Research & Reviews in BioSciences

Research | Vol 11 Iss 3

Toxicity of Lead on Femoral Bone in Suckling Rats: Alleviation by Spirulina

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Received: July 27, 2016; **Accepted:** August 28, 2016; **Published:** September 17, 2016

Abstract

This study was aimed at evaluating the toxic effects of a prenatal exposure to lead acetate (Pb) on bone tissue of newborn rats, and potent protective effects of spirulina (*Arthrospira platensis*) added to diet. Female rats were given a normal diet or a diet enriched with spirulina. Additionally, lead acetate was administered to one half through drinking water from the 5th day of gestation to day 14 post-partum. In the Pb group, a decrease in body and femur weights as well as in femur length of pups was noted. Lipid peroxidation was increased, while superoxide dismutase, catalase and glutathione peroxidase activities in femur were decreased. Our results also showed lead deposition in the blood and femurs of newborns. Moreover, lead caused a significant decrease in calcium and phosphorus levels in bone, yet, in plasma, they increased and decreased inversely. Besides, plasma total tartrate-resistant acid phosphatase was enhanced, while total alkaline phosphatase was reduced. Bone disorders were confirmed by femur histological changes. Conversely, no such damages or biochemical changes were found in neonates from spirulina fed lead-poisoned mothers. These results strongly suggest that beneficial effects of spirulina proceeded through the reduction of the lead-induced oxidative stress and related damages.

Keywords:* *Bone; Lead poisoning; Oxidative stress; Spirulina; Suckling pups

Introduction

Lead (Pb) is a persistent air pollutant which can be released into the environment via numerous routes, mainly by industrial activities. This element is found in all parts of environment and enters human body and animals via air, water and foods [1,2]. It is categorized as carcinogenic metal for humans and its toxicity is one of the major public health issues, especially for fetuses/newborn. Previous studies have suggested that lead, even at low concentration and short periods of exposure induce cell abnormalities and death [3]. The most important organs that target to lead intoxication are central and peripheral nervous system, hematopoietic system, kidneys and reproductive system [2,3]. One of the important targets of lead toxicity is bone marrow that plays a critical role in blood cells production and its efficiency is essential for proper function of circulatory, lymphatic and immune systems [4]. Moreover, Aufderheide and Wittmers [5] demonstrated that during infancy and childhood, lead is deposited in trabecular bone, the most active site of remodeling.

Bone is a specialized connective tissue, which forms the framework of the body. Various physiological conditions can adversely affect femoral bone metabolism. Several mechanisms are involved in this process but the most important is oxidative damage and induction apoptosis that affect consequently various cellular and molecular mechanisms including oxidant-sensitive transcription factors, lipid peroxidation, DNA damage, cell signaling pathway, calcium and sulfhydryl homeostasis as well as toxicity on DNA and nuclear proteins [2,6], and inactivation of anti-oxidant enzymes [7].

One alternative to prevent lead-induced oxidative damages in animals is to provide a diet enriched with antioxidants. With that respect, natural products have received considerable concern for protection against many diseases, drug and xenobiotics' toxicity [8-10]. Moreover, the natural additives can contribute to the nutrient requirements, stimulate the endocrine system and intermediate nutrient metabolism [11].

Spirulina platensis (SP), blue-green microalgae (photosynthesizing cyanobacteria) have many biological activities. Due to its high content of valuable proteins, amino acids, vitamins, beta-carotene and other pigments, mineral sub-stances, indispensable fatty acids and polysaccharides, SP has been found to be suitable for use as a bioactive feed additive [12]. Nutritional values of SP are known to slightly vary depending on the production system. Moreover, the anti-inflammatory, hepatoprotective, neuroprotective, and anticancer activities of SP were examined [11-16].

To our knowledge, little is known about the persistent effects of Pb when administered during specific stages of embryonic development. Moreover, there are no studies carried out on suckling rats describing lead-induced oxidative stress and bone disorders during late pregnancy and early postnatal periods. Besides, the protective role of spirulina on lead-induced femur toxicity has not yet been investigated. Consequently, the present experiment is the first attempt to evaluate bone biochemical parameters, oxidative stress and histopathological changes in the bone of suckling rats following a subchronic exposure of adult rats to lead during pregnancy and lactating periods and, subsequently determined, the ability of spirulina supplementation to improve and protect bone maturation and development.

Materials and Methods

Reagents

All reagents used in the present study were of analytical grade. Lead (in the acetate form) was obtained from SD Fine Chemicals, Bhoisar, Mumbai, India. All current chemicals along with 5,50-Dithiobis (2-nitrobenzoic acid) (DTNB), and L-Glutathione (reduced form) were purchased from Sigma Chemical Co., (St. Louis, MO, USA).

Plants

Spirulina (*Arthrospira platensis*) variety Lenor (in powder form) was obtained from the University of Liege in Belgium.

