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Title: The protective role of folic acid against testicular dysfunction in lead-intoxicated rat model

Running title: Folic acid, testicular dysfunction, and lead-intoxicated rat

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Abstract

There is an increasing concern over male reproductive toxicity caused by lead exposure. Folic acid (FA) is supposed to be a promising therapeutic strategy against lead toxicity. Therefore, the aim of this experimental study was to shed light on the potential protective role of FA on lead-induced testicular dysfunction in rats and its possible underlying mechanistic pathways. Rats (n = 24) were divided into four equal groups: the control, the FA, the lead, and the FA & lead groups. After 4 weeks, lead intoxication resulted in a marked reduction in the relative testicular weight and the serum level of testosterone, an impairment in the characters of semen analysis, and an increased content of lead, malondialdehyde and both interleukin-6 and -10 and decreased antioxidant enzyme levels in the testicular tissue homogenate. Furthermore, marked degenerative histological changes and an increased expression of nuclear transcription factor kappa B were also noticed in the testicular tissue of the lead group. Supplementation of FA in association with lead considerably alleviated these adverse outcome responses most probably owing to its cytoprotective ability as emerged from combating the oxidative stress and inflammatory reactions. We concluded that FA could act as a highly effective fighting approach against lead-associated testicular toxicity.

Keywords: folic acid; lead; NF-κB; oxidative stress; semen analysis

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The protective role of folic acid against testicular dysfunction in lead-intoxicated rat model

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Abstract

There is an increasing concern over male reproductive toxicity caused by lead exposure. Folic acid (FA) is supposed to be a promising therapeutic strategy against lead toxicity. Therefore, the aim of this experimental study was to shed light on the potential protective role of FA on lead-induced testicular dysfunction in rats and its possible underlying mechanistic pathways. Rats (n = 24) were divided into four equal groups: the control, the FA, the lead, and the FA & lead groups. After 4 weeks, lead intoxication resulted in a marked reduction in the relative testicular weight and the serum level of testosterone, an impairment in the characters of semen analysis, and an increased content of lead, malondialdehyde and both interleukin-6 and -10 and decreased antioxidant enzyme levels in the testicular tissue homogenate. Furthermore, marked degenerative histological changes and an increased expression of nuclear transcription factor kappa B were also noticed in the testicular tissue of the lead group. Supplementation of FA in association with lead considerably alleviated these adverse outcome responses most probably owing to its cytoprotective ability as emerged from combating the oxidative stress and inflammatory reactions. We concluded that FA could act as a highly effective fighting approach against lead-associated testicular toxicity.

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Introduction

The causative link between heavy metals exposure and health burden remains a topic of research for many years and represents a scientifically attractive area for investigators due to a gradual rise in occupational and environmental poisoning risks. The non-biodegradable nature, prolonged persistence in the environment and variable ways of exposure (Carocci et al., 2015) direct the attention towards lead as a major globally hazardous pollutant with general negative health effects (Sun et al., 2016a) especially on the male reproductive organs (Elgawish and Abdelrazek, 2014).

In Egypt, the control of lead exposure is not efficient and hence, environmentally and occupationally exposed urban Egyptian men were found to have erectile dysfunction and impaired semen parameters (Anis et al., 2007; El-Zohairy et al., 1996). Induction of oxidative stress and inflammation were supposed to be important mechanistic avenues of lead-associated testicular dysfunction (Barbhuiya and Sengupta, 2015; Elgawish and Abdelrazek, 2014). Oxidative stress could enhance the activation of nuclear transcription factor kappa B (NF-κB), which in turn controls the transcription of genes involved in the immune response, inflammation and apoptosis (Lee et al., 2014). However, plenty of controversies still emerged about its testicular toxicity relative to strain/age differential response (Elgawish and Abdelrazek, 2014) providing a driving force for continuation in exploring this area of research with respect to the multifactorial nature of testicular impairment.

The search for agents having a powerful ameliorative impact against this widely spread toxicant with potential natural biological occurrence and predictably having no side effects is worthwhile. In this regard, a still not fully researched folic acid (FA) is considered a highly promising candidate owing to its anti-apoptotic, anti-inflammatory and anti-oxidant activities (Hwang et al., 2011; Majumdar et al., 2010; Zhao et al., 2013) giving a rationality to block the multiple toxicological targets of lead. Dietary FA deficiency acts as a predisposing

factor in increasing the susceptibility for lead intoxication (Rader et al., 1982). Up to our knowledge, there is one available literature about the combating effects of this therapeutic agent against neurotoxicity induced by lead exposure in rat pups (Quan et al., 2015). Therefore, the mission of this study is to shed light on the potential protective role of FA on lead-induced testicular dysfunction in rat and its possible underlying mechanistic pathways.

Material and Methods

Animals and experimental design

Twenty-four adult male Wistar albino rats aged 7-8 weeks (120-135 g in weight) obtained from the Animal House of Assiut University were used in this study. They were kept at room temperature in polypropylene cages and were exposed to a natural 12 h light/dark cycle with free access to standard laboratory chow and water *ad libitum*. Experimental procedures in this study were conducted in accordance with the internationally accepted principles for the Care and Use of Laboratory Animals and were approved by our institutional ethics committee.

After 7 days of acclimatization, rats were randomly divided into four groups (six animals each): the control group received distilled water (DW) daily by oral gavage, the FA group treated daily with FA (CAS: 59-30-3, Oxford Lab. Co., India) at a dose of 2 mg Kg^{-1} body weight (BW) by oral gavage (Ajeigbe et al., 2011) for 4 weeks, the lead group administered lead acetate (CAS: 6080-56-4, Oxford Lab. Co., India) 5 days weekly at a dose of 10 mg Kg^{-1} BW intraperitoneal (i.p.) (Hamed et al., 2014) for 4 weeks, and the FA & lead group received the same previous doses of both FA and lead acetate for the same duration.

