**RESEARCH ARTICLE**

# The Effect of Boric Acid on Immune Response in Mice Vaccinated by *Proteus Vulgaris* Antigen

Saif M. Abed[1], May A. Hamed[2], Hussein T. Abdulabbas[3]

[1] *M.Sc. Microbiology science, Samawah, Iraq*
[2] *M.Sc. Microbiology at Department of Microbiology, College of Medicine, University of Al-Muthanna, Samawah, Iraq*
[3] *M.Sc. Microbiology, Lecturer at College of Medicine, University of Al-Muthanna, Samawah, Iraq*

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**SUMMARY**

This study was carried out to investigate the immunomodulatory effects of the Boric acid on the immune responses of mice that vaccinated by *Proteus Vulgaris* antigens. The study was included six groups; the first group (I) was treated with distilled water. The II group was mice treated with *Proteus Vulgaris antigens* only, (III) group: injected subcutaneously with a dose of (600 ug/Kg), IV group was injected subcutaneously with a dose of (400 ug /Kg) boric acid. The V and VI groups were treated with *p. Vulgaris* and boric acid. All these groups were carried out at day 1; then, the mice were killed on day 8 to estimate the parameter. The phagocytic activity index was estimated by Eliza reader. While on day I4 the lymphocyte transformation was estimated by Lymphocyte Transformation Test (MTT) index and by Eliza reader. In this regard, all groups of mice showed different significant increases ($p \leq 0.05$) in the Nitro blue Tetrazolium (NBT) index as compared with group I, the control group, which was injected with distilled water and group 5 and 6 showed increasing significantly compared with group II. III. IV. On the other side of the study, the results of the lymphocyte transformation index in mice of all groups were showed different significant as compared to group I. The best treatment efficiency was recorded in-group V, VI compared with treated groups II. III. IV). The results demonstrated a clear immunomodulatory effect of boric acid (enhancement of nonspecific immunity, and adaptive immunity) of the treated mice immunized with *P. Vulgaris*.

**Keywords:** Immune Response, Mice Vaccinated, MTT, NVT.

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## INTRODUCTION

Boric acid (H3BO3) is one of the boron compounds which soluble and circulates in plasma.[1] boric acid has been used extensively for many industrial purposes also its salts have been used for medications as an antiseptic against the microbial organisms such as bacteria and fungi. The toxicity of boric acid is low and non-volatile mineral with insecticidal, fungicidal, and herbicidal properties.[2] Essentials of the boron in plants appeared in 1923 when Warrington found signs of Boron deficiencies in several leguminous plants. Soon after Warrington's report,[3] also reported that Boron was essential for the life cycle many plants to be complete, it has been discovered that Boron easily forms complexes biological compounds that contain *cis*hydroxyl groups. Boron binding and linking to hydroxyl-containing compounds, such as phosphoinositides, glycoproteins, and glycolipids are the foundation of evidence that boron has a role in membrane composition.[4] These studies mostly assumed that supplemental boron has a positive effect on animal performances five including the immunity, the immune system consists of a complex array of cellular responses originating from bone marrow and the lymphatic and mucosal systems.[6] Many pieces of research from a number of animal studies discovered that boron is of nutritional importance and affects various immune mechanisms and processes such as inflammatory responses and cytokines production and proliferation of lymphocytes (T cell, B cell).[7-9]

*Proteus vulgaris* is gram-negative facultative anaerobe bacteria grow in 37C and exists in the intestines of humans and animals, also in environments such as manure, soils, and polluted waters. *P. vulgaris* is opportunistic and associated with cases of bacteremia, pneumonia, and lesions. It has been described as one of the opportunistic etiological agents in infections of wounds, burns, skins, eyes, ears, nose, throat, and the pulmonary system as well as in gastroenteritis from the consumption of contaminated meat or other food.[10]

## MATERIAL AND METHODS

All experiments were done in the laboratories of the College of Medicine in Al–Muthanna University, and research on

**Author for Correspondence: hussbiooo@gmail.com*

male and female albino mice (Blab-c), which were supplied by this college. Their age ranged between 6–8 weeks, and their average weight was 22–25 grams at the beginning of the experiments, the animals were left in separate cages for one week to experience the acclimatization period.

