REVERSIBILITY OF AGE-RELATED IMMUNE DYSFUNCTION IN MAMMALS BY A COMBINED TREATMENT OF SOME TRACE ELEMENTS AND VITAMINS

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SUMMARY: Immune responses normally reduced during aging may be resulted to different extends by the intake of several essential micro- and macronutrients. The aim of the present study was to investigate the in vivo effect of different doses of hepanox on humoral and cell-mediated immune responses of aged-mice in a trial to improve age-associated immune dysfunction.

Different doses of hepanox (0.4, 2 or 10 µg/mouse) were orally administrated to aged mice daily for one month. Control mice were treated with 0.2 ml of the sesame oil only. Our study shows that total leukocyte counts from PBL, Thy, PLN, MLN and BM as well as total and differential counts of PEC were significantly decreased in aged mice. Treatment of aged mice with hepanox preparations (0.4, 2 or 10 µg/mouse) caused a progressive increase in the total numbers of leukocytes from all lymphoid organs studied.

The present study shows also that the phagocytic function of PEC decreased with age of mice, while treatment of aged mice with hepanox preparation elicited a progressive increase in the scavenger activity of PEC. In old mice, there is a significant decline in PFC response to in vivo immunization with SRBC while the treatment of aged mice with hepanox preparation elicited a gradual increase in PFC response. Also, the number of T-lymphocytes decreased significantly with advancing age of mice. Treatment of aged mice with hepanox preparations (2 and 10 µg/mouse) elicited a progressive increase in the number of T-lymphocytes. While in vitro addition of hepanox preparations (0.002, 0.01 or 0.05 µg/well) to old mice splenocytes, stimulated with Con A mitogen, significantly increased T-cells proliferation. The treatment of aged-mice with hepanox might be capable of maintaining and restoring the aged-related reduction in the immune efficiency.

Key words: Hepanox, aging, lymphocytes, immunization, immune efficiency.

INTRODUCTION

As age advances, the zinc pool undergoes progressive reduction as shown by the low zinc plasma levels and the negative crude zinc balance (1). Zinc levels in granulocytes and lymphocytes are also decreased in elderly subjects and appear to be a significant problem in free-living peoples (2). Zinc supplementation in the elderly has been attempted with use of several forms of zinc. In most of these studies, zinc was administered with other vitamins or a multivitamin/mineral supplement. Several studies indicate zinc may enhance the number of $CD3^+$, $CD4^+$, $CD16^+$ and $CD56^+$ lymphocytes, NK cell activity or DTH responses in elderly subjects (3, 4). However, others demonstrate no effect upon DTH responses (5) or antibody production in response to influenza vaccine (6).

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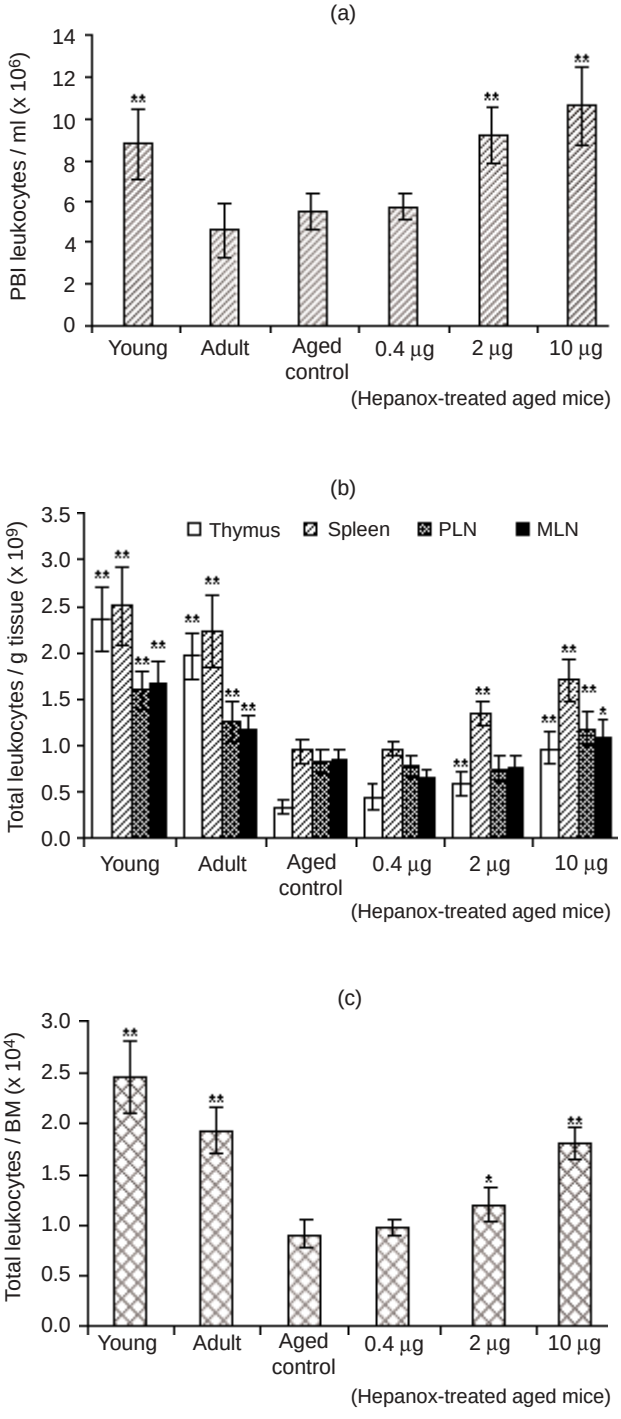
In aged populations, under nutrition induces lower immune responses, particularly in cell-mediated immunity. A micronutrient deficit, namely selenium (Se), which is prevalent in aged populations, is associated with decreased lymphocyte proliferation, reduced cytokine release, and lower antibody response to vaccine. Because aging and malnutrition exert cumulative influences on immune responses, many elderly people have poor cell-mediated immune responses and therefore a high risk of infection (7). Girodon *et al.* (8) found that low-dose supplementation of Se provides significant improvement in elderly patients by increasing the humoral response after vaccination and could have considerable public health importance by reducing morbidity from respiratory tract infections. In a prospective, randomized study design, Wood *et al.* (9) found that Se enhanced immune function (NK cell cytotoxicity) and phenotypic expression of T-cell subsets.

