Journal of Trace Elements in Medicine and Biology 52 (2019) 222–231

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Toxicology

Fatty acid levels alterations in THP-1 macrophages cultured with lead (Pb)

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ARTICLE INFO

Keywords:
Lead
Pb
Fatty acids
Macrophages
Atherosclerosis

ABSTRACT

Objective: As cardiovascular events are one of the main causes of death in developed countries, each factor potentially increasing the risk of cardiovascular disease deserves special attention. One such factor is the potentially atherogenic effect of lead (Pb) on lipid metabolism, and is significant in view of the still considerable Pb environmental pollution and the non-degradability of Pb compounds.
Methods: Analysis of saturated fatty acids (SFA) (caprylic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), and behenic acid (C22:0)), monounsaturated fatty acid (MUFA) (palmitoleic acid (C16:1), oleic acid (18:1w9), *trans*-vaccenic acid (C18:1 *trans*11)), and polyunsaturated fatty acid (PUFA) (linoleic acid (C18:2n6), *gamma*-linolenic acid (C18:3n6), arachidonic acid (C20:4n6)), was conducted by gas chromatography. Analysis of stearoyl-CoA desaturase *(SCD),* fatty acid desaturase 1 (*FADS1)* and fatty acid desaturase 2 (*FADS2)* expression was performed using qRT-PCR. Oxidative stress intensity (malondialdehyde - MDA concentration) was measured using spectrophotometric method. Intracellular generation of reactive oxygen species (ROS) in macrophages was visualized by fluorescence microscopy and quantitatively measured by plate reader.
Results: Pb caused quantitative alterations in FAs profile in macrophages; the effect was Pb-concentration dependent and selective (i.e. concerned only selected FAs). In general, the effect of Pb was biphasic, with Pb levels of 1.25 μg/dL and 2.5 μg/dL being stimulatory, and 10 μg/dL being inhibitory on concentrations of selected FAs. The most potent Pb concentration, resulting in increase in levels of 9 FAs, was 2.5 μg/dL, the Pb-level corresponding to the mean blood Pb concentrations of people living in urban areas not contaminated by Pb. Pb was found to exert similar, biphasic effect on the expression of *FADS1*. However, Pb decreased, in a concentration-dependent manner, the expression of *SCD* and *FADS2*. Pb significantly increased MDA and ROS concentration in macrophages.
Conclusion: Environmental Pb exposure might be a risk factor resulting in alterations in FAs levels, oxidative stress and increased MDA concentration in macrophages, which might lead to the formation of foam cells and to inflammatory reactions.

1. Introduction

Classified as the 2nd most dangerous environmental poison [1], lead (Pb) is still widespread in the environment, posing a significant threat to humans [2,3]. Pb exposure is known to be a risk factor for aortic atherosclerotic plaque burden [4–9]. Atherosclerotic cardiovascular disease (to which ischemic heart disease and cerebrovascular disease belong) is the leading cause of mortality worldwide. Ischemic heart disease and stroke (being the world's first and third causes of death, respectively) accounted for 247.9 deaths/100000 population in 2013 [10].

During chronic exposure to Pb, an increase in the level of total cholesterol and triglycerides occurs in the human body [11,12]. Pb causes lipid oxidation [13–15]. The oxidised lipids accumulate in macrophages, which leads to the formation of foam cells and to

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https://doi.org/10.1016/j.jtemb.2019.01.003
Received 23 October 2018; Received in revised form 16 December 2018; Accepted 4 January 2019
0946-672X/

inflammatory reactions [16–19]. During these processes, the accumulation of esterified cholesterol takes places, a process in which fatty acids (FAs) accumulated in the macrophages also participate [20,21].

Increased concentration of fatty acids in the blood and in macrophages may be an important factor in the development of atherosclerosis. First, fatty acids affect receptors for oxLDL in macrophages: lectin-like oxidized low-density-lipoprotein receptor-1 (LOX-1) [22–24] and CD36 [25–27]. The expression of these receptors is enhanced by SFA and reduced by MUFA and PUFA.

Oxidized lipids in macrophages have a pro-inflammatory action, which is an important property in the development of atherosclerosis [28]. Fatty acids can modulate inflammatory reactions. SFA can induce and increase the existing inflammatory reactions [29–34]. MUFA and PUFA abolish the effects of SFA and have anti-inflammatory effects [29,31,34,35]. Thanks to this, high SFA concentration is a predictive factor in the development of atherosclerosis. Unlike SFA, MUFA and PUFA inhibit inflammatory reactions and thereby inhibit the development of this disease.

The results of studies on the influence of Pb on lipid metabolism and Pb contribution to atherosclerosis development concerned exposure to high Pb concentrations which was usually associated with occupational exposure [36,37] or residing in areas highly contaminated with Pb [7,38,39]. In the present study, we investigate, for the first time, the effects of Pb at concentrations corresponding to low blood Pb levels detected in people environmentally exposed, on i) the levels of fatty acids in THP-1 macrophages, ii) the expression of desaturases, enzymes involved in synthesis of FAs, and iii) the concentrations of ROS and MDA in these cells.

2. Materials and methods

2.1. Cell culture and treatment

The experiments were conducted on macrophages derived from the human monocytic cell line THP-1 (ATCC, Rockville, USA). The differentiation of THP-1 cells into macrophages was achieved by administration of 100 nM phorbol myristate acetate (PMA) and further incubation for 24 h [40]. The adherent macrophages were washed three times with PBS and then incubated with one of various Pb acetate (PbAc) solutions for 48 h at 37 °C in 5% CO_2. The following concentrations of Pb were used in this study: 1.25 μg/dL and 2.5 μg/dL - concentrations recorded in the blood of people environmentally exposed to Pb in non-polluted areas, based on a study on young women and their newborns from the city of Szczecin (West Pomerania, Poland) [41]; 5 μg/dL - permissible concentration of Pb in children and pregnant women; 10 μg/dL - permissible concentration of Pb in the blood in adults) [42]. As a control cells were incubated in RPMI medium with 10% FBS. After 48 h, the cells were harvested by scraping and the pellets were obtained by centrifugation (800 G, 10 min). The viability of the cells was determined by trypan blue exclusion with the use of a Bright Line Hemacytometer (Sigma-Aldrich, Poznan, Poland). Cell cultures with a viability above 97% were used in the experiments [43].

