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Chromosome fragility in river buffalo cows exposed to dioxins

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Abstract Fifty river buffalo (*Bubalus bubalis*, 2n=50) cows reared in two different provinces of Campania (southern Italy) underwent cytogenetic investigations to ascertain possible differences in their chromosome stability. One group (Caserta province) was under legal sequestration due to the presence in the milk mass of higher mean values of dioxins [21.79 pg/g of fat as sum of polychloro-dibenzo-dioxins (PCDDs), polychloro-dibenzo-furans (PCDFs) and dioxin-like polychlorobiphenyls (DL-PCBs)] than both those permitted (6.0 pg/g of fat as WHO-TEQ) and those (1.3 pg/g of fat as WHO-TEQ) observed in the control group raised in Salerno province. Two types of peripheral blood cell cultures were performed: without (normal cultures for the chromosome abnormality (CA) test: chromatid breaks, chromosome breaks, fragments) and with the addition of BrdU for the sister chromatid exchange (SCE) test). The CA test revealed a significantly ($P<0.01$) higher chromosome fragility in the exposed cows compared to the control. Indeed, mean values of CA/cell were 1.26±1.15 in exposed cows and 0.37±0.71 in the control. Mean SCE was higher in exposed cows (8.50±3.35) than that (8.29±3.51) found in the control but the difference was not significant. Comparison within the same group of cows at first (FL) and multiple (ML) lactations revealed significantly ($P<0.01$) higher mean values of CA/cell in exposed ML-cows vs FL-cows while no statistical differences were found between ML-cows and FL-cows in the control farm. By contrast, significantly ($P<0.01$) higher mean values of SCE were found in both groups of FL-cows versus ML-cows. Comparisons with other previous studied species (sheep and cattle) were also performed.

Keywords Chromosome abnormality · Chromosome fragility · Dioxin · River buffalo · SCE

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Introduction

Dioxins are considered highly toxic pollutants. While it has been difficult to establish specific health effects in humans due to the lack of controlled dose experiments, studies in animals have shown that dioxins cause a wide variety of toxic effects. They are divided into three main groups: polychloro-dibenzo-dioxins (PCDDs), polychloro-dibenzo-furans (PCDFs) and dioxin-like polychlorobiphenyls (DL-PCBs). Among hundreds of molecules belonging to these chemical congeners, 17 (PCDDs+PCDFs) and 12 (DL-PCBs) chemicals are normally investigated during food control. Of these, tetrachloro-dibenzo-*p*-dioxin (TCDD) has been considered the most toxic, being reported to be teratogenic, mutagenic, carcinogenic, immunotoxic, and hepatotoxic (Bock and Kohle 2004; Steenland et al. 2004). The most sensitive effects, observed in many species, appear to be developmental, including effects on the developing

immune, nervous, and reproductive systems (Mandal 2005). TCDD has a toxicity equivalent of 1, all remaining 28 molecules (among PCDDs, PCDFs, DL-PCBs) having lower toxicity equivalent (TEQ) coefficients. Each of these chemicals is detected in pg/g of fat and this value is then corrected by a specific TEQ-coefficient so as to obtain a final value expressed in whole toxicity equivalent (WHO-TEQ).

The toxicity of dioxins is also amplified by their high level of persistence in the environment, especially when entering the human or animal body due to their ability to be absorbed by fat tissue. Their half-life in the body is estimated to be between seven to 11 years (Wolfe et al. 1994), although more recent studies report lower durations (Ogura 2004). Hence 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. In milk these values are 3.0 pg/g of fat for PCDDs+PCDFs as WHO-TEQ and 6.0 pg/g of fat as the sum of PCDDs+PCDFs+DL-PCBs as WHO-TEQ.

