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Research Article

Immune Cell Counts, Plasma Immunoglobulin Contents and INF-γ Gene Expression in Rats Exposed to Bisphenol A

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Abstract

The purpose of this study was to evaluate the effect of low-dose graded of bisphenol A (BPA) on immune cell counts, immunoglobulin contents and gamma-interferon (INF-γ) gene expression in rats. Bisphenol A was injected intraperitoneally to male Wistar rats at doses of 15, 30 and 45 µg/kg body weight for 5 weeks. Rats were anesthetized with diethyl ether and blood samples collected, plasma separated and spleen inserted in liquid nitrogen. Total and differential white blood cells, antibody titers, and gene expression of INF-γ were measured. Plasma malondialdehyde levels increased linearly as BPA doses increased. Bisphenol A at dose of 45 µg/kg body weight resulted in significant increase of plasma cortisol level as compared with other treatments. Red blood cell counts decreased linearly as doses of BPA increased. There were no significant differences among treatment for eosinophil, monocytes and basophil counts. The gene expression of INF-γ increased as doses of BPA increased. Rats received 45 µg BPA/kg body weight had 3.2 folds higher gene expression of INF-γ than the control group. The plasma IgG and IgA levels increased linearly as bisphenol doses increased. There was no significant difference among treatments for plasma IgM level. Based on the results of this study, BPA at low doses may result in increase of immune responses in a dose dependent manner.

Keywords: *bisphenol A, Blood cells counts, INF-γ, Immune response, Rat*

Bisfenol A'ya Maruz Bırakılmış Sıçanlarda İmmun Hücre Sayısı, Plazma İmmunglobulin İçeriği ve INF-γ Gen Ekspresyonu

Özet

Bu çalışmanın amacı, sıçanlarda immun hücre sayısı, immunoglobulin içeriği ve gama-interferon (INF-γ) gen ekspresyonu üzerine bisfenol A'nın (BPA) kademeli düşük doz etkisini değerlendirmekti. Bisfenol A Erkek Wistar ratlara 5 hafta boyunca 15, 30 ve 45 ug/kg canlı ağırlık (c.a.) dozlarında intraperitonal olarak enjekte edildi. Sıçanlar dietil eter ile anestetize sonra kan örnekleri toplandı, plazma ayrıldı ve dalak sıvı azot içine konuldu. Toplam ve diferansiyel beyaz kan hücreleri, antikor titreleri, ve INF-γ gen ekspresyonu ölçüldü. Plazma malondialdehit düzeyleri BPA dozları arttıkça doğrusal olarak arttı. Bisfenol A'nın 45 ug/kg c.a. dozu, diğer tedaviler ile karşılaştırıldığında plazma kortizol seviyesinde önemli bir artışla sonuçlandı. BPA dozları arttıkça kırmızı kan hücresi sayımları da doğrusal azaldı. Tedavi grupları arasında eozinofil, monosit ve bazofil sayıları yönünden anlamlı bir fark saptanmadı. BPA dozları arttıkça INF-γ gen ekpresyonu da arttı. 45ug/kg c.a. BPA verilen sıçanlar kontrol grubuna göre 3.2 kat daha yüksek INF-γ gen ekpresyonuna sahipti. Plazma IgG ve IgA düzeyleri bisfenol dozları arttıkça doğrusal olarak arttı. Plazma IgM düzeyi için tedavi grupları arasında anlamlı bir fark yoktu. Bu çalışmanın sonuçlarına göre; düşük dozlardaki BPA, doz-bağımlı bir şekilde immun tepkilerin artmasına neden olabilir.