2.2. Extraction and analysis of fatty acids

Lipids from the samples of cells were extracted using Folch mixture [44]. Then, the samples were hydrolyzed to give fatty acids which were transformed into fatty acid methyl esters (FAMEs) [45]. 0.5% solution of BHT was added to secure against oxidation. FAME in hexane solution was injected onto the capillary column (CP-SIL88 50 M × 0.25 mm ID, film thickness 0.2 μm, Varian) of the 6890 M Agilent gas chromatograph equipped with an autosampler. FAMEs were mobile in the column in an atmosphere of hydrogen as a carrier gas. The initial temperature was about 100 °C and maintained for 1 min, then the temperature was increased at a rate of 10 °C/min to 180 °C, then at a rate of 3 °C/min to 205 °C, and then at a rate of 10 °C/min to 220 °C. Identification of geometric and positional fatty acids isomers was carried out on the basis of a comparison of retention times with Sigma-Aldrich fatty acid standards. The concentrations of fatty acids were determined based on standard curves and expressed in mg/mL.

Further analyses took into account the following fatty acids: caprylic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (18:1w9), *trans*-vaccenic acid (C18:1*trans*11), linoleic acid (C18:2n6), *gamma*-linolenic acid (C18:3n6), arachidonic acid (C20:4n6), behenic acid (C22:0).

2.2.1. Quantitative real time polymerase chain reaction (qRT-PCR)

Quantitative analyses of stearoyl-CoA desaturase (*SCD*), fatty acid desaturase 1 (*FADS1*) and fatty acid desaturase 2 (*FADS2*) genes were performed by two-step reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from cells using an RNeasy Kit (Qiagen, USA). cDNA was prepared from 1 μg of total cellular RNA in 20 μl of reaction volume using a FirstStrand cDNA synthesis kit and oligo-dT primers (Fermentas, USA). The quantitative assessment of mRNA levels was performed by quantitative real-time qRT-PCR using an ABI 7500Fast instrument with Power SYBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95 °C (15 s), 40 cycles at 95 °C (15 s), and 60 °C (1 min). According to melting point analysis, only one PCR product was amplified under these conditions. Each sample was analyzed in two technical replicates, and the mean Ct values were used for further analysis. The relative quantity of the target, normalized to the endogenous control GAPDH gene and relative to a calibrator, is expressed as 2-ΔΔCt (-fold difference), where Ct is the threshold cycle, ΔCt = (Ct of target genes) – (Ct of endogenous control gene), and ΔΔCt = (ΔCt of samples for target gene) – (ΔCt of calibrator for the target gene). The following primer pairs were used: SCD Forward: TTCCTACCTGCA AGTTCTACACC; SCD Reverse: CCGAGCTTTGTAAGAGCGGT,

FADS1 Forward: CCAACTGCTTCCGCAAAGAC; FADS1 Reverse: GCTGGTGGTTGTACGGCATA; FADS2 Forward: TGACCGCAAGGTTTA CAACAT; FADS2 Reverse: AGGCATCCGTTGCATCTTCTC.

2.3. Oxidative stress assay

2.3.1. Imaging of intracellular reactive oxygen species (ROS) generation

The intracellular generation of ROS was visualized by fluorescent marker 2′,7′-dichlorofluorescein diacetate DCFH-DA (Sigma-Aldrich, Poland) as previously described [46,47]. Cells were loaded with 5 μM DCFH-DA. After incubation, the cells were washed with culture medium at room temperature and the preparations were examined under a confocal microscope. When DCFH-DA is oxidized to DCF by hydrogen peroxide within the cell, it becomes fluorescent (excitation at 495 nm, emission at 525 nm).

2.3.2. Quantitative evaluation of intracellular ROS generation

Cells were pre-incubated with DCFH-DA in conditions as for the microscopic study. The intensity of fluorescence coming from DFC was measured using a microplate reader and normalized to protein levels measured by Micro BCA assay [48,49].

2.4. Lipid peroxidation measurement

The malondialdehyde (MDA) concentration was measured using the Bioxytech LPO-586 Assay Kit (OxisResearch, Poland), according to manufacturer's instruction.

2.5. Protein assay

All the above-mentioned results were calculated from the protein content in the samples. Protein concentration was measured using a Micro BCA Protein Assay Kit (Thermo Scientific, Pierce Biotechnology, USA) and plate reader (UVM340, ASYS) [50].

2.6. Statistical analysis

The statistical analysis of obtained results was conducted using Statistica 10 software (Statsoft, Poland). The results were expressed as arithmetical means ± standard deviation (SD). The distribution of results for individual variables was obtained with the Shapiro-Wilk W test. As most of the distributions deviated from the normal distribution, non-parametric tests were used for further analyses. To assess the differences between the groups studied, the non-parametric Wilcoxon matched-pair test was used. The Spearman correlation rank coefficient was used to determine the strength of correlations between the parameters. A probability at $p \leq 0.05$ was considered as statistically significant.

3. Results

Our study demonstrated a clear effect of Pb on fatty acids profile in THP-1 macrophages, i.e. Pbinduced alterations in levels of selected FAs. The effect of Pb varied depending on the Pb concentration used and the type of analyzed FAs. The most potent in induction of FAs levels alterations appeared Pb concentration of 2.5 μg/dL.

Pb at concentration of 2.5 μg/dL produced significant changes in concentrations of 9 FAs (both SFAs, MUFAs and PUFAs), Pb concentration of 1.25 μg/dL resulted in significant alterations of levels of 6 FAs (only SFAs), while 5 μg/dL and 10 μg/dL Pb caused significant changes in levels of 3 FAs (only SFAs) and 2 FAs (1 SFA and 1 PUFA), respectively (Tables 1 and 2).

3.1. Saturated fatty acids

In the present study, the concentrations of 10 saturated fatty acids (SFAs) were determined. The proportions of total SFAs were found to be significantly increased due to 1.25 μg/dL; 2.5 μg/dL and 5 μg/dL Pb treatment, with the highest increase for 2.5 μg/dL Pb ($p = 0.026$; $p = 0.022$; $p = 0.064$ respectively) (Fig. 1). The highest tested Pb concentration caused insignificant decrease in proportions of total SFAs relative to control ($p = 0.072$). However Pb at concentration of 1.25 μg/dL significantly increased the concentrations of C8:0, C13:0, C14:0, C16:0, C17:0 and C18:0 ($p = 0.021$; $p = 0.034$; $p = 0.022$; $p = 0.025$; $p = 0.012$ respectively) (Table 1). The concentration of Pb 2.5 μg/dL Pb resulted in marked enhancement in the levels of C12:0, C14:0, C16:0, C17:0 and C18:0 ($p = 0.042$, $p = 0.031$, $p = 0.015$, $p = 0.030$; $p = 0.022$ respectively). Pb at concentration of 5 μg/dL caused significant increase in concentrations of C14:0 and C16:0, and significant decrease in concentration of C17:0 ($p = 0.045$; $p = 0.022$; $p = 0.046$). The highest tested Pb concentration (10 μg/dL) produced significant decrease in the levels of C10:0 ($p = 0.036$) with no effect on other SFA levels (Table 1).

