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*Science of the Total Environment 433(2012): 450-455*
http://dx.doi.org/10.1016/j.scitotenv.2012.06.100

# Analysis of plasma indices of redox homeostasis in dairy cows reared in polluted areas of Piedmont (northern Italy)

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*Abbreviations:* Ret, retinol; Toc, alpha-tocopherol; Asc, ascorbic acid; N-Tyr, nitro-tyrosine; PC, protein-bound carbonyls; LPO, lipid hydroperoxides; SOD, superoxide dismutase; GPx, glutathione peroxidase; PCDDs, polychlorinated dibenzo-p-dioxins; PCDFs, polychlorinated dibenzofurans; PCBs, polychlorinated biphenyls; WHO, World Health Organization; TEQ, toxic equivalents.

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## HIGHLIGHTS

► The effect of environmental pollutants on blood redox status of bovine was evaluated.
► Pollutant exposure negatively affects blood redox homeostasis of bovine cows.
► Blood redox status represents a biomarker of the extent of animal exposure to environmental pollutants.
► Lipophilic compartment is a selective target of oxidative damage in dairy cows.
► The extent of oxidative damage is correlated to the degree of milk dioxin contamination.


## ABSTRACT

Steel manufacturing is responsible for the emission of pollutants, including dioxins and transition metals, inducing reactive oxygen species generation and DNA damage. Dioxin pollution represents the major cause of milk and dairy product contamination, in Italy, and is associated with oxidative stress-related processes, that may impair health and performance of cows. We evaluated the effect of exposure to different concentrations of pollutants derived from steel manufacturing on blood redox homeostasis of bovine cows. We analyzed two groups of dairy cows (A, B), reared in two different polluted areas, and a control group of cows bred in an industry free area. The extent of exposure to contaminants was defined by measuring dioxin level in bulk milk samples collected from animals of each farm. This level was lower in milk of group A than in group B. Plasma concentrations of retinol, alpha-tocopherol and ascorbate, the total antioxidant capacity, and the activities of superoxide dismutase and glutathione peroxidase were higher in control group than in exposed groups. In particular, retinol and tocopherol levels were higher in the group with lower milk dioxin level. Plasma titers of protein-bound carbonyls (PC), nitro-tyrosine, and hydroperoxides were lower in control group than in A or B. Hydroperoxides and PC plasma concentrations were increased in the group with higher milk concentration of dioxin. Our results demonstrate that, irrespective of the nature of chemicals inducing oxidative modifications, the extent of damage to plasma protein and lipid, is correlated with the concentration of dioxin in milk. So, the characterization of blood redox status might be a useful tool for identifying animals exposed to environmental pollutants. Plasma concentrations of retinol, alpha-tocopherol, PC and hydroperoxides could therefore represent good indices of the extent of animal exposure, as they significantly change in groups with different milk concentrations of dioxin.

## 1. Introduction

Iron and steel manufacturing plants are responsible for the emission of noxious pollutants, such as dioxins, sulfur oxides, nitrogen oxides, hydrocarbons, carbon monoxide and transition metals, that can exert genotoxic and/or carcinogenic effects (Borska et al., 2003; Desoize, 2003; Mateuca et al., 2005; Cavallo et al., 2008). Waste incineration and illegal disposal of industrial waste represent major sources of environmental and food chain pollution by dioxins (Brambilla et al., 2004). In particular, dioxin pollution has occurred in Italy, especially

