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Immunotoxic effect of sodium nitrate in immunized mice with *Brucella melitensis*

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Key words: *Brucellamelitensis*, enzyme-linked immunosorbent assay test, interferon gamma, IgG, phagocyte test.

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Abstract

Brucella melitensis causes a worldwide zoonosis. It is one of the major causes of abortions in sheep and goats. The organism is secreted in the milk of infected animals. This study aimed to determine the immunotoxic effects of sodium nitrate and to make a comparison between direct immunization by Brucella vaccine and Vitamin E. A total of forty eight adult males Swiss Albino mice, aged 8 weeks, were divided into four groups as follows: the 1stgroup administrated orally with drinking water 1/10LD50 (273.84mg/kg b.w.) of sodium nitrate daily for 6 weeks and at the same time inoculated by I/P with 0.4 ml Brucella Rev1 vaccine two doses for two weeks interval. The 2nd group was immunized and administrated with sodium nitrate as 1st group and at same time vitamin E (0.15 I.U./kg). The 3rd group was immunized as 1st group. The 4th group was considers as control negative group. Blood samples were collected from (1st, 2nd, 3rd and 4th) groups for measuring serum of INF-γ and IgG using ELISA Kit. The result showed that the sodium nitrate caused inhibition of both cellular and humoral immune response in addition to decrease the phagocytic activity. Result also, showed that the mean value of skin thickness in G3 at 24 hr. ($p<0.05$) was a significantly ($P<0.05$) lower in G2 and in G1 and G4 at 48 hr. Also, the result of ELISA tests for detection levels of (IgG) titer in G3 showed a significant decreasing ($p<0.05$) compared with G2, G1 and G4. The result of ELISA tests for detection levels of (INF-γ) titer in G3 ($p<0.05$) revealed a significant decreasing ($P <0.05$) as compared with G2, G1 and G4.

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Introduction

Brucella melitensis causes a worldwide zoonosis. It is one of the major causes of abortions in sheep and goats, and the organism is secreted in the milk of infected animals. People contract the disease by direct contact with contaminated fetal membranes or, more commonly, as a result of the consumption of contaminated unpasteurized milk and cheese products. The organisms are small, gram-negative coccobacilli that grow in the host as no obligatory intracellular pathogens of the reticuloendothelial system. (Elberg, 1981).Further, *Rev-1* is virulent to humans (Blasco and Díaz, 1993). Another drawback rooted in *Rev-1* is that immunization can stimulate strong anti-O antigen antibody titters (Marín *et al.,* 1999). IgG plays an important role in antibody-dependent cell-mediated cytotoxicity (ADCC) and intracellular antibody-mediated proteolysis, in which it binds to TRIM21 (the receptor with greatest affinity to IgG in humans) in order to direct marked virions to the proteasome in the cytosol; (Mallery *et al.,* 2010). Immunoglobulin IgG (Janeway *et al.,* 2001). Interferon gamma (IFN-γ) is a dimer zed soluble cytokine that is the only member of the type II class of interferons. (Gray and Goeddel, 1982). IFN-γ, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. IFN-γ is an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression. IFN-γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops. (Schoenborn and Wilson, 2007).IFN-γ is also produced by non-cytotoxic innate lymphoid cells (ILC), a family of immune cells first discovered in the early 2010s (Artis *et al.,* 2015). (Ustyugova IV, 2002; Kozliuk *et al.*, 1989) reported the effect of nitrate/nitrite ingestion on immune system. The effect of nitrate ingestion on human immune system indicated that nitrate had no effect on lymphocyte growth, but nitrite decreases proliferation of lymphocytes. Fibroblast growth remains unaffected. A decreased production of Th1 cytokines (interleukin-2, interferon-gamma, and tumour necrosis factor-beta), which is responsible for resistance to a variety of infectious diseases was noted (CDFA, 1989).

(Porter *et al.,* 1999) investigated that nitrate not effecton the production of the Th2 cytokine interleukin-10, which is responsible for disease susceptibility, was noted. Because nitrate/nitrite shifted the balance from a Th1 to a Th2 response in some individuals, exposure to these compounds may decrease these people's responsiveness to infectious diseases. The levels of nitrate used in this study are relevant to human health because they are present in the liquid portion (no breast fed) of some 2-month-old infant diets in rural Romania. Animal studies also reported an immune suppression due to high nitrate ingestion (Porter *et al.,* 1999).