Anahtar sözcükler: *bisfenol A, Kan hücre sayımı, INF-γ, İmmun yanıt, Sıçan*

INTRODUCTION

Bisphenol A (BPA) is a monomer of plastics and epoxy resins that is pervasive in the soil, water and food. Recently, exposure of human and animals to BPA has been increased. The presence of BPA in urine of 95% of human urine samples was reported [1], which indicates that environmental exposure is widespread. In the last two decades an increasing interest can be observed on biological effects of this compound. Most studies on endocrine disrupters including bisphenol A have been focused on reproductive toxicology and carcinogenesis [2-5]. It acts as endocrine disrupter with estrogenic activities in the body. Estrogen has stimulatory effects on humoral immune responses [6,7]. More recent studies demonstrate that estrogen increases the secretion of interferon-γ (IFN-γ) from splenic lymphocytes, which play a major role in regulating the function of all key immune cells [8,9]. Yoshino et al.[10] suggested that

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prenatal exposure to BPA result in the increase of IFN-γ secretion and up-regulation of immune responses. Youn et al.[11] reported that IFN-γ production induced by BPA treatment after it suppressed IL-4 production. It was not clear that these effects occur in mRNA formation of IFN-γ.

In addition to estrogenic activity, BPA could initiate nitrosative and oxidative stress. Rats prenatally exposed to a human-relevant exposure dose of bisphenol showed increased reactive nitrogen species [2,12] and it also increased reactive oxygen species [3,13,14]. Reactive nitrogen species act together with reactive oxygen species to damage cells, causing nitrosative and oxidative stress. This type of stress is one of the factors that cause immune dysfunctions [14].

Therefore, it is of interest to determine whether BPA influences the immune system in a manner similar to estrogen or reduce immune response because of inducing the stress. There are discordances in the literature and some controversy over the resulting about the effect of BPA on immune parameters. Moreover, information about its effect on blood cells counts and expression of immune genes is also scarce. Therefore, the purpose of this study was to evaluate the effect of low range doses of BPA on immune cell counts, immunoglobulin contents and gene expression of INF-γ in rats.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch (approval date: 30.06.2014; no: 93530).

Chemicals

Bisphenol A was purchased from Sigma Chemical Company (CAS Registry No. 80-05-7). BPA was dissolved in 5% ethanol solution. Malondialdehyde kit was also provided by Pars Azmoon Company (Tehran, Iran). Immunoglobolins kits were provided from Life Diagnostics Inc. (West Chester, PA, USA). Cortisol ELISA Kit was provided from Mono Bind Company (Lake Forest, CA, USA). All other materials were of analytical grade, obtained from standard sources.

Animals and Experimental Design

Twenty male Wistar albino rats (50-55 g body weight) were purchased from the Razi Institute (Karaj, Iran). The animals were housed in polycarbonate cages, fed a standard laboratory diet and water *ad libitum*. Rats were exposed to a 12 h light/dark cycle, and maintained at 20±2°C. After one week of acclimatization to the animal house, rats were weighted and randomly divided into four experimental groups (5 rats in each). The first group served as control group and was injected 5% ethanol solution intraperitoneally. Rats of the second, third and fourth groups received BPA at doses of 15, 30 and 45 µg/kg body weight four times per week for 5 weeks. The doses of bisphenol were calculated according to the animal's body weight before each injection.

Blood Sampling and Measurements

At the end of experiment, rats (weight, 170 g; age, 8 weeks) were anesthetized with diethyl ether and blood samples were collected into vacuum tubes from heart. Immediately after collection, 500 µL of blood were transferred to micro-tube containing 100 µL sodium citrate solutions (3.85 mg/100 µL) and immediately mixed. These tubes transferred to laboratory (Kharazmi Lab., Tehran, Iran) for counting red blood cells and total and differential white blood cells. Remainder of blood sample was transferred to glass tubes containing heparin and centrifuged at 1500 × *g* for 15 min. Plasma was obtained and stored at -20°C until analyses of malondialdehyde (MDA), cortisol and antibody titers.

Plasma malondialdehyde level was determined using commercial kit (Pars Azmoon, Tehran, Iran) based on method described by Dropper et al.[15]. Red blood cells, total and differential white blood cells counts were done in hemocytometer (T-890, Culter, USA). Giemsa-stained blood films were used for differential white blood cell (WBC) counts.

The rat IgG, IgA and IgM ELISA kit (Life Diagnostics Inc., PA, USA) was used for measurement of plasma IgG, IgA and IgM. The assay uses goat anti-rat IgG, IgA or IgM for solid phase immobilization and horseradish peroxidase (HRP) conjugated goat anti-rat IgG, IgA or IgM antibodies for detection (Life Diagnostics Inc., PA, USA). Plasma cortisol level was measured by rat cortisol EIA Kit (Mono Bind Co., CA, USA).