Food preparation

Standard diet provided to the rats consisted of pellets containing a mixture of wheat, alfalfa, soybean, vitamins and minerals. Alternatively, a diet enriched with plants was prepared by mixing plant powder with food pellets in distilled water so as to obtain a homogenous paste. That mixture was cut into pellets and allowed to dry before starting the experiment.

A preliminary study, using different plant doses in the diet (i.e., 0% to 15%, w/w), did not reveal any toxic effects or oxidative stress in adult females treated with spirulina at doses up to 5%. Higher doses resulted in the occurrence of toxicity, diarrhoea and reduced growth, but were not lethal to rats [13].

Animals and treatment

Wistar rats weighing 170g to 180g, aged between 7 and 8 weeks were obtained from the "Central Pharmacy of Tunis" (SIPHAT). They were kept in cages in a breeding farm at a temperature of 21°C ± 1°C with alternating periods of 14 h/10 h of darkness/illumination and a relative humidity around 40%. All animals had free access to drinking water. The basic food consisted of 15% protein industrial pellets provided by the Industrial Society of Concentrate (SICO, Sfax, Tunisia).

The experimental procedure was carried out according to the general guidelines on the use of living animals in scientific investigations [17] and approved by the Ethical Committee of the Faculty of Science of Sfax.

After one-week acclimatisation in the laboratory conditions, adult females were placed with males on the proestrus night and the presence of spermatozoa in the vaginal smear was noted as day 0. Pregnant females were individually housed in plastic cages in a temperature-controlled nursery (22°C to 24°C).

Thirty-two pregnant rats were randomized into two sets of 16 rats. The first set consisted of control animals drinking distilled water. The second set was given water containing 6 g/L lead acetate [18]. Each group was then separated into two subgroups of eight animals. Among the animals not intoxicated with lead, rats belonging to C (control) and S (spirulina) subgroups were given a normal diet and a diet enriched with 5% spirulina. Similarly, two subgroups treated with lead acetate were given either a normal diet (Pb) or a diet enriched with spirulina (S Pb). All groups were treated from day 5 of gestation to day 14 of lactation, development being strongly sensitive to environmental pollutants during this period [19].

At birth, pups from each mother were weighed and each litter was reduced to 8 pups (4 males and 4 females) in order to maximise lactation performance [20]. During the lactating period, the dams' food and water intake was measured daily at the same time: the daily amount of ingested diet was calculated as the difference between the weight of feed placed in the food bin (D1) and that remaining the day after (D2). All the recorded data were then used to calculate the daily average feed intake over the whole experiment. Using that method, quantities of Pb and S ingested by each lactating dam were calculated from water and diet intake.

Organ sampling

On day 14 after delivery, pups (control and treated rats) were anesthetized with chloral hydrate by intra-abdominal injection. The body weights of pups were recorded and blood samples were collected in heparin tubes by brachial artery. Plasma samples were drawn from blood after centrifugation at 2,500 x g for 15 min. They were kept at -20°C until analysis.

Femurs were dissected out and the surrounding muscles and connective tissues were removed. All samples were weighed. Some of them were intended for histological examination, others were mineralized to serve for calcium and phosphorus determination and others were homogenized with 2 ml of 0.1 M Tris-HCl buffer (pH 7.2) using a mortar and pestle according to Ramajayam et al. [21]. The homogenates were centrifuged at 10,000 x g for 30 min at 4°C and supernatants were used for biochemical assays.

Evaluation of lead content

Mineralisation of blood, femurs contents and pellets was carried out at 200°C in Kjeldahl tubes in the presence of a nitric acid/perchloric acid (2:1 v/v) mixture. Lead contents were then determined using a fast sequential atomic absorption spectrometer (220 FSAA, Varian).

Accordingly, no lead was detected in food pellets. Calcium and phosphorus contents in the plasma and femurs were determined using an ion assay with a disodium salt solution of ethylene diaminetetraacetic acid (EDTA) at pH between 12 and 13 [13].

Biochemical assays

Plasma total alkaline phosphatase (ALP) and acid phosphatase (ACP) levels: The plasma levels of total alkaline phosphatase (ALP) and total tartrate-resistant acid phosphatase (ACP) were determined, respectively, by a colorimetric method (Elitech diagnostics SEES FRANCE, Ref PASL-0501; Biomerieux FRANCE, Ref 746419901).

Protein quantification: Bone protein contents were measured according to Lowry' et al. [22] method using bovine serum albumin.

Determination of antioxidant enzymes activities and lipid peroxidation level: Levels of lipid peroxidation in bone was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to Yagi's [23] method. Superoxide-dismutase (SOD) activity was determined in bone homogenate according to Beyer and Fridovich [24] method. Catalase (CAT) activity was measured using the method of Aebi [25], and glutathione-peroxidase (GPX) activity was measured according to the method of Flohe and Gunzler [26].

Histological studies

For histological studies, other femurs were cleaned from adhering soft tissue and immediately decalcified at room temperature in acetic acid 1.7 mol/L for 3 days according to Talbott et al. [27]. Bones were fixed in bouin solution. After hydration in graded ethanol series and in toluene, the bone samples were embedded in paraffin. A 5 ml section was prepared, paraffin removed with toluene, dehydrated, and stained with hematoxylin-eosin [28]**.** Then, lead deposits were evidenced by rhodizonate staining through characteristic dark brown colour [29].

