

## OXIDATIVE INDICES CORRELATE WITH DYSLIPIDEMIA AND PRO-INFLAMMATORY CYTOKINE LEVELS IN FLUORIDE-EXPOSED RATS

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The aim of this study was to establish the effects of fluoride on lipid metabolism and attendant inflammatory response by exposing rats to 50 mg L<sup>-1</sup> and 100 mg L<sup>-1</sup> of fluoride through drinking water for seven weeks. Both concentrations led to hypercholesterolemia while the 100 mg L<sup>-1</sup> concentration induced hypertriglyceridaemia. High density lipoprotein (HDL) cholesterol levels dropped in the exposed rats while interleukin 2 (IL-2) increased more than 1.5-fold ( $p < 0.05$ ) and IL-6 and plasma TNF- $\alpha$  more than 2.5 fold ( $p < 0.05$ ). Fluoride-exposed rats also had significantly higher levels of liver malondialdehyde (MDA) and plasma lipid hydroperoxide (LOOH) but lower plasma paraoxonase (PON1) activity. Oxidative stress indices correlated with pro-inflammatory cytokines and plasma cholesterol. In contrast, pro-inflammatory cytokines inversely correlated with plasma triglyceride, HDL cholesterol and PON1. Our results suggest that the association between fluoride exposure with cardiovascular diseases may be related to its ability to disturb lipid homeostasis, and trigger pro-inflammatory cytokines and oxidative stress.

**KEY WORDS:** *cardiovascular disorders, IL-2, IL-6, paraoxonase, plasma lipid hydroperoxide, TNF- $\alpha$*

Fluorine and fluoride compounds are constituents of minerals in rocks and soils, and the main sources of fluoride exposure for humans include foodstuffs, fluoride supplements, fluoride dentifrices, and water contaminated with high concentrations of fluoride compounds from geological sources (1, 2).

Fluoride compounds are being utilised in the life-science industry, crops, pharmaceuticals, hygiene, cosmetics, and domestic commodities. Their production has been increasing steadily over the years (3). Excessive chronic fluoride intake results in fluorosis, characterised by a vast array of symptoms and pathological changes such as dental mottling, crippling

deformities, osteoporosis, and osteosclerosis (4,5). Endemic fluorosis has now become a global concern (6).

Reports on the effects of fluoride poisoning on lipid metabolism are conflicting. Kanbur et al. (7) reported a lowering in plasma cholesterol and triglyceride levels, whereas Czerny et al. (8) reported increase. Some have associated fluoride poisoning with impaired myocardial cell function (9), while others (10) have demonstrated an association between markers of subclinical inflammation and cardiovascular disease. Cytokines have also been implicated in various pathological conditions associated with the

disruption of lipid metabolism such as progression of atherosclerosis and increased risk of heart attack and stroke (11).

Our intention was to investigate the effects of subchronic exposure to fluoride through drinking water on the lipid metabolism and markers of inflammatory response in rats and try to shed some light on the association between disturbances in lipid metabolism and cytokine activity, as we feel that little is still known about them and the underlying mechanisms.

## MATERIALS AND METHODS

### *Materials*

Sodium fluoride, paraoxon, triphenylphosphine (TPP), butylhydroxytoluene (BHT), and xylenol orange were produced by Sigma-Aldrich (Munich, Germany), sodium dodecyl sulphate (SDS), trichloroacetic acid (TCA) and thiobarbituric acid (TBA) by Qualigens Fine Chemicals (Mumbai, India), the kits for the detection of total cholesterol, triglyceride, and phospholipids by Chemelex (Barcelona, Spain), and the ELISA kits by RayBiotech Inc. (Norcross, GA, USA).

### *Animals and treatment*

For the experiment we used 24 male Wistar rats [bred at the Animal House of the Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Nigeria] aged between 9 and 10 weeks and weighing 120 g in average. The rats were housed in clean plastic cages and maintained on a standard pellet diet and distilled water *ad libitum*. After they were allowed to acclimatise for two weeks, their body weights were recorded, and the rats were then randomly distributed in three groups of eight animals. Group one served as control, while the groups two and three were exposed to subchronic concentrations of 50 mg L<sup>-1</sup> and 100 mg L<sup>-1</sup> fluoride in the form of sodium fluoride through drinking water for 7 weeks. Water exposure mimicked epidemiological exposure and the concentrations were chosen based on studies of fluoride-induced toxicity (12-15).

