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Investigation on Cytotoxic Effect of *Brucella abortus* on Cattle and the Role of Cytokines in Regulation of Intracellular Bacterial Infection

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Abstract

This study was performed on cattle farm in Egypt country which was endemic with *Brucella*, these animals were suffered from abortion in late three months of pregnancy with retained placenta. Study group was comprised of 18 cattle aged between 2-3 years, all animals of this farm were exposed to serological test to determine the infected animals. Field diagnosis Rose Bengal test was performed and confirmed by ELISA IgG test to determine the type of *Brucella*, Serological tests detected 12 animals were positive *Brucella abortus* and remain were negative by these serological tests, so these negative animals were considered as control negative. This experiment was performed in Vet. Teaching Hosp, Moshtohor, Benha University. Biochemical tests were performed to determine oxidant and antioxidant changes associated to infection by measuring indicators As Malondaldhyde (MDA), Lactate Dehydrogenase (LDH) and Nitric Oxide(NO), Total Protein(TP), Albumin (AL) and Total Antioxidant Capacity(TAC), associated with *B. abortus* infection. ELISA tests to measure alterations in cytokines IL-10, IL-1β and TNF ∝that associated to infection, this investigation pointed to the role of these parameters in regulation of intracellular bacterial infection as response of body immunity system to infection.

Keywords: Biomarkers; *Brucella*; Cytokines Immunity (IL-10, IL-1β and TNF∝) oxidants and antioxidants

Introduction

Brucella spp. are facultative intracellular bacteria that cause brucellosis in a variety of animals and undulant fever in humans. The disease is one of the most widespread common diseases in the world, especially in developing countries. Six species have been described, but only *Brucella melitensis*, *B. abortus*, and *B. suis* pose that threat public health. *Brucella* causes serious economic losses as abortion and infertility in cattle [1,2]. Therefore, these species have been classified as category B agents that can be used as biological arms. Brucellosis called thousand faces diseases because it has long lasting side effects, *Brucella* has developed various strategies to evade innate and adaptive immune response so became the commonest chronic bacterial diseases worldwide, which can establish intercellular for long term survival and replication [3-6]. From previous we concluded that clinical signs alone are not sufficient for brucellosis diagnosis but we need to sensitive, specific, rapid and inexpensive method to detect infection early because early accurate diagnosis of disease give good prognosis and eradication of disease [7]. As we explained above *Brucella* stimulates both humeral and cellular immunity but the main mechanism of recovery is cellular immunity science elimination of brucellosis depend on conjunction with activated macrophages which induced by the cell mediate immunity cytokines that release during this stimulation [8,9]. The immune response against *Brucella* depend on activating of macrophages by bacterial infections and

potentiates the apoptotic death of infected macrophages this immune response was considered the pivotal role of macrophages, Pathogenesis is the product of a complex series from interaction between bacteria and different components of immune system special macrophages that is considered the main cell of *Brucella* residence in the host [3,10,11-13]. Highlight on *Brucella* infection, it associated with acute inflammatory reaction, which represents the principal local defense against spread of infection, many of the severe complications of bacterial infections result from excessive immune activation so we can say the maximal pathogen control does not necessarily lead to the minimal disease for example free radicals by-products which produced continuously during many normal cellular reactions can cause various damages in the organisms [14-16].Protective response against *Brucella* infection requires Th1-type cytokines such as Inter Feron-gamma (IFN-Y),Tumor Necrotic Factor (TNF-∝))and activated macrophages [5,17] Interleukin-1 beta (IL-1β) also known as leukocytic pyrogenic, mononuclear cell factor, lymphocyte activating factor and other names. The synthesis of IL-1β precursor is induced by stimulation of innate and cellular immunity by exposure of macrophages cells to pathogen-associated molecular pattern wherever IL-1β is synthesized as a precursor form protein only, which is considered as another group of appalling [18].

Material and Methods

Animals

This study was performed on cattle farm in Egypt which was endemic with *Brucella*, these animals were suffered from abortion in late three months of pregnancy with retained placenta. Study group of cattle consisted of 18 cows aged between 2-3 years, all animals of this farm were exposed to serological test to determine the infected animals. Field diagnosis Rose Bengal test and confirmed by ELISA IgG test to determine type of *Brucella*, serological test detected12 animals were positive to *Brucella abortus* and 6 out of 18 were negative by all serological tests so considered the negative cows control (Figure 1).

