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Short communication

Antioxidant status and biomarkers of oxidative stress in bovine leukemia virus-infected dairy cows

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ABSTRACT

Bovine leukemia virus (BLV) is among the most widespread livestock pathogens in many countries. Despite advances in understanding the pathogenesis of this disease, little is known about the involvement of oxidative stress. Therefore, this study examined the antioxidant status and the markers of oxidative stress in BLV-infected dairy cows. BLV infection was associated with an increase in triacylglycerol levels, a decrease in glutathione peroxidase (GSH-Px) activity and a tendency toward lower superoxide dismutase activity in the infected animals. No significant difference was observed in other markers of oxidative stress (i.e., conjugated dienes, hydroperoxides and malondialdehyde) in the infected animals compared to controls. A novel method for the analysis of oxidative stress, Z-scan based on the measurement of the mean-value of θ in low density lipoprotein indicated that the infected animals had low-density lipoprotein particles that were slightly less modified than those from the healthy group. Thus, we conclude that BLV infection is associated with a selective decrease in GSH-Px activity without any alteration in the common plasma markers of oxidative stress.

1. Introduction

Bovine leukemia virus (BLV) is a member of the family Retroviridae and the genus Deltaretrovirus that is genetically and structurally similar to primate T-lymphotropic viruses types 1–5 (i.e., HTLV-1–4). Although it has been successfully eradicated in some regions of Europe, BLV is among the most widespread livestock pathogens in many countries, especially in dairy herds. BLV infection in cattle may remain clinically silent or may emerge as persistent lymphocytosis (PL); more rarely, BLV may develop into B-cell lymphoma. PL is characterized as a chronic elevation in the number of circulating B-lymphocytes, and it is found in approximately 20–30% of BLV-infected cattle (Gillet et al., 2007).Oxidative stress has drawn attention because reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) are generated during infections. Oxidative stress has been defined as the imbalance between the generation of ROS and/or RNS and the antioxidant systems (Bouzar

Abbreviations: BLV, bovine leukemia virus; GSH-Px, glutathione peroxidase; PL, persistent lymphocytosis; ROS, reactive oxygen species; RNS, reactive nitrogen species; TG, triacylglycerol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SOD, superoxide dismutase; Hb, hemoglobin; TAS, total antioxidant status; ZS, Z-scan technique; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances; FOX, ferrous oxidation in the xylenol orange; PAF, platelet-activating factor.

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et al., 2009). Evidence indicates that ROS may play a role in the inhibition of apoptosis in B-cells isolated from sheep experimentally infected with BLV (Alcaraz et al., 2004; Bouzar et al., 2009). ROS also influence BLV expression in infected B cell lines (Bondzio et al., 2001, 2003). Despite advances in understanding the pathogenesis of this disease, little is known about the involvement of oxidative stress, especially the antioxidant system, in control of BLV infection.Studies have demonstrated that chronic and acute infection may alter lipid metabolism (Steiger et al., 1999; Calza et al., 2008). During infection, the most commonly observed alterations are increased levels of triacylglycerol (TG) and low-density lipoprotein (LDL) and decreased high-density lipoprotein (HDL) cholesterol (Sammalkorpi et al., 1988; Kushibiki et al., 2002; Monteiro et al., 2009). Few studies have characterized the bovine lipid profile (Wendlandt and Davis, 1972; Steiger et al., 1999; Kushibiki et al., 2002), and to our knowledge, this is the first study to analyze the lipid profile during BLV infection. Thus, the aim of present study is to investigate whether BLV infection is associated with the markers of oxidative stress and the antioxidant defenses, and to evaluate the physical properties of the LDL particle and possible alterations in lipid profile.

2. Materials and methods

2.1. Animals and serological detection of BLV

Fifteen clinically healthy, lactating Holstein dairy cows were selected from a commercial herd with more than one hundred lactating dairy cows. These selected animals had an average daily milk yield of 27.87 kg/day, and their parity range was 2–3. All animals received the same basal diet, formulated to meet their requirements (NRC, 2001). The animals were fed with corn silage as the roughage and corn meal, citrus pulp, soybean meal, soybean grain and urea as the concentrates, as well as vitamins and mineral supplements.