Quantification of INF-γ Gene Expression

At the end of experiment, spleen were removed and immediately stored in liquid nitrogen for messenger RNA (mRNA) extraction using extraction kit (Vivantis Company, Malaysia). cDNA synthesis was done by reverse transcriptase according to the kit (Vivantis Company, Malaysia). Real time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA).

Statistical Analysis

Collected data were analyzed using completely randomized design using ANOVA procedure of SAS (SAS Institute, Cary, NC). To evaluate the differences between the control and treatments, significant means were analyzed using Duncan's multiple range tests. In all cases, $P \leq 0.05$ were considered significant.

RESULTS

In the present study, BPA was administered intraperitoneally in 5% ethanol solution every forty-eight hours for 5 weeks on male Wistar rats. Body weight of rats

received different doses of BPA did not show significant differences and when compared to the control group.

The effect of different treatments on plasma MDA concentration is shown in *Fig. 1*. Duncan's test showed significant differences among treatments for plasma MDA concentration. The lowest MDA concentration was found in control group and the highest was for rats received 45 µg BPA/kg body weight. Based on orthogonal contrast, MDA concentration increased linearly as doses of bisphenol A increased.

There was no significant difference for plasma cortisol level among rats in control group and those in groups received 15 and 30 µg BPA/kg body weight *(Fig. 2)*. Administration of 45 µg BPA/kg body weight to rats resulted in significant increase of plasma cortisol level as compared with other treatments.

Red blood cell counts decreased linearly as doses of BPA increased *(Table 1)*. The highest counts of white blood cells were observed in rats received 30 µg BPA/kg body weight and the lowest one found in control group. There was no significant difference for white blood cells count among rats received different doses of bisphenol A, but difference among these groups and control group was significant. The highest lymphocyte count was found in rats received 15 µg BPA/kg body weight and the lowest count found in rats received 45 µg BPA/kg body weight. There was significant difference for neutrophil count

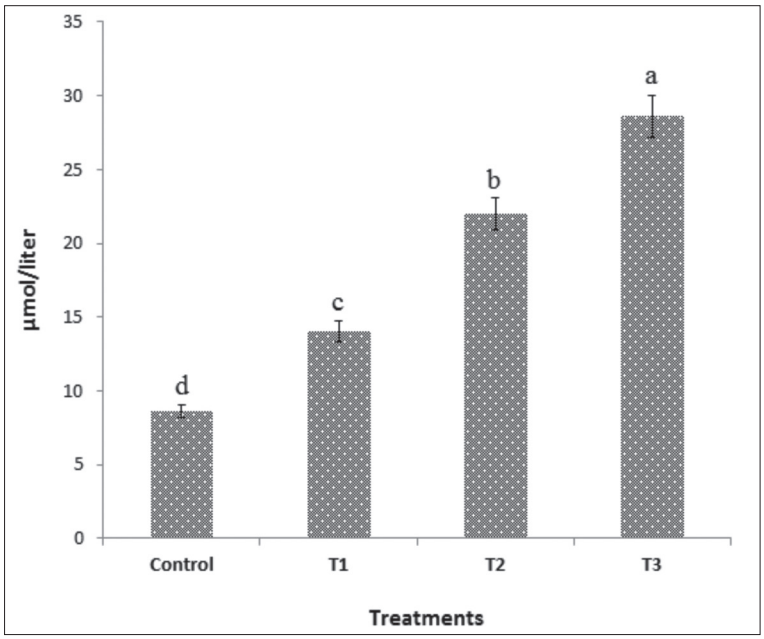

Fig 1. The effect of graded doses of bisphenol A on plasma malondialdehyde level of rats. Control: control group injected i.p. 5% ethanol solution; T1, T2 and T3: injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively

