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Docosahexaenoic Acid (DHA, C22:6, ω-3) Composition of Milk and Mammary Gland Tissues of Lactating Mother Rats Is Severely Affected by Lead (Pb) Exposure

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Abstract

Docosahexaenoic acid (DHA, C22:6, ω-3), an ω-3 polyunsaturated fatty acid (PUFA), is critical for brain growth, development, and cognitive ability. It is consumed by offspring via milk during lactation. However, the toxic heavy metal lead (Pb) readily passes into the mammary glands of mother animals and then to offspring through milk. Here, we investigated whether DHA composition of milk and mammary gland tissues is affected by Pb exposure. Mother rats were exposed to Pb via drinking water (0.1%). The fatty acid profile and levels of reduced glutathione (GSH), lipid peroxide (LPO), and pro-inflammatory TNF-α in milk and mammary tissues were measured. Levels of DHA and antioxidant GSH decreased ($P < 0.05$), while LPO and TNF-α levels increased ($P < 0.05$) both in milk and mammary tissues. Our results suggest that toxic Pb exposure can upset the level of milk DHA, which may affect brain growth and development, and hence cognitive ability in adulthood and later life.

Keywords DHA · Lead toxicity · Milk-mammary gland · LPO · TNF-α · Brain cognition

Introduction

Docosahexaenoic acid (DHA, C22:6, ω-3) is the most abundant ω-3 polyunsaturated fatty acid (PUFA) in the brain. DHA plays important roles in neurogenesis [1], synaptogenesis [2], neurotransmission [3], as well as growth and development of the brain [4, 5]. The biophysical properties of the membrane lipid bilayer are more prominent with DHA than with other PUFAs [6–10]. The increase of DHA content in cellular bilayer membranes enhances the activities of membrane-bound enzymes, membrane permeability, signal transduction, transmitter release, endocytosis, and exocytosis [5, 7, 11, 12]. Highest concentrations of DHA are found in brain synaptosomal membranes, retinal outer segment membranes, and sperm mitochondrial membranes [13–16]. Deficiency in DHA impairs visual acuity and cognitive ability in premature infants [17]. Furthermore, a decline in brain DHA levels is associated with learning and memory deficits in young [18] and adult rats [19], elderly humans [20], and those affected with aging-related neurodegenerative diseases, such as Alzheimer's disease (AD) [21, 22]. Maternal DHA, which the offspring receives during lactation, is linked with their cognitive development and immunomodulation. Numerous reports suggest that milk not only provides essential nutrients to the offspring but also contains growth factors, cytokines, including tumor necrosis factor alpha (TNF-α) that have regulatory roles on the mammary gland itself or immunomodulating effects on recipient neonates [23, 24]. Moreover, de novo synthesis of DHA takes place at an extremely slow rate, as compared with the rate of its accretion in brain neurons [25]. The developing fetus, thus, has to depend on maternal source of DHA [26]; the offspring is fully dependent on DHA in mother's milk during the pre-weaning period.

Milk is synthesized in the specialized mammary alveolar cells that take up the blood-borne nutrients by a host of cellular processes, including endocytosis through the bilayer membranes at the basal side of the duct system; it is then secreted by exocytosis via the bilayer membranes of the apical side [12]. Yang et al. [27] reported that DHA enrichment in cellular membranes increased the secretory capacity of cells. Thus, it is conceivable that

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DHA content of the milk-secretory cells of mammary gland tissues may influence milk secretion/milk content by the process of exocytosis. Pb exposure in early life may result in late-life neuropathological changes similar to those of AD [28]. Oral administration of DHA improves behavioral impairments and antioxidative defense with a concurrent reduction of oxidative stress in the hippocampus, cerebellum, and cortex in Pb-administered rats [29]. We have also reported previously that DHA increases antioxidative defense in these brain regions of aged rats [30]. All these studies, thus, suggest that evaluation of the effect of Pb on milk DHA levels deserves special attention. Although there are a number of reports on the oxidative effects of Pb on the liver [31], kidneys [32], spleen [33], cardiovascular tissues [34], erythrocytes [35], reproductive systems [36], and brain development [37, 38], information on the effects of Pb exposure on oxidative stress in milk and mammary gland tissues is still inadequate. The aim of this study was, therefore, to investigate whether Pb exposure has any influence on DHA composition, oxidative status, and TNF-α content in milk and mammary tissues of lactating mother rats.