in Campania and in industrial areas of Piedmont, Lombardy, Tuscany and Puglia (Biasioli and Ajmone-Marsan, 2007; Ingelido et al., 2009; Turrio-Baldassarri et al., 2009), leading to contamination of dairy milk in sheep, cattle and river buffaloes (Iannuzzi et al., 2004; Perucatti et al., 2006; Cirillo et al., 2008; Esposito et al., 2009). Dioxins are polyhalogenated aromatic hydrocarbons, highly toxic and persistent, present at low level in air, soil, water, feed as well as in foods such as dairy products (Matsumura, 2003). Dioxins share a common toxicity mechanism, that is mediated via binding to a specific intracellular receptor, the aryl hydrocarbon receptor (AhR) (Alsharif et al., 1994; Mandal, 2005), whose activation is responsible for the enhanced expression of genes coding for cytochrome P450 1 family enzymes in the liver of several species, including cattle (Safe, 1986; Whitlock, 1990; Machala et al., 1998; Matsumura, 2003; Guruge et al., 2009). Dioxin exposure promotes highly reactive oxygen species (ROS) production (Slezak et al., 2000; Nebert et al., 2000; Dalton et al., 2002), and depression of several ROS quenching systems (Ishida et al., 2009), thus inducing increased DNA fragmentation, as well as production of superoxide anion, thiobarbituric acid reactive substances, and hydroperoxides (Shertzer et al., 1995; Zhao and Ramos, 1998; Slezak et al., 2000, 2002). Dioxin-dependent sustained oxidative conditions (Shertzer et al., 1998; Senft et al., 2002a, 2002b) overwhelm antioxidant defences, leading to oxidative stress-related processes (Cadenas and Davies, 2000; Halliwell and Gutteridge, 2000; Mandal, 2005; Pelclova et al., 2011). In physiological conditions, the antioxidant defence system scavenges oxygen and nitrogen-reactive species, thus limiting or preventing oxidative damage (Halliwell, 2012). The defence system includes enzymatic (superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic antioxidants, such as ascorbic acid (Asc), retinol (Ret), and alpha-tocopherol (Toc) (Halliwell and Gutteridge, 2000; Galli and Azzi, 2010; Halliwell, 2012). Oxidative stress occurs as a consequence of an imbalance between ROS production and neutralizing capacity of antioxidant mechanisms, and is associated with modifications of physiological and metabolic functions (Halliwell and Gutteridge, 2000). In particular, it was reported that oxidative stress impairs health, fertility and zootechnical performance of dairy cows (Miller et al., 1993b), and is involved in the etiology of several diseases and metabolic disorders (Harrison et al., 1984; Smith et al., 1984; Gröhn et al., 1989; Lomba, 1996; Bernabucci et al., 2002, 2005). Feedstuffs are the major source of dioxin intake by dairy animals, as a consequence of the pollution of pastures and other feed ingredients (Brambilla et al., 2004). Dioxins accumulate in adipose tissue, liver, and muscles, and can be also transferred into milk and eggs, thus animal productions represent by far the major source of exposure for humans (Thomas et al., 1999).

The objective of this study was to analyze the effect of exposure to different concentrations of environmental pollutants derived from steel manufacturing on blood redox homeostasis of bovine cows, in order to define the potential targets of oxidative damage in blood, and to identify biomarkers useful for evaluating both the extent of animal exposure to environmental contaminants and the milk quality. Plasma concentrations of Ret, Toc and Asc, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured, and used as indices of the antioxidant defence system. Also, plasma concentrations of nitro-tyrosine (N-Tyr) and protein-bound carbonyls (PC) were used as markers of oxidative damage to protein, and plasma level of lipid hydroperoxides (LPO) was used as index of the extent of lipid peroxidation induced by the interaction of free radicals with polyunsaturated fatty acids. It is worth mentioning that N-Tyr level represents the footprint of protein oxidative damage induced by peroxynitrite (Halliwell, 1997), while PC may be introduced into proteins by direct oxidative attack to proteins themselves (Kristal and Yu, 1992), or by reactions with aldehydes originated during lipid peroxidation processes (Uchida and Stadtman, 1993). These parameters were analyzed in plasma collected from bovine cows, reared in two different dairy farms localized in two different areas, near a high-temperature steel production plant, and in plasma obtained from dairy cows reared in the same valley, but in a farm located in an industry free area. Although steel manufacturing is associated with the emissions of numerous dangerous pollutants, the extent of exposure to environmental contaminants was quantified by measuring bulk milk dioxin and dioxin-like polychlorinated biphenyls (PCBs), expressed as toxic equivalents (TEQ). Indeed, due to the presence of dioxins in the food chain, TEQ measurement is widely used, in the context of animal production, to assess milk safety, and to evaluate the contamination of raw milk and dairy products (Cirillo et al., 2008; Esposito et al., 2009), according to European Union Regulation (Commission Regulation 1259/201 1/EC).

