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*Original article*

# ACUTE PHASE BIOMARKERS, OXIDANTS, ANTIOXIDANTS, AND TRACE MINERALS OF MOBILE SHEEP FLOCKS NATURALLY INFECTED WITH BRUCELLOSIS

N. A. SHALBY[1], A. M. ABO EL-MAATY[2], A. H. ALI[2] & M. ELGIOUSHY[3]

[1]Animal Health Research Institute, Agriculture Research center, Dokki, Giza, Egypt;
[2]Animal Reproduction and Artificial Insemination, Veterinary Research Division, National Research Centre, Dokki, Giza (Reproductive Physiology), Egypt;
[3]Department of Animal Medicine, Faculty of Veterinary Medicine, Aswan University, Aswan, Egypt

**Summary**

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This study assayed the acute phase responses of sheep seropositive to *Brucella*. Sera collected from ewes (n=160) were subjected to serological tests of *Brucella*, Rose Bengal plate agglutination test (RBPAT), buffer acidified plate agglutination test (BAPAT), and complement fixation test (CFT). Results revealed that CFT was the most predictive test of brucellosis followed by BAPAT then RBPAT. The moderate predictive blood biochemical parameters were zinc and ascorbic acid. Ewes with low CFT titre (chronic) had low fibrinogen, copper, NO, and GPx. Seropositive animals had high blood concentrations of ascorbic acid and zinc.

**Key words**: acute phase reactants, *Brucella*, serological tests, sheep, trace minerals

## INTRODUCTION

The bacterial infection is the major initiator of acute phase response in animals. The macrophage is the main cell responsible for the release of several mediators, reactive oxygen species and regulatory proteins during infection. The bacterial endotoxin (lipopolysaccharide, LPS) is the primary inducer of the acute phase response (Koj, 1996). As Gram-negative bacteria, the members of genus *Brucella* are facultative intracellular pathogens that cause brucellosis of livestock, producing serious economic losses. Brucella LPS plays an essential role during infection and can be used as a target for new vaccine strategies (Zhao *et al.*, 2018).

The acute phase proteins (APP) are a group of blood proteins mainly produced by the liver that relate to defense from pathological damage, to restoring homeostasis and limiting microbial growth in an antibody-independent manner in animals

subjected to infection (Ceciliani *et al.*, 2012). APPs can also be used as indicators of the health status in flocks and as prognostic indicators because their levels are associated with disease severity (Miglio *et al.*, 2018). In response to infection, inflammation, and internal or external challenges, APPs concentrations either increase (positive) or decrease (negative) in the plasma providing valuable diagnostic and prognostic information during infection and inflammation with substantial variations between different species (Ceciliani *et al.*, 2002; Gruys *et al.*, 2005). The positive APPs that undergo an increase in the course of the AP response include haptoglobin (Hp) and serum amyloid A (SAA), where Hp is the primary APP in ruminants (Ceciliani *et al.*, 2002). In small ruminants, some APPs levels change similarly in both sheep and goat, whereas other APPs show different magnitudes of response between the two species (Gonzalez *et al.*, 2008). In ewes, haptoglobin (Hp) is one of the major APPs whose serum concentrations are normally very low but increase markedly in response to acute and chronic challenges and increase moderately in sub-clinical inflammation (Meling *et al.*, 2012; Miglio *et al.*, 2013), related to the severity of the disorder and the extent of the tissue damage (Murata *et al.*, 2004; Ceciliani *et al.*, 2012; Iliev & Georgieva, 2016). Functionally, haptoglobin is an acute-phase protein that binds to heme preventing it from serving as a nutrient for pathogens and initiating deleterious oxidation reactions resulting in a rapid inflammatory response (Matson *et al.*, 2006; 2012). Hp tended to increase with the reduction of ewe feed intake to function as an antioxidant for preventing oxidative tissue damage (Gabay & Kushner, 1999).

Fibrinogen is a plasma glycoprotein originating from the liver. It is not only involved in the blood clotting (Jayachandran *et al.*, 2016), but its levels are elevated during any form of inflammation (Page & Schroeder, 1976). In Egyptian dairy cows (Abou ElAzab, 2015), Indian sheep (Kumar, *et al.*, 2015), and horses (Gull *et al.*, 2013) the increase of ALT, AST, CK, TP, γ-globulin, globulin, cholesterol, and LDH activities with a decrease of alkaline phosphatase, albumin and glucose were recorded in animals, serologically positive for *Brucella*. The changes in the concentrations of blood biochemical parameters and enzyme profiles show the impact of brucellosis on the vital organs' functioning in the body and thereby help understanding the health status of any individual (Radostits *et al.,* 2007).

