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Cardio protective effects of *Nigella sativa* oil on lead induced cardio toxicity: Anti inflammatory and antioxidant mechanism

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The present study aimed to evaluate cardio-protective effect of *Nigella sativa* oil (NSO) on lead induced cardio toxicity. Forty five albino adult rats were randomly divided into 3 groups: control lead (Pb) group that received lead acetate (20 mg/kg/day) 3 times weekly for 8 weeks and PB + NSO group (rats pretreated with *Nigella sativa* oil (4 ml/kg) orally for 1 h before administration of lead acetate (given as in Pb group). Myocardial injury was assessed by laboratory and pathological studies, and heart rate was recorded in all animals. Lead intake resulted in significant increases in cardiac high-sensitivity C-reactive protein (hs-CRP), interlukin-6 (IL-6), E-selectin, troponin I, malondialdehyde (MDA) and serum creatine kinase-MB (CK-MB). The cardiac apelin, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione (GSH) levels significantly decreased in Pb group compared to the control. Currently, heart rate and ST segment increased significantly after lead intake. Heart lesions as a result of lead treatment were in the form of hemorrhage, myocardial necrosis, mononuclear cell infiltration and fibrosis. Immuno histochemical results of the heart revealed positive cyclooxyenase-2 (Cox-2) expressions in Pb-treated group. NSO administration produced significant normalization of the physiological parameters as well as restored the histological structure and decreased the COX-2 expression of the heart compared to Pb group. In conclusion, NSO intake has cardio protective potential through its ability to decrease pro inflammatory cytokines, oxidative stress and cardiac tissue damage in lead-induced cardio toxicity.

Key words: *Nigella sativa* oil, lead acetate, cardio toxicity, inflammation.

INTRODUCTION

Cardiovascular diseases (CVD) have become the most frequent cause of death worldwide and their incidence continually rises. There is an association between CVD, inflammation and oxidative stress (Roshan et al., 2011). Lead (Pb) is a non-essential toxic heavy metal widely distributed in the environment; various physiological, biochemical and behavioral dysfunctions are induced by chronic exposure to low levels of pb (Ahamed and Siddiqui, 2007; Alghazal et al., 2008).

A link between ambient air pollutants and health has been reported (Makri and Stilianakis, 2008). Previous studies have reported that exposure to low level Pb has been associated with several disease outcomes such as cardiovascular disease and hypertension. It has been proposed that one possible mechanism of Pb toxicity is generate inflammation (Boris et al., 2008) and the disturbance

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of prooxidant and antioxidant balance by generation of reactive oxygen species (ROS) (Alghazal et al., 2008; Roshan et al., 2012). Adipokines such as apelin, C-reactive protein (CRP), tumor necrosis factor TNF-α and E-selectin represent a family of proteins released by adipocytes that affect various biological processes including metabolism, satiety, inflammation and cardiovascular function (Gaeini et al., 2008).

Nigella sativa (NS) Linn. is an annual herbaceous plant of the Ranunculaceae family and grows in countries bordering the Mediterranean Sea, Pakistan, India and Iran (Ali and Blunden, 2003). *N. sativa* seed contains more than 30% fixed oil and 0.4 to 0.45% volatile oil. The fixed oil is composed mainly of unsaturated fatty acids. Thymoquinone (TQ) is the major active ingredient of the volatile oil (Worthen et al., 1998). Thymoquinone has a strong antioxidant potential due to its scavenging activity towards free radicals (Känter et al., 2005). There are many reports on its biological activities including anti-hypertensive, anti diabetic, anti-bacterial, anti-tumour and immunomodulator (Kökdil et al., 2009). Therefore, this study was initiated to investigate the possible cardio protective effects of *N. sativa* oil on lead -induced cardiotoxicity.

