



## Mapping of oxidative stress in immune response induced by polymer gel based *Brucella melitensis* vaccine in mice

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### ABSTRACT

*Brucella melitensis* is an obligate intracellular parasite causing worldwide zoonoses. It has been implicated that oxidative stress induced due to bacterial invasion lead to the niche of *Brucella* in organs like spleen and lymph nodes. The present study was aimed at evaluating the erythrocytic and tissue specific oxidative stress responses induced by intranasal inoculation of killed *Brucella* whole cell protein antigen in conjunction with a polymer gel adjuvant for offering protection against live virulent *Brucella*. The results clearly implicate that both the glutathione mediated nonenzymic antioxidant defense system and catalase play a critical role in 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 in all the vital tissues, viz. liver, kidney, lung and spleen of vaccinated mice were almost at par with the adjuvant inoculated animals. Thus, it may be said that intranasal immunization with polymer gel based *Brucella* vaccine induces an oxidative stress with negligible signs of inflammatory pathophysiology in all the vital organs and supports the development of significant level of immunogenicity against *Brucella* challenge.

**Key words:** *Brucella melitensis*, Immune response, Oxidative stress, Polymer gel, Vaccine

Brucellosis is a common global zoonotic infection caused in small ruminants by a bacterium, *Brucella melitensis* (Kumar *et al.* 2016). Once introduced inside a mammalian host, the bacteria mainly reside within a vacuole in phagocytic cells (Roop *et al.* 2004). The presence of bacteria in the phagocytes induce a clinical or subclinical disease depending on its ability to survive and to multiply by inhibition of blending of phagocytic vacuoles with lysosomal contents and resistance to oxidative killing (Kumar *et al.* 2017). Depending upon the pathogenicity of the bacterial strain and the host factors (Rahal *et al.* 2014), localised/systemic brucellosis share variable clinical manifestations including inflammatory symptoms (Kumar *et al.* 2016) leading to triggering of interleukin (IL)-8, tumor necrosis factor (TNF)-alpha and IL-6. These chemical mediators induce an increased level of oxidative stress and inflammation in the tissues leading to cellular damage along

with perpetuating the desirable immune response (Celli *et al.* 2003). Therefore, reducing the inflammatory response and the oxidative stress, may be an important intercession to thwart the pathological consequences of *Brucella* vaccinations (Baldi and Giambartolomei 2013). Based on this concept and to improve benefit-risk ratio, antigenic molecules of *Brucella* are being coupled with carrier molecules (Minas 2006) like biodegradable hydrophobic polymers as an ideal adjuvant for intranasal delivery of vaccines (Kumar *et al.* 2013). Thus, the present study was aimed at evaluating the erythrocytic and tissue specific oxidative stress response caused by killed *Brucella* whole cell protein antigen unified with a polymer gel adjuvant for offering protection against live virulent *Brucella*.

### MATERIALS AND METHODS

**Vaccine:** The formalized killed polymer gel based *B. melitensis* biovar 3 IND1 vaccine (PGV) was prepared with the addition of polymer gel (Montanide<sup>TM</sup> Gel 1) in the ratio of 1:10 (V/V) to formalized killed virulent *Brucella melitensis* biovar 3 bacterial strain (Accession no. VTCCBAA228). The final vaccine had the 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.

