Effect of vitamin E on liver dysfunction in male rat after exposure to nonylphenol

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Abstract
In the recent decades, many environmental pollutants have received great attention due to their ability to mimic the actions of endogenous estrogens. These pollutants are referred as environmental estrogens and are suspected of causing health hazards in both humans and wildlife through disruption of the endocrine system. Nonylphenol (NP) is considered one of those which has severe estrogenic effects on organisms and consequently on different organs. Therefore, the present study was conducted to monitor general toxicity of p-NP on the liver of male rats. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione reductase (GR), liver enzymes and lipid profile (cholesterol, triglycerides, HDL-c and LDL-c) were assayed. Thus, adult male rats were divided into four groups: one group was gavaged with NP dissolved in corn oil, second one is the control group rats which were gavaged with corn oil alone, vit. E. alone was administrated to the third group and the last group was gavaged with a combination of vit. E. and NP. These treatments lasted 30 days. The activity levels of the SOD, CAT, GR and HDL-c were significantly decreased in NP treated rats when compared to the control group, while the MDA, lipid profiles (all except HDL-c) and liver enzymes were significantly increased. Moreover, vit. E caused significant recovery on the different measured parameters once combined with the nonylphenol. In conclusion, this study revealed that NP has estrogenic potential which disrupt antioxidant balance which leads to oxidative stress. This study also supports the significance of vit. E. as an antioxidant.

[Key words: estrogen; nonylphenol; male rats; liver; vitamine E]

Introduction

Recently, the public concern to the environmental pollutants has been increased significantly [1], [2], [3]. These pollutants are referred as environmental estrogens. These environmental estrogens are classified as endocrine disrupting compounds (EDCs). Endocrine disrupter is defined as an exogenous substance that alters one or more functions of the endocrine system, and consequently causes adverse health effects in intact organisms [4], [5]. One of the most ubiquitous environmental pollutants is nonylphenol (NP). NP is the final product of alkylphenol polyethoxylates (APEOs), which is widely used in the preparation of lubricating oil additives, resins, plasticizers, surface-active agents, detergents, paints, cosmetics and can be found in almost all environmental water matrices [6]. It is also found in polyvinyl chloride which used in the food processing and packaging industry. Human are exposed to NP through contaminated water and food products [7]. NP is shown to possess estrogenic property and the lipophilic nature of NP leads to its accumulation in animal tissues [8]. As it is an endocrine disruptor it causes harmful effects including feminization and carcinogenesis to various organisms [9], [10]. p-NP is persistent and more stable than nonylphenol polyetholate (NPE) [11]. APEs undergo biodegradation process to give short side chain derivatives such as nonylphenol (NP), octylphenol (OP) and butylphenol (BP) in anaerobic conditions in water [12]. It accumulates in fat tissues, which may cause serious health problem [13].

Diverse biological effects have been observed following exposure to NP even at micromolar levels [14] and various molecular mechanisms have been proposed with the help of in vitro assay systems. The liver was proved the major organ of accumulation, biotransformation and degradation of environmental pollutants [15],[16]. Also, liver function tests are routinely used as diagnostic marker for hepatotoxicity. Unfortunately, only a few studies reported the effects of NP at low doses on liver of adult rats [17].

Antioxidants are substances capable of counteracting the damaging effects of oxidation in body tissues. There are two major groups of antioxidants in living cells: enzymatic antioxidants and non-enzymatic antioxidants. These groups are divided into several subgroups. The enzymatic antioxidants are divided into primary and secondary enzymatic defenses [18]. The primary defense is composed of three important enzymes that prevent the formation of and neutralize free radicals: glutathione peroxidase, catalase, and superoxide dismutase. The secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase [19].
Vitamin E is the only major lipid-soluble, chain breaking antioxidant found in plasma, red cells and tissues, allowing it to protect the integrity of lipid structures, mainly membranes [20]. Vitamin E is classified as an antioxidant due to its ability to scavenge lipid radicals and terminate oxidative chain reactions.
Most studies on NP focused on its effect on reproduction and limited information considering the toxic effect on liver are available. Consequently, this study aimed to evaluate the effect of p-nonylphenol on some enzymatic activities and antioxidant enzymes in liver and serum total protein and total bilirubin of treated developing albino rats. Also, studying the possible role of vit. E. in diminishing the detrimental effect of NP on the liver.