## 2. Materials and methods

### *2.1. Materials*

Bovine serum albumin fraction V (BSA), chemicals of the highest purity, Goat anti Rabbit IgG-horseradish peroxidase linked (GAR-HRP), Rabbit anti-dinitrophenylhydrazine (anti-DNP) IgGs, and standards for high performance liquid chromatography (HPLC) were purchased from Sigma-Aldrich (via Gallarate 154, 20151, Milan, Italy). The Nucleosil 100-$NH_2$ column (5 μm particle size, 250x4.6 mm I.D.) and the Nova-PAK C18 column (4 μm particle size, 125x2 mm I.D) were obtained from Macherey-Nagel (distributed by Delchimica Scientific, via Ruffini, 20123, Milan, Italy). Organic solvents were purchased from Romil (distributed by Delchimica Scientific, via fratelli Ruffini, 20123, Milan, Italy). Polystyrene 96-wells plates were purchased from Nunc (distributed by VWR International, via Stephenson, 94,I-20157, Milan). Nitrated BSA and the kit for titration of lipoperoxide of Cayman Chemical, as well as rabbit anti-nitrotyrosine IgG of Covalab were purchased by Vincibiochem (Via Ponte di Bagnolo, 10, 50059 Vinci, Italy).

*2.2. Farm selection and animals*

The study was carried out in the Susa Valley (Piedmont, northern Italy) on 36 dairy cows (mainly PiedmontesexValdostana crossbreds) reared in two different dairy farms (A and B) localized in two different areas, both near a high-temperature steel production plant. These cows were fed on contaminated fodder, and were then regarded as groups exposed to dioxins and other environmental pollutants. Nineteen Valdostana dairy cows reared in a farm located in the same valley, but in an industry-free area, were also included in the study as control group (K). In particular, the extent of exposure to environmental pollutants was defined by measuring toxic equivalent (TEQ) values of polychlorodibenzodioxins (PCDDs), dioxin-like polychlorobiphenyls (DL-PCBs), and polychlorodibenzofurans (PCDFs) in bulk milk samples obtained from animals of group A (N = 18), B (N = 18), and K (N = 19). These values amounted to 8.56 pg/g fat (farm A) and 18.56 pg/gof fat (farm B), thus exceeding those legally permitted (5.5 pg/g fat as the sum of PCDDs, PCDFs and DLPCBs), according to the European Union legislation in force (Commission Regulation 1259/2011/EC). Conversely, TEQ values measured in bulk milk from farm K (1.75 pg/g fat) were lower than those recommended by European Union (1.75 pg/g of fat as the sum of PCDDs and DL-PCDFs/PCBs) (Table 1). The milk sampling was performed by the Regional Veterinary Services, and the analyses of dioxin level in milk samples were carried out by Istituto Zooprofilattico Sperimentale of Piedmont, Liguria, and Valle d'Aosta using a validated High Resolution Gas Chromatography Mass Spectrometry method.

In each farm blood samples were collected, early in the morning, in the same day and under the same environmental conditions, into heparinized tubes. Plasma was obtained by centrifugation (500 *g;* 15 min; 4 °C), and processed, by the same operator, for titration of antioxidants, SOD and GPx activity, total antioxidant capacity (TAC), nitro-tyrosine (N-Tyr), protein-bound carbonyls (PC), and hydroperoxides (LPO).

**Table 1**

Dioxin and DL-PCB levels in bulk milk obtained from dairy cows reared in different areas of Susa Valley (Piedmont, northern Italy).

| | A | B | K |
|---|---|---|---|
| WHO-PCDD/PCDF-TEQ (pg/g of fat) | 1.11 | 1.66 | 1.2 |
| WHO-PCB-TEQ (pg/g of fat) | 7.45 | 16.09 | 0.55 |
| WHO-PCDD/PCDF-PCB-TEQ (pg/g of fat) | 8.56 | 18.56 | 1.75 |

WHO: World Health Organization; TEQ: toxic equivalents; PCDD: polychlorinated dibenzo-*para*-dioxins; PCB: polychlorinated biphenyls; PCDF: polychlorinated dibenzo-furans.
Maximum levels legally admitted of WHO-PCDD/F-TEQ and WHO-PCDD/F-PCB-TEQ are 2.5 and 5.5 pg/g of fat, respectively.
Data are expressed as pg per g of milk fat.

