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Effects of Exposure to Extremely Low Frequency Electro-magnetic Fields on Circadian Rhythms and Distribution of Some Leukocyte Differentiation Antigens in Dairy Cows

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Objective To investigate the effects of extremely low frequency magnetic and electric fields (ELFEMFs) emitted from 380 kV transmission lines on some leukocyte differentiation antigens in dairy cows. **Methods** The study was carried out in 5 cows exposed to 1.98-3.28 μT of ELFEMFs and in 5 control cows exposed to 0.2-0.7 μT of ELFEMFs. Following haematological and immunologic parameters were measured in both groups: WBC, CD45R, CD6, CD4, CD8, CD21, and CD11B leukocyte antigen expression. **Results** Some of the haematological and immunologic parameters under investigation were similar in both groups. However, CD8 (T lymphocyte surface antigen) was higher in the exposed group (1.35 ± 0.120 *vs* 0.50 ± 0.14 × 10^3/mL). Furthermore, the CD4/CD8 ratio (0.84 ± 0.05 and 2.19 ± 0.16 for exposed and not exposed cows respectively) and circadian rhythm were different between the two groups. **Conclusion** Exposure to ELFEMFs is responsible of the abnormal temporal variations and distribution of some haematological and immunological parameters in dairy cows.

Key words: Electro-magnetic fields; Low frequency; Leukocyte; Circadian rhythm; Dairy cow

INTRODUCTION

Man-made electromagnetic fields (50-60 Hz) are many thousands of time greater than natural fields arising from either the sun or the earth. Their frequencies are in a 0-300 Hz range, so that they are defined as extremely low frequency electro magnetic fields (ELFEMFs). Several studies have tried to demonstrate the effects generated by exposing animal organs to ELFEMFs, but most of these works show incongruities and contrasting results[1]. There are two recent hypotheses to explain the interactions of ELFEMFs with cell metabolism and immunitary system, that is either directly on immunosystem cells by interacting with ion channel functionality[2-4], or indirectly by influencing melatonin serum level. The circadian rhythms shown by melatonin serum levels could be influenced either through an action similar to that of the light, assuming that ELFEMFs are able to stimulate the retino-hypothalamic nervous tract and consequently the suprachiasmatic nuclei, or because an enhanced quenching activity by melatonin is requested during ELFEMFs-induced high free radical production[5-10]. In this context, the central effect of exposure to ELFEMFs could be translated into a change of the usual rhythmicity shown by some clinical, haematological and immunitary parameters. Some investigations performed in bovine have discussed the main effects of exposure to ELFEMFs in their epidemiological studies[11-15]. The topics in these works refer, above all, to the effects of exposure to ELFEMFs on the energetic metabolism and its correlated pathological processes, with specific information about the increased level of insulin growth factor-1 (IGF-1), whereas the inhibitory action of melatonin at hypothalamic level

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is absent. The aim of the present work was to investigate whether ELFEMFs emitted from 380 kV transmission lines can affect the health status of dairy cows exposed to field conditions by evaluating the distribution and circadian rhythms of some differentiation leukocyte antigens in white blood cells.

MATERIALS AND METHODS

Farms and Animals

The exposed farm is located in the La Salle territory (Aosta Valley, Italy). The farm was built 7 meters below the 380 kV Albertiville-Rondissone transmission line span. The control farm is located in the Prè St. Didier. In the present study, five clinically healthy Valdostana cows in the exposed farm (A) and five clinically healthy Valdostana cows in the control farm (B) were examined. All animals were between 2 and 4 years of age, between the 186th and 218th day of lactation period. Both farms were similar in structural conditions, in line with the typology of the Valdostana cow-breeding tradition to produce the typical Fontina cheese. The cows were kept in tether stalls according to traditional housing modality which foresees a summer mountain pasture period (commonly for dry and delivery times) and the rest of the year in a cubicle stall to produce milk. The diet was composed of hay and grass mowed and given to the fixed stalling cows, and by a concentrated feed, that was the same in all the farms which harvest the milk for Fontina cheese production. At the moment of the study all cows had been stalled for almost seven months and were pregnant (ranging between the fourth and fifth mounts of gestation).