**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

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

Group	Vaccination, booster 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	56 <sup>th</sup> day
	<i>Mice erythrocytes catalase activity (mM H<sub>2</sub>O<sub>2</sub> utilized/min/mg of protein)</i>					
NPGV	114.59±00.69	140.51 <sup>a</sup> ±01.05	323.60 <sup>a</sup> ±08.27	280.97 <sup>a</sup> ±03.43	255.48 <sup>a</sup> ±14.15	-
NPGVB	114.74±00.22	140.49 <sup>a</sup> ±01.64	323.96 <sup>a</sup> ±03.91	202.55 <sup>b</sup> ±09.02	418.66 <sup>b</sup> ±11.76	639.90 <sup>a</sup> ±23.48
NPG	114.54±00.32	371.57 <sup>b</sup> ±05.45	376.63 <sup>b</sup> ±13.85	406.29 <sup>c</sup> ±05.25	388.01 <sup>b</sup> ±11.28	-
NPGB	114.45±00.47	371.79 <sup>b</sup> ±12.08	376.84 <sup>b</sup> ±14.02	263.06 <sup>b</sup> ±08.57	158.62 <sup>c</sup> ±11.07	153.63 <sup>b</sup> ±03.53
Control	163.31±0.65	160.18 <sup>a</sup> ±6.28	162.25 <sup>c</sup> ±07.93	162.24 <sup>b</sup> ±01.52	139.20 <sup>c</sup> ±02.74	-
CB	163.31±0.65	160.18 <sup>a</sup> ±6.28	162.25 <sup>c</sup> ±07.93	162.24 <sup>b</sup> ±01.52	161.16 <sup>c</sup> ±03.51	162.97 <sup>b</sup> ±05.03
	<i>Mice erythrocytes superoxide dismutase activity (SOD) activity</i>					
NPGV	0.0064±0.0002	0.0057 <sup>a</sup> ±0.0002	0.0038 <sup>a</sup> ±0.0002	0.0042 <sup>a</sup> ±0.0002	0.0033 <sup>a</sup> ±0.0001	-
NPGVB	0.0064±0.0001	0.0057 <sup>a</sup> ±0.0002	0.0039 <sup>a</sup> ±0.0001	0.0036 <sup>b</sup> ±0.0003	0.0043 <sup>b</sup> ±0.0002	0.0050 <sup>a</sup> ±0.0001
NPG	0.0065±0.0001	0.0044 <sup>b</sup> ±0.0002	0.0044 <sup>a</sup> ±0.0003	0.0045 <sup>a</sup> ±0.0003	0.0046 <sup>b</sup> ±0.0001	-
NPGB	0.0065±0.0003	0.0044 <sup>b</sup> ±0.0002	0.0044 <sup>a</sup> ±0.0002	0.0041 <sup>a</sup> ±0.0001	0.0056 <sup>c</sup> ±0.0002	0.0047 <sup>a</sup> ±0.0004
Control	0.0055±0.0003	0.0054 <sup>a</sup> ±0.0002	0.0060 <sup>b</sup> ±0.0002	0.0060 <sup>c</sup> ±0.0002	0.0070 <sup>d</sup> ±0.0002	-
CB	0.0055±0.0003	0.0054 <sup>a</sup> ±0.0002	0.0060 <sup>b</sup> ±0.0002	0.0060 <sup>c</sup> ±0.0002	0.0059 <sup>c</sup> ±0.0002	0.0057 <sup>b</sup> ±0.0003
	<i>Mice erythrocytes glutathione-S-transferase (GST) (mM CDNB conjugate/min/mg protein)</i>					
NPGV	275.13±18.31	192.31 <sup>a</sup> ±07.87	287.96 <sup>a</sup> ±12.90	293.31 <sup>a</sup> ±13.92	206.89 <sup>a</sup> ±21.02	-
NPGVB	277.32±17.18	193.09 <sup>a</sup> ±16.34	290.87 <sup>a</sup> ±23.10	202.61 <sup>b</sup> ±08.04	248.90 <sup>b</sup> ±12.96	194.47±17.90
NPG	274.70±09.26	322.08 <sup>b</sup> ±07.35	315.83 <sup>b</sup> ±02.42	182.83 <sup>c</sup> ±04.64	178.00 <sup>c</sup> ±14.10	-
NPGB	275.59±12.40	322.07 <sup>b</sup> ±11.34	315.13 <sup>b</sup> ±04.23	219.44 <sup>d</sup> ±11.61	275.88 <sup>d</sup> ±14.05	186.86±07.57
Control	203.65±04.23	204.85 <sup>a</sup> ±01.46	200.78 <sup>c</sup> ±03.77	200.68 <sup>b</sup> ±02.57	177.88 <sup>c</sup> ±04.90	-
CB	203.65±04.23	204.85 <sup>a</sup> ±01.46	200.78 <sup>c</sup> ±03.77	200.68 <sup>b</sup> ±02.57	205.63 <sup>a</sup> ±03.21	202.63±03.27
	<i>Mice erythrocytes lipid peroxidation (LPO) (nM MDA/g tissue)</i>					
NPGV	83.41±05.29	90.73 <sup>a</sup> ±07.64	95.04 <sup>a</sup> ±11.63	93.68 <sup>a</sup> ±09.45	105.99 <sup>a</sup> ±15.02	-
NPGVB	82.07±03.83	91.79 <sup>a</sup> ±13.94	90.78 <sup>a</sup> ±00.81	107.75 <sup>a</sup> ±03.28	127.71 <sup>b</sup> ±09.22	131.23 <sup>a</sup> ±07.45
NPG	84.52±05.95	89.23 <sup>a</sup> ±11.35	100.17 <sup>a</sup> ±07.05	120.15 <sup>a</sup> ±13.46	129.03 <sup>b</sup> ±06.59	-
NPGB	86.51±05.02	90.54 <sup>a</sup> ±04.40	97.66 <sup>a</sup> ±05.72	121.82 <sup>a</sup> ±09.64	110.23 <sup>a</sup> ±04.42	98.03 <sup>b</sup> ±05.511
Control	50.45±03.50	59.41 <sup>b</sup> ±04.72	56.36 <sup>b</sup> ±02.29	55.60 <sup>b</sup> ±04.52	127.71 <sup>b</sup> ±06.89	-
CB	50.45±03.50	59.41 <sup>b</sup> ±04.72	56.36 <sup>b</sup> ±02.29	55.60 <sup>b</sup> ±04.52	58.36 <sup>c</sup> ±03.95	59.36 <sup>c</sup> ±7.09
	<i>Mice erythrocytes reduced glutathione (GSH) (mM GSH/g tissue)</i>					
NPGV	0.0551±0.0023	0.041 <sup>a</sup> ±0.0003	0.124 <sup>a</sup> ±0.0138	0.101 <sup>a</sup> ±0.0009	0.042 <sup>a</sup> ±0.0020	-