Materials and Methods

Chemicals and Antibodies

Lead (Pb) acetate of analytical grade was procured from Merck. 1,1,3,3-Tetraethoxypropane (TEP) was purchased from Wako Pure Chemicals, Japan. 3,3′,5,5′-*Tetramethylbenzidine (TMB) solution* and oxytocin peptide hormone were procured from Sigma-Aldrich, St. Louis, USA. The primary anti-rabbit TNF-α antibody was obtained from Santa Cruz Biotechnologies, Inc., CA, USA. HRP (horseradish peroxidase)-conjugated 2° anti-rabbit antibody (Cell Signalling Technology) was procured from Invitrogen (USA).

Animals and Treatment

Animals

Inbred albino Wistar rats were used in this study. They were fed with a standard pellet diet with water supply ad libitum. The rats were maintained under 12-h light:12-h dark conditions, at 25 ± 2 °C temperature and 50 ± 10% humidity. Each pair (1 female and 1 male) rat was cohabited until the presence of copulatory plug was observed in the vagina of the female rats. Positive mating date was considered gestational day 0. The males were immediately isolated from the cage of the pregnant female rats. All the experiments were performed according to the guidelines of the Animal Ethical Committee of Jahangirnagar University, Savar, Dhaka, Bangladesh. As lactation generally lasts *until weaning, the* exposure to Pb was continued until this period.

Study Design

The pregnant rats were divided into 2 groups (Fig. 1): control and Pb-exposed experimental group. Pb exposure was done via drinking water containing 0.1% lead acetate. After delivery of pups, Pb feeding was continued throughout the lactation period. Milk was collected from dams on day 18 of lactation in the morning.

Milking

The mother rats were separated from their pups for 2 h prior to milking. The dams were then injected subcutaneously with oxytocin (4.0 IU/kg BW). After a light anesthesia with pentobarbital, milking was done by using a mechanical suction apparatus, as previously described by Hossain et al. [39]. A part of whole milk was centrifuged at 10,000×*g* to collect the supernatant. The whole milk and the supernatant collected in Eppendorf tubes were stored at − 80 °C until further analysis.

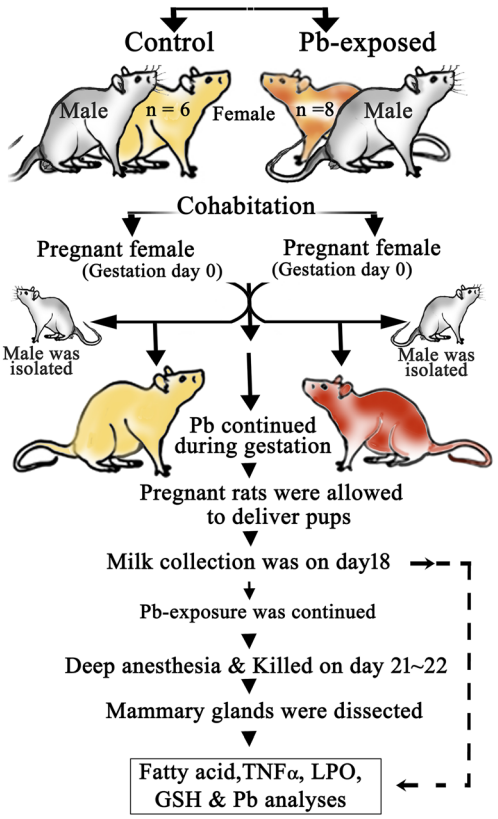

Fig 1 Experimental design

Mammary Gland Tissue Preparation

The mother rats were deeply anesthetized with sodium pentobarbital, the abdomen of the rats was clean-shaved, and rats were then killed. After drawing blood, the mammary glands were carefully dissected. The mammary tissues were immediately chopped in ice-cold phosphate buffer (100 mM, pH 7.4) containing 0.005% PMSF, incubated on ice for 20 min, spun for 5 min at 700×*g* to discard the floating adipose fatty tissues. The resulting tissues were then homogenized in a Polytron homogenizer. The homogenates were centrifuged initially at 1000×*g* to discard the unbroken tissues and debris. The resulting supernatant was assigned as a whole homogenate (WH). WH samples were used for the assays of fatty acid profile and lipid peroxide (LPO). The WH was again centrifuged at 12,000×g and the resulting supernatant fraction was used for TNF-α assay.

