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Increased frequencies of both chromosome abnormalities and SCEs in two sheep flocks exposed to high dioxin levels during pasturage

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During the past four years several livestock farms (sheep, cattle and river buffalo) in the provinces of Naples and Caserta (southern Italy) have been unable to sell their milk and other dairy products due to the levels of dioxins (17 different types) present in the milk mass exceeding the value permitted [3 pg/g of fat, as human WHO 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) equivalent]. While some farms, especially those showing relatively low levels of dioxins, have managed to reduce the dioxins in the milk below the permitted threshold by changing the diet, many, especially sheep farms, have failed to do so. Indeed, about 12 000 head of cattle, river buffaloes and sheep have so far been culled. In the present study, 34 and 42 sheep from two herds raised in the province of Naples (Acerra municipality) and where high levels of dioxins (50.65 and 39.51 pg/g of fat, respectively) were found in the milk mass, were cytogenetically investigated and compared with 20 sheep (control) raised 80 km away from the exposed area. Increases of both chromosome abnormalities (gap, chromosome and chromatid breaks) (17 and 8 times higher in the two exposed herds, respectively) and sister chromatid exchanges (SCEs) were found in both herds when compared with the control, and the differences were highly significant ($P < 0.001$). No statistical differences were found when comparing the frequencies of aneuploid cells of exposed animals (16.4 and 17.8%) and control (17.9%). Furthermore, high levels of mortality and abnormal foetuses were recorded in one of the two farms when compared with the control.

Introduction

During a routine analysis performed in 2002 in search of dioxins in the milk mass of some farm animals raised in Campania (southern Italy), some tested positive [levels of dioxins >3 pg/g of fat as toxic equivalent (TEQ)]. Systematic analyses were then performed in most of the cattle; river buffalo and sheep dairy farms in the provinces of Naples and Caserta; many tested positive, and their milk and other dairy products were unmarketable for a long period of time. Although some farms, especially those showing relatively low dioxin levels, were able to reduce the quantity of dioxins below the permitted limit value by changing animal diets, many farms, especially those raising sheep, did not. Hence, about 12 000 head of cattle, river buffaloes and sheep were culled at the end of 2003. Chemical analyses performed by specialized laboratories and the regional environmental agency (ARPA) in search of dioxins in soil, water and grass established that most of the samples were normal (below the legal threshold).

Dioxins are a group of toxic chemicals which includes both polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Dioxins have been reported to be carcinogenic and to induce toxic and biochemical responses in the endocrine, reproductive and immune systems (1–6). They are not naturally present in the environment because they are produced during the manufacture of herbicides, pulp and paper, by fires involving chlorinated benzenes, by municipal waste incinerators or simply by illegal burning of domestic waste. Dioxins are accumulated in the body, especially in the fat, for a long time (the half-life varies between 7 and 11 years) (7,8). Undoubtedly, milk represents one of the most important sources of dioxin contamination not only in animals but also in humans (9).

The few cytogenetic studies performed in both humans and animals (mouse and monkey) exposed *in vivo* or *in vitro* to dioxins reported conflicting data with or without chromosome damage (reviewed in ref. 10). However, molecular studies revealed damage to the chromatin structure in cells of individuals exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (11). Fletcher *et al.* (12) suggest that even low-dose TCDD exposure can alter the expression of several genes, indicative of cellular stress or DNA damage associated with cell cycle control. The toxic effect of dioxins seems to be mediated through the aryl hydrocarbon receptor (AhR) with a formation of heterodimers with the AhR nuclear translocator (ARNT) which regulate the expression of the genes involved (12,13).

In a preliminary study we found a chromosome fragility in two sheep flocks (24 animals) exposed to relatively low levels of dioxins (5.25 pg/g of fat) and which were also found positive to the *Brucella abortus* (50% of animals), compared with that of unexposed sheep (control) (10). In the present study, a larger sample of animals (76 animals) from two sheep flocks exposed to relatively high levels of dioxins during pasturage and which were *Brucella abortus* free, were investigated and compared with sheep raised far from the contaminated area. High levels of both chromosome abnormalities (gap, chromatid and chromosome breaks, fragments) and sister chromatid exchanges (SCEs) were found in the exposed animals compared with the control.

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Material and methods

Sheep herds

The two sheep herds numbered 330 animals (Farm A) on January 24, 2005 and 450 animals (Farm B) in December 2003. However, the numerical consistency of herd A was 680 animals in 2002. They were extensively raised in province of Naples, especially in the municipality of Acerra territory. They were hybrids of different breeds, one of which (Comisana) was predominant. The sheep used as control (all from the Comisana breed) were raised about 80 km away (Mt. Matese, Benevento province). The age of both exposed animals and control varied between 2 and 5 years. Both exposed and unexposed (control) herds were naturally pastured. Both the exposed herds were under legal sequestration from 2002. While all animals from Farm A were still alive as at October 2005, those of Farm B were culled in December 2003. Animal mortality in Farm A in the last three years (2002–2004) was recorded by the veterinary service of the local health authority (ASL-NA4), while the number of both abortions and abnormal foetuses was recorded by the farmer. The percentages of abortions and abnormal foetuses were calculated on the basis of the total births in the three years. No data concerning the mortality, abortion and abnormal foetuses are available for the herd of Farm B (all animals were culled in December of 2003 and we only received blood samples prior to culling).

Chemical analyses

Chemical analyses to ascertain the presence of dioxins (17 different types of dioxins and furans) in the milk mass of the two herds, as well as in the soil and grass of the pasturage area, were performed by specialized laboratories under the control of the local health authorities.

Cell cultures

Thirty-four ewes from Farm A and forty-two from Farm B were randomly sampled, as well 20 ewes as control, and investigated cytogenetically. Blood samples were collected at two different times in the exposed herds and only once in the control. In the first sample only normal cultures (without the addition of any base-analogue) were performed in the exposed herds, while both normal and 5-Bromodeoxyuridine (BrdU)-treated cell cultures were performed in the second sample, as well as for all 20 control ewes.

