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 = 36) were selected based on mean bulk milk toxic equivalent values of polychlorodibenzoxydioxins (PCDDs) and dioxin-like (DL) polychlorobiphenyls (PCBs) and polychlorodibenzoxyfurans (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 = 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 performed: without (normal cultures for the chromosome abnormality (CA)-test: gaps, chromatid breaks, chromosome 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 compared to controls: CA/cell mean values (without gaps) were 0.65 ± 0.91, 0.51 ± 0.81 and 0.13 ± 0.39 in Farms A, B and controls, respectively, while SCE/cell mean values were 7.00 ± 2.88, 6.39 ± 2.80 and 5.29 ± 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 polychlorodibenzoxydioxins (PCDDs) and dioxin-like polychlorodibenzoxyfurans (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, respectively, 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-hydrocarbon receptor (AhR) resulting in 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 coefficient (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 industrial 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). Applicable 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 fragments) and the sister chromatid exchange (SCE) test, 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-Toxicity 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 came from two different farms (A or B, 18 animals each) located near 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.

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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, 10% fetal calf serum, 1% L-glutamine, 1% antibiotic–antimycotic mixture, 1% and 15 µg/ml Concanavalin A. 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 different types of cell culture were performed: with or without (normal cultures) BrdU. Both cell cultures were gently agitated once a day.

Concemid exposure (0.01 µg/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 acidine orange (0.01% in phosphate buffer), washed with tap and distilled water, mounted in phosphate buffer (pH = 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.

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)

<table>
<thead>
<tr>
<th>Source</th>
<th>WHO-PCDD/PCDF-TEQ (pg/g)</th>
<th>WHO-PCB-TEQ (pg/g)</th>
<th>WHO-PCDD/PCDF-PCB-TEQ (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>1.66</td>
<td>16.09</td>
<td>18.56</td>
</tr>
<tr>
<td>Farm B</td>
<td>1.11</td>
<td>7.45</td>
<td>8.56</td>
</tr>
<tr>
<td>Control</td>
<td>1.2</td>
<td>0.55</td>
<td>1.75</td>
</tr>
</tbody>
</table>

Permitted levels of WHO-PCDD/F-TEQ and WHO-PCDF/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), chromatid 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)

<table>
<thead>
<tr>
<th>Animals (n)</th>
<th>Examined cells (n)</th>
<th>Abnormal cells</th>
<th>Gaps</th>
<th>Chromatid breaks</th>
<th>Chromosome breaks</th>
<th>Fragments</th>
<th>ct + cs + fg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
</tr>
<tr>
<td>Farm A total (18) 900</td>
<td>687</td>
<td>0.76±0.2 ± 0.42</td>
<td>1228</td>
<td>1.36±1.20</td>
<td>497</td>
<td>0.55±0.83</td>
<td>76</td>
</tr>
<tr>
<td>Farm B total (18) 900</td>
<td>555</td>
<td>0.62±0.49</td>
<td>640</td>
<td>0.71±0.91</td>
<td>374</td>
<td>0.42±0.70</td>
<td>83</td>
</tr>
<tr>
<td>Control total (19) 950</td>
<td>226</td>
<td>0.24±0.43</td>
<td>162</td>
<td>0.17±0.44</td>
<td>87</td>
<td>0.09±0.34</td>
<td>33</td>
</tr>
</tbody>
</table>

*Significantly different versus controls (P < 0.01).

b Significantly different versus Farm B (P < 0.01).

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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 organohalogenated 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 cytogenetic tests in food-producing species as a useful tool to indirectly check the presence of mutagens in the food chain.

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