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# Comparative study on tert-butyl hydroperoxide induced chemiluminescence in bovine, equine and canine erythrocyte lysate

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## ABSTRACT

The present investigation was carried out to analyze changes in the time course of chemiluminescence intensity in bovine, equine and canine suspension of lysates erythrocytes induced for incubation with t-butyl hydroperoxide (t-BHP). The addition of t-BHP to canine, equine and bovine suspension of lysates erythrocyte resulted in the peroxidation as evidenced by the emission of light. Light emission shows a characteristic kinetic, when the samples are added with t-BHP the emission increased quickly to reach a maximal level after about 10 min for all species, then declined with time. The total chemiluminescence during incubation of suspension of lysates erythrocyte in the three species with t-BHP was higher in canine and equine than in bovine lysed red cells. The main conclusion of this study is that the determination of chemiluminescence in suspension of lysates erythrocyte is a sensitive assay applied to detect the existence of oxidative stress associated to experimental pathological situations. 

## KEYWORDS

Oxidative stress;
Chemiluminescence;
Erythrocyte;
Tert-butyl hydroperoxide.

## INTRODUCTION

Circulating erythrocytes are the most abundant cells in adult organism, constituting 10% of the total cell volume. In addition, are easy to obtain cells, which makes that erythrocytes are an excellent model for studying the cellular pathophysiology[16, 19], and a suspension of lysed red cells, which can be maintained refrigerated or cryopreserved. However, these cells have limited functions, since, to differentiate and mature, they lose their nucleus, ribosomes and mitochondria, losing, at the same time, its ability to synthesize proteins. It is why the red blood cells have been considered as hemoglobin bags[9]. Circulating erythrocytes are exposed regularly to stress conditions, as in the lung oxidative stress and hyperosmotic conditions in the kidney[16]. Due to the high tension of $O_2$ in arterial blood and the content of Fe, within erythrocyte continuously occur reactive oxygen species (ROS) such as $O_2^{(-)}$, $H_2O_2$, HO, and many physiological and pathological conditions, such as cellular aging and inflammation, are developed through the action of ROS[6, 8, 16], and a posible relationship between oxidative stress and incidence of metabolic disease has been suggested[17, 25]. Oxidative stress has been associated in several inflammatory conditions and incriminated in the pathogenesis of many diseases in cattle[30, 33]. Oxygen

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radicals are produced continually in red cells of all mammals by hemoglobin autooxidation as a result of the gas exchange that occurs when oxyhemoglobin becomes methemoglobin, where the iron of the heme group from hemoglobin reacts spontaneously with oxygen[31, 26, 14] and this process is accelerated upon exposure to a large number of prooxidants agents such as: cumenehydroperoxide[2, 39], t-butyl hydroperoxide (t-BHP)[26, 43, 18] and hydroperoxides of fatty acids[26, 40]. The presence of redox-active hemoglobin residues, with per-oxidative activity, potentially catalyzes the oxidation of the erythrocyte membrane components mainly polyunsaturated fatty acids, phenomenon known as lipid peroxidation[11, 27, 36, 20, 5]. Erythrocytes are especially vulnerable since they have no membrane repair and regenerative capacity[41] and red cell damages by free radicals would probably be associated with haemolysis[37, 29].

Several indicators determine the veterinary relevance of this work. Previous studies indicate that oxidative damage, phosphatidylserine expression and loss of membrane asymmetry of red blood cells are related to the development of several types of anemia in cattle experimentally infected[34, 35, 32]. Changes in circulatory antioxidant status in horses during prolonged exercise with special emphasis on red blood cells have been described[21] as well as the protective effect of erythropoietin on the oxidative damage of goat erythrocyte membrane by hydroxyl radical[7]. Nevertheless, erythrocyte possesses antioxidant enzymes that avoid or minimize the damage caused by the ROS, the most important being the superoxide dismutase and glutathione peroxidase, which act in conjunction to prevent damage to red cell[13, 31, 8, 32]. When in the erythrocyte or cell in general there is a significant increase in the formation of ROS or a decrease in antioxidant mechanisms, condition called oxidative stress, characterized by an increase in the lipoperoxidación and alterations in antioxidant metabolites and, if this condition is not controlled, can cause irreparable damage to the erythrocyte[15, 32]. At the present, we are not aware of comparative studies of lipid peroxidation before and after induced oxidative stress using suspension of lysates erythrocyte. Taken together these considerations, the aim of the present study was to analyze the time-course of t-BHP induced changes in lipid peroxidation, and chemiluminescence intensity in equine, canine and bovine suspension of lysates erythrocyte.

## MATERIALS AND METHODS

### Materials

The tert-BHP was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade.