It was found that Vit E supplementation to healthy elderly humans was associated with increased DTH score, lymphocyte proliferation as well as Interleukin-2 (IL-2) production, and decreased phytohemagglutinin (PHA)-stimulated prostaglandin production by peripheral blood mononuclear cells (10). Moriguchi *et al.* (11) have found that Vit E enhances T-cell differentiation through the increase of not macrophages but thymic epithelial cell function. Meydani and Hayek (12) reported that, even with high Vit E supplementation, there was no effect on subjects' ability to kill ingested *Candida albicans*. Enioutina *et al.* (13) found that dietary supplementation of aged mice with Vit E was capable of restoring their mucosal and systemic humoral immune responses to mature adult levels.

Although both Vit A deficiency and aging are independently associated with alterations in immune function, the effects of marginal Vit A status or Vit A supplementation on the immune system during aging were little studied. Dawson and Ross (14) reported that marginal Vit A status, particularly in older rats, was associated with increases in the percentage of $CD8^+$ T cells, percentage and number of NK cells, and decreases in the CD4/CD8 T-cell ratio and splenic cell IL-2 production. Penn *et al.* (15) have assessed the Vit A status and cell-mediated

Figure 1: Effect of hepanox treatment (0.4, 2 and 10 μg/mouse) daily for one month on the total number of leukocytes from a) peripheral blood (PBl), b) thymus (Thy), spleen (Spl), peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) and c) bone marrow (BM) of mice (*p<0.05; **p<0.01 in comparison with the aged control group).



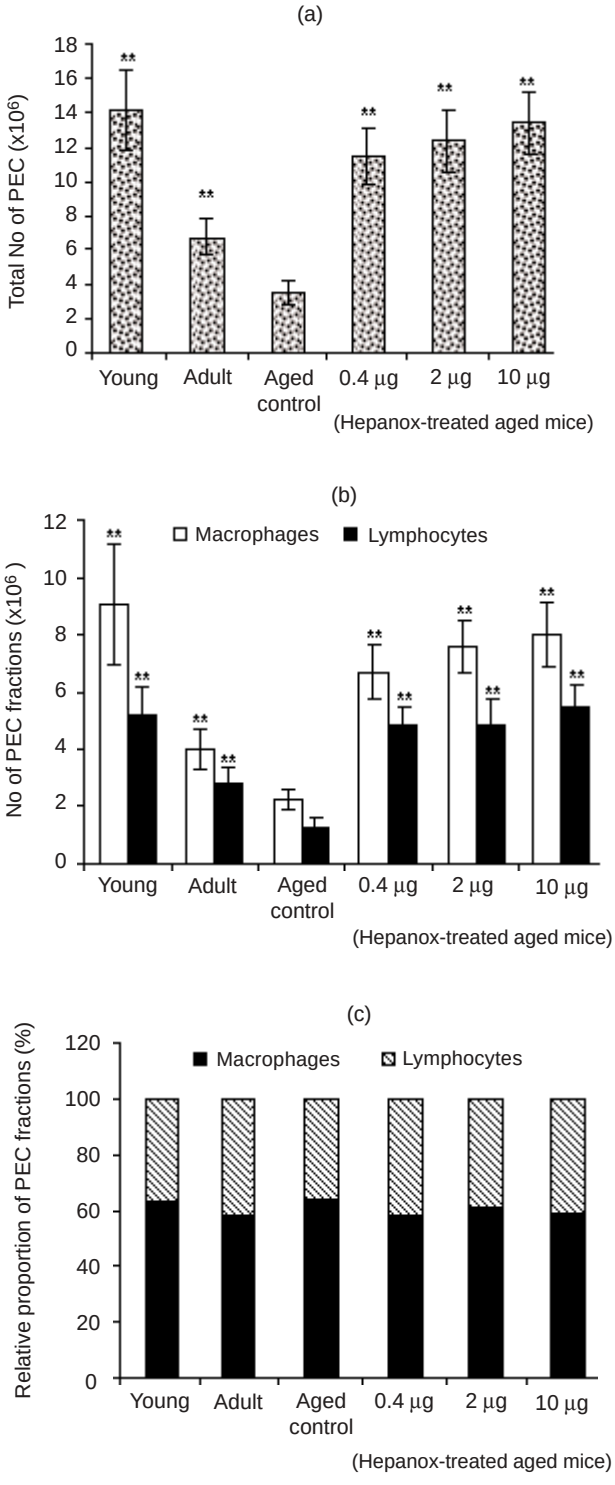
immune function in elderly long-stay patients. Following Vit A supplementation, cell-mediated immune function improved as indicated by a significant increase in the absolute number of T-cells, T4 subsets, T4 to T8 ratio and the proliferation of lymphocytes in response to Phyto-haemagglutinin (PHA) mitogen.