Although dioxins are formed locally, their environmental distribution is global. The highest quantities of these congeners, especially PCBs, seem to be particularly concentrated in the Northern part of the earth: some studies have revealed very high concentrations of PCBs in the Barents sea area where the whole food chain seems to be widely contaminated by these congeners which reach their highest quantities in the Glaucous gull (*Larus hyperboreus)* at the top of the food chain (Bustnes et al. 2003; Erikstad et al. 2011). Dioxins are found throughout the world in practically all media, particularly in some soils, sediments and food, especially dairy products, meat, fish and shellfish. Very low levels are found in plants, water and air.

Since 2006, in addition to PCDDs and PCDFs, DL-PCBs have been investigated in animal products following the EC Regulation No. 199/2006. Most PCDDs, PCDFs and DL-PCBs are produced by both industrial processes and illegal waste burning. The latter has occurred widely in Campania during the last 20 years, thus explaining the presence of dioxins in the milk of animals, especially sheep (Iannuzzi et al. 2004; Perucatti et al. 2006). However, appreciable levels of dioxins have also been found in some industrial areas of other Italian regions, namely Piedmont, Lombardy, Tuscany and Puglia (reviewed in Di Meo et al. 2011).

Cytogenetic tests could be useful to reveal the presence of chromosome damage due to the mutagens present in the food chain by simply monitoring food-producing species. Indeed, several mutagens can cause cancers and high frequencies of chromatid breaks have been found in cell blood from a high percentage of cancer sufferers (Bryant et al. 2004), although the issue of induced chromosome damage in cells exposed in vivo or in vitro to dioxins still remains unclear due to the contradictory results attained so far (reviewed in Iannuzzi et al. 2004; Perucatti et al. 2006).

In previous reports we studied sheep exposed to dioxins (PCDDs+PCDFs only) and cattle exposed to both PCDDs+PCDFs and DL-PCBs, and found pronounced chromosome fragility in exposed herds (especially in sheep), compared to unexposed herds of both sheep and cattle (control) by using both chromosome abnormality (CA: gaps, chromatid breaks, chromosome breaks, fragments) and sister chromatid exchange (SCE) tests (Iannuzzi et al. 2004; Perucatti et al. 2006; Di Meo et al. 2011).

Recently, two river buffalo farms which showed both higher and lower dioxin values than those permitted were investigated to evaluate the effect of exposure to dioxins on the plasma redox status of lactating buffalo cows (Spagnuolo et al. 2011). Statistical differences between the two groups of cows suggest that exposure to dioxins impairs the plasma antioxidant defense system of lactating buffalo cows, and that metabolic processes associated with dioxin detoxification might induce or enhance oxidation of protein and lipids (Spagnuolo et al. 2011).

In the present study we compared the same two groups of river buffalo cows by using both the CA and SCE tests and demonstrate higher chromosome fragility in river buffalo cows exposed to dioxins, compared to that of the control group, but only with the CA test, differences in mean SCE values of the two groups being not statistically significant. Furthermore, we found statistical differences within the same group when comparing cows at the first and multiple lactations.

Material and methods

Animals and dioxin analyses

We studied 50 river buffalo cows (varying from 3-6 years old) randomly sampled from two different farms (25 animals each), located in two different provinces of Campania (southern Italy). Within each group, there were cows at the first (FL) and multiple (ML) lactation. One farm (farm A), located in Caserta province, was under legal sequestration due to the presence in the milk mass of higher dioxin values (sum of DCDDs+DCFFs+DL-PCBs as WHO-TEQ) than both those permitted and attained in the control performed in a farm (farm B) in Salerno province. Chemical analyses in the milk mass (representative of all farm milk production) of the two farms were performed by specialized laboratories under local veterinary health control.