Materials and method

Chemicals and experimental animals

The p-nonylphenol (p-NP, CAS: 8485-2-15-3) used in the present study was purchased from Sigma-Aldrich Inc. (St.Louis, MO, USA). It contains several isomers (a mixture of isomers with differently branched nonyl side chain), with a purity greater than 98%, while the main impurities are traces of 1,000 µg.
Adult male albino rats (n=40, body weight ranges from 150 to 200 g) were obtained from the Animal House, Medical Technology Center, Medical Research Institute, Alexandria University, Egypt. Animals were housed in plastic cages at an environmentally controlled room (constant temperature 25-27 °C, with 12 h light/dark cycle) for two weeks prior to beginning of the experiment for adaptation to the laboratory conditions. They were provided with tap water and standard rat diet (protein 24%, fat 5%, fiber 4%, carbohydrates 55%, calcium 0.6%, moisture 10% and ash 9%).

The animals were randomly divided into 4 equal groups (10 rats each). Each group was housed in separate cage. Oral administration was applied for the four groups as follow: corn oil (0.5 ml, control), vitamin E (100 mg/kg b.wt.), para-nonyl-phenol (0.5 mg/kg b.wt), and vitamin E with a para-nonyl-phenol, respectively.
At the end of experiment which lasted 30 days, rats were sacrificed by cervical decapitation and dissected. Liver from each rat was removed for assay biochemical and enzymatic parameters.

Preparation of liver homogenates

A portion of liver tissues from each rat was stored at -20°C. The reminder portion were minced and homogenized in 5-10 ml cold buffer (i.e, 50 mM potassium phosphate, pH 7.4, 1 mM ethylene diamine tetracetic acid (EDTA). Homogenates were centrifuged at 10,000 rpm for 20 minutes at 4°C [21] and the clear supernatants were used to determine some enzymatic and non-enzymatic antioxidant in liver: Catalase (CAT), Glutathione reduced assay (GSH), and Superoxidedismutase assay (SOD).
Also, this homogenates were used to determine lipid peroxidation level by malondialdehyde (MDA) assay.

Assessment of enzymic antioxidants

Superoxide dismutase (SOD) was assayed by the method of Nishikimi et al. (1972)[22]. Briefly, this assay relies on the ability of the enzyme to inhibit the phenazine metho sulphate mediated reduction of nitro blue tetrazolium dye. The assay mixture contained 50 mM phoshate buffer, 1 mM nitroblue tetrazolium (NBT), 1 mM/L NADH, 0.1 mM/l phenazinemethosulphate (PMS) and extraction reagent. Measure the increase in absorbance at 560 nm for 5 min. for control ($\Delta A_{control}$) and for sample (ΔA_{sample}) at 25°C.

Calculation:

$$\text{Percent inhibition} = \frac{\Delta A_{control}}{\Delta A_{control} - \Delta A_{sample}} \times 100$$

$$\text{SOD activity(U/g tissue)} = \frac{\% \text{ inhibition} \times 3.75 \times 1}{\text{gm tissue used}}$$

Catalase (CAT) was assayed as previously mentioned by Aebi, 1984[23]. Briefly, the assay mixture contained 2.40 ml of phosphate buffer (100 mM, pH 7.0), 500 mM/L hydrogen peroxide and peroxidas enzyme source and preservative. Catalase reacts with a known quantity of H_2O_2. The reaction is stopped after exactly one minute with catalase inhibitor. Incubate 10 min. at 37°C, read sample (A_{sample}) against sample blank and standard ($A_{standard}$) against standard blank at 510 nm.

Glutathione peroxidase (GPx) was assayed by the method of Beutler et al. (1963)[24]. Briefly, the method based on the reduction of 5, 5' dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm

Lipid peroxidation (LPO), Malondialdehyde (MDA), formed as an endproduct of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid to generate a colored product that can be measured optically at 532 nm. A break down product of LPO, thiobarbituric acid reactive substance was measured by the method of Ohkawa et al. (1979)[25]. Briefly, the stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25 N HCl and 2-thiobarbituric acid 0.37% (w/v) in 0.25 N HCl. One volume of the test sample and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min on a boiling water bath. After cooling on ice the precipitate was removed by centrifugation at 1000 g for 15 min and absorbance of the supernatant was measured at 532 nm against blank containing all the reagents except test sample. The value is expressed as µmol of malondialdehyde equivalent formed/min per mg protein.

