Evaluation of Immunomodulatory Effects of Antiepileptic Drug Phenytoin

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Abstract

The immunomodulatory effects of phenytoin (PHN), used as an antiepileptic agent were investigated *in vivo* using female Balb/c mice. The main aim of this study was to investigate the immunotoxicity of PHN. Animals were divided into six groups, eight animals per group. Group I, served as a control group, received only the vehicle. Groups (II-VI) were received a daily intraperitoneal dose of PHN (2.5, 5, 10, 15 and 25 mg/kg, respectively) over a period of 21 days. PHN has shown a significant decrease in the animal body weight. The relative weights of animal's spleens were also decreased significantly at doses of (10, 15, and 25 mg/kg). PHN showed a significant decrease in the percentage of circulating neutrophils and lymphocytes and an increase in the percentage of circulating eosinophils. The result showed a marked suppression in antibody production capacity as a humoral immune response and a suppression in the Delayed type hypersensitivity response as a cell mediated immune response in PHN-treated mice compared to the control group. Detectable changes have also been noticed in the histology of the footpad tissue and spleen.

1. Introduction

The Immune system is a collection of organs, cells and tissues that work together to protect our body from potentially harmful infectious agents and certain tumor cells (Ponce *et al.*, 2009). Immunotoxicology is an important portion of the safety evaluation of drugs and chemicals (Descotes, 2006). Immunotoxicology studies focuses on the modulation of the immune system following exposure to environmental chemicals and drugs. The modulation may include immunosuppression (non- specific), immunostimulation, hypersensitivity, or autoimmunity (Veraldi, 2006; Descotes, 2005). There is increasing evidence that many toxic effects on the immune system components and their functions takes place as a result of drug treatments or chemical exposure (Rooney *et al.*, 2008). Therefore, it is very important to identify and evaluate the potential effects of chemical compounds that produce immunotoxicity during the processes of drug development (Spanhaak, 2006). Determining mechanism of immunotoxicity is an important issue in understanding the clinical relevance of the observed adverse effects. For instance, changes in blood cellular elements could suggest immunosuppression (Schulte, 2006).

Phenytoin (PHN) is an antiepileptic drug which can be useful in the treatment of epilepsy .This drug acts to suppress abnormal brain activity that has been seen in seizure by reducing electrical conductance among brain cells by stabilizing the inactive state of voltage-gated sodium channel, PHN known as (5,5-diphenylimid-azolidine-2,4-dione).Molecular formula: $C_{15}H_{12}N_2O_2$. It is white crystalline powder or granule with melting point 296 c°, insoluble in water, while it is soluble in ethanol, acetone and ether (Bernaskova *et al.*, 2010).

The usual human therapeutic dosage of PHN is (1 g) orally divided in 3 doses (400 mg, 300 mg, 300 mg) given at 2 hour intervals (Ratanakorn *et al.*, 1997). Due to risk of serious side effects of PHN, it must be titrated gradually over several weeks to reach this target dose range (Keppel, 1998) PHN is associated with adverse side effects, depending on dosage , side effects occurred in 1/3 of the patients, especially those whose serum concentration was more than 20µg/ml (Beier *et al.*, 1978), increasing with higher serum concentrations and in combination therapy, including diplopia, nystagmus. Allergic skin rash, change in blood counts such as leukopenia , and impairment of hepatic function are seen and may return to normal after dose reduction (Walia, 2004). In some cases, serious allergic reaction, such as

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skin inflammation with large area flaking (exfoliative dermatitis) (Pelekanos *et al.,* 1991). Hematological side effects of antiepileptic drugs occur infrequently but remain a potential cause of severe toxicity. It is recommended that patients receiving PHN are necessary to have a complete blood count (CBC) every two weeks. According to the international monitoring guidelines, total white blood cells count (WBCs) and differential WBC count must be performed, with PHN treatment.Other hematological effects of PHN include eosinophilia also has been reported in 34% of PHN treated patients (Yang *et al.*, 2011; Bjornsson *et al.,* 2007; and Fararjeh *et al.*, 2008). The aim of the present study was to investigate the toxic effect of PHN on immune system cells and tissues in mice by assessing humoral and cell mediated immunity. Routine hematological parameters, screening for changes in organ and body weight**,** hemagglutination titer (HA), and delayed type hypersensitivity (DTH) in PHN-treated animal groups were tested. Histological examination of the spleen and inflamed tissue was also evaluated for each animal (Putman *et al*., 2003).

