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Chromosome instability in lymphocytes of Friesian cows naturally exposed to dioxins being raised close to a metallurgic factory area in southern Italy

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ABSTRACT

Dioxins (PCDDs, PCDFs and DL-PCBs) are a large family of congeners that are considered highly toxic and are reported to be teratogenic, mutagenic, carcinogenic, immunotoxic and hepatotoxic, also affecting the nervous and reproductive systems. Farm animals are particularly exposed to these chemicals when they are fed with grass produced close to polluted areas such as those located in vicinity of metallurgic factories. Cytogenetic tests can be very useful to check genetic damage occurring to domestic animal cells exposed to these chemicals. Fifty-two randomly selected Italian Friesian cows (*Bos taurus*, $2n = 60$) from two farms located in the vicinity of and (as a control) far from the a metallurgic industrial area underwent cytogenetic investigations to ascertain possible differences in their chromosome fragility. One farm was under legal sequestration due to the presence in the milk mass of higher mean values of dioxins (24.78 ± 3.19 pg g^{-1} of fat as sum of PCDD + PCDF + DL-PCBs as WHO-TEQ (World Health Organization-Toxic Equivalent Quantity), with DL-PCBs being the main chemical component) than those permitted (5.5 pg g^{-1} of fat as WHO-TEQ). Cytogenetic analyses, performed by using both the chromosome abnormality (CA) test (chromosome and chromatid breaks) and sister chromatid exchange (SCE) test, revealed a significantly ($p < 0.01$) higher chromosome fragility in cells of exposed cows (26 cows) compared to those of the control (23 cows).

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Dioxin; DL-PCB; Friesian cattle; chromosome fragility; metallurgic factory

Introduction

Dioxins are a large family of congeners which can be fitted in three main groups: polychloro-dibenzo-dioxins (PCDDs), polychloro-dibenzo-furans (PCDFs) and dioxin-like polychlorobiphenyls (DL-PCBs). These chemicals are considered highly toxic, especially tetrachloro-dibenzo-*p*-dioxin (TCDD) which has been reported to be teratogenic, mutagenic, carcinogenic, immunotoxic, hepatotoxic and affecting also the nervous and reproductive systems (Mandal 2005; Bock and Kohle 2006; Steenland et al. 2014). Dioxins are also highly persistent in the environment, especially when entering in the human or animal body due to their ability to be absorbed by fat tissue where they can remain for long time; their half-life in the body varies from 7 to 11 years (Wolfe et al. 1994; Ogura 2004).

For this reason, international committees have established very low levels of permitted dioxins in both animal and fish fat, although these values vary among species and type of food as established in the most recent EC Regulation No.1259/2011. According to this EC regulation, in animal milk these values are 2.5 pg g^{-1} of fat for PCDDs + PCDFs as WHO-TEQ (World Health Organization-Toxic Equivalent Quantity) and 5.5 pg g^{-1} of fat as the sum of PCDDs + PCDFs + DL-PCBs as WHO-TEQ.

Most PCDDs and PCDFs are produced by both industrial processes and illegal waste burning, while DL-PCBs are produced during some industrial processes, e.g. steel production.

PCBs have been used in the past in various industrial applications. Due to their toxicity, many countries have forbidden their use (in Italy since 1985). PCB levels seem to be particularly high in the northern hemisphere, e.g. as revealed by studies performed in Barents Sea, which showed very high levels (36 mg g^{-1} of fat) in the Glaucous Gull (*Larus hyperboreus*) which is at the top of food chain in that area (Bustnes et al. 2003; Erikstad et al. 2011).