Materials and methods

Forty eight adult male Swiss Albino mice and aged 8 weeks and weighed range (20-25g), supplied from animal house of the College of Vet. Med. University of Baghdad were used in present study. They were housed and maintained in a conventional animal facility, with controlled conditions of temperature (20 ± 5°C). The animals were fed on special formula of food pellets and given water *ad libitum*. Throughout the experiments, each group of mice was housed in plastic cage containing hard-wood chip as bedding. The bedding was changed continuously to ensure a clean environment. The experiment is University of Baghdad College of Veterinary Medicine Department of pathology and poultry.

Experimental design

Forty eight adult males Swiss Albino mice, aged 8 weeks, were divided into four groups.

1st group administrated orally with drinking water 1/10LD50 (273.84mg/kg b.w.) of sodium nitrate daily for 6 weeks and at the same time inoculated by I/P with 0.4 ml Brucella Rev1 vaccine two doses, two weeks interval.

2nd group was immunized and administrated with sodium nitrate as 1st group and at same time vitamin E (0.15 I.U./kg) as 2nd group.

3rd group was immunized as 1st group. 4th group was considers as control negative group. At day 28 post-immunization (PI), DTH skin test was done using *Brucellin* soluble antigen. At 30 days serum samples were collected from all groups of mice and used for detection of PHA test; IFN-γ IgG by ELISA, in addition to phagocytic assay.

Median lethal dose (LD_{50})

"Up-and-down" method.(Dixon, 1980).was used for determination of median lethal dose (LD50) of Sodium Nitrate which fifty albino mice weighed (20-25) gm were used in this study in which Sodium Nitrate was prepared at concentration 27 mg / ml by dissolving powder Sodium Nitrate in distilled water. The volume of doses was calculated according to animal weight, Sodium Nitrate was given orally to the animals at range of doses (2800) mg /kg B.W and the difference in doses was (400mg / kg B.W) . LD50 was calculated after 24 hours observation of lethality in the dosed animals. The LD50 is calculated by using the equation in Appendix- 1.

Brucellin preparation

This Ag was used for immunization animals Prepared according to (Saleh, 1999) as follow:

The *Brucella melitensis* vaccine life attenuated.
Add 30x10⁹ *Brucella melitensis* with 15 ml PBS.

The solution suspension sonication: the universal tube that contained *Brucella melitensis* suspension was put in cold environment (ice) and placed in the ultra-sonicator (type Karl Klob – Germany) with 2 minutes intervals between them, for 30 minutes in alkindi company to production of vaccines and veterinary medicines.

The suspension was centrifuged in cold centrifuge at (10000 rpm/4c⁰ / /30 min) then the supernatant was taken in sterile method.

The supernatant was filtered by Millipore filter 0.22nm.

The sample sonicated was tested by culture. The supernatant considered the *Brucillin* and the protein concentration were evaluated by biurat procedure.

The Brucellin diluted to become 0.5 mg/ml PBS, and stored in -20° till use and injection of mice 0.1 ml in pad foot.

Immunological tests

(Skin test) DTH: was carried out at the 28th day post 1st immunization, and the procedure was adapted by Hudson and Hay (Hudson and Hay 1980).

Detection of serum IFN-γ concentration in mice sera was assessed using commercial available enzyme-linked immunosorbent assay (ELISA) Kit obtained from Elabscience. (U.S.A.). The results were reported as pictogram/milliliter (Pg/mL). This test was carried out according to the manufacture assay protocol.

Phagocyte test (PHA): test was done according to Herbert (Herbert, 1978). Moreover, phagocytic assay in serum was estimated by the carbon clearance assay according to Cheng and Lamont (Cheng and Lamont, 1988).

Statistical analysis

Statistical analysis was applied by two way ANOVA and the mean difference was significant at the ($P \leq 0.05$) level by SPSS (statistical package for social sciences).

Results

Acute toxicity study

This study revealed that the LD50 of Sodium Nitrate according to Dixon method was mg / kg B.W with toxicity rate which is considered as moderately toxic as in Table 1. The acute toxicity symptoms which were observed after dosing the animals include, hyperactivity, staggered gait, tremors, myosis, hunched posture then death (the severity of symptoms was positively proportional to the dose).