Magnetic Field Measurements

Measurements of ELFEMFs were performed using an electric and magnetic field gauge (EMDEX II, ENERTECH Consultants, USA) with measurement intervals of 10-15000 V/m and 0.01-300 μT for electric field and magnetic field, respectively. The typical accuracy was ± 1%. This gauge has two accessories: a magnetic field automatic acquisition system (LINDA system, ENERTECH Consultants, USA) and an electric field probe (E-PROBE system ENERTECH Consultants, USA). Magnetic spot measurements inside the stalls, electric field spot measurement outside the stalls, and spatial continuity plotting on one trace inside and on one outside the stalls were carried out. Moreover, a 24-hour plotting was evaluated using EMDEX LITE (ENERTECH consultants, USA) with 4 seconds of magnetic field frequency acquisition.

Sampling

Venous blood samples were collected simultaneously from all animals in the two farms at 3 h intervals (at 12.30 a.m.; 3.30 p.m.; 6.30 p.m.; 9.30 p.m.; 12.30 p.m.; 3.30 a.m.; 6.30 a.m.; 9.30 a.m.) by jugular puncture using vacutainer system tubes with EDTA as anti-coagulant. During the dark period, a red-filtered flashlight was used to facilitate venipuncture and avoid retino-hypothalamic tract stimulation.

Leukocyte Differentiation Antigens

Blood samples were immediately refrigerated (4℃) using a portable refrigerator. White blood cells (WBC) from the venipuncture were estimated within 36 h using a haematology analyzer (Advia 120, BAYER AG, Germany). Subsequently blood samples were subjected to a flow cytofluorimetric analysis (Coulter Epics XL, BEKMAN COULTER INC., USA) to determine the following leukocyte antigens: CD45R+ leukocyte common antigen (OBT4893, SEROTEC LTD, Oxford, UK); CD4+ expressed by T lymphocytes (OBT4003, SEROTEC LTD., Oxford, UK); CD8+ expressed by T lymphocytes (OBT4890, SEROTEC LTD, Oxford, UK); CD6+ expressed by lymphocytes (OBT4889, SEROTEC LTD, Oxford, UK); CD11B+ expressed by granulocytes and also called MAC-1 (OBT4891, SEROTEC LTD, Oxford, UK); CD21+ expressed by B lymphocytes, dendritic cells, and immature thymocytes (OBT4892, SEROTEC LTD, Oxford, UK). The samples were treated with the addition of species-specific monoclonal immunoglobulins and differential clusters in the proportion indicated by the procedures. The erythrolysis took place 20 minutes after the incubation using NH_4CL for 10 minutes, followed by a centrifugation at 1200 rpm for 10 minutes and a washing with PBS. The difficulty to find monoclonal immunoglobulins directly conjugated with a fluorescent compound induced us to use secondary immunoglobulin conjugated to fluorescein isothiocyanate (incubation time of 10 minutes) (STAR9B, SEROTEC LTD, Oxford, UK) for all the samples. A cytofluorimetric analysis was conducted with a regulation of the acquisition grills, based on morphological parameters and the presence of the differential marker. The results, expressed as percentage of the number of events acquired by the cytometer (15.000), were reported also as the absolute number considering the WBC value.

Statistic Analysis

Analysis of variance of the considered parameters was carried out using the GLM procedure

of the SIGMASTAT 2.03 software. A trigonometric statistical model was also applied to the average values of each time series, so as to describe the periodic phenomenon analytically, by individuating the main rhythmic parameters according to the single cosinor procedure[16]. This method clarifies if the temporal variations of a set of data can be described by a sinusoid, defining the following parameters. Midline estimating statistic of rhythm (MESOR) represents the intermediate value between the highest and smallest values of a function used to describe a rhythm, expressed in the unit of the relatively considered parameter and with the confidence interval at 95%. Amplitude identifies the difference between the maximum and minimum level in a determinate period, expressed in the same unit of the relative MESOR. Acrophase is expressed in hours, with a confidence interval at 95%.