For the diagnosis of brucellosis, cultural, serological and molecular methods are usually used (Sahin *et al.,* 2008). To minimise the laborious, time-consuming analyses and danger to laboratory workers associated to the traditional isolation and characterisation of organism by cultural methods and the high cost of the molecular techniques, serological methods as Rose Bengal Plate Test (RBPT), Buffered acidified plate antigen test (BAPAT) that are easy to perform, highly sensitive for individual diagnosis and can be done in the field itself to screen the flocks, are used. The RBPT can be used in all animal species but positive results should be confirmed by a quantitative test. Regular scanning policy is important for the culling of infected animals and vaccinations of non-infected ones.

Keeping in mind the above facts, the present work was undertaken to assess and compare the serum zinc, copper and iron in association with the acute phase res-

ponse (haptoglobin and fibrinogen) parameters, SOD (superoxide dismutase), glutathione peroxidase (GPx), ascorbic acid and nitric oxide (NO) of native mobile female sheep infected with brucellosis with different titres to reflect the effects of the disease on animals' health and performance.

## MATERIALS AND METHODS

### *Ethical approval*

The study was performed by following the Institutional Animal Ethical Committee of the National Research Centre for collecting blood from animals who underwent this study. All sheep breeders provided consent for the livestock to be included in this study and to inform the sheep owners in case of detecting any seropositive animal to perform the regular preventive measures to protect humans and other animals from getting the infection.

### *Animals and sample collection*

Blood samples were collected from the sheep of different mobile sheep flocks. Native ewes (N=160) belonging to several breeders were subjected to blood sampling in plain vacuum tubes. The serum was stored at –20 °C for biochemical analysis.

### *Brucella serological tests*

Sera were tested using buffered acidified plate antigen test (BAPAT) and Rose Bengal plate test (RBPT) as screening tests and complement fixation test (CFT) as a confirmatory test for detection of *Brucella* antibodies (Alton *et al.*, 1988; OIE, 2016).

### *Biochemical analyses*

Haptoglobin, was assayed by means of quantitative immuno-turbidimetric assay with sensitivity limit 2.9 mg/dL, within-run and run-to-run precisions of 2.1% (Ben S.r.l. Biochemical Enterprise). Fibrinogen was measured using an immuno-turbidimetric diagnostic kit with sensitivity 4.5 mg/dL (Salucea, The Netherlands).

Superoxide dismutase, glutathione peroxidase, ascorbic acid, nitric oxide, copper, iron, and zinc were estimated using commercially available kits (Biodiagnostics, Egypt).

### *Statistical analysis*

To compare the levels of acute-phase reactants to infection (seronegative *vs*. seropositive) and their interaction, generalised mixed linear models were designed separately for each parameter. Each individual was included as a random factor with a normal distribution and an identity link. According to the complement fixation test, animals were classified into seronegative, mild (CFT<10) and severe (CFT>10 to 320), then simple one-way ANOVA was processed. Duncan's multiple range test was performed to differentiate between significant means at $P<0.05$. The Independent Sample Student t-Test was also performed between all diseased and negative animals determined by CFT.

For the selection of cut-off points that optimise sensitivity and specificity for each parameter, the non-parametric receiver operating characteristic curve (ROC) was used. The ROC curves were constructed by plotting 1-specificity (*x*-axis) versus sensitivity (*y*-axis) for all possible threshold values of the evaluated parameter. The area under the curve (AUC) indicates the overall accuracy of the tested parameter. A biomarker without predictive value would have an AUC of 0.5 (represented by the diagonal line in the ROC plot), while a biomarker with perfect ability to predict disease would

have an AUC of 1.0. Values of AUC between 0.5 and 1.0 were interpreted as having low (0.5 >AUC ≤ 0.7), moderate (0.7 >AUC ≤ 0.9), or high (0.9 >AUC <1) accuracy (Swets, 1986; 1988). The two-graph ROC (TGROC) plot was also used to illustrate variation of sensitivity and specificity of biomarkers across a range of cut-offs. The sensitivity and specificity of the RBPAT and BAPAT were counted regarding the positive samples of the CFT. All analyses were done using SPSS 20.