3.2. Unsaturated fatty acids

In the current study, the concentrations of 6 unsaturated fatty acids (UFAs) were determined: 3 monounsaturated fatty acids (MUFAs) and 3 polyunsaturated fatty acids (PUFAs). The effect of Pb on the concentrations of UFAs was not uniform. Exposure of THP-1 macrophages to 1.25 μg/dL; 2.5 μg/dL; and 5 μg/dL Pb resulted in insignificant increase in the proportions of total UFAs, while the highest tested Pb concentration (10 μg/dL) appeared to insignificantly decrease the proportions of total UFAs, relative to control (Fig. 2).

The lowest tested Pb concentration (1.25 μg/dL) had no significant effect on the concentrations of analyzed UFAs (Table 2). Pb at concentration of 2.5 μg/dL markedly increased the levels of most tested UFAs, i.e. C18:1w9, C18:1trans11, C18:3n6 and C20:4n6 ($p = 0.032$; $p = 0.022$; $p = 0.021$; and $p = 0.038$, respectively). The concentration of 5 μg/dL Pb had no effect on the levels of analyzed UFAs ($p = 0.058$). The highest tested Pb concentration (10 μg/dL) was found to significantly decrease the concentration of C18:3n6 ($p = 0.045$), with no effect on the other analyzed UFAs ($p = 0.065$) (Table 2).

3.3. Desaturases

In the present study, Pb significantly affected the expression of desaturase genes (i.e. *SCD*, *FADS1* and *FADS2*) in THP-1 macrophages (Fig. 3).

At all tested concentrations, Pb decreased the expression of *SCD* relative to the control ($p = 0.028$). This effect was proportional to Pb concentration. At a concentration of 10 μg/dL, the decrease in *SCD* expression was 2.5 times relative to the control ($p = 0.028$).

Pb had a much stronger effect on the expression of *FADS2*. At the lowest concentration of 1.25 μg/dL, Pb halved the gene expression ($p = 0.028$). At the highest concentration of 10 μg/dL, Pb reduced the expression of *FADS2* by almost 20 times ($p = 0.028$).

However, the effect of Pb on *FADS1* expression was biphasic, with Pb levels of 1.25 μg/dL, 2.5 μg/dL and 5 μg/dL being stimulatory ($p = 0.043$, $p = 0.043$ and $p = 0.043$, respectively), and 10 μg/dL being inhibitory ($p = 0.045$).

3.4. Intracellular ROS synthesis

Images from the confocal microscope showed the increased level of green fluorescence coming from DCF (thereby ROS synthesis in the cytoplasm of macrophages) from Pb-treated cells vs control (Fig. 4). The

Table 1
The effect of Pb on saturated fatty acids (SFA) concentration in THP-1 macrophages.

Fatty Acids [μg/mg protein]	Control	1.25 μg/dL Pb	2.5 μg/dL Pb	5 μg/dL Pb	10 μg/dL Pb
C8:0	0.20 ± 0.02	0.29 ± 0.05 (C, 10)	0.31 ± 0.15 (10)	0.23 ± 0.06	0.15 ± 0.05
C10:0	0.78 ± 0.27	0.91 ± 0.29 (10)	0.88 ± 0.26 (10)	0.76 ± 0.18 (10)	0.56 ± 0.17 (C)
C12:0	0.13 ± 0.04	0.18 ± 0.04 (10)	0.20 ± 0.04 (C, 5, 10)	0.14 ± 0.04 (10)	0.11 ± 0.03
C13:0	0.48 ± 0.11	0.65 ± 0.15 (C)	0.63 ± 0.19	0.72 ± 0.28 (10)	0.51 ± 0.26
C14:0	0.87 ± 0.18	1.25 ± 0.25 (C, 10)	1.19 ± 0.25 (C)	1.08 ± 0.16 (C)	0.88 ± 0.33
C15:0	0.17 ± 0.04	0.27 ± 0.08	0.22 ± 0.08	0.25 ± 0.12	0.21 ± 0.10
C16:0	17.86 ± 3.79	25.03 ± 4.84 (C, 10)	24.32 ± 4.74 (C, 10)	21.52 ± 2.70 (C)	17.23 ± 5.57
C17:0	0.18 ± 0.06	0.27 ± 0.06 (C, 10)	0.26 ± 0.07 (C, 10)	0.17 ± 0.03 (C)	0.19 ± 0.06
C18:0	19.20 ± 4.23	26.75 ± 5.09 (C, 5, 10)	26.21 ± 5.26 (C, 10)	20.48 ± 4.80	16.60 ± 5.76
C22:0	0.05 ± 0.08	b.d.	b.d.	b.d.	b.d.

(C) - statistically significant difference in the concentration of the acid in relation to control ($p \leq 0.05$).
(5) - statistically significant difference in the concentration of the acid compared to 5 μg/dL Pb ($p \leq 0.05$).
(10) - statistically significant difference in the concentration of the acid compared to 10 μg/dL.
Pb ($p \leq 0.05$).
b.d. – below detection level.
Data represent the means ± SD for 6 independent experiments each analysed in triplicate.

Table 2
The effect of Pb on total unsaturated fatty acids (UFA) concentration in THP-1 macrophages.