**Isolation and identification *Proteus vulgaris***

The bacterial isolate was obtained from the Central Health Laboratory in Al-Muthanna province and activated by Nutrient broth and cultured on Nutrient agar for antigen preparation.

**Preparation P. *vulgaris* Antigen whole-cell antigen.**

Prepared *P. vulgaris* antigen whole-cell antigen according to a method presented by I. Motive, *et al*,[11] by using the Ultra soncater system. As following

*P. vulgaris* inoculate on Nutrient agar for 24 hour and collected from the Nutrient agar then was centrifuged (3000 rpm) for 30 minutes with PBS and discharged the supernatant, repeatedly washed three times

Suspended the precipitate cells in PBs and sonicate the suspension by the mixture was blended in an Ultra soncater system (15 KHZ/Sec (for a total of 5 minutes with intervals (one minute) in an ice bath for 30 minutes at 4ºC.

The mixture was blended in a vortex for a total of 5 minutes with intervals (one minute) in an ice bath. The supernatant was centrifuged in a cooling centrifuge at 10000 rpm for 30 minutes, and passed through Millipore filter (0.45 µm), and tested for sterility. On MacConky agar. The supernatant froze at –20 ºC

**Preparion of boric Acid and Determine LD50**

The boric acid powder (1 grams) was dissolved in 100 ml of Distiller water and shaken well at room temperature (25ºC), then prepared many doses (6 doses) to determine the LD50 by using six groups of mice each group included five animals as in Table 1.

**Vaccination by *P. vulgaris* antigen program**

There were four groups in this experiment, which was designed to evaluate the level of the immunoglobulin IgG, IgM, IgA in the serum of the mice which inoculated with whole protein vaccine, using radial immunodiffusion (RID). The total number of animals in these groups was included 20 mice (5 mice in each group). For the purpose of measuring the concentration of the immunoglobulins which present in their serum, the following steps were done.

- Group I: injected subcutaneously with **normal saline** (0.2ml)**.**

**Table 1:** Doses of boric acid that were used in the assessment of LD50.

| *Dose/mouse* | *Dose/Kg* | *Number of animals* | *Mortality rates (%)* |
|---|---|---|---|
| 100 µg | 4 mg | 5 | 0.0 |
| 200 µg | 8 mg | 5 | 0.0 |
| 300 µg | 12 mg | 5 | 0.0 |
| 400 µg | 16 mg | 5 | 0.0 |
| 500 µg | 20 mg | 5 | 0.0 |
| 600 µg | 24 mg | 5 | 0.0 |

- Group II: injected subcutaneously with a single dose of 40 *ug/ml* of *P. vulgaris* vaccine in day 1.
- Group III: injected subcutaneously with a single dose of 60 *ug/ml of P. vulgaris* vaccine in day 1.
- Group IV: injected subcutaneously with a single dose of 80 *ug/ml* of *P. vulgaris* vaccine in day 1.

The first and second booster doses of the *P. vulgaris* vaccine were injected after the first week and second week, respectively, after the time of vaccination. The mice were sacrificed after the vaccination after one month of the second booster dose, and the serum is obtained and froze in -20ºC.

**Radial Immunodiffusion**

Radial Immunodiffusion (RID) was used following a procedure according to the instruction of the company (Inter medical) as follow:

Five aliquot of the frozen serum was thawed at (in room temperature) for around 15 minutes, and then wait, as it has been completely adsorbing and incubated the plate for 72 hours. The examined proteins, diffusing in agarose gel that containing a specific antibody, will form an immune – complex, as a visible ring around the well, Measure the precipitating ring by the conversion table which is present with materials the test.

**Groups of study**

This current study was included 6 groups each group included 5 mice as following

- Group I Injected with normal saline.
- Group II Injected with *P. vulgaris* vaccine.
- Group III Injected with a high dose (600 µg/kg) of boric acid.
- Group IV Injected with a moderate dose (400 µg/kg) of boric acid.
- Group V: Injected with *P. vulgaris* vaccine+ high dose (600 µg / kg) of boric acid.
- Group VI: injected with *P. vulgaris* vaccine + moderate dose (400 µg / kg) of boric acid.