Supplementation of healthy elderly persons with β-carotene has been considered a way to enhance immune response. Jyonouchi *et al.* (16) examined the effect of β-carotene on antibody production using spleen cells from young and old B6 mice. Wood *et al.* (9) investigated the effects of β-carotene supplementation in a healthy elderly population, focusing on NK activity. Elderly persons had significantly lower NK activity than did middle-aged subjects. Elderly persons supplemented with β-carotene had significantly greater NK activity when compared with elderly persons receiving placebo. No significant difference was seen in NK activity due to β-carotene supplementation in the middle-aged group (17). On the other hand, Santos *et al.* (18) showed that short- and long-term supplementation of β-carotene did not have an enhancing or suppressive effect on T cell-mediated immunity of healthy elderly.

Elderly people are known to be lacking in Vit C, primarily because their diet is poor. Elderly people had only half the level of ascorbic acid in their blood plasma, as did younger subjects. Men and women over 65 need a daily dose of about 150 mg to maintain a plasma level of 1.0 mg/dl (19). An important epidemiologic study by Singh *et al.* (20) showed that men who took Vit C supplements lived, on average, 6 years longer than men who relied on normal dietary sources of Vit C. The effect of Vit C supplementation on the age-related decline in immune function was studied by Buzina-Suboticanec *et al.* (21). Vit C supplementation for a period of 10 weeks significantly improved the biochemical parameters for the age-related decline in the DCH test. De la Fuente *et al.* (22) have investigated the effect of supplementation of the diet with Vit C and Vit E on several functions of the immune response of aged women.

Optimal amounts of GSH are needed for maintenance of immune response during aging. This need might be more critical, however, in aged persons. The

Figure 2: Effect of hepanox treatment (0.4, 2 and 10 μg/mouse) daily for one month on a) total cellularity of PEC, b) absolute number of PEC fractions and c) relative proportion (%) of PEC fractions of mice. (*p<0.05; **p<0.01 in comparison with the aged control group).



well known age-related increase in free radical formation and lipid peroxidation contributes, at least in part, to this phenomenon (23). However, Meydani *et al.* (24) reported that GSH was able to modulate cytokine production and T-cell-mediated function in the aged. A decline in tissue and serum of GSH contents and GSH-metabolizing enzymes with age has been implicated in increasing susceptibility to carcinogens, and in infectious and autoimmune diseases which occur with advanced age (25). The potential of dietary GSH to alter immune response in aging mice was studied by Furukawa *et al.* (26). Dietary GSH supplementation reversed the age-associated decline in Con A-stimulated splenocyte proliferation, depression of T-cell- mediated immune function and reduction of spleen GSH levels.

Alpha-lipoic acid, a mitochondrial co-enzyme, may be involved in aging. In old animals, mitochondrial membrane potential, cardiolipin levels, respiratory control ratio, and overall cellular oxygen consumption are lower than in young animals, and the level of oxidants is higher. Feeding old animals with α-lipoic acid, restores mitochondrial function, and lowers oxidants to the level of young animals (27). Both glutathione and Vit C levels declined with age, but their loss was completely reversed with α-lipoic acid (28). Various *in vitro* and animal studies have proved that α-lipoic acid restores the age-associated decline in endogenous low molecular weight antioxidants and mitochondrial enzymes (29), and exerts protective effects against oxidative stress during skin aging (30). Alpha-lipoic acid also attenuated the increased mitochondrial-induced oxidative stress during heart aging (31).

The aim of this study was to evaluate the *in vivo* effect of different doses of hepanox on humoral as well as cell-mediated immune responses of aged-mice.

MATERIALS AND METHODS

Animals

The experimental animals used in this study were young (1 month old), adult (3 months old), aged (18 months old) mice and were obtained from Helwan Research Animal Center, Cairo, Egypt. They were maintained in a quite room at 28°C with a light period of 12 hours alternating with a dark period of 12 hours. Mice received food and water *ad libitum* and were allowed a period of 10 days, prior to initiation of experiments, to acclimatize to the laboratory conditions.

Hepanox treatment

Hepanox, a capsule containing a mixture of glutathione, α-Lipoic acid, N-acetylcysteine, selenium, zinc, vitamin A, vitamin C, vitamin E and β-Carotene (Natural Pharmaceutical Co., Cairo, Egypt) was dissolved in sesame oil, and doses of 0.4, 2 or 10 μg/mouse were orally administrated to aged mice daily for one month. Control mice were injected with 0.2 ml of the sesame oil only.

Determination of lymphoid cell counts

Young, adult, aged and hepanox-treated aged mice were sacrificed 24 h after the last injection, and thymuses (Thy), spleens (Spl), peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) were excised, cleaned, defatted and weighed. Single cell suspensions were prepared by gentle squeezing of the respective organs between two slides in Hank's balanced salt solution (HBSS), followed by filtering through a nylon sieve. Bone marrow (BM) was obtained from femurs and tabiae, suspended in HBSS and filtered. Red blood cells from all these tissues, in addition to peripheral blood (PBl) were lysed by addition of Tris/NH_4Cl buffer (0.17 M Tris-hydroxymethyl aminomethane and 0.16 M NH_4Cl, pH 7.2). The respective cell suspensions were washed three times, resuspended in HBSS, and cells were counted with a haemocytometer and calculated per gram of tissues.

