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Exocyclic DNA adducts in sheep with skeletal fluorosis resident in the proximity to the Portoscuso-Portovesme industrial estate on Sardinia Island, Italy

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Running head: M_1dG adducts in sheep with fluorosis living near non-ferrous metallurgy complexes on Sardinia Island, Italy

Key-words: sheep, skeletal fluorosis, exocyclic DNA adducts, cadmium, lead

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Abstract

The mechanisms by which fluoride produces its toxic effects are still not clear. Therefore, we conducted a cross-sectional study to evaluate the fluoride-induced toxicity on randomly selected sheep with skeletal fluorosis resident near the large non-ferrous metallurgy Portoscuso-Portovesme industrial estate and the Carbonia and Gonnessa towns (control district) in respect to animals from a remote site without industrial settlements, on Sardinia Island, Italy. We measured the prevalence of 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one deoxyguanosine (M_1dG) adducts, a biomarker of oxidative stress and lipid peroxidation, in the soft organs of the study-animals using the ^{32}P-postlabelling assay. Then, we analysed the association between M_1dG adducts and sheep-farm localization. Additionally, cadmium and lead levels were measured in the same matrices by mass-spectrometry. The histopathology analysis showed that the sheep resident near the industrial site and in the control district presented typical manifestations of fluorosis. The statistical analyses using log-normal regression models indicated that there was a significant association between exocyclic DNA adducts and skeletal fluorosis. The Mean Ratios, adjusted for age, of renal and hepatic M_1dG for the sheep with fluorosis were of 5.09, 95% C.I. 1.67-15.53, and 2.04, 95% C.I. 0.91-4.57, $p = 0.009$, and $p = 0.078$, respectively. After stratification for sheep-farm localization, the renal and hepatic levels of M_1dG adducts were significantly higher in the sheep resident nearby the industrial estate as compared to controls. Intermediated amounts of DNA damage were observed in the kidney of the sheep living in the control district. As expected, the levels of cadmium and lead were significantly increased in the sheep with fluorosis as compared to controls. Also, the concentrations of heavy-metals were driven from the distance of the sheep-farms to the industrial site. Our results broaden knowledge about the role of exocyclic DNA adducts in the etiology of skeletal fluorosis. Fluoride generated from the non-ferrous metallurgy complexes may increase the intracellular amounts of oxidative stress and ROS within the soft organs. A continuous attack to DNA may contribute to the general decline of cellular functions, from disturbance of DNA metabolism, triggering cell-cycle arrest and apoptosis, up to inducing necrosis and cellular degenerative changes.

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Introduction

Fluoride is naturally part of the environment, nevertheless excessive exposure to this metal from various anthropogenic sources may cause fluorosis, [1] a progressive degenerative disorder, known to affect predominantly the skeletal systems, teeth and the structure and function of skeletal muscle, brain, and spinal cord. [2] Adverse health effects, including clinical signs of fluorosis, have been associated to environmental exposure to fluoride as well in aluminum smelting and steel foundries, [3-5] as well in the surrounding residential area. [6] The Portoscuso-Portovesme industrial estate, Cagliari province, Sardinia Island, Italy, hosts the largest non-ferrous metallurgy complexes with facilities for processing aluminium and other metals in the Mediterranean area. [7] A number of pollutants may be emitted from aluminum smelting complexes, including gaseous and particulate fluorides, [2, 8] cadmium (Cd), lead (Pb), [9, 10] formaldehyde and phenol, [11] that may cause adverse health effects for workers, [3] as well as people and domestic animals. [6, 12-14]

As in the case of many chronic degenerative diseases, increased production of reactive oxygen species (ROS) and subsequent DNA damage and peroxidation of lipids (LPO) is considered to play a relevant role in the etiology of fluorosis. [15-17] ROS are highly reactive chemicals capable to interact with DNA and lipids, leading to oxidation of deoxyribose and LPO by-products, [18] as well to reduce antioxidant enzyme activities. [19] ROS may cause the generation of malondialdehyde (MDA) or β-hydroxyacrolein, a reactive by-product of LPO, [20-22] together with the production of base-propenals, compounds that are MDA analogs. [23] MDA is an aldehyde capable of interacting with DNA forming 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one deoxyguanosine (M_1dG) adducts. [21, 23] Base-propenal intermediates are also considered main sources of M_1dG. [23] If not repaired, M_1dG may block cell replication and cause base pair and frameshift mutations in repeated sequences. [20, 24] M_1dG production has been associated to the loss of DNA methylation in the Long Interspersed Nuclear Element-1 repeated sequences, and in the promoter region of the inflammatory cytokine *interleukin-6* gene. [25, 26] Hospital based studies have also indicated that high M_1dG levels are associated to cancer development and tumor progression. [27-31]

