UNIVERSITÀ DEGLI STUDI DI TORINO

Chromosome fragility in dairy cows exposed to dioxins and dioxin-like PCBs

G.P. Di Meo, A. Perucatti, V. Genualdo, A. Caputi-Jambrenghi, R. Rasero, C. Nebbia and L. Iannuzzi

Mutagenesis, 26(2):269-72, 2011.

doi: 10.1093/mutage/geq082.

Chromosome fragility in dairy cows exposed to dioxins and dioxin-like PCBs

G.P. Di Meo, A. Perucatti, V. Genualdo, A. Caputi-Jambrenghi, R. Rasero, C. Nebbia and L. Iannuzzi

In this study, we compared cross-bred dairy cows in the Susa Valley (Piedmont, northern Italy), reared either near a high-temperature steel production plant (Farms A and B) or in an industry-free area (control). Exposed cows (n 536) were selected based on mean bulk milk toxic equivalent values of polychlorodibenzodioxins (PCDDs) and dioxin- like (DL) polychlorobiphenyls (PCBs) and polychlorodi- benzofurans (PCDFs) equal to 18.56 pg/g fat and 8.56 pg/g of fat in dairy cows from Farms A and B, respectively, exceeding both those permitted by the legislation in force (6 pg/g fat PCDDs and DL-PCDFs/PCBs), and those measured in dairy cows (n 5 19) of the farm used as control (1.75 pg/g of fat PCDDs and DL-PCDFs/PCBs). Two types of peripheral blood cell cultures were per- formed: without (normal cultures for the chromosome abnormality (CA)-test: gaps, chromatid breaks, chromo- some breaks and fragments) and with addition of bromodeoxyuridine [for the sister chromatid exchange (SCE)-test]. Both tests revealed a significant (P £ 0.05) higher chromosome fragility in the exposed cattle com- pared to controls: CA/cell mean values (without gaps) were 0.65 6 0.91, 0.51 6 0.81 and 0.13 6 0.39 in Farms A, B and controls, respectively, while SCE/cell mean values were 7.00 6 2.88, 6.39 6 2.80 and 5.29 6 2.51. Although the role of other pollutants (e.g. heavy metals) in the genesis of the recorded chromosome alterations cannot be ruled out, our results confirm the findings of previous research into dioxin-exposed sheep.

Introduction

Dioxins are a large family of anthropogenic chlorinated compounds including polychlorodibenzodioxins (PCDDs) and dioxin-like polychlorodibenzofurans (DL-PCDFs) and dioxin-like polychlorobiphenyls (DL-PCBs). Among the hundredths of structurally related congeners, only 17 and 12 compounds between PCDD/PCDFs and DL-PCBs, respec- tively, are considered dangerous and monitored in food and feed commodities. All dioxin-like (DL)-compounds share the ability to bind, albeit with different affinity, to the cytosolic aryl-hydrocabon receptor (AhR) resulting in the transcriptional activation or repression of a wide array of genes. This property is believed to represent the key event in the toxicity of such chemicals affecting the immune, endocrine and reproductive systems (1). Assuming a similar mechanism of action (binding to the AhR), the toxic potency of each DL-compound can be expressed as a fraction of the most potent one, the 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD), to which a toxicity co- efficient (toxicity equivalent factor [TEF]) equal to 1 has been assigned. For the remaining molecules, specific TEFs were therefore determined (,1) so that their concentrations in picograms are multiplied for their relative coefficients to get the 'toxicity equivalent' (TEQ) to TCDD for each molecule and for all of them (whole values of dioxins and DL-compounds).

They are classified among the most dangerous environmental pollutants due to their extremely high environmental persistence and a long biological half-life in living organisms, ranging from several months up to 20 years in humans, according to the nature of the compound and the characteristics of exposure (2). In most cases, such molecules occur as trace contaminants in several industrial processes, including those leading to the production of steel, lubricant oils or chlorinated phenols used as pesticides. In recent years, legal or illegal waste incineration and illegal disposal of industrial waste have become major causes of environmental and food chain pollution (3).