In Italy, after the famous Seveso disaster (10 July 1976) where a very high quantity of TCDDs (30 kg) were spread in the environment, many controls conducted

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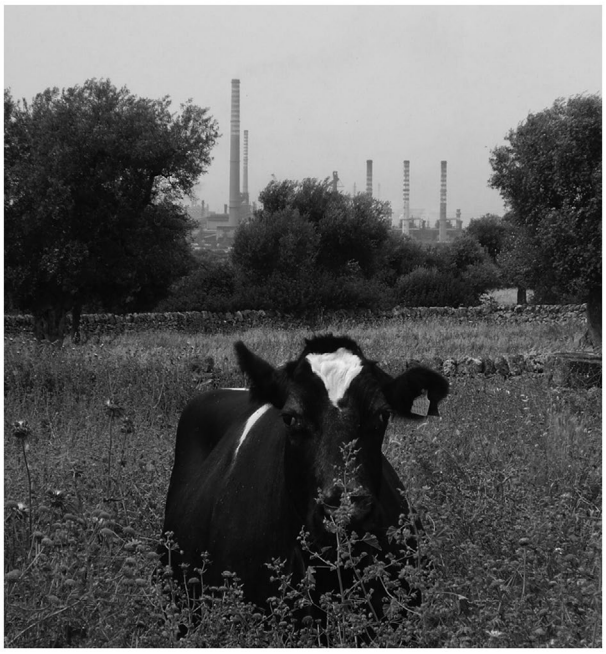

Figure 1. Farm raising Friesian cattle very close to the metallurgic industrial area of Taranto city.

in farm animals to check the transmission of dioxins to humans have established the presence of dioxins in animals raised in various regions such us Piedmont, Lombardy, Tuscany and Apulian regions (Biasioli and Ajmone-Marsan 2007; Ingelido et al. 2009; Di Meo et al. 2011).

The Apulian region (southern Italy), and more specifically the metallurgic Taranto city industrial area, has been in the spotlight because it was indicated as responsible for the production of environmental pollution (i.e. air pollution, reviewed in Mangia et al. 2013), dioxins, especially DL-PCBs, increasing cases of mortality and cancers (Mitis et al. 2005; Martinelli et al. 2009; Casale 2011). For this reason, the veterinary service of the local health sanitary control performed analyses in search of dioxins in a farm raising Friesian cattle relatively close to the metallurgic industrial area (Figure 1). In this farm, under legal sequestration for long time, levels of dioxins (mainly DL-PCBs) were found to be much higher than those permitted in the milk of dairy cattle (5.5 pg g^{-1} of fat as a sum of PCDDs + PCDFs and DL-PCBs).

Cytogenetic tests are very useful to check the presence of chromosome damage due to the mutagens present in the environment (Lovreglio et al. 2014; Mrdjanovic et al. 2014), in the food chain and more specifically in livestock animals, which are below humans in the food chain (Iannuzzi et al. 2004; Perucatti et al. 2006; Di Meo et al. 2011). In addition, high frequencies of chromatid breaks have been found in blood cells from a high percentage of cancer patients (Bryant et al. 2004). Indeed, 40% of breast cancer cases show elevated "chromatid radiosensitivity" in contrast to only some 6% in a similar matched group of normal (non-cancer) surgical cases (Bryant et al. 2004).

PCBs have been found to produce sex chromosome disomy in sperms, especially YY and XY (McAuliffe et al. 2012). Indeed, dietary PCB exposure seems to have a negative impact on the sperm chromatin integrity of adult males (Spanò et al. 2005). A small chromosome region duplication has been found in humans exposed to PCB (in particular to PCB95) (Mitchell et al. 2012). *In vitro* studies, using some types of PCBs, have revealed a very high percentage of tetraploid cells and significant increasing number of sister chromatid exchanges, compared to those achieved in the control (Flor and Ludewig 2010). Some PCBs, especially the volatile ones (PCBs 28 and 52), reduced significantly the chromosome telomerase activity (Senthilkumara et al. 2011).

In previous reports we found chromosome fragility in cells of livestock naturally exposed to dioxins (PCDDs + PCDFs only), i.e. sheep (Iannuzzi et al. 2004; Perucatti et al. 2006) and to both PCDDs + PCDFs and DL-PCBs (i.e. cattle, northern Italy – Piedmont-Valdostana hybrid cattle breeds) (Di Meo et al. 2011); or river buffalo (Genualdo et al. 2012) compared to that found in cell populations of control animals.

In the present paper we have studied, for the first time, the most famous dairy cattle breed in the world (Friesian) by comparing two groups of sample cows raised very close to, and (as a control) far from the largest European metallurgic factory, which is located in the industrial area of Taranto city (Apulian region, southern Italy). We performed the study using two different cytogenetic tests and found a significant higher chromosome fragility in cells of the exposed cows compared to that of the control group.