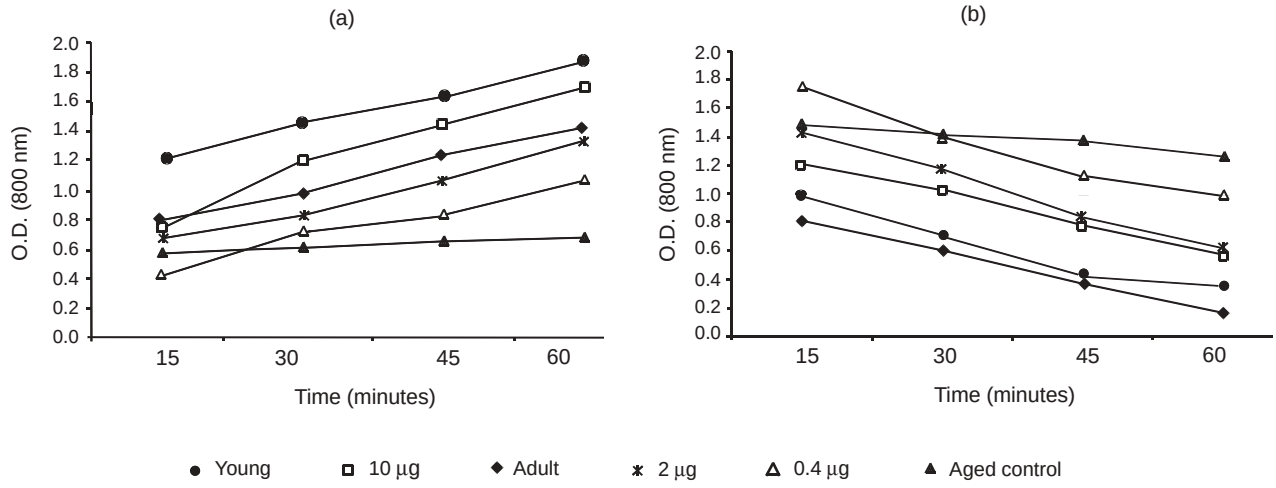
Harvesting of peritoneal exudate cells

To obtain inflammatory peritoneal phagocytes, young, adult, aged and hepanox-treated aged mice were intraperitoneally (i.p.) injected with 2.0 ml of starch suspension. Three days later, mice were killed and the peritoneal exudate cells (PEC) were obtained by peritoneal lavage with 5.0 ml of HBSS. Cells were washed three times by centrifugation (160 r.p.m. for 10 min.) and resuspended in HBSS. Total and differential counts of PEC were determined using haemocytometer, by the uptake of 1% W/V neutral red in saline (32).

Carbon clearance assay

Phagocytic activity of PEC was measured by using Pelikan special biological ink (Pelikan-Werke, Hannover, Germany). The original suspension was diluted 1:1 with 0.9% NaCl solution, and 0.2 ml of the diluted ink was i.p. injected into young, adult, aged and hepanox-treated aged mice after stimulation with 2.0 ml of starch suspension, which was i.p. injected three days earlier. Carbon challenged animals were killed 15, 30, 45 and 60 minutes after carbon injection. Five ml of 0.1% EDTA-saline solution was i.p. injected, and peritoneal lavage was collected and centrifuged (700 r.p.m. for 5 min). The resultant supernatant was decanted into another tube, and the precipitated cells were resuspended in 1 ml of equal volumes of gelatin (2% gelatin in

Figure 3: Effect of hepanox treatment (0.4, 2 and 10 μg/mouse) daily for one month on the scavenger activity of macrophages. a) carbon uptake by peritoneal exudate cells, and b) carbon particles remained in peritoneal fluid as measured by optical density (O.D.).



saline) and ethanol potassium saline (5% KOH in 70% ethanol), and incubated overnight at 37°C. Optical densities of both supernatant and digested cells were measured using a spectrophotometer (Spectronic 20, Bausch and Lomb Inc., Rochester, NY, USA).

Detection of plaque-forming cells (PFC)

The procedure was performed as described by Broussaeu *et al.* (33). Primary humoral immune responses against sheep red blood cells (SRBC) were measured after one i.p. injection of $1x10^8$ SRBC in 0.2 ml saline. Five days later, young, adult, aged and hepanox-treated mice were killed and spleens were excised and cleaned. Single cell suspensions were prepared, washed twice by centrifugation at 200 g and resuspended in HBSS to a concentration of $2x10^6$/ml. The liquid assay mixture was prepared by adding 50 μl of 25% SRBC and 50 μl of guinea pig complement to 100 μl of spleen cell suspension. The assay mixture was plated to a slide chamber and incubated for 30 to 45 min at 37°C. The plaques were scored microscopically and calculated per million mononuclear cells.

Detection of E rosette-forming lymphocytes

The procedure was performed as described by Hsu *et al.* (34). Seven days before they were killed, the mice received an i.p. injection of $1x10^8$ SRBC in 0.2 ml saline. Spleens from young, adult, aged and hepanox-treated mice were excised and cleaned. Single cell suspensions were prepared, washed twice by centrifugation at 200 X g and resuspended in HBSS to a concentration of $2x10^6$/ml. 0.2 ml of spleen cell suspension were mixed with an equal volume of 0.5% SRBC in a glass tube and incubated for 2-4 hours at 37°C. The tubes containing the mixture were gently shaken to resuspend the cells in the pellet. The rosettes were counted in a haemocytometer and calculated per million mononuclear cells. The cells surrounded by three or more SRBC were counted as E-rosette-forming T-cells.