Peripheral blood samples from both exposed animals and control were cultured for about 72 h in RPMI medium (Gibco-BRL), foetal calf serum (10%), penicillin-streptomycin (1%) and Concanavalin A (15 μg/ml) (Sigma) as mitogen. Two types of cell cultures were performed: without (normal cultures) and with the addition of BrdU (10 μg/ml) during the last two cell cycles for the SCE test. In the latter, BrdU was added 30 h before harvesting (this is the best time in our lab to reach almost all cells at the second cell cycle of replication in the presence of BrdU). Colcemid (0.5 μg/ml) was added 1.5 h before harvesting. Hypotonic treatment (20 min) and three fixations (the third overnight) in methanol-acetic acid (3:1) followed.

Slides obtained from normal cultures were stained for 10 min with acridine orange (0.01% in phosphate buffer), washed in tap water and distilled water, air dried, mounted with a glass coverslip using a phosphate buffer and sealed with rubber cement. Slides from BrdU-treated cells were first stained with Hoechst 33258 (25 μg/ml) for 10 min, washed with distilled water and mounted with a glass coverslip using a phosphate buffer (0.8 ml for slide) with no pressure of coverslip. Slides were exposed to UV light for 30 min, then washed again with distilled water, air dried and stained with acridine orange as reported above. Slides from both types of cell cultures were observed a day later (or more) under two fluorescence microscopes (Nikon E-1000 and Aristoplan Leica) connected with digital cameras (Coolsnap CF, Photometrics). At least 50 and 30 cells were studied in the normal and BrdU-treated cells, respectively. For the SCE test only cells which replicated twice in the presence of BrdU were studied. Only chromatid gaps were considered to avoid errors due to the presence of less stained chromosome regions on both chromatids.

Statistical analysis

Statistical analyses among exposed animals and control were performed by determining whether or not there was evidence that the differences among the three cytogenetic tests we applied—aneuploidy, chromosome abnormalities (gap, chromatid and chromosome breaks, fragments) and SCE—were other than 0. The hypothesis test statistics all had a P value of 0.001 to better support possible differences between animals exposed to dioxins and animals unexposed (control).

Results

Chemical analyses

Chemical analyses revealed high values of dioxins in the milk mass of both herds (50.65 and 39.51 pg/g of fat in Herd A and B, respectively) compared with the legal value permitted (3 pg/g of fat as WHO-TE.) (Table I). Also the values of only TCDD were very high, reaching 7.32 and 8.13 pg/g of fat, respectively, compared with the background values (0.1 and 1.0 pg/g of fat) found in Italy and the Campania region, respectively. Both dioxin and polychloro-biphenyl (PCB) values were below the permitted limits in the soil (mean values among four different sites), while in grass, dioxins were higher (1.33 pg/g) and PCBs lower (0.31 pg/g) than those permitted (0.75 pg/g), even though they were obtained at only one site.

Abortions, abnormal foetuses and mortality

Sheep mortality (925 young and adult) on Farm A during the last three years was very high, while in the control no deaths were recorded in the same period. Also, the percentages of both abortion (12%) (mostly at the fourth month) and abnormal foetuses (4%) were higher when compared with the control (Table II). We were able to examine only one abnormal foetus. It showed an apparent normal body conformation, except for the head which was completely deformed: the two eyes were not completely separate, the anterior part was completely crushed, the nose was elongated like a small elephant proboscis and the mouth apparatus was very small.

Cytogenetic analyses

The percentages of abnormal cells which include aneuploidy (almost all cells were hypoploid), gaps, chromatid breaks, chromosome breaks and fragments, were considerably higher in both herds (95.9 and 84.5%) compared with the control (40.4%) (Table III). These differences were much more evident when considering the percentages of chromosome abnormalities (aneuploidy, gap, chromatid and chromosome breaks, fragments) on both the abnormal cells and total examined cells (Table IV). Indeed, when dividing the various chromosome abnormalities, while no differences were noticed between the percentages of aneuploid cells of exposed herds (16.4 and 17.8%) and control (17.9%), all other abnormalities showed higher percentages in the exposed herds compared

Table I. Results of the chemical analyses for searching 17 types of dioxins

Source	Dioxins+furans WHO-TE (pg/g)	PCB WHO-TE (pg/g)	TCDD (pg/g)
Milk-mass (herd A)	50.65 (3.0)		7.32 (0.1)
Milk mass (herd B)	39.51 (3.0)		8.13 (0.1)
Soil[a]	4.94 (10.0)	0.08 (1.0)	
Grass[b]	1.33 (0.75)	0.31 (0.75)	

Dioxins grouped as dioxins+furans and PCB's and expressed in 'Human-WHO-TCDD Equivalent' (WHO-TE) and TCDD alone on milk mass of two sheep herds, as well as on soil and grass in the area of Acerra Municipality where sheep are pastured. Maximum values permitted of dioxins and PCBs, as well as the mean national values of TCDD are reported in parentheses.
[a]Mean values between four sites.
[b]One site.