At the end of the seven-week exposure, the rats were fasted overnight and their body weights recorded again. Blood was collected into heparinised tubes by cardiac puncture under light diethyl ether anaesthesia and the samples centrifuged at 3,000g for 10 min to

obtain plasma. The liver was removed and homogenised in ice-cold KCl (150 mmol L<sup>-1</sup>). The obtained 10 % homogenate was then stored at -20 °C until biochemical analysis. The LAUTECH Department of Biochemistry guidelines for the care and use of laboratory animals were followed throughout the experiment.

### *Determination of plasma lipids*

Plasma concentrations of total cholesterol, triglyceride, and phospholipids were determined with commercial kits (Chemelex®, Barcelona, Spain). HDL cholesterol was analysed with the commercial kit for total cholesterol after very low density lipoproteins (VLDL) and LDL were precipitated with heparin-MnCl<sub>2</sub> solution as described by Gidez et al. (16). LDL cholesterol was determined as described by Friedewald et al. (17).

### *Determination of thiobarbituric acid-reactive substances (TBARS)*

Lipid peroxidation (LPO) was determined in liver homogenate using the method of Ohkawa et al. (18). Briefly, to the reaction mixture consisting of 0.2 mL of 8 % sodium dodecyl sulphate (SDS), 1.5 mL of 20 % acetic acid, and 0.6 mL of distilled water we added 0.2 mL of tissue homogenate. Reaction was initiated by adding 1.5 mL of 1 % thiobarbituric acid (TBA) and terminated by adding 10 % trichloroacetic acid (TCA). It was then centrifuged and absorbance was read at 532 nm with a Genesys 10S UV-VIS spectrophotometer (THERMO SCIENTIFIC, Madison, WI, U.S.A.). LPO was expressed in nanomoles of MDA formed per milligram of tissue using the molar extinction coefficient of 1.56x10<sup>5</sup> L mol<sup>-1</sup> cm<sup>-1</sup>.

### *Determination of plasma lipid hydroperoxides*

Lipid hydroperoxides (LOOH) in plasma were determined using the method of Naurooz-Zadeh et al. (19). Samples of plasma (90 µL) were mixed with either 10 µL of 10 mmol L<sup>-1</sup> triphenylphosphine (TPP) in methanol or with 10 µL of methanol and incubated at room temperature for 30 min. Then, 900 µL of FOX2 reagent (250 µmol L<sup>-1</sup> ammonium ferrous sulphate, 100 µmol L<sup>-1</sup> xylenol orange, 25 mmol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> and 4 mmol L<sup>-1</sup> BHT in 90 % methanol) was added, and the sample was incubated for another 30 min. The mixture was then centrifuged at 12,000g for 10 min to remove flocculated material, and the absorbance was read with the same Genesys

spectrophotometer at 560 nm. The absorbance of the sample with TPP was subtracted from the sample without TPP and hydroperoxide concentration calculated from the standard curve prepared using different concentrations of  $H_2O_2$  ( $1 \mu\text{mol L}^{-1}$  to  $20 \mu\text{mol L}^{-1}$ ).

#### Determination of plasma paraoxonase activity

Paraoxonase activity of PON1 was determined using paraoxon (O,O-diethyl-o-p-nitrophenylphosphate) as the substrate. We measured increase in absorbance at 405 nm due to the formation of 4-nitrophenol following the hydrolysis of paraoxon as described by Furlong and Richter (20). Plasma (20  $\mu\text{L}$ ) was added to 760  $\mu\text{L}$  of the assay buffer containing  $0.132 \text{ mol L}^{-1}$  Tris-HCl, pH 8.5, and  $1.32 \text{ mmol L}^{-1}$   $\text{CaCl}_2$ . The substrate, paraoxon in  $50 \text{ mmol L}^{-1}$  Tris-HCl (200  $\mu\text{L}$ ), was added to initiate the reaction. The change in absorbance was continuously monitored on the Genesys 10S UV-VIS spectrophotometer for 3 min. Molar extinction coefficient of  $18,050 \text{ L mol}^{-1} \text{ cm}^{-1}$  was used to calculate enzyme activity. One unit of paraoxonase activity was defined as enzyme quantity that disintegrated one micromole of paraoxon substrate in 1 min at  $25^\circ\text{C}$ .