Fatty acids [μg/mg protein]	Control	1.25 μg/dL Pb	2.5 μg/dL Pb	5 μg/dL Pb	10 μg/dL Pb
C16:1	0.05 ± 0.03	0.04 ± 0.04	0.04 ± 0.06	0.06 ± 0.05	0.06 ± 0.04
C18:1w9	0.39 ± 0.10	0.51 ± 0.13	0.54 ± 0.07 (C, 10)	0.49 ± 0.03 (10)	0.39 ± 0.11
C18:1*trans*11	0.15 ± 0.06	0.22 ± 0.09	0.22 ± 0.05 (C, 10)	0.18 ± 0.02	0.13 ± 0.04
C18:2n6	b.d.	b.d.	0.15 ± 0.11	0.16 ± 0.07 (10)	0.04 ± 0.07
C18:3n6	0.13 ± 0.03	0.18 ± 0.08	0.16 ± 0.05 (C, 10)	0.16 ± 0.09 (10)	0.09 ± 0.03 (C)
C20:4n6	0.17 ± 0.07	0.22 ± 0.09	0.29 ± 0.03 (C, 10)	0.28 ± 0.12 (10)	0.14 ± 0.06

(C) – statistically significant difference in concentration of the acid relative to control ($p \leq 0.05$).
(5) - statistically significant difference in the concentration of the acid compared to 5 μg/ dL Pb ($p \leq 0.05$).
(10) - statistically significant difference between the concentration of the acid compared to 10 μg/dL Pb ($p \leq 0.05$);
b.d. – below detection level.
Data represent the means ± SD for 6 independent experiments each analysed in triplicate.

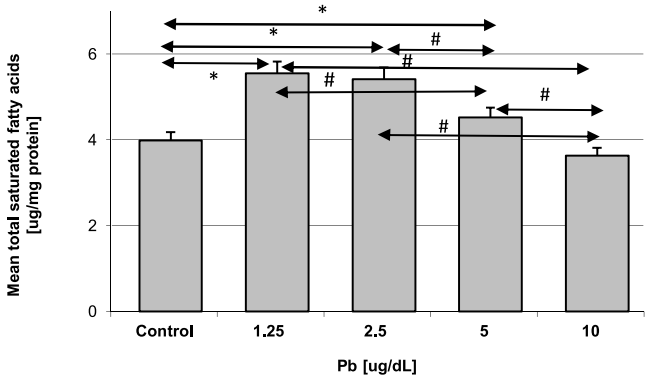

Fig. 1. The effect of Pb on total saturated fatty acids (SFA) concentration in THP-1 macrophages.
Data represent the means ± SD for 6 independent experiments each analysed in triplicate. PMA–activated macrophages of the THP-1 cell line were cultured with Pb at concentrations of 1.25 μg/dL and 2.5 μg/dL (concentrations recorded in the blood of people environmentally exposed to Pb in non-polluted areas), 5 μg/dL (permissible concentration of Pb in children and pregnant women), and 10 μg/dL (permissible concentration of Pb in the blood in adults). As a control cells were incubated in RPMI medium with 10% FBS. After 48 h of incubation, the cells were scraped and analyzed using a gas chromatograph.
* statistically significant difference in the amount of fatty acid in macrophages relative to control, $p \leq 0.05$;
# statistically significant difference in the amount of fatty acid in macrophages between two concentrations of Pb, $p \leq 0.05$.

quantitative measurement of fluorescence intensity confirmed the statistically significant increase in intracellular ROS level in Pb-treated macrophages vs control cells (Table 3), (by 15% with 1.25 μg/dL Pb, $p = 0.021$; by 18% with 2.5 μg/dL Pb, $p = 0.001$; by 21% with 5 μg/dL Pb, $p = 0.001$; by 49% with 10 μg/dL Pb, $p = 0.001$). ROS level was strongly positively correlated with Pb concentration ($Rs = 0.75$, $p = 0.001$).

3.5. MDA concentration

Lipid peroxidation product, MDA concentration was statistically significantly higher in macrophages cultured with 1.25 μg/dL Pb (by 5%, $p = 0.042$), 2.5 μg/dL Pb (by 8%, $p = 0.040$), and 5 μg/dL Pb (by 12.5%, $p = 0.001$) vs control. The highest Pb concentration used in our study, corresponding to permissible concentration of Pb in the blood of adults (10 μg/dL Pb), resulted in the statistically significant increase in MDA concentration (by 21%, $p = 0.001$) in comparison to control. MDA concentration was strongly positively correlated with Pb concentration ($Rs = 0.65$, $p = 0.001$).

4. Discussion

The influence of Pb exposure on macrophages can have detrimental effects throughout the body, resulting in the increased risk of cancer, hypertension, anemia, tissue and organ damage (for instance, cardiac damage, neuronal damage), and decreased pathogen killing [51].

The effect of cadmium exposure on FAs levels in THP-1 macrophages has been shown [52]. To our knowledge, this is the first study that examines the effect of Pb at concentrations relevant to human blood Pb levels, on FAs profile in THP-1 macrophages. The results demonstrate that Pb influenced the concentrations of FAs: the effect was Pb concentration dependent and selective (i.e. not the same for all analyzed FAs). Pb effect was, in general, biphasic, with Pb levels of 1.25 μg/dL and 2.5 μg/dL being stimulatory and 10 μg/dL Pb being inhibitory on selected FA levels. PbAc at concentrations 1.25 μg/dL and 2.5 μg/dL appeared to increase the levels of 6 and 9 FAs, respectively, 5 μg/dL Pb increased concentrations of 2 FAs and decreased concentration of 1 F A, while the highest tested Pb concentration (10 μg/dL) decreased levels of 2 FAs. The most potent Pb concentration was 2.5 μg/dL, inducing increase in levels of both SFAs, MUFAs and PUFAs (Table 4).

Regarding the effect of Pb at concentrations of 1.25–5 μg/dL on SFAs, our results seem to be consistent with studies results of Donaldson

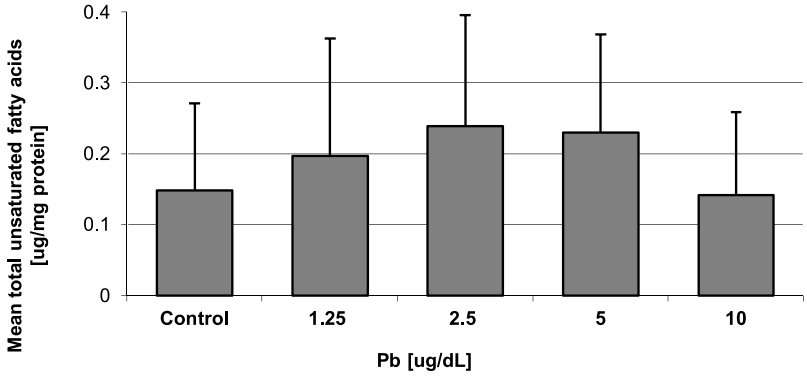

Fig. 2. The effect of Pb on total unsaturated fatty acids (UFA) concentration in THP-1 macrophages.
Data represent the means ± SD for 6 independent experiments each analysed in triplicate. PMA–activated macrophages of the THP-1 cell line were cultured with Pb at concentrations of 1.25 μg/dL and 2.5 μg/dL (concentrations recorded in the blood of people environmentally exposed to Pb in non-polluted areas), 5 μg/dL (permissible concentration of Pb in children and pregnant women), and 10 μg/dL (permissible concentration of Pb in the blood in adults). As a control cells were incubated in RPMI medium with 10% FBS. After 48h of incubation, the cells were scraped and analyzed using a gas chromatograph. There were no statistically significant differences in the concentration of fatty acid in macrophages relative to control or between two concentrations of Pb.