Materials and methods

Animals and dioxin analyses

We studied 52 Friesian cows (3–5 years old) randomly sampled from two different farms: one (29 animals) located close to the steel manufacturing industrial area of Taranto city, Apulian region (southern Italy) and the other one (23 animals) 65 km away in the same province and used as control. Both farms used the same animal feeding: natural pasture in the area of the farm and additional food in the box, especially during lactation.

The farm located close to the industrial area of Taranto city was under legal sequestration for a long time due to the presence in the milk mass of higher dioxin values (sum of DCDDs + DCFFs + DL-PCBs as WHO-TEQ) than those permitted. Chemical analyses in search of dioxins were also extended to both pasture and soil of the farm located close to the industrial area. All chemical analyses were performed by specialized laboratories under local veterinary sanitary health control.

Table 1. Levels of PCDD + PCDFs, DL-PCBs and sum of PCDD + PCDF + DL-PCBs in the milk mass of the farm under legal sequestration, as well as in the grass and soil of the same farm. Permitted values of dioxins are reported between parentheses.

Source	PCDD + PCDF WHO-TEQ (pg g^{-1})	DL-PCBs WHO-TEQ (pg g^{-1})	PCDD + PCDF+ DL-PCBs WHO-TEQ(pg g^{-1})
Milk mass	4.22 ± 0.58 (2.5)	20.56 ± 2.53	24.78 ± 3.19 (5.5)
Grass	0.83 (0.75)	1.54	2.37 (1.5)
Soil	2.49 ± 0.53 (10)	0.23 ± 0.04	

Blood samples and cell cultures

Peripheral blood samples were collected by veterinarians of the local health unit by using sterile vacutainer tubes containing sodium heparin. Cell cultures from whole blood were performed at 37.8°C in RPMI (Roswell Park Memorial Institute) medium, enriched with fetal calf serum (10%), L-glutamine (1%), antibiotic-antimycotic mixure (1%) and concanavalin A (15 μg ml^{-1}) as mitogen. Two different cell cultures were performed: normal cultures (duration time 48 h) and cultures treated with 5-bromodeoxyuridine (BrdU) 28 h before harvesting (duration time 72 h). Colcemid (0.01 μg ml^{-1}) lasted 1.5 h for both cell cultures. Slides obtained from normal cultures were used for chromosome abnormality (CA) tests such as chromatid breaks and chromosome breaks, while those treated with BrdU were used for sister chromatid exchange (SCE) tests. Slides from both types of cell cultures were stained for 10 min with acridine orange (0.01% in phosphate buffer pH = 7.0), washed with tap and distilled water, mounted in P-buffer and sealed under slide coverslips. The slides were observed a day later (or more) under a fluorescence microscope NIKON E-1000 connected to a digital camera. At least 50 cells for the CA test and 35 for the SCE test were studied for each animal (exposed and control animals). All images were recorded and later carefully examined by two expert cytogeneticists.

Statistical analysis

Mean values and standard deviations of both CA and SCE were calculated for single animals and animal groups. Statistical analyses were performed between the two cow groups by using a non-parametric test (Mann–Whitney), and differences were considered significant if $p \leq 0.05$.

Results

Chemical analysis

Chemical analysis in search of dioxins revealed higher levels of dioxins (24.78 pg g^{-1} of fat as the sum of PCDDs + PCDFs + DL-PCBs as WHO-TEQ) in the milk mass of cows raised in the farm located close to the metallurgic area than those permitted (5.5 pg g^{-1} of fat as WHO-TEQ) (Table 1). The same situation was found in the dioxins in the grass, with higher levels (2.37 pg g^{-1}) than those permitted (1.5 pg g^{-1}) (Table 1). Conversely, levels of dioxins in the soil were lower (2.72 pg g^{-1}) than those permitted (10 pg g^{-1}) (Table 1).