Fatty Acid Profile

Fatty acid analyses of the whole milk (i.e., milk as it is) and mammary tissue whole homogenates were determined, according to the methods of Hashimoto et al. [40]. To 50 μl of milk samples and mammary tissue whole homogenates, 2.0 ml methanol–*n*-octane (4:1, v/v) containing 10 μg tricosanoic acid as internal standard and 200 μl acetyl chloride were added. The mixture was incubated at 100 °C for 60 min and cooled, then neutralized with 0.5 N aqueous NaOH containing 10% sodium chloride. The neutralized mixture was shaken for 10 min at room temperature and centrifuged at 1800×*g* for 5 min. The octane phase with the fatty acid methyl esters was directly subjected to gas chromatography. The gas chromatography separation was done on a Model 5890II (Hewlett–Packard, Avondale, PA, USA) equipped with a flame ionization detector and an automatic sampler Model 7673. A 30 m × 0.25 mm capillary column (DB-WAX P/N 122-7032, J & W Scientific, CA, USA) was initially maintained at 100 °C for 1 min, raised to 180 °C at 2 °C/min, then raised to 240 °C at 28 °C/min, further raised to 260 °C at 4 °C/min, and maintained for 5 min. The chromatograms were identified and quantified by a JEOL JMA-2000S mass data analysis system (Nippon Denshi).

Lipid Peroxide Test

LPO was measured by determining the thiobarbituric acid–reactive substances (TBARs), as previously described [6, 7, 21]. The mammary gland tissue whole homogenate and/or whole milk (0.1 ml) samples were added to 0.1 ml of sodium dodecyl sulfate (8.1%, w/v) and 2 ml of 0.4% thiobarbituric acid prepared in 20% acetic acid (pH 3.5) and 0.1 ml H_2O. Tubes were then tightly capped and heated at 95 °C for 1 h. After cooling with tap water, 2.0 ml of *n*-butanol-pyridine (15:1, v/v) was added and shaken for 10 min. Following centrifugation at 1200×*g* for 10 min at room temperature (digital centrifuge, DSC-1512SD), absorbance of the organic layer was measured at 532 nm against 1,1,3,3-tetraethoxypropane (TEP) standard.

Reduced Glutathione Assay

Glutathione (GSH) levels were determined according to the methods described previously [30, 41]. Appropriate volumes from mammary gland whole homogenates and/or whole milk were added to 500 μl of freshly prepared 10% trichloroacetic acid and incubated at 4 °C for 30 min. Afterward, all samples were centrifuged at 10,000×*g* for 10 min. The supernatant was used for the fluorometric measurement of GSH. Briefly, 50 μl of supernatant was incubated in 0.1 M sodium phosphate-0.005 M EDTA buffer (pH 8.0) in the presence of 100 μl of ortho-phthalaldehyde for 15 min in the dark. Fluorescence at 420 nm was determined at the excitation wavelength of 350 nm in a fluorometer against purified GSH as a standard.

Tumor Necrosis Factor Alpha Assay

We determined the milk TNF-α levels according to the methods of Etem-Piskin et al. [42] and Lehtolainen et al. [43], but with slight modifications and after adaptation to our laboratory setting. The thawed milk supernatant was centrifuged at 10,000×*g* for 30 min at 4 °C to remove the final remnants of fat. The resulting aqueous supernatant was used for TNF-α assay (ELISA). The multi-well plate was coated with 20 μl of mammary gland tissue cytosolic fraction and milk supernatant in 180 μl of 100 mM sodium bicarbonate buffer (pH 9.6) and incubated at 4 °C for 12 h. Afterwards, the plate was washed and the wells were blocked with BSA (1%) in Tris-buffered saline (TBS) for 4 h. The anti-rabbit TNF-α (1° antibody at 1:1000 dilutions) was added to each well and incubated overnight at 4 °C. On the next day, HRP-coupled 2° anti-rabbit antibody was added to each well and the plate was incubated for 2 h at room temperature. At the end of incubation, the plate was washed thrice and tetramethylbenzidine was used to develop color. The color reaction was stopped after 30 min by adding 0.1 N HCl. Wells coated with only 0.1 M carbonate buffer (pH 9.6) were used as blank. The absorbance of the plate was determined at 450 nm. The absorbance of the wells of the control rats was considered 100%. Finally, all absorbance values were normalized to protein concentration. All samples were tested in triplicate.

Pb Assay

Pb levels in milk and mammary tissues were determined by atomic absorption spectroscopy (AAS, Varian AA240 Atomic Absorption Spectrometer), as described previously [44].

Protein concentration of whole milk and whole tissue homogenates and/or their supernatant fractions was determined by the Pierce™ BCA kit.

Statistical Analysis

Results are expressed as mean ± standard error of mean (SE) (n = 6~7 rats per group during LPO and Pb determination; n = 5 per group during fatty acid determination). Data were analyzed using Student's t test. The correlation between the levels of LPO of milk versus mammary gland tissues was performed by a simple regression analysis. $P < 0.05$ was considered significant. Statistical software used was StatView 4.1 (Abacus Ltd., CA, USA).