RESULTS

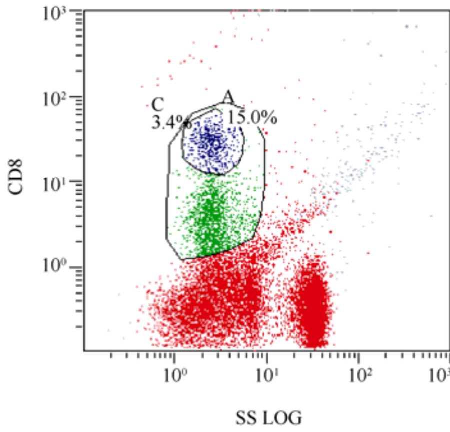
Magnetic field values measured in the A farm varied from 1.98 to 3.28 μT whereas the values measured in the B farm were lower than detectable (0.01 μT), except in brief periods (3 minutes × 4 times/day) in which the obtained measures varied from 0.2 to 0.7 μT (possibly due to the ignition of the automatic feed distributor). The values of immunological parameters are reported in Table 1. The total number of leukocytes could be considered in a normal range ($4.0\text{-}12.0\times10^3/\mu L$) in both farms. It appears that the mean values of some leukocyte antigens were different in the A farm compared to the B farm (the differences were statistically significant if $P<0.05$). However, the differences were significant whether the percentage or the absolute values of CD8+ and of CD6+ were higher in the A farm (exposed) than in the B farm (not exposed), whereas the value of CD11b+ showed an opposite result. Therefore, the CD4+/CD8+ ratio seemed to be the most exemplifying of the leukocyte antigen values. In fact the value found in the exposed farm was clearly lower than that found in the not-exposed farm (Fig. 1). Cytofluorimetric analysis showed that two typologies of CD8+, called Dim and Bright, in function of the fluorescence, were evident only in the exposed farm, whereas the population remained non-divided in the not-exposed farm. Application of a periodic model showed the circadian trend of some parameters (for example the CD4+/CD8+ ratio in the not-exposed farm) in the studied temporal series (Table 2). This model was applied only to the parameters showing no intra-group differences in the considered different temporal points.

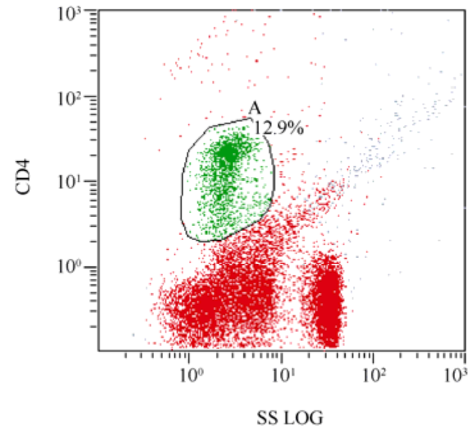
TABLE 1

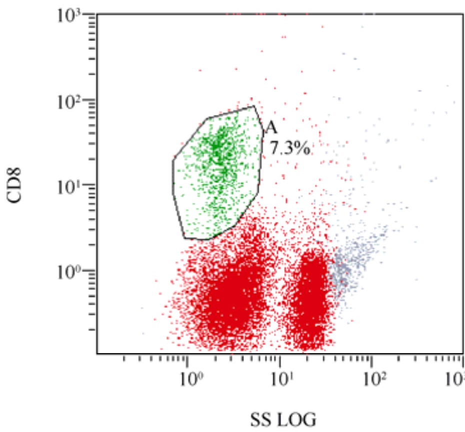
Leukocyte Subpopulations ($\bar{x} \pm s$) in a (Exposed) and B Farms (Not-exposed)

Leukocyte Antigens	Farm A	Farm B	*P*<0.05
CD45R + (%)	22.17 ± 0.0113	20.24 ± 0.0131	
CD4 + (%)	10.09 ± 0.00531	10.23 ± 0.00614	
CD8 + (%)	15.72 ± 0.0132	5.24 ± 0.0152	*
CD6 + (%)	15.78 ± 0.0125	10.39 ± 0.0144	*
CD11B + (%)	57.99 ± 0.0209	63.79 ± 0.024	*
CD21 + (%)	26.22 ± 0.0742	35.94 ± 0.0857	
WBC ($\times 10^3/\mu L$)	8.62 ± 0.353	10.05 ± 0.407	*
CD45R + ($\times 10^3/\mu L$)	1.91± 0.137	2.01 ± 0.158	
CD4 + ($\times 10^3/\mu L$)	0.86 ± 0.0510	0.99 ± 0.0589	
CD8 + ($\times 10^3/\mu L$)	1.35 ± 0.120	0.51 ± 0.138	*
CD6 + ($\times 10^3/\mu L$)	1.33 ± 0.101	0.95 ± 0117	*
CD11B + ($\times 10^3/\mu L$)	4.99 ± 0.306	6.48 ± 0.354	*
CD21 + ($\times 10^3/\mu L$)	2.21 ± 0.612	3.19 ± 0.707	
CD4/CD8	0.84 ± 0.0521	2.19 ± 0.162	*