NPGVB	0.0538±0.0013	0.041 <sup>a</sup> ±0.0009	0.137 <sup>a</sup> ±0.0087	0.101 <sup>a</sup> ±0.0010	0.088 <sup>b</sup> ±0.0025	0.041 <sup>a</sup> ±0.0019	0.031 <sup>a</sup> ±0.0029
NPG	0.0552±0.0025	0.135 <sup>b</sup> ±0.0041	0.131 <sup>a</sup> ±0.0064	0.099 <sup>a</sup> ±0.0020	0.079 <sup>b</sup> ±0.0032	-	0.110 <sup>b</sup> ±0.0003
NPGB	0.0551±0.0015	0.129 <sup>b</sup> ±0.0071	0.128 <sup>a</sup> ±0.0075	0.180 <sup>b</sup> ±0.0088	0.173 <sup>c</sup> ±0.0079	0.173 <sup>b</sup> ±0.0072	0.110 <sup>b</sup> ±0.0003
Control	0.036±0.0015	0.039 <sup>a</sup> ±0.0035	0.037 <sup>b</sup> ±0.0031	0.043 <sup>c</sup> ±0.0015	0.026 <sup>a</sup> ±0.0011	-	0.028 <sup>b</sup> ±0.0021
CB	0.036±0.0015	0.039 <sup>a</sup> ±0.0035	0.037 <sup>b</sup> ±0.0031	0.043 <sup>c</sup> ±0.0015	0.038 <sup>a</sup> ±0.0029	0.041 <sup>a</sup> ±0.0015	0.028 <sup>b</sup> ±0.0021
<i>Mice erythrocytes total protein content (TP) (ng/ml)</i>							
NPGV	0.057±0.004	0.071 <sup>a</sup> ±0.007	0.072 <sup>a</sup> ±0.003	0.071 <sup>a</sup> ±0.003	0.056 <sup>a</sup> ±0.008	-	0.065 <sup>a</sup> ±0.001
NPGVB	0.056±0.004	0.071 <sup>a</sup> ±0.004	0.072 <sup>a</sup> ±0.002	0.080 <sup>b</sup> ±0.005	0.082 <sup>b</sup> ±0.005	0.075 <sup>a</sup> ±0.010	0.065 <sup>a</sup> ±0.001
NPG	0.057±0.007	0.055 <sup>b</sup> ±0.005	0.056 <sup>b</sup> ±0.001	0.070 <sup>a</sup> ±0.001	0.065 <sup>c</sup> ±0.003	-	0.048 <sup>b</sup> ±0.002
NPGB	0.057±0.002	0.055 <sup>b</sup> ±0.003	0.055 <sup>b</sup> ±0.001	0.065 <sup>a</sup> ±0.001	0.070 <sup>c</sup> ±0.006	0.058 <sup>b</sup> ±0.005	0.048 <sup>b</sup> ±0.002
Control	0.042±0.002	0.047 <sup>c</sup> ±0.002	0.050 <sup>b</sup> ±0.001	0.053 <sup>c</sup> ±0.002	0.062 <sup>c</sup> ±0.002	-	0.063 <sup>a</sup> ±0.004
CB	0.042±0.002	0.047 <sup>c</sup> ±0.002	0.050 <sup>b</sup> ±0.001	0.053 <sup>c</sup> ±0.002	0.049 <sup>a</sup> ±0.008	0.051 <sup>b</sup> ±0.003	0.063 <sup>a</sup> ±0.004

NPGV, Intra-nasally vaccinated with polymer gel vaccine; NPG, Intra-nasally administered with polymer gel; NPGVB, Intra-nasally vaccinated with booster dose of polymer gel vaccine; NPGB, Intra-nasally administered with booster dose of polymer gel; Control, Intra-nasally administered with PBS; CB, Intra-nasally administered with booster dose of PBS. All the mice were challenged 28<sup>th</sup> day post vaccination and booster vaccination. Booster was given 21<sup>st</sup> day of initial vaccination and mice were sacrificed 7<sup>th</sup> day after challenge (35<sup>th</sup> day of initial vaccination and booster vaccination). Data are presented as mean±SE in each group of five inbred BALB/c mice. Mean with different superscript in each column differ significantly (P<0.05).

maintained as per the good management practices (GMP) of CPCSEA. 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.

