ARTICLE

Immune Modulating Effects of Malathion with the Role of Bradykinin Potentiating Factor in Juvenile Male Rats

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Abstract
Background: The use of malathion as an insecticide, is accompanied by the appearance of many adverse effects. **Objective**: To verify the immunological characteristics of malathion and to determine whether Bradykinin Potentiating Factor (BPF) is able to counteract these effects, and to emphasize the affected pathophysiological mechanisms. **Material and methods:** Juvenile rats were divided into three groups: animals received a vehicle (control group), malathion (malathion group), or malathion then BPF (BPF group). Each group included three subgroups, which were sacrificed two days after two, four, and six weeks of malathion exposure respectively. BPF subgroups were exposed to malathion, then treated with single, double, and triple successive IP injections of BPF respectively. **Results**: The concentration of total globulin, total immunoglobulins, IgG, IgM, circulatory immune complexes, total number of RBC & platelets, and hemoglobin concentrations decreased significantly in malathion-exposed animals. The number of total leucocytes and lymphocytes increased. Histopathological changes of bone marrow and spleen after malathion exposure were consistent with these findings. Recovery of bone marrow and splenic changes, normalization of peripheral blood elements, reduction of elevated proinflammatory markers (IL-2, IL-4 and TNF-α), total plasma peroxide and oxidative stress index (OSI) while increased total antioxidant capacity (TAC) were observed after double and triple injections of BPF. Our results suggest that exposure to malathion has negative effects on immune function that was mediated through alteration of cytokines, antioxidants and direct damage of BM. Also, BPF can ameliorate both physiological and morphological changes.

Keywords: Malathion, Bradykinin Potentiating factor, Immunity parameters, IL-2, IL-4, TNF-α, OSI and Acetylcholinesterase

Abbreviations
BPF, Bradykinin Potentiating factor; IL and TNF-α, interleukin, tumor necrosis factor-alpha; OSI, oxidative stress index; TAC, total antioxidant capacity; ACE, Acetylcholinesterase; BM, Bone marrow

Introduction
Malathion is sprayed in mega doses and is most commonly used to control mosquitoes, flies, household insects and human lice (1). It is lipophilic and readily absorbed through the skin, respiratory system, or gastrointestinal tract, with absorption enhanced if malathion is in liquid form (1). Analysis of dietary exposure to malathion indicates that this pesticide was found in 75.2% of solid food samples (2). The high food contamination rates and intended uses of malathion are likely to result in severe environmental and occupational pollution (3) that may lead to adverse effects on many organs (4). The known mechanism of organophosphate toxicity is inhibition of acetylcholinesterase (5), however, other mechanisms are still debated.

Kinins are members of peptide release from kininogens by the action of kallikreins (6). BPF of natural or synthetic origin activates acetylcholinesterase in the blood (6). Although Bradykinin is implicated in the development of immunity against parasites (7), it can induce lung inflammation and bronchoconstriction (8). The exact role of BPF on immunity is controversial and unclear. Accordingly, there is a critical need to verify the possible role of BPF on immune systems of malathion-exposed animals. In this study, we hypothesized that BPF may have a role in immune suppression induced by malathion. The rationale behind this investigation was to characterize the alteration in immune response following administration of one of the most common environmental pollution agents, malathion, and the potential role of BPF in immune suppression animal models, as well as the possible pathophysiological mechanisms involved.

Material and Methods
Many studies have used highly purified malathion (2), while this study investigated the effects of a commercial formulation of it that is similar to the malathion used in the location. Immune suppression model was induced by malathion and the effects of BPF were studied by evaluating: (i) total globulin, immunoglobulins (total, IgG, IgM) and circulating immune complex; (ii) number and differentiation of leucocytes in peripheral blood and bone marrow (BM), (iii) number of RBCs, hemoglobin concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and number of platelets, (iv) Cytokine (IL-2, IL-4, TNF-α). (v) TAC, total peroxide and OSI, and (vi) histological changes in BM and spleen.

Chemical
Malathion (Sigma Chemical, Co., St Louis, MO), a 57% formulation of malathion, was evaluated for effects on immune status in rats. This formulation is used to control insect pests in the home and garden. Bradykinin Potentiating factor was obtained from (Sigma Chemical, Co., St Louis, MO).

Animal care and maintenance
White male Albino rats (n = 54) six weeks old, disease free, weighing 100 ± 10 g, were procured and maintained in the Animal Nutrition and Care House, Assiut University. They were randomly assigned to treatment cages (two per cage) and subsequently allowed to acclimatize for one week. The rats were housed in polycarbonate shoebox-style cages which were changed every third day. Rodent chow and water were supplied ad libitum. The housing facility was maintained at 25 °C with a 12:00 h light, 12:00 h dark cycle. Food and water consumption as well as body weight gain were monitored for the duration of each experiment. Proper hygiene conditions were maintained in the cages. The animals were treated in accordance with the published guidelines established by the Assiut Council on Laboratory Animal Care, and the experimental protocol was approved by the Institutional Animal Use Committee of Faculty of Medicine, Assiut University, Assiut, Egypt.

Exposure to malathion and treatment with BPF
Malathion prepared in a saline vehicle was administered to rats at does of 10 mg/kg by oral gastric tube at 8 a.m. (9) on alternate days. This dosing schedule was selected in an attempt to copy the intermittent nature of pesticide exposure. The animals were alienated randomly and equally into three (I, II and III) groups. Group II (malathion group) was divided equally into three subgroups (A, B and C) that were exposed to malathion for two, four and six weeks respectively, while group I (control) received the vehicle only. Animals in group III (malathion then BPF group) were exposed to the same dose and duration of malathion after that, subgroups A, B, and C were treated with single, double, and triple IP injections of 1 ug/ gram body weight of BPF/ day (8 a.m.), in successive days, after 2, 4 and 6

weeks of malathion exposure respectively (10). The body weight of the animals was recorded weekly. The dose of malathion was revised accordingly and increased as the animals gained weight.

Body weight
All animals were weighed at the beginning of the experiment (baseline) and weekly at fixed times (8 a.m.) throughout the entire course of the experiment. The initial body weight of different groups was not significantly different.

Laboratory measurements
Blood sample collection
Two days after the last medication, the animals were anesthetized, peripheral blood samples were taken immediately before decapitation from the ophthalmic vein using sterile 1-ml polypropylene syringes equipped with 26-gauge/12- mm or 27-gauge/20-mm needles. Each blood sample was divided into three portions. One portion was taken in EDTA tubes without freezing for leucocytes, RBCs, platelet count, and differentiation. The second part was placed into EDTA tubes and centrifuged at 3000 rpm for 10 min. to separate plasma and to detect IL2 & 4 & TNF-α, total antioxidant capacity& oxidant status, oxidative stress index and acetylcholine activity. The third portion of blood was left in a clean tube for clotting then serum was separated by centrifugation for 30 minutes at 3000 rpm and stored at – 20 ^{0}C until used for estimation of total globulin, circulating immuncomplex, and immunoglobulins (total immunoglobulin, IgG and IgM)

*Total globulin was determined as following:
Globulin= total protein – albumin gm/dl. Determination of total serum protein and albumin were performed according to Peters (11) and Doumas et al., (12) methods respectively by using a spectrophotometer.

*Total immunoglobulins were estimated by ammonium sulphate precipitation (13).

*Circulating immune complexes were estimated by PEG precipitation (14).

*IgM and IgG were estimated by radial immunodiffusion technique (15).

*WBCs, RBCs and platelets were counted according to the method described by Harris et al., (16) in which hematological analysis was carried out by means of Veterinary Hematology Analyzer (Medonic CA 620, Sweden). A drop of blood was spread on blood film, then stained by Giemsa stain for differential leukocyte counts.

*Enzyme-linked immunosorbent assays (ELISA) were performed to measure concentrations of TNF-α (Cat. No. KAC 1571), IL-2 and IL-6 (Cat. No. M1916 and M 1915) using Biosurce Europe commercial kits with monoclonal antibodies against each substance and following the instructions supplied with each kit. The apparatus used was Ansoth 2000, manufactured in Austria.

*Measurement of the TAC of plasma: This was performed by reaction of antioxidants in samples with a defined amount of exogenously provided hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminated a certain amount of the provided hydrogen peroxide. The residual (H_2O_2) is determined colorimeterically by an enzymatic reaction which involves the conversion of 3, 5-dichloro-2-hydroxybenzensulphonate to a colored product (17).