Cell cultures

Peripheral blood cell cultures were performed at 37.8 °C in RPMI medium, enriched with fetal calf serum (10 %), L-glutamine (1 %), antibiotic-antimycotic mixure (1 %) and Concanavalin A (15 μg/ml) as mitogen. Two different types

Table 1 Results of the chemical analyses for searching PCDDs, PCDFs and PCBs in milk mass of exposed and control river buffalo cows. The values between parentheses are those permitted

Source	WHO-PCDD/F-TEQ pg/g	WHO-PCB-TEQ pg/g	WHO-PCDD/F-PCB-TEQ pg/g
Exposed	17.00 (3.0)	4.79	21.79 (6.0)
Control	n.d.	n.d.	1.30 (6.0)

of cell cultures were performed for 48 h (normal cultures) and 72 h, the latter with the addition of 5-Bromodeoxyuridine (BrdU) 28 h before harvesting. Colcemid (0.01 μg/ml) lasted 1.5 h for both cell cultures. Slides obtained from normal cultures were used to study chromosome abnormalities (CA test), namely gap, chromatid breaks, chromosome breaks, and fragments, while those treated with BrdU were used to study sister chromatid exchanges (SCE test). Slides from both types of cell cultures were stained for 10 min with acridine orange (0.01 % in P buffer), 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 connected to a digital camera. At least 50 cells for the CA test and 35 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 and standard deviations of both CA and SCE were calculated for both single animals and animal groups (farm A, farm B, cows at the first lactation, cows at multiple lactations). Statistical analyses were performed among group A (exposed) and B (control), as well as between cows at the first and multiple lactations within the same farm by using a 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 (21.79 pg/g of fat as the sum of PCDDs+PCDFs+DL-PCBs as WHO-TEQ) in group A than both those permitted and those reached in the control (farm B) (1.3 pg/g of fat as WHO-TEQ) (Table 1). Moreover, most of the dioxins present in the exposed cows were essentially due to the presence of a PCDDs+PCDFs component (17.0 pg/g of fat as WHO-TE), being DL-PCBs 4.79 pg/g of fat as WHO-TEQ (Table 1).

Cytogenetic analysis

The mean value of abnormal cells (with at least one chromatid break, chromosomal break and fragment) was significantly ($P < 0.01$) higher in farm A animals (0.70±0.46) than the control (0.28±0.45) (Table 2). Significant differences were found between the two groups of animals when comparing mean CA values (chromosome breaks+chromatid breaks+fragments, Fig. 1) which were 1.26±1.15 and 0.37±0.71 in farm A and B (control), respectively (Table 2). Significant differences were found between farms A and B even when considering

Table 2 Number of animals studied, examined cells, abnormal cells, chromatid breaks (ct), chromosome breaks (cs) and fragments (fg) in river buffalo cows exposed to dioxins and control cows as well as in cows at the multiple (ML) and first (FL) lactations within the same farm

Animals (*n*)	Examined cells (*n*)	Abnormal cell *n* Mean ± SD	Chromatid breaks *n* Mean ± SD	Chromosome breaks *n* Mean ± SD	Fragments *n* Mean ± SD	ct+cs+fg *n* Mean ± SD
Exposed total FL+ML (25)	1250	886 0.70[a]±0.46	1277 1.02[a]±0.99	282 0.23[a]±0.50	17 0.01±0.12	1576 1.26[a]±1.15
Control total FL+ML (25)	1250	344 0.28±0.45	316 0.25±0.52	135 0.11±0.37	14 0.01±0.11	465 0.37±0.71
Exposed ML (10)	500	375 0.75[a]±0.43	581 1.16[a]±1.04	129 0.26[a]±0.53	8 0.02±0.12	718 1.44[a]±1.22
Control ML (15)	750	208 0.28±0.45	195 0.26±0.55	92 0.12±0.40	12 0.02±0.13	299 0.40±0.77
Exposed FL (15)	750	501 0.67[a]±0.47	696 0.93[a]±0.94	153 0.20[a]±0.47	9 0.01±0.11	858 1.14[a]±1.08
Control FL (10)	500	136 0.27±0.45	121 0.24±0.48	43 0.09±0.32	2 0.00±0.06	166 0.33±0.61
Exposed ML (10)	500	375 0.75[b] ±0.43	581 1.16[b]±1.04	129 0.26±0.53	8 0.02±0.12	718 1.44[b]±1.22
Exposed FL (15)	750	501 0.67±0.47	696 0.93±0.94	153 0.20±0.47	9 0.01±0.11	858 1.14±1.08
Control ML (15)	750	208 0.28±0.45	195 0.26±0.55	92 0.12±0.40	12 0.02±0.13	299 0.40±0.77
Control FL (10)	500	136 0.27±0.45	121 0.24±0.48	43 0.09±0.32	2 0.00±0.06	166 0.33±0.61