2. Materials and Methods

2.1. Animals

Adult female balb/c mice (6-8 weeks old; 18-20 g weight) were obtained from The Hashimate University animal house, Zarqa', Jordan. Animals were housed in plastic cages containing saw-dust bedding and adapted for 1 week in the lab prior to usage. The animal's room was maintained at a temperature of (23 ± 2 °C) with relative humidity (50 ± 20 %) under a 12 hour light/dark cycle (lights on at 07:00). All animals were maintained at standard laboratory food and tap water ad libitum. Animals were cared for in accordance with the guide to the care and use of experimental animals. All procedures involving animals were reviewed and approved by the institutional review board (IRB) issued by The Hashemite University.

2.2. Chemicals

One hundred milligram of phenytoin sodium (Goedecke AG/Germany) was purchased from local drug store (Jordan). Phosphate buffered saline (PBS), RBC lysing buffer, Sheep- RBCs (SRBC) and RPMI-1640 medium were purchased from Sigma (USA).

2.3. Phenytion Administration

Phenytoin (One hundred mg) was dissolved in 8 ml of absolute ethanol (12.5 mg/ml). PHN was made up in a concentrated stock solution and diluted to an appropriate dilution with PBS for intraperitoneal (i.p.) injection each day of experiment (Tomson *et al*., 2007; Wyllie *et al*., 1991).

2.4. Dose and Exposure Schedules

Mice were randomly divided into six groups (I-VI), each of 8 animals. Group I (control group) received Phosphate buffer saline (PBS) and the same percentage of drug solvent (ethanol). Groups II–VI (treatment groups) received the corresponding doses of PHN. Animals were treated with PHN in PBS intraperitoneally for 21 consecutive days. Phenytion was administered at a volume of 10 ml/kg. The drug doses were 0.5,

1.0, 2.0, 3.0 and 5.0 X times of the human therapeutic dose, and were equal to 2.5, 5, 10, 15, and 25 mg/kg respectively. Mice were sacrificed by cervical dislocation 4 hours after the administration of the last drug dose.

2.5. Organ and Body Weight Ratio

The weights of animals were recorded at the beginning of the study and 4 hours after the last day of treatment (day twenty one). On the day of experiment, animals were sacrificed by cervical dislocation and body organs such as spleen, liver, kidneys and thymus were removed, and weighed. Connective and adipose tissue were removed from these organs before weighing. The relative weight of the organs of each mouse was calculated as organ weight (mg)/ body weight (g).

2.6. Determination of the Hematological Parameters

Blood was collected from the retro-orbital plexus of each mouse before being sacrificed using heparinized capillary tubes. 0.2 ml of blood were collected in a sterile (K_2-EDTA) anticoagulated tubes (Minicollect®, Impromini , China) so that the blood to anticoagulant ratio was 1:0.075 v/v. Routine hematological parameters were assessed including hemoglobin content, packed cell volume percent (PCV%), red blood cell (RBC) count, and WBC count. Blood films were prepared and stained with Gimsa stain for each treated animal for differential WBCs counts, slides were observed under light microscope (Nikon, China). Blood samples analyses were confirmed by Mindray BC 2800 hematology analyzer (Jiangsu, China) at Hamdan institution for medical equipment.

2.6.1. Serum Antibody Titer: Hemagglutination (HA) Titer Assay

Seven days before ending the treatments, mice were immunized by i.p injection of $10^8/50$ µl Sheep-RBCs (SRBCs) in PBS (Lee, 2004). Phenytoin treatments were continued up to 21 days. At the end of experimentation (day 21), sera were prepared from peripheral blood samples, then 25 µl of twofold diluted sera in PBS were placed in the wells of a U-shape 96-microtiter plates. All samples were challenged with 25 µl of 5% v/v SRBCs suspension and mixed according to (Fararjeh *et al*., 2008). The plates were incubated at 37 °C for 1 h. Hemagglutination was then observed according to (Riahi *et al.,* 2010).