At the end of the experiment, animals were weighed and fasting venous blood samples were collected in plain tubes from the retro-orbital vein in anesthetized rats by an experienced laboratory technician. Then, animals were sacrificed by cervical dislocation. Blood samples were centrifuged at 3000 rpm for 15 minutes and the clear supernatant sera were removed and

kept at -20 ºC until use. Orchidectomy was performed by open castration method through a midline incision and the testis was milked out of the incision site, weighed and rapidly exposed by incising the tunica vaginalis and the cauda epididymis was quickly removed and used for semen analysis. The relative testicular weight was calculated as the percentage ratio of testicular weight to body weight. One testis was kept at -80 ºC until use for further analysis and the other testis was used for histopathological examination.

Measurement of the serum testosterone hormone level

Testosterone was estimated by the enzyme-linked immunosorbent assay kit (Cat. No. BC-1115, BioCheck, Inc., Foster City, USA) according to the manufacturer's instructions. The serum testosterone level was expressed as ng mL^{-1}.

Semen analysis

Semen was collected as previously described (Oyeyemi and Ubiogoro, 2005). Briefly, cauda epididymis was opened longitudinally with a pair of fine-pointed scissors, and epididymal content was squeezed into a sterile watch glass. Sperm motility was examined microscopically as explained previously (Zemjanis, 1977). The spermatozoa were counted by a hemocytometer using the improved Neubauer chamber (LABART, Germany) (Pant and Srivastava, 2003). Sperm morphology was assessed using alkaline methyl violet stain. Briefly, the stain was poured off on slides containing diluted semen sample (1:4) and the stain was left for 5-10 minutes. Thereafter, slides were washed with DW, dried with filter paper and examined under the light microscope (Olympus CH, Japan) where the spermatozoa appeared violet in colour. A total of 200 spermatozoa from each rat were examined for morphological changes and the percentage of morphologically normal sperms was recorded. The viability of sperms was assessed using Eosin & Nigrosine stain. The stain could pass only through the non-living cell membrane and hence, non-viable sperms absorbed the stain and

appear red and viable sperms appear colorless. Viability was reported as the percentage of total number of colorless sperms (Bjorndahl et al., 2003).

Measurement of testicular lead concentration

Testicular tissue was digested with concentrated nitric acid and was placed in an oven at 40 °C for 30 minutes. Then, digests were diluted to a constant volume with DW. The diluted tissue samples were analyzed using atomic absorption spectrophotometer (Buck model 210 VGP, East Norwalk, CT, USA), and the results were reported as µg L^{-1}.

Measurement of testicular oxidative stress biomarkers

Testicular tissues from all animals were homogenized in phosphate buffer saline to give 10% (wt/v) homogenate. The homogenate was centrifuged for 15 minutes at 5000 g at 4 ºC. Then, homogenates were used for estimation of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and malondialdehyde (MDA) using commercially available colorimetric kits (Bio-Diagnostics, Egypt, Cat. No. SD2521, GP 2524, CA 2517, and MD 2529; respectively) according to the manufacturer's protocol. Briefly, measuring SOD was based on its ability to decrease the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye to form a red product (Nishikimi et al., 1972). The oxidation of NADPH to $NADP^+$ is associated with a decrease in absorbance at 340 nm providing spectrophotometric means for measuring GSH-Px levels (Paglia and Valentine, 1967). CAT was determined based on the fact that 3,5-dichloro -2-hydroxybenzene sulfonic acid could rapidly terminate the degradation reaction of hydrogen peroxide catalyzed by CAT and react with the residual hydrogen peroxide to generate a yellow product (Aebi, 1984). The reaction of thiobarbituric acid with MDA in acidic medium to form thiobarbituric acid reactive pink product was the principle for estimation of MDA (Ohkawa et al., 1979). Total protein in testicular homogenate samples was assayed using biuret reagent (Gornall et al., 1949). Results

of SOD, GSH-Px and CAT were expressed as U mg^{-1} protein, and results of MDA were reported as nM mg^{-1} protein.

Measurement of testicular interleukin (IL)-6 and -10

Testicular homogenates were used for estimation of IL-6 and IL-10 concentrations according to the manufacturer's instructions (Koma Biotech Inc., Seoul, Korea; K0112464 and K0332134, respectively). Briefly, samples were added to 96-microwell ELISA plates, precoated with a monoclonal antibody directed against each cytokine. Standards of known rat cytokines concentration and unknown samples were pipetted into these wells. After 3 hours of incubation, the IL-6 or IL-10 antigen binds to the coating antibody. After washing, a biotinylated (detection) antibody specific for both cytokines was added to bind to the IL-6 or IL-10 antigens captured during incubation. After incubation, plates were washed and a streptavidin-horseradish peroxidase (Colour Development enzyme) was added. This enzyme binds to the detection antibody. After incubation for 30 minutes and further washing, colour development solution was added and the plates were read using a microplate reader at 450 nm. A standard curve was created to obtain the concentration of the unknown samples. Their levels were reported as ng mg^{-1} protein.

Histopathology and immunohistochemistry

Specimens from the testis of all animals were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. 5 µm thick sections were cut and stained with Hematoxylin and Eosin (H&E) as described previously (Bancroft and Gamble, 2008).

Immunohistochemical staining of NF-κB/p65 was performed by the avidin biotin immunoperoxidase complex technique (Ultravision plus detection system anti-polyvalent HRP/DAB, Thermo scientific Corporation Fremont, CA, USA). Immunohistochemistry was performed according to the manufacturer's protocol. Briefly, 4 µm sections were deparaffinized in xylene and rehydrated with graded alcohol series. Endogenous peroxidase

was blocked by immersion of the sections in 3% hydrogen peroxide solution for 10 minutes. Sections were then subjected to antigen retrieval by immersing them in 10 mM citrate buffer for 10 minutes followed by placing them in the microwave for 8 minutes. Then, samples were incubated with NF-κB p65 at a dilution of 1:100 for one hour at 4 °C. After the application of a secondary antibody, slides were covered by adding one drop of 3-3′-diaminobenzidine chromogen. Finally, sections were counterstained with Mayer's hematoxylin. Negative control slides were prepared by omitting the primary antibody.