*Measurement of total plasma peroxide concentration: This was done by using the ferrous oxidation in xylenol orange by spectrophotometer (18). The coefficient of variation for individual plasma samples was <5%.

*Measurement of OSI: The ratio percentage of the total peroxide to the total antioxidant potential gave the OSI, an indicator of the degree of oxidative stress (19).

*Estimation of plasma AChE activity: Blood samples were collected in the morning, between 5:30 and 9:30 a.m. to minimize possible diurnal variation in enzyme activities (20).Time from blood collection to storage of plasma never exceeded 4 hr and a constant temperature of 25 °C was maintained for the entire procedure to eliminate temperature-induced changes in enzyme activity. The AChE levels were determined colorimetrically in plasma according to the method of Venkataraman et al. (21).

Histological assessment
BM was aspirated by sterilized syringe from the femur for light microscopic and ultrastructural examinations. Specimens from the spleen were trimmed and fixed in neutral buffered formalin and stained for general light microscopic examination.

Statistical analysis
Data are expressed as mean ± SD for all parameters.

The data were analyzed by using GraphPad Prism data analysis program (GraphPad Software, Inc., San Diego, CA, USA). For the comparison of statistical significance between different groups, Student Newman-Keuls t-test for unpaired data was used. For multiple comparisons, one-way analysis of variance (One-Way-ANOVA) test followed by Least Significant Difference (LSD) was used. Values were accepted as being statistically significant if a P value was <0.05.

Results

A summary of results is shown in Tables 1-2, and figures 1-5.

Exposure to malathion was associated with an insignificant increase of body weight after two, four, and six weeks in all subgroups (145± 16.43, 181.7 ± 8.2, 225 ±10.5 gm) when compared with corresponding age- and sex-matched controls (140±8.94, 176.7± 8.17, 217 ± 12.11 gm). Similarly, administration of BPF after malathion exposure did not modify body weight (146.7±17.8, 180 ± 6.325 and 231.7 ± 14.72 gm) in different subgroups when compared with

Table 1: Comparison mean value ± SD of total globulin, total immunoglobulin, IgM, IgG and circulating immune complex after treatment by BPF in juvenile male rats exposed to malathion.

Groups Items	Control (I)	Malathion (II)	Malathion then BPF (III)
Total globulin (gm/dl)	(I A) 2.50 ± 0.3 (I B) 2.60 ± 0.1 (I C) 2.63 ± 0.22	(II A) 2.0 ± 0.21** (II B) 1.8 ± 0.15 *** (II C) 1.5 ± 0.3 ***	(III A) 2.0 ± 0.21 (III B) 1.95 ± 0.08 (III C) 1.57 ± 0.2
Total immuno- globulin (gm/dl)	(I A) 0.98 ± 0.14 (I B) 1.10 ± 0.31 (I C) 1.10 ± 0.31	(II A) 0.82 ± 0.17 NS (II B) 0.75 ± 0.11 * (II C) 0.67 ± 0.12 *	(III A) 0.83 ± 0.12 (III B) 0.90 ± 0.06 € (III C) 0.87 ± 0.082 €€
IgM (IU/L)	(I A) 1.68 ± 0.26 (I B) 1.6 0± 0.33 (I C) 1.65 ± 0.27	(II A) 0.83 ± 0.22*** (II B) 0.80 ± 0.14*** (II C) 0.62 ± 0.15 ***	(III A) 0.78 ± 0.21 (III B) 1.03 ± 0.14 € (III C) 0.9 ± 0.19 €
IgG (IU/L)	(I A) 14.49 ± 0.39 (I B) 14.27 ± 0.3 (I C) 14.25 ± 0.36	(II A) 13.05 ± 0.61*** (II B) 12.00 ± 0.63 *** (II C) 11.80 ± 0.47 ***	(III A) 13.05 ± 0.18 (III B) 13.15 ± 0.42 €€ (III C) 13.20 ± 0.7 €€
Circulating immune complexes (g/dl)	(I A) 1.90 ± 0.14 (I B) 1.80 ± 0.12 (I C) 1.79 ± 0.10	(II A) 1.95 ± 0.19 (II B) 1.50 ± 0.301 * (II C) 1.40 ± 0.19 ***	(III A) 1.90 ± 0.24 (III B) 1.90 ± 0.17 € (III C) 1.97 ± 0.19 €€€

Means ± SD were analyzed by a Student–Newman–Keuls t-test , Subgroup A, B and C of rats, sacrificed after 2,4 and 6 weeks after exposure to malathion. Brady kinin poteniating factor, BPF subgroups (A, B and C) exposed to malathion then treated with BPF one, double and triple successive injections respectively. (n=6 in each subgroup) , (*) Significance versus control group. (€) Significance of values in BPF subgroup versus its corresponding malathion subgroup, ***/ (€)(€)(€) significant difference at P<0.001, , **/(€)(€) significant difference at P<0.01, ,*/ (€)) significant difference at P<0.05. The remaining difference was not significant (P>0.05).

malathion subgroups.

Results of the measured laboratory parameters
Relation to total globulin, total immunoglobulin, IgM, IgG and circulating immune complex (Table 1, Figure 1):
Serum levels verified a decrease in all malathion exposed subgroups and the reduction was significant (P < 0.001) especially in subgroup B and C that were exposed to malathion for four and six weeks when compared to corresponding age- and sex-matched control subgroups. Treatment with BPF ameliorated this reduction and elevated serum levels significantly (P < 0.001), especially in subgroups B and C that received two and three successive doses of BPF after four and six weeks of malathion exposure

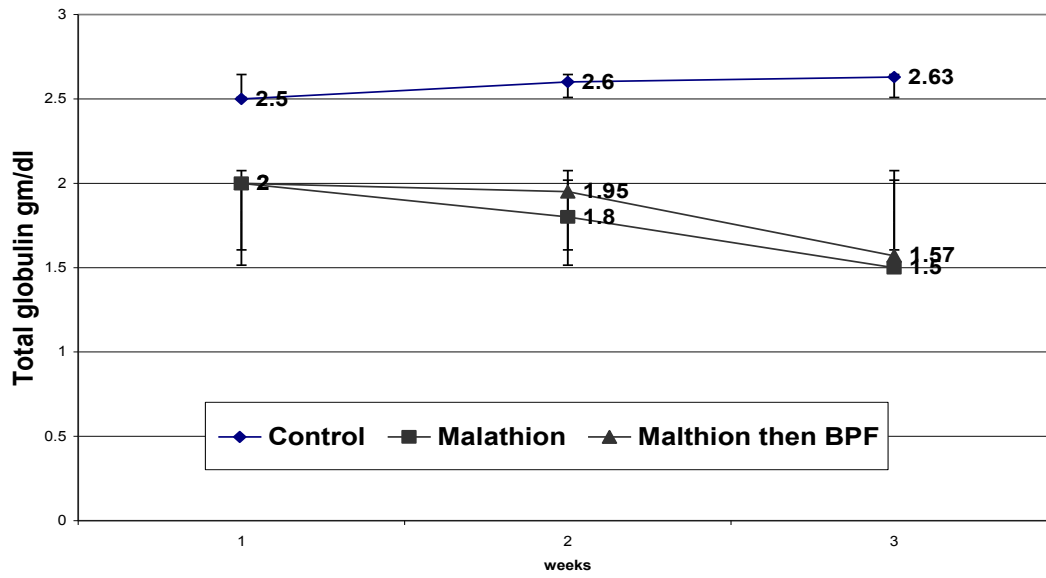| Table 2: The effects of treatment by BPF on mean value ± SD of erythrocytes (RBCs) number , hemoglobin (Hgb) concentration, packed cell volume (PCV%), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC %) and platelet numbers in juvenile male rats exposed to malathion.		
Groups	Control (I)	Malathion (II)	Malathion then BPF (III)
RBCs X 10^6	(I A) 5.5 ± 0.37 (I B) 5.2 ± 0.56 (I C) 5.3 ± 0.23	(II A) 4.2 ± 0.17*** (II B) 3.2 ± 0.6 *** (II C) 2.8 ± 0.5 ***	(III A) 4.4 ± 0.31 (III B) 4.5 ± 0.2 *** (III C) 4.7 ± 0.6 ***
Hgb(gm/dl)	(I A) 11.88 ± 0.4 (I B) 11.72 ± 0.2 (I C) 11.70 ± 0.26	(II A) 8.9 ± 0.63 *** (II B) 7.7 ± 0.82 *** (II C) 7.2 ± 0.49 *	(III A) 8.92 ± 1.5 (III B) 10.28 ± 0.77 *** (III C) 10.52 ± 1.1 ***
PCV (%)	(I A) 30.33±1.6 (I B) 30±1.4 (I C) 29.17± 1.7	(II A) 25.8± 1.8 ** (II B) 21.7± 1.6 *** (II C) 18.5± 1.3 ***	(III A) 27.17 ± 1.6 (III B) 26.80 ± 1.6 *** (III C) 27.80 ± 1.8 ***
MCV (fl)	(I A) 50.3 ± 1.2 (I B) 50.1 ± 1.4 (I C) 49.8 ± 1.5	(II A) 48.7 ± 2.16 (II B) 48.5 ± 1.38 (II C) 48 ± 1.4	(III A) 49.17± 2.32 (III B) 49± 1.6 (IIIC) 49.5± 1.9
MCH (pg)	(I A) 20.1 ± 0.91 (I B) 19.9 ± 1.3 (I C) 20.4 ± 1.2	(II A) 19.4 ± 0.6 (II B) 19.1 ± 0.96 (II C) 19.4 ± 1.1	(III A) 19.3± 1.0 (III B) 19.98 ± 1.5 (III C) 19.87 ± 1.4
MCHC g/dl	(I A) 39.7 ± 1.0 (I B) 39.5 ±1.0 (I C) 40.33± 1.4	(II A) 40.33± 1.6 (II B) 39.5 ± 1.1 (II C) 39.8± 1.5	(III A) 40.5 ± 1.1 (III B) 39.33 ± 1.6 (III C) 40.5 ± 1.4
Mean platelets X 10^3/ mm^3	(I A) 482 ± 3.9 (I A) 483 ± 2.5 (I A) 483 ± 2.3	(II A) 481 ± 6.1 (II A) 430 ± 5.5 *** (II C) 405.3 ± 3.5 ***	(III A) 477.7 ± 6.9 (III A) 442.7 ± 8.98 * (III A) 422 ± 4.0 ***
Means ± SD were analyzed by a Student–Newman–Keuls t-test , Subgroup A, B and C of rats, sacrificed after 2,4 and 6 weeks after exposure to malathion. Brady kinin poteniating factor, BPF subgroups (A, B and C) exposed to malathion then treated with BPF one, double and triple successive injections respectively. (n=6 in each subgroup) , (*) Significance versus control group. (€) Significance of values in BPF subgroup versus its corresponding malathion subgroup, ***/ (€)(€)(€) significant difference at P<0.001, , **/(€)(€) significant difference at P<0.01, ,*/ (€)) significant difference at P<0.05. The remaining difference was not significant (P>0.05)		