2.7. Delayed-type Hypersensitivity Response (DTH)

Delayed-type hypersensitivity response (DTH) was determined for all groups using a modified method of Bin-hafeez *et al.,* 2003. On the 14th day of the treatment, animals were immunized with $10^8/50$ µl SRBCs. After seven days of immunization (day twenty of the experiment), all animals were again challenged with a booster dose of $10^8/50$ µl SRBCs in the right hind footpad according to (Bin-hafeez *et al.,* 2003; and Fararjeh *et al*., 2008). The left hind footpad was injected with an equal volume of PBS to serve as trauma control for nonspecific swelling. Increased volume of footpad was measured 24 h after the last challenge with SRBCs using digital plethysmometer LE 7500 (Harvard, UK) and the differences between right and left hind footpad volumes were calculated (Dietert and Holsapple, 2007).

2.8. Histological Examination

The collected tissues of right footpad and spleen of each treatment group of mice were fixed in 10% formalin and sectioned using Shandon rotary microtome (Egenolf, 2011). Various sections (4-5 μm thickness) were prepared and stained with Hematoxylin–Eosin (H–E). Histological changes in these organs were examined under light microscope (Nikon, china) by an experienced pathologist and scored according to the degrees of changes in cellular infiltration and architectural distortion as described by (Kugelberg *et al.*, 2005; and Fararjeh *et al.*, 2008).

Scoring for the presence of the mononuclear and polymorphonuclear leukocytes in the dermal layer was considered as follow; 0, neither mononuclear nor polymorphonuclear leukocytes were present; 1, (1-3 cells per field); 2, (5-7 cells per field); and 3, abundant occurrence of mononuclear and polymorphonuclear leukocytes (more than 7 cells per field) (Kugelberg *et al.,* 2005). The presence of the mononuclear and polymorphonuclear leukocytes in the muscular layer was also scored in a similar fashion. Histological changes in spleen were scored according to the white pulp, red pulp, and trabecular changes. Negative sign (-) indicates no changes observed; +, minimal changes; and ++, readily detectable changes.

2.9. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) of eight independent experiments. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test using Graphpad Prism version 5 software package. In the figures, asterisks represent a statistically significant, increase or decrease, compared to control, where $* = p < 0.05$, $** = p < 0.01$ and $*** = p < 0.001$.

3. Results

3.1. Effect of PHN on Organ and Body Weight

At the beginning of the study, all animals have almost comparable body weights since none of the groups showed any significant difference between groups as calculated by multiple comparison test. None of the used doses of PHN caused mortality in the treated animal groups. Animals treated with PHN showed a significant decrease in their body weight at the highest used doses (15 and 25 mg/kg) when compared to the control group . However, neither the subtherapeutic nor therapeutic doses of PHN (2.5, 5 and 10 mg/kg, respectively) showed any significant difference to the control group .On the other hand, PHN at the doses of (10, 15 and 25 mg/kg) caused a decrease in the relative (% body weight) of spleen .However, only (15 and 25 mg/kg) PHN doses showed a significant decrease in relative weight of thymus .No effects were observed in liver and kidney relative weights at any given doses when compared to the control group (Table 1) .

Table 1. Effects of PHN on Red blood cells (RBCs) parameters of different treatment groups of Balb /c mice females.

PHN (mg/kg)	RBCs parameters		
	RBCs count ($\times 10^6$/mm^3)	PCV (%)	Hemoglobin (g/dl)
Control	8.70±1.28	45.99±3.82	14.66±1.27
2.5	8.69±1.19	43.54±6.66	14.51±2.22
5	8.56±0.89	47.55±4.35	15.85±1.45
10	8.67±0.93	45.00±5.33	15.00±1.77
15	7.05±1.60	44.63±4.43	14.88±1.47
25	7.03±1.53*	45.64±5.99	15.21±1.99

Data are means± SEM of eight animals. (PHN; phenytoin, PCV; packed cell volume, RBC; Red blood cells). $* P < 0.05$ when compared to control.

3.2. Effect of PHN on Hematological Parameters

Hematological tests of the peripheral blood revealed that mice treated with PHN caused a significant decrease in erythrocytes count at the highest dose 25 mg/kg (Table 1). Also, PHN caused a significant decrease in leukocytes count at the doses (15, 25 mg/kg), while doses (2.5, 5, 10 mg/kg) of PHN did not show any significant change compared to the control group (Table 2). Moreover, PHN caused a significant decrease in the percentage of circulating peripheral blood neutrophils and lymphocytes at 15, 25 mg/kg doses. However, there is no significant difference in the percentage of peripheral blood leukocytes among the lower three doses. Interestingly as shown in Table 2, PHN caused a significant dose dependent increase in the percentage of eosinophils. A significant increase was also found between 5, 10, 15 and 25 mg/kg doses. While none of the PHN doses used appears to have any effect on other hematological parameters (Table 1 and Table 2).