Of the different dioxin types, DL-PCBs are the main chemical component in both milk mass (20.56 ± 2.53 pg g^{-1}) and grass (1.54 pg g^{-1}), while PDDDs and PCDF are prevalent in the soil (2.49 ± 0.53), although levels of total dioxins in the soil are below those permitted (10 pg g^{-1}) (Table 1). No analyses of dioxins have been performed in the control farm by the local veterinary health control because the area where it was located was very far from sources of pollution, including dioxins.

Cytogenetic analyses

The mean value of abnormal cells, with at least one chromatid break or chromosomal break (Figure 2), was significantly higher ($p < 0.01$) in the exposed cows (0.52 ± 0.49) than those of the control (0.16 ± 0.37) (Table 2). This is due to the presence of a significantly ($p < 0.01$) higher mean number of chromatid breaks (CT) (0.72 ± 0.86) and chromosome breaks (CS) (0.07 ± 0.28) in the exposed cows, compared to those (0.15 ± 0.39 and 0.02 ± 0.15, respectively) achieved in the control (Table 2).

SCE/cell mean value (Figure 3) was significantly higher ($p < 0.01$) in the exposed cows (7.41 ± 3.02) compared to the control group (5.30 ± 2.61) (Table 3).

Data analysis

Table 4 provides some descriptive statistics for our variables. They are all count variables and descriptive statistics show consistently larger values in the exposed animals compared with the control sample, as also shown in Figure 4 which demonstrates a no normal distribution of variables.

To confirm that data are not normally distributed, we test for normality with the Shapiro–Wilk test (Table 5). The hypothesis H_0 is rejected for all variables both for control and for exposed animals. In this case, a non-parametric test is more appropriate than using a *t*-test. The Mann–Whitney test (Table 6) is implemented to verify whether means of CS, CT, CT + CS and SCE of exposed cell animals are larger than those achieved in the control sample. All H_0 are rejected and the exposed cell variable shows a larger effect. This means that exposed cell variables are always greater in the mean than in the control. We can also test whether other sample statistics are different between exposed and control samples. It is interesting to verify whether the two samples have different dispersion parameters by using a permutation test (Table 7). We use the ratio of the mean deviance as a statistic. In this case, H_0 is rejected in all cases. This

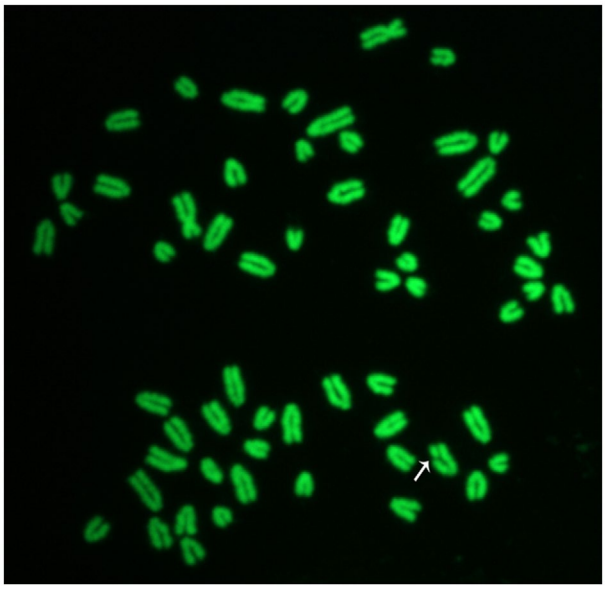

Figure 2. Female cattle metaphase plate showing a chromatid break (arrow). Slides were stained with acridine orange and later observed under a fluorescence microscope connected with a digital camera.