Statistical analysis

All data were expressed as mean ± SD. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test after investigating the data for normality using Shapiro-Wilk test to be sure that the data are normally distributed using SPSS program version 16 (SPSS Inc., Chicago, USA). Differences of $P < 0.05$ were considered to be significant.

Results

The relative testicular weight and serum testosterone levels

Table (1) showed that there was insignificant difference between the relative testicular weight of the control and FA groups (0.97 ± 0.11% versus 0.85 ± 0.13%). Lead administration resulted in a significant decrease of the relative testicular weight (0.55 ± 0.16%) when compared with the control and FA groups. Treatment with FA associated with lead administration significantly increased relative testicular weight to 0.91 ± 0.24% in comparison with the lead group. The relative testicular weight of the FA & lead group showed insignificant difference when compared with both the control and FA groups.

Figure (1) revealed the absence of significant difference between the serum testosterone levels of the control group (0.33 ± 0.03 ng mL^{-1}) versus those of the FA-supplemented one (0.29 ± 0.03 ng mL^{-1}). Lead intoxication was manifested by a significant

reduction of the serum testosterone levels (0.15 ± 0.01 ng mL^{-1}) when compared with both the control and FA groups. FA treatment in association with lead intoxication significantly succeeded in elevation of the serum testosterone levels to 0.22 ± 0.01 ng mL^{-1} in comparison with the lead group. However, it was still significantly lower than those of the control and FA groups.

Semen analysis

Figure (2) showed that there were insignificant differences in all tested parameters of semen analysis; sperm motility, sperm count and the percentages of morphologically normal and viable spermatozoa; between control (80.0 ± 4.08, 48.8 ± 5.56, 82.25 ± 3.86 and 84.25 ± 2.98%) and FA (83.0 ± 3.55; 51.8 ± 6.60, 85.0 ± 4.08 and 88.50 ± 2.38%) groups. Lead administration altered semen analysis in the form of significant reductions of sperm motility, sperm count and the percentages of morphologically normal and viable spermatozoa when compared with both the control and FA groups (8.00 ± 2.94, 4.50 ± 1.73, 3.50 ± 1.29, and 10.0 ± 3.55%). FA treatment in combination with lead intoxication resulted in a marked improvement in the form of significant increases in all tested parameters of semen analysis (47.5 ± 6.45, 18.0 ± 4.83, 44.25 ± 5.31, and 60.0 ± 7.07%) in comparison with the lead group. However, in comparison with the control and FA groups, the FA & lead group showed a significant reduction in all tested parameters of semen analysis.

Testicular lead concentration

Regarding lead concentration in testicular homogenates, there was no significant difference between control (0.396 ± 0.08 µg L^{-1}) and FA (0.411± 0.07 µg L^{-1}) groups. A significant increase of lead content in testicular tissue of the lead group (0.656 ± 0.03 µg L^{-1}) was found when compared with both the control and FA groups. Treatment with FA in combination with lead supplementation resulted in a marked reduction of lead content to 0.518 ± 0.07µg L^{-1} in comparison with the lead group. However, there was no significant

increase in lead concentration of testicular tissue of the FA & lead group in comparison with both the control and FA groups (Table 1).

Testicular oxidative stress biomarkers

No significant differences could be found in the testicular enzymatic antioxidant levels; namely SOD, CAT and GSH-Px of the control rats (203.13 ± 45.89, 1.68 ± 0.33 and 42.08 ± 9.13 U mg^{-1} protein; respectively) versus the FA-supplemented ones (201.70 ± 38.18, 1.68 ± 0.36 and 43.58 ± 8.96 U mg^{-1} protein; respectively). The testis of animals of the lead group showed a significant reduction in SOD, CAT and GSH-Px levels reaching 65.55 ± 10.67, 0.55 ± 0.13 and 14.13 ± 2.70 U mg^{-1} protein; respectively in comparison with both the control and FA groups. A significant elevation was observed in SOD, CAT and GSH-Px levels to 126.85 ± 11.76, 1.05 ± 0.13 and 24.40 ± 1.82 U mg^{-1} protein, respectively following FA administration to lead-intoxicated rats, but their levels were still significantly below those in the control and FA groups (Figure 3; A, B, C).

Testicular MDA level of the control group (2.63 ± 0.57 nM mg^{-1} protein) exhibited no significant difference when compared with the FA group (2.5 ± 0.47 nM mg^{-1} protein). Testicular tissue of the lead group was characterized by a significant elevation in MDA levels reaching 5.53 ± 0.66 nM mg^{-1} protein as compared with the control and FA groups. FA treatment of rats intoxicated with lead was efficient in reducing lipid peroxidation as evident by a significant decline in MDA level to 3.88 ± 0.29 nM mg^{-1} protein. Moreover, MDA level of the FA & lead group was significantly higher than that in the control and FA groups (Figure 3; D).

Testicular IL-6 and IL-10

Insignificant differences were noted in the testicular levels of both IL-6 and IL-10 between the control (127.5 ± 5.68 and 130.3 ± 6.37 ng mg^{-1} protein) and FA (127.9 ± 9.08 and 126.3 ± 6.68 ng mg^{-1} protein) groups. However, lead administration resulted in a

significant increase of both IL-6 (207.6 ± 23.94 mg^{-1} protein) and IL-10 (276.6 ± 27.10 mg^{-1} protein) levels in testicular tissue in comparison with both the control and FA groups. Treatment with FA in combination with lead administration resulted in a significant decrease in the levels of both IL-6 (158.1 ± 19.45 ng mg^{-1} protein) and IL-10 (210.7 ± 23.06 ng mg^{-1} protein) in comparison with the lead group. However, in comparison with both the control and FA groups, levels of both IL-6 and IL-10 in the FA & lead group were significantly higher (Table 1).