*Study design:* The three groups of female BALB/c mice, designated as group A (10), B (10) and C (6) were inoculated 10 µl of vaccine through nasal routes designated as nasal polymer gel vaccine (NPGV); adjuvant (1:10 diluted polymer gel in PBS; pH 7.4) (PG) and PBS (pH 7.4) (Control), respectively. In all three groups, 50% of mice were challenged with live virulent *B. melitensis* biovar 3 IND1 cultures (10<sup>9</sup> CFU) through I/P route (EP, 5.0) on 28<sup>th</sup> day post vaccination. The remaining half of the mice in each group received booster dose of vaccine, adjuvant and PBS (pH 7.4) (dose and routes as described previously) on 21<sup>st</sup> day of initial vaccination and were, later on challenged on 28<sup>th</sup> day of booster vaccination i.e. 49<sup>th</sup> day of initial 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 and post booster vaccination (28<sup>th</sup>, 35<sup>th</sup>, 49<sup>th</sup> and 56<sup>th</sup> day of first vaccination) from retro-orbital plexus of mice with the help of glass capillary tubes (Sorg and Buckner 1964).

The mice were sacrificed as per the standard guidelines (OIE 2010) on 7<sup>th</sup> day of challenge (35<sup>th</sup> day post vaccination and 56<sup>th</sup> day for booster 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.

*Estimation of oxidative stress parameters:* The extent of lipid peroxidation (LPO) was evaluated in terms of malondialdehyde (MDA) production (Rehman 1984). Glutathione-S-transferase (GST) was estimated as described previously (Habig *et al.* 1974). Reduced glutathione (GSH) was estimated by the 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) method (Prins and Loos 1969). The method of Bergmeyer (1983) was used for the estimation of catalase in erythrocyte and superoxide dismutase (SOD) was estimated as per the method described by Madesh and Balasubramanian (1998). The method of Lowry *et al.* (1951) was used for protein estimation. Oxidative stress biomarkers in kidney, liver, lung and spleen tissues were estimated as described for erythrocytes except for GSH. GSH was determined by estimating free SH groups, using DTNB method (Sedlak and Lindsay 1968).

*Statistical analysis:* Various parameters, oxidative stress related biochemical indices and serum enzymes were expressed as mean±SE (Snedecor and Cochran 1989).

## RESULTS AND DISCUSSION

Administration of vaccine in the respiratory tract led to the exposure of the nasal mucosa to the *Brucella* whole cell antigen as well as polymer gel adjuvant molecule, which is likely to induce a protective immune reflex in the MALTs. During the reflex process, the rise in ROS level as well as the translation of defensive enzymes is a normal phenomenon. The excessive ROS may also be associated

with the local tissue damage which may further contribute to the rise in total protein levels (Table 1). With regard to the immune system, high levels of ROS, as suggested by the high LPO levels, can be advantageous; neutrophils generate ROS and play an essential role in the TLR4-dependent innate clearance of bacteria (Thoren *et al.* 2007).

The cell membrane damage is measured as lipid peroxidation, which in turn is calculated as the rise in malondialdehyde concentrations, a major oxidative product of peroxidized polyunsaturated fatty acids. The increase in LPO levels (Table 1) by vaccine, adjuvant, and challenge with virulent *B. melitensis* indicated significant induction of oxidative stress resulting in marked cellular damage with subsequent release of ROS. Further, prolonged and persistent oxidative stress leads to histoarchitectural damage in the form of carbonylation of protein, lipid peroxidation and oxidation of DNA as reported earlier in ewes (Al-Khafaji and Al-Farwachi 2012). However, the booster doses of vaccine showed a further rise in LPO while booster inoculation of adjuvant showed decreasing pattern 7th day onward (Table 1). With regard to the immune system, high levels of ROS, as suggested by the high LPO levels, can be advantageous; neutrophils generate ROS and play an essential role in the TLR4-dependent innate clearance of bacteria (Thoren *et al.* 2007). Therefore, the marked rise in LPO following booster vaccination is a desirable one and in accordance with the targets of the vaccination. The rise in LPO following the challenge (Table 1) assures the competency of the immune system in tackling the virulent bacteria. Thus, the overall stimulation of LPO seems to be within the manageable range of the animal body with no visible signs of disease.

Glutathione (GSH) is a tri-peptide nonenzymic antioxidant that is employed to neutralize any excess ROS by the body. It also serves as a critical cofactor for glutathione-dependent antioxidant enzymes (Pacitti *et al.* 2013). GSH levels after vaccination, booster vaccination and challenge in erythrocytes are given in Table 1. 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 and differentiation, and to maintain the active immune response (Pacitti *et al.* 2013). Further 2–3-fold increase in GSH values after vaccination was lower than the increase after adjuvant inoculation was expected as adjuvant lacked the antigenic component and therefore, better host defence response was generated. The post challenge sharp decline in GSH level in all groups (Table 1) was in accordance with the sharp rise in LPO and total protein following the challenge. Several studies implicate the role for GSH in T-cell functioning as thiols play an important role in T-cell function. Depletion of GSH in human and murine T cells have been correlated to a direct affiliation between iGSH levels and T-cell proliferation (Suthanthiran *et al.* 1990). It is now recognized that changes in intracellular levels of ROS or altered redox state, as well as levels at the interface

between APC and T cells at the cell surface, can impact on T-cell activation, proliferation and differentiation, thereby modulating their function (Yan *et al.* 2010). Therefore, it may be interpreted from the present findings that the sharp rise in the GSH levels are indicative of excellent T cell and dendritic cell activation which are a prerequisite for development of acquired immunity.