Table II. Number of births, abortions, abnormal foetuses and deaths (mortality) recorded

Herds (2002–2004)	Births *N*	Abortions *N* (%)	Abnormal foetuses *N* (%)	Deaths *N*
Exposed A	1600	192 (12.0)	64 (4.0)	925
Control	400	5 (1.2)	—	—

Table III. Percentages of normal and abnormal cells in herds exposed to dioxins and control animals

Animals (*n*)	Examined cells (*n*)	Normal cells		Abnormal cells	
		n	(%)	*n*	(%)
Exposed A					
1	50	0	0.0	50	100.0
2	50	1	2.0	49	98.0
3	51	9	17.6	42	82.4
4	50	0	0.0	50	100.0
5	50	4	8.0	46	92.0
6	50	0	0.0	50	100.0
7	50	9	18.0	41	82.0
8	50	1	2.0	49	98.0
9	51	11	21.6	40	78.4
10	51	0	0.0	51	100.0
11	51	0	0.0	51	100.0
12	53	1	1.9	52	98.1
13	52	0	0.0	52	100.0
14	50	1	2.0	49	98.0
15	51	1	2.0	50	98.0
16	50	0	0.0	50	100.0
17	50	0	0.0	50	100.0
18	50	4	8.0	46	92.0
19	50	1	2.0	49	98.0
20	50	2	4.0	48	96.0
21	50	2	4.0	48	96.0
22	50	4	8.0	46	92.0
23	51	0	0.0	51	100.0
24	50	5	10.0	45	90.0
25	52	0	0.0	52	100.0
26	50	0	0.0	50	100.0
27	50	0	0.0	50	100.0
28	50	7	14.0	43	86.0
29	50	5	10.0	45	90.0
30	50	0	0.0	50	100.0
31	50	0	0.0	50	100.0
32	51	0	0.0	51	100.0
33	50	0	0.0	50	100.0
34	50	2	4.0	48	96.0
Total (34)	1714	70	4.1	1644	95.9
Exposed B					
1	54	3	5.6	51	94.4
2	51	9	17.6	42	82.4
3	44	1	2.3	43	97.7
4	53	9	17.0	44	83.0
5	51	6	11.8	45	88.2
6	49	8	16.3	41	83.7
7	46	6	13.0	40	87.0
8	65	17	26.2	48	73.8
9	52	15	28.8	37	71.2
10	56	6	10.7	50	89.3
11	61	6	9.8	55	90.2
12	50	8	16.0	42	84.0
13	61	10	16.4	51	83.6
14	50	7	14.0	43	86.0
15	67	1	1.5	66	98.5
16	59	1	1.7	58	98.3
17	49	4	8.2	45	91.8
18	48	1	2.1	47	97.9
19	52	1	1.9	51	98.1
20	60	4	6.7	56	93.3
21	50	5	10.0	45	50.0
22	51	1	2.0	50	98.0
23	57	8	14.0	49	86.0
24	73	6	8.2	67	91.8
25	48	1	2.1	47	97.9
26	58	2	3.4	56	96.6
27	66	12	18.2	54	81.8
28	64	18	28.1	46	72.3
29	72	17	23.6	55	76.4
30	83	34	41.0	49	59.0
31	72	44	61.1	28	39.9
32	79	28	35.4	51	64.6
33	68	11	16.2	57	83.8
34	49	18	36.7	31	63.3
35	63	6	9.5	57	90.5
36	47	19	40.4	28	59.6
37	38	4	10.5	34	89.5
38	55	1	1.8	54	98.2
39	58	3	5.2	55	94.8
40	63	4	6.3	59	93.7
41	56	2	3.6	54	96.4
42	60	7	11.7	53	88.3
Total (42)	2408	374	15.5	2034	84.5
Control					
1	59	42	71	17	28.8
2	55	28	50.9	27	49.1
3	54	41	75.9	13	24.1
4	55	41	74.5	14	25.5
5	57	40	70.2	17	29.8
6	65	43	66.2	22	33.8
7	58	45	77.6	13	22.4
8	50	34	68.0	16	32.0
9	50	21	42.0	29	58.0
10	50	19	38.0	31	62.0
11	50	24	48.0	26	52.0
12	50	11	22.0	39	78.0
13	55	23	41.8	32	58.2
14	52	33	63.4	19	36.6
15	55	35	63.6	20	36.4
16	59	34	57.6	25	42.4
17	52	35	67.3	17	32.7
18	50	32	64.0	18	36.0
19	55	30	54.5	25	45.5
20	57	37	64.9	20	35.1
Total (20)	1088	648	59.6	440	40.4

with those of the control (Table IV). For example, the percentages of gaps were 329.4 and 151.9% in the cells of exposed herds compared with only 13.3% of the control. This is more evident on comparing data obtained in the diploid, aneuploid and total cells (Table V). Indeed, mean chromosome abnormalities per cell were 3.96 ± 2.31 and 1.88 ± 1.44 in exposed A and B herds, respectively and only 0.24 ± 0.5 in the control. These values were 17 and 8 times higher in exposed Herds A and B, respectively, than those observed in the control and clearly the differences were highly significant ($P < 0.001$).

The most common chromosome abnormalities in both exposed herds were the gaps, followed by chromatid breaks, aneuploidy, chromosome breaks and fragments. In the control, aneuploidy was the most common chromosome abnormality, followed by gaps, chromatid breaks, chromosome breaks and fragments. The occurrence of chromosome abnormalities in the two exposed herds was also significantly higher ($P < 0.001$) than that earlier observed in two herds exposed to lower levels of dioxins (5.27 pg/g) (10).

Also, when applying the SCE test we observed higher mean SCEs in the exposed herds than in the control (Table VI). Indeed, mean SCEs were 11.07 ± 3.77 and 11.03 ± 3.10 in exposed Herds A and B, respectively, when compared with the control (mean SCE 7.90 ± 3.10) and the differences were highly significant ($P < 0.001$). We also found mean SCEs in the exposed herds to be significantly higher ($P < 0.001$) than those found previously in sheep exposed to lower levels of dioxins (10).