Histopathology and immunohistochemistry

Microscopic examination of sections of the testis of both the control and FA groups showed the normal histological structure of the testis (Figure 4; A and B). Sections of the lead group revealed the presence of oedematous seminiferous tubules with irregular contour, abnormal architecture and acidophilic degenerated hyalinized center. Furthermore, degenerated or hyalinized interstitial tissue was also present. Spermatogenic, Sertoli and Leydig cells appeared disorganized, degenerated and apoptotic. There were also sloughed apoptotic germ cells in the center of the seminiferous tubules, reduced spermatogenic cells count and reduced height of germinal epithelium (less than 3 layers). In some areas of the seminiferous tubules, there was a complete loss of spermatogenic cells which were replaced by degenerated tissue (Figure 4; C). Treatment with FA in combination with lead administration resulted in a marked histological improvement as seminiferous tubules appeared at different stages of improvement as some seminiferous tubules appeared with a large and others with a small acidophilic fibrosed center. Seminiferous tubules in advanced stages of improvement had a continuous basement membrane and contained nearly healthy multilayered spermatogenic cells at different stages of spermatogenesis. The interstitial tissue and Leydig cells appeared more or less normal (Figure 4; D).

Immunostaining of sections of the testis of the control and FA groups showed moderate cytoplasmic expression of NF-κB in spermatogonia, primary spermatocytes and myoid cells in many of the cross-sections of the seminiferous tubules. In contrast, the majority of the cross-sections showed no nuclear NF-κB expression except for a weak nuclear staining of few Sertoli cells. Identification of Sertoli cells was based on their typical localization in the seminiferous epithelium and their characteristic oval nucleus. Moderate cytoplasmic staining of NF-κB was also observed in Leydig cells (Figure 5; A and B). However, immunostaining of sections of testis of the lead group revealed an intense nuclear immunostaining of NF-κB in the germ cells. Immunostaining of sections of the FA & lead group showed a moderate cytoplasmic and nuclear staining of NF-κB in spermatocytes and spermatid and an intense nuclear and moderate cytoplasmic immunostaining of NF-κB in few germ cells (Figure 5; C and D).

Discussion

In the current study, FA dietary intervention efficiently mitigates lead-induced testicular dysfunction via combating oxidative stress and inflammatory signs due its antioxidant and anti-inflammatory activities. These findings paving the road towards its utilization as a protective therapy against lead-associated reproductive anomalies and left the door open in front of further investigations for its mechanistic molecular approaches.

Normal testicular weight is largely dependent on the mass of the differentiated spermatogenic cells that highly correlate with testosterone level (Reshma Anjum and Sreenivasula Reddy, 2015). Thus, the decreased relative testicular weight of the lead group of the present study might be explained by the withdrawal of testosterone trophic action and the degenerative histological patterns found in their testicular tissues. The marked reduction of the serum testosterone level in the lead group of the current study was reported previously (Kresovich et al., 2015) and was supported by the presence of degenerated and apoptotic

Leydig cells in the histopathological sections of the testis in our study. Inhibition of testicular steroidogenesis biosynthetic enzymes and gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) release (Ji et al., 2015; Klein et al., 1994) could be implicated in this outcome response. It is worthy to note the causative involvement of the hypothalamic-pituitary axis, as a major target for lead toxicity, in the testicular degeneration (Doumouchtsis et al., 2009). However, conflict data arise from the literature regarding the modulatory effects of lead on this axis, most probably reflect the variation in dose and duration of exposure (Allouche et al., 2009; Sokol et al., 2002).

Normal spermatogenesis and also normal secretory functions of the testis depend on the normal production of the testosterone hormone that enhances testicular growth and increases the number of spermatogonia and Sertoli cells (Elgawish and Abdelrazek, 2014; Reshma Anjum and Sreenivasula Reddy, 2015). Hence, decreased testosterone level might be the reason for the observed significant deterioration in semen outcome measures in the lead group of the present study. Our results were in agreement with other studies (Jensen et al., 2006; Reshma Anjum and Sreenivasula Reddy, 2015). Other contributory factors involved in the mechanism of lead-induced alteration of parameters of semen analysis include accessory gland dysfunction, cellular nutrition depletion, sperm chromatin condensation disturbance, germ cell DNA fragmentation and necrosis of germinal epithelium and Sertoli cells (Haouas et al., 2015; Naha and Manna, 2007; Shaban El-Neweshy and Said El-Sayed, 2011).

Lead can cross the blood-testis barrier, accumulate in the testis, and damage germinal cells at various levels of differentiation (Apostoli et al., 1999). This could explain the significantly increased lead concentration noticed in testicular tissue of the lead group vs. other groups of the present study.

Administration of lead, in the current study, shifted the oxidant/antioxidant profile towards oxidant side as manifested by the marked exhaustion of the enzymatic antioxidants

together with the accumulation of lipid peroxidation product in the testicular tissue homogenate. This was in corroboration with earlier reports (Ansar et al., 2016; Hasanein et al., 2016). Lead interrupted redox homeostasis through slow clearance of hydrogen peroxide, stimulation of membrane lipid peroxidation, inhibition of delta-aminolevulinic acid dehydratase and upregulation of production and oxidizing potential of oxidant species (Adonaylo and Oteiza, 1999a, 1999b; Robinson et al., 2015). The increased oxidative stress was incriminated in playing the main role in the pathogenesis of lead toxicity (Mohammadi et al., 2014). Since the membranes of spermatozoa are rich in polyunsaturated fatty acids, they are vulnerable to oxidative stress damage (Abd-Ellah et al., 2016) which might be one of the leading causes of the observed disturbed parameters of semen analysis in the lead group of the present study.