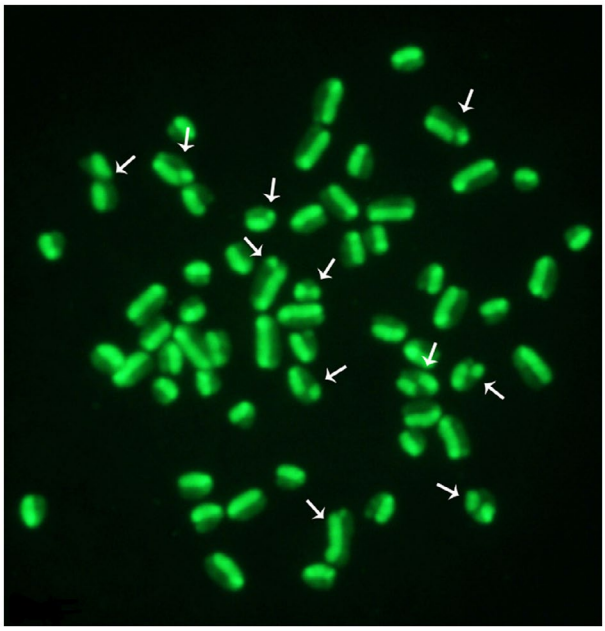

Figure 3. Female cattle metaphase plate showing several SCEs (arrows). Slides were stained with acridine orange and later observed under a fluorescence microscope connected with a digital camera.

means that not only is there a larger effect on the variable in the exposed cell samples, but also a higher variance compared with the control. In two cases there are similar values for mean and variance in the samples. Therefore, we decided to test the goodness-of-fit for the variables as a Poisson distribution. CT and CS + CT have a Poisson distribution for the control sample, but the variables measured in the exposed samples reject always the H_0 to be distributed as a Poisson. This is important because we can also implement a parametric test that can show differences in all distributions between exposed and control samples (Figure 5).

Discussion

Chromatin damage can be induced by several environmental mutagens (Bryant et al. 2004). Although cytogenetic tests applied to both human and animals exposed to dioxins have generated contradictory results (reviewed in Iannuzzi et al. 2004; Perucatti et al. 2006), all studies performed so far in domestic animals revealed an higher chromosome fragility in animal cells naturally exposed to dioxins (PCDD, PCDF and DL-PCBs), compared to that achieved in the controls (same species, breed, age and dietary habits) (Iannuzzi et al. 2004; Perucatti et al. 2006; Di Meo et al. 2011; Genualdo et al. 2012). In addition, chromatin revealed the presence of localized and discontinuous changes due to dioxin (TCDD) (Okino and Whitlock 1995). In particular, PCBs were found to produce sex chromosome disomy in sperms, especially YY and XY (McAuliffe et al. 2012), probably because PCB exposure has been reported to have a negative impact on the sperm chromatin integrity of adult males (Spanò et al. 2005). Furthermore, small chromosome region duplications have been found in humans exposed to PCBs (Mitchell et al. 2012), while *in vitro* studies on male Chinese hamster V79 lung fibroblasts revealed a very high percentage of tetraploid cells and significant increasing number of sister chromatid exchanges using some types of PCBs, compared to those achieved in the control (Flor and Ludewig 2010). In addition, the chromosome telomerase activity appeared significantly reduced in human skin keratinocytes when using some PCBs, especially the volatile ones (PCBs 28 and 52) (Senthilkumara et al. 2011).

Considering that chromatin is the main component of chromosomes, damage at the chromosomal level, especially when double DNA breakages occur, may denote chromosome fragility with a subsequent increasing

Table 2. Number of animals studies, examined cells, abnormal cells (AC), chromatid breaks (CT), chromosome breaks (CS), and total CT + CS in cattle reared in dioxin-contaminated and control areas of Apulian region (southern Italy).

		Abnormal cells (AC)		Chromatid breaks (CT)		Chromosome breaks (CS)		CT + CS	
Animals (*n*)	Examined cells(*n*)	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	*n*	mean ± SD
Exposed – total (28)	980	508	0.52 ± 0.49[a]	704	0.72 ± 0.86[a]	69	0.07 ± 0.28[a]	773	0.79 ± 0.92[a]
Control – total (20)	700	112	0.16 ± 0.37	105	0.15 ± 0.39	17	0.02 ± 0.15	122	0.17 ± 0.42

[a]Significantly higher versus controls ($p < 0.01$)

Table 3. Number of examined cells and SCE mean values in Friesian dairy cows reared in dioxin-contaminated and control areas of Apulian region (southern Italy).

		SCE/cell	
Animals (*n*)	Examined cells (*n*)	*n*	Mean ± SD
Exposed (29)	870	6450	7.41 ± 3.02[a]
Control (23)	690	3660	5.30 ± 2.61

[a]Significantly higher versus control ($p < 0.01$)

Table 4. Descriptive statistics.