#### Determination of plasma interleukins 2 and 6 and tumour necrosis factor alpha

Plasma concentrations of interleukin 2 (IL-2), 6 (IL-6), and tumour necrosis factor-alpha (TNF- $\alpha$ ) were determined using commercial rat ELISA kits (RayBiotech, Inc., Norcross, GA, USA), following the instructions in respective kit manuals. The absorbance was monitored on a Hawksley HA-1600 microplate reader (Hawksley, London, U.K.).

#### Statistical analysis

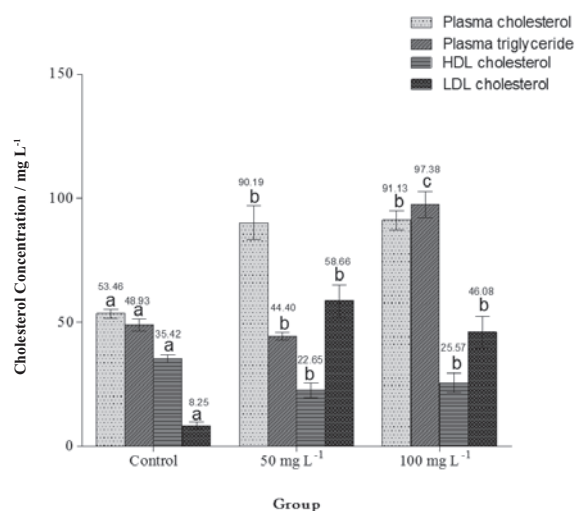
Results, expressed as mean  $\pm$  SD were analysed using one-way analysis of variance (ANOVA) and Tukey's test. The power of  $<0.05$  was considered significant. Associations between the parameters were tested using Pearson's correlation coefficient. All data analyses were run using the Graph Pad Prism version 5.0 statistical package (GraphPad Software, Inc., La Jolla, CA, U.S.A.).

## RESULTS

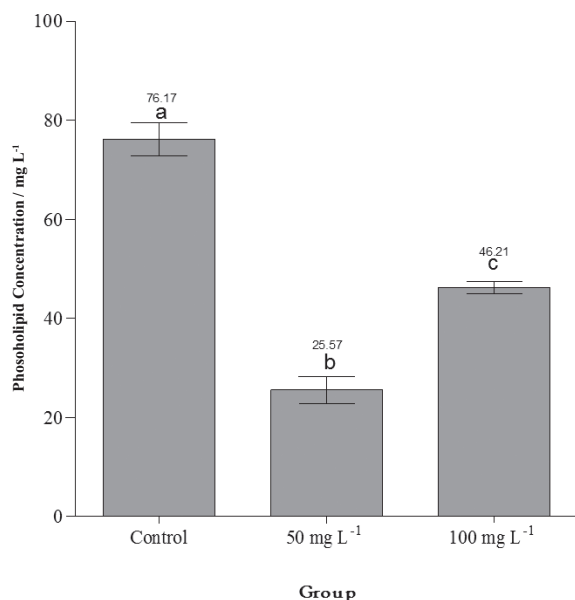
Rat exposure to fluoride through water significantly increased total plasma cholesterol and LDL cholesterol

(Figure 1). In contrast, HDL cholesterol significantly dropped. Interestingly, triglyceride concentrations responded differently to fluoride concentrations used; the lower concentration lowered plasma triglycerides whereas the higher increased them.

Another interesting observation is that plasma phospholipids (Figure 2) dropped significantly more in rats treated with fluoride in the lower than in the higher concentration.