In the current experiment, increased levels of both IL-6 and IL-10 following lead exposure were in the same line with other previous studies (Li et al., 2015; Valentino et al., 2007). Their increased levels indicated the ability of lead to trigger inflammation which might occur in response to increased oxidative stress through activation of NF-κB (Heeba and Hamza, 2015). Interestingly, increased IL-6 could inhibit testosterone production by Leydig cells (Bini et al., 2015). Elevated levels of the anti-inflammatory IL-10 could control inflammation via inhibition of activation of immune cells such as macrophages (Sinuani et al., 2013) and prevention of the pathological effects of the inflammatory cytokines (Carocci et al., 2015). A negative correlation between cytokine levels in the semen and the standard semen parameters was previously reported (Sanocka et al., 2003). Furthermore, an increased level of IL-6 was reported to be present in the semen of infertile men (Camejo, 2003) confirming the important role played by these cytokines in the disturbed semen analysis found in the lead group.

Testis of the lead group in the present study showed the presence of severe degenerative and apoptotic changes in harmony with previous studies (Elgawish and Abdelrazek, 2014; Shaban El-Neweshy and Said El-Sayed, 2011). These histological changes could be related to the damaging effects of the accumulated lead and the increased oxidative stress and inflammatory cytokines recorded in this group. In fact, the primary mechanism by which lead produced cellular damage was found to be through increasing the level of reactive oxygen species which altered the physicochemical properties of cell membranes and induced protein degradation and DNA damage (Sun et al., 2016b). Moreover, oxidative stress stimulated the progression of inflammation which might end in apoptosis of cells (Hu et al., 2015). This explained the increased apoptotic changes seen in the testis of the lead group of the current study. The reduced level of testosterone, necessary to maintain the normal structure and function of the testis (Abd-Ellah et al., 2016), could be also attributed to these histopathological changes. Lead-induced morphological changes might result in complete arrest of spermatogenesis (Shaban El-Neweshy and Said El-Sayed, 2011) which could explain the severely altered semen analysis of this group.

Interestingly, sections of the testis of the lead group in this study showed also an intense nuclear expression of NF-κB transcription factor in agreement with the findings of Rodriguez-Iturbe et al. (2005). NF-κB activation in our study might be the result of the increased oxidative stress (Heeba and Hamza, 2015). NF-κB played a central role in inflammation as it triggered the transcription of proteins involved in the inflammatory response (Sun et al., 2016a) as IL-6 (Taniguchi and Karin, 2014). This might be a reason for the increased levels of interleukins in the lead group of the present study.

The increased relative testicular weight and serum testosterone level following concurrent administration of FA to lead-challenged rats might be caused by the notable return of the histological structure of the testicular cells towards the normal pattern. Consequently,

this group showed an improved sperm quality which was in harmony with the previously reported benefits of FA supplementation on sperm health condition and male fertility (Ibrahim et al., 2011; Shalaby et al., 2010). Low sperm count found in the control rats in comparison with other studies might be related to seasonal and age variations (Guraya, 1987). By acting as an essential building block in DNA synthesis and as a dietary antioxidant, FA is considered a central denominator in germ cell development and protection of cellular membrane and DNA from free radical damage (Ebisch et al., 2007; Joshi et al., 2001). The protective effects of FA against lead intoxication might be attributed to its involvement in a large number of biochemical processes, especially remethylation pathway of homocysteine in the testis which is essential for normal spermatogenesis and normal semen parameters (Shalaby et al., 2010). Furthermore, FA reduced the testicular lead accumulation when compared with the lead group. Binding of lead by FA and its increased excretion (Quan et al., 2015) can underly this effect and contribute to the protective effect of folic acid.

According to the findings of the present study, FA successfully alleviated the SOD, CAT, and GSH-Px depletion and MDA elevation occurred with lead intoxication. This is in agreement with several lines of evidence emerged from other animal models about its broad scope of protection against oxidative damage (Alférez et al., 2015; Majumdar et al., 2010). The current study revealed also that co-administration FA and lead resulted in attenuation of the inflammation associated with lead administration evidenced by a significant decrease of both IL-6 and IL-10. In line with our results, different experimental studies confirmed the anti-inflammatory effect of FA (Tousoulis et al., 2014; Zhao et al., 2013).

Sections of the testis of rats given FA concurrently with lead intoxication in the current study revealed a markedly improved histological architecture. Similar results were obtained by Shalaby et al. (2010) who used FA against methomyl insecticide in rats. Being antioxidant and anti-inflammatory, FA could protect cells against damage caused by lead

administration. FA is important for normal cell multiplication and differentiation processes (Williams et al., 2011), especially during periods of rapid cell division and growth because it is required to synthesize and repair DNA (Ibrahim et al., 2011). Therefore, FA supplementation affected positively spermatogenesis (Forges et al., 2007). The observed anti-apoptotic effects of FA supplementation in the current study was also reported previously (Ajeigbe et al., 2011).

Sections of the testis of the FA & lead group also showed a decreased expression of NF-κB in comparison with the lead group. This was in agreement with other studies (Au-Yeung et al., 2006; Zhao et al., 2013). The oxidative stability, produced by the supplementation of FA to lead-intoxicated rats of the current study; might be the cause of the downregulation of NF-κB activation as reported previously (Ebaid et al., 2013). Being a link between oxidative damage and inflammation (Heeba and Hamza, 2015), the inhibition of NF-κB would suppress the inflammatory cascade and the inflammatory cytokines (Ebaid et al., 2013) which explained the anti-inflammatory effect of FA in the present study. Moreover, an earlier study indicated an essential role played by NF-κB in testicular apoptosis induced by testicular ischemia-reperfusion injury (Minutoli et al., 2009) hence, the downregulation of NFκB activation by FA might also explain its observed anti-apoptotic effect.

In conclusion, lead is one of the most attention-commanding heavy metals which altered testicular homeostasis in the present animal model. However, co-administration of FA and lead antagonized its testicular toxicity as reflected on the enhancement of the relative testicular weight and testosterone secretion, alleviation of oxidative stress and inflammatory pathogenic responses together with cytoprotection of the testis and downregulation of NF-κB signaling pathway.