Although DL-PCBs have been banned in industrial processing since 1986 in many countries (including Italy), they are still present in the food chain. For this reason, starting from 2006, DL-

PCBs, in addition to DL-PCDDs and PCDFs, are being investigated in the context of animal production according to EC Regulation No. 199/2006. Aside from the internationally known case of toxic release in Seveso in 1976, lesser-known outbreaks of dioxin pollution, mostly originating from in- dustrial processes and illegal waste burning, have occurred in the last 20 years in Italy, especially in Campania, leading to contamination of dairy milk in sheep, cattle and river buffaloes (4,5). Appreciable levels of dioxins have also been found in some industrial areas of other Italian regions like Piedmont, Lombardy, Tuscany and Puglia (6–8). Cytogenetic tests can be useful to reveal the presence of chromosome damage due to mutagens occurring in the food chain by simply monitoring livestock species. Indeed, several mutagens display carcinogenic properties and elevated frequencies of chromatid breaks have been found in blood cells from a high proportion of cancer patients (9). Despite the large number of studies supporting the ability of dioxins to induce chromosome damage under in vivo or in vitro conditions, the mutagenicity of such derivatives is still a matter of debate due to the contradictory results achieved so far (reviewed in refs 4,5). As assessed by using both chromosome abnormality (CA: gaps, chromatid breaks, chromosome breaks and frag- ments) and the sister chromatid exchange (SCE) tests, a pronounced chromosome fragility was reported in flocks of sheep grazing on PCDD- and PCDF-contaminated pastures compared to unexposed sheep, based on the World Health Organization-Toxcitity Equivalent (WHO-TEQ) values of the bulk milk (4,5).

In the present study, we applied the same tests to blood samples from cattle reared in a dioxin-contaminated area of Piedmont (northern Italy) and showed higher chromosome fragility in the exposed animals compared to that found in the controls. To our knowledge, this is the first study performed on dioxins and DL-PCB-exposed cattle by using two different

cytogenetic tests.

Materials and methods

Farm selection

The study was performed in the Susa Valley (Piedmont, northern Italy) on 55 dairy cows, 36 of which (mainly Piedmontese Valdostana cross-breeds) came from two different farms (A or B, 18 animals each) located near a contaminated area and exhibiting mean bulk milk WHO-TEQ values exceeding legal thresholds (6 pg/g fat as the sum of PCDDs, PCDFs and DL- PCBs) according to the European Union legislation in force (Commission Regulation 1881/2006/EC). For comparison, 19 Valdostana dairy cows reared in a similar manner in a farm located in the same valley, but displaying bulk milk WHO-TEQ levels well below those permitted by law, were included in the study and regarded as controls (Table I). Data concerning milk contamination were obtained from Regional Veterinary Services and the 'Istituto Zooprofi- lattico Sperimentale' of Piedmont, Liguria and Valle d'Aosta.

Cell cultures

Blood samples were collected by venipuncture using lithium heparin as an anticoagulant, put in refrigerated boxes and shipped to the laboratory within 24 h. Blood cell cultures were performed at 37.8 C for 72 h in RPMI medium, enriched with foetal calf serum (10%), L-glutamine (1%), antibiotic– antimycotic mixture (1%) and Concanavalin A (15 lg/ml) as mitogen. Two different types of cell culture were performed: with or without (normal cultures) 5-bromodeoxyuridine (BrdU), which was added 30 h before harvesting at the concentration of 10 lg/ml. Both cell

