



## Interplay of oxidative stress and antioxidant bio markers in oil adjuvant *Brucella melitensis* vaccinated and challenged mice



Amit Kumar<sup>a,\*</sup>, V.K. Gupta<sup>b</sup>, Rajesh Mandil<sup>c</sup>, Anu Rahal<sup>d</sup>, A.K. Verma<sup>e</sup>, S.K. Yadav<sup>f</sup>

<sup>a</sup> Department of Immunology & Defense Mechanism, College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, 250110, India

<sup>b</sup> CADRAD, Indian Veterinary Research Institute, Izatnagar, 243122, India

<sup>c</sup> Department of Veterinary Pharmacology, College of Veterinary and Animal Sciences, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, 250110, India

<sup>d</sup> Division of Goat Health, Central Institute for Research on Goats, Farha, Mathura, 281122, India

<sup>e</sup> Department of Veterinary Medicine, College of Veterinary and Animal Sciences, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, 250110, India

<sup>f</sup> Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, DUVASU, Mathura, 281001, India

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### ABSTRACT

The intracellular nature of *Brucella* leads to rise in oxidative stress due to bacterial invasion, particularly at the site of predilection spleen and lymph nodes. The present study aimed to evaluate the erythrocytic and tissue specific oxidative stress responses induced during oil adjuvant killed *Brucella melitensis* vaccination. The results of the study clearly implicated a significant increase in level of catalase, and superoxide dismutase (SOD) activity and lipid peroxidation (LPO), and total protein content in erythrocytes after vaccination. The activity of glutathione-S-transferase (GST) was unaltered during the period of experiment. The catalase activity and GSH content was significantly increased in lung and spleen tissues. The tissues GST levels increased significantly in all tissues, while tissue SOD level increased significantly only in lung tissues. Thus, it can be inferred that oil adjuvant based *Brucella* vaccine induces negligible signs of inflammatory pathophysiology and supports the development of significant level of protection against virulent *Brucella* challenge.

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### 1. Introduction

Brucellosis is a re-emerging zoonotic infection in small ruminants. It is caused by a bacterium, *Brucella melitensis* [1]. The survival of the bacteria within a vacuole [2–4] of phagocytic cells [5] makes treatment in- or less effective. It survives and multiply within host phagocytes [6] and induces a clinical or subclinical disease. The survival mechanism involves inhibition of blending of phagocytic vacuoles with lysosomal contents and resistance to oxidative killing [7,8]. This causes an uninterrupted elevated level of oxidative stress, leading to triggering of proinflammatory cytokines in the tissues leading to cellular damage along with perpetuation of the desirable immune response [4].

The establishment of infection or disease depends upon the virulence of the bacterial strain and the host factors including stress [9]. Further, any significant change in the stress level or the associated inflammatory pathophysiology in the tissues is likely to induce/inhibit the settlement of the brucellae in the host [3,4]. The infusion of a live vaccine also induces a low-grade inflamma-

tion with prolonged intracellular persistence and multiplication in infected tissues leading to long-lasting inflammatory response that mediates different pathways of tissue damage [3,4,10]. Therefore, for a successful vaccination, approach should include efforts to minimize intracellular survival of the bacterium along with maximized immunological response with minimum or no oxidative stress [11]. The addition of adjuvants should enhance antibody response in both time and space [12] with minimum inflammation and tissue destruction [13] and that can be assessed by estimating level of oxidative stress markers [14,15]. Despite the importance and success of adjuvants in vaccine, little is known about the mechanisms of oxidative stress and antioxidant defenses involved in oil adjuvant *Brucella* vaccines. Thus, the present study aimed to assess the erythrocytic and tissue specific oxidative stress response produced by oil adjuvant *Brucella melitensis* vaccine against a challenge of live virulent brucella.

### 2. Material and methods

The experiment was conducted under Biosafety Level- 2 (BSL2) facility with strict biosafety procedures during isolation and challenge trials. Mice were kept under BSL2 bio-safety cabinets and

\* Corresponding author.

E-mail address: [balyan74@gmail.com](mailto:balyan74@gmail.com) (A. Kumar).

due permission was taken from Institutional Animal Ethics Committee (IAEC).

### 2.1. Vaccine

Virulent *Brucella melitensis* biovar 3 (Accession no. VTCCBAA228) was used as the vaccine candidate. The full genome sequence of the organism has already been submitted to NCBI, Data Bank and has been assigned as *Brucella melitensis* biovar 3IND1. The vaccine was prepared using Montanide™ VSG (Seppic, France) as oil adjuvant. The Montanide™ adjuvant and its components have been considered as safe by the Committee for the Veterinary Medical Products (CVMP) for the use in immunological products and are included as authorized substance in the annex of the European Council Regulation no. 470/2009 (previously 2377/90/EC) requiring no further MRL studies ([www.seppic.com](http://www.seppic.com)). The vaccine candidate *B. melitensis* biovar 3 IND1 was grown on sterile Brucella selective agar (HiMedia, Mumbai) and harvested aseptically to obtain a final concentration of  $10^{11}$  CFU/ml by addition of sterile PBS (pH 7.4). The brucellae were then killed by the addition of 1.25% (V/V) formalin and formalized killed polymer gel based *B. melitensis* biovar 3 IND1 vaccine (PGV) was prepared with the addition of oil adjuvant (Montanide™ VSG) in the ratio of 1:1 (V/V) to bacterial suspension as per the recommendations of Seppic, France ([www.seppic.com](http://www.seppic.com)). The final vaccine had a bacterial load of  $1.41 \times 10^8$  CFU per shot (i.e. 10  $\mu$ l). The vaccine was tested for its sterility, stability and safety prior to start of trial.

### 2.2. Experimental animals

For *in vivo* trial, female inbred BALB/c mice were procured from Indian Toxicological Research Centre (ITRC), Lucknow, India. All the mice were maintained as per the good management practices (GMP) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were provided feed and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of U.P. Pt Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya, Mathura, UP, India.