Culture procedure

Mice were sacrificed by cervical dislocation and the spleens were aseptically removed and pooled. Spleens were dispersed gently by using two sharp forceps in serum free RPMI-1640 medium. The splenocytes were then seeded into 96-well culture plates (Falcon, Oxnard, CA) at a density of 1.5×10^5 splenocytes/well in RPMI-1640 medium supplemented with 5% fetal calf serum (Gibco, Grand N.Y.), 50 μM β-mercaptoethanol (Sigma Chemical Co.) and antibiotics. The cells were treated with 0.04 and 0.2 μg/well concanavalin A (Con A) in the presence of hepanox (0.002, 0.01 or 0.05 μg/well) and subsequently incubated in a humidified 5% CO_2 environment. Three days later, 150 μl of the medium were removed from each well and stored at 4°C for the IL-6 bioassay. The extent of spleen cell proliferation was determined using the tetrazolium salt MTT (3[4,5-dimethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co.), which adheres to active mitochondria to form a dark blue formazan product (35). MTT (5 μl of 5 mg/ml) was added to each well and incubated at 37°C for 4 h. The dark blue crystals were dissolved by the addition of 150 μl of 0.04 M HCl/isopropanol. After an overnight incubation in the dark, the plates were inserted into a Dynatech MR580 microelisa spectrophotometer and optical densities were obtained using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Histological study

Aged and hepanox-treated aged mice were sacrificed 24 h after the last dose. Thymuses were excised and fixed separately

in 10% neutral buffered formalin. The specimens were then dehydrated in ascending grades of ethyl alcohol, cleared in terpinol, washed in benzene, embedded in paraffin wax, sectioned at 5 μ, and stained with haematoxylin and eosin (36).

Statistical analysis

All *in vivo* results are expressed as means ± SD of groups consisting of 6 mice. The *in vitro* data are also expressed as means ± SD of groups consisting of four wells. Each experiment was performed independently at least three times. All data were analysed for significance using Student's *t*-test. Values of $p<0.05$ and $p < 0.01$ were considered statistically significant.

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RESULTS

Effect of aging and hepanox treatment on lymphoid cell count

Figure 1a shows that peripheral blood leukocytes (PBL) from aged mice were significantly decreased when compared with those from young ($p < 0.01$) but not adult mice. While treatment of aged mice with the lower dose of hepanox preparation (0.4 μg) caused a slight increase in the total number of PBL, treatment of aged mice with the higher doses of hepanox (2 and 10 μg) caused a statistically significant increase in the number of PBL. The percentage of this increase reached about 168% and 193% of the corresponding aged control respectively.

Figure 1b shows that the total number of leukocytes from thymus (Thy), spleen (Spl), peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) of aged mice were significantly decreased when compared with those of the corresponding young and adult mice ($p < 0.01$). Treatment of aged mice with the lower dose of hepanox (0.4 μg) caused a slight increase in the total number of leukocytes form both Thy and Spl, as well as a slight decrease in the total number of leukocytes from both PLN and MLN. However, treatment with hepanox (2 μg) caused a statistically significant increase in the total number of leukocytes from both Thy and Spl ($p < 0.01$). Moreover, treatment with the higher dose of hepanox (10 μg) caused a statistically significant increase in the total number of leukocytes from Thy, Spl, PLN and MLN ($p < 0.05$; $p < 0.01$).

Figure 1c shows that aged mice bone marrow (BM) leukocytes were significantly less than those from both young and adult mice ($p < 0.01$). While treatment of aged mice with hepanox (0.4 μg) caused a slight increase in the total number of BM leukocytes, the higher doses of hepanox (2 and 10 μg) caused a statistically significant increase in the total number of BM leukocytes ($p < 0.05$; $p < 0.01$).

Effect of aging and hepanox treatment on total and differential PEC counts

As shown in Figures 2a and b, the total number of PEC as well as the absolute number of both peritoneal macrophages and lymphocytes of aged mice were significantly decreased ($p < 0.01$) when compared with those of young and adult mice. Treatment of aged mice with hepanox (0.4, 2 and 10 μg for 30 consecutive days) caused a statistically significant increase in the total number of PEC as well as the absolute number of both peritoneal macrophages and lymphocytes ($p < 0.01$). On the other hand, Figure 2c shows that the relative proportion of both peritoneal macrophages and lymphocytes of aged mice did not differ significantly from that of young, adult or hepanox-treated aged mice.

Effect of aging and hepanox treatment on phagocytic function of PEC

To study the phagocytic function of PEC, carbon uptake by PEC as well as carbon particles remaining peritoneal fluid were measured in young, adult, aged and hepanox-treated aged mice after 15, 30, 45 and 60 minutes of injection. Figures 3a and b show that carbon uptake by PEC of aged mice was less than that of young and adult mice. However, treatment of aged mice with hepanox (0.4, 2 and 10 μg for 30 consecutive days) caused a progressive increase in the scavenger activity of PEC. On the other hand, carbon particles remaining in peritoneal fluid of aged mice were more than those of both young and adult mice. Treatment of aged mice with hepanox caused a gradual decrease in carbon contents in the peritoneal fluid, although it did not reach the values of young mice.