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In the present study, we evaluated the mechanisms of fluoride-induced toxicity on the soft tissues of randomly selected sheep with skeletal fluorosis living in the proximity to the largest aluminum smelting complexes in Sardinia Island, and in a control district at 14 km away to the industrial site. Our approach consisted in the conduction of a cross-sectional study aimed to compare the prevalence of M_1dG adducts, a biomarker of oxidative stress and LPO, [32-35] in the soft tissues, such as the liver and kidney, [36, 37] of the sheep with fluorosis exposed to different levels of metal-containing emissions generated from the non-ferrous metallurgy complexes as compared to controls from a remote site without industrial settlements. Then, we evaluated the association of exocyclic DNA adducts with the proximity of the sheep-farms to the industrial site to analyze the variations of M_1dG in groups of animals experiencing various degrees of air pollution exposures. The renal and hepatic levels of M_1dG adducts were measured by the ^{32}P-DNA postlabelling technique. Additionally, the levels of Cd and Pb, that were, respectively, classified as human carcinogen and probably carcinogenic to humans by the International Agency for Research on Cancer, [38, 39] were measured in the same matrices using the inductively coupled plasma mass spectrometry technique. [40]

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Results

Histopathology

The results of the histopathology analysis are presented in Figure 1. Our findings showed that various clinical manifestation of skeletal fluorosis were present in the soft tissues of the study-animals living in the area surrounding the industrial estate and in the control district, such as depletion of bone matrix, dark pigmentation, fracture of teeth, and chondrosis of articular cartilage. [4] The anatomo-histopathological observations revealed also diffuse degenerative alterations typical of chronic damage, such as necrosis and cellular degenerative changes. Then, there were severe compromise of cellular membrane and increase of citoplasma size, such as cytoplasmic cloudy swelling and hydropic degeneration. These lesions were present in large renal and hepatic areas, indicating serious state of tissue sufferance. Signs of chronic inflammations were generally present in the soft organs.

Reference standard

There were 5.0 M_1dG adducts ± 0.6 per 10^6 nn in the MDA-treated calf-thymus DNA samples based on the ^{32}P-postlabeling technique. The presence of the M_1dG adduct in the MDA treated DNA was confirmed by MALDI-TOF-MS, as previously reported, [41, 42] and we are using the nomenclature reported by Goda and Marnett for M_1dG. [43] A calibration curve was set up by diluting the reference standard with control DNA and measuring the decreasing amounts of M_1dG adducts, r-squared = 0.99.

M_1dG adducts

To look for DNA adduct formation in the study-animals, we measured the levels of M_1dG adducts using the ^{32}P-postlabeling technique. [18, 34, 44] A characteristic pattern of M_1dG adduct spot was detected in all the chromatographic plates. The presence of M_1dG adducts in the DNA of the study-animals was then confirmed by co-chromatography. Our findings showed that the intensity of M_1dG was stronger in the chromatograms of the sheep with fluorosis as compared to controls from the remote site (Table I). The statistical analyses using log-normal regression models showed that there was a significant association between the generation of exocyclic DNA adducts and fluorosis. The Mean Ratios (MR), adjusted for age, of renal and hepatic M_1dG for the sheep with fluorosis were of 5.09, 95% C.I. 1.67-15.53, and 2.04, 95% C.I.

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0.91-4.57, p = 0.009, and p = 0.078, respectively. After stratification of the sheep-farms for geo-localization, [45] we found that the renal and hepatic levels of M_1dG adducts were significantly higher in the sheep resident nearby the industrial estate as compared to controls. In the case of the kidney, the MR, adjusted for age, of M_1dG adducts were of 4.58, 95% C.I. 1.31-16.00, and 5.90, 95% C.I. 1.57-22.13, for the sheep living nearby the industrial complexes and in the control district, p = 0.022, and p = 0.014, respectively. Intermediated renal DNA damage levels were also observed in the sheep living in the control district. A borderline significant trend was observed, p = 0.091. In the case of the liver, the MR, corrected for age, of M_1dG adducts were of 2.57, 95% C.I. 1.04-6.36, and 1.73, 95% C.I. 0.74-4.06, in the animals resident nearby the industrial complexes and in the control district, p = 0.042, and p = 0.188, respectively. A significant trend was found, p = 0.035.