Note. *$P<0.05$.







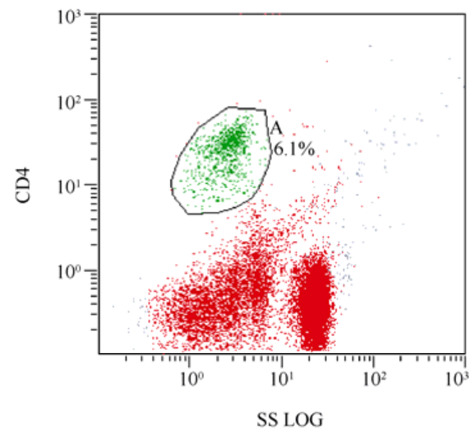


FIG. 1. Example plots of leukocyte antigen distributions in exposed and not exposed cows.The two typologies of CD8+, Dim (green distribution) and Bright (blue distribution) were evident in exposed cows.

TABLE 2

Parameters Showing a Circadian Rhythm in a (Exposed) and B Farms (Not-exposed)

Parameter	Farm	MESOR	A	Φ	C.I. (95%)
CD4 + (%)	A	10	1.45	13.24	10.20-16.28
	B	-	-	-	-
CD8 + (× 10^3/μL)	A	-	-	-	-
	B	0.50	9.76	16.08	12.00-20.16
CD4 +/CD8+	A	-	-	-	-
	B	2.19	0.17	5.44	00.00-11.28
WBC (× 10^3/μL)	A	4.42	0.33	17.56	13.52-22.00
	B	-	-	-	-

Note. Application of the single cosinor statistic model showing effects only on parameters with no significant interindividual statistical differences.

DISCUSSION

CD45R (a CD45 isoform) preferentially expressed by T lymphocytes can be used as an index of blastogenesis[17]. No significant difference was found in CD45R between the two farms, suggesting that it may be in a normal range. Lymphocyte CD4+ functions as a co-receptor, to activate T lymphocytes restricted for the histocompatibility major complex II (MHCII) whereas CD8+ functions as a co-receptor, to activate T lymphocytes restricted for the MHCI. In farms A and B, the reported data concerning the percentages of CD4+ and CD8+ values were lower than those in the peripartum of cows, when the animals were subjected to the physiological stress of delivery and early of lactation[18]. The obtained values could represent the environmental situation that is inadequate for the welfare condition of the tested animals. However, forced containment may reduce the immune response[19]. Very interesting is the feature observed only in the A farm (exposed cows) in which animals present two different populations of CD8+ in relation to the different fluorescence. These different populations are named Dim and Bright according to the different fluorescence (Fig. 1). If the A farm (exposed cows) was considered, the percentages of CD8+ Dim were higher than those of CD8+ Bright ($7.8 \pm 0.0063 \times 10^3/\mu L$ *vs* $4.5 \pm 0.0057 \times 10^3/\mu L$), the usual Dim/Bright ratio in humans is about 2[20]. We can suspect that an external agent may induce this condition. In fact, the ratio in the exposed animals was about 0.51. In this case, the difference between the two farms was evident, considering that CD8+ Bright could represent T suppressor lymphocytes and CD8+ Dim could represent T cytotoxic lymphocytes. Comparisons between ruminants and men seem to be reasonable because until now only small differences in functional, biochemical and cellular distribution patterns have been reported[21]. CD4+ is expressed by T helper lymphocytes whereas CD8+ by T cytotoxic and suppressor lymphocytes, therefore an increase or a decrease of these clusters and their ratio variation are indicative of cell-mediated immune trafficking response derangement. The statistically significant differences were observed in CD8+ between the two farms (Table 1). These data may suggest an increased tendency to cellular apoptosis due to the cytotoxic activity of CD8+ in animals showing CD4+/CD8+ in favour of CD8+, considering also the data reported by other authors[18]. In fact, CD8+ lymphocytes can interact with almost all nucleate cells (all the cells express MHC-I molecules)[22-23], whereas CD4+ lymphocytes only recognize antigens expressing MCH-II molecules, and only interact with antigen presenting cells.