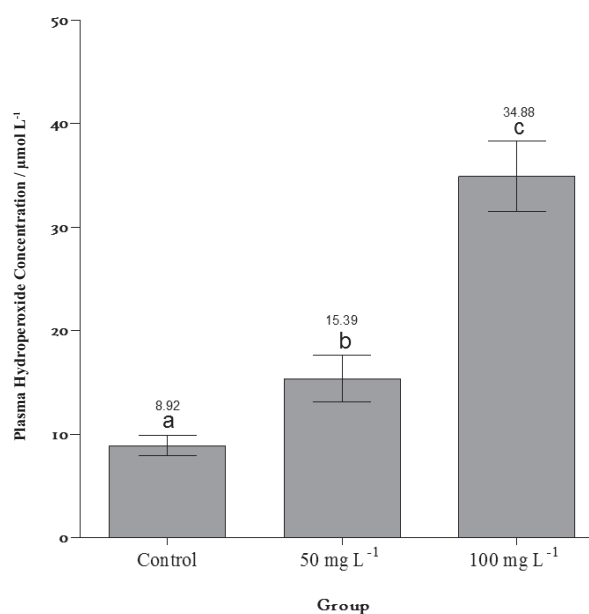


**Figure 1** Plasma lipids and lipoproteins in rats exposed to fluoride vs. controls. Different bar letters denote significant difference between the groups ( $p < 0.05$ ).



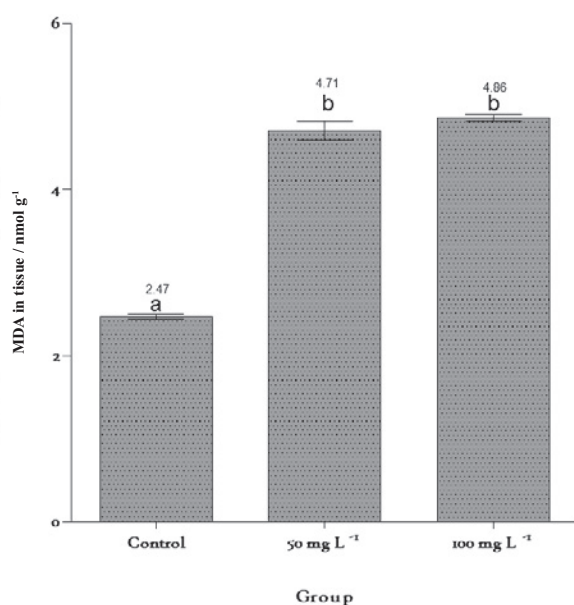
**Figure 2** Plasma phospholipid concentrations in rats exposed to fluoride vs. controls. Different bar letters denote significant difference between the groups ( $p < 0.05$ ).

Plasma hydroperoxides significantly increased in the concentration-dependent manner (Figure 3) whereas liver MDA levels showed a similar increase



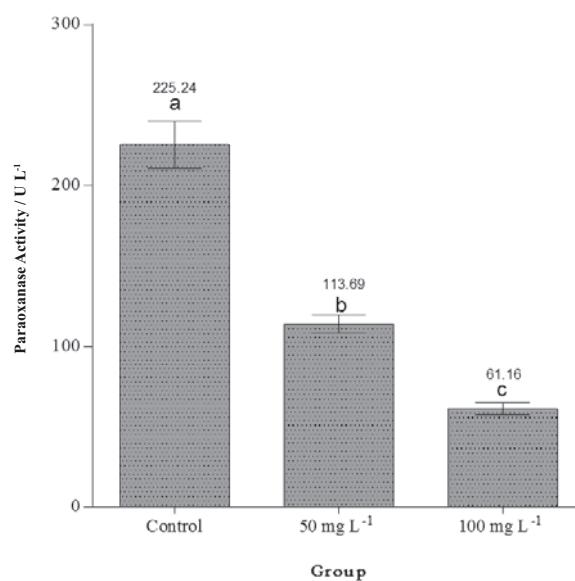
**Figure 3** Plasma lipid hydroperoxide concentrations in rats exposed to fluoride vs. controls. Different bar letters denote significant difference between the groups ( $p < 0.05$ ).

in both treated groups (Figure 4). Plasma paraoxonase, in turn, showed a significant, concentration-dependent drop (Figure 5).