Şekil 1. Sıçanlarda bisfenol A'nın kademeli dozlarının plazma malondialdehit düzeyi üzerine etkisi. Kontrol: %5 i.p. etanol çözeltisi enjekte edilen kontrol grubu; T1, T2, T3: sırası ile 15, 30 ve 45 ug/kg c.a. dozunda bisfenol A enjekte edilmiş

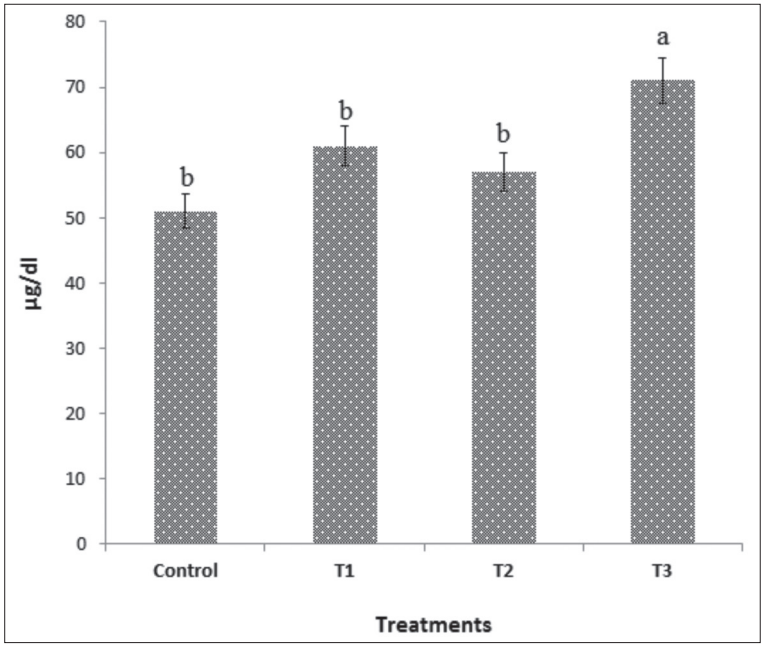

Fig 2. The effect of graded doses of bisphenol A on plasma cortisol level of rats. Control: control group injected ip 5% ethanol solution; T1, T2 and T3: injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively

Şekil 2. Sıçanlarda kademeli bisfenol A dozlarının plazma kortizol seviyesi üzerine etkisi. Kontrol: % 5 i.p. etanol çözeltisi enjekte edilen kontrol grubu; T1, T2, T3: sırası ile 15, 30 ve 45 ug/kg c.a. dozunda bisfenol A enjekte edilmiş

Table 1. *Plasma immunoglobulin contents of rats exposed to graded doses of bisphenol*

Tablo 1. *Bisfenol'ün farklı dozlarına maruz bırakılan sıçanlarda plazma immünoglobulin içeriği*

Treatments*	IgG (mg/L)	IgM (mg/L)	IgA (mg/L)
Control	1113^{c}	788	90^{b}
T1	1565^{b}	831	97^{b}
T2	1752^{b}	846	101^{b}
T3	1980^{a}	855	121^{a}
SEM	117.2	36.8	6.79
Linearity	0.001	0.20	0.002

^{abc} *Means without a common superscript letter differ within each part of a column (P<0.05); * Control: control group injected ip 5% ethanol solution, T1, T2 and T3 injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively*

The gene expression of INF-γ increased as doses of PBA increased *(Fig. 3)*. Rats received 45 µg BPA/kg body weight had 3.2 folds higher gene expression than the control group.

The plasma IgG level increased linearly with increases in BPA doses *(Table 2)*. There was no significant difference among treatments for plasma IgM level. Increase in doses of bisphenol resulted in significant linear increase in plasma IgA level.