The first line of antioxidant enzymic defense consists of the antioxidant enzymes SOD and CAT, which converts superoxide radicals into hydrogen peroxide and then into water and molecular oxygen (Dorval and Hontela 2003). The induction of catalase activity was higher with adjuvant in comparison to vaccine after the initial dose (Table 1), perhaps due to the presence of antigenic component in the vaccine, some ROS signaling must have been utilized in producing a hyper inflammatory state, in an effort to remove the antigen. After the booster dose, the catalase activity was higher in vaccinated animals, the animals being already sensitized to the antigen, not much effort were required to take care of it. The CAT activity showed a decline after the challenge in all the groups with an exception of booster adjuvant; may be due to sudden boost up in the inflammatory cascade in the cellular milieu. Reduction in SOD (Table 1) implies comparatively reduced levels of peroxides, as was desired by the body for effective immune development so catalytic activity of catalase might be responsible for H<sub>2</sub>O<sub>2</sub> generation to maintain an optimum level. Moreover, SOD plays a role in moderating a low level of oxidative stress. Once the body shows severe stress, down regulation of expression of SOD is an observable fact, whereas CAT becomes more important in protecting against severe oxidant stress. The down-regulation of expression of SOD and SOD mRNA might have also been accelerated by the H<sub>2</sub>O<sub>2</sub> upsurge post-challenge (Lu and Daret 2009). According to the oxidative stress theory of aging, the loss of SOD activity results in increased sensitivity to oxidative stress, which is desired for eliciting a most potent immune response. Over-expression of SOD has already been reported to demolish the protective effect of BCG against tuberculosis by modulating innate and adaptive immune responses (Jain *et al.* 2011).

There was an increase in overall oxidative stress following challenge (Table 1). This was in accordance with the prospects, as during the course of phagocytosis for antigens, glycolytic reactions are activated in the host which in turn increase the consumption of oxygen and induce the production of ROS to kill the bacterial microbes (Lambeth 2004). A study of *Brucella abortus* had earlier shown a direct relationship between catalase activity and virulence of the strain. Exogenous catalase or SOD has also been found to protect *Brucella* organisms from phagocyte killing *in vitro* (Jiang and Baldwin 1993). The over expression of antioxidant enzyme catalase (Table 1), may be viewed as an adaptive response and protective mechanism against a vaccine or polymer gel-induced oxidative stress.

The glutathione-S-transferase (GST) is an enzyme that is involved in detoxification as well as antioxidant pathway.

Table 2. Effect of vaccination, booster vaccination and *Brucella melitensis* bv 3 IND1 challenge on oxidative stress biomarkers in kidney, lung, liver and spleen of BALB/c mice