### 2.3. Study design

The three groups of female BALB/c mice, designated as group A (n = 10), B (n = 10) and C (n = 6) were inoculated 10  $\mu$ l of vaccine through intramuscular route with oil adjuvant vaccine (OAV); adjuvant (1:1 diluted oil adjuvant in PBS; pH 7.4) and PBS (pH 7.4), respectively. In all three groups, mice were challenged with live virulent *B. melitensis* biovar 3 IND1 cultures ( $10^9$  CFU) through intraperitoneal route (EP, 5.0) on 28<sup>th</sup> day post vaccination. Blood samples were collected on 0, 7<sup>th</sup>, 14<sup>th</sup>, 28<sup>th</sup> and 35<sup>th</sup> day of initial vaccination from retro-orbital plexus of mice with the help of glass capillary tubes [16]. The mice were sacrificed as per standard procedures [17,18] on 7<sup>th</sup> day of challenge (35<sup>th</sup> day post vaccination). The postmortem was conducted immediately after sacrifice of mice and vital organs viz., lung, liver, spleen and kidney were collected to estimate oxidative stress marker enzymes.

### 2.4. Estimation of oxidative stress parameters

From whole blood, erythrocytes were separated and the resulting erythrocyte pellet was washed thrice with 0.15 M NaCl. The 33% dilution of the packed RBC was made in PBS (pH 7.4) [19] and kept at 4°C till further estimation of lipid peroxidation and glutathione-S-transferase. The 1:10 dilution of packed erythrocytes in PBS (pH 7.4) was used for the estimation of reduced glutathione, catalase, superoxide dismutase and total protein. The

extent of lipid peroxidation was evaluated in terms of malondialdehyde (MDA) production as determined by the method of Rehmaan [20]. Glutathione-S-transferase was estimated as per the method of Habig et al. [21]. GSH was estimated by the 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) method [22]. Similarly, the level of Catalase [23], Superoxide dismutase (SOD) [24] and protein [25] were estimated in erythrocytes. Oxidative stress biomarkers were also estimated in kidney, liver, lung and spleen tissues collected during postmortem on 35<sup>th</sup> day of initial vaccination and 7<sup>th</sup> day of challenge with the method described for erythrocytes except for GSH. GSH was determined by estimating free -SH groups, using DTNB method [26].

### 2.5. Statistical analysis

Various oxidative stress related biochemical indices are expressed as mean  $\pm$  SE and the statistical difference between the groups were analyzed by analysis of variance (ANOVA). The statistical difference showing  $P < 0.05$  were considered as significant [27].

## 3. Results

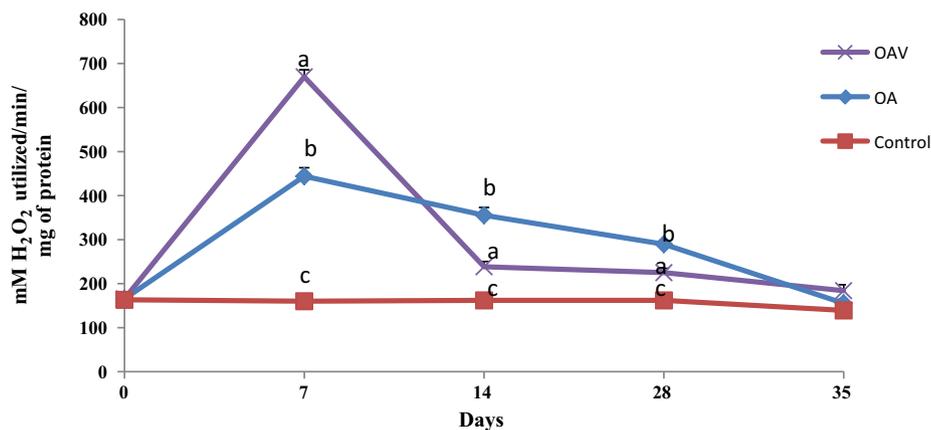
The erythrocytic catalase activity showed significant increase ( $P < 0.05$ ), initially on 7<sup>th</sup> day post vaccination and then decreased significantly ( $P < 0.05$ ) on 14<sup>th</sup> day onward post vaccination in comparison to adjuvant inoculated group (Table 1, Fig. 1a). The SOD activity level in erythrocytes also increased significantly ( $P < 0.05$ ), after vaccination and challenge in vaccinated and adjuvant inoculated mice. The increase was higher in vaccinated mice as compared to adjuvant inoculated mice group where the increase was slow, but persistent (Table 1, Fig. 1b). The GST level showed significant ( $P < 0.05$ ) variation in all the mice on 7<sup>th</sup> day post vaccination and remained unchanged after challenge in both vaccinated and adjuvant inoculated mice (Table 1, Fig. 1c). The LPO levels were increased in vaccinated and adjuvant inoculated animals significantly ( $P < 0.05$ ). The challenge with virulent *B. melitensis* further increased LPO levels in adjuvant inoculated and control mice. The highest increase in LPO ( $P < 0.05$ ) was observed in vaccinated mice from other groups and as expected; the highest level was recorded after challenge in non vaccinated mice, indicating significant induction of oxidative stress resulting in marked cellular damage (Table 1, Fig. 1d). Maximal GSH levels were observed on 7<sup>th</sup> day of adjuvant inoculation while on 14<sup>th</sup> day postvaccination. Thereafter, it was maintained almost at par with the adjuvant group and finally, it declined after the challenge (Table 1, Fig. 1e). During the study, the total protein content of erythrocytes showed a rise after vaccination and challenge with subsequent slight decline (Table 1, Fig. 1f). The rise was highest in vaccinated mice with significant ( $P < 0.05$ ) difference to control group.

The estimation of oxidative stress markers in vaccinated and/or challenged vital organ tissues revealed different patterns in different organs (Table 2, Fig. 2a, Fig. 2b, Fig. 2c, Fig. 2d, Fig. 2e). The catalase activity was non-significantly ( $P < 0.05$ ) increased in lung, liver and kidney tissues in comparison to control group (Table 2, Fig. 2a). It was further reduced significantly ( $P < 0.05$ ) in spleen tissues. The activity of SOD was maintained in all the tissues except in lungs where it was significantly ( $P < 0.05$ ) lower as compared to control animals (Table 2, Fig. 2b). The tissue GST level increased significantly ( $P < 0.05$ ) in kidney and liver but reduced significantly ( $P < 0.05$ ) in spleen tissues (Table 2, Fig. 2c). Little variation was observed in tissue LPO level except in liver. In liver, LPO level increased significantly ( $P < 0.05$ ) postvaccination (Table 2, Fig. 2d). The GSH concentration in tissues of vaccinated group