Heavy-metals

Table II reports the results of the analysis of heavy-metals in the soft tissues of the study-animals according to fluorosis status and sheep-farm localization. As expected, the levels of Cd and Pb were significantly increased in the soft organs of the sheep with fluorosis as compared to controls. Furthermore, the concentrations of heavy-metals were driven from the distance of the sheep-farms to the industrial site. In particular, we observed that the values of Cd were significantly increased, up to more than 50 fold, in the kidney of the sheep resident near the non-ferrous metallurgy estate in respect to controls, with intermediate values in the control district. Indeed, the level of Cd distinguished the area nearby the industrial estate, the control district, and the remote site from each other.

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Discussion

The accumulation of fluorides in the organism is usually a long-term process, and skeletal fluorosis appears only after many years of exposure. In the present study, we addressed the use of randomly selected sheep resident in an area on Sardinia Island considered at high risk of environmental crisis [7] as experimental model to study the etiology of fluorosis. The histopathology analysis showed that majority of the sheep, aged six year old, living nearby the industrial complexes and in the control district have typical manifestations of fluorosis. Then, we asked whether sheep with fluorosis experienced increased levels of M_1dG adducts, a biomarker of oxidative stress and LPO. [32-35] Our most striking finding showed that increased levels of M_1dG adducts were detectable in the soft organs of the sheep with fluorosis in comparison to the controls. This is keeping with previous results, showing increased levels of MDA in rodents with fluorosis. [46] Also, other studies reported relationships between fluoride exposure and oxidative stress and LPO. [36, 37, 47] For an example, increased oxidative stress and alterations of anti-oxidant defence systems were found in the soft organs of rodents treated with fluoride. [36, 48] A number of fluoride-induced pathological changes, including LPO modifications, apoptosis, and necrosis, were found to be associated with oxidative stress and ROS. [49] In the present article, our results showed that fluoride intoxication may increase the intracellular amounts of oxidative stress and ROS within renal cells and hepatocytes, leading to the oxidation of DNA and LPO, which generate biological aldehydes, including MDA and base propenals, causing the generation of M_1dG adducts.

Next result showed that the generation of M_1dG adducts was significantly associated to sheep-farm localization. In particular, the renal levels of M_1dG adducts were significantly increased in the sheep resident near the industrial complexes in respect to controls, with intermediate levels in the control district at 14 km away to the industrial site. Interestingly, adverse renal effects were also detected in people resident in the Portoscuso town, where a significant excess in hospital-based prevalence for renal failure (males and females, SR: 1.90; 90% CI 1.79–2.00) was found. [7] Increased levels of exocyclic DNA adducts in the sheep resident near the large non-ferrous metallurgy complexes and in the control district may reflect the inhalation of metal-containing emission generated from the industrial facilities and transported by dust through the ambient air, as well as the consumption of contaminated drinking water and food. Indeed, the

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present study suggested that the metal-emissions from the industrial estate might be the causative factor of the observed increment of M_1dG adducts in the domestic animals living in the surrounding areas, however, further studies, are necessary to confirm the present finding.

The quality of exposure assessment is a main determinant of the overall quality of toxicological studies, [50] thus, we measured the tissue levels of Cd and Pb generated from the facilities present in the non-ferrous metallurgy complexes. The tissue levels of heavy-metals were significantly increased in the soft tissues of the sheep with fluorosis in respect to controls, indicating that the domestic animals were grazing on contaminated area. An exposure gradient was found with the highest levels in the animals living in the proximity to the industrial site, with intermediate levels in those of the control district, and with the lowest concentrations in the animals coming from the remote site. Furthermore, there were levels of heavy-metals that exceeded the regulatory limits of the European Commission for food components [Commission Regulation 466/2001 of 8 March 2001], setting the maximum renal and hepatic levels of Cd and Pb of 1.0; 0.5 μg/mg and 0.5; 05 μg/mg, respectively. Our measurements provide evidence of exposure to metal-containing industrial emissions in animals resident in the proximity to the Portoscuso-Portovesme industrial estate, in keeping with previous studies. [12-14]