Results

Pb Levels in Milk and Mammary Gland Tissues

Pb levels in milk from control and Pb-exposed mother rats were 3.4 ± 0.10 μg/l and 13.5 ± 0.65 μg/l, respectively. Pb-exposed mother rats thus had ~ 4-fold increase in milk Pb levels, when compared with those of the control mother rats. Pb levels in the mammary tissue whole homogenates of control and Pb-exposed mother rats were 4.52 ± 0.10 and 15.6 ± 0.65 μg/mg protein, respectively. Pb levels hence increased by ~ 3.45-fold in the mammary gland tissues of Pb-exposed mother rats.

Food Intake and Body Weight Gain in Rats

Food intake between the control and Pb-exposed mother rats was not significantly different (controls vs. Pb-exposed mother rats: 18.25 ± 1.0 and 17.95 ± 1.2 g, respectively). Body weight (BW) of the control and Pb-exposed mother rats was 193 ± 7.8 and 201 ± 5.8 g, respectively, at day 0; at the end of the study, their BW was 272 ± 12 g and 247 ± 7.0 g, respectively. BW increased by 42% and 23%, respectively, in the control and Pb-exposed rats. Body weight gain was, therefore, significantly lower in the Pb-exposed mother rats, when compared with that of the control animals at the end of the study.

Fatty Acid Profile of Milk and Mammary Tissues

Fatty acid profile of milk and mammary gland tissues of the control and Pb-exposed mother rats is shown in Table 1. DHA levels decreased ($P < 0.05$) both in the milk and mammary gland tissues of the Pb-exposed mother rats when compared with those of the control rats. In contrast, levels of arachidonic acid (AA) increased ($P < 0.05$) in the milk and mammary tissues of the Pb-treated rats than those in the control rats (Table 1). This resulted in a significant decrease in DHA/AA ratio both in the milk and mammary tissues of Pb-exposed mother rats. Levels of eicosapentaenoic acid (EPA) also declined in the milk and mammary tissues of Pb-exposed mother rats. On the other hand, levels of ω-6 linoleic acid (LLA) and ω-3 alpha-linolenic acid (LNA) increased ($P < 0.05$) both in the milk and mammary tissues of Pb-treated mother rats. Pb exposure increased the levels of monounsaturated fatty acids, palmitoleic acid (POA) and oleic acid (OLA), only in the mammary tissues. Compared with the control rats, the levels of total saturated fatty acids (palmitic acid+stearic acid+tricosanoic acid) increased significantly in the mammary tissues of the Pb-exposed rats. Finally, all these changes in the fatty acid composition brought about a significant reduction in the ω-3 unsaturation index (USI) both in the milk and mammary gland tissues of the Pb-exposed rats. Concomitantly, Pb exposure gave rise to an elevation ($P < 0.05$) of ω-6 USI both in the milk and mammary gland tissues of the Pb-exposed rats, as compared with those of the control rats.

Oxidative Stress in the Milk and Mammary Tissues

The effects of Pb exposure on the levels of lipid peroxide (LPO) and GSH in the milk and mammary tissues are shown in Fig. 2. LPO levels increased significantly ($P < 0.05$) both in the milk (by 24%) and mammary tissues (by 3-fold) of Pb-exposed lactating mother rats (Fig. 2a, b). Levels of reduced glutathione (GSH), on the other hand, decreased by 34% in the milk and by ~ 37% in the mammary gland tissues of Pb-exposed mother rats, when compared with those of the control rats.

Pro-inflammatory Tumor Necrosis Factor Alpha in Milk and Mammary Tissues

TNF-α levels increased ($P < 0.05$) by > 43% in the milk of Pb-exposed mother rats, when compared with those of the control rats (Fig. 3a). However, its levels increased by 26% in the mammary gland tissues, as compared with those of the controls (Fig. 3b).

Correlation Between Milk LPO Versus Mammary Gland LPO

The correlation between the levels of milk and mammary gland LPOs was tested by subjecting the corresponding data to a simple regression analysis (Fig. 4). The analysis revealed a significant positive correlation between LPO levels of milk versus mammary gland tissues ($P < 0.05$, $R^2 = 0.48$).