**Blood Samples Collection**

The mice were killed after the vaccination after one month of the second booster dose; around two milliliters of blood was obtained by cardiac puncture and transferred to Eppendorf tubes. Then the blood left at room temperature to clot for 15 minutes and centrifuged (2000 rpm) for 15 minutes. the serum was collected, divided into many aliquots (50 µL) and kept at –20ºC until use for laboratory assessments

**Laboratory assessments**

*Nitro blue Tetrazolium (NBT) Index*

The assay was carried out according to a method presented by[12] for all mice.

*Lymphocyte Transformation Test (MTT)*

The procedure of MTT assay (3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) measured by Elisa to assess the lymphocytes transformation percentage after an *in vitro* stimulation with a specific antigen (*P. vulgaris* vaccine) or PHA.[12]

### Statistical Analysis

Statistical analysis values of the investigated parameters were given in terms of means ± standard errors (S.E.), and differences between means also were assessed by ANOVA, LSD test by using the computer programmer SPSS. The differences were considered significant when the probability value was equal to or less than 0.05.

## RESULTS

The bacterial isolate was obtained from Central Health Laboratory in Al-Muthanna province and has been identified according to the morphological character. *P. vulgaris* is characterized by its swarming phenomenon in young culture on blood agar according to,[13] and the identification was achieved by Ap20 index too. The isolated were activated and cultured on Nutrient broth and nutrient agar, respectively, because those media are enrichment[14] to increase the growth of bacteria for harvesting and preparing the antigen.

The results of vaccination program were revealed the immunoglobulin concentration IgG was a significant increasing ($p \leq 0.05$) animals group III (60 *ug/m*L) compares with other groups, these the variations between group III and the other groups were very high as observed in Table 2.

The immunological parameter results were included. The NBT and MTT tests were revealed the NBT results of the current study were revealed the significant differences between group Group VI and Group I, and also there an increase between Group VI and group IV, but no significant differences.

Also, the results of this study was showed group V have significant differences against group I and group II and group III.

On the other hand of the result, comparison of Group IV and Group III with Group I showed significant differences, while Group II was compared with all the other studied groups showed significant differences, as in Table 3. While the results of MTT, showed the group VI (400Boric Acid+ Vaccine) and group V have significant differences ($p < 0.05$) among all studies, as in Table 3.

**Table 2:** IgG concentration rate (mean) of *P. vulgaris* antigen:

| *Groups* | *Dosage* | *IgG Mg/dL* |
|---|---|---|
| Group I | 0.2 mL | $97 \pm 1.8^{e}$ |
| Group II | 40 *ug/m*L | $222.2 \pm 3.8^{c}$ |
| Group III | 60 *ug/m*L | $4421.4 \pm 6.18^{a}$ |
| Group IV | 80 *ug/m*L | $3501.3 \pm 6.3^{b}$ |

*The different letters denoted that significant differences among the groups $< p \leq 0.05$

## DISCUSSION

This study was focused on investigating the effects of boric acid on the immune response (nonspecific immunity and adaptive immunity) by measure the phagocyte activity index and transformation lymphocyte index, respectively. Previous studies have used the boron for this purpose, and other researchers have used the dietary boron effect on the immune system. Therefore our results and discussion will be compared to studies that used the boron to investigate the effect on immune system because, first the boric acid is one of boron compounds and second lacking the references that used the boric acid as way we used in our study.