Table 2. Effects of PHN on White blood cells (WBCs) parameters of different treatment groups of female Balb/c mice

PHN (mg/kg)	WBCs parameters				
	WBCs count ($\times 10^3$/mm^3)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
Control	7.21± 0.97	23.33± 2.54	65.81± 2.86	10.09± 1.69	0.75± 0.71
2.5	7.22± 1.99	22.48± 5.71	62.16± 9.71	9.34± 1.85	1.25± 0.46
5	5.89± 1.17	22.44± 2.70	61.9± 14.06	9.66± 0.79	2.5± 1.19*
10	5.94± 1.21	22.69± 3.17	56.13± 17.41	10.15± 1.48	2.38± 1.51*
15	5.03± 1.41**	19.21± 2.17*	43.3± 12.9**	10.18± 1.83	3.5± 0.9***
25	3.7± 0.9***	16.4± 2.2***	41.8± 8.8**	8.99± 1.66	4.1± 1.3***

Data are means± SEM of eight animals. (PHN; phenytoin, WBC; white blood cells).$* p< 0.05$ when compared to control, $**p< 0.01$ when compared to control,$*** p< 0.001$ when compared to control.

3.3. Effect of PHN on Serum Antibody titer: Hemagglutination (HA) titer

Hemagglutination titer at the doses of (15, and 25 mg/kg) showed significant inhibition ($p<0.001$) in the concentration of the anti-SRBCs antibodies expressed as

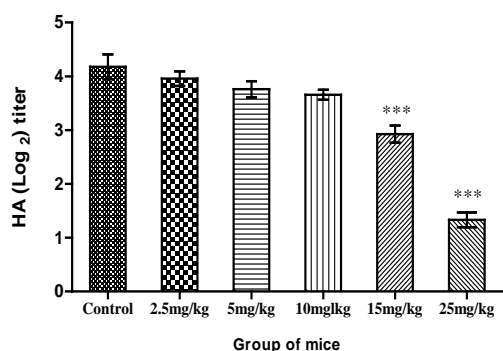antibody titer when compared with the control group (Figure 1).

Figure 1. Effect of PHN on the anti-SRBCs antibodies production assessed by the hemagglutination $\log_2$ titer assay. Data are means ± SEM (n=8). ***p <0.001 when compared to control animals

3.4. Effect of PHN on Delayed- type Hypersensitivity Response (DTH)

The subtherapeutic dose 2.5mg/kg and the therapeutic dose 5 mg/kg PHN did not show any significant change on DTH response compared to the control group. However, a significant suppression of the DTH response was determined after a subsequent injection of antigen (SRBCs) at 10 mg/kg dose or more (Figure 2).
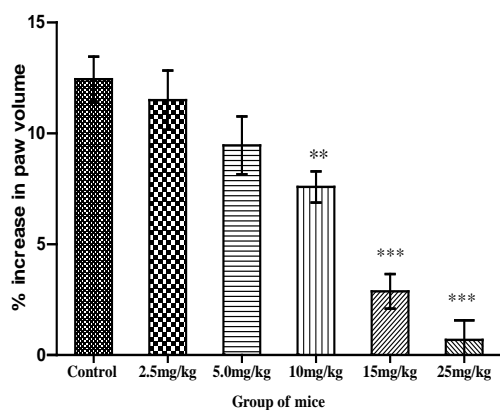

Figure 2. Effect of PHN on delayed-type hypersensitivity (DTH) measuredas the difference between SRBC- injected and PBS-injected hind footpad volumes. Mice received daily i.p injection of the indicated doses of PHN for 21 days. Data are means ±SEM of eight animals. **p < 0.01 and ***p <0.001 when compared to control animals.

3.5. Effect of PHN on Histology of Footpad Tissue

Microscopic examination of the footpad tissues showed an inflammatory infiltrate of leukocytes (polymorphonuclear and mononuclear leukocytes) after being challenged with SRBCs, in both hypodermis and muscle layers.

Treatment with PHN caused a significant decrease in the inflammatory infiltrate at the doses of 15 and 25mg/kg . No effects were observed in other inflammatory parameters at all doses of PHN (Table 3 and Figure 3).