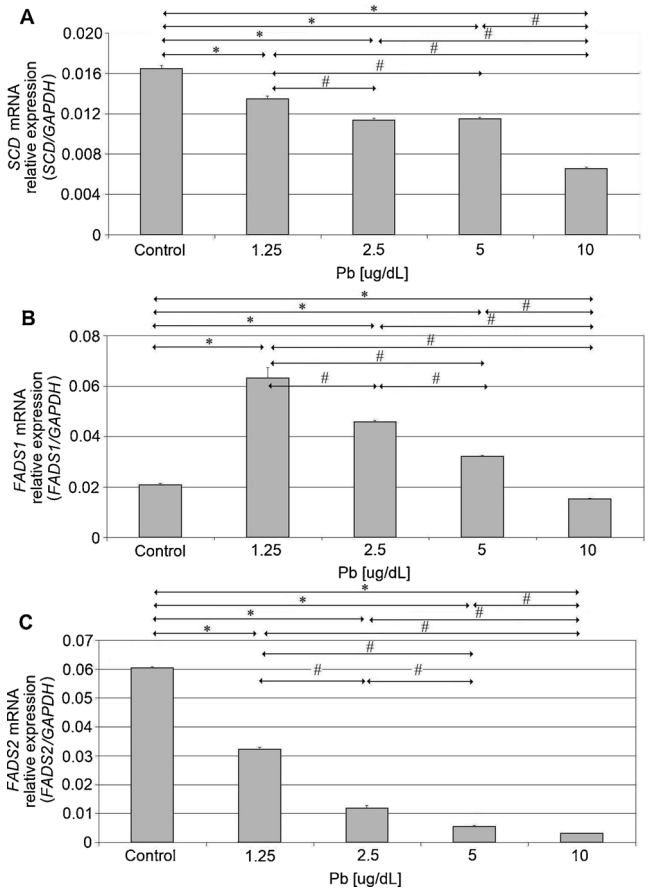

Fig. 3. The effect of Pb on *SCD*, *FADS1* and *FADS2* gene expression in THP-1 macrophages.

Data represent the means ± SD for 6 independent experiments each analysed in triplicate. PMA–activated macrophages of the THP-1 cell line were cultured with Pb at concentrations of 1.25 μg/dL and 2.5 μg/dL (concentrations recorded in the blood of people environmentally exposed to Pb in non-polluted areas), 5 μg/dL (permissible concentration of Pb in children and pregnant women), and 10 μg/dL (permissible concentration of Pb in the blood in adults). As a control cells were incubated in RPMI medium with 10% FBS. After 48 h of incubation, the cells were scraped and analyzed using a qRT-PCR.

* statistically significant difference in the amount of fatty acid in macrophages relative to control, $p \leq 0.05$;

statistically significant difference in the amount of fatty acid in macrophages between two concentrations of Pb, $p \leq 0.05$.

and Leeming [53], Mateo et al. [54] and Jung et al. [55], who demonstrated Pb-induced increased concentrations of C16:0 [54,55] and C18:0 [53–55] in different experimental models and experimental conditions. In our study, also other SFAs levels were significantly increased by Pb: C8:0 (by 1.25 μg/dL Pb), C12:0 (by 2.5 μg/dL Pb), C13:0 (by 1.25 μg/dL Pb), C14:0 (by 1.25, 2.5 and 5 μg/dL Pb) and C17:0 (by 1.25 and 2.5 μg/dL Pb). The effect of Pb on C17:0 levels in our study is in disagreement with the results of Mateo et al. [54] and Jung et al. [55], who demonstrated lack of significant alterations in C17:0 levels in mallards [54] and mice [55] exposed to Pb. The stimulatory effect of Pb on C14:0 levels found in the present study is in contrast to the results of Jung et al., who found that C14:0 levels were not altered by Pb in brains of mice fed Pb in drinking water [55]. The highest tested Pb concentration significantly reduced the levels of only one SFA, i.e. decanoic acid (C10:0), with no significant effect on other SFAs.

As regards the influence of Pb on UFAs in our study, 2.5 μg/dL Pb appeared to significantly increase concentrations of four UFAs: two MUFAs (oleic acid and trans-vaccenic acid) and two PUFAs (γ-linolenic acid and arachidonic acid). Available literature demonstrates different effects of Pb on oleic acid levels: significant increase in brains of mice exposed to Pb via drinking water [55], lack of significant alteration in liver of mallards fed on diets containing Pb [54] or significant reduction in liver of chicks exposed to Pb via diet [56]. Concerning the influence of Pb on C20:4n6 levels in THP-1 macrophages, only exposure to 2.5 μg/dL Pb resulted in significant increase in C20:4n6 concentration. The stimulatory effect of Pb on C20:4n6 levels was reported in many studies, involving chicks [14,53,56–59], turkeys [60], mallards [54], rodents [59,61] and humans [59]. However, one study showed that AA concentrations were not significantly altered in mouse brain due to Pb exposure [55].

Pb influenced the expression of *FADS1* and *FADS2*. It reduced the expression of *FADS2*, however, its effect on the expression of *FADS1* was biphasic: Pb at low concentrations increased expression of that gene, while at a concentration of 10 μg/dL Pb caused a decrease in *FADS1* expression. *FADS1* and *FADS2* genes encode Δ^5- and Δ^6-desaturase, respectively [62,63]. Δ^6-desaturase is responsible for the formation of C18:3n6 from C18:2n6. The Δ^5-desaturase is found in the pathway of synthesis of C20:4n6 from C18:3n6. Both enzymes also participate in the processing of FAs of n-3 family. To our knowledge, this is the first study that investigates the effect of Pb on the expression of *FADS1* and *FADS2,* therefore it is impossible to refer to literature data.