[a] Significantly different versus controls ($P<0.01$)

[b] Significantly different versus FL ($P<0.01$)

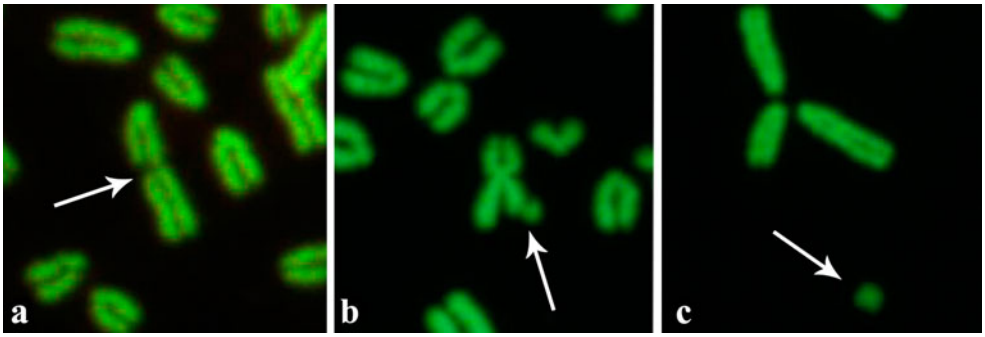

Fig. 1 Details of river buffalo metaphase plates showing chromosome breaks (**a**), chromatid breaks (**b**) and fragments (**c**) (arrows)

chromatid breaks and chromosome breaks separately (Table 2), differences between fragments being not significant.

Comparison between cows at the multiple and first lactations of the same farm revealed significantly ($P<0.01$) higher mean values of CA in cows at multiple lactations than those at the first lactation but only in farm A (exposed animals), differences not being significant between cows at first and multiple lactations in farm B (control).

SCE (Fig. 2) mean value/cell was higher (8.50±3.35) in the exposed cows compared to the control group (8.29±3.51) but the difference was not significant (Table 3). Statistical differences ($P<0.01$) were found between SCE mean values when considering cows at the first and multiple lactations of both groups: SCE mean values were 8.28±3.39 and 8.75±3.29 in ML- and FL-cows, respectively, of farm A, and 7.92±3.51 in ML and 8.84±3.43 in FL-cows of farm B.

Table 4 shows the comparison between data obtained in the present study as well as in previous studies performed in sheep (Perucatti et al. 2006) and cattle (Di Meo et al. 2011) by using both CA- and SCE-test. River buffalo shows a higher chromosome fragility in both exposed and control groups than that found in both sheep and cattle when observing data on abnormal cells (0.70 in river buffalo, 0.40/0.22 in sheep and 0.46/0.36 in cattle exposed animals, as well as 0.28 in river buffalo, 0.10 in sheep and 0.11 in cattle control animals) and CA-test (1.26 in river buffalo, 0.58/0.33 in sheep and 0.64/1.1 in cattle exposed animals, as well 0.37 in river buffalo, 0.11 in sheep and 0.13 in cattle control animals). The highest SCE-values were found in exposed sheep herds (11.07/11.03) compared to both exposed cattle (7.0/6.38) and river buffalo (8.50) cows, while the highest control SCE value was found in river buffalo (8.29) being SCE-mean values in control sheep and cattle lower (7.90 and 5.20, respectively).