### 2.3. *Determination of antioxidants and lipid hydroperoxides (LPO)*

Plasma samples were processed for determination of Asc concentration as previously described (Spagnuolo et al., 2011), and analyzed by HPLC using an anion exchange column (Nucleosil 100-$NH_2$, 5 μm, 250x4.6 mm i.d.; Macherey-Nagel). Ret and Toc levels were measured according to a published procedure (Spagnuolo et al., 2003), and analyzed by HPLC, using a reverse phase C1 8 column (Nova-PAK C1 8, 4 μm, 125x2 mm i.d.; Macherey-Nagel). LPO concentration was measured by a colorimetric quantitative assay, using the Lipid Hydroperoxide Assay Kit of Cayman Chemical, according to the manufacturer's instructions.

### 2.4. *Determination of total antioxidant capacity*

The plasma total antioxidant capacity (TAC) was measured by the Trolox Equivalent Antioxidant Capacity Assay (TEAC), according to Miller et al., 1993a. Plasma samples were reacted with the radical 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) [ABTS], and the antioxidant capacity was measured as the decrease of the absorbance at 734 nm, and expressed as μM concentration of Trolox equivalents (Miller et al., 1993a; Spagnuolo et al., 2001).

### 2.5. *Determination of nitro-tyrosine (N-Tyr) in plasma samples*

Nitrated protein levels in plasma samples were measured by ELISA, as previously reported (Spagnuolo et al., 2001). Plasma samples were diluted (1:500, 1:1000, 1:3000, 1:9000, and 1:15,000) with coating buffer (7 mM $Na_2CO_3$,17 mM $NaHCO_3$,1.5 mM $NaN_3$, pH 9.6), and incubated in the wells of a microtiter plate overnight at 4 °C. Standard curves were obtained with serial dilutions of nitrated BSA. N-Tyr was detected by incubation with Rabbit anti-N-Tyr antibody (1:1000 dilution in 130 mM NaCl, 20 mM Tris-HCl, 0.05 % Tween 20, pH 7.3, supplemented with 0.25% BSA; 1 h, 37 °C), followed by GAR-HRP diluted 1:2000 as the primary antibody. Color development was monitored at 492 nm, as previously described (Spagnuolo et al., 2003). Data were reported as nmol of N-Tyr per mg of proteins.

### 2.6. *Determination of protein-bound carbonyls*

PC concentration in plasma samples was titrated by ELISA, according to Buss et al. (1997). Protein derivatization was carried out with a dinitrophenylhydrazine (DNP) solution (10 mM in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5), to a final protein concentration of 3 mg/ml. Samples were incubated at room temperature for 45 min, vortexing every 10-15 min. Each sample was then diluted (1:1000-1:20,000) with10 mM sodium phosphate buffer, pH 7.0, containing 140 mM NaCl, and incubated (overnight at 4 °C) in the wells of a microtiter plate. PC were detected by incubation (1 h, 37 °C) with Rabbit anti-DNP antibody (Sigma-Aldrich, St Louis, MO, USA) diluted 1:1500 with PBS supplemented with 0.2% gelatin and 0.05% Tween 20, followed by GAR-HRP antibody (diluted 1:3000 as the primary

antibody). Color development was monitored at 492 nm, as previously described (Spagnuolo et al., 2003). A six-point standard curve of oxidized BSA was included in each plate. A blank for DNP reagent in PBS without protein was subtracted from each absorbance. Data were reported as nmol of carbonyls per mg of proteins.

### *2.7. Evaluation of plasma activity of glutathione peroxidase (GPx) and superoxide dismutase (SOD)*

GPx activity was measured indirectly by a coupled reaction with glutathione reductase (GR), using the glutathione peroxidase assay kit of Cayman Chemical, according to the manufacturer's instructions. GPx activity was expressed as nmol of NADPH oxidized per minute per ml of sample.

SOD activity was measured with the superoxide dismutase assay kit of Cayman Chemical, according to the manufacturer's instructions. SOD activity was expressed unit/ml. One unit of SOD is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical.

### *2.8. Statistical analysis*

The samples for measurement of SOD and GPx activities, PC, N-Tyr, or LPO concentration were processed in triplicate. The titration of Toc, Ret, and Asc was carried out on duplicates. Values were expressed as mean±SEM. Significance of statistical differences was evaluated by one-way ANOVA, followed by Tukey's test for multiple comparisons, using the Graph Pad Prism 3 program (Graph Pad Software, San Diego, CA, USA).

## 3. Results

### *3.1. Analysis of antioxidants and total antioxidant capacity*

Plasma concentration of Ret, Toc, and Asc, the total antioxidant capacity (TAC), and GPx and SOD activities were measured, and used as markers of the antioxidant defence system. Data are reported in Table 2.