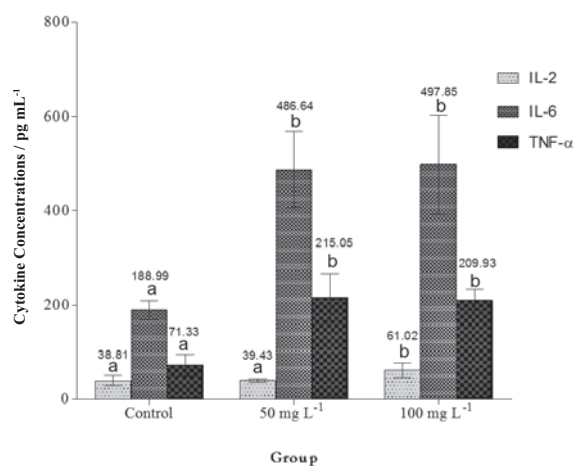


**Figure 4** Liver MDA levels in rats exposed to fluoride vs. controls. Different bar letters denote significant difference between groups ( $p < 0.05$ ).

IL-2 increased significantly only with the higher fluoride concentration, whereas IL-6 and TNF- $\alpha$  showed a similar behaviour with either concentration (Figure 6).



**Figure 5** Plasma paraoxonase activity in rats exposed to fluoride vs. controls. Different bar letters denote significant difference between the groups ( $p < 0.05$ ).



**Figure 6** Plasma IL-2, IL-6, and TNF- $\alpha$  levels in rats exposed to fluoride vs. controls. Different bar letters denote significant difference between the groups ( $p < 0.05$ ).

Associations between all parameters turned out to be significant (Table 1). HDL-cholesterol levels negatively correlated with the pro-inflammatory cytokines, IL-6, and TNF- $\alpha$  levels, while positive associations were observed between plasma triglyceride and the cytokines, IL-2, and IL-6. Strong positive correlations were found between the two oxidative stress parameters, liver MDA and plasma LOOH, on the one hand and the cytokines on the other. Plasma cholesterol also showed positive association with the cytokines. Plasma PON1 activity negatively correlated with IL-2, IL-6, TNF- $\alpha$ , liver MDA, plasma LOOH, and HDL-cholesterol.

**Table 1** Strength of association between oxidative stress indices, pro-inflammatory cytokines, and lipid parameters in rats exposed to fluoride

Parameter	Correlation coefficient / <i>r</i>	<i>p</i> -value
HDL cholesterol vs. liver MDA	-0.865	<0.001
HDL cholesterol vs. IL-6	-0.873	<0.001
HDL cholesterol vs. TNF- $\alpha$	-0.861	<0.001
Liver MDA vs. IL-2	0.544	0.011
Liver MDA vs. IL-6	0.981	<0.001
Liver MDA vs. plasma cholesterol	0.971	<0.001
Liver MDA vs. plasma phospholipids	-0.882	<0.001
Liver MDA vs. plasma PON	-0.957	<0.001
Liver MDA vs. TNF- $\alpha$	0.968	<0.001
Total plasma cholesterol vs. IL-2	0.496	0.022
Total plasma cholesterol vs. IL-6	0.981	<0.001
Total plasma cholesterol vs. plasma LOOH	0.658	0.001
Total plasma cholesterol vs. TNF- $\alpha$	0.961	<0.001
Plasma LOOH vs. HDL cholesterol	-0.496	0.022
Plasma LOOH vs. Liver MDA	0.716	<0.001
Plasma LOOH vs. IL-2	0.899	<0.001
Plasma LOOH vs. IL-6	0.672	0.001
Plasma LOOH vs. TNF- $\alpha$	0.643	0.002
Plasma PON vs. HDL cholesterol	0.778	<0.001
Plasma PON vs. IL-2	-0.708	<0.001
Plasma PON vs. IL-6	-0.936	<0.001
Plasma PON vs. plasma LOOH	-0.855	<0.001
Plasma PON vs. TNF- $\alpha$	-0.902	<0.001
Plasma triglyceride vs. IL-2	0.910	<0.001
Plasma triglyceride vs. IL-6	0.436	0.048
Plasma triglyceride vs. plasma LOOH	0.924	<0.001

IL-6 = interleukin-6; TNF- $\alpha$  = tumour necrosis factor- $\alpha$ ; IL-2 = interleukin-2; LOOH = lipid hydroperoxides; MDA = malonyldialdehyde; PON = paraoxonase

## DISCUSSION

Studies on the effects of fluoride on lipid metabolism are few and contradictory. Unlike Kanbur et al. (7), who reported a drop in both plasma cholesterol and triglyceride, fluoride exposure of rats in our study induced dyslipidemia, which is a major risk factor for the development of cardiovascular diseases (21). This is quite significant, as it has recently been reported that increased fluoride uptake by coronary arteries may increase cardiovascular risk (22).