Statistical analysis

The data were analysed using the statistical package program Stat Graphics plus 5.1 (stats graphics). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (FLSD) test as a post hoc test for comparison between groups. Differences were considered significant at different levels ($P<0.05$, $P<0.01$, $P<0.001$).

Results

Effects of lead on general health

Death was not observed in any experimental groups during the treatment period (21 days). However, in lead treated group, few clinical signs such as reduced activity, increasing weakness were observed.

Food intake

Lead exposure of mothers caused a decrease in the consumption of food (-24.8%) and drinking water (+30%). Co-treatment with spirulina improved food and water consumptions by mothers, reaching normal values (TABLE 1).

Body weight, femur weight and length

In Pb group, a significant decrease ($P<0.01$) in the weight of 14-day-old rats was noted on euthanasia day compared to control males and females, respectively (TABLE 2).

TABLE 1. **Effect of lead exposure and/or spirulina consumption of mother rats on their body weight. Daily food consumption, water intake and body weight of control (C) or treated mothers with 6 g/L lead acetate (Pb) and with 5% of spirulina in feed (S Pb) from day 5 of pregnancy to day 14 after delivery.**

Parameters	Mothers (n=8)		
	C	Pb	S Pb
Food consumption (g/day/dam)	37.78 ± 4.25	28.40 ± 2.45*	34.85 ± 1.62^{+}
Water intake (ml/day/dam)	70.18 ± 7.85	41.26 ± 5.19*	76.81 ± 5.28^{+}
Body weight (g)	180.70 ± 5.51	167.53 ± 2.94**	171.91 ± 5.44^{+}

Values are expressed as means ± SD; Significant differences between groups were mentioned as follows: Pb or S Pb group compared to control (C): *P<0.05; *P<0.01; ***P<0.001; S Pb group compared to Pb group: $^{+}$P<0.05; $^{++}$P<0.01; $^{+++}$P<0.001.

TABLE 2. **Body weight, femur weight and length of 14 day-old rats from control (C) or treated mothers with 6 g/L of lead acetate (Pb) and 5% of spirulina in feed (S Pb), from the 5th day of pregnancy until day 14 after delivery.**

Measurments	Male			Female		
	C	Pb	S Pb	C	Pb	S Pb
Body weights (g)	19.51 ± 0.21	17.62 ± 0.47**	18.77 ± 0.47^{+}	18.72 ± 0.29	16.93 ± 0.360*	18.47 ± 0.57^{+}
Femurs weights (g)	0.15 ± 0.003	0.10 ± 0.004**	0.13 ± 0.002^{++}	0.15 ± 0.005	0.10 ± 0.006**	0.14 ± 0.008^{+}
Femurs lengths (mm)	13.25 ± 0.22	12.07 ± 0.21**	12.83 ± 0.25^{+}	13.03 ± 0.16	11.93 ± 0.25**	12.58 ± 0.20^{+}

Values are expressed as means ± SD; Significant differences between groups were mentioned as follows: Pb or S Pb group compared to control (C): *P<0.05; **P<0.01; ***P<0.001; S Pb group compared to Pb group: $^{+}$P<0.05; $^{++}$P<0.01; $^{+++}$P<0.001.

Moreover, the addition of spirulina in the food of pregnant and lactating mothers resulted in highly significant increase in their body weights (+10.6%, P<0.05) and those of 14 day-old males and females as compared to control. Supplementation of spirulina to the diet of control mothers had no effect per se on the body weight of newborn (data not shown).

Lead administration in pregnant and lactating mothers resulted in a highly significant decrease in femur weight and length of 14 day-old males (-28.47% and -8.49%, respectively) and females (-26.41% and 8.41%, respectively) as compared to controls TABLE 2. whereas supplementation of spirulina to the diet of control mothers has no effect per se on the femur weight and length of newborn bone (data not shown), the addition of spirulina to rat diet restored lead effect on femur weights and length of males and female’s pups.

Effects of treatments on lead concentration in neonate blood and bone

Blood Pb levels were measured in control and intoxicated groups TABLE 3. Lead concentration in blood of intoxicated pups aged 14 days exhibited 8.2- and 7.5- fold increases compared to that in control males and females, respectively. Addition of spirulina to the diet of control mothers had no effect per se on the blood lead level of newborns (data not shown). In the same group, a significant increase in lead activity in the bone of male and female off springs (by 12- and 11- fold, respectively) as compared to control animals.

Moreover, supplementation of the diet of lead-intoxicated mothers with spirulina reduced significantly by 50% (P<0.01), the accumulation of lead in the blood and bone circulation of neonates.