MATERIALS AND METHODS

Chemicals

Lead acetate was purchased from Sigma Aldrich (St. Louis, MO, USA). *N. sativa* seeds were obtained from local market in Assiut, Egypt. They were authenticated by Pharmacognsy Department, Faculty of Pharmacy, Assiut University, Egypt. Blackseed essential oil was prepared according to the procedure described by Burits and Bucar (2000); 75 g of blackseed was crushed and extraction was done using about 220 ml of light petroleum ether in a Soxhlet apparatus. The extraction continued for four hours and was repeated until sufficient oil was collected. The oil collected was analyzed for thymoquinone by high performance liquid chromatography (HPLC) according to the procedure described by Ghosheh et al. (1999). The column was Reprosil Gold 120 C18 type (250 × 4.6 mm,5 µm particle size). The isocratic mobile phase consisted of H_2O: methanol: 2-propanol in the ratio of 10: 9: 1 by volume. Column temperature and the flow rate were 28°C and 1 ml/min, respectively. The detector was a DAD (254.4 nm) and the injection volume was 5 µl. The oil was kept in deep freezer at -20°C until it was used.

Animals

The experimental protocol was approved by the Institutional Animal Research Committee of the Faculty of Medicine, Assiut University, Egypt, and the published guidelines and regulations were followed. Forty five adult male Wistar albino rats, 8 weeks of age, weighing about 180 to 200 g were obtained and maintained in The Assiut University Animal Nutrition and Care House. The animals were caged in metabolic cages and kept under standard conventional laboratory conditions at a temperature of 22 ± 2°C, with a relative humidity of 50 ± 5% and a 12-h/12-h light/dark cycle. They had unlimited access to drinking water and rat chow. The rats were randomly divided into three experimental groups of 15 rats each:

1. Control group: rats given only standard rat chow and water for 8 weeks.
2. Pb group: The rats that received lead acetate from Sigma (St. Louis, MO), at a dose of 20 mg/kg in the form of a saline solution (for intraperitoneal [ip] injection), 3 days weekly for 8 weeks (Ghosheh et al., 1999).
3. Pb + NSO group: Rats pretreated with *N. sativa* oil (4 ml/kg) orally for 1 h before administration of lead acetate which is given at the same dose and for the same duration as in Pb group.

Electrocardiography

Recoding of electrocardiogram (ECG) was done at the end of the treatment. It was recorded by needle electrodes which were inserted under the skin of the four limbs of the animals under anesthesia with urethane (1 g/kg, intraperitoneal injection) in lead II position. The needle was connected to an ECG recorder (ECG Cardiofax Nih Onkohn Kohden, Kogyo Co. Ltd, Kogyo, Japan). The QRS, ST and P-R intervals were recorded. The heart rate (HR) was calculated from the P-R intervals by counting them. At the end of the treatment period, the body weights of the animals of each group were measured and recorded. Blood samples were collected from each rat via retro orbital vein. Then, all animals were decapitated under anesthesia with urethane. Blood samples were initially centrifuged at 3000 rpm for 15 min. Serum was kept at -20°C until analysis of Creatine Kinase-MB (CK-MB) levels. The body cavities were then opened and the heart was quickly excised from the aortic root. Heart tissues were weighed.

Tissue preparation

Heart tissues were homogenized in ice-cold 10 mmol/L Tris-HC1, pH 8.2, containing 0.25 mol/L sucrose, 2 mmol/L 2-mercaptoethanol, 10 mmol/L sodium azide and 0.1 mmol/L phenylmethylsulfonyl fluoride with a polytron (4 vol/wt), and centrifuged at 50,000 g (20 min, 4°C). The supernatants were lyophilized and stored at -20°C.

Assay of the cardiac levels of pro-inflammatory cytokines

Enzyme-linked immunosorbent assays (ELISA) were performed for measuring concentrations of Apelin (ELISA kit, Phoenix Pharmaceuticals, Inc.), E-selectin (rat E-selectin ELISA Kit), high sensitive C reactive protein (rat C-reactive protein (CRP) ELISA Kit, e-Biosceince, Inc), interlukin-6 IL-6 (rat ELISA; BioSource, Camarillo, CA); they were assayed in total cell extracts prepared from heart tissues.

Estimation of cardiac biochemical markers and lead levels

CK-MB levels in serum were determined using a commercial kit supplied by Agappe Diagnostics, Kerala, India and Cardaic troponin I in cardiac homogenate was measured by ELISA. The estimation of cardiac levels of lead was done by an atomic absorption spectrophotometer (Perkin-Elmer, 2380) according to the method of Slater and Sawyer (1971).