cultures were gently agitated once a day Colcemid exposure (0.01 lg/ml) lasted 1.5 h for both cell cultures. Cells were then treated with a hypotonic solution (0.56% KCl) for 20 min, and three fixations in methanol–acetic acid (3:1), the last overnight. Three drops of fixed cell suspension were spread on wet and cold slides, air-dried and kept in slide boxes at room temperature. Slides obtained from normal cultures were used to study the chromosome abnormalities (CA-test) (gap, chromatid breaks, chromosome breaks and fragments), while those treated with BrdU were utilised to study the SCEs. Slides from both cell culture types were stained for 10 min with acridine orange (0.01% in phosphate buffer), washed with tap and distilled water, mounted in phosphate buffer (pH 5 7.0) and sealed under slide coverslips. Slides were observed a day later (or more) under a fluorescence microscope connected with a digital camera. At least 50 cells for the CA-test and 35 cells for the SCE-test were studied for each animal. All images were recorded and later carefully examined by two expert cytogeneticists.

Statistical analysis

Mean values of both CA and SCE with standard deviation were calculated for single animal as well as for total cell populations of the three groups of cows (Farms A and B and control). Where appropriate, data were analysed by a non- parametric test (Mann–Whitney test), and differences were considered significant if P 0.05. Milk dioxin content from different farms was not subjected to statistical evaluation as it is derived from bulk milk analysis for each farm (A, B and control) and not from chemical determinations performed on single individuals.

Results

As depicted in Table I, WHO-TEQ bulk milk values from dairy cows belonging to farms in the contaminated area were within the established limits for PCDDs/PCDFs (3 pg/g fat) but non-compliant with the cumulative values including DL-PCBs (6 pg/g fat). Indeed, total values of dioxins and DL-PCBs were

18.56 and 8.56 pg/g of fat in dairy cows of Farms A and B, respectively, and only 1.75 pg/g of fat in dairy cows of the farm used as control.

As regards cytogenetic studies, mean values of abnormal cells were considerably higher in individuals from both Farms A and B (0.76, 0.42 and 0.62, 0.49, respectively) compared with the controls (0.24 0.43) (Table II). These differences were also confirmed by the CA mean values when referring to both single abnormalities (gaps and chromatid breaks) (Fig. 1) and total CA (sum of chromatid breaks, chromosome breaks

and fragments) (Table II). Indeed, statistically higher mean values of CA/cell (P 0.05) were found in samples from exposed cows compared to the control (Table II). Statistical differences (P 0.05) were also noted when comparing the two groups of exposed cows. Finally, there was an increase in chromosome fragility in the exposed animals, showing higher mean SCE values (by 34%) (Fig. 2) with respect to controls (Table III).

Discussion

Although cytogenetic tests applied to samples from humans or laboratory species exposed to dioxins failed to give unequivocal results (reviewed in refs 4,5), studies on hepatoma cells incubated with 1 nM TCDD revealed the presence of localised and discontinuous chromatin

changes (10). It has been suggested that the key event in TCDD and DL-compound toxicity consists in binding to the cytosolic AhR, followed by translocation into the nucleus resulting in enhancing the transcription of genes including cytochrome P450 1A1 (CYP1A1) (3,11). Overexpression of CYP1A contributes to the generation of reactive oxygen species and subsequent oxidative stress in mammalian cells (12), a mechanism that has been related to TCDD-mediated DNA damage in murine neuroblastoma cells (13). Due to a very long biological half-life (14,15), PCDDs and related substances including PCDFs and DL-PCBs are highly persistent in the environment (and in the food chain) and may therefore build up to a significant extent in livestock, animal products representing the main source of exposure to such compounds for humans. In Italy, after the Seveso accident (1976), dioxin-contaminated areas (and animal products) have been found in several regions, including Campania, Piedmont, Lombardy, Tuscany and Puglia (4–8), especially since 2006 when, according to Council Directive 2006/13/EC and Regulation 1881/2006/EC, not only PCDDs and PCDFs but also DL-PCBs must be included in the routine controls for food and feed dioxin contamination. In the case reported here, DL- PCBs accounted for the great majority of the measured WHO- TEQ values in the bulk milk of the examined cows (data not shown). The source of the pollution by DL-PCBs was very likely due to a nearby high-temperature steel production plant; indeed, metallurgical industries are believed to largely contribute to the environmental burden of such organohalo- genated contaminants (16).