DISCUSSION

The reference dose for BPA accepted by the United States Environmental Protection Agency's is 50 µg/kg/day, which is the recommended safe level of exposure. Doses of

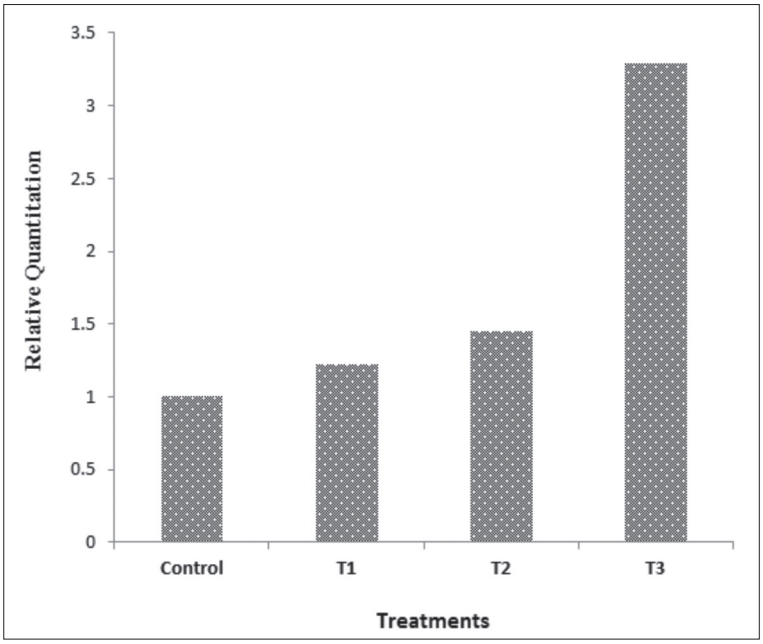

Fig 3. The effect of graded doses of bisphenol A on gene expression of INF-γ. Control: control group injected ip 5% ethanol solution; T1, T2 and T3: injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively

Şekil 3. Bisfenol A'nın kademeli dozlarının NF-y gen ekspresyonu üzerine etkisi. Kontrol: % 5 i.p. etanol çözeltisi enjekte edilen kontrol grubu; T1, T2, T3: sırası ile 15, 30 ve 45 ug/kg c.a. dozunda bisfenol A enjekte edilmiş

Table 2. *Blood cells counts of rats exposed to graded doses of bisphenol A*

Tablo 2. *Kademeli dozlarda bisfenol A'ya maruz bırakılan sıçanların kan hücre sayıları*

Treatments*	RBC ×10^{6}/mm^{3}	WBC ×10^{3}/mm^{3}	Lymph ×10^{3}/mm^{3}	Neutr ×10^{3}/mm^{3}	Eosino ×10^{3}/mm^{3}	Monocyt ×10^{3}/mm^{3}	Basophyl ×10^{3}/mm^{3}
Control	8.0^{a}	3.8^{b}	1.6^{ab}	8.2^{bc}	0.036	0.016	0.007
T1	8.7^{a}	9.8^{ab}	8.6^{a}	5.2^{c}	0.004	0.013	0.007
T2	3.7^{ab}	3.10^{a}	6^{ab}	2.3^{b}	0.032	0.014	0.006
T3	5.6^{b}	7.9^{ab}	6.5^{b}	6.3^{a}	0.029	0.01	0.006
SEM	0.68	0.84	0.42	0.24	0.007	0.007	0.004
Linearity	0.08	0.07	0.053	0.002	0.4	0.8	0.97

^{abc} *Means without a common superscript letter differ within each part of a column (P<0.05); * Control: control group injected ip 5% ethanol solution, T1, T2 and T3 injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively*

.among treatments. The lowest count was for rats received 15 µg BPA/kg body weight and the highest count found in rats received 45 µg BPA/kg body weight. There were no significant differences among treatment for eosinophil, monocytes and basophil counts.

BPA in this study were selected below the reference dose for BPA accepted by this agency and previously tested in a rat study [14]. In the literature, information about the effect of BPA on immune cells and response was scarce, especially effects of important factors such as dose,

duration of exposure and exposure age. Rats used in this study received BPA only for 5 weeks, while humans receive BPA from different sources (metal food cans, water, foods, dental sealants, printed papers, etc.) for a longer period. The main objective of this study was to evaluate the dose effects of BPA on immune cells and response in mature rats (average final weight 170 g, age of 8 weeks) that received BPA from weaning (55 g body weight, age of 5 weeks).