Assay of cardiac lipid peroxidation

Malondialdehyde (MDA), which formed as an end product of the

Table 1. Effect of *Nigella sativa* oil on the body weight, heart weight and heart weight/body weight ratio of the experimental animals.

Parameter	Control	Pb	Pb +NSO
Initial body weight (g)	186±6.27	187±6.16	186±6.27
Final body weight (g)	306.9±16.3	290.3±8.11^{**++}	306±16.16^{NS}
Heart weight (g)	1.07±0.1	1.18 ±0.12^{*+}	1.1±0.08^{NS}
Heart weight/body weight ratio (%)	0.35±0.03	0.4±0.03^{**++}	0.36±0.03^{NS}

All values are presented as mean ±SD. for fifteen rats in each group.* $p < 0.05$, ** $p < 0.01$ as compared to control group.+ $p< 0.05$, ++ $p< 0.01$ as compared NSO group. NS : non significant as compared to control group.

spectrophotometer, as described previously (Ohkawa et al.,1979). The MDA level was expressed as n moles/mg protein.

Assay of endogenous antioxidants

Glutathione (GSH) was assayed in heart tissue homogenates using the Ellmen's reagent (DTNB) method (Elman, 1999). The absorbance was read at 412 nm and results were expressed as µmol of GSH/gm of wet tissue. Glutathione peroxidase (GPx) assay was carried out by the method used by Rotruck et al. (1973) in 10% (w/v) homogenates of heart spectrophotometrically at 420 nm. Superoxide dismutase (SOD) activity was assayed in the tissue homogenates (Lowry et al., 1951) spectrophotometrically at 320 nm. One unit of SOD activity is defined as the enzyme concentration required to inhibit the rate of auto-oxidation of adrenaline by 50% in 1 min at pH 10. The total protein content was determined in heart homogenates (10% w/v) of experimental animals (Lowry et al., 1951).

Light microscopic examination

Immediately after euthanasia, heart specimens were fixed in 10% buffered formalin, embedded in paraffin, prepared as 4 µm thick sections and stained with hematoxylin and eosin (HE) (Bancroft et al., 1996). Stained sections were examined under light microscope (Olympus CX31, Japan) and photographed using digital camera (Olympus, Camedia C-5060, Japan). The most significant histopathological lesions were listed and their incidence (present/not present) was recorded.

Immunohistochemistry (IHC)

Sections of 5 mm thickness were placed on positive charged slides. Briefly, the sections were de-paraffinised and endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H_2O_2) in PBS for 30 min. Antigen retrieval was performed by microwaving the sections in 0.01 M sodium citrate buffer (pH 6.0). The slides were then rinsed in PBS, blocked with normal goat serum and incubated, respectively with the primary antibodies COX2 (diluted 1:200 in PBS, Thermo Fisher Scientific, USA) over night at 4°C.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) for all parameters. The data were analyzed using GraphPad Prism data analysis program (GraphPad Software, Inc., San Diego, CA, USA). For comparison of statistical significance between different groups, Student Newman-Keuls t-test for paired data were used. For multiple comparisons, one-way analysis of variance [(one-way-analysis of variance (ANOVA)] test was done followed by the least significant difference (LST). Correlations were assessed using Spearman's non-parametric correlation coefficient as described by Knapp and Miller (1992). A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

No mortality was observed in animals of any group exposed to lead acetate alone or in combination with *N. sativa* oil during the treatment period of 8 weeks.

Body, heart weight and heart weight/ body weight ratio

Table 1 reveals a significant decrease in the final body weight of Pb group compared to control ($p < 0.01$). However, treatment of rats with NSO significantly attenuated the decrease of the final body weight compared to Pb group ($p < 0.01$). There was no significant difference in body weights between Pb + NSO and control group. The heart weights of Pb group were significantly higher than those of Pb + NSO and control ($p < 0.05$, respectively). Pretreatment with NSO for a period of 8 weeks decreased significantly the weights of hearts as they did not differ from those of the control group. The heart weight/ body weight ratio of Pb group was significantly increased than those of control and Pb + NSO group ($p > 0.01$). However, this ratio decreased significantly after NSO treatment, but was still higher than those of control ($p > 0.05$).