TABLE 3. **Lead concentration in the blood and bone of 14 day-old rats from control (C) or treated mothers with 6 g/L of lead acetate (Pb) and 5% of spirulina in feed (S Pb), from the 5th day of pregnancy until day 14 after delivery.**

Measurements	Male (n=4)			Female (n=4)		
	C	Pb	S Pb	C	Pb	S Pb
Blood lead concentration (μg/ml)	0.19 ± 0.01	1.60 ± 0.13***	0.81 ± 0.14++	0.24 ± 0.03	1.79 ± 0.33***	0.90 ± 0.13++
Bone level (μg/g)	8.86 ± 1.53	68.15 ± 4.61***	34.94 ± 2.97++	6.66 ± 0.82	63.083 ± 3.53***	32.78 ± 2.47++

Values are expressed as means ± SD; Significant differences between groups were mentioned as follows: Pb or S Pb group compared to control (C): *P<0.05; **P<0.01; ***P<0.001; S Pb group compared to Pb group: +P<0.05; ++P<0.01; +++P<0.001.

Calcium and phosphorus levels in bone, plasma

The exposure of mothers to Pb (6g L^{-1}) altered the bone mineral composition of their offspring. Indeed, a decline in calcium -76.5% and -38.8% contents in bone tissues of male and female newborn's, respectively, was noted (TABLE 4). In addition, phosphorus levels decreased by -23% and -39% in bone of male and female offspring's, respectively. Moreover, calcium levels increased by +17% and +18% in plasma. while phosphorus levels decreased by -30% and -28% in plasma (TABLE 4). of male and female newborns, respectively, as compared to control. Addition of spirulina to the diet of control mothers had no effect per se on the calcium and phosphorus levels in bone and plasma of newborns (data not shown).

Supplementation of spirulina modulated significantly the calcium and phosphorus levels in femur and plasma of newborn's (TABLE 4).

TABLE 4. **Calcium and phosphorus levels in bone and plasma of 14 day-old rats from control (C) or treated mothers with 6 g/L of lead acetate (Pb) and 5% of spirulina in feed (S Pb), from the 5th day of pregnancy until day 14 after delivery.**

Parameters	Male (n=4)			Female (n=4)		
	C	Pb	S Pb	C	Pb	S Pb
Bones levels (mg/g)						
Calcium	82.21 ± 5.67	46.58 ± 6.22**	69.95 ± 3.10++	81.89 ± 3.53	49.01 ± 1.77**	69.31 ± 3.76+
Phosphorus	59.36 ± 3.48	38.97 ± 4.10**	57.20 ± 4.24++	58.72 ± 6.62	39.05 ± 5.58**	54.75 ± 2.67++
Plasma levels (mg/L)						
Calcium	58.63 ± 1.29	78.02 ± 1.88***	65.83 ± 6.71+	70.59 ± 5.41	86.61 ± 4.97***	64.31 ± 4.30++
Phosphorus	86.63 ± 4.46	35.79 ± 2.52***	53.55 ± 3.42+++	59.55 ± 4,07	41.72 ± 2.11***	50.79 ± 0,11++

Values are expressed as means ± SD; Significant differences between groups were mentioned as follows: Pb or S Pb group compared to control (C): *P<0.05; **P<0.01; ***P<0.001; S Pb group compared to Pb group: +P<0.05; ++P<0.01; +++P<0.001.

Plasma levels of acid phosphatase (ACP) and alkaline phosphatase (ALP)