**Table 1**  
Effect of vaccination and challenge on oxidative stress biomarkers in erythrocytes of inbred BALB/c mice.

| Groups  | Vaccination and challenge schedule |                               |                              |                               |                               |
|---|------------------------------------|-------------------------------|------------------------------|-------------------------------|-------------------------------|
|   | 0 day                              | 7 <sup>th</sup> day           | 14 <sup>th</sup> day         | 28 <sup>th</sup> day          | 35 <sup>th</sup> day          |
| <b>Mice erythrocytes catalase activity (mM H<sub>2</sub>O<sub>2</sub> utilized/min/mg of protein)</b> |                                    |                               |                              |                               |                               |
| OAV   | 165.60 ± 0.27                      | 669.83 <sup>a</sup> ± 15.93   | 238.26 <sup>a</sup> ± 12.06  | 225.02 <sup>a</sup> ± 9.64    | 184.02 ± 13.45                |
| OA  | 165.63 ± 0.93                      | 444.23 <sup>b</sup> ± 19.15   | 355.28 <sup>b</sup> ± 17.73  | 289.47 <sup>b</sup> ± 7.78    | 155.48 ± 9.42                 |
| Control   | 163.31 ± 0.65                      | 160.18 <sup>c</sup> ± 6.28    | 162.25 <sup>c</sup> ± 7.93   | 162.24 <sup>c</sup> ± 1.52    | 139.20 ± 2.74                 |
| <b>Mice erythrocytes superoxide dismutase activity (SOD) activity</b>                                 |                                    |                               |                              |                               |                               |
| OAV   | 0.0054 ± 0.0003                    | 0.0045 <sup>a</sup> ± 0.0002  | 0.0049 <sup>a</sup> ± 0.0001 | 0.0044 <sup>a</sup> ± 0.0003  | 0.0042 <sup>a</sup> ± 0.00009 |
| OA  | 0.0054 ± 0.0002                    | 0.0040 <sup>a</sup> ± 0.00003 | 0.0034 <sup>b</sup> ± 0.0002 | 0.0035 <sup>b</sup> ± 0.00005 | 0.0036 <sup>a</sup> ± 0.0001  |
| Control   | 0.0055 ± 0.0003                    | 0.0054 <sup>b</sup> ± 0.0002  | 0.0060 <sup>c</sup> ± 0.0002 | 0.0060 <sup>c</sup> ± 0.0002  | 0.0070 <sup>b</sup> ± 0.0002  |
| <b>Mice erythrocytes glutathione-S-transferase (GST) (mMCDNB conjugate/min/ mg protein)</b>           |                                    |                               |                              |                               |                               |
| OAV   | 200.40 ± 1.55                      | 225.62 <sup>ab</sup> ± 2.57   | 201.67 ± 3.60                | 201.27 ± 0.99                 | 201.93 <sup>ab</sup> ± 4.94   |
| OA  | 202.24 ± 18.90                     | 240.24 <sup>a</sup> ± 10.05   | 215.37 ± 8.84                | 215.22 ± 9.31                 | 215.63 <sup>a</sup> ± 8.01    |
| Control   | 203.65 ± 4.23                      | 204.85 <sup>b</sup> ± 1.46    | 200.78 ± 3.77                | 200.68 ± 2.57                 | 177.88 <sup>b</sup> ± 4.90    |
| <b>Mice erythrocytes lipid peroxidation (LPO) (nM MDA/g tissue)</b>                                   |                                    |                               |                              |                               |                               |
| OAV   | 63.04 ± 2.64                       | 90.05 <sup>a</sup> ± 6.46     | 112.53 <sup>a</sup> ± 2.06   | 120.98 <sup>a</sup> ± 11.22   | 106.17 <sup>a</sup> ± 4.317   |
| OA  | 63.39 ± 10.11                      | 72.91 <sup>b</sup> ± 12.63    | 76.81 <sup>b</sup> ± 6.23    | 76.94 <sup>b</sup> ± 7.63     | 106.42 <sup>a</sup> ± 16.46   |
| Control   | 50.45 ± 3.50                       | 59.41 <sup>c</sup> ± 4.72     | 56.36 <sup>c</sup> ± 2.29    | 55.60 <sup>c</sup> ± 4.52     | 127.71 <sup>b</sup> ± 6.89    |
| <b>Mice erythrocytes reduced glutathione (GSH) (mM GSH/g tissue)</b>                                  |                                    |                               |                              |                               |                               |
| OAV   | 0.035 ± 0.0013                     | 0.028 <sup>a</sup> ± 0.0005   | 0.081 <sup>a</sup> ± 0.0070  | 0.074 <sup>a</sup> ± 0.0036   | 0.040 <sup>a</sup> ± 0.0015   |
| OA  | 0.042 ± 0.0039                     | 0.119 <sup>b</sup> ± 0.0108   | 0.088 <sup>a</sup> ± 0.0061  | 0.075 <sup>a</sup> ± 0.0025   | 0.044 <sup>a</sup> ± 0.0018   |
| Control   | 0.036 ± 0.0015                     | 0.039 <sup>a</sup> ± 0.0035   | 0.037 <sup>b</sup> ± 0.0031  | 0.043 <sup>b</sup> ± 0.0015   | 0.026 <sup>b</sup> ± 0.0011   |
| <b>Mice erythrocytes total protein content (TP) (mg/ml)</b>   |                                    |                               |                              |                               |                               |
| OAV   | 0.050 ± 0.004                      | 0.059 <sup>a</sup> ± 0.004    | 0.075 <sup>a</sup> ± 0.003   | 0.077 <sup>a</sup> ± 0.000    | 0.071 <sup>a</sup> ± 0.005    |
| OA  | 0.050 ± 0.001                      | 0.052 <sup>b</sup> ± 0.005    | 0.064 <sup>b</sup> ± 0.002   | 0.069 <sup>a</sup> ± 0.005    | 0.061 <sup>b</sup> ± 0.002    |
| Control   | 0.042 ± 0.002                      | 0.047 <sup>b</sup> ± 0.002    | 0.050 <sup>c</sup> ± 0.001   | 0.053 <sup>b</sup> ± 0.002    | 0.062 <sup>b</sup> ± 0.002    |

OAV- oil adjuvant vaccine administered group. OA- oil adjuvant administered group. All the mice were challenged 28<sup>th</sup> day post vaccination and sacrificed 7<sup>th</sup> day after challenge (35<sup>th</sup> day of vaccination). Various oxidative stress related biochemical indices are expressed as mean ± SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript, <sup>a,b,c</sup> differ significantly in each column ( $P < 0.05$ ).