The generation of M_1dG adducts, a biomarker of oxidative stress and LPO, [18, 32-35] is promoted by a secondary reaction, that may be induced by any reactive species capable of induce oxidative stress. Therefore, the heavy-metals emitted from the non-ferrous metallurgy estate [9, 11] may also play a relevant role in the formation of exocyclic DNA adducts. For an example, Cd, is not redox-active, but it may induce increased levels of oxidative stress, possibly through the inhibitory effect on antioxidant enzymes, as well as by the inhibition of DNA-repair systems. [51] Experimental exposures to Cd in drinking water were also associated to increased levels of LPO by-products and decreased antioxidant defence in rodents. [52] Conversely, the toxicity of Pb, another not redox-active metal, can be mainly attributed to its ability of promoting an unbalance in the oxidant/antioxidant status. [53] Heavy-metals may also cause the production of ROS through inflammation processes, [32] especially by the activation of macrophages and neutrophils that release reactive oxidants. [54, 55] An alternative mechanism of DNA damage induction in the study-animals

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exposed to the emission generated from the industrial site may involve the exposures to formaldehyde, [33] that may cause increments of oxidative stress by the induction of enzymes producing ROS, and by the inhibition of scavenger systems. [33, 56] For instance, formaldehyde is a substrate for cytochrome P-450 monooxygenase system II E1 isozyme, and is oxidized by peroxidases, aldehyde and xanthine oxidases with subsequent production of ROS. [57] Our findings are in line with previous studies that analysed the associations of environmental exposures to air pollutants to various type of DNA damage. [34, 35, 58-65] Recently, we evaluated the association between the residential proximity to large oil-refinery and petrochemical complexes and oxidatively damaged DNA. That study showed that increased M_1dG levels were present in a group of representative children living near the industrial estate as compared to rural controls. [35]

In the attempt of evaluating the molecular mechanisms underlying the pathogenesis of fluorosis, we examined the scientific literature evaluating potential interactions between fluoride and heavy-metals. [66-68] For example, fluoride, a powerful oxidizing agent, can directly bind to and inhibit the metal ions - active centers of enzymes. [69] Sawan et al. investigated whether fluoride co-administered with Pb increased the concentrations of heavy-metals in rodents. [66] In this study, fluoride significantly increased the levels of Pb in peripheral blood as well in calcified bone compared to experimental animals exposed to Pb alone. Also, Leng et al. studied the effects of co-treatment of fluoride and Pb. [67] Co-exposure to fluoride and heavy-metals was found to exacerbate dental fluorosis in rodents, suggesting that co-exposure to Pb may alter the severity of enamel fluorosis, possibly by inhibiting enzymes involved in the enamel formation. [68] It is plausible that fluorotic lesions may be worsened in the presence of heavy-metals, capable of disturbing the metabolism of extracellular matrix components, such as Pb and Cd. [68]

The presence of high amounts of M_1dG adducts in the soft organs of sheep with fluorosis supports the involvement of oxidative stress and redox imbalance in the etiology of this disease. In this manner, the disturbance of the normal redox state of the renal cells and hepatocytes may induce a vicious cycle of ROS formation. Excess ROS may exert their detrimental effects on the soft organs via attack on DNA and inner membrane lipids causing increased LPO and high levels of exocyclic DNA adducts. M_1dG adduct is a form of DNA damage that is quite persistent, with a relatively long half-life of 12.5 days. [70] If unrepaired, M_1dG

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adducts may alter cellular homeostasis by inhibiting transcription at DNA damage sites. Persistent DNA damage would also result in strand breaks and direct initiation of cell death. Thus, one could argue that if the continuous production of ROS and exocyclic DNA adducts persists, increases the risk of decline of cellular functions and apoptotic cell-death up to degenerative lesions.