	Control				Exposed			
Variables	CS	CT	CT+CS	SCE	CS	CT	CT+CS	SCE
Mean	0.0243	0.1500	0.1743	5.0343	0.0704	0.7183	0.7888	7.4138
Var	0.0237	0.1563	0.1756	6.8158	0.0798	0.7398	0.8532	9.1266
Median	0	0	0	5	0	0	1	7
Range	0–1	0–3	0–3	0–24	0–2	0–4	0–5	0–32
n	700	700	700	690	980	980	980	870

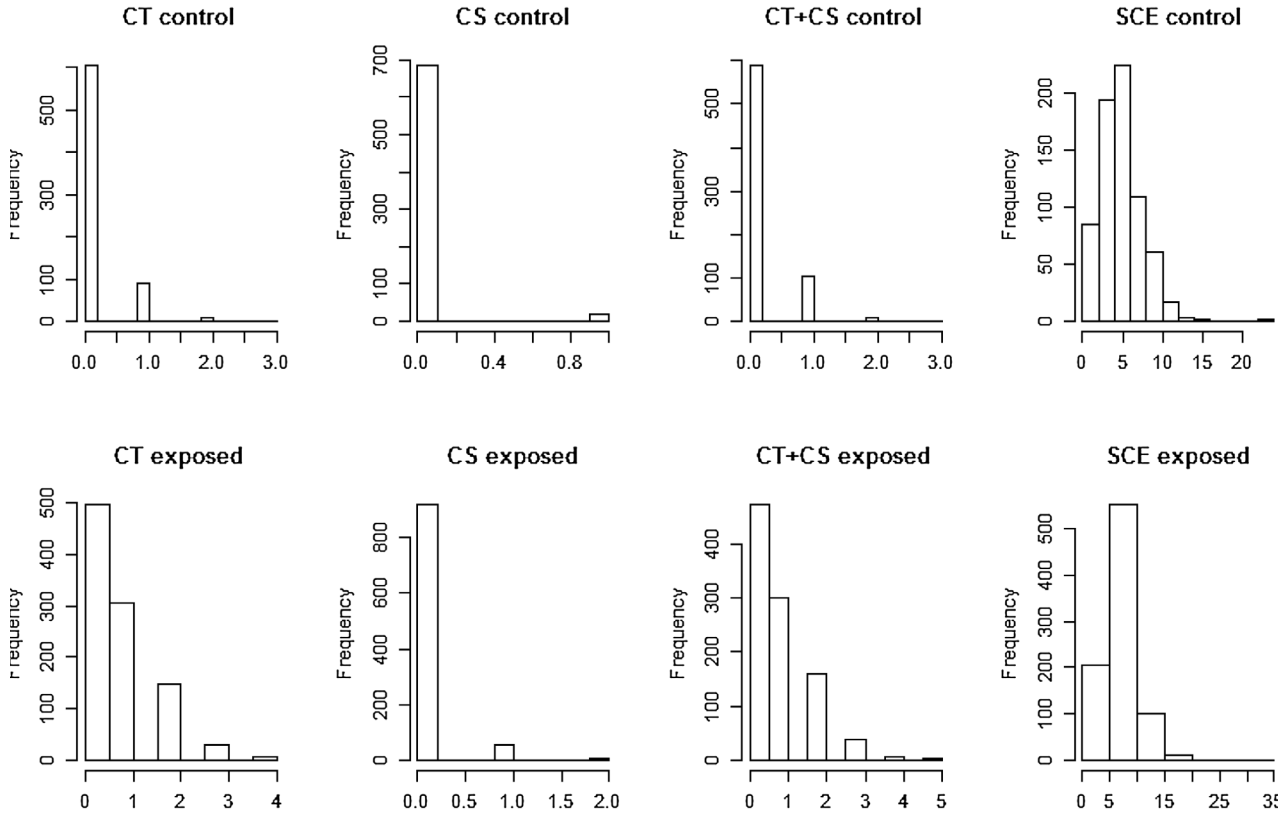

Figure 4. The histograms of variables showing a no normal distribution.

Table 5. Shapiro Wilk test for normality.