Table IV. Percentages of chromosome abnormalities with respect to both abnormal cells (AC) and total cells examined (TC)

Animals (*n*)	Abnormal cells														
	Aneuploidy ($2n < 54$)			Gaps			Chromatid breaks			Chromosome breaks			Fragments		
	n	AC (%)	TC (%)	*n*	AC (%)	TC (%)	*n*	AC (%)	TC (%)	*n*	AC (%)	TC (%)	*n*	AC (%)	TC (%)
Exposed A															
1	9	18	18	233	466	466	20	40	40	1	2	2	3	6	6
2	11	22.45	22	141	287.7	282	20	40.8	40	2	4.1	4	1	2.04	2
3	11	26.19	21.57	71	169	139.2	16	38.1	31.4	2	4.8	3.9	0	0	0
4	7	14	14	264	528	528	24	48	48	1	2	2	2	4	4
5	13	28.26	26	111	241.3	222	27	58.7	54	2	4.3	4	1	2.2	2
6	9	18	18	170	340	340	25	50	50	1	2	2	1	2	2
7	8	19.5	16	51	124.4	102	20	48.1	40	1	2.4	2	2	4.9	4
8	2	4.08	4	112	228.6	224	69	140.8	138	1	2.04	2	0	0	0
9	6	15	11.76	53	132.5	103.9	6	15	11.8	2	5	3.9	1	2.5	1.9
10	6	11.76	11.76	265	519.6	519.6	27	52.9	52.9	1	1.9	1.9	0	0	0
11	11	21.57	21.57	170	333.3	333.3	31	60.8	60.8	4	7.8	7.8	0	0	0
12	10	19.23	18.87	170	326.9	320.7	43	82.7	81.1	5	9.6	9.4	0	0	0
13	3	5.77	5.77	250	480.8	480.8	20	38.5	38.5	1	1.9	1.9	1	1.9	1.9
14	12	24.49	24.00	123	251	246	22	44.9	44	5	10.2	10	0	0	0
15	17	34	33.33	161	322	315.7	11	22	21.6	1	2	1.9	3	6	5.9
16	11	22	22	241	482	482	27	54	54	1	2	2	2	4	4
17	4	8	8	206	412	412	20	40	40	1	2	2	1	2	2
18	6	13.04	12	78	169.6	156	15	32.6	30	1	2.2	2	0	0	0
19	4	8.16	8	180	367.3	360	55	112.2	110	0	0	0	0	0	0
20	5	10.42	10	189	393.7	378	20	41.7	40	1	2.1	2	0	0	0
21	8	16.67	16	112	233.3	224	38	79.2	76	1	2.1	2	1	2.1	2
22	5	10.87	10	86	186.9	172	39	84.8	78	3	6.5	6	0	0	0
23	4	7.84	7.84	224	439.2	439.2	47	92.1	92.1	3	5.9	5.9	3	5.9	5.9
24	3	6.67	6	99	220	198	21	46.7	42	3	6.7	6	0	0	0
25	4	7.69	7.69	168	323.1	323.1	34	65.4	65.4	7	13.5	13.5	0	0	0
26	6	12	12	267	534	534	42	84	84	3	6	6	0	0	0
27	7	14	14	258	516	516	28	56	56	3	6	6	0	0	0
28	2	4.65	4	89	207	178	19	44.2	38	1	2.3	2	0	0	0
29	7	15.56	14	142	315.5	284	23	51.1	46	3	6.7	6	0	0	0
30	11	22	22	225	450	450	6	12	12	2	4	4	4	8	8
31	7	14	14	193	386	386	18	36	36	1	2	2	0	0	0
32	8	15.69	15.69	281	551	551	42	82.3	82.3	5	9.8	9.8	0	0	0
33	31	62	62	139	278	278	4	8	8	1	2	2	0	0	0
34	13	27.08	26	124	258.3	248	18	37.5	36	4	8.3	8	0	0	0
Total (34)	281	17	16.4	5646	343.4	329.4	897	54.5	52.3	74	4.5	4.3	26	1.6	1.5
Exposed B															
1	9	17.6	16.67	71	139.2	131.48	32	62.7	59.2	9	17.6	16.7	0	0	0
2	14	33.3	27.45	53	126.2	103.9	22	52.4	43.14	2	4.76	3.9	0	0	0
3	12	27.9	27.28	64	148.8	145.45	22	51.16	50	11	25.6	25	1	2.32	2.27
4	12	27.27	22.64	56	127.3	105.66	12	27.27	22.64	4	9.09	7.55	0	0	0
5	8	17.8	15.69	76	168.9	149.02	8	17.8	15.7	2	4.44	3.91	0	0	0
6	12	29.3	24.49	58	141.5	118.37	26	63.4	53.06	1	2.4	2.04	0	0	0
7	7	17.5	15.22	52	130	113.04	19	47.5	41.3	5	12.5	10.86	0	0	0
8	9	18.75	13.85	77	160.4	118.46	7	14.6	10.8	0	0	0	0	0	0
9	6	16.2	11.54	44	118.9	84.6	21	56.7	40.4	0	0	0	1	2.7	1.9
10	12	24	21.43	73	146	130.35	18	36	32.14	0	0	0	0	0	0
11	16	29.1	26.23	109	198.2	178.7	10	18.2	16.4	3	5.45	4.9	0	0	0
12	5	11.9	10	65	154.8	130	21	50	42	1	2.38	2	0	0	0
13	11	21.6	18.03	82	160.8	134.4	15	29.4	24.6	2	3.9	3.28	0	0	0
14	3	6.98	6	59	137.2	118	39	90.7	78	3	6.97	6	0	0	0
15	15	22.7	22.39	128	193.9	191.04	18	27.3	26.9	3	4.54	4.47	0	0	0
16	6	10.34	10.17	142	244.8	240.7	27	46.5	45.8	8	13.8	13.55	1	1.72	1.69
17	7	15.5	14.29	96	213.3	195.9	4	8.9	8.16	0	0	0	1	2.22	2.04
18	10	21.28	20.83	110	234	229.2	16	34.04	33.3	3	6.38	6.25	0	0	0
19	5	9.8	9.62	124	243.1	238.5	36	70.6	69.2	6	11.76	11.53	0	0	0
20	12	21.4	20	116	207.1	193.3	24	42.8	40	2	3.57	3.33	1	1.78	1.67
21	8	17.8	16.32	121	268.9	246.9	23	51.1	46.9	3	6.7	6.1	0	0	0
22	7	14	14	117	234	234	30	60	60	2	4	4	0	0	0
23	17	34.7	29.83	58	118.37	101.7	4	8.16	7.02	1	2.04	1.75	1	2.04	1.75
24	14	20.9	19.18	151	225.37	206.8	12	17.9	16.4	1	1.49	1.37	3	4.48	4.11
25	7	14.9	14.58	95	202.13	197.9	17	36.17	35.42	1	2.12	2.08	0	0	0
26	11	19.64	18.97	129	230.36	222.4	35	62.5	60.34	4	7.14	6.89	4	7.14	6.89
27	11	20.37	16.67	85	157.4	128.8	6	11.1	9.1	2	3.7	3.03	7	1.85	1.51
28	17	36.96	26.98	53	115.22	84.13	2	4.35	3.17	0	0	0	0	0	0
29	12	21.81	16.67	79	143.6	109.7	7	12.7	9.7	2	3.63	2.78	2	3.63	2.78
30	20	40.8	24.1	62	126.5	74.7	10	20.4	12.05	1	2.04	1.20	3	6.12	3.61