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Methods

Study-animals

Representative study-animals with manifestations of skeletal fluorosis resident in the sheep-farms located in the rural area surrounding the Portoscuso-Portovesme industrial estate were identified and randomly selected by veterinary hygiene inspectors. Only control animals without exposures to known or suspected environmental carcinogens were eligible to the study. In total, 15 sheep, 5.7 ± 2.2 years of age, from 11 flocks were included in the study. There were 12 sheep with fluorosis, 6.2 ± 2.2 years of age, and 3 sheep, 4 ± 0.0 years of age, from a remote site without industrial settlements, used as "control-farm". The remote site was in the Sassari province, Sardinia Island, Italy at more than 200 km away to the non-ferrous metallurgy estate. After geo-localization of the sheep-farms, [45] the study-animals were sub-grouped in : *a*) sheep resident at approximately 4 km, range 2.7-7.6 km, away to the industrial estate; b) sheep living in the control district near the Carbonia and Gonnessa towns at 14 km, range 9.6-19.6 km, away to the industrial site; c) and sheep living in the "control-farm". After categorisation for sheep-farm localisation, there were 5 sheep, 5.4 ± 1.9 years of age, living in the area surrounding the industrial estate; 7 sheep, 6.7 ± 2.4 years of age, resident in the control district; and 3 sheep, 4 ± 0.0 years of age, from the remote site. Animal procedures were performed in accordance with the guidelines of the Helsinki convention for the use of animals. The study was approved by the Institutional Review Board of the University of Sassari, Sassari, Italy.

Sample processing and histopathology

For each animal, after the post mortem examination, tissue samples were collected by veterinary hygiene inspectors at the closer municipal slaughterhouse. All samples came from regions of kidney and liver with macroscopic lesions. Tissue samples of adequate size were collected in double aliquot; a portion was packed in labelled plastic bags, immediately transported in a cooler to the laboratory and stored at -80°C until laboratory analysis, whereas the other portion was fixed in 10% neutral buffered formalin for 24–48 h and embedded in paraffin according to standard methods. Hematoxylin-eosin-stained sections of kidney and liver of the study-animals were examined for clinical signs of fluoride intoxication by a pathologist blinded to the study.

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Reference adduct standard

A reference adduct standard was prepared as follow: calf-thymus DNA was treated with 10 mM MDA (ICN Biomedicals, Irvine, CA, USA), as already reported. [33] Then, MDA treated DNA was diluted with untreated DNA to obtain decreasing levels of the reference adduct standard to generate a calibration curve.

DNA extraction and purification

DNA was extracted and purified using a method that requires digestion with ribonuclease A, ribonuclease T_1 and proteinase K treatment and extraction with saturated phenol, phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1) and ethanol precipitation. [71, 72] DNA concentration was measured spectrophotometrically. Coded DNA was stored at -80°C until laboratory analysis.

Mass-spectrometry

Determination of Cd and Pb in the kidney and liver tissues were assessed by inductively coupled plasma-mass spectrometry (Perkin-Elmer DRC II) after acid mineralization of the stored sample in a microwave oven, as previously reported. [40, 73] Conversely, the presence of DNA adducts in the MDA treated calf-thymus DNA sample was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Voyager DE STR from Applied Biosystems, Framingham, MA), as reported elsewhere. [41, 42]

^{32}P-DNA postlabelling

The levels of M_1dG adducts were measured by the ^{32}P-DNA postlabelling technique. [34] This assay is an highly sensitive chromatographic technique widely used for the analysis of DNA damage caused from a large number of environmental carcinogens, including ROS-generating chemicals. [44, 74-77] Briefly, DNA (2 µg) was hydrolyzed by incubation with micrococcal nuclease (21.45 mU/µl) and spleen phosphodiesterase (6.0 mU/µl) at 37°C for 4.5 h. [74] Hydrolyzed DNA was treated with nuclease P1 (0.1 U/µl) at 37°C for 30 min. The samples were incubated with 25 µCi of carrier-free [γ-^{32}P]ATP (3000 Ci/mM) and polynucleotide kinase T4 (0.75 U/µl) to generate ^{32}P-labeled adducts at 37°C for 30 min, [74] followed by spotting onto