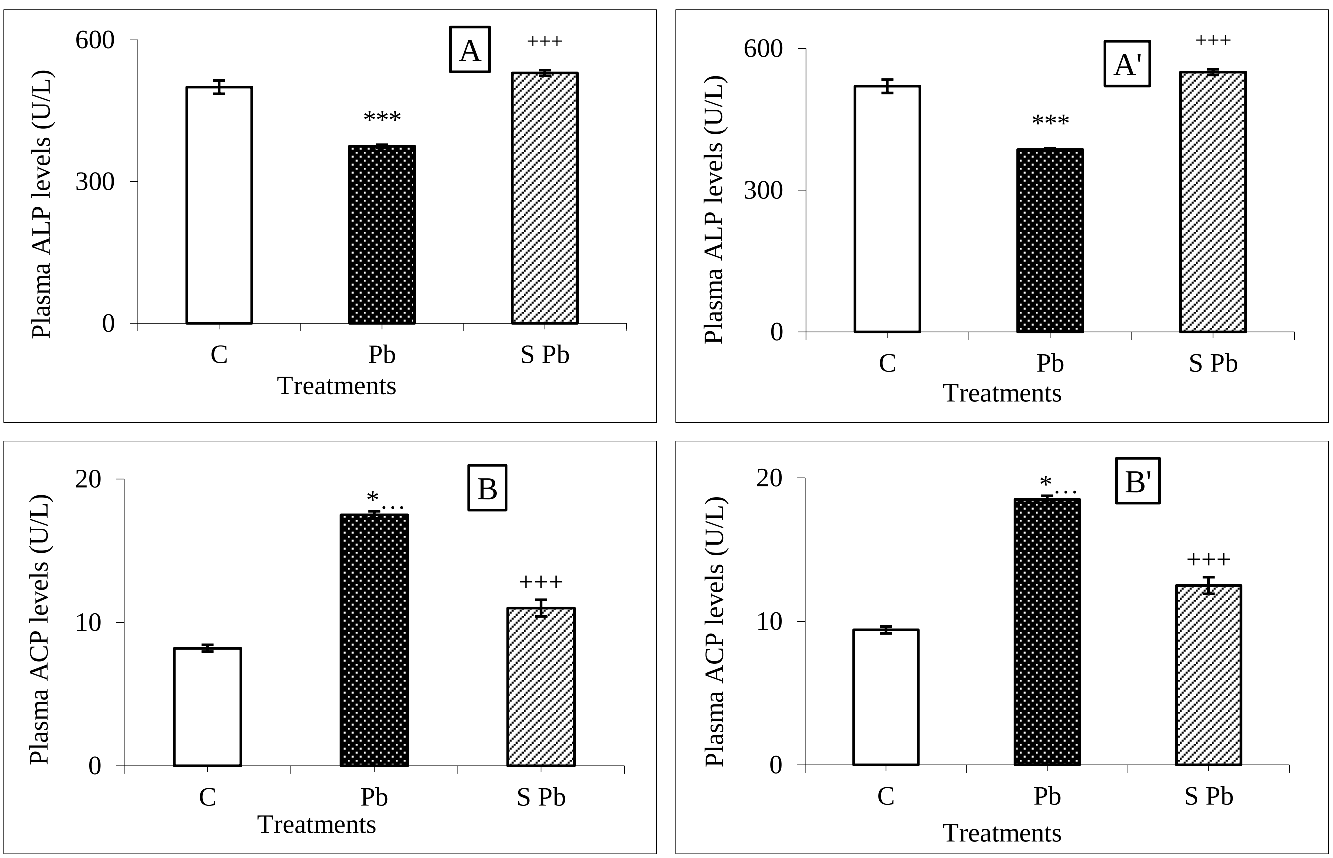
Biochemical markers such as total tartrate-resistant acid phosphatase (ACP), which reflected bone resorption, increased by 58%, and 60% in male and female offspring's, respectively; while total alkaline phosphatase (ALP), which reflected bone formation, was significantly reduced ($p<0.05$) (FIG. 1).

The supplementation of spirulina corrected the femur biochemical markers, reaching normal values.

Lipid peroxidation in bone

Lead intoxication of mothers resulted in +14% and 15% increases of TBARS concentrations in the femur homogenates of male and female newborns, respectively, as compared to negative control. Here again, addition of spirulina to the diet of control mothers had no effect per se on the lipid peroxidation level of newborns (data not shown) (TABLE 5).

However, dietary spirulina supplementation to the lead-poisoned mothers significantly reduced the peroxidation level in femur of newborns towards control levels ($P<0.05$).



Data are means ± SE of eight rats per group; Significant differences between groups are mentioned as follows: Pb or S Pb group compared to control (C): $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; S Pb group compared to Pb group: $^{+}P<0.05$; $^{++}P<0.01$; $^{+++}P<0.001$.

FIG. 1. **Plasma levels of total tartrate-resistant acid phosphatase (ACP) and total alkaline phosphatase (ALP) in the femur of 14 day-old rats (A, B: male and A', B': female) from control (C) or treated mothers with 6 g/L of lead acetate (Pb) and 5% of spirulina (S Pb) in feed, from day 5 of pregnancy to day 14 after delivery.**

Antioxidant enzyme activities in bone

In the femur homogenate of lead-treated rats, glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) activities decreased significantly by -27.6%, -22% and -52% of male offspring when compared to negative controls (TABLE 5). Similar observation could be made in female neonates (-30%, -25% and -54%, respectively, in femur).

The administration of spirulina ameliorated enzyme activities (CAT, GPx and SOD) in (Pb+S) group.

TABLE 5. **TBARS levels and activities of CAT, SOD and GPX in femur of 14 day-old rats from control (C) or treated mothers with 6 g/L lead acetate (Pb) and 5% of spirulina (S Pb) in feed, from day 5 of pregnancy until day 14 after delivery.**

Parameters	Male (n=4)			Female (n=4)		
	C	Pb	S Pb	C	Pb	S Pb
TBARS	0.49 ± 0.09	0.60 ± 0.04***	0.52 ± 0.05^{+++}	0.47 ± 0.01	0.62 ± 0.09***	0.48 ± 0.07^{+++}
Superoxyde dismutase	16.15 ± 1.01	7.30 ± 0.51**	13.16 ± 1.18^{+++}	17.16 ± 0.09	7.33 ± 0.81***	14.18 ± 0.01^{++}
Catalase	17.98 ± 5.83	13.30 ± 2.37**	15.79 ± 4.98^{++}	18.86 ± 2.91	13.75 ± 2.44**	17.43 ± 3.37^{++}
Glutathione peroxidase	33.47 ± 2.35	22.40 ± 1.44**	34.31 ± 2.69^{+}	33.36 ± 3.50	25.84 ± 3.21**	34.25 ± 1.63^{++}

Data are means ± SE of 4 determinations; TBARS: nmol/mg protein; Superoxide dismutase activity: U/mg protein; Catalase activity: µmol H_2O_2/min/mg protein; Glutathione peroxidase activity: nmol GSH/min/mg protein; Significant differences between two groups are mentioned as follows: Pb or (Pb+S) group compared to control group: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; (Pb+S) group compared to Pb group: $^{+}P<0.05$; $^{++}P<0.01$; $^{+++}P<0.001$.