	Control				Exposed			
Variables	CS	CT	CT+CS	SCE	CS	CT	CT+CS	SCE
Score	0.1373	0.4140	0.4511	0.9455	0.2601	0.7735	0.7865	0.9531
P-value	2.2e-16	2.2e-16	2.2e-16	3.084e-15	2.2e-16	2.2e-16	2.2e-16	5.055e-16

H_0: The variable is distributed as a normal.

Table 6. Mann–Whitney non-parametric test for equality of means.

	Control versus exposed			
Variables	CS	CT	CT+CS	SCE
SCORE	356429.5	472458.5	475338.5	427537.5
P-value	9.35e-05	2.2e-16	2.2e-16	2.2e-16

H_0: Control and exposed groups are from the same population and have the same mean.

Table 7. Permutation test for equality of deviance.

	Control versus exposed			
Variables	CS	CT	CT+CS	SCE
SCORE	2.9	4.8	4.3	1.2
P-value	1e-04	1e-04	1e-04	3e-04

H_0: Control and exposed groups are from the same population and have the same deviance.

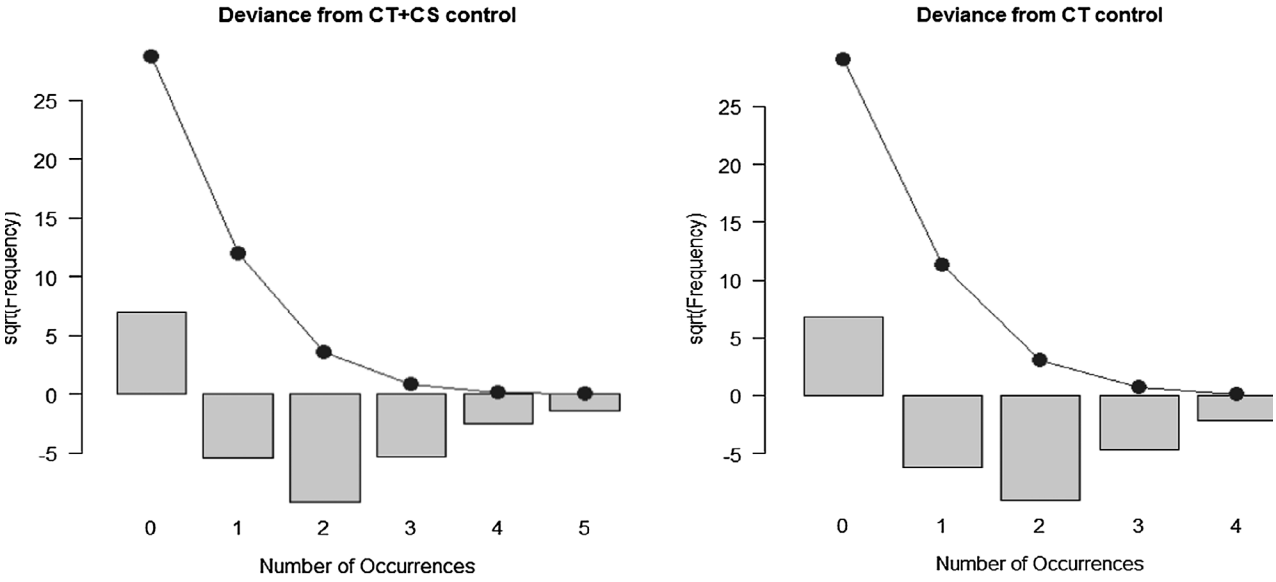

Figure 5. Deviance of exposed CT+CS and CT from the control sample distributions.

probability of originating unbalanced gametes during meiosis, and unbalanced embryos, which can die in early embryonic life. Alternatively, the animal may have an abortion or abnormal fetuses as those occurring in sheep exposed to relatively high levels of dioxins (Perucatti et al. 2006).