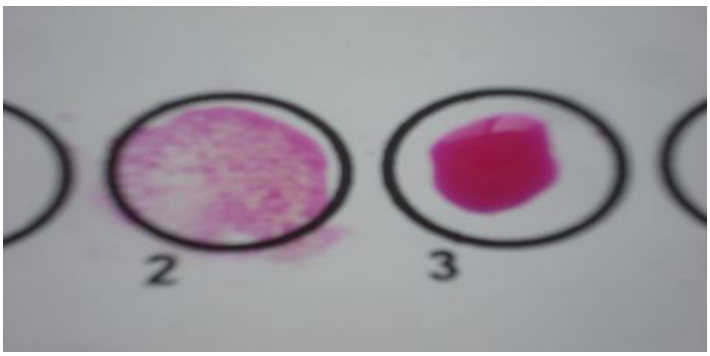

Figure 1: Rose Bengal test showing negative and positive agglutination sample.

This experiment was performed in Vet. Hosp. Moshtohor Benha University. Biochemical tests were performed to determine biochemical change associated with *B. abortus* infection. ELISA Kit which was performed to measure Bovine *Brucella* Ab IgG(BAL) ELISA Kit Cat No: MBS753249. Serum samples were evaluated for various parameters, namely tumor necrosis factor-alpha (TNF-α) ELISA kits were utilized is Cat. No. E11-807, interleukin IL-10 the kits were utilized is Cat. No. MBS 703712 and interleukin IL-B the kits were utilized is Cat. No. MBS 703996 while Lactate Dehydrogenase (LDH) measured by using kit of SPINREACT, S.A.U. Ctra. Santa Coloma, 7 E-171716 SANT ESTEVE DE BAS (GI) SPAIN, moreover spectrophotometric method using commercial kits (bio-diagnostic, Egypt) was used for measuring oxidative and anti-oxidative biomarkers as serum Malondialdehyde (MDA), Nitric Oxide (NO), Total protein, Albumin and Total Antioxidant Capacity (TAC) to determine the role of these parameters in regulation of intracellular bacterial infection and response of body immunity system.

Results

In this study animals positive to infection from animals suffered from clinical signs suggested *Brucella* and positive stereological testes were 12 with Rose Bengal and ELISA tests while the remain six were negative and considered as control negative group. (Table 1) showed that infection with brucellosis had significant increase in NO, MDH and LDH in another side notice non-significant change in TP, AL and TAC. Table 2 were showed that significant increase in IL-10 and IL1β in infected group while TNF was showed non-significant change between infected and non-infected animals.

Group Parameter	Control negative	Infected
Albumin	2.508±0.1537	2.082±0.1346
TAC	66.91±10.0 8	56.86±9.517
T. protein	5.590±0.3721	5.113± 0.2807
MDA	60.69±9.447	117.2.6±17.17***
NO	53.45±9.109	88.8±9.865***
LDH	349.9±20.32	355.9±20.05**
** Value of p < 0.01 and *** value of p< 0.001.		

Table 1: Serum MDA and NO and some antioxidants levels in infected animals with *Brucella abortus* and healthy cattle.

Group Parameter	IL-10	IL-1β	TNF-α
Control	58.62± 12.79	62.65±2.05	0.120±0.02
Infected	25.31±4.88***	176.01±34.55***	0.19±0.015
** Value of p < 0.01 and *** value of p< 0.001.			

Table 2: IL10, TNF and IL1β in healthyand infected animals with *Brucella abortus*.

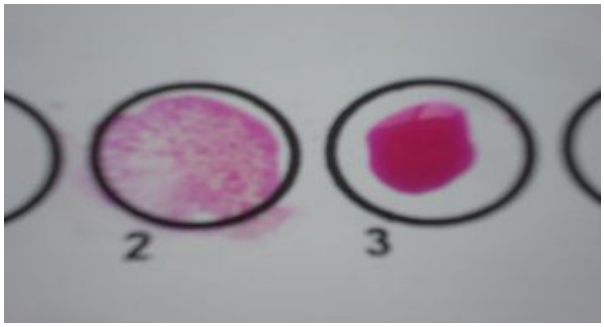

Figure 1: Rose Bengal test showing negative and positive agglutination sample.

Static

The statistics was applied by means of SPSS software (SPSS ver. 16, Inc., Chicago, IL). T-test was used for each group at a significant value at $p<0.05$ [19].