Table IV. *Continued*

Animals (*n*)	Abnormal cells														
	Aneuploidy ($2n < 54$)			Gaps			Chromatid breaks			Chromosome breaks			Fragments		
	n	AC (%)	TC (%)	*n*	AC (%)	TC (%)	*n*	AC (%)	TC (%)	*n*	AC (%)	TC (%)	*n*	AC (%)	TC (%)
31	22	78.57	30.98	34	121.4	47.9	5	17.8	7.04	0	0	0	0	0	0
32	13	25.5	16.45	74	145.1	93.7	5	9.8	6.33	0	0	0	0	0	0
33	18	31.6	26.47	99	173.7	145.6	10	17.5	14.7	4	7.01	5.89	2	3.5	2.94
34	8	25.8	16.33	49	158.06	100	0	0	0	1	3.22	2.04	0	0	0
35	7	12.3	11.11	123	215.8	195.24	8	14.03	12.7	1	1.75	1.59	0	0	0
36	6	21.43	12.76	55	196.4	117.02	1	3.6	2.13	1	3.57	2.13	0	0	0
37	11	32.3	28.95	58	170.6	152.6	0	0	0	1	2.94	2.63	4	11.76	10.5
38	3	5.55	5.45	136	251.8	247.27	12	22.2	21.8	1	1.85	1.81	0	0	0
39	7	12.7	12.07	105	190.9	181.03	10	18.18	17.2	0	0	0	0	0	0
40	12	20.34	19.05	135	228.8	214.3	8	13.56	12.7	0	0	0	0	0	0
41	5	9.26	8.93	100	185.2	178.6	49	90.7	87.5	6	11.1	10.7	0	0	0
42	2	3.8	3.33	86	162.3	143.3	9	16.9	15.0	2	3.77	3.3	0	0	0
Total (42)	429	21.1	17.8	3659	179.9	151.9	660	32.4	27.4	99	4.9	4.1	31	1.5	1.3
Control															
1	9	52.9	15.25	8	47.05	13.56	2	11.7	3.38	0	0	0	0	0	0
2	11	40.7	20	7	25.9	12.7	3	11.1	5.45	3	11.1	5.45	0	0	0
3	7	53.8	12.96	4	30.8	7.4	4	30.8	7.4	1	7.7	1.8	1	7.7	1.8
4	7	50.0	12.73	7	50	12.7	2	14.3	3.63	0	0	0	0	0	0
5	10	58.8	17.54	7	41.2	12.3	2	11.8	3.5	1	5.8	1.7	0	0	0
6	11	50	16.92	11	50	16.9	3	13.6	4.6	0	0	0	0	0	0
7	7	53.8	12.07	6	46.15	10.34	0	0	0	0	0	0	0	0	0
8	4	25	8	3	18.75	6	9	56.25	18	0	0	0	0	0	0
9	7	24.13	14	11	37.9	22	11	37.9	22	0	0	0	0	0	0
10	13	41.9	26	11	35.5	22	7	22.6	14	0	0	0	0	0	0
11	17	65.4	34	3	11.5	6	5	19.2	10	1	3.8	2	0	0	0
12	14	35.9	28	9	23.1	18	16	41.0	32	0	0	0	0	0	0
13	10	31.25	18.18	9	28.13	16.36	6	18.75	10.91	0	0.00	0.00	0	0.00	0.00
14	15	78.9	28.85	8	42.11	15.38	10	52.63	19.23	0	0.00	0.00	0	0.00	0.00
15	7	35	12.73	5	25.00	9.09	0	0.00	0.00	1	5.00	1.82	1	5.00	1.82
16	17	68	28.81	6	24.00	10.17	7	28.00	11.86	1	4.00	1.69	0	0.00	0.00
17	7	41.2	13.46	7	41.18	13.46	9	52.94	17.31	0	0.00	0.00	1	5.88	1.92
18	7	38.9	14.00	9	50.00	18.00	5	27.78	10.00	1	5.56	2.00	0	0.00	0.00
19	6	24	10.91	3	12.00	5.45	4	16.00	7.27	0	0.00	0.00	0	0.00	0.00
20	9	45	15.79	11	55.00	19.30	2	10.00	3.51	1	5.00	1.75	0	0.00	0.00
Total (20)	195	44.3	17.9	145	32.9	13.3	107	24.3	9.8	10	2.3	0.9	3	0.7	0.3