Group	Kidney	Lung	Liver	Spleen
<i>Mice tissue catalase activity (mM H<sub>2</sub>O<sub>2</sub> utilized/min/mg of protein)</i>				
NPGV	16018.62 <sup>a</sup> ±172.66	14714.15 <sup>a</sup> ±174.32	16758.55 <sup>a</sup> ±515.64	28059.07 <sup>a</sup> ±1084.13
NPGVB	16482.71 <sup>a</sup> ±062.67	14412.75 <sup>a</sup> ±216.26	13159.63 <sup>b</sup> ±434.10	34611.86 <sup>b</sup> ±1973.27
NPG	16303.92 <sup>a</sup> ±435.59	16365.51 <sup>b</sup> ±429.57	17227.18 <sup>a</sup> ±413.12	31749.33 <sup>±</sup> 1508.71
NPGB	17329.59 <sup>b</sup> ±332.66	19278.48 <sup>±</sup> 307.68	12192.02 <sup>b</sup> ±117.28	35419.24 <sup>b</sup> ±1621.34
Control	14920.69 <sup>±</sup> 205.41	18162.03 <sup>±</sup> 228.40	12746.71 <sup>b</sup> ±150.09	36752.35 <sup>b</sup> ±1423.58
<i>Mice tissue glutathione-S-transferase (GST) (mM CDNB conjugate/min/mg protein)</i>				
NPGV	27.92 <sup>a</sup> ±1.96	31.08 <sup>a</sup> ±1.11	50.58 <sup>a</sup> ±0.86	40.37 <sup>a</sup> ±2.16
NPGVB	47.87 <sup>b</sup> ±1.99	45.67 <sup>b</sup> ±0.55	19.40 <sup>b</sup> ±0.77	50.57 <sup>a</sup> ±2.33
NPG	34.55 <sup>c</sup> ±3.57	38.50 <sup>c</sup> ±1.27	62.85 <sup>c</sup> ±1.56	24.87 <sup>b</sup> ±1.82
NPGB	33.21 <sup>c</sup> ±1.45	51.06 <sup>±</sup> 1.09	13.23 <sup>b</sup> ±0.50	65.29 <sup>a</sup> ±1.78
control	20.58 <sup>d</sup> ±1.13	43.32 <sup>b</sup> ±0.59	18.46 <sup>b</sup> ±0.38	102.01 <sup>c</sup> ±5.11
<i>Mice tissue lipid peroxidation (LPO) (nM MDA/g tissue)</i>				
NPGV	373.05 <sup>a</sup> ±3.02	349.36 <sup>a</sup> ±0.78	407.00 <sup>a</sup> ±2.05	370.93 <sup>a</sup> ±1.78
NPGVB	307.58 <sup>b</sup> ±2.99	404.46 <sup>b</sup> ±1.51	385.42 <sup>b</sup> ±1.32	445.82 <sup>b</sup> ±1.10
NPG	411.97 <sup>c</sup> ±1.91	343.01 <sup>a</sup> ±1.32	404.67 <sup>a</sup> ±1.52	408.80 <sup>c</sup> ±1.00
NPGB	317.10 <sup>b</sup> ±1.37	395.68 <sup>b</sup> ±3.48	352.85 <sup>c</sup> ±1.67	401.82 <sup>c</sup> ±1.39
Control	352.42 <sup>a</sup> ±2.82	351.58 <sup>a</sup> ±1.49	386.37 <sup>b</sup> ±1.98	411.76 <sup>c</sup> ±1.00
<i>Mice tissue superoxide dismutase activity (SOD) activity</i>				
NPGV	0.0437 <sup>a</sup> ±0.0008	0.0477 <sup>a</sup> ±0.0011	0.0588 <sup>a</sup> ±0.0014	0.0264 <sup>a</sup> ±0.0009
NPGVB	0.0516 <sup>b</sup> ±0.0008	0.0505 <sup>a</sup> ±0.0012	0.0718 <sup>b</sup> ±0.0026	0.0225 <sup>b</sup> ±0.0012
NPG	0.0405 <sup>c</sup> ±0.0011	0.0549 <sup>b</sup> ±0.0018	0.0551 <sup>a</sup> ±0.0011	0.0266 <sup>a</sup> ±0.0011
NPGB	0.0504 <sup>b</sup> ±0.0014	0.0487 <sup>a</sup> ±0.0008	0.0834 <sup>c</sup> ±0.0014	0.0263 <sup>a</sup> ±0.0009
Control	0.0445 <sup>a</sup> ±0.0009	0.0452 <sup>c</sup> ±0.0009	0.0720 <sup>b</sup> ±0.0015	0.0284 <sup>a</sup> ±0.0009
<i>Mice tissue reduced glutathione (GSH) (mM GSH/g tissue)</i>				
NPGV	0.5340 <sup>a</sup> ±0.0038	0.4530 <sup>a</sup> ±0.0040	0.4390 <sup>a</sup> ±0.0040	0.4173 <sup>a</sup> ±0.0031
NPGVB	0.4111 <sup>b</sup> ±0.0030	0.4308 <sup>a</sup> ±0.0052	0.4743 <sup>b</sup> ±0.0052	0.4135 <sup>a</sup> ±0.0034
NPG	0.4131 <sup>b</sup> ±0.0037	0.4961 <sup>b</sup> ±0.0023	0.4075 <sup>c</sup> ±0.0023	0.4974 <sup>b</sup> ±0.0037
NPGB	0.4123 <sup>b</sup> ±0.0023	0.5081 <sup>b</sup> ±0.0029	0.4985 <sup>b</sup> ±0.0029	0.4745 <sup>b</sup> ±0.0036
Control	0.4061 <sup>b</sup> ±0.0055	0.5927 <sup>c</sup> ±0.0015	0.4266 <sup>a</sup> ±0.0015	0.5000 <sup>b</sup> ±0.0032
<i>Mice tissue total protein content (TP) (mg/ml)</i>				
NPGV	0.0724 <sup>a</sup> ±0.0006	0.0791 <sup>a</sup> ±0.0009	0.0685 <sup>a</sup> ±0.0017	0.0404 <sup>a</sup> ±0.0017
NPGVB	0.0801 <sup>b</sup> ±0.0007	0.0800 <sup>a</sup> ±0.0008	0.0801 <sup>b</sup> ±0.0011	0.0495 <sup>b</sup> ±0.0014
NPG	0.0664 <sup>c</sup> ±0.0008	0.0553 <sup>b</sup> ±0.0006	0.0700 <sup>a</sup> ±0.0016	0.0363 <sup>a</sup> ±0.0012
NPGB	0.0676 <sup>c</sup> ±0.0013	0.0591 <sup>c</sup> ±0.0005	0.0797 <sup>b</sup> ±0.0006	0.0394 <sup>a</sup> ±0.0013
Control	0.0762 <sup>a</sup> ±0.0010	0.0625 <sup>c</sup> ±0.0006	0.0742 <sup>c</sup> ±0.0016	0.0312 <sup>c</sup> ±0.0013