Discussion

Bovine brucellosis is mainly caused by *Brucella abortus* which is clinically characterized by abortion in the three late months of gestation period and infertility in cows while caused orchitis and inflammation of the accessory sex organs in bulls. Natural *B. abortus* infection in cattle occurs primarily through penetration of mucosal membrane of the oropharynx followed by uptake with macrophages (MQ) and transport to the regional lymph nodes [20]. Macrophages are the primary target cells in which *Brucella* organisms multiply and cause persistent infection, the bacteria also invade trophoblast cells and cause abortion in ruminants as the front line of the innate immune response; macrophages ingest and kill invading pathogens, produce various cytokines, and perform antigen presentation to develop adaptive immunity [21]. In our study we used field diagnosis rose Bengal test to determine infected animals because clinical signs only insufficient to detect positive animals after that use ELISA test to confirm the result of positive *Brucella* infected animals this came agree with Molavi [7]. who reported that Using new methods such as ELISA has higher sensitivity and specificity than positive complement fixation, which can show both G and M immunoglobulins. It is also suitable for examining certain class of immunoglobulin and added that It can also prevent the complexity created by glucan or incomplete antibodies therefore, acute brucellosis can be easily diagnosed from chronic brucellosis using ELISA method so when interpreting agglutination test is met with confusion, the result can be confirmed using ELISA test. It should be noted that IgG, IgM (IgG1, IgG2), IgA and partial amount of IgE are produced in Brucellosis humoral immunity response. IgG is particularly involved in serological tests. IgM appears on the fifth to seventh day of brucellosis infection and reaches the final amount during 13 to 21 days after bacteria penetrated the body. Low amount of IgA is also generated in the interval between emergences of above two immunoglobulins. IgG titer is higher and more durable during the disease. This is significant in serological survey of brucellosis when the serum is tested. If infected serum in the first week was tested, no immunoglobulin may be observed. Thus, the test result will be negative. IgM level increases in second week. IgG is generated between the second and third weeks. IgG reaches the maximum level after three weeks. This level is still high during infection all this fact support our studies that have shown ELISA is a complete method in vitro for detection of chronic samples, especially when other tests results are negative. In addition to this method, all unique and specific immunoglobulin in tested serum appear with high speed and accuracy. In current investigation we recorded significant increase in NO in infected animals group than control negative group because toxic effects of pathogens produce activation of oxidative bactericidal activity which protect the host, this result came in agreement [16,22,23-26]. Who concluded that nitric oxide (NO) is an important free radical molecule which is a general characteristic of activated macrophages**,** fundamentally, NO is a substantial factor in the elimination of *Brucella* infection. Immediately after engulfment of the bacteria, inducible nitric oxide synthase enzyme is expressed in the phagocytic cells and the levels of NO sharply increase. In fact, production of nitric oxide contributes to Impairment of *Brucella* growth in macrophages [27,28]. Gross A [29] presumed that the chronicity of infection lead to inability of macrophages to produce NO during *Brucella* infection.NO is a cytotoxic effector molecule which regulate of apoptosis and lymphocyte migration, it modulates the Th1/Th2 balance and is involved in the regulation of vascular tone, wound repair and other processes. In our study we noticed significant increase in Malondialdehyde (MDA) in infected group than control negative one which is considered as a by-product of lipid peroxidation and used as an index of the rate of tissue reaction chain since it presents in membrane of cells this result came in contact with McCord JM [30], Marnett LJ [31] and Czuczejko J [32], Jimenez de Bagues MP [33], Couper KN [15], Madebo T [34**]**. Who concluded that increased oxygen free radical is related to antioxidant consumption causes oxidative stress production and increase Malondialdehyde (MDA) which has been reported as one of the major products of lipid peroxidation that promote cross-linking bonds in the cell membrane and leads to unfavorable effects such as changes in ion permeability and enzyme activity due to lipid peroxidation react with biological structures such as proteins, lipids, carbohydrates and DNA and cause damage to them. In this study we aimed to investigate the changes in NO and MDA levels in cattle infected with B. abortus this results came in contact with Cevat Nisbet [35] who reported that the increase in serum NO levels in cattle infected with *B. abortus* is due to the increased NO synthesis in the macrophages by bacterial lipopolysaccharides. On the other hand, increased MDA may be a result of excessive production of radical secondary