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Figure legends

Figure 1: Serum testosterone level in the various experimental groups. Values are expressed as means ± SD. [a] Denotes that the data are significantly different from the control group ($P < 0.05$), [b] Denotes that the data are significantly different from the folic acid group ($P < 0.05$), and [c] Denotes that the data are significantly different from the lead group ($P < 0.05$).

Figure 2: Semen analysis outcomes in the various experimental groups. (A): Sperm motility; (B): Sperm count; (C): Sperm morphology, and (D): Sperm viability. Values are means ± SD. [a] Denotes that the data are significantly different from the control group ($P < 0.05$), [b] Denotes that the data are significantly different from the folic acid group ($P < 0.05$), and [c] Denotes that the data are significantly different from the lead group ($P < 0.05$).

Figure 3: Levels of enzymatic antioxidants and malondialdehyde (MDA) in testicular tissue homogenate of various experimental groups. (A): Superoxide dismutase (SOD); (B): Glutathione peroxidase (GSH-Px); (C): Catalase (CAT) and (D): MDA. Values are means ± SD. [a] Denotes that the data are significantly different from the control group ($P < 0.05$), [b] Denotes that the data are significantly different from the folic acid group ($P < 0.05$), and [c] Denotes that the data are significantly different from the lead group ($P < 0.05$).

Figure 4: A photomicrograph of H&E-stained sections of the testis of rats in various experimental groups. (A): The control group showed the normal architecture and contour of seminiferous tubules (ST) with healthy, multi-layered spermatogenic cells (SC) and normal vascular interstitial tissue (IST) with Leydig cells (arrow head) and blood vessels (BV) (X 100). (B): The folic acid (FA) group showed the normal histological appearance of testis as the control group (X 100). (C): The lead group showed (1): disorganized SC, apoptotic germ cells (arrow) in the center of the seminiferous tubules and apoptotic Leydig cells (arrow head) and degenerated hyalinized interstitial tissue (HIT) (X 200), (2): seminiferous tubules with acidophilic degenerated hyalinized center (HC), in some areas of the seminiferous tubules

there was complete loss of spermatogenic cells (forked tail arrow) and apoptotic or degenerated Leydig cells (arrow) were also observed (X 200). (D): The FA & lead group showed (1): seminiferous tubules in different stages of improvement; seminiferous tubules with large acidophilic fibrosed center (STL) and other seminiferous tubules with small acidophilic fibrosed center (STs). Note the normal IST with Leydig cells (X 100). (2): seminiferous tubules appeared more or less normal with nearly healthy SC at different stages of spermatogenesis (X 200).

Figure 5: A photomicrograph of NF-κB/p65-immunostained sections of the testis of rats in various experimental groups. (A): The control group showed the cellular localization of the NF-κB/p65. In many cross-sections of the seminiferous tubules (ST), moderate cytoplasmic expression of NF-$_K$B was observed in spermatogonia (G), primary spermatocytes (arrow) and myoid cells (forked tail arrow). The majority of the cross-sections showed no nuclear NF-κB/p65 expression except for weak nuclear staining of few Sertoli cells (Se). Moderate cytoplasmic staining of NF-κB/p65 was also observed in Leydig cells (arrow head) (X 200). (B): The FA group showed moderate cytoplasmic staining of NF-κB/p65 in spermatogonia (G), primary spermatocytes (P) and spermatid (S). Note no nuclear NF-κB/p65 expression was detected (X 400). (C): The lead group showing intense nuclear immunostaining of NF-κB/p65 in the germ cells (arrow), degenerated germ cells (arrow head) (X 200). (D): The FA & lead group showing moderate cytoplasmic and nuclear NF-κB/p65 expression in spermatocytes (arrow head) and spermatid (S), intense nuclear and moderate cytoplasmic immunostaining of NF-κB/p65 in few germ cells (arrow) (X 400).

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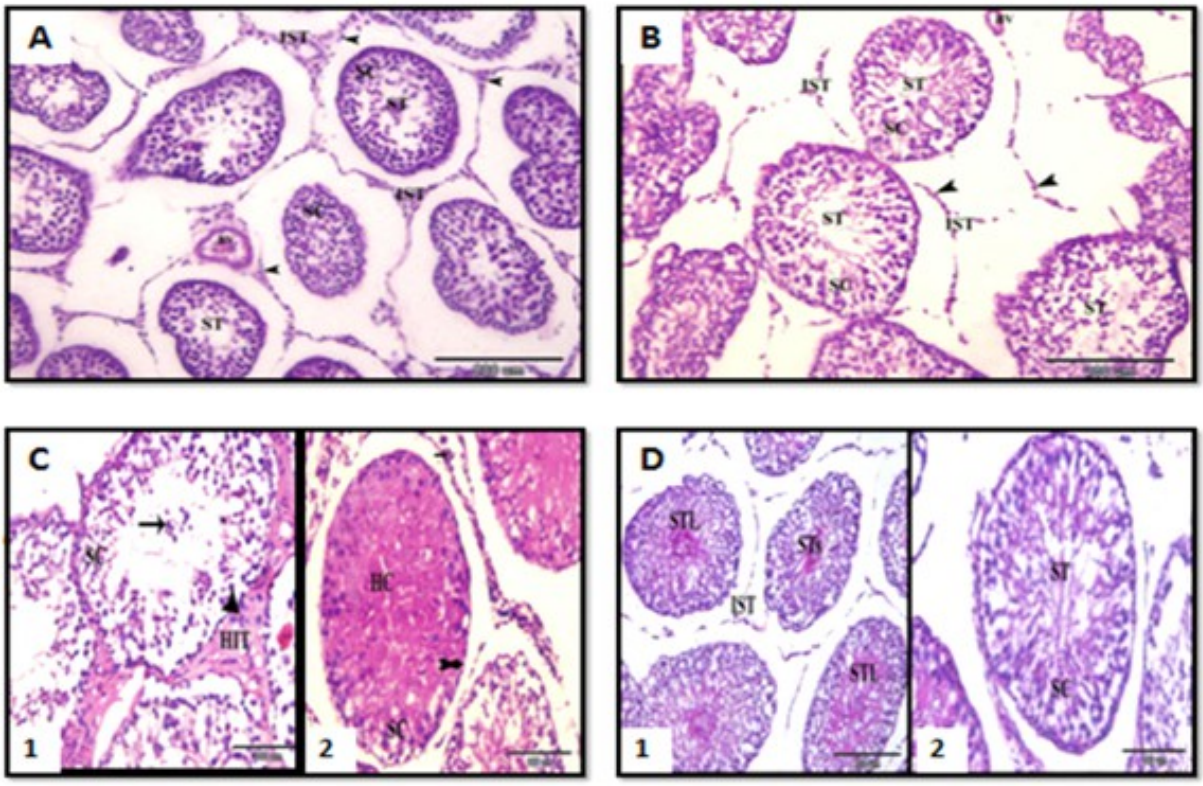