Histological studies

The biochemical modifications cited above were correlated with our histological studies (FIG. 2). In fact, in the femur sections of lead-treated rats, proliferating chondrocytes failed to form discreet columns. Also, hypertrophic chondrocyte differentiation and neovascularization in this region were greatly diminished (FIG. 2B). Treated rats displayed fewer, thinner, and fragmented bone trabeculae (FIG. 2B). compared to the control group where the proliferative zone (PZ) contained flattened chondrocytes in columns or clusters parallel to the growth axis; the largest proliferative cells differentiated to form hypertrophic chondrocytes (FIG. 2A). In addition, bone trabeculae in the primary spongium of control rats were markedly developed and were organized parallel to the columns of proliferating chondrocytes (FIG. 2A). When spirulina was supplemented to the diet of lead-treated rats (FIG. 2C). the histological aspects of the femur sections were partially reversed when compared to the control group. In Spirulina treated group, the histological aspects of the femur were similar to those of controls (data not show).

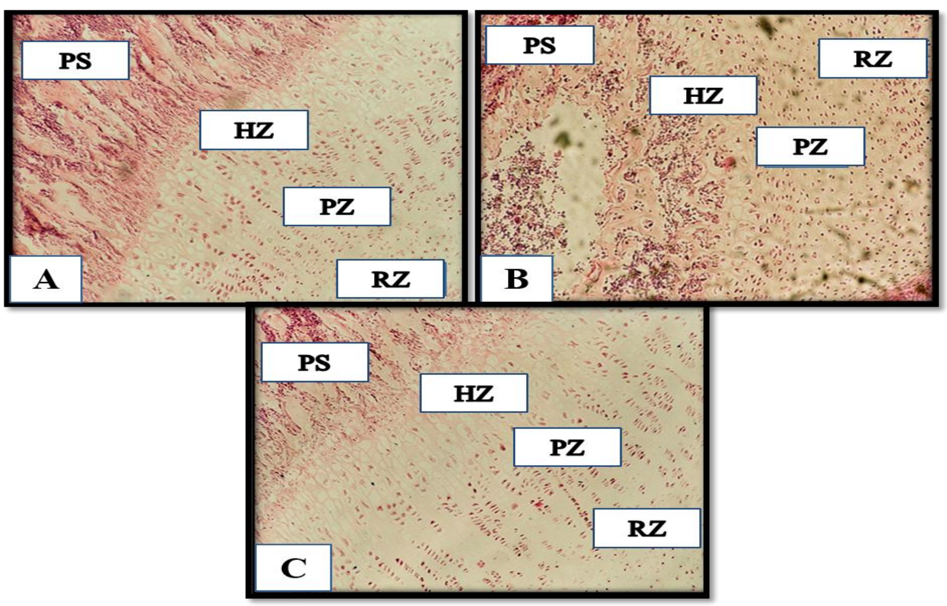
The rhodizonate staining (FIG. 3) revealed the presence of lead chelates in the femurs of young rats born from mothers intoxicated with lead (Pb group) (FIG. 3B). while these chelates were absent in control rats (FIG. 3A). Addition of spirulina to the diet of lead-intoxicated mothers resulted in a strong reduction of the density of lead deposits in femur compared to rats of the Pb group (FIG. 3C).

Discussion

Many studies have indicated that lead exposure induces a wide range of biochemical and physiological dysfunction in humans and laboratory animals [30]. More precisely, pregnant, infants and young children are mostly affected by lead exposure [31].

In our experimental study, exposure of female rats to Pb during late pregnancy and early postnatal periods decreased the body weight of their suckling pups. This could be explained by a decrease of food intake by lactating rats. In humans, a pregnant lady can transfer her body burden of lead to the growing fetus, as there is no placental barrier for a heavy metal like lead [32]. Thus, offspring from adults exposed to lead during gestation have elevated blood Pb level at birth, even higher than that of dams sampled at the same time point [32,33]. In our study, we found high levels of lead (exceeding 1.5 mg/L) in young rat blood and stomach contents. In general, mother and fetus can be considered a unique system that remains in equilibrium through pregnancy. Therefore, at the end of pregnancy, similar lead has been accumulated slowly in the body and even low doses can eventually lead to produce a variety of toxic effects i.e., disturbances of the central nervous system, bones and soft tissues of both the mother and the child [34,35]. To our knowledge, this is the first report which investigates the toxic effects of lead on bone maturation, mineral composition and histoarchitecture in suckling rats.

