease in *Brucella* infected groups. The levels of serum interleukins 10 (IL-10) were significantly increased in the infected groups. Under the field of this investigation, authors concluded that the significant elevation of several stress biomarkers in the infected groups may contribute to the resistance of the *Brucella* to oxidative damage during intracellular proliferation. Besides, the significant decrease of IFN-γ accompanied by a significant increase in IL-10 in a response to stimulation by *Brucella* antigens is reflecting the capability of *Brucella* to survive intracellularly under the harsh condition for a long term. Accordingly, further studies of immune mechanisms against *Brucella* may help in the development of novel therapeutic or new vaccine approaches.

**1. INTRODUCTION**

Brucellosis is an infectious disease of animals caused by a number of host-adapted species of Gram negative intracellular bacteria of the genus *Brucella* (Mantur et al., 2007; Byndloss and Tsolis, 2016). Brucellosis is characterized by a late term of abortion, retained placenta, drop in milk production (10-20%) and in male animals by orchitis, epididymitis and less frequently arthritis (Adams, 2002). Brucellosis is a worldwide zoonotic disease that is recognized as a major cause of heavy economic losses to the livestock industry, and also poses serious human health hazards (FAO/WHO, 1986).

Brucellosis caused by *Brucella melitensis* has a high incidence in developing countries, and the World Health Organization considers brucellosis one of the seven neglected zoonoses that contribute to the perpetuation of poverty (Maudlin and Weber-Mosdorf, 2005; Maudlin et al., 2009).

Stealthy brucellae exploit strategies to establish infection, including (i) evasion of intracellular destruction by restricting fusion of type IV secretion system-dependent *Brucella* containing vacuoles with lysosomal compartments, (ii) inhibition of apoptosis of infected mononuclear cells, and (iii) prevention of dendritic cell maturation, antigen presentation, and activation of naive T cells (De Figueiredo et al., 2015).

Yet, the gold standard of diagnosis of brucellosis is based on the isolation of the organism from infected animals but this is a cumbersome and time-consuming task, due to the fact that these fastidious organisms grow slowly on primary isolation (Meyer, 1981). Moreover, it is not possible to isolate *Brucella* every time even from an infected individual (Ray, 1979), therefore, assessment of
antibody response employing serological test plays a major role in the routine diagnosis of brucellosis which supported by bacteriological examination (Alton et al., 1988).

The immune response against Brucella involves both humoral (Th2) and cell-mediated (Th1) immunity (Zhan and Cheers, 1993). Resistance to Brucella species relies on cell-mediated immunity, which involves activation of the bactericidal mechanisms of antigen-presenting cells (macrophages and dendritic cells) and the subsequent expansion of antigen-specific CD4+ T-cell clones towards a Th1 phenotype. The Th1 cytokines (TNFα and IFNγ) enhance the anti-Brucella mechanisms of macrophages (Mφ) and induce the CD8+ CTL-mediated cytotoxicity against Brucella-infected Mφ (specific cellular immunity) (Dorneles et al., 2015).

The Th2 response activates B lymphocytes for antibody production, facilitating the phagocytosis of Brucella through opsonization (specific humoral immunity). The Th2 cytokines (e.g. IL-10) inhibit the action of Th1 cytokines (e.g. IFNγ) and vice versa (Skendros and Boura 2013).

Table 1. Description of animal groups used in this study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of examined serum samples</th>
<th>Number of selected serum samples</th>
<th>Description of selected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>245</td>
<td>24</td>
<td>Positive cows’ sera</td>
</tr>
<tr>
<td>Group B</td>
<td>205</td>
<td>18</td>
<td>Positive buffaloes’ sera</td>
</tr>
<tr>
<td>Group C</td>
<td>480</td>
<td>20</td>
<td>Negative cows’ sera</td>
</tr>
<tr>
<td>Group D</td>
<td>502</td>
<td>13</td>
<td>Negative buffaloes’ sera</td>
</tr>
</tbody>
</table>
2.4. Serum biomarkers of oxidative stress/ and the activity of antioxidant enzymes:

Serum levels of Catalase (Cayman chemical company, USA), Superoxide dismutase (Cayman chemical company, USA) and Malondialdehyde (Northwest Life Science Specialties, LLC) were measured according to the instructions of the manufacturers of the kits.

2.5. Immunological parameters:

Serum levels of IL-10 (Genorise Scientific, INC), IFN-γ (ELISpot, R&D Systems, Inc. USA), IgM (Bethyl Laboratories, Inc), IgG (Bethyl Laboratories, Inc) and c-reactive protein were measured with enzyme linked immunosorbent assay (ELISA) Kits according to the instructions of the manufacturers.