**Fig. 1a.** Catalase activity in erythrocytes of vaccinated and challenged mice. The biochemical indices are expressed as mean ± SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).

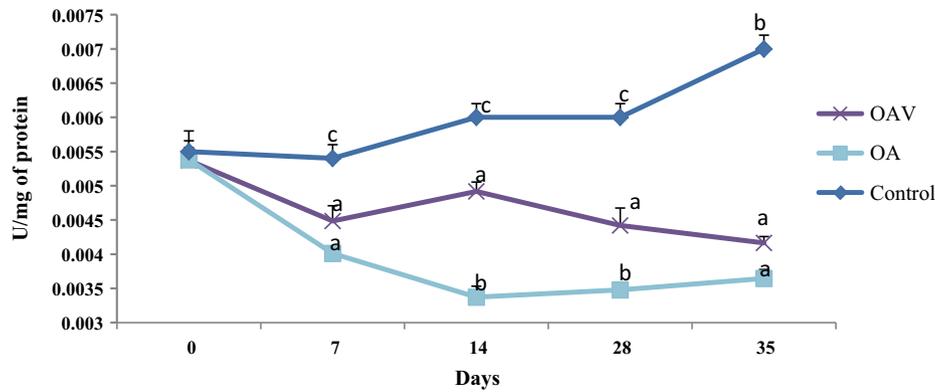
showed variable pattern with significant ( $P < 0.05$ ) increase in kidney, liver, spleen but a significant ( $P < 0.05$ ) reduction in lungs as comparison to control group (Table 2, Fig. 2e). The total protein level showed dual pattern with lower levels in liver and kidney, and significantly ( $P < 0.05$ ) higher content in spleen (Table 2, Fig. 2f).

#### 4. Discussion

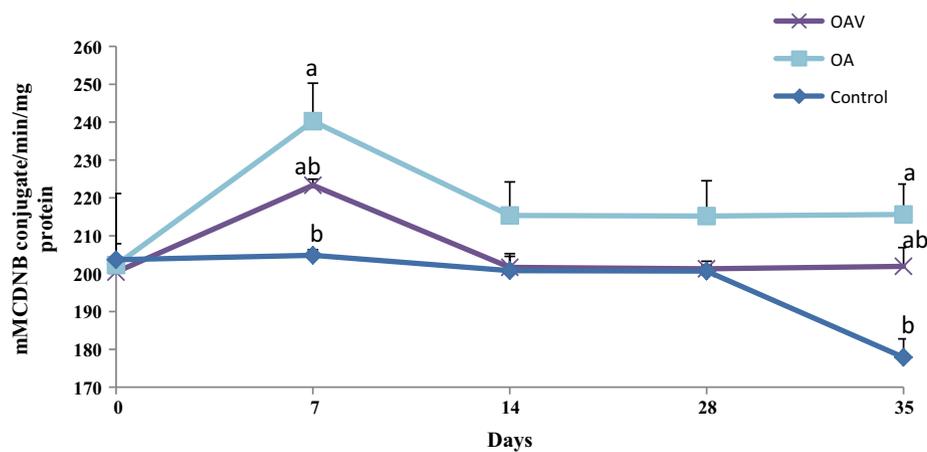
Endurance and replication of brucellae in host phagocytic cells are key mechanism of their survival and virulence [6]. The host immune response target many bacterial stress proteins during initiation of infections [28–31]. It might be because their immunodominance is related to bacterial load within antigen-presenting

cells under conditions of oxidative stress [29,30,32]. Vaccine/adjuvant-induced interplay of antioxidant system (GSH/ROS scavengers) modulate it in favour of achieving the mandate of immunization as vaccination of the host aims at achieving antigen specific protection without altering the body physiological and biochemical processes [33]. The inoculation of vaccine or adjuvant into a living system leads to generation of reactive oxygen radicals (ROS) as a part of the body defence mechanism. The ROS generation can be measured by estimating levels of different biomarkers like catalase, GST, LPO, SOD and GSH in circulating erythrocytes or in body tissues.

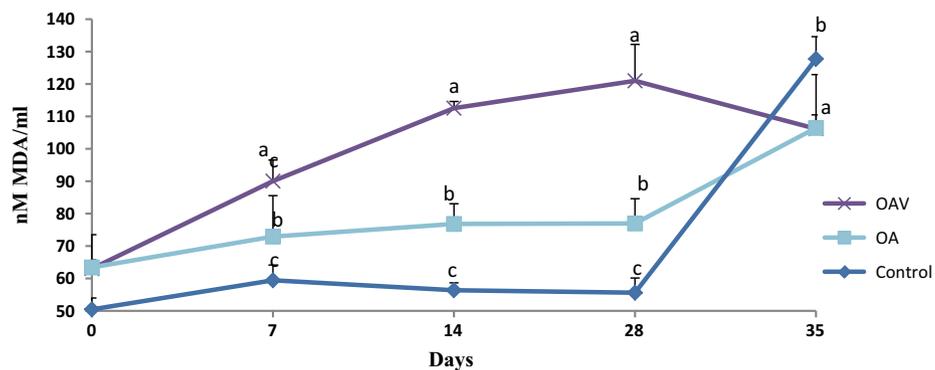
The SOD and catalase are the first line of antioxidant enzymic defense that convert superoxide radicals into hydrogen peroxide and hydrogen peroxide further into water and molecular oxygen [34]. Catalase in association with other enzymatic antioxidants like



**Fig. 1b.** SOD activity in erythrocytes of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript<sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).



**Fig. 1c.** GST activity in erythrocytes of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript<sup>a,b</sup> differ significantly ( $P < 0.05$ ).