Plasma concentration of Ret was positively correlated with Toc concentration in each group ($r = 0.618$, $P = 0.005$, group K; $r = 0.821$, $P \leq 0.0001$, group A; $r = 0.884$, $P \leq 0.0001$, group B; data not shown), as expected since they are both dietary liposoluble antioxidants.

The concentrations of Toc and Ret were found significantly lower (Pb0.0001) in plasma collected from cows reared in the two exposed farms (A and B) than in control group (K). Further, the concentration

**Table 2**

Markers of the antioxidant defence system and markers of oxidative stress in plasma of dairy cows exposed (A and B) or not exposed (K) to environmental pollutants.

| | A | B | K |
|---|---|---|---|
| Retinol (μg/ml) | $0.58 \pm 0.01^{***}$ | $0.50 \pm 0.01$ | $0.69 \pm 0.01^{a,b}$ |
| α-Tocopherol (μg/ml) | $1.55 \pm 0.02^{**}$ | $1.31 \pm 0.01$ | $1.86 \pm 0.02^{a,b}$ |
| Ascorbate (μM) | $6.23 \pm 0.06$ | $6.28 \pm 0.14$ | $7.99 \pm 0.13^{a,b}$ |
| GPx (nmol/min/ml) | $115.70 \pm 8.91$ | $107.30 \pm 9.60$ | $152.0 \pm 10.88^{a,b}$ |
| SOD (U/ml) | $1.16 \pm 0.07$ | $1.10 \pm 0.08$ | $1.48 \pm 0.07^{a,b}$ |
| PC (nmol/mg p) | $13.63 \pm 0.63^{*}$ | $15.84 \pm 0.58$ | $7.22 \pm 0.22^{a,b}$ |
| N-Tyr (nmol/mg p) | $9.36 \pm 0.13$ | $9.65 \pm 0.15$ | $5.47 \pm 0.13^{a,b}$ |
| LPO (μM) | $6.80 \pm 0.19^{*}$ | $7.66 \pm 0.32$ | $2.83 \pm 0.11^{a,b}$ |
| TAC (μM) | $92.66 \pm 2.60$ | $88.29 \pm 2.03$ | $130.7 \pm 3.16^{a,b}$ |
| N | 18 | 18 | 19 |

Concentration of antioxidants, PC, N-Tyr, LPO, and TAC in plasma samples of bovine cows exposed (group A and group B; N = 18) or not exposed (group K; N = 19) to environmental pollutants derived from steel manufacturing. PC, protein-bound carbonyls (nmol per mg of protein); N-Tyr, nitro-tyrosine (nmol per mg of protein); LPO, lipid hydroperoxides (μM); TAC, total antioxidant capacity (μM concentration of Trolox equivalents); GPx, glutathione peroxidase activity (expressed as nmol of NADPH oxidized per min per ml); SOD, superoxide dismutase (U/ml).
K vs A, $^{a}P<0.01$.
A vs B, $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.
K vs B, $^{b}P<0.01$.

of liposoluble antioxidants was higher in group A than in group B (Pb0.001). As shown in Table 2, plasma concentration of Asc, and TAC were higher in group K than in groups A and B ($P \leq 0.0001$), while no differences were observed between group A and group B. In addition, TAC was found positively correlated with plasma concentration of Asc in each group (group K, $r = 0.640$, $P = 0.003$; group A, $r = 0.755$, $P = 0.0001$; group B, $r = 0.761$, $P \leq 0.0001$; data not shown), but not with the concentration of Ret or Toc. Also, GPx and SOD activities were higher in the control group than in the exposed groups (A and B, Pb0.01), while they did not differ between group A and group B.

Also, GPx and SOD activities were lower in the exposed groups (A and B) than in the control group (Pb0.01), but did not differ between group A and group B.

### *3.2. Analysis of PC, N-Tyr and LPO*

The extent of oxidative damage to proteins and lipids was evaluated by measuring plasma concentrations of PC, N-Tyr, and LPO. As shown in Table 2, plasma levels of both N-Tyr and PC were significantly lower in group K than in group A and group B (Pb0.0001). In addition, plasma concentration of PC was higher in group B, showing higher milk TEQ value, than in group A ($P = 0.02$), while plasma level of N-Tyr was not different between the two groups. LPO plasma concentration was found significantly lower (Pb0.0001) in the control cows than in the exposed groups. Also, by comparing group A with group B, we found a higher level of LPO in the plasma from animals of group B ($P = 0.02$) than in group A.