Figure 1A
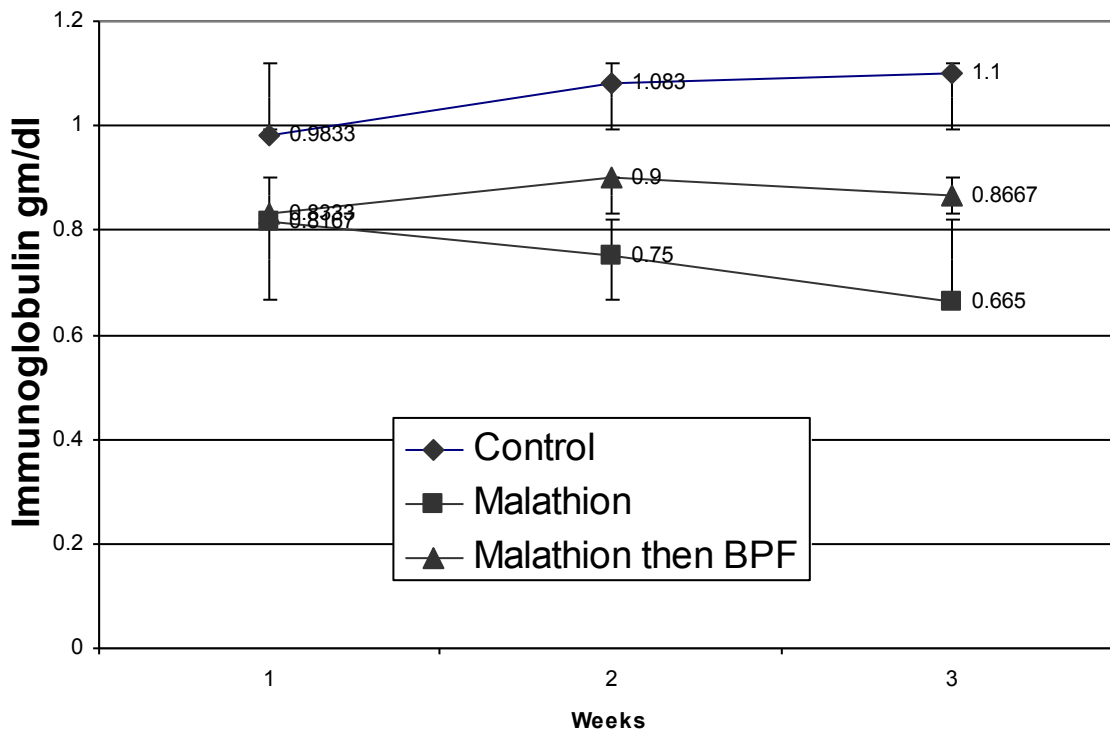
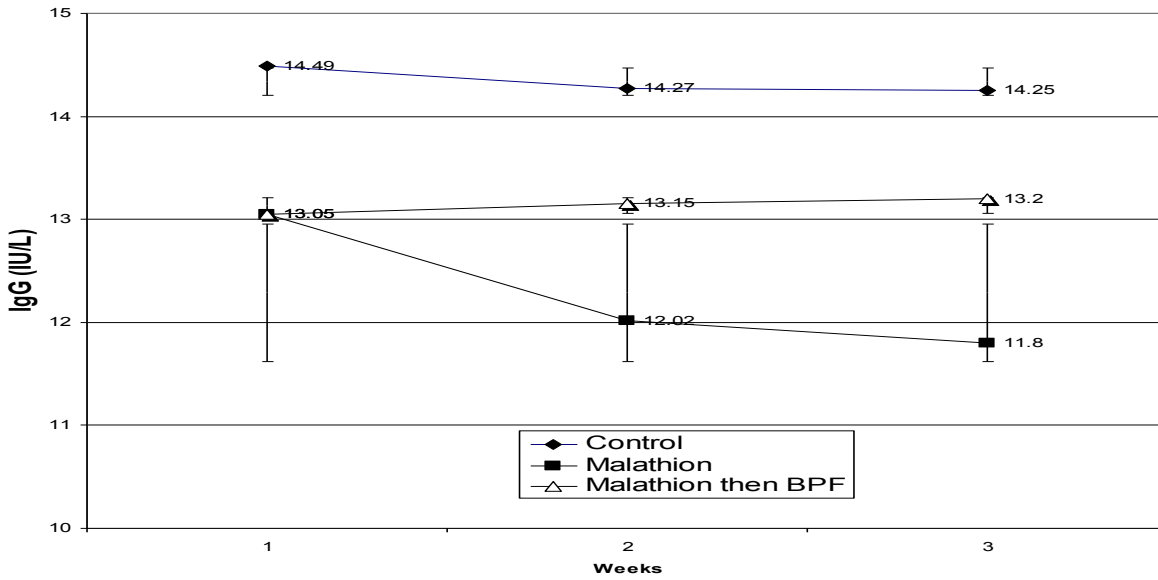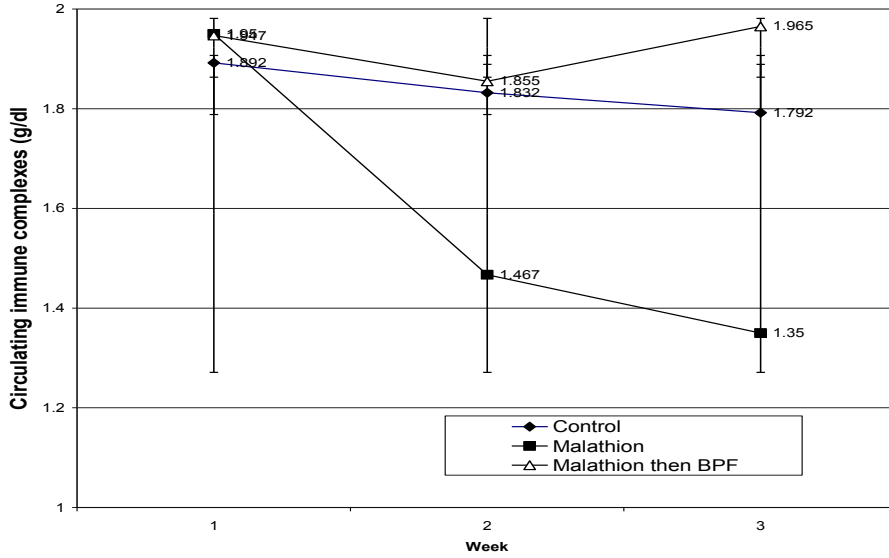**Figure 1B**
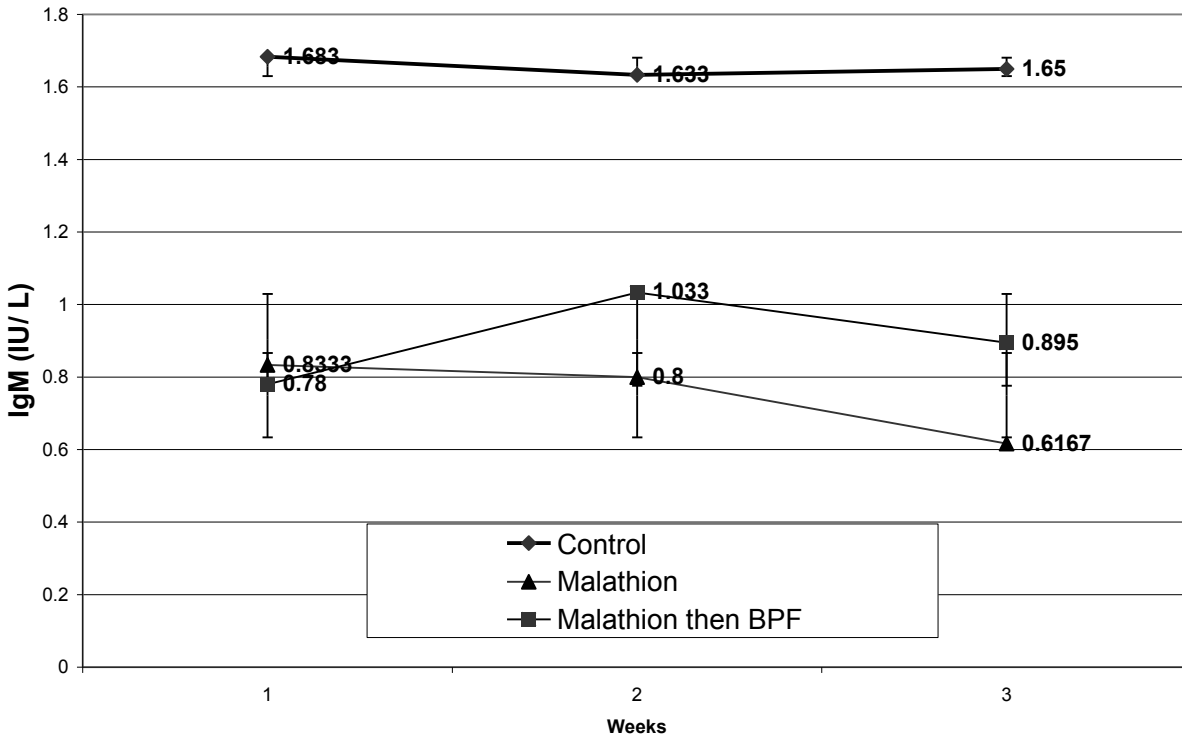
Figure 1C