Table 1. Oral LD50 of Sodium Nitrate in mice according to up and down method.

Initial dose mg/kg.B.wt.	Final dose mg/kg.B.wt.	Differences between doses mg/kg.B.wt.	Number animals	of Out come after 24 hours
2000	2800	400	5	KOOOO

(skin test) DTH:

At 24 hours post-test, the results showed that the mean values of skin thickness against *Brucella* was significantly (p≤ 0.05) high in the G3(B0.84±0.07b) mm as compared with the G2 and G1 (B0.50±0.08b), (A0.41±0.16a) mm, at 48hours post examination, the mean values of skin thickness were declined in the G1group (B 0.43±0.05 b). and negative control group G4 (0), as revealed in Table 2.

Table 2. Difference Skin thickness (mm) of different immunized mice groups at 24 and 48 hours post examination.

Groups	24 hours	48 hours
G1	A 0.41±0.16 a	B 0.43±0.05 b
G2	B 0.50±0.08b	A 0.71±0.16 a
G3	B 0.84±0.07b	A 0.97±0.08 a
G4	0	0

Means with a different small letter in the same column significantly different (P<0.05)
Means with a different capital letter in the same row significantly different (P<0.05).

Elisa test for detection levels of (INF-γ) The current result showed that the mean values of serum INFγ (pg./ml), at 30 day post immunization in the G3 was higher (557.76±32.40) than those values in the G2 and G1 group (432.88±65.16),(418.38±13.90) and negative control group G4 (0), as revealed in Table 3.

Table 3. The mean value of serum INF-a 30 day post immunization of different mice groups immunization at 4 weeks by Elisa test.

Groups	INF-γ (pg./ml) (Mean ± SE) at 4 weeks
G1	418.38±13.90 C
G2	432.88±65.16 B
G3	557.76±32.40 A
G4	0

Elisa test for detection levels of IgG titter

The current result showed that the mean values of serum IgG (g/L), at 30 day post immunization in the G3was higher (126.84±1.10), than those values in the G2 and G1 group (42.55±1.09), (11.80±1.22)and negative control group G4 (0),as revealed in Table 4.

Phagocytes assay

The current result showed that the mean values of serum phagocyte assay ratio(%), 30 day post immunization in the G3 was significant (P≤0.05) higher (17.70 ±0.10A) than those values in the G2 (12.06 ± 0.07B), also G1 showed significant (P≤0.05) increase to reach (9.19 ± 0.04C) while the negative control group represent with the normal(0), Table 5.

Discussion

The current study revealed that the mean values of skin thickness in immunized animals treated with sodium nitrate were lower than those value in immunized animals only, this result may indicate that *Rev-1* Ags stimulated cell mediated immunity and sodium nitrate diminished the immune response elicited by this Ags, this observation also may give indication that treatment with sodium nitrate

associated with decreased activity of vaccine program against brucellosis in farm animals.

The result of skin test is agreed with that of serum levels of INF-y and titter of IgG supported the idea that sodium nitrate can cause immunotoxic and genotoxic effects of nitrate in immune cell, that led to decreased host defense mechanism and increase bacterial invasion, which occurred to release many endogenous antioxidant enzymes, this indicated that nitrate may be release ROS and endogenously transformed into nitrite which in turn can react with amines and amides to produce nitrosamines and free radicals (Singhal *et al*., 2001; Manassaram *et al*., 2006). ROS have been recognized as contributing to vascular dysfunction, through mechanisms including endothelial dysfunction, vascular smooth muscle cell growth, lipid peroxidation, and inflammation (Touyz, *et al*., 2004), these result in agreement with (Sindler *etal.,* 2011) who reported that dietary nitrite supplementation was shown to modulate age-related inflammatory cytokines in mice.

Table 4. The mean value of serum IgG 30 day post immunization of different mice groups immunization at 4 weeks by Elisa test.