Discussion

The levels of dioxins in the milk mass of the two herds were some of the highest found during chemical analyses performed by the local veterinary service. Interestingly, when examining the data of the 17 different types of dioxins separately, the quantity of TCDD alone was very high in the milk mass of both herds (7.3 and 8.1 pg/g of fat in Farms A and B, respectively) (Table I) when compared with both total dioxins permitted (3 pg/g of fat) and the normal background value of TCDD alone (0.1 and 1.0 pg/g) found in Italy and the Campania region, respectively. The quantity of dioxins in the soil and grass was below and above the limit values, respectively. However, as grass was only examined at one site, it seems inappropriate to draw hard and fast conclusions. Certainly, the sheep are only naturally pastured, which suggests the need to better investigate the pasturage areas with further chemical analyses of both grass and soil. Special attention should be given to the quantity of dioxins in the soil as ~12% of the daily food is represented by soil for naturally pastured animals, and as the limit of dioxins permitted in the soil (10 pg/g) is much higher than that permitted in grass (0.75 pg/g).

The data concerning mortality, abortion and abnormal foetuses appear very high in Farm A. It is difficult to understand the real cause of such a high number of deaths (Table 1). According to the sheep farmer, very probably, in addition to the dioxins, one of the causes could be the presence of a chemical plant very close to the pasturage area and the sheep pens. The situation on the farm has improved considerably since the closure of the chemical plant in 2004.

In a previous study (10) we also observed an increasing number of both abortions and abnormal foetuses in two sheep herds exposed to relatively low levels of dioxins (5.3 pg/g of fat). However, the high percentage of abortions found in the herds investigated previously (25%) was mainly due to the presence of *Brucella abortus* (~50% of sheep tested positive). Since the present herds were *Brucella abortus* free, the high percentage of abortions found in this study must be due to other factors which could include the high level of dioxins. Other reasons can be the altered expression of numerous genes involved in exposure to TCDD (12) or other contaminants in the environment, although the altered gene expression may not be directly related to congenital abnormalities.

Both cytogenetic tests (Tables III–VI) gave clear indications of high levels of chromosome fragility in the two herds exposed to high doses of dioxins compared with the control. In particular, the percentages of abnormal cells were very high in the exposed herds (95.9 and 84.5%) compared with that (40.4%) of the control. The chromosome fragility found in

Table V. Number of chromosome abnormalities (CA) in diploid cells, aneuploid cells and total cells of animals exposed to dioxins and controls

Animals (*n*)	Diploid cells				Aneuploid cells ($2n < 54$)				Total cells			
	n	CA			*n*	CA			*n*	CA		
		n	Mean/cell	±SD		*n*	Mean/cell	±SD		*n*	Mean/cell	±SD
Exposed A												
1	41	213	5.20	1.57	9	44	4.89	1.76	50	257	5.14	1.59
2	39	141	3.62	1.71	11	23	2.09	1.22	50	164	3.28	1.73
3	40	68	1.70	1.34	11	21	1.91	1.22	51	89	1.74	1.31
4	43	263	6.12	1.90	7	28	4.00	1.73	50	291	5.82	2.01
5	37	104	2.81	2.00	13	37	2.85	2.03	50	141	2.82	1.99
6	41	154	3.76	2.29	9	43	4.78	2.11	50	197	3.94	2.27
7	42	63	1.5	1.29	8	11	1.38	0.74	50	74	1.48	1.22
8	48	179	3.73	1.73	2	3	1.50	2.12	50	182	3.64	1.78
9	45	54	1.20	1.00	6	8	1.33	0.52	51	62	1.22	0.94
10	45	263	5.84	2.31	6	30	5.00	1.90	51	293	5.75	2.26
11	41	169	4.10	1.79	10	36	3.60	1.84	51	205	4.02	1.79
12	43	183	4.26	2.07	10	35	3.50	2.12	53	218	4.11	2.08
13	49	260	5.31	2.35	3	12	4.00	1.00	52	272	5.23	2.31
14	38	120	3.16	1.67	12	30	2.50	2.15	50	150	3.00	1.80
15	33	126	3.82	1.98	18	50	2.78	1.48	51	176	3.45	1.87
16	39	208	5.33	1.88	11	63	5.73	2.45	50	271	5.42	2.00
17	46	208	4.52	1.11	4	20	5.00	1.63	50	228	4.56	1.15
18	44	87	1.98	1.11	6	7	1.17	1.17	50	94	1.88	1.14
19	46	215	4.67	1.83	4	20	5.00	0.82	50	235	4.70	1.76
20	45	193	4.29	2.47	5	17	3.40	2.61	50	210	4.20	2.47
21	42	128	3.05	1.79	8	24	3.00	1.07	50	152	3.04	1.69
22	45	116	2.58	1.85	5	12	2.40	2.30	50	128	2.56	1.88
23	47	252	5.36	1.82	4	25	6.25	1.26	51	277	5.43	1.79
24	47	116	2.47	1.54	3	7	2.33	0.58	50	123	2.46	1.50
25	48	193	4.02	1.71	4	16	4.00	1.41	52	209	4.02	1.65
26	44	271	6.16	1.90	6	41	6.83	1.94	50	312	6.24	1.90
27	43	252	5.86	2.23	7	37	5.29	1.50	50	289	5.78	2.14
28	48	104	2.17	1.46	2	5	2.50	0.71	50	109	2.18	1.44
29	43	147	3.42	2.01	7	21	3.00	1.53	50	168	3.36	1.95
30	39	209	5.36	2.58	11	28	2.55	2.02	50	237	4.74	2.72
31	43	178	4.14	1.73	7	34	4.86	1.21	50	212	4.24	1.67
32	43	274	6.37	2.72	8	54	6.75	1.49	51	328	6.43	2.56
33	19	59	3.11	1.10	31	85	2.74	1.24	50	144	2.88	1.19
34	37	115	3.11	1.66	13	31	2.38	1.45	50	146	2.92	1.63
Total (34)	1433	5685	3.96[a]	2.31	281	958	3.40[a]	2.11	1714	6643	3.87[a]	2.29
Exposed B												
1	45	88	1.96	1.19	9	24	2.67	3	54	112	2.07	1.61
2	37	55	1.49	1.19	14	22	1.57	1.4	51	77	1.51	1.24
3	32	69	2.16	1.32	12	29	2.42	1.98	44	98	2.23	1.51
4	41	51	1.24	0.97	12	21	1.75	1.6	53	72	1.36	1.15
5	43	73	1.7	1.5	8	13	1.63	1.06	51	86	1.69	1.44
6	37	68	1.84	1.61	12	17	1.42	0.9	49	85	1.73	1.47
7	39	62	1.59	1.31	7	14	2	1.41	46	76	1.65	1.32
8	56	73	1.3	1.22	9	11	1.22	1.09	65	84	1.29	1.2
9	46	57	1.24	1.18	6	9	1.5	1.87	52	66	1.27	1.25
10	44	68	1.55	1.04	12	23	1.92	1.08	56	91	1.63	1.05
11	45	96	2.13	1.82	16	26	1.63	1.5	61	122	2	1.74
12	45	80	1.78	1.38	5	7	1.4	1.14	50	87	1.74	1.35
13	50	78	1.56	1.37	11	21	1.91	1.7	61	99	1.62	1.43
14	47	93	1.98	1.78	3	8	2.67	2.52	50	101	2.2	1.8
15	52	117	2.25	1.34	15	32	2.13	1.73	67	149	2.22	1.42
16	53	163	3.07	1.89	6	15	2.5	1.64	59	178	3.01	1.86
17	42	91	2.17	1.23	7	10	1.43	1.13	49	101	2.6	1.23
18	38	99	2.61	1.5	10	30	3	1.56	48	129	2.69	1.5
19	47	149	3.17	1.62	5	17	3.4	1.34	52	166	3.19	1.58
20	48	121	2.53	1.47	12	22	1.83	1.47	60	143	2.38	1.49
21	42	122	2.9	1.74	8	25	3.13	1.89	50	147	2.94	1.74
22	45	134	2.98	1.27	6	15	2.5	0.55	51	149	2.92	1.21
23	40	52	1.3	0.99	17	12	0.71	0.99	57	64	1.12	1.02
24	59	130	2.2	1.57	14	37	2.64	1.39	73	167	2.29	1.54
25	41	97	2.37	0.94	7	16	2.29	1.25	48	113	2.35	0.98
26	47	136	2.89	1.63	11	36	3.37	1.27	58	172	2.97	1.57
27	55	81	1.47	1.21	11	19	1.73	1.56	66	100	1.52	1.27
28	48	45	0.94	0.89	16	10	0.63	0.72	64	55	0.86	0.85
29	60	76	1.26	0.96	12	14	1.18	0.98	72	90	1.25	0.96
30	63	59	0.94	0.91	20	17	0.85	1.09	83	76	0.92	0.95
31	50	35	0.71	0.81	22	4	0.14	0.36	72	39	0.54	0.75