NPGV, Intra-nasally vaccinated with polymer gel vaccine; NPG, Intra-nasally administered with polymer gel; NPGVB, Intra-nasally vaccinated with booster dose of polymer gel vaccine; NPGB, Intra-nasally administered with booster dose of polymer gel; Control, Intra-nasally administered with PBS; CB, Intra-nasally administered with booster dose of PBS. All the mice were challenged 28<sup>th</sup> day post vaccination and booster vaccination. Booster was given 21<sup>st</sup> day of initial vaccination and mice were sacrificed 7<sup>th</sup> day after challenge (35<sup>th</sup> day of initial vaccination and booster vaccination). Data are presented as mean±SE in each group of five inbred BALB/c mice. Mean with different superscript in each column differ significantly (P<0.05).

Increased GST activity (Table 1) implied that the consumption of GSH through the GST catalyzed reaction may play a role in the detoxification of generated free radicals and electrophiles. The sharp fall in GST levels (Table 1) might be due to inhibition of drug metabolizing enzymes by the antigen and/or polymer gel adjuvant. Several experiments suggest inhibition of drug-metabolizing enzymes owing to vaccines and their adjuvants. A direct, non-specific activation of macrophages with subsequent release of IL1 may account for this inhibition (Tkachenko *et al.* 2014).

Liver is a metabolically active organ with a powerful antioxidant potential. Therefore, higher MDA levels were expected in the liver than in the other tissues as reported

earlier by other researchers (Tkachenko *et al.* 2014). However, lipid peroxidation (LPO) showed almost similar profile in all the groups with minor variation in tissues (Table 2). In contrast to findings of present study, significant increase of oxidative stress biomarkers level in liver and gills of vaccinated fish had also indicated susceptibility of lipid molecules to ROS and appreciable oxidative damage under the influence of vaccination (Tkachenko *et al.* 2014). Moreover, higher expression levels have been detected for genes coding for proteins involved in redox homeostasis and protection against ROS in vaccinated fish (Skugor *et al.* 2009).

Spleen is a secondary lymphoid organ and main predilection site for the bacteria after 2nd week of infection.

It filters the antigens in the blood rather than the lymphatics. Thus, it has a special importance in checking infections that have invaded the blood (Roop *et al.* 2004). The higher value of LPO in the spleen of the NPGVB group is as per the mandate of spleen mass. It is further substantiated by the lowest values of GSH in the spleen of vaccinated animals (Table 2). Both antigen and polymer gel were expected to initiate and promote the free radical production for activation of the lymphocytes for generation of an active immune response. The lung tissues are also involved in the immune defense through the mucosa-associated lymphoid tissues (MALT) that harbour macrophages, neutrophils, lymphocytes, and mast cells/eosinophilic granulocytes (Kirron 2012). The higher levels of LPO in booster groups as compared to initial vaccination and adjuvant inoculation groups prove the active role of MALTs in local inflammatory and immune stimulation mechanisms. Significant rise in LPO from 45th day in plasma, liver and spleen after *Brucella* inoculation have been reported earlier which subsequently declined to basal levels (Melek *et al.* 2006).

In addition to specialised antioxidant enzymes, the most important intracellular low-molecular-weight antioxidant is glutathione (GSH), which has a thiol moiety and is reactive with pro-oxidant species. Non significant differences in the GSH levels (Table 2) are indicative of the excellent management of the antigen and the adjuvant by the host immune system without imposing any threat to the defence homeostasis. The GSH levels in spleen were comparable to control values in adjuvant treated groups and slightly lower in vaccinated group. Expression of thiols on cell surface of T cells and release of cysteine into the extracellular space by dendritic cells to create a reducing environment, thereby facilitating an immune response has been considered as an integral part of active immunization to induce Tregs mediated immunosuppressive effect as an interference with this process (Yan *et al.* 2010). Therefore, maintenance of GSH levels in the spleen and lungs are indicative of efficient activation of lymphocytes for producing the desired immunological response. Liver is the main site of detoxification in the body. The higher level of GSH in liver of booster groups may be attributed to the previously induced enzyme systems leading to efficient management of xenobiotics and reduction of free radicals. The raised levels of GSH in kidney of single vaccinated animals were unexpected and needs to be further studied. The importance of the glutathione-mediated antioxidant defense system in protection against endosulfan-induced oxidative stress was also demonstrated in adrenocortical cells of rainbow trout (Dorval and Hontela 2003).