We observed hypercholesterolemia and hypertriglyceridemia in the rats, which is in agreement with the report of Czerny et al. (8). The increase in plasma cholesterol may result from enhanced hepatic cholesterologenesis and/or reduced cholesterol clearance from the system, an effect that has been suggested for other metals (23, 24). Furthermore, lower clearance of LDL precursor particles could result in a persistence

of cholesterol, resulting in hypercholesterolemia (25). Consistent with this is the elevated LDL-cholesterol fraction observed in this study. The hypertriglyceridemic effect of fluoride may be related to lower hydrolysis of triglyceride (TG), attributable to fluoride-induced reduction in the activity of lipoprotein lipase (LPL) (26). Other possible contributors to hypertriglyceridemia include stimulation of hepatic synthesis of very low density lipoprotein (VLDL), which is a consequence of increased hepatic fatty acid (FA) synthesis, activation of adipose tissue lipolysis, and/or suppression of FA oxidation and ketogenesis (27). HDL-C inversely correlates with the risk of coronary heart disease (CHD) and its reduction by fluoride reflects changes in HDL metabolism that could lead to defective reverse cholesterol transport, eventually promoting atherogenesis. The dyslipidemic effect of fluoride reflected in hypercholesterolemia, hypertriglyceridemia, increased levels of triglyceride-

rich lipoproteins, and HDL depletion may well contribute to its atherogenic tendencies, as dyslipidemia has been well implicated in cardiovascular disease (21).

Generation of reactive oxygen species (ROS) and enhanced lipid peroxidation are important pathological mediators in many disorders (28). In our study, fluoride-exposed rats exhibited a high level of lipid peroxidation (through 90 % and 98 % increase in hepatic MDA at low and high doses, respectively) and four times higher plasma LOOH concentrations at the higher dose. The increased oxidative stress in the animals may be the consequence of depletion of the antioxidant scavenger system (29, 30). Our results are in accordance with previous studies showing close association between chronic fluoride toxicity and increased oxidative stress (31, 32).

However, the effect of fluoride toxicity on paraoxonase activity has drawn our attention even more. This is because paraoxonase activity has been implicated in a number of pathological conditions, including coronary artery disease (33), but, to our knowledge, no study before ours has investigated the effects of fluoride on this enzyme.

Paraoxonase, a high-density lipoprotein (HDL)-associated enzyme has been demonstrated to protect serum lipids from oxidative modification, to reduce macrophage foam cell formation, and to attenuate atherosclerosis development (34). Lowered PON1 in animals may result in greater oxidative modification of HDL-C and LDL-C and eventually to plaque formation and atherosclerosis. Its inhibition, along with dyslipidemia, suggest possible mechanisms of fluoride toxicity in the development of cardiovascular diseases.

Inflammation-related molecules, such as IL-2, IL-6, and TNF- $\alpha$  are considered good predictors of CHD, even in people with no conventional risk factors (35). Studies reviewed by Berg and Scherer (36) have demonstrated that increase in the levels of inflammatory markers such as, IL-6 and TNF- $\alpha$ , may translate to accelerated atherogenesis. Our data have confirmed the increasing effect of fluoride on IL-2, IL-6, and TNF- $\alpha$  levels in rats. In contrast, a recent human *ex vivo* study revealed lower expression of IL-2 in peripheral blood mononuclear cell obtained from humans exposed to fluoride (37, 38). In our study, however, IL-2 increase following fluoride intoxication points to immunological response of T cells, as evidenced elsewhere (39). Normally, the antigen binding to the T cell receptor stimulates the secretion