Table V. *Continued*

Animals (*n*)	Diploid cells				Aneuploid cells ($2n < 54$)				Total cells			
	n	CA			*n*	CA			*n*	CA		
		n	Mean/cell	±SD		*n*	Mean/cell	±SD		*n*	Mean/cell	±SD
32	66	66	1	0.93	13	13	1	0.71	79	79	1	0.89
33	50	87	1.74	1.35	18	28	1.56	1.15	68	115	1.69	1.3
34	41	41	1	1.05	8	9	1.13	0.83	49	50	1.02	1.01
35	56	126	2.25	1.24	7	9	1.29	0.76	63	135	2.14	1.23
36	41	51	1.24	1.41	6	6	1	1.55	47	57	1.21	1.41
37	27	49	1.81	0.88	11	14	1.27	0.9	38	63	1.66	0.91
38	52	141	2.71	1.27	3	8	2.67	1.15	55	149	2.71	1.26
39	51	98	1.92	1	7	17	2.43	0.98	58	115	1.98	1
40	51	122	2.39	1	12	21	1.75	0.87	63	143	2.27	1
41	51	143	2.8	1.41	5	12	2.4	1.52	56	155	2.77	1.41
42	56	88	1.57	0.99	4	6	1.5	0.58	60	94	1.57	0.96
Total (42)	1979	3730	1.88[a]	1.44	429	719	1.68[a]	1.47	2408	4449	1.84[a]	1.44
Control												
1	50	9	0.18	0.48	9	1	0.11	0.33	59	10	0.17	0.46
2	44	12	0.27	0.54	11	1	0.09	0.30	55	13	0.24	0.51
3	47	8	0.17	0.43	7	2	0.29	0.76	54	10	0.19	0.48
4	48	8	0.17	0.43	7	1	0.14	0.38	55	9	0.16	0.42
5	47	8	0.17	0.43	10	2	0.20	0.42	57	10	0.18	0.43
6	54	11	0.20	0.45	11	3	0.27	0.90	65	14	0.22	0.54
7	51	6	0.12	0.33	7	0	0.00	0.00	58	6	0.10	0.31
8	46	10	0.22	0.51	4	2	0.50	1.00	50	12	0.24	0.56
9	43	21	0.49	0.74	7	1	0.14	0.38	50	22	0.44	0.70
10	37	14	0.38	0.68	13	4	0.31	0.48	50	18	0.36	0.63
11	33	5	0.15	0.36	17	4	0.24	0.44	50	9	0.18	0.39
12	36	18	0.50	0.70	14	7	0.50	0.76	50	25	0.50	0.71
13	48	14	0.29	0.50	6	2	0.33	0.52	54	16	0.30	0.50
14	42	7	0.17	0.44	14	1	0.07	0.27	56	8	0.14	0.40
15	41	10	0.24	0.54	10	3	0.03	0.48	51	13	0.25	0.52
16	50	11	0.22	0.51	5	5	1.00	0.71	55	16	0.29	0.57
17	45	9	0.20	0.50	10	2	0.20	0.42	55	11	0.20	0.49
18	50	13	0.26	0.53	7	1	0.14	0.38	57	14	0.25	0.51
19	33	10	0.30	0.59	18	3	0.17	0.51	51	13	0.25	0.56
20	48	14	0.29	0.54	8	2	0.25	0.46	56	16	0.29	0.53
Total (20)	893	218	0.24	0.5	195	47	0.24	0.55	1088	265	0.24	0.5