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Table 1 Fatty acid profile of milk and mammary gland tissues of control and Pb-exposed rats

	Milk		Mammary gland tissues	
μg/mg protein	Control	Pb-exposed	Control	Pb-exposed
PLA $_{(C16:0)}$	82 ± 5.0^a	64 ± 3.5^b	140 ± 7.5^a	319 ± 12^b
STA $_{(C18:0)}$	39 ± 2.0^a	29 ± 1.50^b	38 ± 1.5^a	68 ± 3.0^b
TCA $_{(C24:0,\ ω-9)}$	1.4 ± 0.07^a	1.2 ± 0.10^a	4.7 ± 0.50^a	5.2 ± 0.60^a
POA $_{(C16:1,\ ω-9)}$	2.8 ± 0.1^a	3.0 ± 0.40^a	24.0 ± 1.0^a	54 ± 2.0^b
OLA $_{(C18:1,\ ω-9)}$	55 ± 3.8^a	57 ± 1.90^a	189 ± 8.50^a	543 ± 22^b
LLA $_{(C18:2,\ ω-6)}$	20 ± 1.2^a	25 ± 1.0^b	131 ± 5.50^a	458 ± 20^b
αALA $_{(C18:3,\ ω-3)}$	4.2 ± 0.2^a	6.2 ± 0.30^b	7.0 ± 0.50^a	20 ± 1.0^b
AA $_{(C20:4,\ ω-6)}$	1.6 ± 0.03^a	2.3 ± 0.12^b	5.0 ± 0.30^a	21 ± 0.90^b
EPA $_{(C20:5,\ ω-3)}$	2.3 ± 0.08^a	1.3 ± 0.03^b	5.0 ± 0.30^a	3.5 ± 0.15^b
DPA $_{(C22:5,\ ω-3)}$	1.5 ± 0.04^a	0.70 ± 0.03^b	1.7 ± 0.07^a	1.0 ± 0.07^a
DHA $_{(C22:6,\ ω-3)}$	9.0 ± 0.35^a	6.0 ± 0.25^b	1.3 ± 0.03^a	0.90 ± 0.03^b
NVA $_{(C24:1,\ ω-9)}$	0.2 ± 0.02^a	0.25 ± 0.02^a	1.3 ± 0.05^a	0.25 ± 0.01^b
DHA/AA molar ratio	5.0 ± 0.20^a	2.4 ± 0.12^b	0.2 ± 0.0^a	0.04 ± 0.0^b
ω-3 USI	0.33 ± 0.01^a	0.29 ± 0.01^b	0.10 ± 0.0^a	0.06 ± 0.0^b
ω-6 USI	0.20 ± 0.01^a	0.30 ± 0.01^b	0.50 ± 0.0^a	0.70 ± 0.0^b

Results are mean ± SEM ($n = 5$) for duplicate determinations. Values (of either milk or mammary tissue columns) with different superscript letters are significantly different at $P < 0.05$ (Student's t test)

PLA, palmitic acid ($C_{16:0}$); *STA*, stearic acid ($C_{18:0}$); *TCA*, tetracosanoic acid ($C_{24:0}$); *POA*, palmitoleic acid ($C_{16:1}$); *OLA*, oleic acid ($C_{18:1}$); *LLA*, linoleic acid ($C_{18:2,\ ω-6}$); *α-ALA*, alpha-linoleic acid ($C_{18:3,\ ω-3}$); *AA*, arachidonic acid ($C_{20:4,\ ω-6}$); *EPA*, eicosapentaenoic acid ($C_{20:5,\ ω-3}$); *DPA*, docosapentaenoic acid ($C_{22:5,\ ω-3}$); *DHA*, docosahexaenoic acid ($C_{22:6,\ ω-3}$); and *NVA*, nervonic acid ($C_{24:1}$); *DHA/AA*, molar ratio of DHA and AA; *ω-3 USI* (ω-3-unsaturation index) = ∑ [mol% of each ω-3 polyunsaturated fatty acid × number of double bond(s) per ω-3 polyunsaturated fatty acid] / 100. *ω-6 USI* (ω-6 unsaturation index) = ∑ [mol% of each ω-6 polyunsaturated fatty acid × number of double bond(s) per ω-6 polyunsaturated fatty acid] / 100

Discussion

The results of this study provide clear evidence that DHA levels decrease significantly both in the milk and mammary gland tissues of Pb-exposed lactating mother rats. The decline in the levels of DHA was accompanied by increase in the levels of AA, LPO, and TNF-α, concurrently with decrease in GSH levels and DHA/AA molar ratios in the milk/mammary tissues of Pb-exposed mother rats. ω-3-DHA and ω-6-AA are both highly polyunsaturated fatty acids (PUFAs); however, DHA is more unsaturated than AA. Halliwell and Gutteridge [45] reported that fatty acids containing zero to two double bonds are more resistant to oxidative stress than the PUFAs with more than two double bonds. This means that the more polyunsaturated a fatty acid is, the more susceptible it is to oxidation. The notion was corroborated after incubation of linoleic (LA, C18:2, ω-6), alpha-linolenic (ALA, C18:3, ω-3), and arachidonic acid (AA, C20:4, ω-3) with Pb solution, in which the concentration of the LPO increased with the increase in the number of double