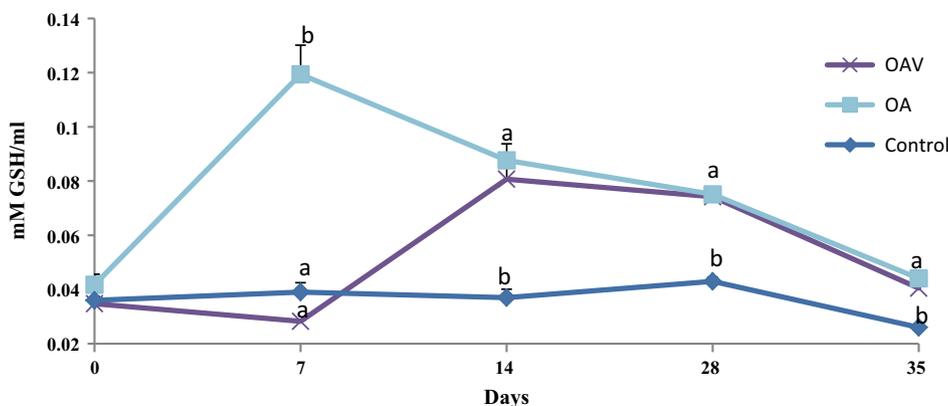


**Fig. 1d.** LPO concentration in erythrocytes of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript<sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).

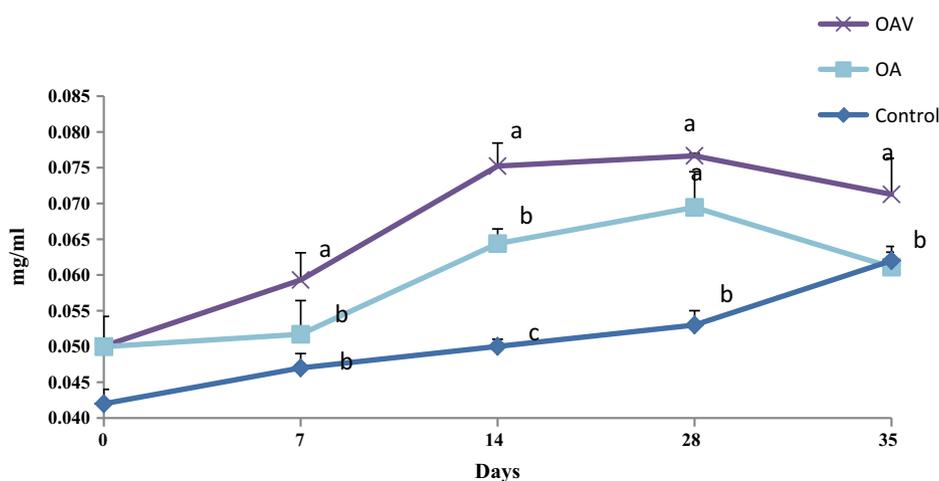
peroxidases and SOD neutralizes or scavenges ROS and with the GSH redox cycle, catalase is the primary cellular enzymatic defense system [35]. The increase in erythrocytic catalase activity on 7<sup>th</sup> to 14<sup>th</sup> day post vaccination and 7<sup>th</sup> day post challenge are suggestive of positive antioxidant mechanism. The catalase activity initially increased and then returned to almost normal levels at 28<sup>th</sup> day post vaccination (Table 1, Fig. 1a), playing in synergism of desired action. Similarly, increased catalase activity in kidney and liver tissues (Table 2, Fig. 2a) also suggests the increased ROS generation in liver and kidney. It might be because of adjuvant which is mainly

metabolized in liver and then metabolites are filtered through kidney creating high levels of free radicals. The increased catalase activity might be associated with the defense mechanism to overcome these free radicals.

SOD is a metalloenzyme that catalyzes the dismutation of superoxide to hydrogen peroxide [36]. These hydrogen peroxide molecules are further metabolized by catalase therefore increased levels of SOD post vaccination might have been further involved with the increased catalase activity during this period to combat free radicals produced by SOD. These increased levels of SOD



**Fig. 1e.** GSH concentration in erythrocytes of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b</sup> differ significantly ( $P < 0.05$ ).



**Fig. 1f.** Total protein concentration in erythrocytes of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).

(Table 1, Fig. 1b) were desirable for eliciting a most potent immune response as reduction in SOD activity rapid the aging process and SOD is supposed to maintain for anti aging process [37]. The tissue SOD levels were slightly elevated in comparison to control (Table 2, Fig. 2b). The observations of present study are suggestive of induction of constructive SOD activity as SOD level is required to moderate a low level of oxidative stress and in severe stress, down regulate expression of SOD [38]. Simultaneous over-expression of SOD is reported to knock down the protective effect of BCG against tuberculosis by altering innate and adaptive immune responses [37].

There was an increase in overall oxidative stress as suggested by post challenge catalase and SOD levels in erythrocytes (Table 1, Fig. 1a, Fig. 1b). This was in harmony with the prospects, as during the process of phagocytosis for antigens, glycolytic reactions are initiated in the host to increase the consumption of oxygen and encourage the production of ROS to kill the bacteria [39,40]. The overexpression of antioxidant enzyme catalase (Table 1, Fig. 1a, Fig. 1b), may be viewed as an adaptive response and protective mechanism [40].

The glutathione-S-transferase (GST) is an enzyme that is involved in antioxidant pathway leading to detoxification. Increase in post vaccination GST activity (Table 1, Fig. 1c) imply that the processing of adjuvant used in study is safe and produced lower level of free radicals and electrophiles as GST catalyze reaction in the detoxification of generated free radicals and electrophiles. Post

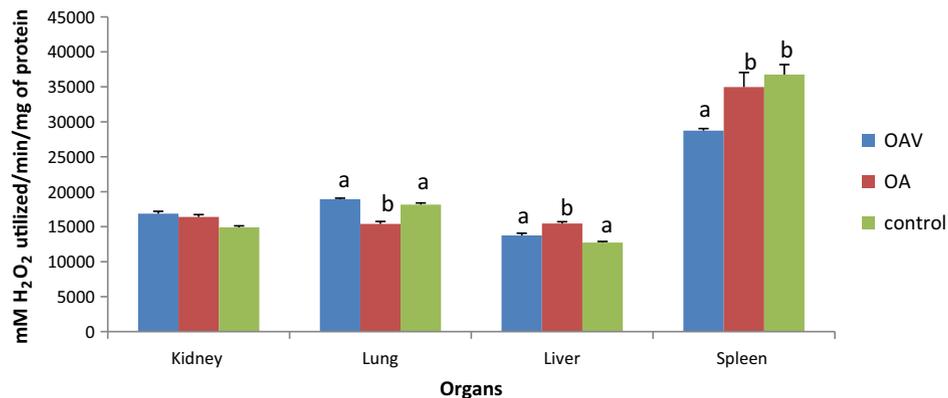
challenge tissue GST levels were increased in kidney and liver (Table 2, Fig. 2c) suggesting detoxification of adjuvant and antigen in kidney and liver, respectively. The decline in 14<sup>th</sup> day post vaccination, erythrocytes and post challenge lung and spleen GST levels (Table 1, Fig. 1c) might be due to inhibition of metabolizing enzymes by the antigen and/or oil adjuvant as earlier experiments also suggested inhibition of metabolizing enzymes due to vaccines and their adjuvants. This inhibition is associated with release of interleukin-1 by non-specific activation of macrophages [41].