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polyethyleneimine (PEI) cellulose thin layer chromatography (TLC) plates (Macherey-Nagel, Germany). ^{32}P-labeled samples were applied on PEI-cellulose TLC plates (Macherey-Nagel, Germany) and analyzed as previously described. [22] This modification of the ^{32}P-postlabeling technique was developed from our laboratory for the specific detection of M_1dG adducts in DNA [22] by using a low-urea solvent system known to be effective for the detection of low molecular weight and highly polar DNA adducts. ^{32}P-labeled samples were spotted to the origin of chromatograms and developed with 0.35 $MgCl_2$ up to 2.0 cm filter paper wick. Chromatograms were then developed in the opposite direction with 2.1 M lithium formate, 3.75 M urea, pH 3.75, and then run at the right angle to the previous development with 0.24 M sodium phosphate, 2.4 M urea, pH 6.4. Visual detection and quantification of M_1dG adducts and normal nucleotides were performed using storage phosphor imaging with intensifying screens from Molecular Dynamics (Sunnyvale, CA, USA). The intensifying screens were scanned by a Typhoon 9210 (Amersham). The software used for processing the data was ImageQuant (version 5.0) from Molecular Dynamics. After background subtraction, the levels of M_1dG adducts were expressed such as relative adduct labelling (RAL): pixels for adducted nucleotides / pixels for normal nucleotides. The RAL levels were corrected across experiments based on the recovery of the reference standard. Higher specificity of the ^{32}P-labeling assay is obtained when this method is combined with the use of reference standards, [72, 78, 79] thus, co-chromatography on PEI-cellulose TLC plates was employed to confirm the identity of adduct spots observed in the study-animals. In detail, ^{32}P-labeled samples, e.g. study-animal and reference standard samples, were spotted onto the same PEI-cellulose TLC plates and examined by the following solvent system: 2.1 M lithium formate, 3.75 M urea, pH 3.75 (first direction) and 0.24 M sodium phosphate, 2.4 M urea, pH 6.4 (second direction) or 0.24 M sodium phosphate, 2.7 M urea, pH 6.4 (second direction). After storage phosphor imaging with intensifying screens, the chromatographic mobility of the adduct spots of the study-animals was compared with that of the reference standard onto PEI-cellulose TLC plates.

Statistical methods

All statistical analyses were performed on log-transformed data to stabilize the variance and normalize the distribution of heavy-metals and M_1dG adducts. We initially performed a descriptive analysis to explore the levels of M_1dG adducts and Cd, and Pb in the soft tissues of the study-animals according to

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skeletal fluorosis (presence/absence), and sheep-farm localization. Log-normal regression models, including age as predictive variables, were used to analyze the association between fluorosis, sheep-farm localization and the levels of M_1dG adducts in the soft tissues of the study-animals. The regression parameters estimated from the models were interpreted as ratios between the means (MR) of DNA adducts of each level of the categorical variables with respect to the reference level, adjusted by age. The MR was used as a measure of effect. The multivariate analysis was also performed using log-normal regression models to estimate the association between fluorosis, sheep-farm localization, and renal and hepatic Cd and Pb levels, adjusting for the age (continuous). All statistical tests were two-sided and $p<0.05$ was considered to be statistically significant. The data were analysed using SPSS 13.0 (IBM SPSS Statistics, New York, NY).

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Conclusions

Our findings broaden knowledge about the importance of exocyclic DNA adducts in fluoride-induced toxicity. Fluoride generated from the non-ferrous metallurgy complexes may increase the intracellular amounts of oxidative stress and ROS within renal cells and hepatocytes. A continuous attack to DNA may contribute to the general decline of cellular functions, from disturbance of DNA metabolism, such as transcription and replication, triggering cell-cycle arrest and apoptosis, up to inducing necrosis and cellular degenerative changes.

Declaration of Interest

The authors declare no conflict of interest. The study was partially supported from the Tuscany Region, and the Italian Ministry of Education and Scientific Research (PRIN20072S2HT8 Grant).

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Fig. 1. Anatomo-histopathological observations of the soft organs of fluorotic sheep. A) Evident fluorosis symptom: dark pigmentation and fracture of teeth (white arrow). B) Glenohumeral joint: chondrosis of articular cartilage (black arrow). C) Liver: centrolobular degeneration (black arrow) with cytoplasmic cloudy swelling and hydropic degeneration of hepatocytes (higher magnification in E). D) Kidney: severe jalina degeneration with necrosis of tubular cell (black arrow).

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Table I. Mean levels of 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one deoxyguanosine, M_1dG adducts per 10^8 normal nucleotides, and Mean Ratios (MR) and 95% CI, in the soft organs of sheep with fluorosis as compared to controls, and according to sheep-farm localization.