Figure 1D

Figure 1E

Figure 1. Time course alteration in mean values ± SD of total plasma globulin (A), total immunoglobulin (B), IgM (C), IgG (D) and circulating immune complex (E) after treatment with BPF in juvenile rats exposed to malathion. Animals were divided into three groups: animals received a vehicle for 6 weeks (control group), or malathion orally for 6 weeks (malathion group), or malathion then BPF (1 ug/gm IP) (BPF group). Malathion subgroups A, B and C of rats, sacrificed 2 days after 2,4 and 6 weeks of exposure to malathion. BPF subgroups (A, B and C) exposed to malathion for 2, 4 and 6 weeks respectively then treated with BPF one, double and triple successive injections respectively. (n=6 in each subgroup). Treatment with double and triple injections of BPF ameliorated the reduction in immune parameters and elevated their serum levels significantly (P < 0.001).
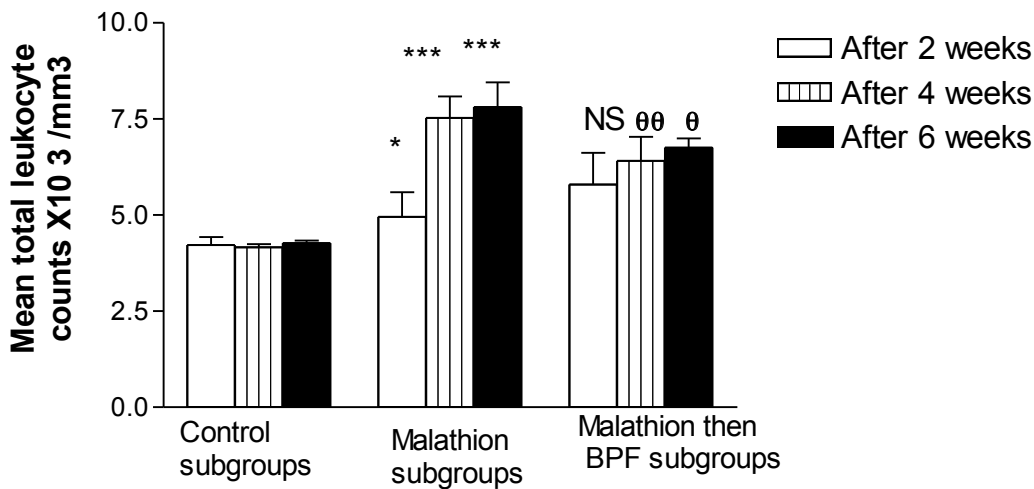
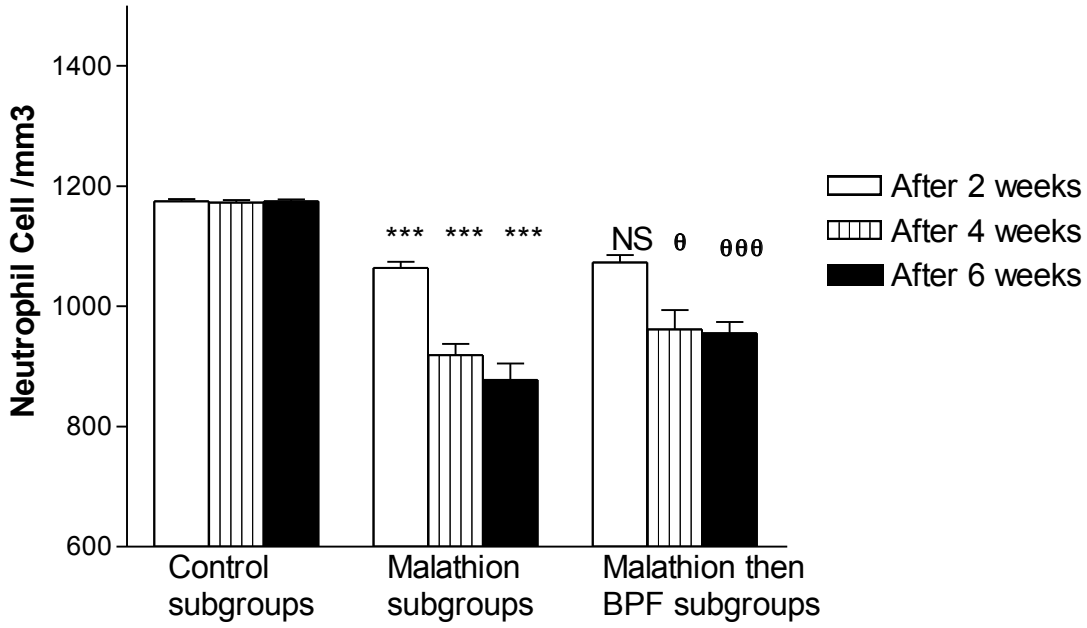