The cytogenetic investigation we performed revealed significant high mean values of abnormal blood cells (Table

II), which can be mainly attributed to higher chromosome fragility. Indeed, the number of CA was significantly higher in the exposed animals compared to that of the controls (Table II).

This behaviour was confirmed even when considering chromatid breaks and chromosome breaks alone, although statistically significant changes occurred for chromatid breaks only (Table II). Similar results were obtained in sheep,

although the CA-test revealed higher levels of chromosome fragility in such species (4,5).

The increased chromosome fragility in exposed cows was

also confirmed by the SCE test (Table III), in agreement with the results obtained in sheep under similar conditions (4,5).

Significant differences (P 0.05) in most of the examined

parameters were also observed when comparing the two groups of exposed cows (Table II). This may support the hypothesis of a correlation between chromosome damage and the extent of dioxin contamination, as previously reported in sheep exposed

to different levels of dioxins (4,5). Interestingly, higher chromosome fragility revealed by both CA and SCE tests has also been found in river buffalo calves affected by limb

malformations (transversal hemimelia) compared to that recorded in normal calves (17).

Since a variety of chemical and physical agents (e.g.

radiation) may induce chromosome damage (for review, see ref. 18) and in the examined cows, only dioxins were investigated; further research is warranted using more specific

tests (e.g. the expression profile of target genes) to ultimately confirm the major involvement of PCDDs, PCDFs and DL- PCBs in the cytogenetic alterations observed in the present study. However, irrespective of the nature of chemicals, which may have induced chromosome fragility, results from this investigation reinforce the importance of performing cytoge- netic tests in food-producing species as a useful tool to indirectly check the presence of mutagens in the food chain.

Funding

National Research Council (CNR) of Rome, Italy, (DG.RSTL.083.001); Regione Piemonte, Direzione Sviluppo Agricoltura, Progetto DIOSSIPOLI, Turin, Italy 'Ricerca di biomarcatori di esposizione a diossine e policlorobifenili diossino- simili in specie animali produttrici di alimenti provenienti da allevamenti ad alto rischio di contaminazione'; Italian Ministry of University and Research 'PRIN-2008—Contaminazione da diossine e composti diossino-simili: ricerca di biomarcatori in popolazioni bovine e bufaline esposte'.

Acknowledgements

The authors wish to thank D. Incarnato for his excellent technical assistance and Dr Giuseppe Ru and the Regional Veterinary Services for providing data on milk dioxin contamination.

Conflict of interest statement: None declared.

References

1. Mandal, P. K. (2005) Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. J. Comp. Physiol. B., 175, 221–230.

2. Ogura, I. (2004) Half-life of each dioxin and PCB congener in the human body. Organohalogen Compd., 66, 3329–3337.

3. Brambilla, G., Cherubini, G., De Filippis, S., Magliuolo, M. and Di

Domenico, A. (2004) Review of aspects pertaining to food contamination by polychlorinated dibenzodioxins, dibenzofurans, and biphenyls at the farm level. Anal. Chim. Acta., 514, 1–7.

4. Iannuzzi, L., Perucatti, A., Di Meo, G. P. et al. (2004) Chromosome fragility in two sheep flocks exposed to dioxins during pasturage. Mutagenesis, 19, 355–359.

5. Perucatti, A., Di Meo, G. P., Albarella, S., Ciotola, F., Incarnato, D.,

Caputi-Jambrenghi, A., Peretti, V., Vonghia, G. and Iannuzzi, L. (2006) Increased frequencies of both chromosome abnormalities and SCEs in two sheep flocks exposed to high dioxin levels during pasturage. Mutagenesis,

21, 67–75.

6. Biasioli, M. and Ajmone-Marsan, F. (2007) Organic and inorganic diffuse contamination in urban soils: the case of Torino (Italy). J. Environ. Monit.,

9, 862–868.