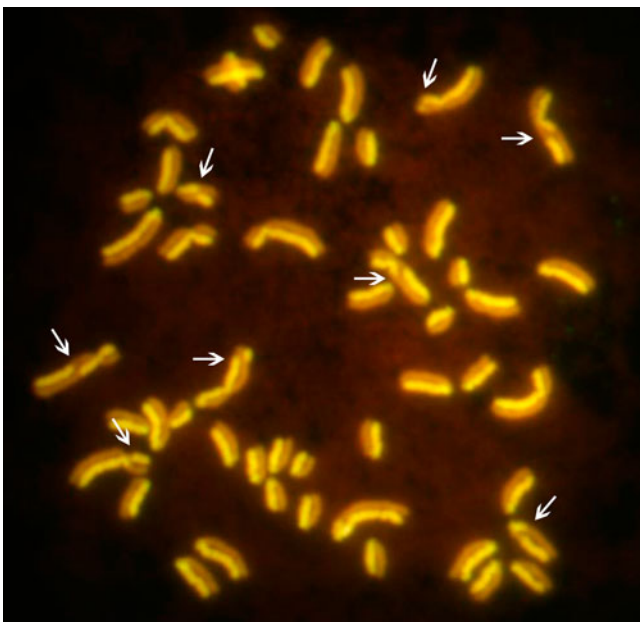

Fig. 2 Female river buffalo metaphase plate showing several SCEs (arrows)

Discussion

Chromatin damage can be induced by several environmental mutagens (Bryant et al. 2004). As the 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 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

Table 3 Cells examined and SCE mean values in exposed and control animals, as well as in cows at the first (FL) and multiple (ML) lactations

Animals (n)	Examined cells (n)	SCE/cells		
		n	Mean	±SD
Exposed (25)	875	7440	8.50	3.35
Control (25)	875	7253	8.29	3.51
Exposed FL (12)	420	3674	8.75 [a]	3.29
Exposed ML (13)	455	3766	8.28	3.39
Control FL (10)	350	3093	8.84[a]	3.45
Control ML (15)	525	4160	7.92	3.51

[a] Significantly different versus ML ($P<0.01$)

Table 4 Number of studied animals, examined cells, abnormal cells, CA (ct+cs+fg) and SCEs observed in exposed (and control) sheep (Perucatti et al. 2006), cattle (Di Meo et al. 2011) and river buffalo cows (present study)

Species	Chromosome abnormalities (CA)				Sister chromatid exchange (SCE)		
	Animal/group (n)	Examined cells (*n*)	Abnormal cell *n* Mean ± SD	CA (ct+cs+fg) *n* Mean ± SD	Animal/ group	Examined cells (*n*)	SCEs *n* Mean ± SD
Sheep	I group exp (42)	2408	535 0.22±0.42 [a]	788 0.33±0.75 [a]	I group exp (23)	715	7883 11.03±3.61 [a]
	II group exp (34)	1714	694 0.40±0.49 [a]	997 0.58±0.84 [a]	II group exp (29)	1228	13593 11.07±3.77 [a]
	Contr (20)	1088	109 0.10±0.30	123 0.11±0.36	Contr (20)	600	4744 7.90±3.10
Cattle	I group exp (18)	900	386 0.43±0.49[a]	581 0.65±0.91[a]	I group exp (18)	630	4418 7.00±2.87 [a]
	II group exp (18)	900	328 0.36±0.48 [a]	462 0.51±0.81[a]	II group exp (18)	630	4022 6.38±2.80 [a]
	Contr (19)	950	106 0.11±0.31	123 0.13±0.40	Contr (19)	665	3455 5.20±2.50
Buffalo	I group exp (25)	1250	881 0.70±0.46 [a]	1576 1.26±1.15 [a]	I group exp (29)	875	7440 8.50±3.35
	Contr (25)	1250	344 0.28±0.45	465 0.37±0.71	Contr (23)	875	7253 8.29±3.51

[a] Significantly different versus controls ($P<0.01$)

abnormal foetuses such as those occurring in sheep exposed to relatively high levels of dioxins (Perucatti et al. 2006).

Cytogenetic testing applied to both human and animal populations exposed to dioxins generated contradictory results (reviewed in Iannuzzi et al. 2004; Perucatti et al. 2006), although studies on the chromatin revealed the presence of localized and discontinuous changes due to dioxin (TCDD).