Status of tissue leads content

In this study, lead levels in the rat cardiac tissue of Pb group were significantly higher compared to the control (p

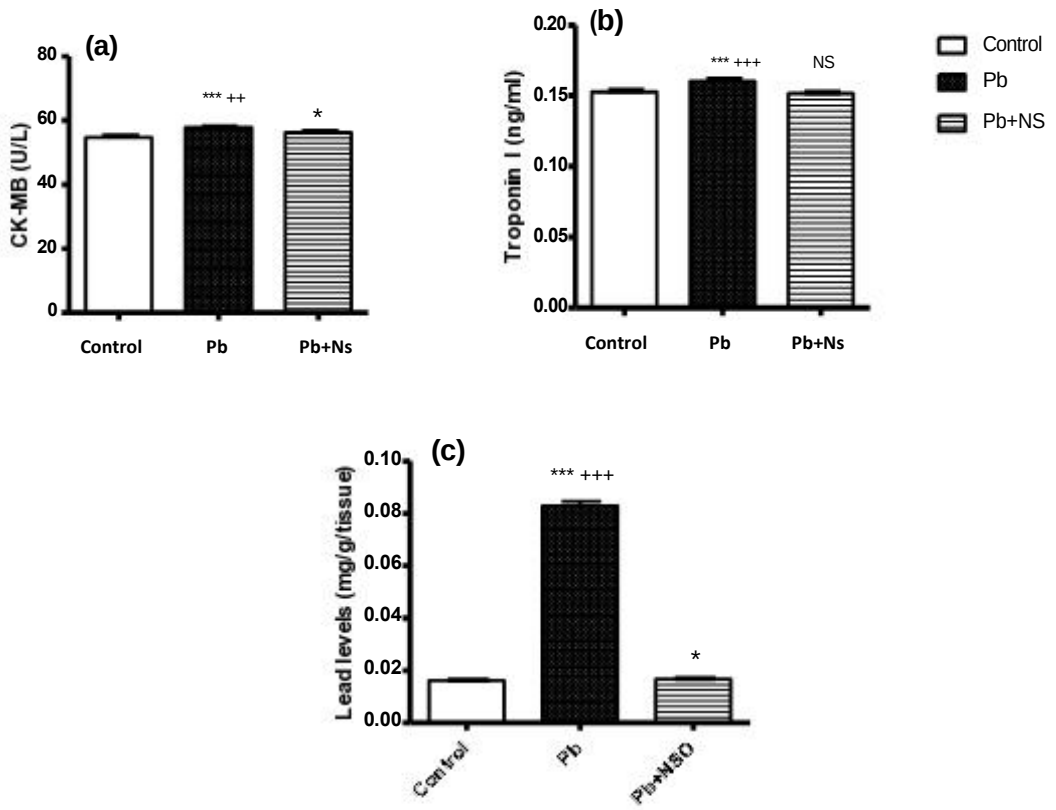

Figure 1. Cardiac biochemical markers and lead levels. a. Serum CK-MB levels (U/L). (b) Cardiac levels of troponin I(ng/ml). (c) Cardiac levels of lead (mg/g tissues). Data are presented as the mean± SD (15 rats for each group), * p< 0.01 and ***p< 0.001 versus the control group. ++ p< 0.01 and +++ p<0.01 versus Pb+NSO group.

< 0.001). However, when the rats were pre-treated with NSO, the cardiac tissue lead content was found to be reduced significantly compared to Pb ($p < 0.001$) and ($p < 0.05$) control groups (Figure 1).

The effect of *N. sativa* oil on changes of heart rate and electrocardiogram patterns induced by lead acetate

The mean values of heart rate in the rats of Pb group increased significantly than those of the control ($p < 0.01$) and Pb + NSO groups ($p < 0.01$). The treatment with NSO (Pb + NSO group) prevented the increment in heart rate and there were no significant differences of heart rate values between Pb + NSO and those of the control rats. Electrocardiogram patterns of normal and experimental animals are shown in Table 2. Lead acetate induced rats showed a significant ($p < 0.01$) increase in ST-segment compared to the control group. NSO treated group showed decreased ST-segment significantly but still higher than those of control rats ($p < 0.05$). The differences in the QRS interval were not significant in any group compared to the controls.