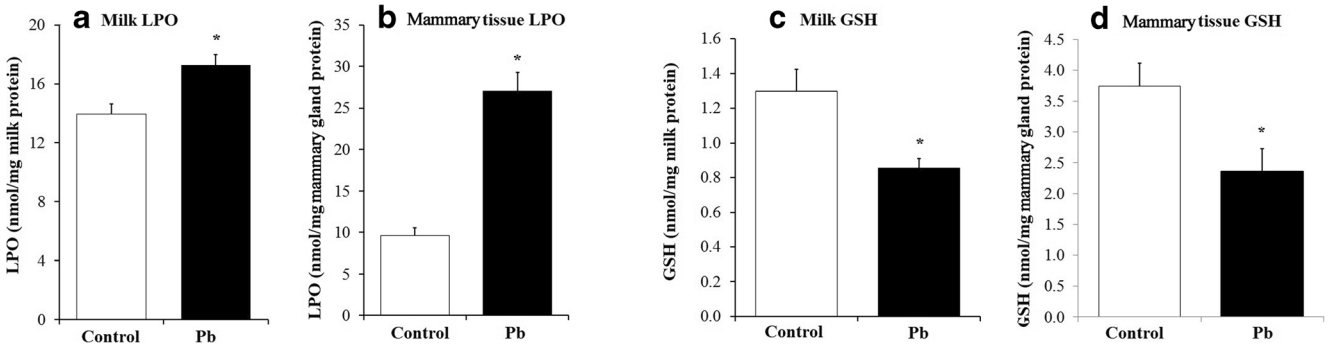

Fig 2 Effect of Pb on the levels of lipid peroxide (**a**, **b**) and GSH (**c**, **d**) of milk and mammary gland tissues. Results are mean ± SE of 6–7 rats per group. Bar with asterisk (*) symbol in each figure (**a**–**d**) is significantly different at $*P < 0.05$. Data were analyzed by Student's t test

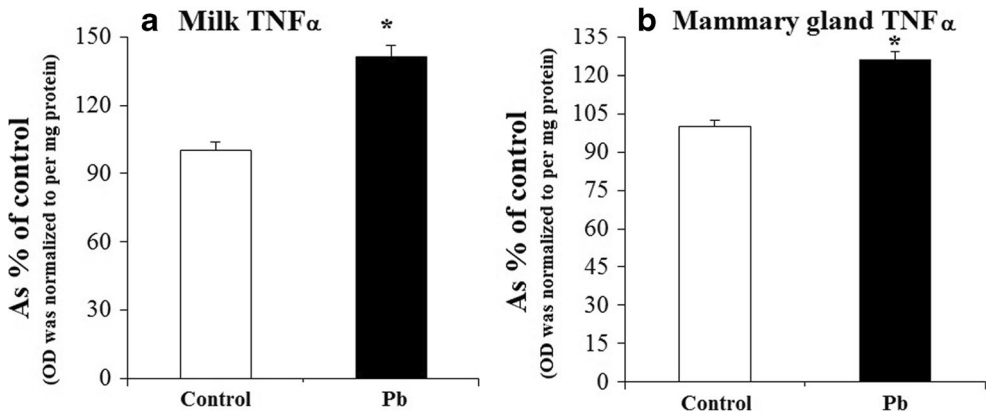

Fig 3 Effect of Pb on the tumor necrosis factor alpha (TNF-α) of milk (**a**) and mammary gland tissues (**b**). Results are mean ± SE of 6–7 rats per group. Bar with asterisk (*) symbol in each figure (**a**–**d**) is significantly different at $^*P < 0.05$