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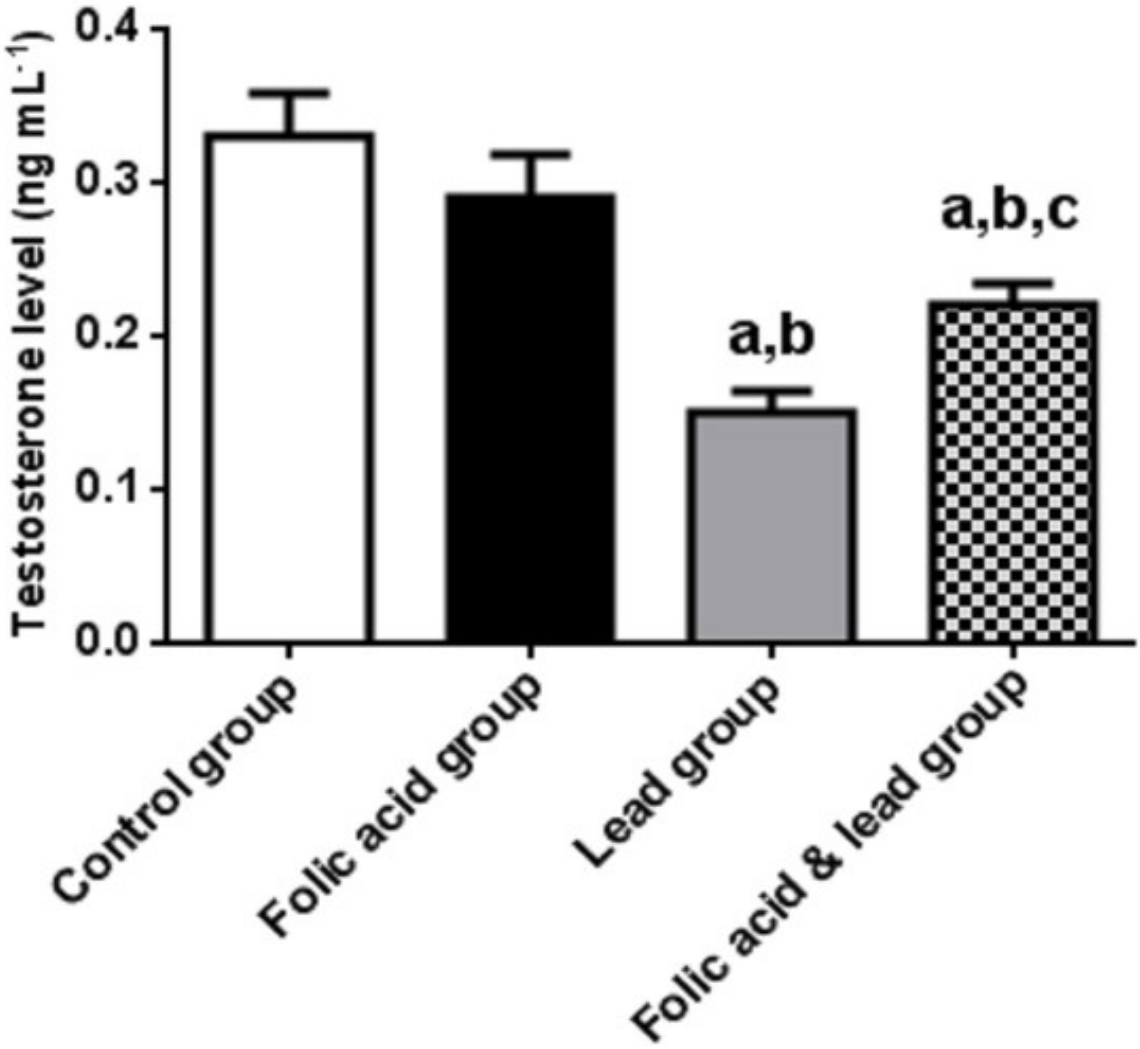