The effect of *N. sativa* oil on the pro-inflammatory cytokines of cardiac tissues

The protective effect of *N. sativa* oil on the levels of the inflammatory cytokines in chronic exposure to pb at the concentration of 20 mg/kg 3 times per week for 8 weeks was assessed in male Wistar rats. As shown in Table 2 the values of apelin were significantly lower ($p < 0.01$) while HS-CRP, E-selectin and IL-6 were significantly higher ($p < 0.001$) in the Pb group than controls. Furthermore, data in this study indicated that after 8 weeks of treatment with NSO, the values of E-selectin, HS-CRP and IL-6 reduced ($p < 0.01$, $p < 0.05$, $p < 0.05$, respectively), while the values of apelin increased in Pb + NSO group significantly than those of Pb group ($p < 0.05$); but they were still significantly different from those of the control group ($p < 0.05$) (Table 3).

Table 2. Effect of NSO treatment on electrocardiographic parameters in lead induced cardio toxicity in rats.

Parameter	ECG parameter		
	Heart rate (beat/min)	QRS duration (ms)	T interval (ms)
Control	371.5±4.29	4.59±0.09	10.16±0.11
Pb	379.9±8.98^{**++}	4.56±0.1^{NS}	10.39±0.37^{***+}
Pb+ NSO	372.1± 3.84^{NS}	4.58±0.09^{NS}	10.39±0.37*

Data are presented as mean ±SD.***p< 0.001,** p< 0.01 and * p<0.05 versus the control group.$^{++}$p< 0.01 and $^{+}$p<0.05 versus Pb+NSO group. NS: non significant compared to control group.

Table 3. The levels of pro-inflammatory markers in cardiac tissues of different groups at the 8th week.

Parameter	Control	Pb	Pb+NSO
E-selectin (ng/ml)	71.11±3.53	79.13±2.72^{***++}	74.86±5.08*
HS-CRP (mg/ml)	0.054±0.003	0.089±0.035^{***++}	0.06±0.0067*
Apelin (pg/dl)	3.65±0.44	2.74±0.56	3.25±0.49
IL-6 (pg/mg)	633.9±5.15	641.4±4.48	637.5±4.3

Data are presented as the mean± SD (15 rats for each group), HS-CRP: high sensitive c-reactive protein,IL6: interlukin -6. * p< 0.01 and ***p< 0.001 versus the control group.+ p< 0.05 and ++ p<0.01 versus Pb+NSO group.

Table 4. Effect of *Nigella sativa* oil on cardiac tissues levels of MDA, SOD, GSH and GPx in lead acetate induced cardiac toxicity in rats.

Parameter	Control	Pb	Pb+NSO
MDA(nmol/mg protein)	1.31±0.06	2.54±1.32^{***++}	1.59±0.41*
SOD (U/mg protein)	7.38±0.39	6.35±0.54^{***++}	6.96±0.45*
GSH (µm/mg protein)	4.22±0.26	3.02±0.49^{***++}	3.85±0.54*
GPx (µg of GSH utilized/min/mg)	0.433±0.046	0.298±0.041^{***++}	0.378±0.076*

Data are presented as the mean± SD (15 rats for each group), One U of GPx = µg of GSH utilized/min/mg protein. * p< 0.01 and ***p< 0.001 versus the control group.+ p< 0.05, ++ p< 0.01 and +++ p<0.01 versus Pb+NSO group.

The effects of *N. sativa* oil on cardiac tissue damage markers

In the present study, lead acetate administration induced damage to the myocardium. Changes in cardiac tissue troponin I and serum CK-MB levels are presented in Figure 1. It was found that cardiac damage marker (CK-MB and troponin I) levels increased in Pb group significantly ($p < 0.001$ and $p < 0.01$, respectively) compared to the control rats; whereas *N. sativa* oil treatment significantly reversed the elevated CK-MB levels, as they became insignificant to those of control group. On the other hand, cardiac troponin I levels after NSO treatment decreased significantly but they are still higher than those of the control rats (Figure 1).