2.6. Bacteriological study:

Trials were done to isolate Brucella spp. from fetal stomach content and fetal spleen samples of recently aborted cows and buffaloes in the five private farms from which the positive serum samples were selected. The samples were cultured into Brucella agar media (Becton, Dickinson and company, Sparks, MD, USA). The inoculated media were incubated at 37°C for 3-5 days in presence of 5% CO2 (OIE, 2009). The growth was checked daily. Brucella suspected pure culture was stained with Gram’s staining method. Suspected Brucella colonies were identified at genus level by colonial morphology and growth characteristics, Gram stain, catalase, urease, and oxidase. Then the isolates were identified at species and biovar levels by Brucella phages lysis, H₂S production, CO₂ requirement, growth in the presence of thionine, and basic fuchsin and slide agglutination testing with monospecific A and M antisera. Brucella isolates were typed according to (Alton et al., 1988).

2.7. Statistical analyses:

Using SPSS® Statistics Version 21, one-way ANOVA was performed to study the significant difference in the means of several stress biomarkers either oxidant (malondialdehyde) or antioxidant (Superoxide dismutase and catalase) as well as some immunological parameters namely; C-reactive protein, IgG, IgM, Interferon gamma and Interleukin 10 between Brucella infected cattle and buffaloes and non-infected groups.

3. RESULTS AND DISCUSSION

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Number of examined animals</th>
<th>RBPT</th>
<th>SAT</th>
<th>Riv.T</th>
<th>CFT</th>
<th>Bacteriological examination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of reactors</td>
<td>% positive</td>
<td>No. of reactors</td>
<td>% positive</td>
<td>No. of reactors</td>
<td>% positive</td>
</tr>
<tr>
<td>Group A</td>
<td>245</td>
<td>24</td>
<td>9.8%</td>
<td>24</td>
<td>9.8%</td>
<td>24</td>
</tr>
<tr>
<td>Group B</td>
<td>205</td>
<td>18</td>
<td>8.8%</td>
<td>18</td>
<td>8.8%</td>
<td>18</td>
</tr>
<tr>
<td>Group C</td>
<td>480</td>
<td>1</td>
<td>0.2%</td>
<td>2</td>
<td>0.4%</td>
<td>0</td>
</tr>
<tr>
<td>Group D</td>
<td>502</td>
<td>0</td>
<td>0%</td>
<td>1</td>
<td>0.19%</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2.** Results of serological and bacteriological examinations of Brucella infected and negative groups of animals.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>MDA µmol/L</th>
<th>CAT nmol/min/ml</th>
<th>SOD U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>3.66 ± 0.74 A</td>
<td>33.44 ± 3.36 A</td>
<td>109.11 ± 6.26 A</td>
</tr>
<tr>
<td>Group B</td>
<td>3.71 ± 0.54 B</td>
<td>34.96 ± 3.99 B</td>
<td>117.00 ± 5.44 B</td>
</tr>
<tr>
<td>Group C</td>
<td>2.05 ± 0.13 ab</td>
<td>20.93 ± 1.65 ab</td>
<td>93.22 ± 2.43 ab</td>
</tr>
<tr>
<td>Group D</td>
<td>1.50 ± 0.41 ab</td>
<td>20.51 ± 0.54 ab</td>
<td>92.89 ± 2.11 ab</td>
</tr>
</tbody>
</table>

The mean difference is significant at p <0.05. Small letters against capital letters in the same column indicate significant difference between groups (using ANOVA test)
Brucellosis is worldwide zoonotic disease, which has important effects on both human health and animal reproduction. It causes great economic losses to the livestock industries through abortion, infertility, births of weak and dead offspring (Ozan et al., 2011). The resistance of animals to Brucella infection is influenced by sex, age, and reproductive status. Pregnant animals are more susceptible to infection than young one. Brucella organisms are first localized in regional lymph node, they proliferate within reticuloendothelial cells with subsequent entry into lymphatics and localized in different tissues like the spleen and reproductive organs (Jones et al., 1999).

Smooth lipopolysaccharide of Brucella contains a lipid A anchor to the cell wall, in the intermediate core region, and an immunodominant O-polysaccharide (OPS) which has been chemically defined as a homopolymer of 4,6-dideoxy-4-formamide-alpha-D-mannose linked via glycosidic linkages (Bundle et al., 1989). Because all smooth species share common epitopes in the OPS, virtually all serological tests for an antibody to these bacteria use B. abortus antigen in the form of whole cells, SLPS or OPS (OIE, 2008). It has long been recognized that the LPS is the major antigen of the surface of smooth brucellae upon which the immunoglobulins are provoked (Zygmunt et al., 1994) and the relevant molecule in classical diagnostic tests. Rose Bengal Plate Test (RBPT), serum agglutination test (SAT), Rivanol test (Riv.T) and Complement Fixation Test (CFT) are utilized in this study for the detection of antibodies specific to smooth Brucella species.