## RESULTS

From the three serological tests used to diagnose mobile sheep with *Brucella*, CFT had the highest sensitivity and specificity, so it detected low seropositive and high seronegative animals compared to RBPAT and BAPAT and the false positive. The false negative percentages of BAPAT were higher than those of the RBPAT (Table 1).

The *Brucella* seronegative animals had lower (P=0.0001) blood ascorbic acid than those with acute or chronic infection (Table 2). In contrast, NO (P<0.001) and GPx (P=0.029) concentrations of infected sheep were lower compared to the non-infected animals. Though SOD concentrations of seropositive animals were lower (P<0.05) than those of seronegative ones, SOD levels of acutely infected animals were not significantly lower compared both to chronically infected and seronegative ones. Concentrations of both copper (P<0.05) and zinc (P<0.002) of diseased animals were significantly higher than respective levels in non-diseased animals. The concentrations of fibrinogen of infected animals in the acute stage were significantly higher (P<0.0001) than those in non-infected animals. Haptoglobin showed a nonsignificant increase of acutely infected sheep compared to the healthy ones and animals with chronic form of the disease (Table 2).

When ROC curve was plotted for all studied parameters (Table 3), the serological complement fixation test (CFT) had the largest area under the curve (AUC) of 1.0 proving that the CFT was the most accurate test to differentiate between diseased and non-diseased animals (Fig. 1). By comparing the two serological screening tests, the buffered acidified plate antigen test (BAPAT) had a larger AUC of 0.97 than the Rose Bengal plate

**Table 1.** Prevalence, sensitivity and specificity of serological tests in sheep infected with *Brucella* (n=160)

| Total number | Serological tests | | | | | |
|---|---|---|---|---|---|---|
| | RBPAT | | BAPAT | | CFT | |
| | N | % | N | % | N | % |
| Seropositive | 115/160 | 71.9 | 119/160 | 74.4 | 91/160 | 56.9 |
| Seronegative | 45/160 | 28.1 | 41/160 | 25.6 | 69/160 | 43.1 |
| False negative | 24/69 | 34.78 | 28/69 | 40.58 | | |
| False positive | 24/91 | 26.37 | 28/91 | 30.77 | | |
| Sensitivity | 91/115 (79.13%) | | 91/119 (76.47%) | | 100% | |
| Specificity | 45/69 (65.22%) | | 41/69 (59.42%) | | 100% | |

Number (N), RBPT (Rose Bengal plate agglutination test), BAPAT (Buffer acidified plate agglutination test), CFT (Complement Fixation Test.

antigen test which had an AUC of 0.96 (Table 3). Only the zinc (Fig. 2) and ascorbic acid (Fig. 3) levels of diseased animals showed moderate accuracy where their AUC were 0.72 and 0.89, respectively. The other parameters (Fig. 4) exhibited a low accuracy with AUC ranging from ≤0.5 to <0.7 (Table 3).

**Table 2.** Acute phase proteins, oxidants, antioxidants and trace minerals in ewes infected with *Brucella* (mean±SD)

| Parameters | Positive according to CFT | | | Negative | P value |
|---|---|---|---|---|---|
| | Low (Chronic) <10 | High (Acute) >10–320 | Total | | |
| Number of cases | 44 | 47 | 91 | 69 | |
| BAPA | 2.4±0.1$^{b}$ | 3.1±0.1$^{c}$ | 2.8±0.08 | 1.0±0.1$^{a**}$ | 0.0001 |
| RBPAT | 1.9±0.1$^{b}$ | 2.8±0.1$^{c}$ | 2.5±0.08 | 0.76±0.07$^{a**}$ | 0.0001 |
| CFT | 6.9±0.4$^{a}$ | 148±18.5$^{b}$ | 97.9±13.5 | 0.00±0.00$^{a**}$ | 0.0001 |
| Ascorbic acid, mg/L | 2222±289$^{b}$ | 2765±242$^{b}$ | 2562±187 | 986±108$^{a**}$ | 0.0001 |
| NO, µmol/L | 27.7±1.4$^{a}$ | 28.2±2.2$^{a}$ | 27.9±1.39$^{a}$ | 52.3±9.3$^{b**}$ | 0.001 |
| GPx, mU/mL | 183±63$^{ab}$ | 49±7$^{a}$ | 88.7±20 | 258±123$^{b*}$ | 0.029 |
| SOD, U/mL | 316±61 | 273±19 | 290±27 | 369±72$^{*}$ | NS |
| Fibrinogen, mg/dL | 8.04±0.451$^{a}$ | 10.17±0.46$^{b}$ | 9.38±0.36 | 8.82±0.45$^{ab}$ | 0.0001 |
| Haptoglobin, mg/dL | 2.86±0.69 | 3.39±0.73 | 3.20±0.52 | 2.95±0.42 | NS |
| Zinc, µg/dL | 25.60±1.37$^{b}$ | 27.29±1.18$^{b}$ | 26.66±0.90 | 21.55±0.51$^{a**}$ | 0.002 |
| Iron, µg/dL | 143.69±11.57 | 147.70±8.06 | 146.21±6.61 | 138.95±11.37 | NS |
| Copper, µg/dL | 76.69±9.38$^{ab}$ | 102.52±12.13$^{b}$ | 92.91±8.52 | 68.82±4.12$^{a\#}$ | 0.05 |