Plasma level of PC was negatively correlated with plasma titer of Asc (Fig. 1, panel A; group K, r=-0.694; P = 0.001; group A, r=-0.721; P = 0.0003; group B, r=-0.636, P = 0.003), and with total antioxidant capacity (Fig. 1, panel B; group K, r= —0.662; P = 0.002; group A, r=-0.647, P = 0.004; group B, r=-0.597, P = 0.006). We also found a negative correlation between plasma level of N-Tyr and Asc both in group K (r=-0.660, P = 0.002) and in group A (r= —0.654, P = 0.002), according with the recognized role of Asc as scavenger of peroxynitrite (Halliwell, 1997).

Plasma concentration of N-Tyr was negatively correlated with total antioxidant capacity in group K (r= —0.511, P = 0.03) and in group A (r= -0.578, P = 0.008; Fig. 2, panel A), but not in group B. A negative correlation between SOD activity and plasma concentration of N-Tyr was found in group K (r= —0.888, P = 0.0001) and in group A (r = -0.528, P = 0.02; Fig. 2, panel B), but not in group B. Finally, SOD activity was positively correlated with plasma concentration of Asc in bothgroup K (r = 0.616, P = 0.005) and group A (r = 0.479, P = 0.04).

## 4. Discussion

Epidemiological and bio-monitoring studies previously showed that steel manufacturing is associated with production of several pollutants, including dioxins, sulfur oxides, nitrogen oxides, hydrocarbons, carbon monoxide, and transition metals, that may induce ROS generation, DNA damage and genome mutations (Danadevi et al., 2003; Goulart et al., 2005; Cavallo et al., 2008; Matés et al., 2010).

This study was carried out on two groups of bovine cows reared in two different farms, located near a high-temperature steel production plant, and exposed to a different degree of environmental contamination. Data were compared with those obtained from cows bred in an industry free area. Although different contaminants in addition to di-oxin might be present in the examined areas, the extent of exposure to environmental pollutants was evaluated by measuring mean bulk milk TEQ values of dioxin and dioxin-like PCBs, as this index is widely used for monitoring milk safety. On the basis of this evaluation, the two exposed farms were designed as A (lower milk TEQ values) or B (higher TEQ values). The blood redox status of the cows showing a different degree of milk dioxin-contamination was characterized