Several effects of dioxins, including those of DL-PCBs, are mediated by the aryl hydrocarbon receptor (AhR), an intracellular protein which binds the dioxin molecule and transports them into the nucleus where AhR forms a complex with ARNT (AhR nuclear translator), which is able to promote and regulate the transcription of specific genes (i.e. *ARNT*, *AHR*, *CYP1A1*, *CYP1A2*, *CYP1B1* and *AHRR*) (Mandal 2005; Beischlag et al. 2008; Hung et al. 2013). These loci have recently been FISH mapped in domestic bovids, including cattle (Genualdo et al. 2011). AHR-KO rats had lower basal expression of transcripts for these genes and also accumulated ~30–45-fold less TCDD in the liver at seven days post-exposure. In untreated animals, AHR-KO mice, but not AHR-KO rats, had alterations in serum analytes indicative of compromised hepatic function, patent ductus venosus of the liver and persistent hyaloid arteries in the eye. Furthermore, AHR-KO rats, but not AHR-KO mice, displayed pathological alterations to the urinary tract: bilateral renal dilation (hydronephrosis), secondary medullary tubular and uroepithelial degenerative changes and bilateral ureter dilation (hydroureter) (Harrill et al. 2013).

As shown in Table 1, the main component of dioxins present in the milk mass of the exposed cows are DL-PCBs, further supporting their origin from industrial processes such as those producing steel in the Taranto city industrial area (metallurgic factory).

Cytogenetic analyses performed in the exposed Friesian cows revealed a significantly higher number of abnormal cells in exposed animals (0.52 ± 0.59) than those of the control (0.16 ± 0.37) (Table 2). This is due to a significantly increasing mean number of both chromatid and chromosome breaks per cell of exposed cows compared to those of the control (Table 2). Indeed, significant differences ($p < 0.01$) were also found when comparing the two groups of cows by examining the total mean number of CA (0.79 ± 0.92 in the exposed cows and 0.17 ± 0.42 in the control), as well as by examining the chromatid breaks (0.72 ± 0.86 in the exposed cows and 0.15 ± 0.39 in the control) and chromosome breaks (0.07 ± 0.28 in the exposed cows and 0.02 ± 0.15 in the control) alone (Table 2).

The CA values found in the present study in both exposed and control animals are similar to those found in cells of other breeds (cattle) and species (sheep and river buffalo) naturally exposed to dioxins (reviewed in Genualdo et al. 2012). The mean CA value in control animal cells of the present study does not differ greatly from those reported in some human control lymphocytes (Bryant et al. 2004), although most studies in human lymphocytes (Liou et al. 1999; Bonassi et al. 2008;

Costa et al. 2014) reported lower CA values than those achieved in the present study and in other previous studies on the same topic in domestic animals (reviewed in Genualdo et al. 2012). The higher CA values found in both exposed and control animal cells, compared to those achieved in human control cells, can in part be explained considering that (i) the animals are below humans in the food chain; and (ii) the soil may be present in the diet, especially during pasturage. In the soil matrix the mutagens are present in higher quantities than in the plants (grass). Dioxins are permitted at higher values in the soil (10 ng kg^{-1}) than in grass (0.75 ng kg^{-1}) (Table 1). Also the acridine orange staining used in this and our previous studies can in part explain the higher CA values compared to CA values reported using Giemsa staining. However, only using acridine orange staining in human cells we can draw final conclusions about this technical aspect.

The chromosome fragility found in the Friesian cows in the present study using the CA test was also confirmed when applying the SCE test, as the SCE mean value was significantly higher ($p < 0.01$) in the exposed cows (7.41 ± 3.02) than in the control (5.30 ± 2.61) (Table 3).

In conclusion, both cytogenetic tests used for the first time on Friesian cows exposed to dioxins (mainly DL-PCBs) showed a pronounced chromosome fragility in the cells of exposed cows compared to that found in the control group cells. It is possible that increasing chromosome damage observed in Friesian cows could originate from synergic action of both dioxins (mainly PCBs in the present case) and other mutagens present in the industrial area of Taranto city. This suggests that farm animals should be raised far from industrial areas to avoid accumulation of toxic chemicals in their products. At the same time, domestic animals can be considered important environmental sentinels of the food chain. Indeed, systematic controls of animal products for the presence of chemicals, accompanied by genetic tests, like those performed in this study, are very useful to organize a correct food chain so to reduce the risk for human populations, especially those living close to industrial areas.

Disclosure statement

No potential conflict of interest was reported by the authors.

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