Figure 2A

Figure 2B

respectively.   *Relation to RBC numbers, hemoglobin (Hgb), PCV%,*
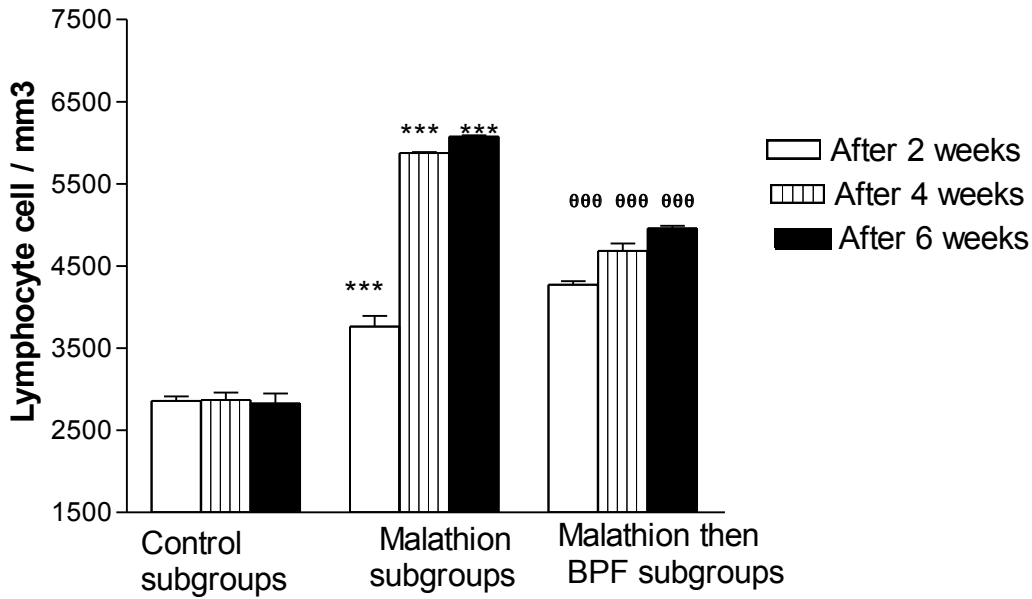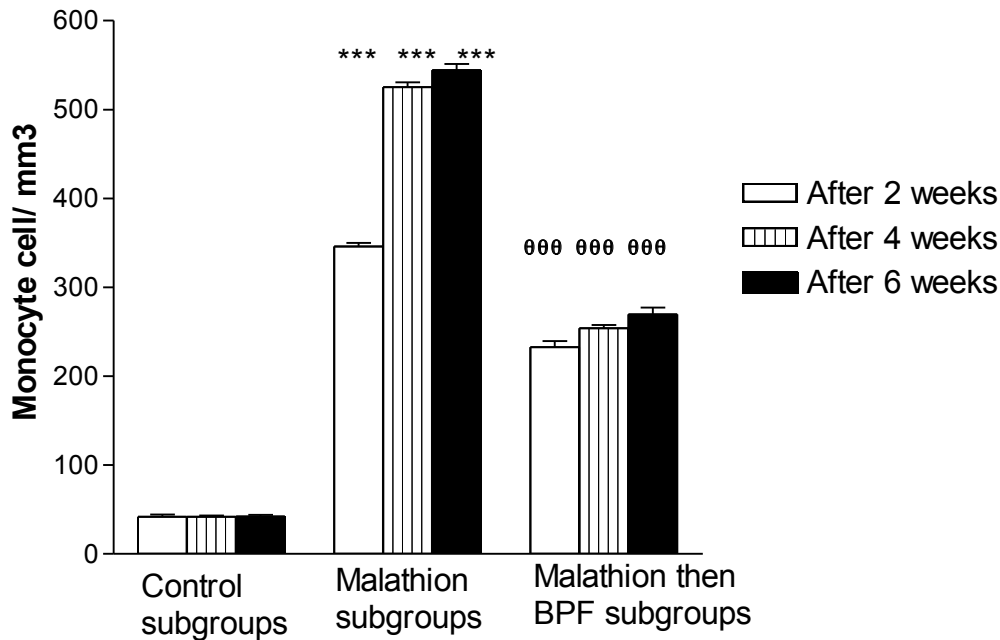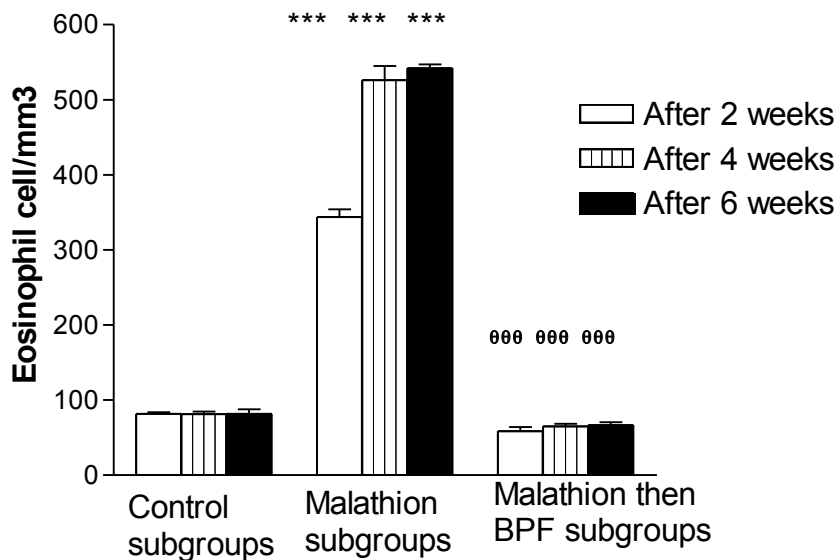