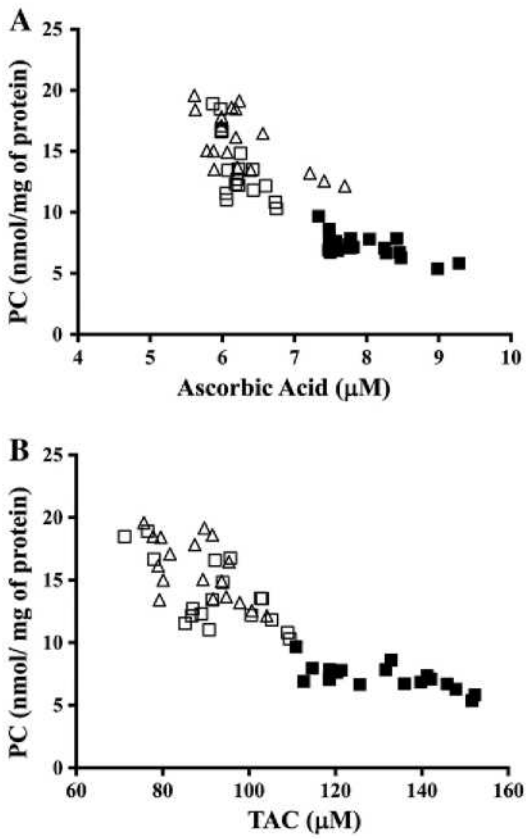


**Fig.** 1. Correlation between the concentration of PC and the concentration of ascorbate or the total antioxidant capacity in the plasma of dairy cows. Panel A. The levels of Asc and PC were measured in plasma samples from bovine cows of control group (group K, full squares), and in plasma from cows exposed to environmental pollutants derived from steel manufacturing (group A, open squares; group B, open triangles). Asc was isolated from deproteinized plasma samples by HPLC, and detected in the effluent material by a UV-spectrophotometer. PC level was measured by ELISA and expressed as nmol/mg of protein. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over *5%* from the mean were not found. The statistical program Graph Pad Prism 3 performed the regression analysis and the calculation of P (group K, r = — 0.694; P=0.001; group A, r= -0.721; P=0.0003; group B, r=-0.636, P=0.003). Panel B. The total antioxidant capacity was measured by the Trolox Equivalent Antioxidant Capacity Assay (TEAC), in plasma samples from bovine cows of control group (group K, full squares), and in plasma from cows exposed to environmental pollutants derived from steel manufacturing (group A open squares; group B, open triangles). The antioxidant capacity was measured as decrease of the absorbance at 734 nm, and expressed as *μM* concentration of Trolox equivalents. PC level was measured by ELISA and expressed as nmol/mg of protein. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over 5% from the mean were not found. The statistical program Graph Pad Prism 3 performed the regression analysis and the calculation of P (group K, r= —0.662; P=0.002; group A r= —0.647, p = 0.004; group B, r= -0.597, P = 0.006).

by evaluating specific indices of blood redox status. A cytogenetic investigation previously carried out on the bovine cows analyzed in the present study detected an increased chromosome fragility in the groups exposed to environmental pollutants, thus suggesting a higher DNA oxidative damage in blood cells of the exposed cows (Di Meo et al., 2011). Transition metals, dioxins and other pollutants produced during steel manufacturing are responsible for the induction of ROS production (Stohs and Bagchi, 1995; Ercal et al., 2001; Matsumura, 2003), thus entailing increased consumption of antioxidants, accumulation of toxic compounds in blood and tissues, and subsequent oxidative stress in mammalian cells (Knerr et al., 2006).

Plasma concentrations of Ret, Toc and Asc, as well as the total antioxidant capacity and the activities of both SOD and GPx were found significantly higher in cows reared in the industry free area, thus suggesting that changes of these antioxidant defence system markers might be correlated with the degree of exposure to environmental pollutants, as assessed by milk TEQ values. These findings agree with