The results of vaccination program were revealed the immunoglobulin concentration IgG was a significant increasing ($p \leq 0.01$) animals group III (60 *ug/m*L) compares with other groups, These the variations between group III and the other groups were very highly significant. The reason behind the dose of group III (moderate dose) was showed a significant difference with other groups because the antigen dose was able to induce the immune response while the third group was a low does and Fourth group which is a high dose were showed no significant difference due to both those induce tolerance.[15]

On the other side of the study, the result of the boric acid LD50 showed no mortality Rates in studied groups; all the doses were showed no observed symptoms of the side effect in all studied groups, depending on this results the study used the highest two dose which is 400 and 600 in next study experiment. This agreed with many studied that revealed the effect of boron to various metabolic processes, nutritionals processes, hormonal processes, and physiological processes, which indicated the boron may be essential to plants,[16,17] but boron is essential for humans and animals as well as the boron performs many functions in mineral metabolisms, also in immunity system, and in the endocrine. Furthermore, it is important for bone growth and health.[17-20]

The reason those doses showed no any serious effects or mortality rates due to the levels boron normally exists in most organisms, and is generally non-toxic, beside the animals have a natural ability to keep maintaining the homeostatic control of boron levels in their bodies.[4]

Furthermore, the group of this study was included six group to assessment the nonspecific and adaptive immunity and as following, the first group (I) was treated with distilled water. II group was mice treated with *Proteus vulgaris* antigens only, group (III): injected subcutaneously with a dose of (600

**Table 3:** The Result of NBT index and MTT test

| *Assayss* | *Control* | *Vaccine* | *600 µg/kg Boric Acid* | *400 µg/kg boric acid* | *600 µg/kg Boric Acid+ P. vulagris antigen* | *400 µg / kg Boric Acid+ P. vulagris antigen* |
|---|---|---|---|---|---|---|
| MTT | $0.211 \pm 0.07^{b}$ | $0.279 \pm 0.086^{d}$ | $1.32 \pm 0.06^{c}$ | $1.42 \pm 0.18^{c}$ | $1.41 \pm 0.17^{c}$ | $1.13 \pm 0.08^{a}$ |
| NBT | $0.94 \pm 0.44^{c}$ | $2.68 \pm 1.22^{c}$ | $2.52 \pm 0.27^{d}$ | $1.69 \pm 0.65^{e}$ | $1.57 \pm 0.44^{d}$ | $2.04 \pm 0.7^{e}$ |

Letters represent as the following: A significant differences against all the other studied Groups $p < 0.05$; b, significant differences against all the other studied Groups $p < 0.05$ (in exception of one group); c, significant differences against all the other studied Groups $p < 0.05$ (in exception of two groups); d, significant differences against only two studied Groups $p < 0.05$; e, significant difference against only one studied groups $p < 0.05$. The results are shown as a Mean ± SD.

ug/Kg), IV group was injected subcutaneously with a dose of (400 ug/Kg) boric acid only, the V, VI groups were treated with combination of *P. vulgaris* and boric acid.

The reason behind the second group was injected with antigen is only to induce the immune-response while the group III and group IV were treated with boric acid to investigate the effect of boric acid to the immune system not have been induced, and group V, Group VI were treated with antigen combined to boric acid to investigate the immunomodulation ability of the boric acid to the induced immune response because this, as we mentioned above this, is to investigate the effect of boric acid on immune response to consider as a Immunomodulator, and the Immunomodulators are biological materials that mediate the effectors mechanism of the immune system through immune stimulation to given antigens.[21]

The NBT index is to measure the phagocytic activity of nonspecific immunity cells, according to result of this study the group VI and group V showed phagocytic activity more than group II as both those groups are treated with antigen to induce the immune response and these results agreed with[22] who studied dairy effects of boron on the mice. and agreed with,[9] due to boric acid have different modes to effect immune process and mechanisms and this agreed with,[23,24] the ability of boric to increase and stimulate the phagocytic cells attributed to many level of mechanism, such as the boric helped to increase and change inflammatory response although this modifications are not well understood but suggested that increase concentration of cytokines production[22] such as TNF α and INF gamma through studied effects of diary boron in injected mice with LPS (Lipopolysaccharides). Tumor necrosis factor-alpha is a pro-inflammatory cytokine that induces the production of other anti-inflammatory cytokines. The acute phase response plays a role as these increased plasma proteins are well known as acute-phase proteins, and are stimulated by the pro-inflammatory cytokines TNF-α, IL-6, and IL1.[6]