Effect of aging and hepanox treatment on B cell function

The number of plaque-forming cells (PFCs) per million splenocytes was determined 5 days after i.p. immu-

nization of mice with $1x10^8$ SRBC. As shown in Figure 4a, the number of PFCs of aged mice was significantly reduced when compared to the number of PFCs in young and adult mice ($p < 0.01$). The magnitude of this reduction was about 80% and 67% respectively. Although treatment of aged mice with the lower dose of hepanox (0.4 μg) caused a slight increase in PFC response, treatment of aged mice with the higher doses of hepanox (2 μg and 10 μg) caused a statistically significant increase in PFC response. The percentage of this increase reached about 49% and 312% of the corresponding aged control respectively.

Effect of aging and hepanox treatment on T cell number

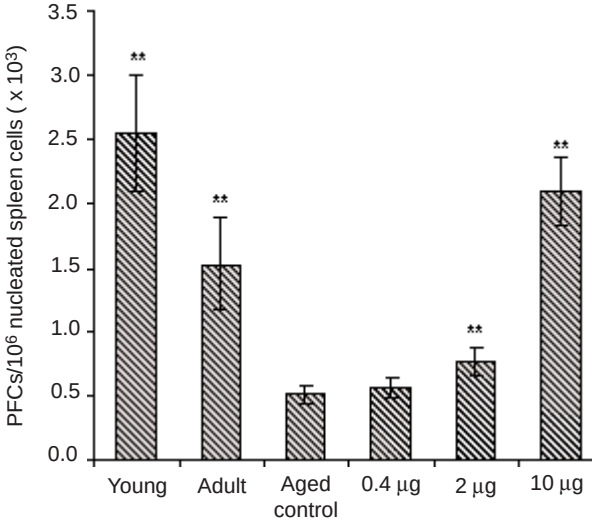
The number of rosette-forming cells (RFCs) per million splenocytes was determined 7 days after i.p. immunization of mice with $1x10^8$ SRBC. As shown in Figure 4b, the number of RFCs in aged mice was significantly decreased when compared with that of young and adult mice ($p < 0.05$; $p < 0.01$). The percentage of decrease reached about 59% and 25% respectively. However, although treatment of aged mice with the lower dose of hepanox (0.4 μg) caused a slight increase in RFC response, treatment of aged mice with the higher doses of hepanox (2 μg and 10 μg) caused a statistically significant increase in RFC response. The percentage of this increase reached about 61% and 168% of the corresponding aged control respectively.

***In vitro* effect of hepanox treatment on T lymphocyte mitogenesis of aged mice**

In order to examine the *in vitro* effect of hepanox preparation on concanavalin A (Con A)-stimulated splenocytes from aged mice, cultured splenocytes ($1.5x10^5$ splenocytes/well) were exposed to culture medium (Control), Con A (0.04 μg/well) and Con A (0.2 μg/well) in the presence of hepanox (0.002, 0.01 and 0.05 μg/well) for 72 hours. As shown in table and Figures 5a, b and c, in the absence of Con A, hepanox by itself had no significant mitogenic activity under the cultured conditions described. In the presence of Con A (0.04 μg/well), hepanox at concentrations of 0.002 μg/well ($p < 0.05$), 0.01 μg/well and 0.05 μg/well ($p < 0.01$) significantly stimulated the proliferation of splenocytes. The percentage of this stimulation

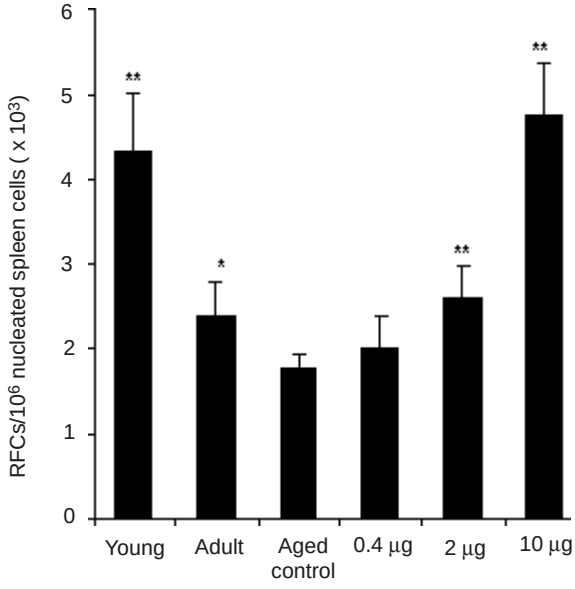
Figure 4: Effect of hepanox treatment (0.4, 2 and 10 μg/mouse) daily for one month on a) PFCs / $1x10^6$ nucleated spleen cells and b) RFCs / $1x10^6$ nucleated spleen cells. Mice were immunized i.p. with 0.2 ml of $1x10^8$ SRBC 4 days before killing (*p<0.05; ** p<0.01 in comparison with the aged control group).

(a)



(Hepanox-treated aged mice)

(b)



(Hepanox-treated aged mice)

reached about 58%, 64% and 92% respectively. Moreover, in the presence of Con A (0.2 μg/well), hepanox preparation (0.002, 0.01 and 0.05 μg/well) caused a significant increase in the proliferation of splenocytes. The increment of this increase was about 45%, 50% and 68% of the control value respectively.