Table 3. Effects of PHN treatment on right footpad tissue in mice receiving (2.5, 5, 10, 15 and 25 mg/kg), as daily i.p. injection for 21days.

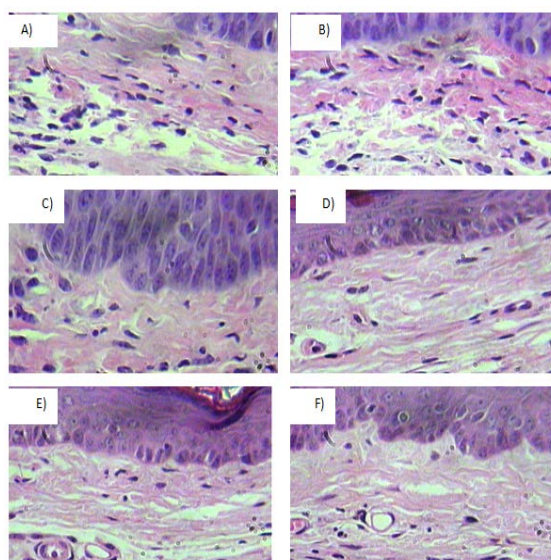| Inflamed tissue	Inflammation score (Presence of mononuclearand polymorphonuclear Leukocytes)	
Treatment mg/kg	Dermal layer	Muscular layer
Control	2.12±0.22	1.37±0.26
2.5	2.12±0.29	1.37± 0.18
5	1.62±0.26	1.25±0.16
10	1.62±0.26	1.0±0.32
15	1.12±0.22*	0.87±0.22
25	0.5±0.18***	0.5±0.18*

Figure 3. Inflamed footpad representative tissue sections of control and PHN-treated animals stained with hematoxylin eosin shown at high dry power field (40 x). (A); right footpad tissue of control group showing infiltration of polymorphonuclear and mononuclear leukocytes after SRBCs challenge. (B-F); PHN treated groups (2.5, 5, 10, 15 and 25 mg/kg, respectively) showing a decrease in the inflammatory infiltration of polymorphonuclear and mononuclear leukocytes.

3.6. Effect of PHN on Histology of Spleen

Microscopic examination of spleen showed a decrease in the density (atrophy) of the spleen white pulps at doses of 15 and 25 mg/kg (Figure 4). No other significant changes in other spleen histological parameters were seen at any used doses of PHN.
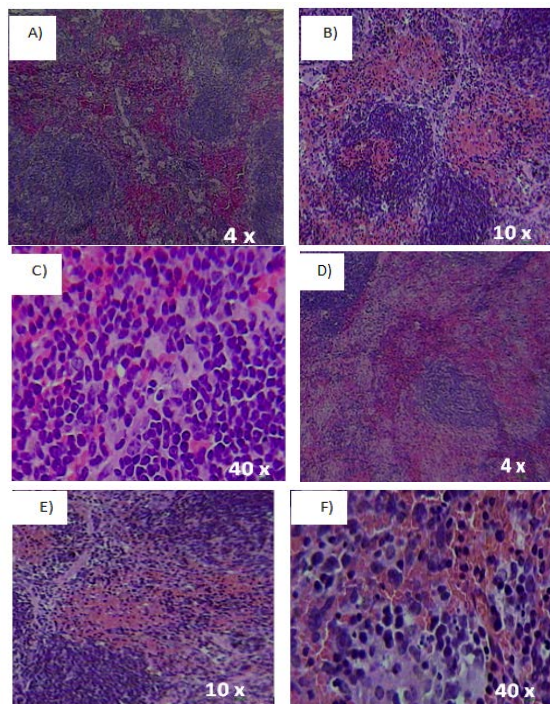
Figure 4. Spleen sections of control and PHN-treated animals stained with hematoxylin- eosin shown at different magnifications. (A-C) normal spleen (control); (D-F); 25 mg/kg PHN- treated animal showing white pulp atrophy.