The other possible modes of action of boric may alter inflammation leads to important in regulating the respiratory burst the same action of boron.25 Some evidence suggests that boron may also reduce tissue damage from inflammation by accelerating the destruction of reactive oxygen species (ROS) via increasing the activities of antioxidant enzymes.[25] A recent study was revealed boron was caused increasing in NO, as well as the expression of iNOS, (which helps to promote inflammations and immune responses) and increase Cytokines production by the LPS-primed macrophages, (specifically the M1 macrophages), which is possibly to act through the Toll-like receptors, the boric acid as the boron that can potentiate the LPS-induced responses of the macrophage, suggested that a possible role of boron in macrophage polarization and increase the phagocytosis activity.[9] While the group II was showed there are a phagocytic activity because it is treated by antigen and lead to induce the immune response as normal[26] (but lower than the group V and group VI that treated with antigen and boric acid.

In other side of the result of, Lymphocyte transformation index test results in this current study was revealed the , Group VI (400Boric Acid + *P. vulagis* antigen) showed significant differences ($p < 0.05$) among all the studied and also Group V (600Boric acid + *P. vulagis* antigen) was showed significant differences against all the other studied Groups $p < 0.05$.due to the boric acid has activity on the adaptive immunity and this result agreed with[27] and with[6,9] that boron may help to proliferative the lymphocytes through stabilizing the Ag-receptor complexs on the lymphocyte surface, which lead to increase in response. Also the boron can stabilize the structure of lymphocyte enhancer factor-1, , which has been implicated in signaling and lymphocyte proliferations.[28]

Another mechanism that explains the effects of boron on lymphocyte proliferation is antioxidant potentials of boron compounds due to boron might act by counteracting the inhibitory effect of oxidative stress on lymphocyte proliferation. (Oxidative stress that prevents the transition from the G0 to the G1 phase in the cell cycle and leads to inhibit the lymphocyte proliferation response to phytohaemagglutinin).[29] The effect of boron on lymphocyte proliferation, also attributed due to its ability to counteract the inhibitory effects of oxidative stress on lymphocyte proliferation produced by *p. vulgaris antigen*, and this agreed[9] with who used OVA to stimulate the immune response.

Boron might help the lymphocytes to proliferate by increasing the antioxidant potential of the cell. In earlier studies, some substances like mercury and 1,25-dihydroxyvitamin D3 have reported to enhance OVA and ConA-induced proliferation of the lymphocytes.[30,31] The, *P. vulgaris* antigen-stimulated the T helper cell (CD4) and Cytotoxic T cell (CD8) T cell subsets in immunized animals[32,30] as in group II but in group V and VI were treated by boric acid and antigen showed increase Lymphocyte transformation because suggested the boric acid as the boron which capable to bind and stabilize the complex molecular structures, due to it acts as small molecules lead to increase strength of TCR (T Cell Receptor). Similarly, act to the BCR (B Cell Receptor) also CD19. The role of CD19 is a regulatory role in the proliferation and differentiation of the B cells.[33,34] CD19, in particular, regulates the basal signaling thresholds and accelerates BCR signals.[35] So the boric acid could act by modulating expression and/or functions of CD19, which may explain its effect on the host immune response as boron did finally these findings suggested the possible role of boric acid is same role of boron through acting as a costimulatory molecule lead to increase the strength of T and B cell receptors, thus augmenting the immune response and to increase in CD4 cells population. CD4 play important roles in immunity, particularly in the the adaptive immunity (Cellular immunity and humoral immunity) by helping other immune cells to release the cytokines, that are essential in B cells antibody class switching, also the activation and growth of CD8 (Cytotoxic T cell). CD4 also play a role in increasing the activity of phagocytes, as the macrophages.[34]

## CONCLUSION

Results of the present study revealed that boric acid had a positive immunomodulatory effect against immune response

in mice immunized with the whole-cell antigen of *P. vulgaris* against. As shown in The results of nitro blue tetrazolium index, lymphocyte transformation, strongly support such conclusions. The results of this study indicated boric acid was acted as a nonspecific immunostimulant, and it selectively activates the cell-mediated immune (CMI) mechanisms. The researchers continue their study to investigate the immunological effect of boric acid by using the same procedure and groups but using bacteria more pathogenic.

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