Liver function biomarkers in the serum

Transaminases namely alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the method of Teitz (1976)[26] where Lactate dehydrogenase (LDH) was assayed according to the method of Henry (1974) [27]. LDH specifically catalyzes the oxidation of lactate to pyruvate with the subsequent reduction of NAD to NADH. The rate at which NADH formed is proportional to LDH activity. The method descrbed determines NADH absorbance increase/minute at 340 nm. Serum biomarkers were determined using the method of Gronall et al (1949)[28], by the presence of an alkaline cupric sulfate, the protein produces a violet color, and the intensity of which is proportional to their concentration. This shows a maximum absorbance reagent blank at 550 nm. Total bilirubin was measured according to the method of Young (2001)[29] depending on the reaction between bilirubin in the sample and the diazonium salt of sulphanilic acid to produce azobilirubin which shows a maximum absorption at 535 nm in an acid medium.

Respecting to the activity of alkaline phosphates, it was measured according to Moss et al. (1987)[30]. In this method, alkaline phosphates (ALP) hydrolyzes p-nitrophenylphnsphate (p-NPP) to p-nitrophenol and phosphate. The alkaline phosphatase (ALP) activity was calculated as follows: U/1=5454 X (ΔA 405 nm/min), Where: ΔAbs is the change in the absorbance and 5454 is theoretical factor.

Determination of lipid profile in the serum

The cholesterol is determined after enzymatic hydrolysis and oxidation by the method of Allain et al. (1974)[31]. The quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The absorbance then measured at 500 nm.
Low-Density Lipoproteins (LDL) were calculated mathematically using the equation of Fruchart (1982)[32].
Triglycerides present in the sample were determined according to Fossati and Prencipe (1982)[33]. As triglycerides break down into glycerol and fatty acids. These glycerols are converted into Quinoneimine. Measure the absorbance of the sample (A_{sample}) and the standard ($A_{standard}$) against blank at 505 nm, and the calculation is done according to the equation:-

Triglycerides concentration (mg/dl) = $\frac{A_{standard}}{A_{sample}} \times 200$, Where 200 is the standard concentration in mg/dl.

Results

3.1. Effect of p-nonylphenol, vitamin E, and their combination on antioxidant enzymes in liver

Table 1 indicated that liver antioxidant enzymes levels in vitamin E treated group were not significantly different compared to control group. Meanwhile, treatment with p-NP alone significantly ($p<0.05$) decreased the enzymes levels compared to control group. The combination of p-NP and vit. E caused recovery of the enzymes levels but there is still significant differences compared with the control group except in case of CAT. Also, the results of lipid peroxidation end product (MDA) indicated that treatment with p-NP alone significantly ($p<0.05$) increased MDA level while treatment with vitamin E alone significantly ($p<0.05$) decreased the MDA compared with the control group. Treatment with vitamin E and p-NP in combination group caused significant ($p<0.05$) decrease in liver compared with the p-NP treated group.
3.2. Effect of p-nonylphenol, vitamin E, and their combination on enzymes activities in liver
Results of liver enzymes in vitamin E treated group have shown significant ($p<0.05$) decrease in ALT compared to control group, while AST, LDH and ALP have presented values with no significance compared with the control group. Treatment with p-NP show a significant ($p<0.05$) increase in all enzymes levels. The combination of p-NP and vit.E. caused significant ($p<0.05$) decrease in only LDH level compared with the control group while the other parameters have been recovered once the combination of p-NP and vit.E occurs (Table 2).

3.3. Effect of p-nonylphenol, vitamin E, and their combination on total protein and total bilirubin in liver

Respecting to the total bilirubin and total protein, no significance was recorded between the different treated groups except in case of total protein in p-NP treated group (Table 3).

3.4. Effect of p-nonylphenol, vitamin E, and their combination on Lipid profile in liver

Lipid profile most frequently includes measurements of total cholesterol (TC), triglycerides (TAG), high density lipoprotein- cholesterol (HDL-c) and low density lipoprotein- cholesterol (LDL-c). Data listed in Table 4 showed that treatment with p-NP alone caused significant ($p<0.05$.) increase in cholesterol and triglyceride and significant decrease in HDL, while no significant change was found in LDL-c compared to the control group. On the other hand treatment with vitamin E and P-NP in combination group significantly ($p<0.05$) reversed the undesired effect, meaning that increased the HDL-c level and decreased cholesterol and triglycerides levels compared with p- NP treated group.