The effects of *N. sativa* oil on cardiac lipid peroxidation and endogenous antioxidants

Table 4 shows that lead intake for 8 weeks caused a significant ($p < 0.001$) increase in levels of lipid peroxidation as measured by the levels of MDA, as well as a significant decrease ($p < 0.001$) in the levels of the enzymatic antioxidant SOD, GSH and GPx compared to the controls ($p < 0.001$). The treatment with NSO significantly inhibited the increase in the levels of MDA ($p < 0.01$) and caused a significant increase in the levels of the endogenous antioxidants ($p < 0.01$), respectively than those of Pb group. But the levels of MDA and endogenous antioxidants of Pb + NSO group were still significantly different from those of the control group ($p < 0.05$).

Table 5. Incidence of heart lesions in treated rats.

Parameter	Control	Pb-treated	Pb+NSO-treated
Hemorrhage	None	++++	+
Myocardial necrosis	None	+++	+
Mononuclear cell infiltration	None	++++	+
Fibrosis	None	++++	None

+ Lesion observed in 1 to 3 rats; ++ Lesion observed in 4 to 7 rats; +++ Lesion observed in 8-11 rats and ++++ Lesion observed in 12 to 15 rats.

Histopathological results

The histopathological examination of HE-stained heart sections revealed that most of the significant pathological lesions were found in Pb-treated rats. On the other hand, co-administration of NS with lead markedly improved the incidence and severity of these reported pathological lesions (Table 5).

Heart lesions as a result of lead treatment were in the form of hemorrhage, myocardial necrosis, mononuclear cell infiltration and fibrosis. Interstitial hemorrhage was observed in most of Pb-treated rats (Figure 2a). The myocardial muscles revealed signs of myocardial necrosis in the form of cogulative necrosis, hypereosinophilia of the myocyte, loss of striation and karyopyknosis of the nuclei (Figure 2b). Fibrosis in the interstitium with infiltration of mononuclear cells such as lymphocytes was seen (Figure 2c to e). The heart sections of Pb + NSO treated group showed improve-ment of the myocardial muscle which resembles the normal myocardial muscles in most cases (Figure 2f).

Immunohistochemistry results

Immunohistochemical results of Cox-2 in the heart revealed positive Cox-2 expression in Pb-treated group, weak Cox-2 expression in Pb + NSO treated group and negative expression in control group (Figure 3).

DISCUSSION

This study was carried out to observe the cardio-protective effects of *N. sativa* oil on lead induced cardio-toxcity in rats. The reduction of weights observed due to lead toxicity in Pb group was almost similar to the findings of some other researchers such as Haque (2005) who reported reduced weight gain after intoxication with lead acetate. Seddik et al. (2010) found that lead intake caused decrease in growth rate of rats which may be due to the imbalance of metabolism produced by impairing zinc status in zinc dependent enzymes which are neces-sary for many metabolic processes.

In this study, the detected elevation in the heart weight and body weight (HW/BW) ratio of Pb group was thought to be due to necrosis which was attributed to accumula-tion of lipid (Badalzadeh et al., 2008). This is consistent with our pathological finding as lead acetate intake caused myocardial necrosis, mononuclear cell infiltration and interstitial hemorrhage of pb group. Our study revealed a highly significant content of Pb in the cardiac tissue of the experimental rats following treatment with lead acetate; and this is consistent with the findings of Patra et al. (2011). The high amount of lead in the tissues might have brought about oxidative stress-induced damages. Pre-treatment of rats with NSO reduced the concen-tration of lead significantly. This may indicate that NSO could remove lead from the organ either by chelating lead or by increasing the clearance of the heavy metal from organs by mechanism(s) not yet clearly known.

The present results showed that lead acetate admini-stration increased the heart rate and caused prolonged ST segment without causing any significant alternation of QRS duration. These results are consistent with the findings of Badalzahed et al. (2008). The increased heart rate in Pb group was probably due to observed central sympathetic nervous system hyperactivity, reduced baroreflex sensitivity and vagal hypotonia in rats treated chronically with lead (Carmignani et al., 2000), while NSO treatment decreased the heart rate to control level and reduced the ST segment significantly; this indicates that NSO has heart rate-reducing effect, which may occur by activating cholinergic mechanisms (El-Tahir et al.,1993; Hamed et al., 2010).