Induction of oxidative stress usually precipitates NF- $\kappa$ B mediated 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 (Mitra *et al.* 2012). In general post vaccination reduction in superoxide dismutase (SOD)

activity was recorded with highest values in liver tissues in all the groups (Table 2) followed by lungs and kidneys. The liver being the main xenobiotic biotransforming organ was expected to induce the free radical generation to persuade the xenobiotic metabolizing enzymes to remove the *Brucella* antigen as well as adjuvant gel (Mitra *et al.* 2012). The kidney works in support of liver to remove the metabolized water soluble xenobiotic metabolites and so, was also expected to show an increase in SOD activity in the booster groups in comparison to non booster and control group. The spleen and may be the lung tissues, being lymphoid organ, revealed lowest SOD activity among four tissues, perhaps, to make possible the activation of antigen presenting cells and thus, facilitate the generation of immune response (Table 2).

The  $H_2O_2$  generated during neutralization of superoxide and peroxide radicals is a potent oxidant and induces oxidative damage to lipid, proteins, and DNA (Bergmeyer 1983). Its breakdown in the tissue is catalysed by the intracellular catalase activity. The decreased CAT activities (Table 2) indicate the reduced capacity to scavenge hydrogen peroxide produced in spleen and lung of vaccinated mice. In the absence of antigen, adjuvant elicited a better redox potential to stimulate the B and T lymphocytes and induce the immune response, which is a highly desirable feature for an adjuvant to be selected for vaccine production and that might be the reason for the highest activity in liver was shown by NPG group followed by NPGV and NPGVB (Table 2). In an earlier report, the *B. melitensis* infection did not change the antioxidant enzyme activities in the plasma, liver and spleen tissues with an exception of significantly increased catalase activity between days 30 and 45 post-infection in the liver (Melek *et al.* 2006). Increased catalase and SOD activities ( $P \leq 0.05$ ) have also been observed in *Brucella* affected buffaloes as compared to healthy buffaloes (Kataria *et al.* 2012). Very low concentration of  $H_2O_2$ , as usually observed at the early phase post-challenge or vaccination can't be eliminated (Kataria *et al.* 2012). Probably, with the slow diffusion of  $H_2O_2$  into cells, the accumulated  $H_2O_2$  can accelerate the degradation rate of SOD protein leading to reduced activity of the enzyme. After 2nd week of infection, liver has a minimal role in clearing the *Brucella*, this might have accounted for a reduced GST activity for the booster doses as compared to the initial vaccination or inoculation. In kidney tissues, minor variations were observed amongst the groups (Table 2). The GST levels showed trend similar to GSH in the various tissues and reflected their good antioxidant status. This carries special significance in case of spleen and liver which are the main sites for the *Brucella* organism.

As the route of inoculation was intranasal, all the groups showed a decline in antioxidant status in lungs, minimal levels being observed in vaccinated groups, perhaps due to release of local mediators against antigenic exposure. Intra nasal administration of vaccine as a rule leads to a vigorous local and systemic response with direct presentation to

NALT mucosal cells, facilitation of specific T and B-memory cells leading to ample production and release of IgA. There is preferentially induction of regulatory T cells that secrete IL-10 and TGF- $\beta$  at the anatomic site where the mucosally administered antigen is located, a phenomenon termed bystander suppression (Dan *et al.* 2004). Nasal antigen also generates a Th2 (IL-4/IL-10) - or a Th3 (TGF- $\beta$ )-type response which prove to be suppressive for Th1 and other immune cells (Faria and Weiner 1999).

Endurance and replication of brucellae in host phagocytes are key mechanism of their virulence (Roop *et al.* 2004). Vaccine/ adjuvant-induced increase in LPO depleted lipid soluble antioxidant system GSH/ROS scavengers and further disrupt the antioxidant enzymes activity but the modulation of parameters seems to be in favour of achieving the mandate of immunization. The spleen, considered as main predilection site, maintained a good antioxidant status after challenge and none of the parameters showed a likelihood of severe tissue stress that suggests effective vaccination against brucellosis.

From the present study, it is clear that both the glutathione mediated nonenzymic antioxidant defense system and endogenous CAT play a critical role in 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 in all the vital tissues, viz. erythrocytes, liver, kidneys, lungs and spleen of vaccinated mice were at par with the polymer gel adjuvant, probably due to maintenance of excellent redox homeostasis in these organs; liver and lungs being metabolically active, kidney being the main excretory organ and spleen playing a vital role in maintaining the immunological homeostasis in the body. Thus, it may be said that immunization with *Brucella* vaccine prepared using whole cell protein antigen with a polymer gel induces an oxidative stress with negligible signs of inflammatory pathophysiology in all the vital organs and can be further explored for intranasal inoculation in animals.

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