of IL-2 and the expression of IL-2 receptors. The IL-2/IL-2R interaction then stimulates the growth, differentiation, and survival of antigen-selected cytotoxic T cells by activating the expression of specific genes (40, 41). Since there has not been any report describing fluoride antigenicity, and our study does not evidence any infection, the increase recorded in plasma IL-2 concentration points to an indirect response, whose aim is to contain the cause of systemic and/or peripheral inflammation, as reported earlier (38, 42). The marked, greater than twofold increase in plasma IL-6 in rats exposed to fluoride - even though not dose-dependent - suggests that IL-6 could have acted as a pro-inflammatory cytokine and supports the IL-2 finding, strengthening further the hypothesis about immunological response triggered by systemic and/or peripheral inflammation (43). TNF- $\alpha$  is a cytokine involved in systemic inflammation, and its overexpression has been reported as the underlining factor in chronic inflammatory response (44). High blood levels of TNF- $\alpha$  have been reported to increase the risk of heart disease by 79 % and that of heart failure by 121 % (45).

Increased production of reactive oxygen species (ROS) has been demonstrated to stimulate the expression and synthesis of inflammatory cytokines (46). Our results have confirmed that the levels of the oxidative stress markers, hepatic MDA, and plasma LOOH correlate positively with pro-inflammatory cytokines. On the other hand, PON1 inversely correlated with all the pro-inflammatory cytokines, confirming earlier reports that ROS may mediate lipid peroxidation and production of cytokines in fluoride exposure (47). Considering the contributions of both dyslipidemia and inflammation to CHD (48), it is interesting to note the atherogenic potential of lipids and their association with pro-inflammatory cytokines established in our study. The positive correlation between plasma triglycerides and the pro-inflammatory cytokines, IL-2 and IL-6, on the one hand, and between plasma cholesterol and IL-2, IL-6, and TNF- $\alpha$  on the other may indicate an interaction between dyslipidemia and inflammation in the pathogenesis of fluoride-induced atherosclerosis. Although the effect of fluoride poisoning is evident, it will take more research to shed light on the mechanism by which ROS induces their release, that is, if their production is indeed an indirect response to systemic inflammation. In addition, further research should focus on the role of PON1 in fluoride poisoning, as it may help to understand the involvement of the metal in lipid dysfunctional states.

To sum up, our findings have confirmed earlier findings that fluoride exposure disturbs lipid metabolism (7, 8, 49), but unlike earlier studies, it has also demonstrated the association between dyslipidemia and pro-inflammatory cytokines.

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### **Sažetak**

#### **INDIKATORI OKSIDACIJE KORELIRAJU S DISLIPIDEMIJOM I RAZINAMA UPALNIH CITOKINA U ŠTAKORA IZLOŽENIH FLUORIDU**

Svrha je ovog istraživanja bila utvrditi djelovanje fluorida na metabolizam lipida i upalni odgovor tako što smo štakore izložili fluoridu u pitkoj vodi u koncentracijama od 50 mg L<sup>-1</sup> i 100 mg L<sup>-1</sup> sedam tjedana. Obje su koncentracije dovele do hiperkolesterolemije, a viša je koncentracija inducirala i hipertrigliceridemiju. U odnosu na kontrolnu skupinu, u izloženih su se štakora razine lipoproteina visoke gustoće (HDL-a) smanjile, a interleukin 2 (IL-2) se povećao više od 1,5 put ( $p < 0,05$ ), kao i IL-6 i čimbenik tumorske nekroze alfa (TNF- $\alpha$ ) u plazmi, i to više od 2,5 puta ( $p < 0,05$ ). Usto su izloženi štakori iskazali značajno više razine malondialdehida (MDA) u jetrima te lipidnog hidroperoksida (LOOH) u plazmi, odnosno sniženu aktivnost paraoksonaze (PON1) u plazmi. Indikatori oksidacijskog stresa korelirali su s upalnim citokinima i kolesterolom u plazmi, a upalni citokini inverzno su korelirali s trigliceridima, HDL kolesterolom i PON1 u plazmi. Rezultati istraživanja upućuju na to da povezanost između izloženosti fluoridu i bolesti srca i krvožilja možda potječe od toga što fluorid remeti lipidnu homeostazu te pokreće upalne citokine i, posljedično, oksidacijski stres.

**KLJUČNE RIJEČI:** *bolesti srca i krvožilja, IL-2, IL-6, lipidni hidroperoksid, paraoksonaza, TNF- $\alpha$*

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