Groups	IgG (g/L) (Mean ± SE)at 4 weeks
G1	11.80±1.22 C
G2	42.55±1.09 B
G3	126.84±1.10 A
G4	0

The upregulation of the inflammatory response is the consequence of a remodeling of the innate and acquired immune system with a chronic inflammatory cytokine production (Baylis, 2013). As well as NaNO3 that induced oxidative injury through the generation of free radicals, as well as the toxic agents induced biological changes in tissue and body fluid of the host cell (Mourad *et al*., 2005). since DTH reaction was considered one arm of cell mediated immunity which was dependent on Th1 producing cytokines particularly INF-y, this evidence is in consistence with this evidence is in consistence with (Oliveira and Splitter, 1995) who demonstrated that CD4+ and CD8+ T cells, a main effectors cells of CMI, produced INF-y, this cytokines activated and attract immune cells particularly macrophages to site of Ags inoculation that lead to DTH reaction.(Oliveira and Splitter, 1995).

The present study showed that immunized animal treated with Sodium nitrate expressed significantly low levels of serum antibodies titer (IgG) as compared with immunized animals only, this result may indicate that Sodium nitrate also induced suppression of humoral immune response, according to result of DTH reaction and serum levels of INF-y in this group, it was suggested that correction between CMI and humoral immunity.

Th2 cytokines were essential factor that help proliferation and differentiation of B cells into plasma cells producing Abs, these mechanisms were dependent on activity of IL-2 producing by Th1 immune response (Maecker *et al*., 1998), also the current suggestion is in agreement with (Zhan and Cheer, 1995) who reported that immunized mice with soluble Brucella antigens stimulated spleen cells to produce Th2 response, CFB Ags. In the present study, were contain secretion products of *Brucella* which were protein in nature that a good stimulator of CMI this idea was agreement with (Mahajan *et al.,* 2005) who found that both CMI and humoral immune responses can elicited by surface proteins of *Brucella melitensis*.

According to above evidence, it was suggested that sodium nitrate alter activity of macrophage to produced IL-12 which stimulated NK cells to produced INF-y, and this cytokine play crucial role in

differentiation of Th0 toTh1 cells that produced IFN-y again (CDFA, 1989), who suggested that sodium nitrate may be inhibit proliferation and attraction of macrophages and lymphocytes to the site of examination, as well as nitrate had effect on lymphocyte growth, but nitrite decreases proliferation of lymphocytes. Fibroblast growth remains unaffected. A decreased production of Th1 cytokines (interleukin-2, interferon-gamma, and tumor necrosis factor-beta), which is responsible for resistance to a variety of infectious diseases was note.

On the base of this observation, it was suggested that sodium nitrate may be inhibit proliferation and attraction of macrophages and lymphocytes due to increase in ROS production, free oxygen radicals react with macromolecules that contain protein, lipid and DNA and cause oxidative damage(Urrutia *et al.,* 2000). ROS released from phagocytes alters the function of amino acids present in enzymes and kinases such as methionine, tyrosine and cysteine and causes tissue and cell damage (Halliwell, 1994; Pernet *et al.,* 1999; You *et al.,* 2003).

Table 5. Shows phagocyte assay ratio of different groups immunization at 30 days.

Groups	Phagocytic test (Mean ± SE)at 4 weeks
G1	9.19 ± 0.04 C
G2	12.06 ± 0.07 B
G3	17.70 ± 0.10 A
G4	0

Means with different capital letters means significant (P≤0.05) different among groups.

Moreover, membrane-related signaling and gene expression are critical in maintaining normal function of immune cells and their ability to defend against various foreign antigens. These functions are, however, highly sensitive to ROS. As a result, it seems appropriate that immune system cells generally have higher concentrations of antioxidant micronutrients than other cells (Hatman and Kayden, 1979).

However, immunized animals fed diet supplement with Vitamin E expressed high value of thickness of skin and Abs titter as comparing with those values in immunized animals only, this result supported the idea that Vitamin E play role in the stimulation of humoral immune response, this result supported the idea mentioned by (McDowell, 2000).Who reported Vitamin E functions as a chain-breaking antioxidant, neutralizing free radicals and preventing oxidation of lipids within membranes Vitamin E serves as the 1st line of defense against peroxidation of phospholipids. The principal vitamin E form with antioxidant and immune functions is α-tocopherol. However, although studies are limited, non α-tocopherol and tocotrienols have important functions. γ-tocopherol has been shown to be a more effective inhibitor of peroxy nitrite-induced lipid peroxidation (McCormick and Parker, 2004). Also, γ-tocopherol is more effective at inhibiting inflammatory reactions. Tocotrienols possess excellent antioxidant activity in vitro and have been suggested to suppress ROS more efficiently than tocopherols (Schaffer *et al.,* 2005).