Means with different superscripts (a, b, c) within a row are significantly different at P<0.05; non-significant (NS). Significant differences between total positive and negative are indicated as followed: # P>0.05, * P<0.05, **P<0.001.

**Table 3.** Area under the curve (AUC) of the ROC curve for each assayed blood parameter

| Test result of variables | Area | Standard error[a] | Asymptotic significance[b] | Asymptotic 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| BAPA | 0.972 | 0.012 | 0.000 | 0.949 | 0.996 |
| RBPAT | 0.964 | 0.013 | 0.000 | 0.938 | 0.990 |
| CFT | 1.000 | 0.000 | 0.000 | 1.000 | 1.000 |
| Iron | 0.538 | 0.087 | 0.652 | 0.366 | 0.709 |
| Copper | 0.501 | 0.085 | 0.993 | 0.335 | 0.667 |
| Zinc | 0.720 | 0.070 | 0.008 | 0.583 | 0.857 |
| Ascorbic acid | 0.896 | 0.041 | 0.000 | 0.815 | 0.977 |
| NO | 0.203 | 0.071 | 0.000 | 0.063 | 0.343 |
| SOD | 0.521 | 0.090 | 0.804 | 0.344 | 0.698 |
| GPX | 0.599 | 0.098 | 0.239 | 0.407 | 0.791 |
| Fibrinogen | 0.537 | 0.065 | 0.555 | 0.410 | 0.665 |
| Haptoglobin | 0.415 | 0.060 | 0.179 | 0.297 | 0.532 |

The test result of variables copper, zinc has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased. a. Under the nonparametric assumption; b. Null hypothesis: true area = 0.5.

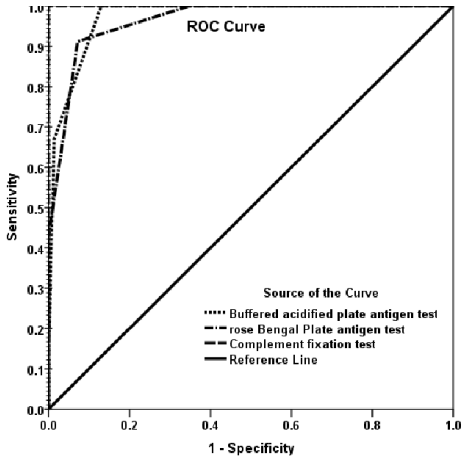


**Fig. 1.** ROC curves of Buffered acidified plate antigen test, Rose Bengal plate antigen test and complement fixation test. The reference line refer to the CFT classification of animals.

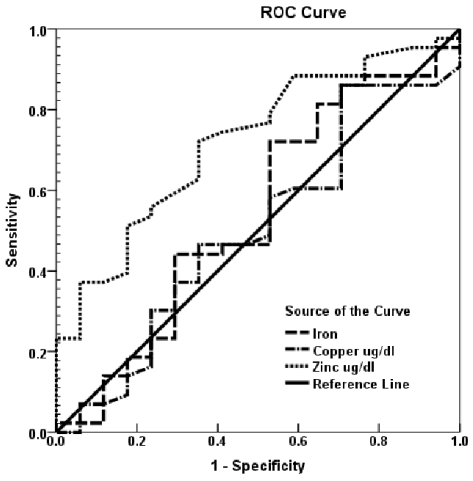


**Fig. 2.** ROC curves of iron, copper and zinc of animals with or without brucellosis. The reference line refer to the CFT classification of animals.