bonds of the fatty acids [46]. However, these reports made no remarks on the effects of Pb incubation on DHA, which contains two more double bonds than AA. In the current study, higher levels of DHA were accompanied by decrease in LPO levels in the milk of control rats, while the decrease in DHA levels was associated with elevated levels of LPO in the milk of Pb-exposed rats. These contrasting changes in the levels of DHA versus AA gave rise to a significant increase and decrease in the DHA/AA molar ratios, in the milk of control and Pb-exposed mother rats, respectively (Table 1). However, the question of which of the PUFAs (DHA vs. AA) contributes to a greater change in LPO levels in in vivo scenarios remains unanswered. We propose that the mere presence of DHA, at least in in vivo scenario, could not be attributed to the increased levels of LPO. We have previously reported that DHA/AA molar ratios were inversely related to LPO levels in the brain tissues of DHA-administered rats [19, 47]. The results of decreased DHA/AA molar ratios and increased levels of LPO in the milk and mammary tissues of Pb-exposed rats are thus consistent with these earlier findings. Differences in the physicochemical properties such as in situ 3d structure, axial rotation, vibrational spring-like motion, molecular surface volume, volume per unit area, and accessibility of oxidants to extractable hydrogen atoms between DHA and AA may relate to their relative sensitivity to oxidation. Other confounding factors, including eicosanoids derived from AA and docosanoids derived from DHA, might contribute to the increased levels of LPO in the milk/mammary tissues of Pb-exposed mother rats. For example, some oxidation products of ω-6 AA are themselves free radical species with higher oxidative potential [48]; these may have an indirect correlation with the increase in LPO levels in the milk/mammary tissues of Pb-exposed rats. Consistent with our findings, Pb exposure increased AA levels in rats [49], birds, rodents, preschool children [50], and adult humans [51]. Whatever is the mechanism, AA levels increased significantly, while DHA levels or DHA/AA molar ratios decreased, concomitantly with upsurges in LPO levels in the milk/mammary tissues of Pb-exposed mother rats. Again, our results of increased LPO levels are consistent with other reports, where Pb exposure enhanced LPO levels in liver tissues of rats [52] and mice [53], and blood of human subjects [54].

Moreover, levels of reduced glutathione (GSH), which plays a defensive role against the action of oxidative species both directly and indirectly as a cofactor of glutathione peroxidase (GPx), reduced significantly in the milk/mammary tissues of Pb-exposed mother rats (Fig. 2). The mechanism of action explaining how an increase in LPO levels induces a reduction in GSH level is not yet clearly known. However, we may look at the affinity of Pb for the sulfhydryl (SH) group. Pb has a high affinity for sulfhydryl (SH) groups; it can alter antioxidant activities by inhibiting functional –SH groups of antioxidative enzymes such as SOD, CAT, and GPx [55]. Sing et al. [29] also reported that Pb exposure decreases levels of antioxidative enzymes in brain tissues. Synthetic free radical scavenger butylated hydroxytoluene (BHT) inhibits Pb-catalyzed peroxidation of unsaturated fatty acids and levels of resulting LPO [46]. We have also previously reported that the natural antioxidant α-tocopherol inhibits Pb-induced increase in LPO levels in erythrocyte plasma membranes [44]. Pb-induced decrease in GSH levels or

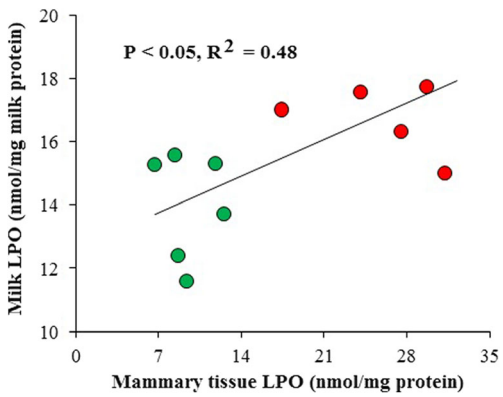

Fig 4 Correlation between the levels of lipid peroxide (LPO) of the milk versus mammary gland tissues of the control (●) and Pb-exposed rats (■). The correlation was performed by a simple regression analysis

weakening of the antioxidative defense system in milk-producing cells may, therefore, contribute to the increase in LPO levels in the mammary tissues, and hence in the milk. As expected, milk LPO levels correlated significantly with those of the mammary gland tissues (Fig. 4).

In our experiment, the decrease in DHA levels in the milk of Pb-exposed mother rats coincided with increase in TNF-α levels both in milk and mammary tissues. Reports on the effect of Pb exposure on milk TNF-α levels are lacking. Milk does not have a TNF-α-synthesizing machinery, specifying that milk is not the immediate source of TNF-α. It is, thus, possible to assume that the increased levels of milk TNF-α were derived from the alveolar and associated inflammatory cells of milk ducts, and/or it might have been accumulated from the plasma. Our results are quantitatively consistent with those of Cheng et al. [56], who also reported increased levels of TNF-α in the liver tissues of Pb-exposed rats. Based on these findings, we speculate that the toxic heavy metal Pb triggered a pro-inflammatory stress in the ductal cells, increasing thereby the intracellular levels of TNF-α, which accumulated in milk through the tributaries of ductal canals. However, the physiological significance of TNF-α and its relationship with DHA in milk need to be clarified. Liu et al. [57] reported that TNF-α from mother's milk had a significant influence on the immune function, neurobiology, and behavior of the offspring. They reported that TNF-α-knockout (KO) mother mice had decreased levels of TNF-α in the mammary glands and milk. The pups that ingested lower levels of TNF-α via milk had increased number of cells in the hippocampus and had a better spatial memory in adulthood. More interestingly, blocking of TNF-α production in wild-type (WT) mothers with a specific antibody had the same effect as if these WT mothers were genetically KOs. These reports thus demonstrate that Pb-induced increase in TNF-α levels in the milk and, thereby, the consumption of Pb-intoxicated milk by the offspring would exert a negative impact on the cells of their hippocampus, the brain region that plays crucial roles in learning and formation of memory.