In the current study, chronic exposure of Pb sig-nificantly increased the cardiac HS-CRP, IL-6 ,E-selectin, troponin I and serum CK-MB and significantly decreased apelin levels in the pb group compared to the control and Pb + NSO groups. These cytokines and markers were assayed as indicators of inflammation and tissue damage in heart degenerative cells and serum of rats treated with lead. Various mechanisms were suggested to explain these effects: inhibition of the calcium-pump or a transport protein, disturbances in mineral metabolism, inactivation of several enzymes, etc. (Patrick, 2006). Pathogenesis of Pb poisoning is mainly attributed to lead-

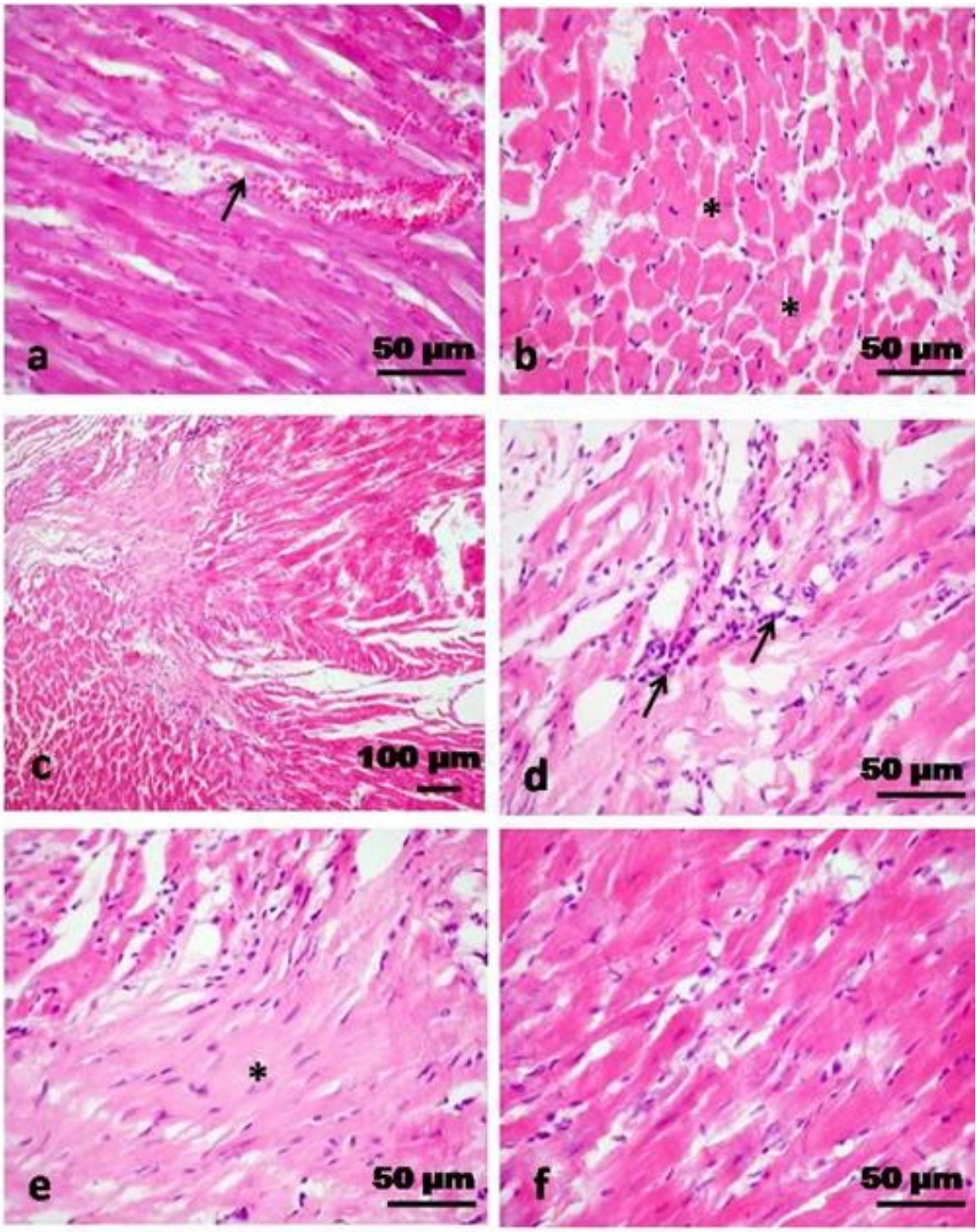

Figure 2. Representative micrograph of the heart from Pb and Pb+NS treated rats. (a) Interstitial hemorrhage (arrow). (b) Myocardial necrosis (asterisk). (c) Myocardial fibrosis. (d,e) Higher magnification showing mononuclear infiltration (arrow) fibrous tissues (asterisk). Section in myocardial muscle in Pb + NSO treated group showing normal appearance HE stain.

induced oxidative stress (Yazdanshenas et al., 2012).

Lead is capable of inducing oxidative damage to brain, heart, kidneys and reproductive organs (Daniel et al., 2004; Gholamhosseini et al., 2009; Wang et al., 2012) and the results of this study support the hypothesis; since there were a significant increase in the level of MDA and a significant decrease in the endogenous antioxidants in the cardiac tissue following intake of lead acetate. This was explained by the high affinity of lead for SH group in several enzymes such as SOD, GSH and GPx; thus it can alter antioxidant activities by inhibiting functional SH groups in these enzymes as these enzymes are potential