These animals were divided uniformly according to sera test results into three groups: negative for BLV infection by agar gel immunodiffusion and ELISA without any hematological alterations (healthy) ($n = 5$), positive in both tests without any hematological alterations known as aleukemic (AL) ($n = 5$) and positive in both tests with PL ($n = 5$). BLV-infected cattle were classified as PL when the lymphocyte counts exceed 10,000/mL and the leukocytes counts exceed 15,000/mL as established by Thurmond et al. (1990). The persistence of lymphocytosis was confirmed after 72 days. All animals were sera tested by agar gel immunodiffusion (Tecpar®, Curitiba, Brazil) and ELISA (VMRD Pullman Inc. Corp®, Pullman, USA, cat. number 284-5) using the glycoprotein gp51 as the antigen. Therefore, no difference was observed in dairy cows infected with BLV with or without PL (data not showed). In face of, we divided the animals according to their serological status for BLV (Healthy $n = 5$, BLV-infected $n = 10$).

Subsequently, each animal was codified and randomized, and further analysis was performed without previous knowledge of the state of the animal from which the sample was drawn. We emphasize that this research complied with the Ethical Principles in Animal Research and was approved by the Bioethics Commission (number 1227/2007).

2.2. Measurement of glutathione peroxidase and superoxide dismutase activity

Measurement of erythrocyte glutathione peroxidase (GSH-Px) activity was performed using a commercial kit (RANSEL® Laboratories, Randox, Crumlin, UK) and used according to the manufacturer. Similarly, a commercially available colorimetric kit (RANSEL® Laboratories, Randox, Crumlin, UK) for measurement of erythrocyte superoxide dismutase (SOD) activity was used. The results were expressed in units per gram of hemoglobin (Hb) for both GSH-Px and SOD. The hemoglobin concentration was accessed by spectrophotometry using a commercial kit (Labtest Diagnostica, Belo Horizonte, Brazil) for the assessment of hemoglobin cyanide, using the protocol supplied by the manufacturer.

2.3. Total antioxidant status

The total antioxidant status (TAS) in the plasma was performed using a commercial kit (RANSEL® Laboratories, Randox, Crumlin, UK) using the protocol supplied by the manufacturer.

2.4. Isolation of LDL

Peripheral blood was collected from each cow, and 20 mL of plasma was obtained after centrifugation at $250 \times g$ at 4 °C for 10 min. Plasma was incubated with benzamidine (2 mM), gentamicin (0.5%), chloramphenicol (0.25%), phenyl-methyl-sulfonyl-fluoride (0.5 mM), and aprotinin (0.1 unit/mL). LDL was isolated by sequential preparative ultracentrifugation (Havel et al., 1955; Wendlandt and Davis, 1972) at $10^5 \times g$, 4 °C, using the 75 Ti rotor of a Hitachi Ultracentrifuge. Briefly, the plasma density was adjusted to 1.019 g/mL with KBr, and samples were centrifuged for 18 h. The supernatant containing very low-density lipoprotein was removed, and the density of the infranatant was adjusted to 1.063 g/mL and further centrifuged for 18 h. The supernatant containing LDL was dialyzed at 4 °C against PBS, pH 7.4, with 0.01% EDTA. The LDL was passaged through a 0.22 mm pore filter (Millipore, Germany). The protein content was determined using the BCA assay (Pierce, USA) using BSA as a standard.

2.5. Z-scan technique

The refractive index of a medium is a measure of the reduction in the speed of light passed through the medium. The Z-scan technique (ZS) is sensitive to small local variations in the index of refraction occurring in a medium. In this technique, a polarized Gaussian beam is focused by a lens and the transmitted intensity of the laser beam is measured in the far field as a function of the sample position (z). The energy absorbed from the beam by the sample is promptly transformed into heat, producing a temperature gradient in the sample. This gradient leads to a change in the refractive index of the sample, generating a thermal

lens within the medium. The thermo-optical coefficient of the medium is the physical quantity that accounts for the modification of the sample index of refraction due to the formation of the thermal lens. It has been demonstrated that the amplitude of the thermal–optical coefficient of LDL solutions strongly depends on the state of the LDL (Gómez et al., 2004). The amplitude of the thermal–optical coefficient of LDL solutions may be considered a signature of the sample related to its state of modification. The dimensionless parameter ΔTpv, measured in a ZS experiment, is proportional to the thermal–optical coefficient. The first successful attempt to apply these concepts to the measurement of human lipoproteins was reported in 2004 (Gómez et al., 2004). To our knowledge, this is the first report of an evaluation of the thermal–optical coefficient of LDL in bovines.

2.6. Malondialdehyde

The malondialdehyde (MDA) content in the plasma was quantified using the thiobarbituric acid reactive substances (TBARS) assay. The sample (50 μL) was mixed with TBARS reagent (200 μL, 1% thiobarbituric acid, 562.5 μM HCl, 15% trichloroacetic acid). The mixture was boiled for 15 min and centrifuged ($10^3 \times g$ for 10 min), and the absorbance of the supernatant was measured at 540 nm using a spectrophotometer and compared to a MDA standard.