Table 1. The (mean ±SE) antioxidant enzymes (glutathione reductase, superoxide dismutase, *malondialdehyde* and catalase) in liver for different groups of male rats exposed to vitamin E, p-NP and their combination for 30 days**.**

Parameters (U/g tissue)	**Experimental groups**			
	Control	**Vitamin E**	**p-NP**	**p-NP +Vitamin E**
GR	175.97±21.67 [a]	258.02±14.49[a]	139.50±13.62 [b]	164.96±22.73[a]
CAT	2.64±0.48 [a]	2.69±0.27 [a]	1.47±0.15 [b]	1.90±0.35[c]
SOD	2358.34±354.49 [a]	2903.62±325.37[a]	1639.40±312.39[b]	2127.23±205.80[a]
(MDA) liver tissue	15.83±1.85 [a]	7.73±0.93 [b]	28.81±2.54 [c]	17.43±1.30[a]

Means followed by different superscript in the same row are significantly different, p<0.05

Table2. The (mean ±SE) of serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in liver of male rats exposed to vitamin E, pNP and their combination for 30 days.

Parameters u/L	**Experimental groups**			
	Control	**Vitamin E**	**p-NP**	**p-NP+ Vitamin E**
AST	174.60±9.96 [a]	174.60±9.96 [a]	210.20±15.35[b]	184.40±10.70 [a]
ALT	131.00±7.22 [a]	109.60±5.05 [b]	155.8±6.02[c]	135.00±13.86[a]
LDH	1053.80±241.88 [a]	1130.00±127.28 [a]	1343.90±335.04[b]	1304.40±127.56[b]
ALP	166.80±12.36 [a]	158.20±6.55 [a]	196.00±18.45[b]	176.20±15.52[a]

Means followed by different superscript in the same row are significantly different, p<0.05

Table 3. The (mean ±SE) of serum total protein and total bilirubin in liver o of male rats exposed to vitamin E, p-NP and their combination for 30days.

Parameters mg/dl	**Experimental groups**			
	Control	**Vitamin E**	**p-NP**	**p-NP+Vitamin E**
Total Bilirubin	0.46±0.10 [a]	0.48±0.12 [a]	0.68±0.07[b]	0.50±0.10 [a]
Total protein	5.94±0.38 [a]	6.04±0.19 [a]	5.38±0.43 [a]	5.24±0.47 [a]

Means followed by different superscript in the same row are significantly different, p<0.05

Table4. The (mean ±SE) of serum total cholesterol (TC),triglycerides (TAG),high density lipoprotein-cholesterol (HDL-c)and low density lipoprotein- cholesterol (LDL-c) in liver of male rats exposed to vitamin E, p-NP and their combination for 30 days.

Parameters (mg/dl)	**Experimental groups**			
	Control	**Vitamin E**	**p-NP**	**p-NP+ Vitamin E**
Cholesterol	84.60±4.48 [a]	83.00±2.89 [a]	97.80±1.40[b]	88.00±5.08[a]
Triglycerides	60.20±3.99 [a]	60.20±3.85 [a]	70.20±2.06[b]	65.20±2.64 [a]
HDL-c	53.80±3. 89 [a]	52.76±3. 80 [a]	17.80±1.66[b]	57.96±6. 65 [a]
LDL-c	22.40±1.21 [a]	23.00±2.52 [a]	60.76±2.05 [b]	20.00±1.77[a]

Means followed by different superscript in the same row are significantly different, p<0.05

Discussion

Subchronic exposure to nonylphenol induced functional and structural changes in the liver, kidney, and spleen of growing male rats and also in total protein, and alkaline phosphatase levels [34]. The liver was proved the major organ of accumulation, biotransformation and degradation of environmental pollutants [15],[16]. To date, there is a controversy about the toxicity of nonylphenol on liver. Meanwhile, only a few studies reported the effects of NP at low doses on liver of adult rats [17]. The present study revealed a disturbance in the oxidative status in rat liver. The correlation between oxidative stress, antioxidant potential and development of chronic disease has become evident. Oxidative stress adversely alters many biological molecules leads to loss of form and function. SOD, GR and CAT constitute a mutually supportive team of antioxidant enzymes which provide a defense system against reactive oxygen species (ROS). The results of the current study reflect the inability of the liver to eliminate free radicals and indicate that NP induces oxidative stress by disturbing the balance between radicals and antioxidant defense system in liver. Korkmaz et al. (2010)[35] showed that NP disturbs the balance between ROSs and antioxidant defense system and causes oxidative stress in the liver of rats. Moreover the results are in agreement with those obtained by Jubendrass et al.(2012)[6]. The change in antioxidant enzyme activities is relevant to the ability of the liver to fight against oxidative stress during p-NP exposure. This study showed that SOD, CAT, GSH-Px levels were significantly decreased in the liver after the treatment of 4-NP compared with the control group. In order to support this hypothesis, this study considered that H_2O_2 and MDA as markers for LPO and as indicator of oxidative liver injury [36]. All these findings indicated that p-NP promote antioxidant activity to induce liver damage.