The RBPAT correlated positively with iron (r=0.20; P=0.03), zinc (r=0.25; P=0.005), copper (r=0.26; P=0.04), fibrinogen (r=0.20; P=0.05), and ascorbic acid (r=0.49; P=0.0001), but showed negative correlations with NO (r= –0.26; P=0.009 and GPx (r= –0.20; P=0.05). BAPAT showed a positive correlation with ascorbic acid (r=0.31; P=0.001) though a negative one with nitric oxide (r= –0.31; P=0.002). The CFT tended to correlate with ascorbic acid (r= –0.16; P=0.079).

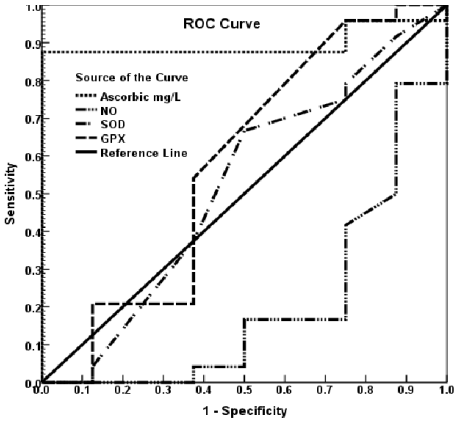


**Fig. 3.** ROC curves of ascorbic acid, nitric oxide (NO), superoxide dismutase (SOD), glutathione peroxidase (GPx) of animals with or without brucellosis. The reference line refer to the CFT classification of animals.

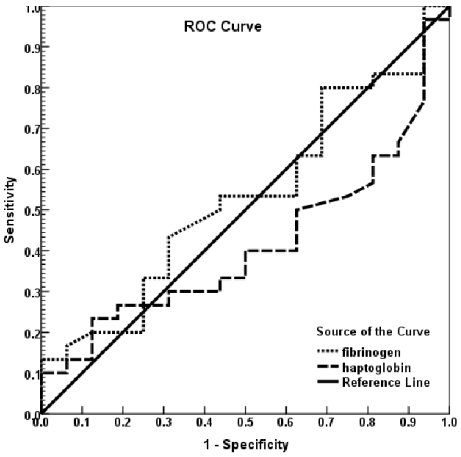


**Fig. 4.** ROC curves of haptoglobin and fibrinogen of animals with or without brucellosis. The reference line refer to the CFT classification of animals.

## DISCUSSION

Sheep and goats get infected not only by the very virulent biotype *B. melitensis* but can contract other types that have major impact on human health (Wang *et al.*, 2017) causing reproductive disorders with significant economic losses in animal husbandry (Antunes *et al.*, 2013; Costa *et al.*, 2016). Many serological tests are widely used for the diagnosis of brucellosis because the infected animal may or may not produce all antibody types at detectable levels. In agreement with the results of serological tests used to detect brucellosis in mobile sheep, RBPT is still of high sensitivity in the diagnosis of bovine and ovine brucellosis. The high sensitivity of RBPT is attributed to its ability to detect very low concentrations of different immunoglobulin classes (IgM, IgG1, and IgG2) up to 5, 50 and 50 µg/mL, respectively, in *Brucella*-infected animals (Diaz-Aparicio *et al.*, 1994). The results of this test did not show a great difference with the results of BAPAT which means that it should be supported by other serological tests. The present results are nearly similar to those obtained by Hamdy *et al.* (2002) and Anwar (1999). There are some problems of the specificity of serological tests for sheep brucellosis since antibodies against *Brucella melitensis* epitopes may be present in the animal population due to vaccination and/or of contacts with other Gram-negative bacteria (mainly, *Yersinia enterocolitica* O:9) sharing cross-reactive epitopes with *Brucella* (Garin-Bastuji *et al.*, 2006). CFT is considered as the gold standard serological test used for the detection of brucellosis as it detects only IgG specific for *Brucella* infection so it overcomes cross-reaction with other similar Gram-negative bacteria and detects no false results. Additionally, the World Organization for Animal Health (OIE) suggested that CFT is a test approved all over the world (OIE, 2016). In sheep of this study, CFT has proved to be the most accurate serological test for the identification of ewes not infected with *Brucella* compared to the other two serological agglutination tests. Several decades ago, CFT was a very useful aid in the serological diagnosis of brucellosis, particularly for differentiating between infected and vaccinated animals (Wisniowski, 1964). According to the results of CFT, animals having a low titre (<10) were classified as chronic and those with high titre (>10 to 320) were included in the acute group which explains the absence of significant differences of some parameters between non-diseased sheep and the chronically diseased animals (with low CFT titre).