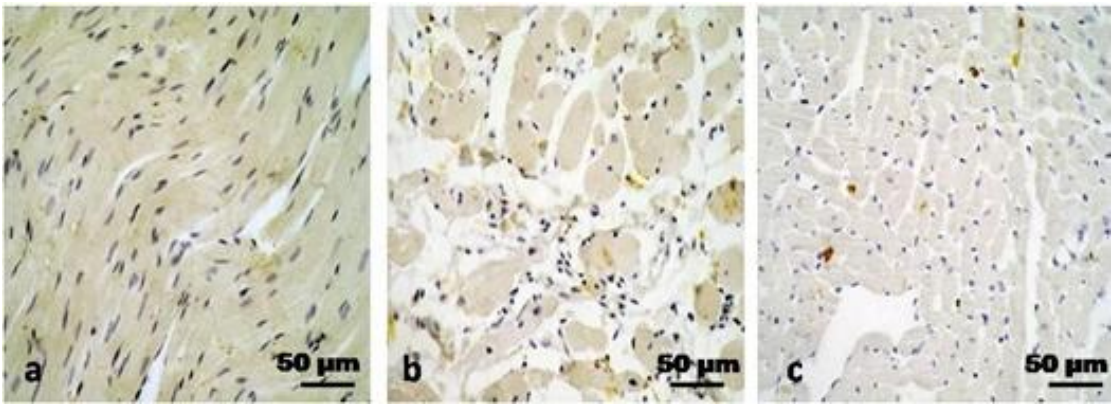

Figure 3. Immunohistochemistry representative micrograph of the heart from Pb, Pb+NSO treated rats and control rats stained Cox-2. (a) Control rat showing negative expression of Cox-2, (b) Pb-treated rats showing positive expression of Cox-2, (c) Pb+NSO treated rats shoeing weak expression of Cox-2.

targets of lead toxicity (Marchlewicz et al., 2007). The present results support the antioxidant activity of NSO. When it was administered, NSO effectively lowered the levels of MDA and caused a significant increase of the endogenous antioxidants in the cardiac tissue.

Our pathological findings confirmed the biochemical data of the present study and showed that lead acetate intake caused cardiac damage in the form of myocardial necrosis, interstitial hemorrhage, mononuclear cell infiltration and fibrosis. Lead acetate increased the cardiac tissues COX -2 expression. However, administration of NSO reduced the cardiac histo pathologic lesions, preserved its structure and decreased the COX-2 expression in Pb + NSO group.

Conclusion

Data from this study suggest that NSO supplementation attenuates lead-induced cardio toxicity by mechanisms related, at least in part, to its ability to decrease the pro inflammatory cytokines, oxidative stress and cardiac tissue damage and preserve the activity of antioxidant enzymes. *N. sativa* could serve as a true functional food and may positively affect health promotion via reducing cardiovascular risk.

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