2.7. Conjugated dienes

The conjugated dienes were estimated by the absorbance at 234 nm and the results were expressed in terms of OD.

2.8. Quantification of hydroperoxides

The plasma peroxide concentrations were determined using the ferrous oxidation in the xylenol orange (FOX)-II method. This test is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides contained in the plasma samples in the presence of xylenol orange, which produces a colored ferric-xylenol orange complex whose absorbance can be measured. Thus, the FOX II reagent was prepared with 90% methanol, 25 mM H_2SO_4, 250 μM ferrous sulfate heptahydrate and 100 μM xylene orange (Wolff, 1994). To facilitate peroxide activity, 100 μL of plasma was incubated for 18 h with 900 μL FOX II reagent. After incubation, the samples were centrifuged at $12{,}000 \times g$ for 10 min. The absorbance of the supernatant at 560 nm was then determined in an ELISA plate reader (GENIOS TECAN, Austria). Experiments were carried out in duplicate, and the results were quantified using a standard curve generated using hydrogen peroxide.

2.9. Determination of triacylglycerol, total cholesterol, high-density lipoprotein and low-density lipoprotein concentrations

The concentration of TG, total cholesterol (TC), HDL and LDL were determined using a commercial kit (Biosystems, Spain) according to the manufacturer's protocol.

2.10. Statistical analysis

A Gaussian distribution was confirmed using the Kolmogorov and Smirnov test. To compare differences between groups (healthy and BLV-infected), the Student's *t*-test for unpaired data was used for data with Gaussian distributions, and the Mann–Whitney test was used for nonparametric data.

Statistical analysis was performed using GraphPad Prism 5.0 software® (GraphPad Software, Inc., San Diego, CA, USA). Results are reported as mean ± SEM when a Gaussian distribution was confirmed. For nonparametric data, the median (minimum–maximum) was presented. Significance was declared at $P \leq 0.05$ unless otherwise indicated.

3. Results and discussion

We investigate the antioxidant status, markers of oxidative stress and lipid profile in BLV-infected dairy cows. In this study, BLV infection was associated with a selective decrease in the concentration of the antioxidant enzyme GSH-Px ($P = 0.04$) and a tendency toward a lower SOD ($P = 0.08$) (Fig. 1). Otherwise, there was no significant difference in the TAS between healthy (1.62 ± 0.07 μmol/L) and

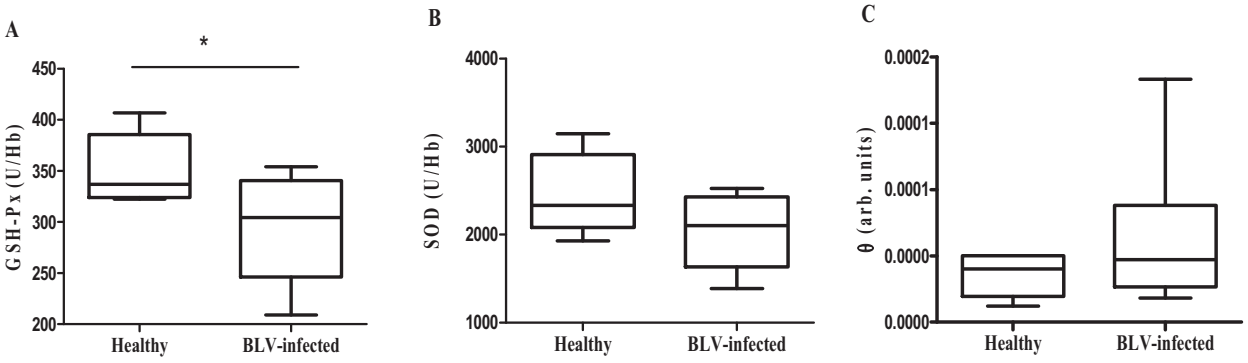

Fig. 1. (A) Glutathione peroxidase activity (GSH-Px) in healthy and bovine leukemia virus (BLV)-infected dairy cows by sera test. (B) Superoxide dismutase activity (SOD) in healthy and BLV-infected dairy cows by sera test. (C) Values of θ from Z-scan measurements in healthy and BLV-infected dairy cows by sera test. $^{*}P \leq 0.05$ (healthy $n = 5$, BLV-infected $n = 10$).

Table 1
Antioxidant status, markers of oxidative stress and lipid profile in healthy and bovine leukemia virus (BLV)-infected dairy cows.