The results of this study show that the levels of MDA increased in dose dependent manner after exposure to BPA. In agreement to our results, a significant increase in the serum MDA level were found in rats exposed to BPA compared to the control group [16]. Also, study of Kourouma et al.[17] showed exposure of rats to BPA resulted in significant increase of liver MDA level. Several studies [2,3,12,13,18,19] demonstrated that exposure to BPA generate reactive oxygen species, but reduce antioxidant content and activity. This condition named oxidative stress. Oxidative stress has proven to be related to BPA toxicity in animal models for years. A study [14] revealed that injection of BPA induces overproduction of hydrogen peroxide in the mouse organs. Hydrogen peroxide is easily converted to hydroxyl radicals. Their results have also revealed decrease in the levels of GSH and increase in the levels of oxidized glutathione by hydroxyl radicals [14]. Therefore, BPA not only increases the free radical formation but also decreases body ability to detoxify reactive oxygen species. So, BPA induces formation of superoxide radicals may cause tissue damage leading to increase in the plasma MDA level.

The plasma level of cortisol increased in group received 45 µg BPA/kg body weight. There are several mechanisms by which BPA disrupts normal endocrine function. BPA can act as a weak estrogen, binding to the estrogen receptor [5] and induce some estrogenic activities. Increases in cortisol level in rats received BPA may be related to estrogenic activity. The study of Edwards and Mills [20] showed that estrogen administration lead to elevated plasma cortisol level. An interesting study [21] demonstrated that bisphenol A, similar to estrogen [22], could increase cortisol production by enhancing phosphorylation of CREB (cAMP response element-binding protein) in normal human adrenocortical cells. Another study [23] showed that BPA could induce corticotropin-releasing hormone expression in the placental cells.

Red blood cells count decreased with increases in doses of BPA. In agreement to our result, Ulutas et al.[24] and Yamasaki and Okuda [25] found that BPA induced a significant decrease in red blood cell count, hemoglobin concentration and packed cell volume. The decrease in the red blood cells may indicate a disruption of erythropoiesis. The administration of estrogens has been known to reduce erythropoiesis in male rats [24]. The present data revealed that BPA induce change in total white blood cells counts or differential count when compared with the control. Inconsistence with our results, Ulutas et al.[24] reported that BPA in rats induce no effect on white blood cells. Increases in reactive oxygen species (as shown by increase in MDA) and also plasma cortisol level of rats received BPA at doses higher than 15 µg BPA/kg body weight may be resulted in decrease of total white blood cells and lymphocyte counts and increase in neutrophil count. BPA changed immune response as it increased gene expression of INF-γ and increased IgG and IgA. In some studies [4,26,27] reported that BPA has multiple actions on patterns of cytokine and antibody production, response to infection, and autoimmune disease progression, T cell subsets, B cell functions, and dendritic cell and macrophage biology. The immunological activities of BPA may be mediated through estrogen receptor signaling, arylhydrocarbon receptor, and the peroxisome proliferator-activated receptor family of nuclear receptors [5]. Estrogen was shown to have immunomodulatory effects, particularly with respect to humoral immunity, and immunosuppressive effects [6,7], particularly with respect to cellular immunity [28]. More recent studies demonstrate that estrogen increases the secretion of IFN-γ from splenic lymphocytes, which play a major role in regulating the function of all key immune cells [8,9]. In a study [28], administration of concanavalin-A, an estrogenic substance, resulted in increase of IFN-γ secretion from thymocytes and splenic lymphocytes. Prenatal exposure to BPA resulted in the increase of IFN-γ secretion and up-regulation of immune responses [10]. Based on report of Youn et al.[11], the production of a strong Th-1 type cytokine (INF-γ) was induced while Th-2 type (IL-4) was suppressed by BPA treatment. Authors [11] suggested that stimulation of prolactin production by estrogenic effects of BPA would affect cytokine profiles, and lead to imbalanced cellular immune response.

In conclusion, bisphenol A could change immune parameters through estrogenic activity and inducing of reactive oxygen species, but its effect on various immune parameters is different. Treatment with BPA decreased count of lymphocytes, but increased IgG, IgA and gene expression of cytokine interferon-γ. Further study is needed to clear the mode of action of bisphenol A on immune parameters.

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