Antibodies against Brucella species were detected by RBPT, SAT, Riv.T, and CFT. The results in (Table 2) showed that 9.8% of cow serum samples (Group A) gave positive results when tested by RBPT, SAT, and CFT but when tested by Riv.T 24 serum samples out of 245 were positive with a percentage of 9.8%. Buffalo serum samples (Group B) showed 8.8% positive results when tested by RBPT, SAT, Riv.T, and CFT. In case of the examined serum samples of Brucella negative cow farms (Group C), none of the cows reacted to CFT and Riv.T except 1(0.2%) and 2(0.4%) of the cows reacted positive to RBPT and SAT respectively. The corresponding pictures of Brucella negative buffalo farms (Group D) showed only 1(0.19%) of the buffaloes reacted to SAT (Table 2).

These findings are in accordance with (Dohoo et al., 1986) in that RBPT was found to be a good screening test, although some authors (Saravi et al., 1990) have found an unacceptable false negative rate with the RBPT. The acidic pH in RBPT diminishes agglutination by IgM resulted in reducing cross-reactions (Alton et al., 1988) in addition the acidic pH in which the stained Brucella cells are preserved encourages agglutination by IgG1 (the predominate immunoglobulin in long lasting infection). SAT is performed at a near neutral pH and therefore detects IgM isotype of antibody efficiently and is therefore very sensitive. The SAT detects IgG less efficiently, especially IgG1, resulting in low assay specificity (Nielsen et al., 1989). Therefore, the SAT is generally not used as a single test but rather in combination with other tests.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>CRP mg/L</th>
<th>IgG mg/dl</th>
<th>IgM mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>8.43 ± 2.66 A</td>
<td>1265.67 ± 11.77 A</td>
<td>108.67 ± 16.46 A</td>
</tr>
<tr>
<td>Group B</td>
<td>7.92 ± 3.99 B</td>
<td>1275.00 ± 7.48 B</td>
<td>86.72 ± 6.20 B</td>
</tr>
<tr>
<td>Group C</td>
<td>3.29 ± 0.19 ab</td>
<td>1167.22 ± 12.05 ab</td>
<td>71.00 ± 1.03 a</td>
</tr>
<tr>
<td>Group D</td>
<td>2.39 ± 0.38 ab</td>
<td>1155.33 ± 10.39 ab</td>
<td>70.42 ± 1.05 a</td>
</tr>
</tbody>
</table>

The mean difference is significant at p < 0.05. Small letters against capital letters in the same column indicate significant difference between groups (using ANOVA test).
False positive serological reactions have been observed with brucellosis screening tests (RBPT and SAT) in animals reared in Brucella negative farms. Potentially Yersinia enterocolitica 0:9 presents the most serious source of this confusion because its O-chain unit is identical to that of Brucella spp. (Nilsen et al., 1989). Poester et al. (2010) reported that these techniques do not distinguish clearly between different stages of Brucella infection and the cross-reactions with related Gram-negative bacteria such as Yersinia enterocolitica, Francisella tularensis and Pseudomonas maltophilia.

For confirmation of brucellosis, Riv.T is suitable and specific detectable test if CFT is not available. The lower incidence than RBRT and SAT may be due to precipitation of IgM, large immunoglobulin molecular weight, that carry high net negative charges if compared to IgG classes by Rivanol solution as recorded by Morgan (1967) and Pietz and Cowart (1980). For this reason, the test only detects IgG1 and IgG2 immunoglobulin.

CFT is considered as the gold standard serological test used for detection of brucellosis as it detects only IgG1 (complement fixing antibody) specific for Brucella infection so it overcomes cross reaction with other similar gram-negative bacteria so no false results were detected. Additionally, World Organization for Animal Health (OIE) suggested that CFT is a confirmatory test approved all over the world (OIE, 2009). This test is considered as a high-quality test when it is used correctly, however, it has lots of practical drawbacks such as time-consuming and difficult to standardize (Abernethy et al., 2012).