	Healthy	BLV-infected
TAS (μmol/L)	1.62 (±0.07)	1.64 (±0.06)
Conjugated dienes	3.3 (±0.07)	3.4 (±0.09)
Hydroperoxides (nm)	1.81 (±0.14)	2.16 (±0.58)
MDA (mM)	10.83 (±0.18)	10.71 (±0.25)
TC (mg/dL)	66.97 (±10.62)	74.45 (±3.44)
TG (mg/dL)	26.84 (±1.34)	31.82 (±1.44)*
LDL (mg/dL)	4.21 (±0.85)	4.40 (±0.49)
HDL (mg/dL)	57.38 (±10.01)	63.68 (±3.56)

Data are presented as mean ± SEM. Healthy $n = 5$, BLV-infected $n = 10$. Total antioxidant status (TAS), malondialdehyde (MDA), total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL).
* $P = 0.047$.

infected (1.64 ± 0.06 μmol/L) animals ($P = 0.91$) (Table 1). A discreet decrease in SOD activity has been described in BLV-transformed fibroblasts (Nemeikaite and Cenas, 1993). Similarly, studies report diminished total selenium content (Khalili et al., 2008), GSH-Px activity (Lei et al., 2007), zinc levels (Khalili et al., 2008) and SOD activity in HIV-infected patients (Treitinger et al., 2000).

In the present study, all steps of lipid peroxidation were evaluated by measuring conjugated diene formation (initial step), quantifying hydroperoxides (propagation step) and MDA adducts (final step), and detecting physical modification (i.e., Z-scan technique) of the LDL particle. However, no differences were observed in the formation of lipid peroxidation products (i.e., MDA adducts conjugated dienes and hydroperoxides) between infected and uninfected cows (Table 1).

The Z-scan curves of the samples investigated presented a peak-to-valley shape (with amplitude ΔT_{pv}), characteristic of samples with negative thermo-optical coefficients. We define the parameter θ as $\theta = (\Delta T_{pv})/(P\, c_0)$. The mean ± SEM and the median of θ for healthy and infected animals were $3.6 \pm 0.73 \times 10^{-5}$ and 4.0×10^{-5}, and $6.5 \pm 1.76 \times 10^{-5}$ and 4.7×10^{-5}, respectively ($P = 0.16$) (Fig. 1). However, the dispersion in the data values of θ from the healthy group is smaller than that of the BLV-infected group. Thus, the initial analysis, based on the inspection of the mean-values of θ of all the animals, indicates that infected animals seem to have LDL particles that are slightly less modified than those from the healthy group. It has been proposed that measurement of the oxidation of the LDL particle by ZS is more sensitive than other assays to measure oxidative stress (Monteiro et al., 2009). Additionally, we report a tendency toward a decrease in the modification in the LDL particle concomitant with an increase in TAS ($r = -0.62$) ($P = 0.06$). This result is particularly relevant because, as a single measure, TAS provides more biologically relevant information that may more effectively describe the dynamic equilibrium between pro-oxidants and antioxidants in the plasma compartment (Ghiselli et al., 2000).

Here, we measured products generated by ROS in circulating blood and found no change between uninfected and infected cattle. The present study indicates that the oxidative "pressure" in herbivores is localized and cell-associated rather than systemic. This could, in part, explain the activation of the cell-associated enzyme-dependent defense system. This scenario is further supported by other studies that demonstrated the involvement of oxidative stress in the pathogenesis of BLV-infected B cells or B cell lines (Bondzio et al., 2001, 2003; Alcaraz et al., 2004; Bouzar et al., 2009) and the absence of any significant change in the markers of oxidative stress measured in the circulating blood of BLV-infected animals reported here.

Another explanation for reduced oxidative stress in the plasma could be the higher concentration of HDL in bovine blood compared to human blood (Wendlandt and Davis, 1972; Sammalkorpi et al., 1988; Monteiro et al., 2009), as HDL is known to counteract lipid and LDL oxidation (Turk et al., 2008; Antončić-Sventina et al., 2010). This may be partly due to the relationship between HDL-associated antioxidative enzymes (apolipoprotein AI, paraoxonase and PAF-acetylhydrolase) and the oxidation and capture of the LDL particle (Turk et al., 2008; Antončić-Sventina et al., 2010).

Additionally, BLV was associated with an increase in TG concentration ($P = 0.047$) (Table 1). Nonetheless, there were no alterations in TC ($P = 0.40$), HDL ($P = 0.95$) and LDL ($P = 0.84$) contents (Table 1). Likewise, alterations in lipid metabolism have been described in HIV-infected subjects (Calza et al., 2008) and has also been associated with others infections (Sammalkorpi et al., 1988; Kushibiki et al., 2002; Monteiro et al., 2009).

Thus, we conclude that BLV infection is associated with a selective decrease in GSH-Px activity without any alteration in the common plasma markers of oxidative stress.

Conflict of interest statement

None declared.

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