In diseased ewes of this study, the increased levels of ascorbic acid, fibrinogen, zinc, and copper, especially during the acute form (high CFT), were associated with a decrease of NO, SOD and GPx. In agreement with our results, the levels of acute-phase proteins are greatly enhanced in the first few hours after exposure to the pathogenic factors during the acute phase of inflammation (Ceciliani *et al.,* 2002), whereas the chronic inflammation is considered as a series of individual inflammatory stimuli and is characterised by longer and slight increase in the serum concentration of APPs as compared to acute inflammation (Murtaugh, 1994; Jain *et al.,* 2011). Also, the *Brucella* infection in ewes was characterised by a significant increase in globulin, cholesterol, aspartate transaminase and alanine transaminase and a decrease in albumin and glucose compared to healthy ewes (Kumar *et al.*, 2015). In agreement with the decrease of NO in *Brucella*-infected animals of this study, healthy mares showed similar patterns of their total proteins, globulins and

NO (Abo El-Maaty *et al.*, 2012). In the serum of *Brucella*-infected animals, a significant decrease of albumin levels and the insignificant decrease of total protein and blood urea nitrogen (BUN) concentrations were recorded in ewes (Kumar *et al.*, 2015), cattle (Nath *et.al.* 2014), ewes, goats and cattle (El-Boshy *et. al.,* 2009; Hamada *et al.,* 2013). Reduced serum albumin and urea concentrations in cattle infected with *Brucella* were referred to damaged liver tissue as expressed by a disturbance in liver enzymes (Al-Hussary *et al.*, 2010, Nath *et al.,* 2014; Kumar *et al.*, 2015). The decreasing pattern of blood nitric oxide in ewes of this study may follow the decreasing pattern of albumin. Albumin is one of the negative acute phase proteins, its concentrations decrease in response to inflammation or infection (Tothova *et al.*, 2014). This decline of serum albumin concentration in the *Brucella* affected ewes was attributed to the loss of albumin through the urine due to kidney damage, decreased feed intake by the affected ewes and reduced production of albumin by the liver due to hepatic damage (Al-Hussary *et.al.*, 2010). Nitric oxide is a gas and a free radical is synthesised enzymatically from the amino acid L-arginine in several tissues using the three isoforms of nitric oxide synthase and the endothelium one is responsible for the regulation of blood flow and the activation of blood platelets (Bruckdorfer, 2005). In the present study, the decrease of NO can be attributed to the lowered feed intake due to *Brucella* which results in lowered synthesis of proteins or to the fact that endothelial NO as a potent vasorelaxant is used to cause vasodilatation during inflammation, a consequence of either damaged/dysfunctional endothelium and the down-regulation of IL-1 β which induces nitric oxide synthase (NOS) and NO synthesis (Rosselli *et al.*, 1998).

All ruminants, horses, and swine can synthesise ascorbic acid from glucose in their liver (Comb, 2008). Vitamin C is also an important water-soluble antioxidant that prevents the oxidation of protein, DNA and nitric oxide (Frei *et al.*, 1989). Ascorbate increases neutrophils protection against oxidative stress induced by free radicals during the oxidative burst (Wolf, 1993), and stimulates interferon production (Goetzl *et al.*, 1974). In contrast to the decrease of plasma ascorbic acid concentrations in sheep infected with *Fasciola hepatica* till week 10 post infection and their subsequent increase (Gameel, 1982), ewes naturally infected with *Brucella* in this study demonstrated high ascorbic acid concentrations. The comparable increase of ascorbic acid may refer to the destructive effects of *Fasciola* on the animal hepatic cells which synthesise ascorbic acid.