The exposure of rats to lead during late pregnancy and early postnatal periods decreased in the pups' femur weight and length. It seemed that over 90% of Pb is eventually stored in bone [36]. In fact, Pascaul et al. [37] showed that the bone matrix is a tissue with high metabolic activity, have already reported this type of observation and several pollutants can disrupt its formation, resulting in a defective osteogenesis which affects both the length and mass of bone. Our results also show a significant accumulation of lead (which exceeds 500% compared with controls) at bone of young rats from the treated dams, and a change in the mineral composition of the bone, particularly in calcium and phosphorus. These data are consistent with recent studies conducted in rats and humans, which showed that pollutants affect bone strength and cause changes in the composition of bone tissue [38,39]. Moreover, our study shows that calcium levels increased in plasma, paradoxically, phosphorus levels decreased, which was reflected by an altered bone mineral composition, especially the decreasing calcium and phosphorus contents. This could be explained, according to Arai et al. [40], by the oxidative stress which probably affected bone mineralization.



RZ: Reserve Zone; PZ: Proliferative Zone; HZ: Hypertrophic Zone; PS: Primary Spongium Region.

FIG. 2. **Histological structure of the femur of 14-day-old rats born from control mothers (2A), mothers treated with lead acetate (2B), mothers intoxicated with lead acetate and fed with spirulina (2C) from the 5th day of gestation. Sections were examined by light microscopy (200x) after haematoxylin-eosin staining.**

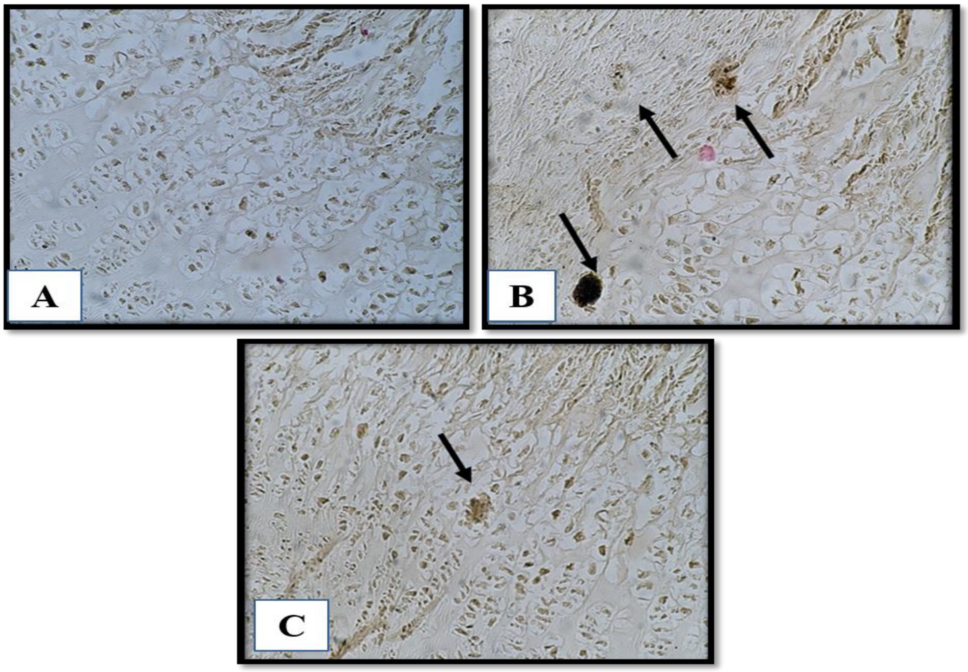


FIG. 3. **Histological structure of the femur of 14-day-old rats born from control mothers (3A), mothers treated with lead acetate (3B), mothers intoxicated with lead acetate and fed with spirulina (3C) from the 5th day of gestation. Sections were examined by light microscopy (200x) after rhodizonate staining.**

Bone is formed and resorbed continuously, starting in the embryo and continuing throughout adult life. This process occurring in adult bone is called bone remodeling, which is carried out by osteoblasts (bone-forming cells) and osteoclasts (bone-resorption cells). In the current investigation, biochemical markers such as total tartrate-resistant acid phosphatase (ACP), which reflects bone resorption, increased, while total alkaline phosphatase (ALP), which reflects bone formation, was reduced in the pups of lead treated group, compared to controls. As the bone resorption was enhanced, the amount of bone loss during the continuously occurring resorption was incompletely restored during its formation, resulting in a net bone loss. Our results agree with previous studies, which found that other metals such as lithium, chrome and uranium inhibited bone formation [41-43]. Others studies have shown that ROS are implicated in bone resorption with a direct contribution of osteoclast-generated superoxide to bone degradation [44-45]. Thus, oxidative stress increases the differentiation and function of osteoclasts [46]. In this respect, Yang et al. [44] showed that osteoblasts can produce antioxidants such as glutathione peroxidase to protect the cell against ROS, whilst osteoclast-generated superoxide contributes to bone degradation. Keeping in mind all such possibilities in bone resorption, the disorders in bone formation observed in our study were probably due to ROS generated after lead intoxication.