However, there are studies which demonstrate that Pb decreases C18:2n6/C20:4n6 ratio [53,56–59], thus affecting the increase in the amount of enzyme product, in relation to the amount of substrate, of the entire pathway in which the desaturases, encoded by the *FADS1* and *FADS2* genes, are found, probably as a result of the increased activity of these enzymes.

Based on our qRT-PCR studies, it is very difficult to determine whether our results are consistent with those observations, because at a concentration of 1.25–5 μg/dL Pb reduces the expression of *FADS2* but increases the expression of *FADS1*. Nevertheless, the results of *FADS1* and *FADS2* expression are reflected in the effect of Pb on the concentration of various PUFAs in THP-1 macrophages.

Pb at a concentration of 2.5 μg/dL and 5 μg/dL increases to a greater extent the concentration of C20:4n6 than the concentration of C18:3n6 relative to the control, which indicates the increase in Δ^5-desaturase activity and *FADS1* expression. However, it should not be forgotten that Pb may also affect the production of PGE_2, which reduces the concentration of C20:4n6. Therefore, the demonstrated differences in PUFAs concentrations should be even greater.

Differences between concentrations of C18:2n6 and C18:3n6 indicate decrease in Δ^6-desaturase activity, which may be the result of the reduction of *FADS2* expression by Pb. In the control and 1.25 μg/dL Pb, the C18:2n6 concentration was determined to be below detection limit, whereas the C18:3n6 concentration was 0.20 μg/mg protein. At higher Pb concentrations, levels of both FAs are comparable. This shows that the differences between the concentrations of these FAs are the smallest at a higher Pb concentration, which indicates decreased Δ^6-desaturase activity and may be the result of decreased *FADS2* expression, proportional to the Pb concentration, as demonstrated by qRT-PCR.

The increase in mRNA expression of the desaturases observed in our study may be related to the direct action of Pb at the level of gene expression regulation. It has been shown, for example, that Pb triggers a signaling pathway leading to the *IL-8* gene induction in human stomach adenocarcinoma cells after Pb administration which induces the epidermal growth factor receptor (EGFR) and phosphorylation of ERK1/2 kinase. The kinase then activates the AP-1 heterodimeric transcription factor (component of which is the c-jun protein), which leads to increased expression of the *IL-8* gene (Lin et al., 2015). Increased *IL-8* gene expression was also observed in endothelial cells isolated from human umbilical vein in response to Pb. The authors of the study demonstrated the activation of the Nrf2 transcription factor by Pb [64]. Nrf2 in the inactive state is bound to KEAP-1 (Kelch-like ECH-associated protein 1) in the cytoplasm. Activation of Nrf2 results in its release from the KEAP-1, translocation to the cell nucleus, where it forms a heterodimer with a small Maf protein and binding to the antioxidant

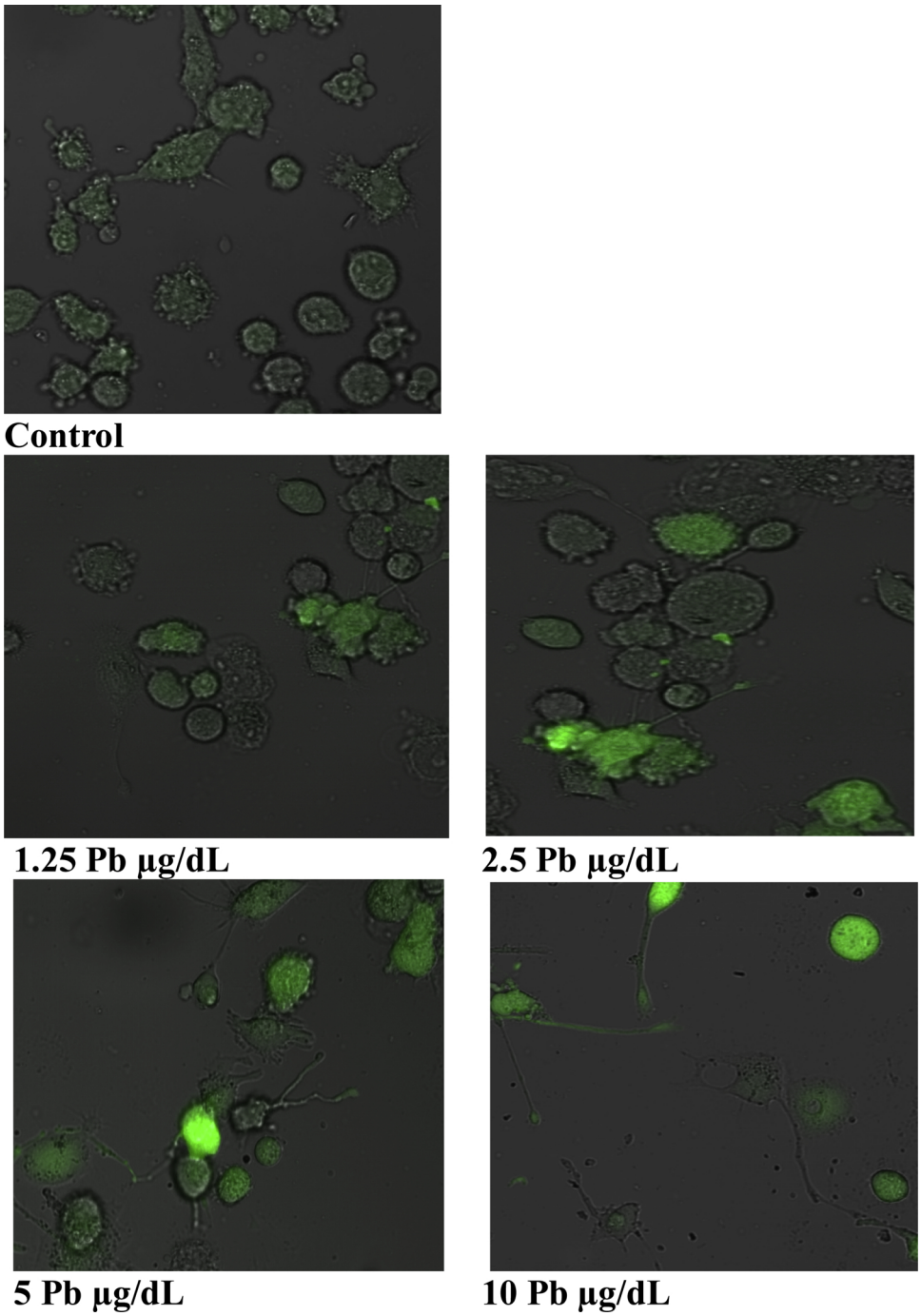

Fig. 4. Imaging of intracellular generation of reactive oxygen species (ROS) by fluorescence microscopy in macrophages cultured with lead.