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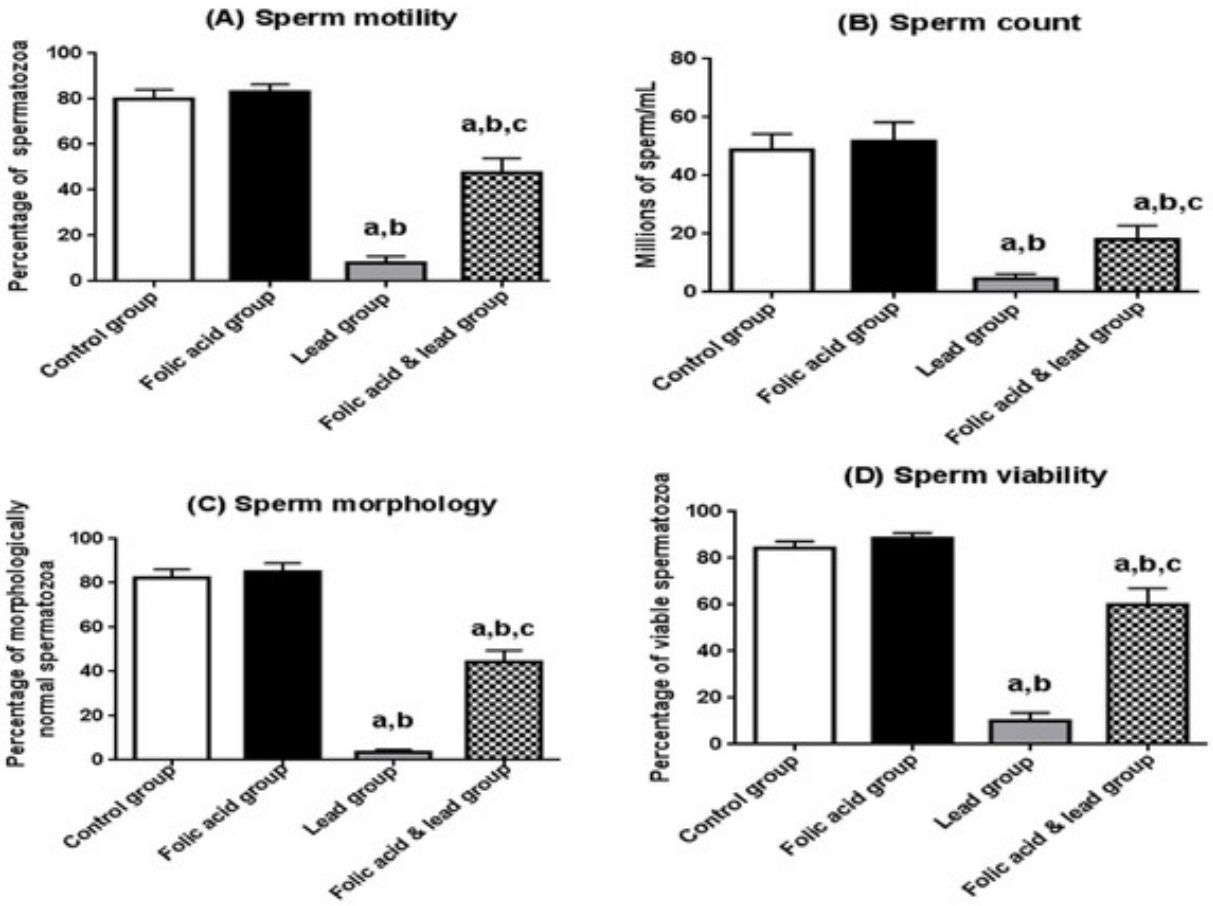

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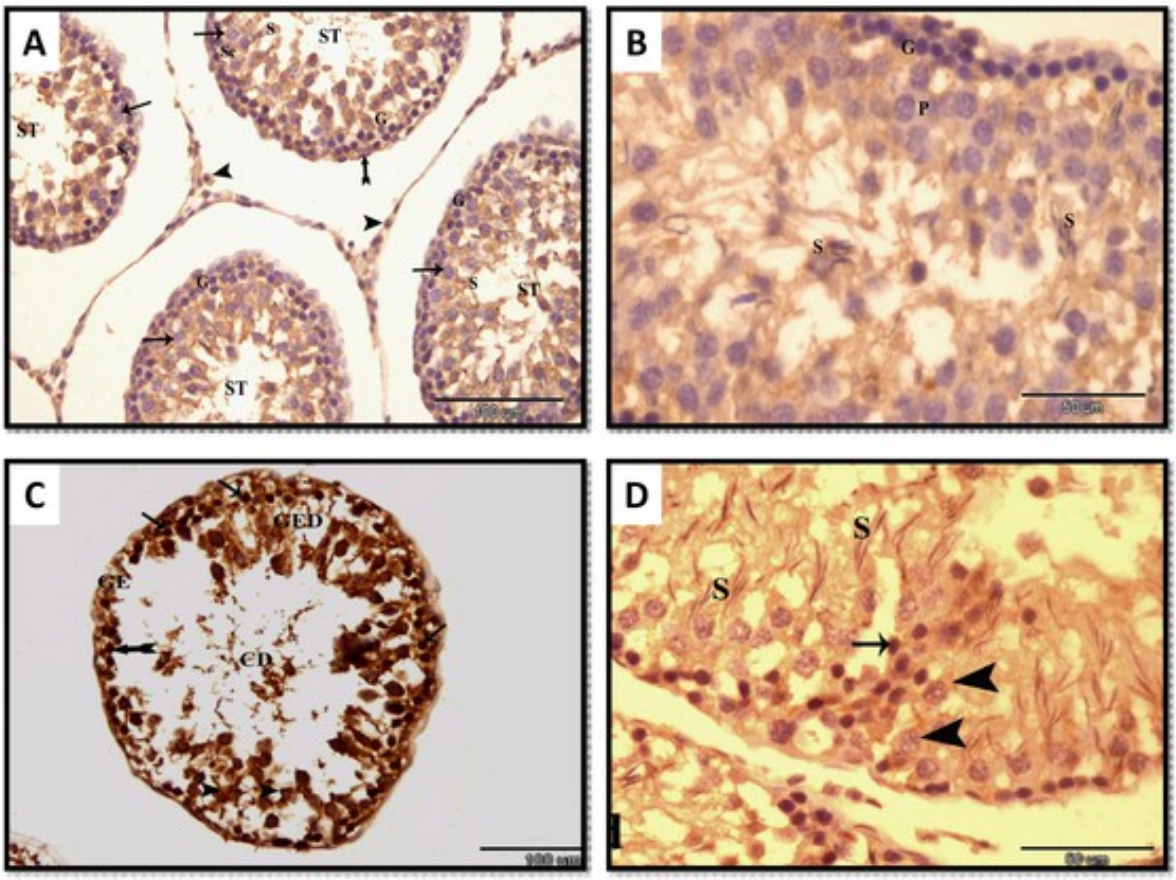

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