Figure 2C

Figure 2D

Figure 2E

Figure 2. Effect of BPF on the mean values of total leukocyte counts X10 3 /mm3 (a), neutrophil Cell /mm3 (b), lymphocyte cell / mm3 (c), monocyte cell/ mm3 (d), Eosinophil cell / mm3 (e) in mice exposed to malathion. Animals were divided into three groups: animals received a vehicle for 6 weeks (control group), or malathion orally for 6 weeks (malathion group), or malathion then BPF (1 ug/gm IP) (BPF group). Malathion subgroups A, B and C of rats, sacrificed 2 days after 2,4 and 6 weeks of exposure to malathion. BPF subgroups (A, B and C) exposed to malathion for 2, 4 and 6 weeks respectively then treated with BPF one, double and triple successive injections respectively. (n=6 in each subgroup). Exposure to malathion increased significantly total leukocyte, Lymphocyte cell, monocyte and Eosinophil while decreased significantly the neutrophil cells. Administration of double and triple injections of BPF normalized significantly (P < 0.001) the variation in WBCs and their differentiation. Values were analysed using a Student–Newman–Keuls t-test and were expressed as mean ± SD.
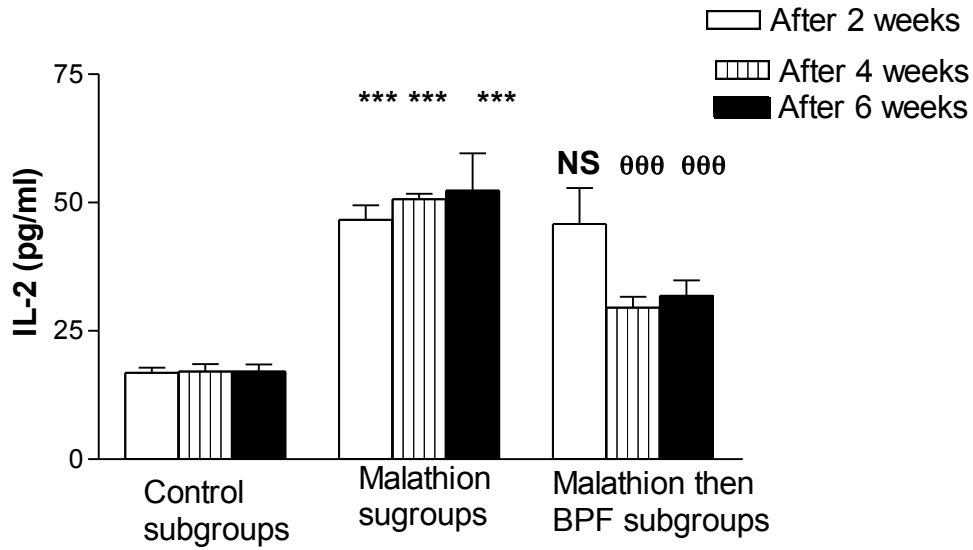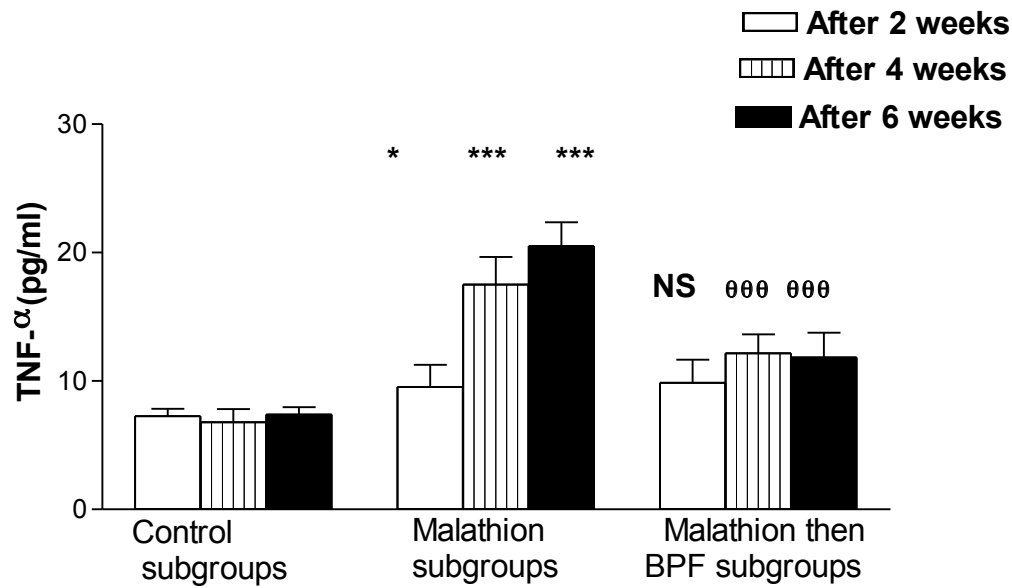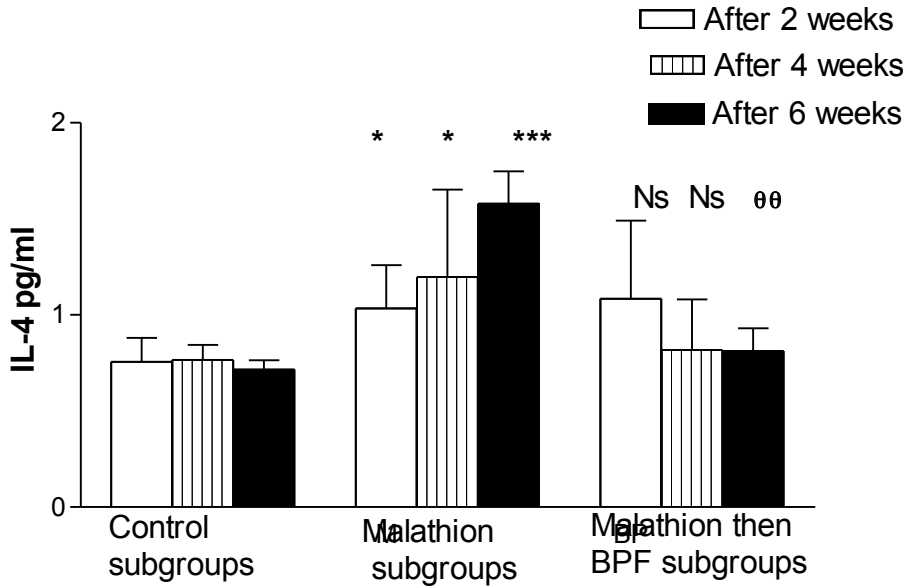
Figure 3A

Figure 3B

Figure 3C

Figure 3. Effect of BPF on the mean values of plasma IL-2 (a), TNF-α (b) and IL-4 (c) in mice exposed to malathion. Animals were divided into three groups: animals received a vehicle for 6 weeks (control group), or malathion orally for 6 weeks (malathion group), or malathion then BPF (1 ug/gm IP) , BPF group. Malathion subgroups A, B and C of rats, sacrificed 2 days after 2,4 and 6 weeks of exposure to malathion. BPF subgroups (A, B and C) exposed to malathion for 2, 4 and 6 weeks respectively then treated with BPF one, double and triple successive injections respectively. (n=6 in each subgroup). Administration of double and triple injections of BPF reduced significantly (P < 0.001) the elevated proinflammatory markers after exposure to malathion. Values were analysed using a Student–Newman–Keuls t-test and were expressed as mean ± SD.
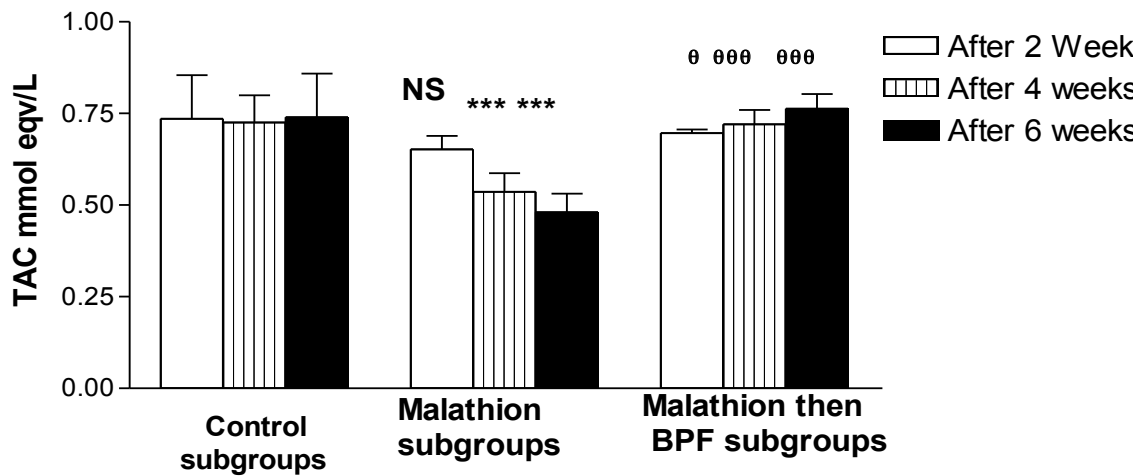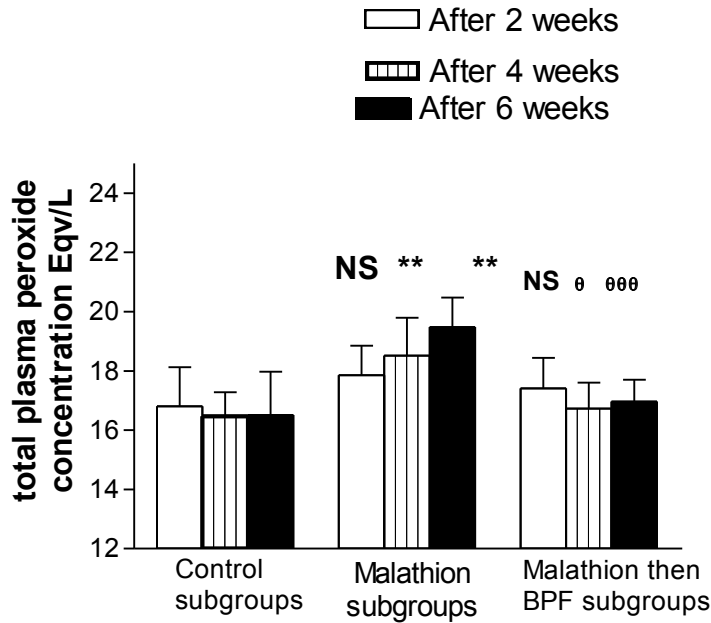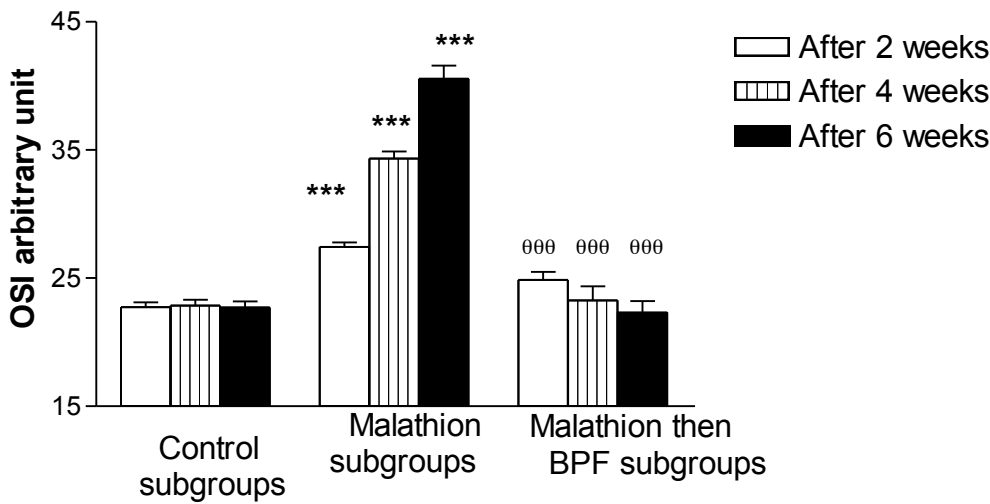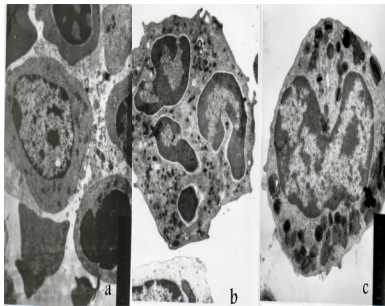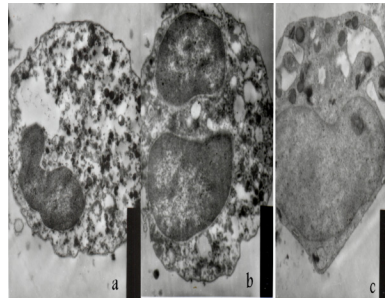