The best known molecular mechanism related to the action of dioxins is that of the aromatic hydrocarbon receptor (AhR), an intracellular protein which binds the dioxin molecule when entering the cells, opening the door to reach the nucleus where TCDD-AhR forms a complex with ARNT (AhR nuclear translator). This TCDD-AhR-ARNT complex activates genes encoding for TCDD metabolizing enzymes (Mandal 2005; Beischlag et al. 2008). The most important loci contacting genes involved in the dioxin metabolism were recently FISH-mapped in domestic bovids, including river buffalo (Genualdo et al. 2011).

In Campania, especially in the provinces of Naples and Caserta, several livestock farms, especially those raising sheep, have been found to have higher levels of dioxins than those legally permitted (Iannuzzi et al. 2004; Perucatti et al. 2006). In river buffalo, with the exception of a few farms in the province of Caserta, almost all analyses performed in search of dioxins in the milk mass revealed dioxin values below those legally permitted, especially in samples of both milk and protected denomination origin (PDO) mozzarella cheese (Santelli et al. 2006).

As shown in Table 1, the main component of dioxins present in farm A comprises PCDDs+PCDFs, further supporting the origin of dioxins in Caserta from illegal waste-burning. By contrast, chemical analysis of dioxin-exposed cattle raised in Piedmont (northern Italy) revealed that DL-PCBs were the main component present in the milk mass (Di Meo et al. 2011).

The cytogenetic investigation we performed in river buffalo cows revealed a significantly larger number of abnormal cells in exposed animals than that of the control (Table 2). This is essentially due to a significantly increasing mean number of CA in cells of exposed cows compared to those of the control (Table 2). Indeed, total mean number of CA was significantly higher in the exposed cows compared to that of the control (Table 2). These significant differences were also found when considering each parameter alone, excluding chromosome fragments (probably due to the small number of chromosome fragments found in this study).

When we consider the cows of each group at multiple (ML) and first (FL) lactations, we found significantly higher mean values of CA in ML-cows than those achieved in FL-cows (Table 2), while no significant differences were found in the CA-mean number of ML and FL control cows (Table 2). This could be explained considering that dioxins are accumulated in the body (fat) and older cows (ML) could have larger amounts of dioxins that those present in younger ones (FL).

As regards the SCE test, SCE mean values were higher in the exposed cows (8.50±3.35) than in the control (8.29±3.51) but the difference was not significant (Table 3). In previous studies performed in both sheep and cattle, both tests revealed significantly higher values of both CA and SCEs in the exposed animals compared to the control (Iannuzzi et al. 2004; Perucatti et al. 2006; Di Meo et al. 2011). However, river buffalo generally show higher levels of SCE compared to other domestic bovids (Iannuzzi et al. 1988), which may have affected the results achieved with the SCE test in both exposed and control cows.

Significantly higher mean values of SCE were found in both exposed and control FL-cows compared to ML-cows. Also here there is a discrepancy with the CA test which shows the opposite result. These discrepancies are probably due to the few ML- and FL-cows studied in each group.

Comparisons between present data and those obtained in previous studies in both sheep (Perucatti et al. 2006) and cattle (Di Meo et al. 2011) (Table 4) show that river buffalo has a higher chromosome fragility (CA-test) in both exposed and control animals than that achieved in both sheep and cattle, while the highest SCE-mean value was found in exposed sheep (Table 4). However, being that the feeding of sheep (natural pasture) is different from that of both cattle and river buffalo (silage and concentrate), it is possible that sheep have ingested other chemicals (i.e., heavy metals) present in the soil (but not determined in the chemical analyses) which could have increased the number of SCEs in the exposed animals.

Conclusion

Although it is difficult to state that chromosome fragility found so far in livestock is due only to the presence of dioxins, this study further supports the hypothesis that chromosome tests can ascertain the safety of the food chain by simply monitoring livestock species. Certainly, chemical analyses on single animals (correlated to the chromosome test which we performed on single animals) could give more precise information about the relation between the presence of dioxins and chromosome fragility.

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