Tocotrienols were found to be more effective for reducing the ageing process and age-related diseases. Considerable attention is directed to the role vitamin E and Se play in protecting leukocytes and macrophages during phagocytosis, the mechanism whereby animals immunologically kill invading bacteria. Both vitamin E and Se may help these cells to survive the toxic products that are produced in order to effectively kill ingested bacteria (Badwey and Karnovsky, 1980).

Macrophages and neutrophils from vitamin E-deficient animals have decreased phagocytic activity. (Lauridsen and Jensen, 2005), reported Vitamin E has been implicated in stimulation of serum antibody synthesis, particularly IgG antibodies (Tengerdy, 1980). Vitamin E supplementation increased α-tocophero status and immune response of weaned

piglets (LeBlanc *et al.,* 2002), who study when given as an adjuvant at vaccination, vitamin E is highly effective in enhancing antibody titers, implying that this may be an effective way of obtaining immunological response following vaccination.

The high percentage of phagocytic activity in immunized animals as compared with those values in the control negative group may indicated that the *Rev 1*Ags were a good stimulator of phagocytic cells, principle cells in the CMI, this result was agrees with high levels DTH reaction and INF-Y, which activated the macrophages, this idea is in consistence with a previous study which recorded that Brucella antigens can stimulate macrophages to synthesize IL-$_{12}$ and TNFα resulting in stimulation of natural killer cells to synthesis of INF-ϒ (Hsieh *et al.,* 1993). As well as (Wu and Kaufman, 1997) who reported that INF-ϒ activation macrophage to killer become bactericidal particularly through the production of nitric oxide. In addition, these cytokines stimulated Th1 producting INF- ϒ that amplifying processiong and presenting antigen activity of the macrophages to CD4+ and CD8+ T cells resulting in active cell mediated immune response that characterized by high phagocytic activity of macrophages.

Also the current finding showed that animals treated with sodium nitrate were very significantly low in the phagocytic activity as compared with the control group, this result may indicate that sodium nitrate induced impairment of host defense mechanism through depressed activity of phagocytic cells which were considered a main cells of innate immune response against infectious and non-infectious agents, this observation was agreement with (Becquet *et al.,*1994), we reported. This decrease phagocytosis by RPE (retinal pigmented epithelial cells) caused by NO may occur as a result of retinal inflammation, and could lead to photoreceptor degeneration. As well as (Hakki *et al.*, 2013), we showed that NO produced by macrophages has a role not only in intracellular killing, but also in phagocytic activity.

Nitric oxide synthase which was responsible for nitric oxide formation and NO play important role in phagocytic activity as well as cytotoxic effect upon tumor cells (Green *et al.,* 2005).

Cytotoxic macrophages and monocytes can also be induced *in vitro* by specific and nonspecific stimuli such as endotoxin treatment or exposure to soluble mediators (lymphokines) released by antigen-ormitogen-stimulated T lymphocytes (Sharma and Piessens 1978; Cameron and Churchill, 1979). As we and others have shown, lyrnphokine treatment also influences a v~U'iety ofother cellular and biologic functions including enhancement of antimicrobial activity and release of oxygen intermediates. Recent studies using PMN's have also demonstrated that the sephagocytes may be potent cytotoxic cells as well (Hafeman and Lucas, 1979; Clark and Kleban, 1979).

The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical.

These are highly reactive species, capable in the nucleus, and in the Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets. Membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids (Young and Woodside, 2001).

Vitamin E, an important antioxidant, plays a role in immunocompetence by increasing humoral antibody protection, resistance to bacterial infections, cell-mediated immunity, the T-lymphocytes tumor necrosis factor production, inhibition of mutagen formation, repair of membranes in DNA, and blocking micro cell line formation. (Sokol, 1988). Hence vitamin E may be useful in cancer prevention

and inhibit carcinogenesis by the stimulation of the immune system.

Conclusion

It was concluded that sodium nitrate induced suppression of humoral and cellular immune responses in immunized mice with *Brucella Rev-1* vaccine.

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