Histological examination of the thymus gland

As shown in Figure 6a, histological examination of the thymus cortex of aged mice exhibited light aggregations of immature thymocytes. However, Figures 6b, c and d showed that the thymus cortex of hepanox-treated aged mice (0.4, 2 or 10 μg) was followed by a gradual increase in the number of immature thymocytes and became normal in appearance with the higher dose of hepanox.

DISCUSSION

Immune responses normally reduced during aging may be resulted to different extends by the intake of several essential micro- and macronutrients. The present study shows that total leukocyte counts from PBL, Thy, PLN, MLN and BM as well as total and differential counts of PEC were significantly decreased in aged mice. Treatment of aged mice with hepanox preparations (0.4, 2 or 10 μg/mouse) caused a progressive increase in the total numbers of leukocytes from all lymphoid organs studied. These results may agree with the findings by Pike and Chandra (37), Lesourd (7) and Buzina-Suboticanec *et al.* (21).

The present study shows also that the phagocytic function of PEC decreased with age of mice, while treatment of aged mice with hepanox preparation elicited a progressive increase in the scavenger activity of PEC. These results are consistent with the results of De la Fuente *et al.* (22) who reported that vitamins C and E intake by aged women resulted in a significant increase in phagocytic function of polymorphonuclear neutrophils. The present results also confirm the results of Del Rio *et al.* (38) who studied the effect of vitamin E, vitamin C, glutathione and N-acetylcysteine on macrophage function *in vitro* and found that all these antioxidants induce stimulation of the phagocytic process of macrophages.

The increase of the phagocytic capacities of macrophages means that these cells are activated regarding their ability to reach and ingest foreign bodies, i.e., to improve their defence function. The ingestion of

Figure 5: Effect of hepanox on aged-mice splenocytes proliferation. Cultured splenocytes (1.5 x 10^5 cells/well) were exposed to a) Cultured medium (control), b) Con A (0.04 μg/well) and c) Con A (0.2 μg/well) in the presence of hepanox (0.002, 0.01 and 0.05 μg/well) for 72 h. The data are expressed as the mean ± SD of groups consisting of four wells (*p<0.05; **p<0.01 in comparison with the aged control group).

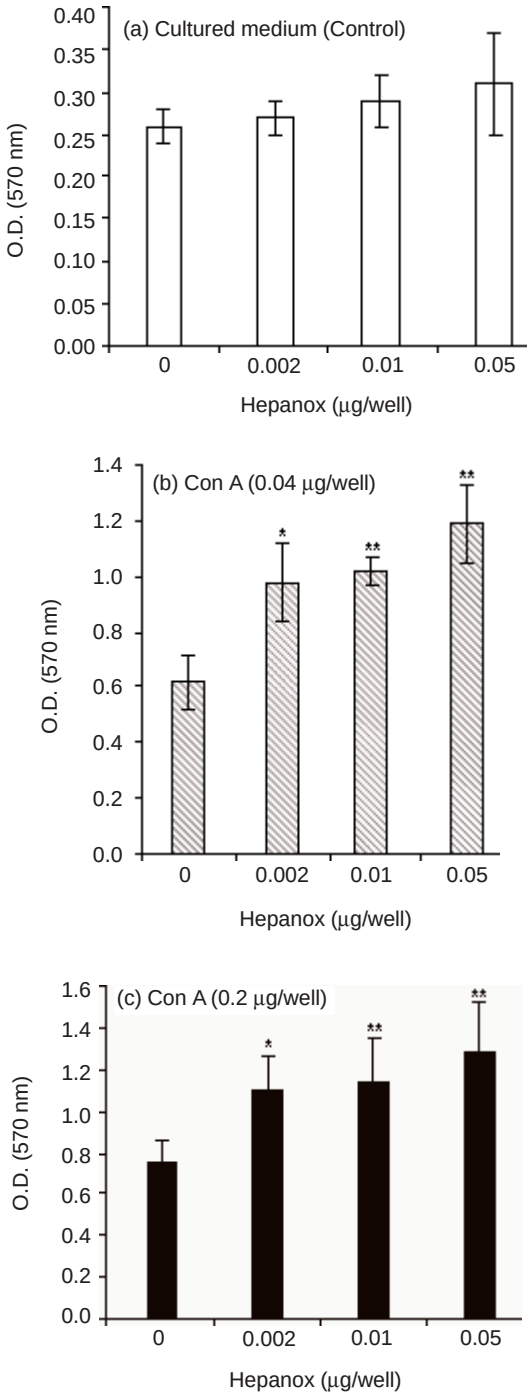
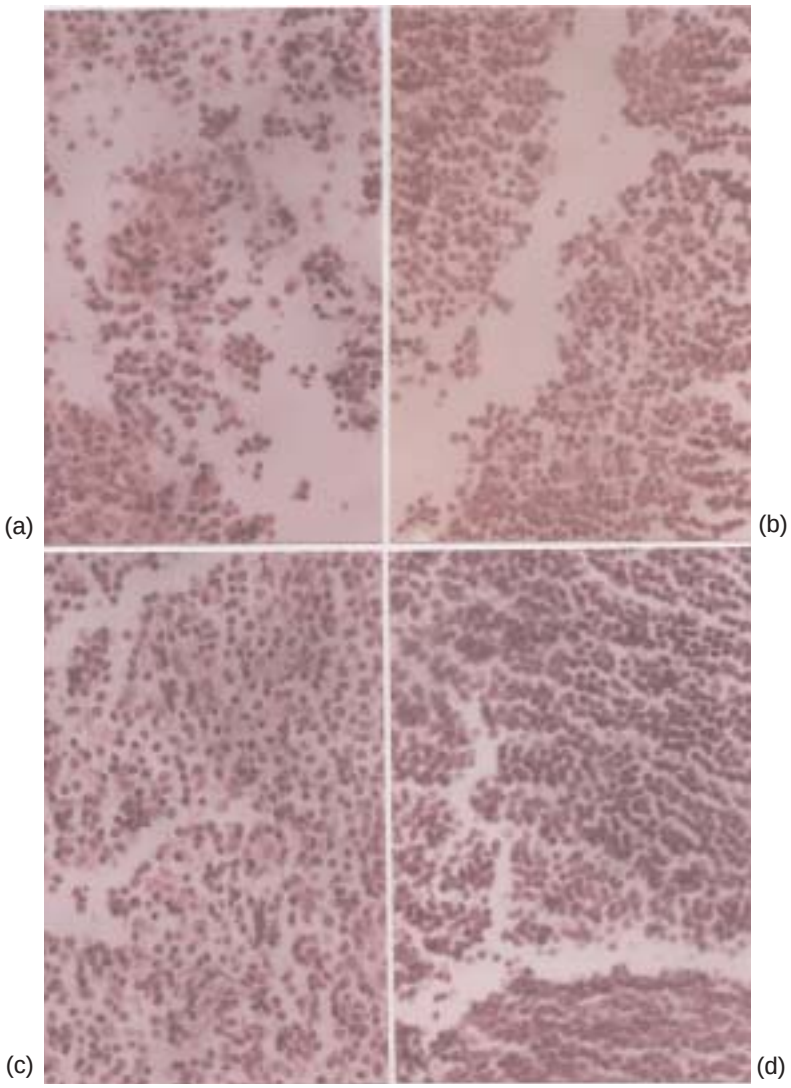