The LPO levels measure the cell membrane damage by calculating the concentration of malondialdehyde, a major oxidative by product of peroxidized polyunsaturated fatty acids [33]. The significant ( $P < 0.05$ ) increase in post vaccination erythrocytic LPO levels (Table 1, Fig. 1d) indicated significant rise in oxidative stress resulting in marked cellular damage with subsequent release of ROS [42]. The increase in ROS has been reported earlier also [42] and required to obtain significant level of protection by stimulation of macrophages, APCs and differentiation of major immune cells like T-cell towards a Th2 phenotype [43,44]. Therefore, the marked rise in LPO following vaccination is a desirable one and is in accordance with the targets of the vaccination [45]. The decrease in erythrocytic LPO following the challenge (Table 1, Fig. 1d) assured the competency of the immune system in tackling the virulent bacteria. The liver is first predilection site of *Brucella* infection followed by spleen whereas kidney are the organs most affected during any xenobiotic exposure [5,6]. Further, liver is a metabolically active

**Table 2**  
Effect of vaccination and challenge on oxidative stress biomarkers in BALB/c mice tissue.

| Groups  | Kidney                       | Lung                           | Liver                          | Spleen                          |
|---|------------------------------|--------------------------------|--------------------------------|---------------------------------|
| <b>Mice tissue catalase activity (mM H<sub>2</sub>O<sub>2</sub> utilized/min/mg of protein)</b> |                              |                                |                                |                                 |
| OAV   | 16869.09 ± 335.94            | 18923.90 <sup>a</sup> ± 150.58 | 13751.78 <sup>a</sup> ± 310.08 | 28734.43 <sup>a</sup> ± 287.83  |
| OA  | 16406.31 ± 322.50            | 15403.42 <sup>b</sup> ± 340.48 | 15452.72 <sup>b</sup> ± 266.52 | 34973.50 <sup>b</sup> ± 274.55  |
| Control   | 14920.69 ± 205.49            | 18162.03 <sup>a</sup> ± 228.50 | 12746.71 <sup>a</sup> ± 150.16 | 36752.35 <sup>b</sup> ± 1424.16 |
| <b>Mice tissue superoxide dismutase activity (SOD) activity</b>                                 |                              |                                |                                |                                 |
| OAV   | 0.0446 ± 0.0012              | 0.0353 <sup>a</sup> ± 0.0004   | 0.0689 ± 0.0009                | 0.0296 ± 0.0006                 |
| OA  | 0.0425 ± 0.0008              | 0.0649 <sup>b</sup> ± 0.0006   | 0.0626 ± 0.0013                | 0.0292 ± 0.0017                 |
| Control   | 0.0445 ± 0.0009              | 0.0452 <sup>c</sup> ± 0.0009   | 0.0720 ± 0.0015                | 0.0284 ± 0.0009                 |
| <b>Mice tissue glutathione-S-transferase (GST) (mMCDNB conjugate/min/ mg protein)</b>           |                              |                                |                                |                                 |
| OAV   | 31.12 <sup>a</sup> ± 1.81    | 40.96 <sup>a</sup> ± 0.72      | 65.22 <sup>a</sup> ± 0.96      | 51.74 <sup>a</sup> ± 1.40       |
| OA  | 25.32 <sup>b</sup> ± 2.32    | 109.79 <sup>b</sup> ± 1.22     | 69.98 <sup>a</sup> ± 0.74      | 35.77 <sup>b</sup> ± 2.91       |
| Control   | 20.58 <sup>c</sup> ± 1.13    | 43.32 <sup>a</sup> ± 0.59      | 18.46 <sup>b</sup> ± 0.38      | 102.01 <sup>c</sup> ± 5.11      |
| <b>Mice tissue lipid per oxidation (LPO) (nM MDA/g tissue)</b>                                  |                              |                                |                                |                                 |
| OAV   | 357.97 ± 1.50                | 352.83 ± 1.45                  | 439.15 <sup>a</sup> ± 1.74     | 373.25 ± 1.82                   |
| OA  | 358.16 ± 1.80                | 348.05 ± 2.81                  | 368.92 <sup>b</sup> ± 0.78     | 363.88 ± 2.87                   |
| Control   | 352.42 ± 2.82                | 347.58 ± 1.49                  | 369.37 <sup>b</sup> ± 1.98     | 381.76 ± 1.00                   |
| <b>Mice tissue reduced glutathione (GSH) (mM GSH/g tissue)</b>                                  |                              |                                |                                |                                 |
| OAV   | 0.4714 <sup>a</sup> ± 0.0028 | 0.4202 <sup>a</sup> ± 0.0037   | 0.4909 <sup>a</sup> ± 0.0037   | 0.5252 <sup>a</sup> ± 0.0033    |
| OA  | 0.4942 <sup>a</sup> ± 0.0030 | 0.5385 <sup>b</sup> ± 0.0028   | 0.4390 <sup>b</sup> ± 0.0027   | 0.4316 <sup>b</sup> ± 0.0040    |
| Control   | 0.4061 <sup>b</sup> ± 0.0055 | 0.5927 <sup>c</sup> ± 0.0015   | 0.4266 <sup>b</sup> ± 0.0057   | 0.5000 <sup>a</sup> ± 0.0032    |
| <b>Mice tissue total protein content (TP) (mg/ml)</b>   |                              |                                |                                |                                 |
| OAV   | 0.0687 <sup>a</sup> ± 0.0013 | 0.0616 <sup>a</sup> ± 0.0005   | 0.0779 <sup>a</sup> ± 0.0011   | 0.0343 ± 0.0004                 |
| OA  | 0.0881 <sup>b</sup> ± 0.0015 | 0.0688 <sup>b</sup> ± 0.0008   | 0.0623 <sup>b</sup> ± 0.0014   | 0.0325 ± 0.0020                 |
| Control   | 0.0762 <sup>c</sup> ± 0.0010 | 0.0625 <sup>a</sup> ± 0.0006   | 0.0842 <sup>c</sup> ± 0.0016   | 0.0312 ± 0.0013                 |