PMA–activated macrophages of the THP-1 cell line were cultured with Pb at concentrations of 1.25 µg/dL and 2.5 µg/dL (concentrations recorded in the blood of people environmentally exposed to Pb in non-polluted areas), 5 µg/dL (permissible concentration of Pb in children and pregnant women), and 10 µg/dL (permissible concentration of Pb in the blood in adults). As a control cells were incubated in RPMI medium with 10% FBS. The intracellular generation of ROS was visualized by fluorescent marker 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Cells were loaded with 5 µM DCFH-DA. After incubation for 15 min, the cells were washed with culture medium at room temperature and the preparations were examined under a confocal microscope. When DCFH-DA is oxidated to DCF by hydrogen peroxide within the cell, it becomes fluorescent (excitation at 495 nm, emission at 525 nm). Increased ROS level in Pb-treated macrophages vs control cells was visible.

Experiments were repeated six times with similar results, thus pictures are representative fields.

response element (ARE) of nuclear DNA through the leucine zipper motif. Nrf2 is responsible for the induction of xenobiotic metabolizing enzyme genes (XMEs), one of which is NQO1 (NAD(P) H: guinone oxidoreductase) [64]. Cheng et al. [65] in hepatocytes co-exposed to LPS + Pb showed increased expression of tumor necrosis factor α (TNF-α) due to the activation of the protein kinase C (PKC) and p42/44 MAPK signaling pathway in the examined cells. Cheng et al. [66] also demonstrated that Pb stimulates PKC to activate p42/44 MAPK, which leads to increased expression of TNF-α in glioblastoma cells. Chang et al. [67] conducted a study in which they demonstrated that in cultures of smooth muscle cells of blood vessels, Pb induces inflammatory mediators (such as prostaglandins) by activating transcription of COX-2 encoding genes. The *COX-2* gene promoter has several potential binding sites for transcription factors such as: cAMP response element (CRE), SP1 (Stimulatory protein 1), NF-kB, NFAT / NF-IL6 (Nuclear Factor of Activated T-cells / the Nuclear Factor Interleukin-6) and the TATA cassette, and therefore many potential sites for Pb action. It was also found that in the brain of exposed rats, Pb raises the mRNA of the early response genes *fos* and *jun* [68] and CREB protein (cAMP Responsive Element Binding protein) which is responsible for the activation of multiple genes and signal transduction [69]. For detailed review see [70,71].

In the available literature, no direct evidence has been found that Pb may affect the sterol-regulatory element binding protein (SREB), which is responsible for regulation of the expression of tested desaturases. SREBP-1c activates the transcription of *SCD*, *FADS2* and probably *FADS1* genes. The SRE-2 sequence, CAGCAG, is conserved in both *SCD* and *FADS2* promoters. Also, SREBP-lc mediates the suppression of desaturase expression by PUFAs [72–74]. However, based on the previously presented studies, one can assume that Pb may affect this factor

Table 3
Effect of Pb on intracellular ROS synthesis in macrophages obtained from the THP-1 monocytic cell line.

Experimental Conditions	DFC fluorescence intensity[#]	% increase *vs* control
Control	60.45 ± 1.50	
1.25 Pb μg/dL	69.52 ± 2.15	+15%[*]
2.5 Pb μg/dL	71.33 ± 1.54	+18%[*]
5.0 Pb μg/dL	73.14 ± 3.60	+21%[*]
10.0 Pb μg/dL	90.07 ± 2.35	+49%[*]

Data represent the means ± SD for 6 independent experiments each analysed in triplicate.
Cells were incubated with 5 μM DCFH-DA and the number of cells exhibiting increased fluorescence of oxidized DCF was measured by microplate reader. The intensity of fluorescence was normalized to protein levels, measured by Bradford assay.
[*] $p < 0.005$, significant difference vs control (Wilcoxon test).
[#] Normalized to total protein levels.

Table 4
Malondialdehyde (MDA) concentration in macrophages obtained from the THP-1 monocytic cell line.

Experimental Conditions	MDA (nmol/mg protein)	% increase *vs* control
Control	5.12 ± 0.95	
1.25 Pb μg/dL	5.38 ± 0.12	+5%*
2.5 Pb μg/dL	5.53 ± 0.11	+8%*
5.0 Pb μg/dL	7.76 ± 0.01	+12.5%[*]
10.0 Pb μg/dL	6.20 ± 0.15	+21%[*]

Data represent the means ± SD for 6 independent experiments each analysed in triplicate.
[**]$p < 0.001$ for the significance of difference vs control (Mann-Whitney test).

of transcriptional regulation and cause the decrease in desaturase expression observed in our study. However, this requires further detailed research.

The initial increase in *FADS1* expression observed at the lowest Pb concentration is difficult to explain. Available data suggest that the induction of *FADS1* desaturase may be caused by altered concentrations of unsaturated fatty acids, which are the regulatory factors for SREB. This hypothesis, however, requires to carry out further detailed studies.

The effect of Pb at concentrations of 2.5 μg/dL and 5 μg/dL on *FADS1* and *FADS2* expression may contribute to the development of atherosclerosis due to the increased activity of the entire C20:4n6 biosynthesis pathway from C18:2n6 in macrophages [75,76]. However, at a concentration of 10 μg/dL, Pb reduced the expression of discussed desaturases and also significantly reduced the concentration of C18:3n6. Moreover, the pro-atherosclerotic effects of Pb are further influenced by its participation in lipid oxidation and in the initiation and propagation of the inflammatory response. The increase in C20:4n6 synthesis at 2.5 μg/dL and 5 μg/dL Pb may also increase the production of leukotrienes and PGE_2, molecules also derived from lipids and involved in the development of atherosclerosis [77–80].