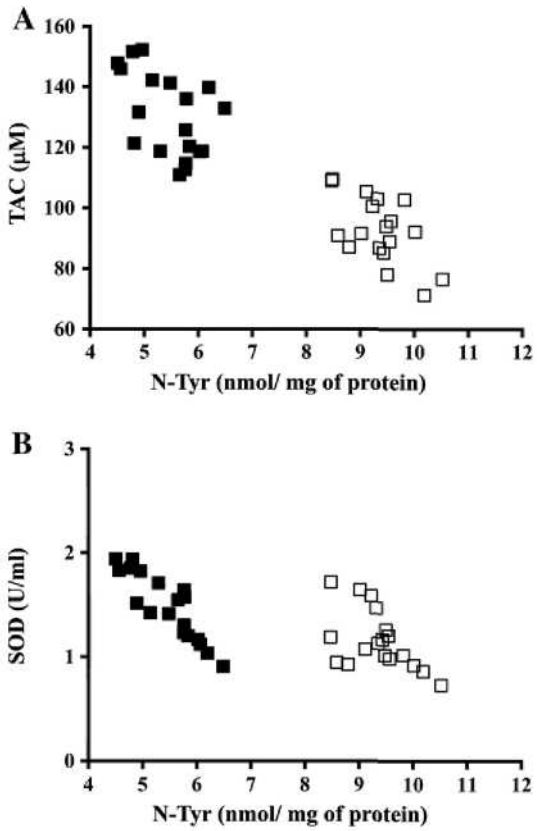


Fig. 2. Correlation between the N-Tyr concentration and the total antioxidant capacity or the SOD activity in the plasma of dairy cows. Panel A. The concentration of N-Tyr and the total antioxidant capacity were measured in plasma samples from bovine cows of control group (group K, full squares), and in plasma from cows exposed to environmental pollutants derived from steel manufacturing (group A, open squares). N-Tyr level was measured by ELISA and expressed as nmol/mg of protein. The total antioxidant capacity was measured by the Trolox Equivalent Antioxidant Capacity Assay (TEAC), and expressed as $\mu M$ concentration of Trolox equivalents. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over *5%* from the mean were not found. The statistical program Graph Pad Prism 3 performed the regression analysis and the calculation of P (group K, r= -0.511, P=0.03; group A, r= -0.578, P=0.008). Panel B. SOD activity was measured in plasma samples from bovine cows of control group (group K, full squares), and in plasma from cows exposed to environmental pollutants derived from steel manufacturing (group A, open squares), and expressed as U/ml. N-Tyr concentration was measured by ELISA and expressed as nmol/mg of protein. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over *5%* from the mean were not found. The statistical program Graph Pad Prism 3 performed the regression analysis and the calculation of P (group K, r = - 0.888, P=0.0001, group A, r = - 0.528, P=0.02).

the data previously obtained in buffalo cows (Spagnuolo et al., 2011), as well as in other laboratory species exposed to either 2,3,7,8 tetrachloro-dibenzo-p-dioxin (Stohs et al., 1984; Hassoun et al., 1998; Slezak et al., 2002) or to DL-PCBs (Lai et al., 2010), and support the hypothesis that the exposure to environmental pollutants, such as dioxins, severely impairs the blood antioxidant defence system. As expected, we found that the lower antioxidant capacity, observed in exposed cows, is associated with a significantly higher extent of oxidative modifications of protein and lipid. By comparing the two groups from the contaminated areas, we found that Ret and Toc plasma concentrations were higher in cows with lower milk TEQlevels (farm A). Conversely, the other markers of the antioxidant defence system did not differ between the two exposed groups. Our results suggest that liposoluble antioxidants play a key role in the protection from environmental pollutants, such as dioxins, and that the lipophilic compartment represents a selective target of damage in cows exposed to steel plant-derived contaminants. Significant differences in the extent of oxidative modifications to plasma proteins and lipids were also found between the two exposed groups. In particular, LPO and PC concentrations were higher in cows exhibiting higher TEQ milk values (farm B), while N-Tyr concentration was similar in the two groups. These results support the hypothesis that lipid peroxidation and its intermediates were crucial in determining oxidative modifications of proteins and lipids in the analyzed groups. In each group, we found that plasma TAC was positively correlated with Asc concentration, and negatively correlated with PC concentration. Furthermore, a negative correlation between plasma concentration of Asc and PC was observed. Our findings suggest that in cows a significant part of the plasma antioxidant capacity, as measured by Trolox Equivalent Capacity, might be accounted for by Asc, and support the hypothesis that circulating Asc plays a major role in preventing protein oxidative modifications induced by carbonyls, as previously reported for humans (Krajcovicova-Kudlackova et al., 2006) and buffalo cows (Spagnuolo et al., 2011). Asc is a well known scavenger of peroxynitrite, and SOD is an enzymatic antioxidant involved in the scavenging of superoxide (Beckman and Koppenol, 1996; Halliwell and Gutteridge, 2000). Interestingly, a negative correlation between Asc and N-Tyr concentration, as well as between SOD activity and N-Tyr concentration, was found only in the control group and in the group with a lower degree of milk dioxin-contamination. Likewise, such groups also exhibited a correlation between SOD activity and Asc concentration, thus demonstrating that the exposure to environmental pollutants, here quantified by assaying milk TEQ levels, negatively affects blood redox homeostasis, and interferes with the protective role of SOD and Asc against peroxynitrite production and damage to protein. We can suggest that, irrespective of the nature of chemicals potentially involved in the induction of oxidative modifications, the extent of protein and lipid damage in plasma of cattle reared in polluted areas, might be correlated with the concentration of dioxins in milk, that we used as index of the degree of environmental contamination. On the basis of these results we propose that the characterization of blood redox status might represent a useful tool for identifying animals exposed to environmental pollutants. In particular, we suggest that plasma concentrations of Ret, Toc, PC and LPO could represent good indices of the extent of animal exposure to environmental contaminants, as these parameters significantly change in groups with different milk TEQ values. So, the above mentioned biomarkers, here selected for evaluating the status of the

antioxidant defence system, might be used for evaluating animal wellbeing, as well as milk quality and safety.

**Acknowledgments**

The study was partially supported by the Italian National Research Council under DG.RSTL.083.001, and by the Italian Ministry of University and Research "PRIN-2008, Contaminazione da diossine e composti diossino-simili: ricerca di biomarcatori in popolazioni bovine e bufaline esposte".

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