OAV- oil adjuvant vaccine administered group. OA- oil adjuvant administered group. All the mice were challenged 28<sup>th</sup> day post vaccination and sacrificed 7<sup>th</sup> day after challenge (35<sup>th</sup> day of vaccination). Various oxidative stress related biochemical indices are expressed as mean ± SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b,c</sup> differ significantly in each column ( $P < 0.05$ ).

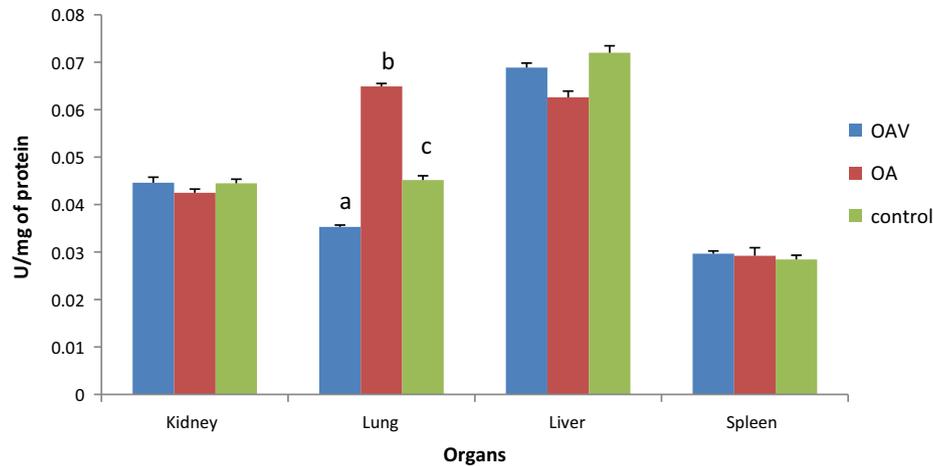


**Fig. 2a.** Catalase activity in tissues of vaccinated and challenged mice. The biochemical indices are expressed as mean ± SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b</sup> differ significantly ( $P < 0.05$ ).

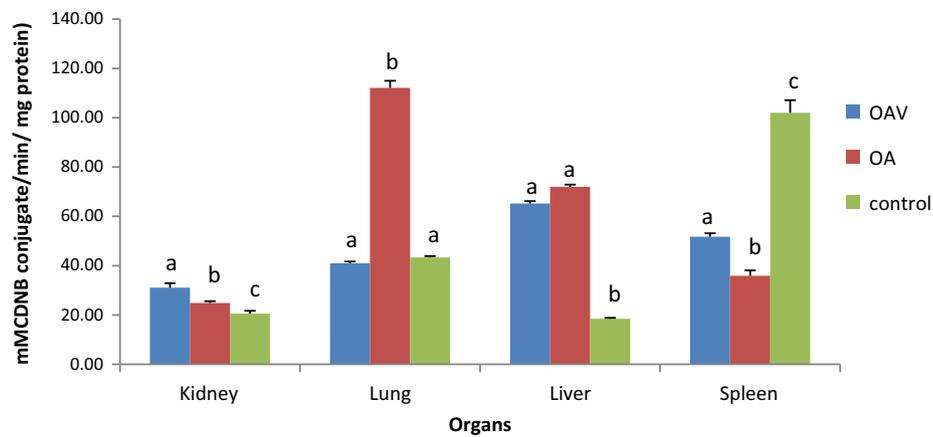
organ to metabolize adjuvant with a powerful antioxidant potential. Therefore, higher MDA levels were as expected (Table 2, Fig. 2d) in the liver and also have been reported previously [45,46]. Similarly, higher expression levels were detected for genes coding for proteins involved in redox homeostasis and protection against ROS in vaccinated fish [47]. No post challenge increase in spleen LPO level are suggestive of adequate protective response of vaccination leading to neutralization of the free radicals produced by virulent Brucella organisms.

Glutathione (GSH) is an intracellular low-molecular-weight antioxidant, that has a thiol moiety to react with pro-oxidant species [48]. Non-significant differences in the erythrocytic GSH levels during initial period of study and even after challenge with significant ( $P < 0.05$ ) difference after 7<sup>th</sup> day post vaccination (Table 1, Fig. 1e) are indicative of the excellent management of the antigen and the adjuvant by the host immune system without imposing any threat to the antioxidant defence homeostasis. The initial fall

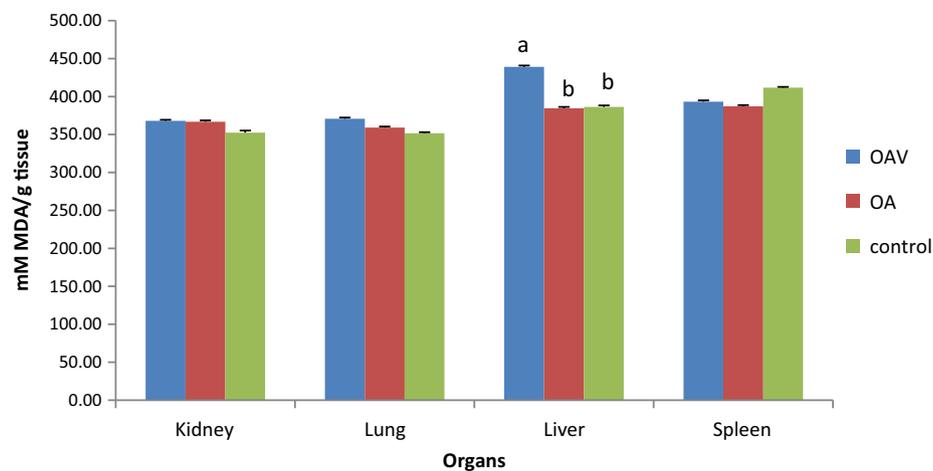
in GSH levels might be due to its participation in the redox cycle to neutralize the excess ROS generated. However, restoration of GSH levels post vaccination was highly desirable for T cell activation, differentiation and to maintain the active immune response [49]. Similar to erythrocytes, GSH levels were maintained in kidneys but reduced in lungs (Table 2, Fig. 2e). The liver and spleen are main predilection sites for brucella. Accordingly, both the organs showed increased GSH levels (Table 2, Fig. 2e). However, the spleen and liver GSH levels were comparable to control and adjuvant treated groups, respectively. These findings are indicative of efficient activation of lymphocytes to produce desirable immune response as expression of thiols on cell surface of T cells [50] and release of cysteine into the extracellular space by dendritic cells [51] are required to create a reducing environment to facilitate immune response. Thus, increased GSH levels are considered as an integral part of active immunization to induce T<sub>regs</sub> mediated immunosuppressive effect [52]. Liver, being main site of detoxifi-