Haptoglobin (Hp) is an acute-phase protein that binds to heme preventing it from serving as a nutrient for pathogens and initiating deleterious oxidation reactions resulting in a rapid inflammatory response (Matson *et al.*, 2006; 2012), and was used as a predictive marker after an endotoxin challenge (Matson *et al.*, 2012). A Hp concentration above 1 mg/mL was considered the approximate cut-off of severe inflammation (Skinner & Roberts, 1994; Wells *et al.*, 2013). In sheep, Hp increases locally through its release by neutrophils in response to the early production of TNF-alpha in inflammatory reactions (Ulutas & Ozpinar, 2006; Lepherd *et al.*, 2009; Bastos *et al.*, 2011). The insignificant increase of haptoglobin in sheep infected with *Brucella* may refer to the chronic infection. Moreover, the sheep breed played an important role in

modulating immune response that was observed when Suffolk ewes and Dorset ewes that received the same lipopolysaccharide, with the Suffolk ewes showing an enhanced acute-phase response demonstrated by increased concentrations of plasma haptoglobin (Hadfield *et al.*, 2018).

Moreover, the age and physiological status of the sheep influenced their immune response (Miglio *et al.*, 2018). Our concentrations of haptoglobin and fibrinogen in ewes infected or not with *Brucella* lied within the normal ranges in healthy ewes (Simplício *et al.*, 2017) which explain the inability of the two major acute-phase proteins to be used as predictive values of the disease. The insignificant decrease of both haptoglobin and fibrinogen concentrations in the serum of ewes with chronic or acute *Brucella* infection was also noted 2 months post-treatment in sheep during a field outbreak of sheep scab (Wells *et al.*, 2013). However, among biochemical parameters, total proteins, fibrinogen, and aspartate aminotransferase did not differ, whereas higher (P=0.0062) alanine aminotransferase and lower (P=0.030) alkaline phosphatase concentrations were noted in *Brucella*-positive horses (Gul *et al.*, 2013).

Similar to the insignificant increase in haptoglobin and fibrinogen of ewes naturally infected with *Brucella*, horses seropositive and seronegative to *Brucella* had the same concentrations of fibrinogen (Gul *et al.*, 2013). In contrast to our results, serum haptoglobin concentrations were significantly higher in sheep with acute ruminal acidosis (Kamr *et al.*, 2017). Furthermore, polymyxin B significantly improved the clinical signs in endotoxaemic animals treated with LPS from *E. coli* serotype O55:B5 at a concentration of 0.5 µg/kg (Hajimohammadi *et al.*, 2018). The insignificant difference in haptoglobin between infected and non-infected ewes with brucellosis was also observed in rams experimentally infected with rough virulent *B. ovis* strain (R-*B. ovis*) where the pro-inflammatory cytokine expression was up-regulated for only 30 days after infection then no changes were observed in the expression of epididymal IL-1α and IL-1β, and testicular IL-12 and INF-γ from day 30 till day 240 after infection indicating that with the development of infection, cytokine gene expression levels decreased, providing evidence of immuno-suppression and evidence of immune evasion that favoured persistence of chronic R-*B. ovis* infection (Antunes *et al.*, 2013). When sheep were subjected to experimental endotoxaemia using LPS, the haptoglobin levels declined significantly in animals treated with tyloxapol and pentoxifylline groups compared to positive control three hours after the start of treatment (Chalmeh *et al.*, 2016). The mouse model experimentally infected with the whole cell of *B. melitensis* or its LPS via the subcutaneous route of exposure demonstrated significant clinical signs and histopathological evidence in *B. melitensis* infected than in LPS-challenged. However, both infected groups showed elevated levels of interleukins (IL-1β & IL6), antibody levels (IgM and IgG) as early as 3 days post-infection with predominance in LPS-infected group (Osman *et al.*, 2018). In contrast to sheep naturally infected with *Pasteurella multocida*, where no significant difference in the area under the curve (AUC) was observed among acute phase proteins and haptoglobin showed better sensitivity and specificity than fibrinogen, the fibrinogen of mobile sheep naturally infected with *Brucella* of this study had higher area under the curve

than haptoglobin (El-Deeb & Elmoslemany, 2016).

The disadvantage of natural infection concerning the determination of Hp responses is that timing cannot be controlled, and the host can have other entero-pathogens altering the expression of cytokines at different stages of the infection causing different profile of APP responses. Previous studies on the changes of serum Hp and SAA concentrations have focused mostly on natural infectious diarrhoea in calves. Experimental infection of neonatal lambs with *Cryptosporidium parvum* showed that Hp concentrations in serum with relatively low values at birth increased 7-fold from day 2 to reach a peak value six days post-infection then started to decrease to the pre-inoculation values 20 days post-inoculation (Dinler *et al.*, 2017). The liver plays important functions of ketone body formation and metabolism and increases the synthesis of fibrinogen and other acute-phase reactants in response to tissue damage (Werner, 1969). Free radicals produced after acute infection affect the cytokine-producing cells directly and modify proteins in the extracellular fluids (Koj, 1996).