to brucellosis itself acting upon membrane lipids. The results have lead to us believe that these can be used as indicators of tissue damage. In our study we found significant different in LDH in two groups this result came in agree with Pagana, &Pagana [36] founded that elevated levels of LD usually indicate some type of tissue damage. LD levels typically will elevate due to cellular damage start reached peak after some time period, and then begin to fall. LD levels are rised in a wide difference of conditions, reflecting its spread tissue distribution. Lactate dehydrogenase (LD or LDH) is an enzyme entail in energy production that is present in most all of the body's cells, with the highest levels found in the cells of the heart, liver, muscles, kidneys, lungs, and in blood cells; bacteria also produce LD. This test measures the level of LD in the blood or sometimes other body fluids. Only a small amount of LD is ordinary detectable in the fluid portion of the blood (serum or plasma). LD is produce from the cells into the serum when cells are breaked down, it may be used, in incorporation with other blood tests, to help controlling cases that lead to tissue damage, such as liver or blood diseases or cancer. In this study we used high `significant rise of a cytokine IL-10 in infected animals when contrast with healthy animals as another diagnostic method, interleukin is cytokine that regulates the balances between pathogen and clearance and immunopathology. *Brucella aborts* is an intracellular bacterium that causes chronic disease in human and domestic animals. Here we evaluate the concentration of IL-10 in host immune response and pathology during *B. abortus*, our investigation demonstrated that IL-10 modulates the proinflammatory immune response to *B. abortus* infection and the lack of IL-10 increase resistance to *Brucella* infection, this result came parallel [7,37]. Who concluded that IL-10 inactivates macrophages that infected with brucellosis Therefore, identification of different forms of IL-10 gene, which effect on production of these cytokines, is considered as effective method for diagnosis of the disease from the above we concluded that limitations of serological methods, which are consuming to time and expensive, as well as importance of early detection of bacteria in epidemic cases, it is recommended to use this new and effective method because many of these methods can overcome limitations of traditional methods. The bactericidal efficiency of IFNY activated macrophages against *Brucella*, TNF-α strong enhancement of the bactericidal activity of phagocytes is controlled by the cytokine IL-10. Neutrophils encounter and kill microbes intracellular upon phagocytosis when antimicrobial granules fuse with the phagosome furthermore release lytic enzymes and Reactive Oxygen s Species (ROS) that destroy pathogen. *Brucella* doesn't replicate within neutrophils [12].Our study pointed to there is no significant change between healthy and infected group in TNF-α concentration because cell death by members of TNFR occurs by apoptotic cell ,unique feature of apoptic cells is that they retain cell membrane integrity even after they have disintegrated into characteristic apoptotic bodies .Apoptotic cells and bodies are phagocytized by active macrophages, thus preventing inflammatory reaction that can result from cell lysis this result came agree with Ming Li and Amer [21], Baldwin CL [3], Jimenez de Bagues MP [4], Martirosyan A [6]. Who recorded that *Brucella* inhibit host cell apoptosis to produce favoring bacterial survival by escaping from host immune surveillance when cells dying by necrosis can lead to inflammation because *Brucella* alter the maturation and function of DCs (critical component of adaptive immunity) prevent infected cell from engaging in their maturation process, impair capacities and failed to release TNF. Jianwupei et al. [38] added that tumor necrotic factor (TNF-α) is cytotoxic for many tumoral cell lines, whereas normal cells generally are considered resistant to this action so it is well accepted that survival of *Brucella* in host macrophages considered virulence and contributes to disease pathogenesis, cytoxic cell death induced by *Brucella* requires healthy macrophage to uptake it, so the previous sections reveal that macrophage killing induced by infection due to requirement of bacteria to synthesis its protein from components of this cell but not due to TNF-α and nitric oxide**,** Cytotoxicity of *Brucella* is macrophage specific and resembles oncosis and necrosis, not apoptosis the mechanism by which *Brucella* killing macrophage by pore formation-mediated lysis due to require of bacteria to synthesis its protein through direct interaction between live bacteria and macrophages [39-41]. Insignificant differences in TNF-α levels compared to control group were related to short half-life of TNF, while high TNF level were associated with proinflammatory mediating features of this cytokine IL-10 an inhibitor of activated macrophages , by acting on macrophages , they inhibit both cytokine release and expression through co-stimulators**.** Our study pointed to there is significant increase IL-1βconcentration in infected group than healthy group and this result came agree with Dinarello [18]. Who concluded that IL-1βis considered as cytokine produce from activated macrophages as proprotein. This cytokine is an essential moderator of the inflammatory reaction, and is entail in a different of cellular activities, ensure cell proliferation, discrimination, and apoptosis so considered as an individual of the interleukin 1 family of cytokines.

Conclusion

This study explains the essential role for immune regulatory components of immune response in limiting pathology among the immune regulatory components IL-10 which is related to prevent the exacerbated proinflammatory response against gram-negative bacteria and considered a control immune regulator antagonizing the excessive Th1 and $CD8^{+}$T-cell responses. Evaluation of antibodies

against *Brucella* by serological tests were considered probable diagnosis of *Brucella* infection. Elisa has higher sensitivity and specificity which can show both G and M immunoglobulins

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