**Fig. 2b.** SOD activity in tissues of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).



**Fig. 2c.** GST activity in tissues of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).

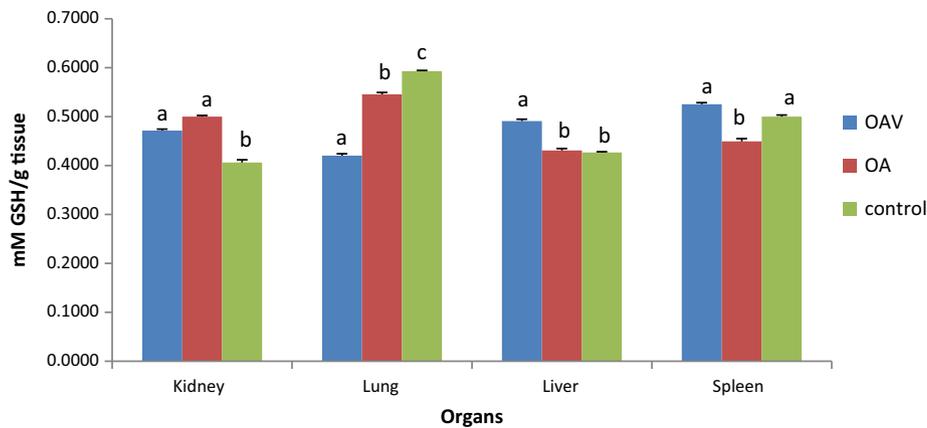


**Fig. 2d.** LPO concentration in tissues of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b</sup> differ significantly ( $P < 0.05$ ).

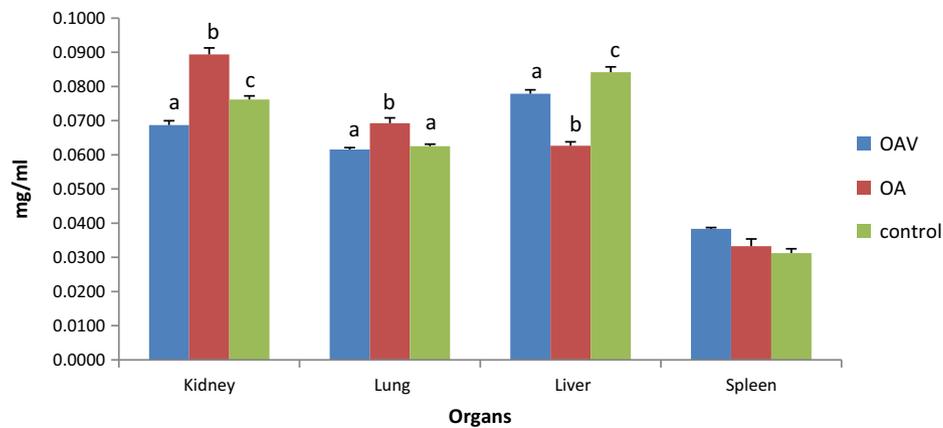
cation, higher level of GSH in liver may be attributed to effective management of xenobiotics to reduce free radicals. Similar to the findings of present study, importance of the GSH-mediated antioxidant defense system has earlier been demonstrated in adrenocor-

tical cells of rainbow trout against endosulfan-induced oxidative stress [35].

ROS are essential for the optimal functioning of the body tissues [53]. Induction of oxidative stress usually precipitates NF- $\kappa$ B medi-



**Fig. 2e.** GSH concentration in tissues of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).



**Fig. 2f.** Total protein concentration in tissues of vaccinated and challenged mice. The biochemical indices are expressed as mean  $\pm$  SE of six inbred BALB/C mice in each group. The statistical difference between the groups was analyzed by analysis of variance (ANOVA). Mean with different superscript <sup>a,b,c</sup> differ significantly ( $P < 0.05$ ).

ated induction of mRNA species of SOD, catalase, and glutathione-S-transferase activities. These antioxidant enzymes convert superoxide anion ( $O_2^-$ ) to  $H_2O_2$  in the presence of SOD and prevent formation of highly pernicious hydroxyl radicals [38]. The inoculation of vaccine/ adjuvants initiated complex interactions at the interface of immunology, physiology, and anatomy *in vivo*. The generation of free reactive radicals resulting in inflammatory sequelae was much below the level required to predispose/ induce the setup of disease in the system. The complex of antigen and adjuvant elicited an excellent constructive redox potential that is required to stimulate the B and T lymphocytes and ultimately to induce the desired immune response.

## 5. Conclusion

From the present study, it is clear that both the glutathione mediated nonenzymic and endogenous catalase and SOD antioxidant defense system play a critical role in maintenance of intracellular antioxidant defense in vaccinated animals along with maintaining an excellent milieu for inducing an active immune potential. At the same time, the antioxidant defenses maintained excellent redox homeostasis in all the vital tissues viz. erythrocytes, liver, kidneys, lungs and spleen of vaccinated mice. The liver and lungs being metabolically active, kidney being the main excretory organ and spleen being a lymphoid organ played a vital role in maintaining the immunological homeostasis in the body. Thus, it

may be said that immunization with the oil adjuvant *Brucella* vaccine OAV induces a constructive oxidative stress with negligible signs of inflammatory pathophysiology in all the vital organs.

## Conflict of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.04.060>.

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