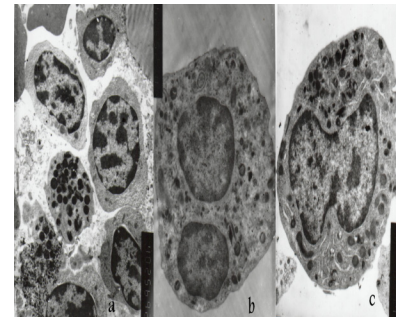**Figure 4A**

Figure 4B

Figure 4C

Figure 4. Effect of BPF on the mean values of TAC (total antioxidant capacity Fig 4 a), total plasma peroxide concentration (Fig. 4 b) and OSI (oxidative stress index Fig. 4 c) in mice exposed to malathion. Animals were divided into three groups: animals received a vehicle for 6 weeks (control group), or malathion orally for 6 weeks (malathion group), or malathion then 1 ug/gm IP injection of BPF, BPF group. Malathion subgroups A, B and C of rats, sacrificed 2 days after 2,4 and 6 weeks of exposure to malathion. BPF subgroups (A, B and C) exposed to malathion for 2, 4 and 6 weeks respectively then treated with BPF one, double and triple successive injections respectively after that they were sacrificed 2 days after. (n=6 in each subgroup). Exposure to malathion increased significantly plasma peroxide concentration and OSI while decreased significantly the total antioxidant capacity. Administration of of BPF compacted significantly (P < 0.001) these changes and decreased oxidative stress. Values were analysed using a Student–Newman–Keuls t-test and were expressed as mean ± SD.
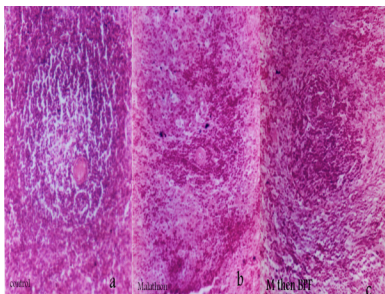
5A

5B

5C

5D

Figure 5A: Electron microscopic examination (EME) of control bone marrow showing (a) a proerythroblast (pro) with less dense cytoplasm and large nucleus with prominent nucleolus (b) segmented neutrophilic granulocyte containing condensed nucleus formed of four lobes (c) eosinophilic granulocyte with bilobed nucleus. (x5000)

Figure 5B: EME of degenerated B.M cells after malathion exposure for 4 weeks showed (a) stab neutrophil granulocyte with small eccentric nucleus and multiple cytoplasmic empty spaces, (b) nutrophilic cell with irregular cytoplasmic granules containing heterogenous contents. Marked dilatation in rER is observed, (c) eosinophil in metamelocyte stage, its cytoplasm shows multiple dilated cisternae of rER and few cytoplasmic granules, Pale kidney shaped nucleus is noticed(N). (x5000)

Figure 5C: EME of BM after treatment with BPF showing partial recovery of BM cells in comparison to Malathion treated BM. (x5000)

Figure 5D: Photomicrograph of white pulp of the (a) control rat spleen showing central artery surrounded by the periarterial lymphoid sheath with normal number and distribution of lymphocytes within the lymphatic nodule, (b) Malathion treated rat showing shrinkage and distortion of the lymphatic follicles with moderate degree of scattered lymphocyte apoptosis, (c) BPF treated rat showing normal appearance of lymphoid follicles. (H&E stain x200).

MCV, MCH, MCHC % and platelet numbers (Table 2):
Exposure to malathion for two, four and six weeks induced a significant (P < 0.001) decrease in mean values of RBCs, Hgb concentration, PCV %, and platelets, however the MCV, MCH and MCHC did not diminish significantly when compared to corresponding controls. In addition, administration of BPF improved significantly (P < 0.001) the number of RBCs, Hgb concentration, PCV%, and platelets in subgroups B and C versus malathion subgroups.
Relation to total leucocytes and their differential cells (Figure 2): Assessment of WBC numbers revealed increasing significance (P < 0.001) of total leucocytes, lymphocytes, monocytes, and eosinophils, while decreasing significantly (P < 0.001) the neutrophils after exposure to

malathion in all subgroups, even after two weeks of malathion exposure ,when compared with corresponding control values. Administration of double and triple injections of BPF normalized significantly (P < 0.001) the WBCs and their differentiation as compared to malathion-exposed subgroups.

Relation to plasma IL-2, IL-4, and TNF-α (Figure 3):
These increased significantly after two, four, and six weeks exposure to malathion versus controls. Treatment with double and triple injections of BPF significantly decreased (P < 0.001) the elevated inflammatory markers when compared to malathion subgroups.

Relation to TAC (total antioxidant capacity): total plasma

peroxide concentration, and OSI (oxidative stress index): Exposure to malathion significantly increased (P < 0.001) total plasma peroxide concentration and OSI while it significantly decreased (P < 0.001) total antioxidant capacity as compared to control levels. After treatment with BPF, these changes corrected significantly (P < 0.001), as shown in Figure 4.

Relation to AChE: Plasma levels of AChE activity revealed a significant (P < 0.01, P < 0.001) unpredicted increase in subgroups A and B (11.73±1.19, 12.07±1.2 U/ml) after exposure to malathion versus the values of corresponding controls (9.35±0.61, 9.367 ±0.5 U/ml). While in subgroup C, the animals exposed to 10 mg/kg malathion on alternate days for six weeks showed dramatic inhibition of acetylcholine activity, which was observed (5.3± 0.82 U/ml) when compared with its corresponding control value (9.8± 0.77 U/ml). Treatment with BPF revealed non-significant changes in all subgroups (11.33±0.61, 11.33±0.98, 5.87±1.1) when compared with malathion-exposed subgroups.

Results of histological examination: It showed degenerated BM cells after malathion exposure for four weeks in subgroup B (Figure5B) when compared to the control group (Figure 5A), while after treatment with BPF partial recovery of BM structure was evident (Figure 5C). In addition, malathion-treated rats showed shrinkage and distortion of the lymphatic follicles of the spleen with a moderate degree of scattered lymphocyte apoptosis, while BPF-treated rats showed normal appearance of lymphoid follicles (Figure 5D).

Discussion

The immune system is very sensitive to a variety of chemical and physical stressors that interfere with its function. Nevertheless, the effects of malathion on the immune system have produced conflicting results as Casale et al. (22) reported that acute oral exposure to high doses of purified malathion produces immunosuppression while exposure to low doses elevated the humoral immune response (23).