CD6+, a T-cell surface glycoprotein that can deliver co-activating signals to mature T lymphocytes, allows adhesion of growing thymocytes to thymus cells rather than co-stimulation of mature T lymphocytes. This antigen is usually expressed by mature T lymphocytes and interactions between CD6 and CD6 ligands (expressed by APC, including cells other than macrophages) may regulate both antigen specific and auto-reactive responses of human T lymphocytes[24]. Very few data are available on the distribution of peripheral blood CD6+ in cows. However, when the total number of CD45R+, or CD8+ and CD4+ was considered, our data showed a difference between A and B farms (Table 1). Consequently, the higher value found in A farm might be due to the increased CD8+ and partially CD45R+. Our data could be used as reference data in future research.

CD11B is the primary integrin of polymorphonuclear (PMN) leukocytes involved in their adhesion, migration and phagocytosis. In quiescent cells, the receptor is stored in intracellular granules from which it is translocated to the cell surface in response to a variety of stimuli. This cluster can mediate interactions of neutrophyles and monocytes with the stimulated endothelium, exerting phagocytosis of immune-complex covered by iC3b or IgG particles[25-26].

In our study, CD11b+ cells were particularly more than other leukocyte populations investigated. Farm A presented a significantly lower level than farm B (Table1), suggesting that the expression of CD11b+ cluster is caused by increased PMN cells expressing the receptor for the complement, whose activation is consequent to inflammatory processes. Detection of increased or decreased CD11b expression depends on the total number of PMN activated and migrated through the endothelium. In our study, PMN activation in both farms and a higher migration rate were observed in exposed cows.

CD21+, belonging to the co-receptor of B lymphocytes (represents an important complex of transduction) in association with CD19 and CD81, shows a strong leukocyte reaction. These results could be related to the naturally high number of B lymphocytes in aged cows[22], or to the increased surface expression post activation in T lymphocytes[27].

The most interesting rhythm observed in our study is the CD4/CD8 ratio in not-exposed farm (Table 2). Mazzacolli *et al.*[20] have described the circadian rhythms of lymphocyte sub-population in humans, particularly CD2, CD4, CD4/CD8 ratio,

CD20, and CD25 with nocturnal acrophase, and CD8 and CD16 with acrophase in the morning. Castrillon *et al.*[28] have described the effects of melatonin supplementation on the onset of circadian rhythms of leukocyte sub-populations of rats which contemporaneously received specific immunostimulants. They observed that circadian rhythmcity of the animals which received only immunostimulants could induce a 10-h shift in comparison with control group.

Melatonin can be used in determine increased CD4 and CD4/CD8 ratio. By comparing our data with the data obtained by Mazzacolli *et al.*[20], the rhythm of humans showed an acrophase at 1:16 in the morning with an amplitude of 0.28, while the rhythm of cows showed an acrophase at 5:44 in the morning withand an amplitude of 0.17, indicating that the rhythm has different internal or external synchronizers which determine a shifting of the same rhythm. Castrillon *et al.*[28] have reported a 10-hour shifting of the rhythm of mitogen activity when an immunological adjuvant or lipopolisaccarids were given.

In conclusion, exposure to ELFEMFs is responsible for abnormal temporal variations of haematologic and immunologic parameters in cows. The markers characterized by a circadian rhythm seem to be the most adequate because ELFEMFs can induce variations in the same rhythm. The CD4+/CD8+ ratio can in fact represent a significant marker for exposure to ELFEMFs. New protocols based on adequate biological markers (for example CD4+/CD8+ ratio) should be developed to estimate the adverse effects of environmental electromagnetic exposure on human and animal heath.

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