In connection with the induction of changes in metabolism and modification of lipids, and the impact on the vascular endothelium, Pb may constitute a considerable atherogenic factor [3,4]. A positive correlation between the concentration of Pb and total cholesterol, LDL, and triglyceride levels, and a simultaneous negative correlation between the concentration of Pb and HDL demonstrated in epidemiological and experimental studies, may be evidence of the mechanisms of atherogenic action of Pb [81]. Elevated LDL promotes the accumulation of lipoprotein under the vascular endothelium and, therefore the formation of atherosclerotic plaque as a result of modifications such as the oxidation of stored lipoproteins. Just as LDL cholesterol has long been known to have an atherogenic effect, HDL has the opposite effect, with its high levels having a protective function against atherosclerosis. This is due to the role of HDL in reverse cholesterol transport to the liver, as well as its ability to prevent LDL oxidation, modulate the endothelial function by stimulating the production of nitric oxide, and anti-atherogenic and anticoagulant action. Therefore, the previously described reduction in HDL levels in the blood serum of individuals or animals exposed to Pb indicates that the exposure to heavy metals including Pb may contribute to the development of atherosclerotic plaque.

In addition, the atherogenic effect of Pb is indicated by Pb-induced hypertriglyceridemia. Triacylglycerols, similar to LDL, also have an atherogenic effect, a result of their participation in the composition of chylomicrons and low density lipoproteins which have a cytotoxic effect involving the formation of cells similar to foam cells. However, regardless of the exact mechanism of Pb influence on lipid metabolism (Pb had no effect on the activity of lecithin-cholesterol acyltransferase), a reduction in HDL cholesterol indicates a potential reduction in the protective anti-atherogenic action of HDL, while the increased concentration of triacylglycerols, increases the risk of the emergence and development of atherosclerosis [81].

As a result of oxidative stress, peroxidation of lipids being the components of the cell membranes occurs, and the main product of this process is MDA having strong mutagenic properties. The research results show an increase in lipid peroxide products (including MDA) in conditions of increased ROS synthesis, which may trigger inflammatory reactions (for review see [82]). Lipid peroxide products can induce the expression of cyclooxygenase (COX-2) in macrophages, there is therefore a relationship between oxidative modification of LDL and activation of the inflammatory potential of macrophages [83,84). In the present study we also found an increase in ROS and MDA concentrations in macrophages cultured with Pb at concentrations corresponding to blood Pb levels detected in people environmentally exposed to this metal.

Lipid peroxidation has been found to be involved in numerous pathological conditions such as inflammation, atherosclerosis, diabetes, ageing, neurodegenerative diseases and cancer [85]. The process of lipid peroxidation is characterized by ROS attack of lipids containing carboncarbon double bonds especially PUFAs and its end-product MDA being one of important biomarkers for oxidative stress [www.sciencedirect.com/topics/agricultural-and-biological-sciences/lipid-peroxidation]. Oxidation of phospholipids containing PUFAs present in plasma lipoproteins results in formation of reactive lipid aldehydes and oxidized phospholipids that convert these lipoproteins to atherogenic particles [86].

Lead is a factor with a widely proven potential prooxidative and pro-inflammatory effect (for review see [70]. A number of studies have suggested involvement of Pb in lipid metabolism and peroxidation [57,87–95].

Soni et al. [96] examined lipid peroxidation (determined by MDA production) in human red blood cells exposed to different concentrations of lead acetate (0.01 mM, 0.1 mM and 1 mM). The authors demonstrated significantly increased lipid peroxidation with increasing concentrations of lead acetate [96]. Kasperczyk et al. [88] examined in their study lipid peroxidation in erythrocytes of Polish workers occupationally exposed to Pb over a long time period. MDA erythrocyte concentration in high Pb exposure group (having blood Pb concentration above 40 μg/dL) increased significantly by 91% as compared to control and by 51% as compared to low Pb exposure group (having blood Pb concentration between 25 μg/dL and 40 μg/dL). Similar results were obtained by Kasperczyk et al. [90] in their next study. Another study by Kasperczyk et al. [90] investigated the effect of occupational Pb exposure on lipid peroxidation. Compared with controls, the level of MDA increased significantly by 13% in low exposure group (with mean blood Pb concentration below 35 μg/dL), by 36% in medium exposure group (with mean blood Pb concentration from 35 μg/dL to 45 μg/dL), and by 41% in high exposure group (with mean

blood Pb concentration above 45 μg/dL). The effect of Pb on lipid peroxidation in human erythrocytes was also examined by Shafiq-ur-Rehnan [87]. MDA concentration increased in a dose-dependent manner with increasing Pb exposure: significantly increased concentration of MDA by 47%, 119% and 177% was found as a result of increased Pb exposure, i.e. 0.5, 2.5 and 5μM, respectively [87]. Chen et al. [97] investigated the effects of Pb on lipid peroxidation in HepG2 cells. HepG2 cells were incubated with various concentrations of Pb for 24 h and lipid peroxidation level was determined by the production rate of thiobarbituric acid reactive substances (TBARS) and was expressed as MDA equivalents. The lowest tested Pb concentration (0.01 μM) had no effect on TBARS formation, however, Pb concentration higher than 0.1 μM resulted in significantly increased TBARS formation in a concentration-dependent manner [97]. Sandhir and Gill [95] examined the effect of Pb on lipid peroxidation in liver of rats. Rats were given lead acetate at a dose of 50 mg/ kg body weight, intragastrically for a period of 8 weeks. Lipid peroxidation (measured as nmol MDA/mg protein) was significantly higher in the liver of lead-treated rats as compared to control [95]. Abd Allah and Badary [94] investigated the effect of Pb given to rats at a dose of 10 mg/kg intraperitoneally for five consecutive days/week for 4 weeks, on lipid peroxidation. Hepatic total peroxide (a biomarker for lipid peroxidation) was significantly higher in Pb-exposed rats compared to control. However, Quinlan et al. pointed to the fact that Pb ions alone did not induce any peroxidation and Pb accelerated lipid peroxidation stimulated by Fe^{2+} ions [93].

4.1. Conclusion

Environmental Pb exposure might be a risk factor resulting in alterations in FAs levels, oxidative stress and increased MDA concentration in macrophages, which might lead to the formation of foam cells and to inflammatory reactions.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Funding

This study was supported by the statutory budget of the Department of Biochemistry and Medical Chemistry Pomeranian Medical University in Szczecin, Poland.

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