4. Discussion

In the current study, the unhealthy effect of PHN has been confirmed by the significant decrease in body weights measured in PHN-treated mice at doses of 15 and 25 mg/kg. In the present study, mice treated with PHN have low food consumption. PHN alter one of the fundamental processes of the human body weight regulation, by altering appetite, metabolism, or absorption of calories. It has been noticed that mice treated with PHN exhibited mild desire for sleep which might indicate a reduction in food intake which results in body weight loss. Moreover, PHN (15, 25 mg/kg) treated mice showed a remarkable signs of behavioral abnormalities manifested by fainting and drowsiness (not calculated). These behavioral abnormalities were associated with reduction in body weights. This effect is consistent with the study of Ben-Menachem (2007) that has reported similar effect suggesting body weight loss.

Phenytoin caused a significant decrease in the relative weight of spleen at doses of (10, 15 and 25 mg/kg), this effect was confirmed by the observation of induced atrophy of the white pulp of spleen in a similar fashion. The spleen is a major lymphoid organ that plays a critical role in the primary humoral and secondary immune response. B lymphocytes partially mature in the bone marrow enter the circulation, and then populate peripheral lymphoid organs, including spleen and lymph nodes, were they complete their maturation process. Spleen white pulp plays also an important part in the initiation of immune responses by B cells to foreign antigens in the blood. This might be the reason behind the relative lymphocytopenia found in peripheral blood of treated mice.

In addition, spleen contains a large number of tissue resident leukocytes, therefore white pulp atrophy will reflect a reduction in the percentage of total leukocytes in the peripheral blood.

It has been shown that the PHN caused a significant decrease in the Red Blood Cells (RBCs) at the highest used dose (25mg/kg) which may be due to suppression of the bone marrow, that's why PHN may reduce the production of erythropoietin (EPO) hormone, thus the erythropoiesis process which is stimulated by EPO can be also affected by PHN. Likewise, PHN caused a significant decreased in the total leukocytes counts and in the percentage of neutrophils and lymphocytes. Leucopenia, neutropenia, and lymphocytopenia may reflect the toxicity or immunomodulatory effects of PHN in treated group of mice. These findings were consistent with the common occurrence of leukopenia in human model by a cohort study which has been conducted by (Blackburn, 1998).

A significant dose dependent increase in the percentage of eosinophils have been demonstrated in PHN treated mice at doses equal to or more than 5 mg/kg which is correspondent to the human therapeutic dose and this was in concordance with the study that has reported similar effect suggesting elevation of IgE with eosinophilia (Chen *et al.,* 2010). The decrease in humoral immunity response to PHN was manifested by suppression of antibodies production, tested as anti-SRBCs antibodies titer which shows a suppression of antibodies at treated doses of 15 and 25 mg/kg. White pulp atrophy, and spleen relative weight decrease observed in this study is a common finding following the administration of certain immunosuppressive drugs, and are accompanied by the decreased ability of the animal immune system to produce antibody. Suppression of DTH reaction by PHN could be not only due to the reduction in the availability of factors essential responsible for the maintenance of T cell proliferation, but also for the recruitment and activation of macrophages. Reduction in DTH may be due to any block in the antigen of SRBCs processing or presentation of these antigens by macrophages for T lymphocytes. The exact mechanism whereby PHN inhibits T cell activation is not known yet. However, it could take place via blocking of antigen specific receptors or acquisition of responsiveness to interleukins. Suppression of the delayed type hypersensitivity response has been demonstrated by PHN in this study at doses of 10, 15, and 25 mg/kg and these results were accompanied by a study by Dietert *et al.* (2010), showing that the inhibitory effects of PHN on cell mediated immune response in animal.

Moreover, DTH responses measured in the skin of the mice footpad have been used to assess cell mediated immunity in vivo. The loss of DTH reaction serves as an indicator of deteriorating cell-mediated immune function. A decreased in the infiltration of the mononuclear and polymorphonuclear leukocytes in the mouse footpad tissue, which has been demonstrated in histological examination at 15 and 25 mg/kg doses of PHN was accompanied by suppression in DTH response. These results might explain the inhibitory effect of PHN on the cell mediated immune responses.

In conclusion, the current study shows that Phenytoin had an inhibitory effect on the innate, adaptive and cell mediated as well as humoral immune response at human subtherapeutic, therapeutic and high doses. These results demonstrate an immunosuppression effect of PHN in mice. The effectiveness of PHN in the treatment of schizophrenia and other related diseases should be counterbalanced by its immunotoxicity. Further research studies should be considered to study the significance of using PHN in patients under long-term treatment. As well, studies on human models should be required to study the toxic effect of PHN on the immune system.

Acknowledgements

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