In this study, trials for isolation of Brucella spp. from infected farms resulted in a recovery of 3 Brucella isolates (2 from cattle and 1 from buffalo) from stomach contents of aborted fetuses (Table 2) that typed as B. melitensis biovar 3. No matter how many Brucella spp. was isolated, it requires only one isolate to declare any farm as infected (Gall and Nielsen, 2004; Elbauomy et al., 2014). The main role of bacteriological examination in this study is to confirm the serological findings since the isolation and typing of Brucella microorganisms from suspected animals is the only diagnostic method that offers a conclusive evidence of brucellosis (OIE, 2016).

Antioxidants play an important role in scavenging free radical and other potentially toxic oxidizing species. When antioxidant defenses are weakened, body cells and tissue become more prone to develop dysfunction and/or disease (Kusano and Ferrari, 2008).

Oxidative stress can be monitored with several biomarkers either oxidents as nitric oxide (NO) and malondialdehyde (MDA) or antioxidants as Superoxide dismutase (SOD), catalase, xanthine oxidase, peroxidase, monoamine oxidase, glutathione reductase, vitamin E, vitamin C, and glutathione (Passi et al., 2001). Malondialdehyde (MDA) is a by-product of lipid peroxidation and it is used as an indicator of oxidative stress in cells and tissues (Kandemir et al., 2002 and Madebo et al., 2003). Our results showed an elevation of MDA level in serum of cows and buffaloes (group A and group B) compared with the Brucella negative animals (group C and group D) (Table 3). It is speculated that this increase might be a result of an elevation of lipid peroxidation in affected tissues of Brucella infected groups. Our results are in accordance with those obtained by (Nisbet et al., 2007) who found a significant increase in MDA levels in cattle infected with brucellosis comparing with the healthy control cattle group. The same results were recorded by (Sharaikhani and Azimzadeh, 2016).

Catalase a common enzyme, that catalyze the decomposition of hydrogen peroxide to water and hydrogen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS) (Teixeira-Gomes et al., 2000).

Superoxide dismutase (SOD) is an important antioxidant defense in nearly all living cells exposed to oxygen. SOD is an enzyme that alternately catalyzes the dismutation of the superoxide (O2−) radical into ordinary molecular oxygen (O2) (Lu et al., 2010).

Regarding our Catalase and SOD activity results, we found a significant increase in the levels of the two enzymes in the groups of cows and buffaloes infected with brucella melitensis (group A and B) if compared with Brucella non-infected groups (C and D) (Table3).

Our results are similar to that obtained by (Kataria et al., 2010) who noticed an elevation in SOD and Catalase activities in Brucella affected cows and buffaloes. It has been reported that B. melitensis possesses SOD and catalase enzyme-encoding genes which are likely to be involved in protecting and contributing to the resistance of the brucellae to ROS toxicity during intracellular proliferation (Doghanay and Aygen 2003). SOD and catalase may also contribute to the resistance of the brucellae to killing by O2− and H2O2 produced by the respiratory burst of phagocytes (Jiang et al., 1993).

Acute-phase proteins (APPs) are a class of proteins whose plasma concentrations increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) in response to inflammation (Abbas et al., 2012). The APPs have been investigated as biomarkers of disease in ruminants and particularly in cattle for a number of decades (Ceciliani et al., 2012).
C-reactive protein (CRP) is one of the major acute phase proteins (APP) and is widely distributed in nearly all vertebrates (Sarikaputi et al., 1991). It is a marker of inflammation and plays a role in innate immunity as an early defense system against infections (Thompson et al., 1999). During infections and under stressful conditions, human and animal mononuclear series cells, including monocytes and macrophages, secrete cytokines; such as, IL-1; IL-6; tumor necrosis factor-a TNF-α; and interferon INF-δ, which stimulate the liver to rapidly synthesize large amounts of CRP (Baumann and Gauldie 1994).

C-reactive protein plays a role in destroying infectious agents, minimizing tissue damage, and facilitating tissue repair and regeneration (Horadagoda et al., 1999). CRP is shown to be beneficial in the diagnosis of acute brucellosis and monitoring the treatment response. However, particularly in the endemic regions, it is difficult to differentiate acute, chronic and recurrent infections (Gunal et al., 2012).

Our results showed slight to moderate significant increase in CRP levels in both groups (A and B) of infected cows and buffaloes comparing with Brucella non-infected groups (C and D) (Table 4). Previous studies in human brucellosis showed a significant increase in CRP level in the patient group compared with control healthy group (Abdollahi et al., 2009 and Togan et al., 2015). Gunal et al. (2014) also found slight-moderate increase in CRP in the group of patients with brucellosis when compared with the group of patients with extracellular bacterial infection.