Additionally, the accompanied decline in the levels of milk DHA may deteriorate further the capacity of memory formation in the offspring. The bases of these speculations are (i) DHA may increase in vitro and in vivo neurogenesis in brain hippocampus [1]; (ii) DHA may enhance levels of memory-related brain proteins, including BDNF, postsynaptic density protein-95 (PSD-95), vesicular acetylcholine transporter (VAchT) [58], synaptosomal-associated protein 25 (SNAP-25) [59], phosphorylated c-AMP response element–binding (pCREB) protein [60], and mRNA levels of NMDA-receptor subunit NR2B [58]; and (iii) DHA may inhibit brain TNF-α levels [61]. We also reported previously that the exposure of mother rats to Pb increased TNF-α levels and suppressed the levels of memory-related proteins, including BDNF, tyrosine receptor kinase B (TrKB), PSD-95, and synaptosomal-associated protein 25 (SNAP-25) in the brain of their offspring [39]. It, thus, suggests that an impairment of memory would occur in the later life of these pups. The inference is further supported by in vivo studies, where Pb exposure instigated an impairment of memory in rats [29, 62] and mice [63]. Since numerous factors can contribute to the elevation of TNF-α levels, understanding the effect of Pb on milk TNF-α is of greater significance to explore the mechanisms by which TNF-α from mother's milk influences the neurodevelopment and behavior of the offspring.

Also, the effect of the alterations in the levels of other fatty acids in the milk and mammary tissues needs to be clarified. Notably, the fatty acids of milk are derived either from de novo synthesis within the mammary gland or from dietary fats or fats mobilized from adipose tissues [64]. The levels of PLA and STA decreased significantly in the milk, while their levels increased significantly in the mammary gland tissues of Pb-exposed mother rats. These findings suggest that Pb exposure per se caused a differential effect on the saturated fatty acid profile in the milk versus mammary gland tissues. A direct role of saturated fatty acids (SFAs) has not been demonstrated in the brain cognition and behavior. Rather, the entry of SFAs in the brain has been ascribed to trigger hypothalamic inflammation [65]. Several studies report that the increase in ω-6 LLA consumption may induce adverse nutritional condition during lactation. It is because the LLA competes with ALA for endogenous conversion to the long-chain EPA/DHA and may also inhibit their incorporation into cellular membranes. Thus, high LA levels in the diet may result in low DHA status, which is critical to the prenatal and postnatal developmental periods of the offspring [66–68]. On the other hand, a high intake of ω-3 PUFAs including DHA in early life is considered beneficial to growth and development, and ultimately metabolic health in later life [67, 69, 70]. In the current study, the degree of the ω-3 USI decreased ($P < 0.05$), and that of the ω-6 USI increased both in the milk and mammary gland tissues of Pb-exposed mother rats. We have previously reported that DHA/AA molar ratio acted as an antioxidant indicator in brain tissues and positively correlated with memory [18, 19, 21, 30]. Pb-induced decrease in the levels of ω-3 PUFAs or increase in the levels of ω-6 PUFAs in milk will, therefore, be expected to have significant effects both on the pre-weaning and post-weaning growth of animals. Finally, DHA, as mentioned earlier, cannot be de novo synthesized adequately; it needs to be pre-formed and supplied by diet to mothers, who in turn provide it to the offspring via milk. Thus, increasing DHA content of milk might confer neurodevelopmental benefits to the recipient offspring. In contrast, if Pb exposure reduces DHA levels in milk, it will obviously affect the growth, development, and neurobehavior of the offspring.

Finally, our study strongly suggests that the levels of DHA significantly decrease in the milk of Pb-exposed lactating mother rats. The DHA/AA molar ratios and GSH levels also

decrease, concomitantly with increases in the levels of the LPO and pro-inflammatory TNF-α. On the basis of the results of the present investigation, we hypothesize that the exposure of pregnant women to Pb might similarly affect DHA content in human milk, and hence DHA-mediated brain cognitive ability.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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