7. Ingelido, A. M., Abballe, A., di Domenico, A., Fochi, I., Iacovella, N., Saragosa, A., Spagnesi, M., Valentini, S. and De Felip, E. (2009) Levels and profiles of polychlorinated dibenzo-p-dioxins, polychlorinated diben- zofurans, and polychlorinated biphenyls in feedstuffs and milk from farms in the vicinity of incineration plants in Tuscany, Italy. Arch. Environ. Contam. Toxicol., 57, 397–404.

8. Turrio-Baldassarri, L., Alivernini, S., Carasi, S. et al. (2009) PCB, PCDD and PCDF contamination of food of animal origin as the effect of soil pollution and the cause of human exposure in Brescia. Chemosphere, 76,

278–285.

9. Bryant, P. E., Gray, L. J. and Peresse, N. (2004) Progress towards understanding the nature of chromatid breakage. Cytogenet. Genome Res.,

104, 65–71.

10. Okino, S. T. and Whitlock, J. P. (1995) Dioxin induces localized, graded changes in chromatin structure: implications for Cyp1A1 Gene Transcrip- tion. Mol. Cell. Biol., 15, 3714–3721.

11. Beischlag, T. V., Morales, J. L., Hollingshead, B. D. and Perdew, G. H. (2008) The aryl hydrocarbon receptor complex and the control of gene

expression. Crit. Rev. Eukaryot. Gene Expr., 18, 207–250.

12. Knerr, S., Schaefer, J., Both, S., Mally, A., Dekant, W. and Schrenk, D. (2006) 2,3,7,8-Tetrachlorodibenzo-p-dioxin induced cytochrome P450s alter the formation of reactive oxygen species in liver cells. Mol. Nutr. Food Res., 50, 378–384.

13. Sul, D., Kim, H. S., Cho, E. K., Lee, M., Kim, H. S., Jung, W. W., Hwang, K. W. and Park, S. Y. (2008) 2,3,7,8-TCDD neurotoxicity in neuroblastoma cells is caused by increased oxidative stress, in- tracellular calcium levels, and tau phosphorylation. Toxicology, 255,

65–71.

14. Wolfe, W. H., Michalek, J. E., Miner, J. C., Pirkle, J. L., Caudill, S. P., Patterson, D. G. and Needham, L. L. (1994) Determinants of TCDD half- life in veterans of operation ranch hand. J. Toxicol. Environ. Health, 41,

481–488.

15. Kerger, B. D., Leung, H.-W., Scott, P., Paustenbach, D. J., Needham, L. L., Patterson, D. G., Gerthoux, P. M. and Mocarelli, P. (2006) Age- and concentration-dependent elimination half-life of 2,3,7,8- tetrachlorodibenzo-p-dioxin in Seveso children. Environ. Health Per- spect., 114, 1596–1602.

16. Choi, S. D., Baek, S. Y. and Chang, Y. S. (2008) Atmospheric levels and distribution of dioxin-like polychlorinated biphenyls (PCBs) and poly-

brominated diphenyl ethers (PBDEs) in the vicinity of an iron and steel making plant. Atmos. Environ., 42, 2479–2488.

17. Albarella, S., Ciotola, F., Dario, C., Iannuzzi, L., Barbieri, V. and Peretti, V. (2009) Chromosome instability in Mediterranean Italian buffaloes affected by limb malformation (transversal hemimelia). Muta- genesis, 24, 471–474.

18. Efferth, T. and Kaina, B. (2008) Chemical carcinogenesis: genotoxic and nongenotoxic mechanisms. In Greim, H. and Snyder, R. (eds), Toxicology and Risk Assessment: A Comprehensive Introduction. John Wiley and Sons Ltd, Chichester, UK, pp. 151–180.