Disorders in bone formation, reflected by changes in its mineralization and the turnover rate, were correlated with histological studies. Indeed, the growth plate in lead-treated rats was grossly disorganized compared to that of controls. In Pb-treated rats, proliferating chondrocytes failed to form discreet columns and the hypertrophic zone was markedly developed and morphologically distinct. Moreover, bone trabeculae in the primary spongium of control rats were organized parallel to the columns of proliferating chondrocytes, reflecting the functional continuity between maturing chondrocytes and mineralizing osteoblasts which were required for normal endochondral ossification, While, in treated rats, we observed a few, thin, fragmented and disorganized bone trabeculae compared to controls.

Noteworthy, our data showed that the consumption of spirulina by mother rats during lead treatment prevented those adverse effects of Pb on neonate bone and plasma. Spirulina is considered as a valuable food source of some macro-and micronutrients including high quality proteins, iron, γ-linolenic acid, carotenoids, phycocyanins and vitamins [47,48]. Moreover, vitamins D were found to protect against osteoporosis, bone fractures, and breaks in older adults. Vitamin D regulates bone metabolism and is known to play an essential role in enhancement of the absorption of calcium and phosphate from the bone [49]. Similarly, deformation and dysfunction of key cells in bone modeling and remodeling impairment has been frequently associated with vitamin A deficiency, and administration of retinoid plays a significant part in bone biology in animals [50]. Therefore, the benefits of spirulina consumption by mother rats on neonate femur development could be due to the vitamin supplementation in the offspring.

In general, oxidative stress is a constant threat to all living organisms and the body to its elimination or attenuation employs an endogenous antioxidant defense system [51]. In the current study, our results confirm a generation of oxidative stress in the femur of suckling rats by lead. Our data demonstrate that intoxication with lead causes a significant increase of lipid peroxidation in the bone of rats. Lipid peroxidation is one of the main manifestations of oxidative damage, which plays an important role in the toxicity of many xenobiotics [52]. Our results corroborate our previous findings [13,14] and those of Soudani et al. [43] who reported an increase of TBARS in bone of suckling pups treated with chrome.

The co-administration of spirulina to Pb-treated group restores MDA levels to near normal values. This could be explained by the important role of spirulina in preventing lipid peroxidation and in protecting the integrity and functioning of tissues and cells [53]. On that point, spirulina has been reported to possess significant levels of primary antioxidants including vitamin C; it is known to be an anti-stress and a powerful reducing agent. It helps in activating several enzymes and acts as an antioxidant for detoxifying toxic substances [54], it protects plasma lipid against lipid peroxidation and has an important role in the regeneration of α-tocopherol [55].

Several studies have demonstrated that lead, associated with oxidative stress and cellular damage is the consequence of both an increased production of free radical and reduced capacity of the antioxidative defense system [13,14,43]. Free radical’s production is believed to induce bone-related diseases by suppressing bone formation and stimulating bone resorption. Indeed, antioxidants deficiency has a negative impact on bone mass [21]. So, there is growing evidence that oxidative stress contributes to bone damage, whereas antioxidants may prevent the undesired oxidative damage induced by reactive oxygen species in the bone tissue [56]. In the present study, the exposure of rats to lead alone during late pregnancy and early postnatal period decreased the enzymatic antioxidant components like catalase (CAT), superoxide dismutase (SOD), and the glutathione peroxidase (GPx). The depletion of these enzymes might be a factor responsible for the lack of elimination of toxic compounds that enter the bone and result in their accumulation, thus aggravating oxidative stress.

Noteworthy, SOD, CAT and GPx activity in neonate femur was ameliorated after consumption of spirulina by nursing mothers. Indeed, spirulina is rich in vitamin c. In fact, previous studies of Padh [57] showed that the primary role of vitamin C, was to neutralize free radicals both inside and outside the cells. According to this author, vitamin C is an excellent source of electrons, which could donate them to free radicals such as hydroxyl and superoxide radicals and quench their reactivity. In addition, a vitamin C levels in femur tissue to a major importance due to the main role of this antioxidant to protect bone formation under chronic exposition to lead.

Conclusions

This is the first report which demonstrates that lead causes oxidative damage in bone tissue of suckling rats. Co-administration of spirulina through diet improved Pb-induced changes in osteomineral metabolism, bone histo-architecture, enzymatic (superoxide dismutase, catalase, and glutathione peroxidase) activities. As a result, spirulina could be a useful method to protect against bone impairment. The protective effect exhibited by Sp supplementation can involve the capability of Sp in activating and protecting various Sp-dependent enzymes.

Acknowledgments

The present work was supported by DGRST grant (Direction Générale de la Recherche Scientifique et Technique-Tunisie — Appui à la Recherche Universitaire de base UR/13 ES-73). The authors also thank Dr Xavier Dauvergne for his help in statistical analysis of data.

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