	M_1dG adducts								
	Kidney					Liver			
	N	Mean ± SE	MR	95%CI	P-value	Mean ± SE	MR	95%CI	P-value
Fluorosis status									
Controls [a,b]	3	1.5 ± 0.1	Ref.	-	-	5.4 ± 1.6	Ref.	-	-
Skeletal fluorosis	12	20.2 ± 5.2	5.09	1.67-15.53	0.009	11.6 ± 2.2	2.04	0.91-4.57	0.078
Sheep-farm localization									
Control district [c]	7	15.8 ± 3.4	5.90	1.57-22.13	0.014	12.8 ± 2.6	1.73	0.74-4.06	0.188
Industrial estate nearby area	5	21.5 ± 10	4.58	1.31-16.00	0.022	11.2 ± 2.8	2.57	1.04-6.36	0.042
P-value for trend					0.091				0.035

[a] Reference level
[b] The controls were living in a remote site without industrial settlements
[c] The farms of the control district were at 14 km away from the industrial site

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Table II. Mean concentrations of heavy-metals in the soft organs of the sheep with fluorosis as compared to controls, and according to sheep-farm localization.

	Cadmium (µg/mg)					Lead (µg/mg)			
	Kidney			Liver		Kidney		Liver	
	N	Mean ± SE	P-value	Mean ± SE	P-value	Mean ± SE	P-value	Mean ± SE	P-value
Fluorosis status									
Controls [a,b]	3	1.7 ± 0.9	-	0.3 ± 0.2	-	0.01 ± 0.0	-	0.01 ± 0.0	-
Skeletal fluorosis	12	48.8 ± 12	0.002	4.9 ± 1.4	<0.001	2.9 ± 1.2	<0.001	2.2 ± 0.8	<0.001
Sheep-farm localization									
Control district [c]	7	18.5 ± 7.8	0.005	1.8 ± 0.4	0.001	3.3 ± 2.0	<0.001	2.1 ± 1.5	<0.001
Industrial estate nearby area	5	91.2 ± 7.0	<0.001	9.1 ± 2.2	<0.001	2.4 ± 0.8	<0.001	2.5 ± 0.4	<0.001

[a] Reference level

[b] The controls were living in a remote site without industrial settlements

[c] The farms of the control district were at 14 km away from the industrial site

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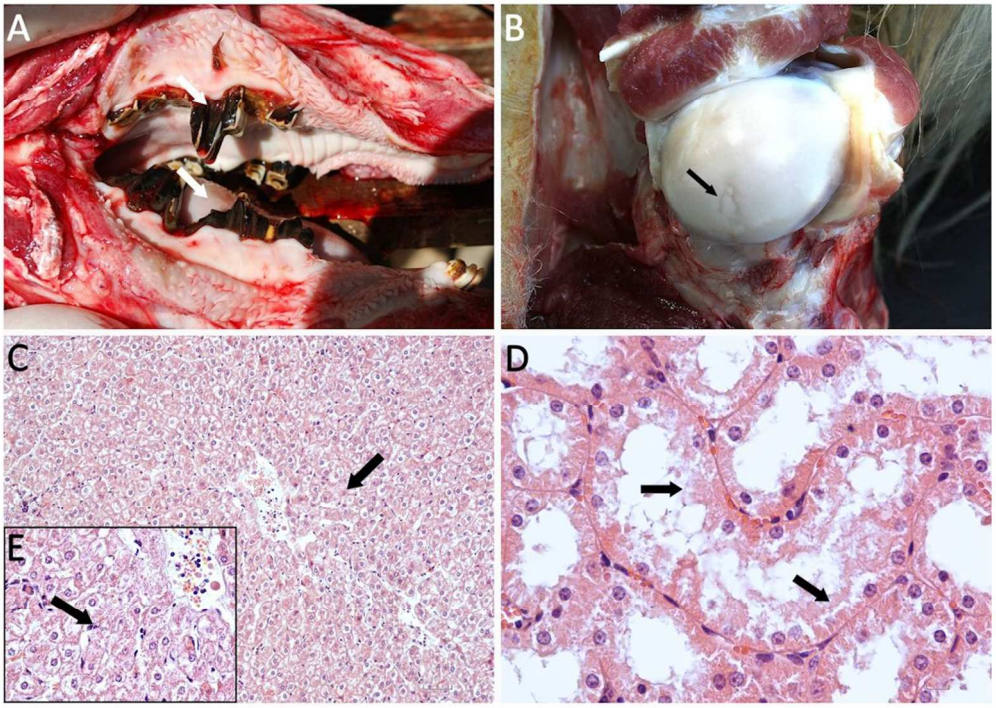

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