Figure 6: (a) Section of the cortex of the thymus gland of aged mice showing the decline in thymocytes production, (b, c and d) sections of the cortex of the thymus gland of Hepanox-treated aged mice (0.4, 2 and 10 μg/mouse) showing the gradual increase in the number of thymocytes (H and E, x400).



antioxidants also appears to slow down the increase in superoxide production by phagocytes that usually occurs with aging (39), an effect that may be beneficial since it prevents an excessive oxidative stress.

The present results show that, in old mice, there is a significant decline in PFC response to *in vivo* immunization with SRBC. Treatment of aged mice with hepanox preparation elicited a gradual increase in PFC response. This increase was statistically significant with the higher doses of hepanox (2 and 10 μg/mouse). These results are consistent with the results of Girodon *et al.* (8) who reported that antibody titers after influenza vaccine in elderly patients were higher in groups that received trace elements and vitamins. Previous studies showed that after primary immunization, antibody responses of aged individuals are characterized by lower, slower, and shorter responses than those observed in young subjects (40, 41). Micronutritional supplementation has been shown to be effective in restoring antibody response to vaccine in the elderly people (42, 43).

The present study showed that the number of T-lymphocytes decreased significantly with advancing age of mice. Treatment of aged mice with hepanox preparation elicited a progressive increase in the number of T-lymphocytes. This increase was statistically significant with the higher doses of hepanox (2 and 10 μg/mouse). The present results agree with the results of Penn *et al.* (15) who found that supplementation of vitamins A, C and E to elderly long-stay patients significantly increased the absolute number of T cells, T4 subsets and T4 to T8 ratio. These results are in good accordance with the results of Lesourd (7) who reported that aging is associated with changes to the equilibrium of peripheral T-lymphocyte subsets, such as decreases in the ratios of mature to immature, naive to memory, and T helper 1 to T helper 2 subsets. Nutritional therapy may improve immune responses of elderly people.

The present study showed that *in vitro* addition of hepanox preparation (0.002, 0.01 or 0.05 μg/well) to old mice splenocytes stimulated with Con A mitogen significantly increased T-cells proliferation. These results are consistent with the results of Penn *et al.* (15) who found that vitamins A, C and E supplementation to elderly long-stay patients significantly increased the proliferation of lymphocytes to PHA mitogen.

One of the central events implicated the development of immune response is the proliferation of activated lymphocytes that results in the clonal expansion of functional antigen-specific cells. The decrease of lymphocyte proliferation in elderly seems to reflect a progressively decreasing proportion of functional T-cells rather that a uniform decline in the function of all cells (44). This decline could be due to the excessive apoptosis of those lymphocytes (45). Since antioxidants are known to inhibit apoptosis (46), this could be the mechanism responsible for the antioxidant-caused improvement of the lymphoproliferative response.

The oxidant-antioxidant balance is an important determinant of immune cell functions, including maintaining the integrity and functionality of membrane lipids, cellular proteins, and nucleic acids. Optimal amounts of antioxidants are needed for maintenance of the immune response across all age groups. This need might be more critical, however, in aged individuals (23). Age-associated dysregulation of immune response is well documented. The well-known age-related increase in free radical formation and lipid peroxidation contributes, at least in part, to this phenomenon (47). Supplementation with micronutrients can play a crucial role in the maintenance of normal immune function in the elderly (37). The protection of cell membranes and other cellular components of immune cells against lipid peroxidation, a very important antioxidant-related mechanism (48), involves prevention of the harmful effects of oxygen free radicals.

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