The role of humoral immunity against intracellular bacterial infections is limited and not protective. Antibody-mediated opsonization by immunoglobulins enhance phagocytic uptake of bacteria, limiting the level of initial infection with Brucella, but has little effect on the intracellular course of Brucella infection. (Baldwin and Goenka 2006). Our results showed a significant increase in total IgG in serum samples of cows and buffaloes infected with Brucella melitensis (groups A and B) on comparing with Brucella non-infected groups, whereas total IgM levels were significantly increased in serum samples of infected cows (group A) only compared with other groups (Table4).

This increase in total IgG and IgM levels in infected animals may be due to host bacterial interaction. A significant increase in serum globulin levels was previously observed in Brucella infected cattle (Nath et al., 2014 and El-Azab, 2015), ewes (Kumar et al., 2015) and goats (Singh et al., 2016). Chronic or subacute bacterial infections can cause increases in globulin fractions, particularly the γ-globulins resulting from the production of different immunoglobulins by plasma cells in response to Brucella antigens stimulation (El-Azab 2015). Del Pozo et al. (2014) reported that IgM antibodies are considered suggestive of acute Brucella infection and appear about a week after the onset of the disease, reaching a peak level 1-3 months later. IgG antibodies appear approximately three weeks after disease onset, reaching a maximum after six to eight weeks. However, detection of IgG antibody was more sensitive than detection of IgM antibody for diagnosing brucellosis cases especially in long lasting infection (Fadeel et al., 2011).

Immunity against Brucella species is principally mediated by cellular immune responses since it is an intracellular pathogen and involves antigen-specific T-cell activation of CD4 and CD8 T cells and humoral responses (Kim et al., 2005). Protection of the host against Brucella infection is thought to be mediated primarily by a Th1 type of immune response (Zhan and Cheers 1993). Interferon-γ (IFN-γ) is an important component of Th1 immune responses against Brucella infection (Kim et al., 2005). On the other hand, Brucella has developed various strategies to evade innate and adaptive immune responses, aimed at the establishment of an intracellular niche for long term survival and replication (Martirosyan et al., 2011).

Our results in Table 5 revealed that the IFN-γ levels displayed a significant decrease in Brucella infected cows and buffaloes compared with Brucella non-infected groups. On the other hand, levels of IL-10 were significantly increased in groups of cows and buffaloes infected with Brucella melitensis compared with brucellosis negative groups (Table 5). IFN-γ plays the predominant role in generating macrophages with strong intracellular Brucella killing activity (Murphy et al., 2001). It was found that IFN-γ induced T-cell responses to have important roles in the immunity to intracellular B. abortus. In both human and animals, IFN-γ has been shown to have a role in the control of brucellosis (Murphy et al., 2001).

Barrionuevo et al., (2008) have demonstrated that infection with Brucella species down-modulates expression of MHC-II and Ag presentation on monocytes/macrophages, decreasing IFN-γ production and persistence of the bacteria in the host for a long time. In relation to our IL-10 results, other studies have estimated IL-10 production following Brucella infection (Hoover et al., 1999; Pasquali et al., 2001). Similar findings were also recorded in camels (Camelus dromedarius) naturally infected either by B. abortus or B. melitensis showing significant elevations in IL-10 and significant decrease IFN-γ levels when compared with non-infected ones (El-Boshy et al., 2009). IL-10 is known to affect the production of
Th1 cytokines, including IFN-γ, by acting on the antigen-presenting capacity of the macrophages (Moore et al., 2001). Fernandes and Baldwin (1995) suggested that IL-10 can inhibit the anti-Brucella effector functions of macrophages as well as decrease the production of the protective cytokine IFN-γ by spleen cells in response to stimulation by Brucella antigens. Both of these mechanisms may contribute to the decreased control of infection in vivo when IL-10 is present.

4. CONCLUSION

In the light of obtained results, authors concluded that:

Significant elevation of several stress biomarkers either oxidant (malondialdehyde) or antioxidant (Superoxide dismutase and catalase) in cattle and buffalo infected groups contribute in the resistance of oxidative damage of brucellae to ROS toxicity during intracellular proliferation.

The significant increase in total IgG in serum samples of cows and buffaloes infected with Brucella melitensis reflecting the endemicity of the disease since it is the predominating antibody that lasts in long lasting infection.

The significant decrease of IFN-γ accompanied by a significant increase in IL-10 in a response to stimulation by Brucella antigens is reflecting the capability of Brucella to survive intracellularly under the harsh condition for a long term.

Accordingly, further studies of immune mechanisms against Brucella are required to help in the development of novel therapeutic or new vaccine approaches.

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