Hp possesses anti-inflammatory and immunomodulatory properties and functions as an antioxidant to prevent oxidative tissue damage (Gabay & Kushner, 1999). Acute bacterial infections and mixed infections lead to higher Hp response than those of viral or parasitic infections and mono-infection (Murata *et al.*, 2004; Gruys *et al.*, 2005; Pourjafar *et al.*, 2011). The differences in the magnitude of the response and the timing of peak values between Hp are related to infectious agent variation and the kinetics of these APPs (Murata *et al.*, 2004; Gruys *et al.*, 2005; Tothova *et al.*, 2014). Similar to the response of mobile sheep to natural infection with *Brucella*, haptoglobin and fibrinogen concentrations and white blood cell counts were not significantly different for seropositive and seronegative sheep to caseous lymphadenitis and their values did not differ significantly among seronegative, acute, and chronic of infection (Bastos *et al.*, 2011). Also, haptoglobin and fibrinogen levels remained within normal limits in lambs infected with ovine lentivirus (de la Concha-Bermejillo *et al.*, 2000). Moreover, various infections and inflammatory processes induced different APP response (Gruys *et al.*, 2005; Tothova *et al.*, 2014).

The decrease of enzymatic concentrations of superoxide dismutase (SOD), glutathione peroxidase (GPx) and nonenzymatic antioxidants (NO) in ewes naturally infected with *Brucella* compared to the non-infected ones could be related to preventing the activation of some cytokines (Ceciliani *et al.*, 2012), or the inactivation of others or the presence of type IV secretion system gene which is responsible for the virulence of *Brucella* by increasing its ability to invade and replicate within the macrophages and the over-secretion of effector proteins causing the death of infected macrophages via activating IRE1α pathway of endoplasmic reticulum stress **(**Li *et al.*, 2017**)**. SOD and GPx activities in treated ewes after challenge with LPS were significantly lower than positive control from one to three hours after induction of treatment (Chalmeh *et al.*, 2016). SOD promotes the conversion of anion superoxide to $H_2O_2$ (Al-Gubory *et al.*, 2010) which is eliminated by catalase (Klotz *et al.*, 1997; Lim *et al.*, 1998). In contrast to the seropositive sheep in the current study, cows infected with *Brucella* had higher nitric oxide concentrations due to stimulation of NO synthesis by macro-

phages following secretion of the bacterial lipopolysaccharides (Nisbet *et al.*, 2007**)**.

The insignificant increase of iron in the blood serum of ewes infected with *Brucella* in the current study could be attributed to the effect of ascorbic acid in increasing iron bioavailability through the reduction of ferric ions to ferrous ions (Wollenberg & Rummel, 1987). The different values of zinc and iron in lactating sheep (Yokus *et al.*, 2004) was attributed to differences in the breed, sex, age, illness, seasonal and physiologic variations, nutritional content of the diet, feeding, environment and geography effects (Kaneko, 1997; García *et al.*, 2000; Yokus *et al.*, 2004). Hypoferremic response of sheep was observed as early as 6 h after LPS challenge and iron reached its lowest level by the 16$^{th}$ h after LPS infusion and this hypoferremia was attributed to the upregulation of hepcidin in sheep liver, spleen and kidney in response to the systemic inflammation (Ridler & West, 2011). Moreover, the administration of LPS reduced serum iron levels via modulating hepcidin (Wallace & Subramaniam, 2015). Similarly to the increased copper concentrations in sheep seropositive to *Brucella*, both men and women infected with *Brucella* had higher serum copper concentrations. In contrast to the increased zinc concentrations in but in agreement with sheep seropositive to *Brucella*, women infected with *Brucella* had significantly low zinc serum concentrations (Mobaien *et al.*, 2010***).***

It could be concluded that the body of the infected ewes reacted to the acute infection with *Brucella* in a trial to overcome it. In the chronic form, it was difficult to differentiate between seropositive and seronegative animals using the acute phase proteins.

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**Correspondence**:

Amal M. Abo El-Maaty
Animal Reproduction and Artificial Insemination, Veterinary Research Division, National Research Centre, Dokki, Giza, Egypt,
e-mail: amalaboelmaaty1@yahoo.com; am.aly@nrc.sci.eg
ORCID ID 0000-0001-8466-613