[a]$P < 0.001$.

both exposed herds, compared with the control, is particularly evident in Tables IV and V where all values were significantly higher in the exposed herds. In particular, chromosome abnormalities/cell were on average 17 and 8 times higher in the cells of the exposed herds when compared with those of the control. The number of aneuploid cells are again high in both exposed herds (16.4 and 17.8) and control (17.9), as found in a previous study (10). We have no explanation for this phenomenon which requires closer investigation also in other species to ascertain whether it is typical for sheep or is a simple technical problem occurring during chromosome treatments. Indeed, since all aneuploid cells were hypoploid, this phenomenon could be just a simple technical artefact.

Moreover, when we applied the SCE test we found significant differences between exposed herds and the control (Table VI). Indeed, mean values of SCE/cell were 11.07 ± 3.77 and 11.03 ± 3.10 in the exposed Herds A and B, respectively, which are not only significantly higher than those detected in the control (7.90 ± 3.10 SCE/cell), but also when compared with previous data performed on sheep exposed to relatively low levels of dioxins (10).

Conclusions

Although it is difficult to establish whether the high chromosome fragility we found in the exposed herds is only related to high doses of dioxins (and of TCDD alone), the higher level of chromosome fragility found in the present herds, compared with that determined both in the control herd and in sheep previously investigated (exposed to lower levels of dioxins and raised in the same area), led us to seriously consider that dioxins may damage the chromosomes (and the DNA). This study suggests the need to examine the whole area for the presence of dioxins and other chemicals. Indeed, soil samples showed dioxin values below the legal threshold and the only grass sample analysed was a minuscule part of the extensive area affected. In addition, police patrols should be carried out frequently in the area as the dioxins mainly originate from the illegal burning of domestic waste. Chemical analyses of the milk of women living in the exposed area should be performed to ascertain the presence and concentration of dioxins, given that other studies have detected high levels of dioxins in women (9). These analyses should be accompanied by epidemiologic studies to establish the frequency of dioxin-related diseases and tumours in the human population (2,3).

This study also shows that animals, especially sheep, may be used as biological indicators of environmental pollution by using cytogenetic tests, such as those we applied or, more generally, using biomarkers of DNA damages. This can give a measure of the biological effects of pollutants before overt disease develops (14).

Table VI. *Cells examined and SCE mean values in exposed and control animals*

Animals (*n*)	Examined cells (*n*)	SCE/cell		
		n	Mean	±SD
Exposed A				
1	48	510	10.63	3.56
2	40	486	12.15	3.75
3	44	449	10.20	3.46
4	50	510	10.20	3.07
5	40	386	9.65	2.60
6	40	411	10.28	3.05
7	40	509	12.73	4.73
8	50	505	10.10	3.16
9	25	223	8.92	3.21
10	50	644	12.88	3.88
11	40	428	10.70	3.78
12	40	463	11.58	3.56
13	50	501	10.02	3.42
14	40	489	12.23	4.87
15	48	482	10.04	3.31
16	35	348	9.94	3.60
17	44	512	11.64	3.42
18	28	307	10.96	5.96
19	37	425	11.49	2.33
20	39	425	10.90	3.98
21	46	572	12.43	3.32
22	44	549	12.48	4.16
23	49	577	11.78	4.14
24	46	548	11.91	3.60
25	45	482	10.71	2.51
26	35	338	9.66	3.24
27	45	483	10.73	4.58
28	50	566	11.32	3.41
29	40	465	11.63	3.87
Total (29)	1228	13593	11.07[a]	3.77
Exposed B				
1	42	386	9.19	3.17
2	39	428	10.97	4.39
3	28	336	12.00	2.77
4	28	301	10.75	3.01
5	19	192	10.11	2.05
6	34	437	12.85	4.75
7	22	196	8.91	3.14
8	22	226	10.27	4.39
9	36	348	9.67	2.97
10	23	243	10.57	4.14
11	27	281	10.41	2.06
12	32	306	9.56	3.30
13	30	341	11.37	4.00
14	18	196	10.89	3.07
15	25	307	12.28	3.09
16	29	374	12.90	4.05
17	40	489	12.23	3.98
18	26	287	11.04	3.79
19	38	404	10.63	3.55
20	50	584	11.68	2.84
21	42	482	11.48	3.43
22	31	334	10.77	2.89
23	34	405	11.91	3.66
Total (23)	715	7883	11.03[a]	3.61
Control				
1	20	155	7.75	3.92
2	20	144	7.20	3.02
3	20	159	7.95	3.30
4	20	132	6.60	2.68
5	20	163	8.15	4.03
6	20	167	8.35	3.51
7	20	116	5.80	2.37
8	20	117	5.85	2.70
9	20	150	7.50	2.26
10	20	151	7.55	2.96
11	20	117	5.85	2.56
12	50	383	7.66	2.52
13	50	512	10.24	3.35
14	50	392	7.84	3.72
15	50	447	8.94	3.58
16	50	459	9.18	3.14
17	30	300	10	3.4
18	30	150	5	2.6
19	40	230	5.75	2.9
20	30	300	10	3.5
Total (20)	600	4744	7.90	3.10

[a] $P < 0.001$.

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