Table I. Results of the chemical analyses for dioxin and DL-PCBs in bulk milk of dairy cows reared in different areas of Piedmont (northern Italy)

	PCDF-TEQ (pg/g)	(pg/g)	PCDF-PCB-TEQ (pg/g)
Farm A	1.66	16.09	18.56
Farm B	1.11	7.45	8.56
Control	1.2	0.55	1.75

Permitted levels of WHO-PCDD/F-TEQ and WHO-PCDD/F-PCB-TEQ are 3.0 and 6.0 pg/g of fat, respectively.

Table II. Number of animals studied, examined cells, abnormal cells, gaps, chromatid breaks (ct), chromosome breaks (cs), fragments (fg) and total ct, cs and fg in dairy cows reared in dioxin-contaminated (Farms A an B) or in control areas from Piedmont (northern Italy)

Animals (n)	Examined cells (n)	Abnormal cells			Gaps			Chromatid breaks			Chromosome breaks			Fragments			ct þ cs þ fg		
		n	Mean	SD	n	Mean/cell	SD	n	Mean/cell	SD	n	Mean/cell	SD	n	Mean/cell	SD	n	Mean/cell	SD
Farm A total (18)	900	687	0.76[a,b]	0.42	1228	1.36[a,b]	1.20	497	0.55[a,b]	0.83	76	0.08	0.30	8	0.01	0.09	581	0.65[a,b]	0.91
Farm B total (18)	900	555	0.62[a]	0.49	640	0.71[a]	0.91	374	0.42[a]	0.70	83	0.09	0.36	5	0.01	0.07	462	0.51[a]	0.81
Control total (19)	950	226	0.24	0.43	162	0.17	0.44	87	0.09	0.34	33	0.03	0.18	3	0.00	0.06	123	0.13	0.39

[a]Significantly different versus controls (P ¸ 0.01).
[b] Significantly different versus Farm B (P ¸ 0.01).

Table III. Number of examined cells and SCE mean values in dairy cows reared in dioxin-contaminated (Farms A and B) and in control areas from Piedmont (northern Italy)

Animals (n)	Examined cells (n)	SCE/cells					
n	Mean	SD	Farm A (17)	595	4165	7.00	2.88a,b
Farm B (17)	595	3805	6.39	2.80a			
Control (17)	595	3145	5.28	2.51			

aSignificantly different from control (P , 0.01).
bSignificantly different from Farm B (P , 0.01).

Fig. 1. Dairy cow metaphase plate stained with acridine orange and showing a chromatid break (large arrow) and two gaps (small arrows).

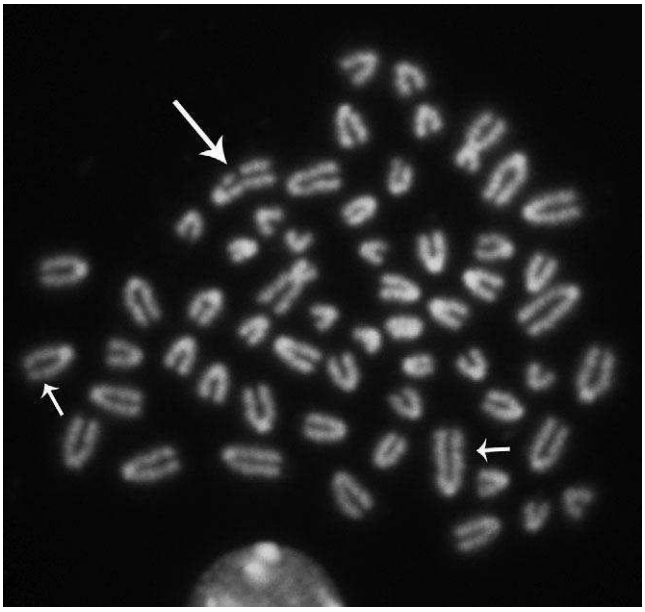

Fig. 2. Dairy cow metaphase plate stained with acridine orange and showing numerous SCEs (arrows).