Although, BPF was implicated in many physiological aspects, its effect on immune function and the mechanisms involved remain obscure. In this work, we hypothesized that BPF may minimize the changes of immune systems in animals exposed to malathion.

Juvenile (six weeks) male rats were used in this study to verify the adverse effects of malathion on immunity since they are more sensitive and were more exposed to mala-

thion. This was supported by findings of Adgate et al. (24) who reported that in human beings urinary levels of malathion in children was higher and detected more frequently than in adults. Reduction of immune related parameters (total globulin, total immunoglobulin, IgM, IgG and circulating immune complex) after every-other-day malathion exposure confirms its immune suppression effects, which were ameliorated to great extent by application of double and triple injections of BPF. This suppression was confirmed by histological examination of BM that show degeneration of most BM cells with shrinkage and distortion of lymphatic follicles of the spleen. This suppression was in line with studies of Banerjee et al. (3), Sodhi et al. (9) and Vandana et al. (25). Normalization effects of BPF on suppressed immunoglobulin, IgM, IgG has been confirmed by Ozturk (26), as BPF can activate glycogen synthetase enzyme in the liver which increases liver glycogen, increases liver protective effects and potentiates detoxification efficiency. Besides, BPF can increase immunoglobulin production from BM or plasma cells either directly or through the cytokine regulation (27).

In the current investigation, the ability of malathion exposure to significantly reduce RBCs, Hgb concentration, PCV %, and platelet numbers is in agreement with the results of Kundu and Roychoudhury (28). However, MCV, MCH and MCHC decreased insignificantly after malathion exposure which suggests the possibility of destruction of BM as the primary cause of hematological abnormalities. It is known that normocytic, normochromic anemias (in which MCH, MCV and MCHC are changed insignificantly,) are caused either by hemolysis, hemorrhage or BM suppression. We excluded hemolysis and hemorrhage by clinical examination and absence of fragmented RBCs in blood film, so the possible suggested cause is BM suppression, which was confirmed by the histopathological degeneration of BM cells recorded in the current study. Moreover, Mansour et al. (29) reported a significant increase in the percentage of chromosomal aberrations in rat bone marrow cells after malathion exposure. Treatment with BPF was effective in restoring the depressed hematological parameters that may be related to modulation in secretion of erythropoietic factors, as IL6, TNF-α that were induced by bradykinin through a receptor-mediated process (30 and 31).

In addition, treatment with BPF caused an increase in platelet count with slight initial recovery after the first dose that progressed after second and third doses. This result was in line with other investigators, such as Shigematsu et al. (32) who found that bradykinin activates newly produced platelets through formation of active mediators

such as platelet activating factor which counterbalances the suppression effects of malathion. More interestingly, a significant increase (P < 0.001) in circulating total leukocyte counts was noted in the present study throughout the malathion exposure period, in addition to lymphocytosis, monocytosis and eosinophilia. This suggests a direct stimulation of the immunological defense due to tissue damage by malathion, as supported by results of Kundu and Roychoudhury (28). Treatment with double doses of BPF ameliorated the changes in leucocyte counts because it enhanced the activity of endogenous bradykinin and its modulation effects on secreted cytokines. Other experimental results supported this finding as noted by Ana et al. (33) reported that Bradykinin receptor 2 is critically required for development of acquired immunity.

In the current investigation, a significant increase in the serum levels of IL-2, IL-4 and TNF-α after exposure to malathion suggests involvement of malathion with lymphocyte proliferation and activation that concurs with the result of Vandana et al. (25). Several investigators support this finding (25,34). Cytokines are the cellular mediators of immunity that synthesize in lymphoid and non-lymphoid cells. Many cytokines induce or inhibit other immune mediators, therefore up-regulation of cytokines is a vital contributor for pathophysiological mechanisms of immune suppression after malathion exposure. Vandana et al. (25) reported that the T helper 1 (Th1) cytokines (IL-2) have important roles in cell mediated immunity and chronic inflammation. The helper CD4 cells regulate the humoral immunity, and B cells produce immunoglobulins (34). Macrophages, lymphocytes, parenchyma cells in pathophysiological situations secrete TNF-α that was not only involved in inflammation and cellular response, but also provided defense against intracellular pathogens. While IL-4 promotes humoral immunity, it is necessary for abolition of extracellular pathogens by stimulation of B cell growth (35). Malathion modulates the immune reaction via enhancement of Th1 response with increases (IL-2 and TNF-α) that induce B cell maturation as well as humoral immunity via IL-4. These increases in leukocytes, lymphocytes and the secreted cytokines may be as a compensatory mechanism for the recorded suppression in BM produced hematological cells and immunoglobulins after malathion exposure. Therefore, these cytokines may be suitable markers for detecting immune suppression effects of malathion. These changes in humoral immunity were likely due to specific actions of malathion or its metabolites on cells of the immune system. Similar results have been reported by the previous study (23). On the other hand, Kaman et al. (7) and Zabrodskii et al. (36) reported that subacute exposure of rats to malathion suppressed both cell and humoral immune responses, and significantly decreased the level of blood cytokines. This contradiction may be related to dose, form, species, purity, and time-dependent factors (23). Treatment with BPF improved the elevated cytokines after malathion exposure in subgroup B and C that received double and triple doses. Huang et al. (37) and Donghoon et al. (38) verified that bradykinin B2 receptors were expressed in human astrocytoma cells and modulated the expression pattern of inflammatory cytokines.

The present study showed that BPF can blunt the oxidative stress after malathion exposure as it decreases this significantly, and in addition decreases total peroxide and OSI, while increasing antioxidant capacity. Sharma et al. (39), Trevisan et al. (40) confirmed that malathion is a pro-oxidative agent that can induce oxidative stress by generating free radicals and altering antioxidant free radical scavenging enzymes. The protective role of BPF against oxidative stress is in line with the studies of Yoshihisa et al. (41) and Hisko et al. (42) who found that bradykinin protects endothelial cells from oxidative stress through bradykinin B2 receptor– and NO-mediated inhibition of DNA damage.

In the current investigation, we found an unexpected increase of plasma ACh E after two and four weeks of malathion exposure, while we noted depressed activity after six weeks. This concurs with results of Trevisan et al. (40) who reported increased hippocampal and cortical AChE activity in rats after repeated low-dose malathion exposure. This finding is in accord with the concept that, after repeated low doses, malathion is able to produce an adaptive response. Also, this pathophysiological increase in AChE activity occurs in order to maintain the homeostasis of the cholinergic system. However, in this study after more extended treatments for six weeks, the AChE inhibition would be more prevalent which was supported by the study of Ramos et al. (43). Treatment with BPF in all subgroups had no significant effect on AChE activity, which predicts the protective role of BPF is not through AChE activity.

Thoroughly, immune suppression effects of malathion exposure proofed in the present study have been mediated by the following possible pathophysiological mechanisms: alteration of oxidant–antioxidant balance system, shift the inflammatory cytokines, and direct injury on BM and splenic tissue. Also, BPF can reverse these mechanisms and protect the body from this frequently used environmental pollution agent.

It is also important to note that body weight remained unaffected after exposure to malathion or application of BPF, and can be excluded as a factor responsible for the observed immune suppression which is in line with other studies (44). In addition, Johnson et al. (44) reported that exposure to malathion did not alter brain acetylcholinesterase activity, body weight gain, organ/body weight ratios or food and water consumption during the treatment periods. However, Gurushankara et al. (45) found that food consumption, growth, and development decreased in L. limnocharis tadpoles with an increase of malathion concentration. This controversy may be related to difference in animal species or doses of malathion used. Gurushankara et al. (45) added that the immune suppression after malathion exposure is possible as it binds with esterase, a vital membrane bound protein that helps immune cells to interact with and destroy foreign organisms (45).

Conclusion

It is evident from the present study that exposure to environmental pollution agents such as malathion can adversely affect immune function. Also, our study clearly demonstrated that intake of BPF was associated with amelioration of both physiological and morphological changes supporting its protective role against immune suppression induced by malathion. This action was mediated by pathophysiological mechanisms such as alteration of immunoglobulins, cytokines, antioxidants and direct damage of BM and splenic bulbs. Therefore, it may be optional that, while long-term exposure to malathion is immuno-oppressive, the supplementation with BPF may partially ameliorate this effect. The clinical ramifications of these findings await further investigation.

Acknowledgements

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