Vitamin E, a strong lipid soluble antioxidant present in the cell, naturally accumulates in the membranes of mitochondria and endoplasmic reticulum and protects cells from lipid peroxidation [37]. Thus, vit. E. showed to reverse the toxic effect of NP on the different examined parameters.

Liver function tests are routinely used as diagnostic marker for hepatotoxicity. Plasma AST and ALT are the most sensitive markers in the diagnosis of hepatic damage. ALT activity is an important index to measure the degree of cell membrane damage while AST is an indicator of mitochondrial damage. Thus, results of the present study indicate that daily administration of NP resulted in hepatic injury as indicated by elevation in the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). The data from other study by Kazemi et al. (2016)[38] indicated that nonylphenol increased alkaline phosphatase level but not changed aspartate aminotransferase and alanine aminotransferase in serum. The results obtained are also in accordance with [35]. On the contrary some other studies have shown no changes in the serum AST and ALT levels even at high doses of NP [39],[40]. The results obtained for oxidative stress, ALT and AST agree with the results obtained by Kourouma et al. (2015)[17] who provide the evidence that 4-NP affects the gene expression related to liver hepatotoxicity, which is correlated with hepatic steatosis. In the

current study, the administration of p-NP for one month resulted in hepatic injury as indicated by elevation in the activity of ALT, AST, ALP and also the level of total bilirubin in the serum. Elevated levels of these markers are familiar indicators for excess cellular leakage and loss of membrane integrity of hepatocytes [41]. These results are in a good agreement with previous studies with similar increases in serum indices for liver damage [35], [42], [43].

The activity of LDH is a biomarker of cell integrity [44]. Elevated plasma levels of LDH have been markedly correlated with chronic oxidative stress, cardiotoxicity, testis carcinoma and many inflammatory conditions [45]. The increase in activity of plasma LDH observed in treated animals suggests that NP exposure causes deterioration of germinal epithelium [46], and consequently may be resulted from breakdown of liver cells.

Liver plays a key role in controlling lipid metabolism. Results of the current study revealed a disturbance of lipid profile as reflected by the significant increase in the level of cholesterol, triglycerides and LDL accompanied with a decrease in HDL.The liver has a network of receptors that regulates the expression of enzymes involved in lipid metabolism [47]. NP has the potential to affect lipid homeostasis in different tissues [48]. Limited studies concerning the effect of NP on lipid profile and the mechanism underlying such acute effect is not fully understood.

Several studies have been shown that supplemental vitamin E has a cytoprotective effect in the liver of rats when subjected to different toxicants [49]. This could be interpreted as vitamin E has the ability to inhibit the formation of the thiobarbituric acid reactive substance, which is used as a measure of free radical mediated-lipid peroxidation in tissue homogenates [50]. In this study, we hypothesized that vitamin E would have a protective role in acute liver injury induced by p-NP. Meaning that, vit. E. showed to reverse the toxic effect of NP on the different examined parameters. Thus, the effect of vit. E. may also explain liver characters in p-NP+Vit.E group compared to p-NP group. p-NP as an endocrine disruptor is an estrogen like component which mimics the effect of estrogen to induce hazardous effects on male rats [51].

Based on the data of the current study, it may be concluded that p-NP has detrimental effect on liver of male rats. Also it supports the possibility that vit. E. plays an efficient role in protecting the liver against p-NP toxicity and oxidative stress in male rats. The potency of vit. E. makes it a possible appropriate candidate and preventive drug for hepatotoxicity resulting from toxicants. More studies are required to investigate the mode of action of such class of chemical which are real threats to the environment.

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