### Animals

Six adult horses, weighing between 450 and 470 kg and belonging to University farm, were used. Horses were maintained on alfalfa bale and tap water ad libitum. We utilized six clinically healthy Aberdeen Angus steers, also belonging to La Plata University, weighing approximately 200 kg each. These animals receive a diet based on natural pasture and water ad libitum. Finally, six healthy dogs, weighting between 25 and 35 kg, and provided by their owners were also considered in the assay.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

### Preparation of erythrocytes

The blood was obtained by jugular venipuncture (bovine and equine) and cephalic venipuncture (canine) with heparinized squirts. Samples were quantified on the basis of hemoglobin concentration, determined by photometry on a Sysmex KX21-N hematology analyzer (Sysmexcorporation, Kobe, Japan). The erythrocytes were isolated from whole blood by centrifugation (1000g for 10 min at 4°C). The buffy coat and plasma were discarded and erythrocytes were washed three times in isotonic phosphate buffer (PBS 5 mM pH 7.4, 150 mMNaCl). The erythrocytes pellet was suspended in isotonic phosphate buffer. Preparation of suspension of lysates erythrocyte was carried out according to the method of Dodge et al. (1963). Briefly, packed, washed erythrocytes were lysed by adding 10 vol of 5 mM phosphate buffer pH 7.4 (at 4°C) while mixing and after leaving on ice for 30 min. Finallyhomogenizing the suspension.

### Peroxidation of erythrocyte analyzed by chemiluminescence

Suspension of lysates erythrocyte were incubated at a final concentration of 0.25 mg/ml total hemoglobin with 2 mM t-BHP for 40 min at 37°C. Identical aliquots of the preparation were incubated for 40 min at 37°C without addition of t-BHP as the control experiment for endogenous peroxidation products in the erythrocyte lysates preparation.

Peroxidation was initiated by adding a small amount of stock solution of t-BHP (80 mM) to each vial that was maintained at 37°C and was measured by monitoring light emission[42] with a liquid scintillation analyzer Packard 1900 TR. Chemiluminescence was determined over a 40 min period and recorded as count per minute (cpm) every 10 min.

### Calculations and statistical analysis

The data are presented as means ± SE of three independent determinations. Analysis of variance and student's t-test. was performed to test the significance of difference ($P<0.05$) between the mean values among groups.

## RESULTS

The addition of t-BHP to canine, equine and bovine suspension of lysates erythrocyte resulted in the peroxidation as evidenced by the emission of light. All results are shown in TABLE 1.

The data for the time-course of light emission are given in Figure 1, showing typical peroxidation experiments with or without t-BHP. Light emission shows a characteristic kinetic, when the samples are added with t-BHP the emission increased quickly to reach a maximal level after about 10 min for all species, then declined with time.

Figure 2 shows the total chemiluminescence during incubation of suspension of lysates erythrocyte in the three species with t-BHP was higher in canine and equine than in bovine lysed red cells.

**TABLE 1: Time course of light emission (cpm x 1000)**

| | Time (min) | 0 | 10 | 20 | 30 | 40 | Total cpm |
|---|---|---|---|---|---|---|---|
| Bovine | With t-BHP | 27.10±3.6$^{a}$ | 41.84±2.1$^{a}$ | 38.59±3.1$^{a}$ | 18.75±1.5$^{a}$ | 14.04±0.8$^{a}$ | 140.31±2.8$^{a}$ |
| | Without t-BHP | 25.58±4.3$^{a}$ | 19.69±2.1$^{b}$ | 14.82±1.6$^{b}$ | 12.34±1.3$^{a}$ | 11.88±1.5$^{a}$ | 84.31±10.5$^{b}$ |
| Equine | With t-BHP | 60.87±17.6$^{a}$ | 78.04±16.0$^{a}$ | 76.63±15.8$^{a}$ | 42.8±10.5$^{a}$ | 30.52±8.9$^{a}$ | 288.87±58.0$^{a}$ |
| | Without t-BHP | 29.69±1.2$^{a}$ | 21.26±2.4$^{b}$ | 17.70±2.1$^{b}$ | 15.63±1.9$^{b}$ | 13.68±1.5$^{a}$ | 97.96±8.6$^{b}$ |
| Canine | With t-BHP | 56.58±6.3$^{a}$ | 80.46±3.1$^{a}$ | 49.01±5.6$^{a}$ | 38.66±3.7$^{a}$ | 29.75±3.5$^{a}$ | 254.47±17.6$^{a}$ |
| | Without t-BHP | 43.27±3.5$^{a}$ | 32.58±4.43$^{b}$ | 27.5±3.5$^{b}$ | 24.77±2.9$^{a}$ | 22.84±2.6$^{a}$ | 150.97±16.1$^{b}$ |

**Data are given as the mean ±SD of six experiments; $^{ab}$ In the same species, means with different superscripts differ significantly at $p<0.05$**

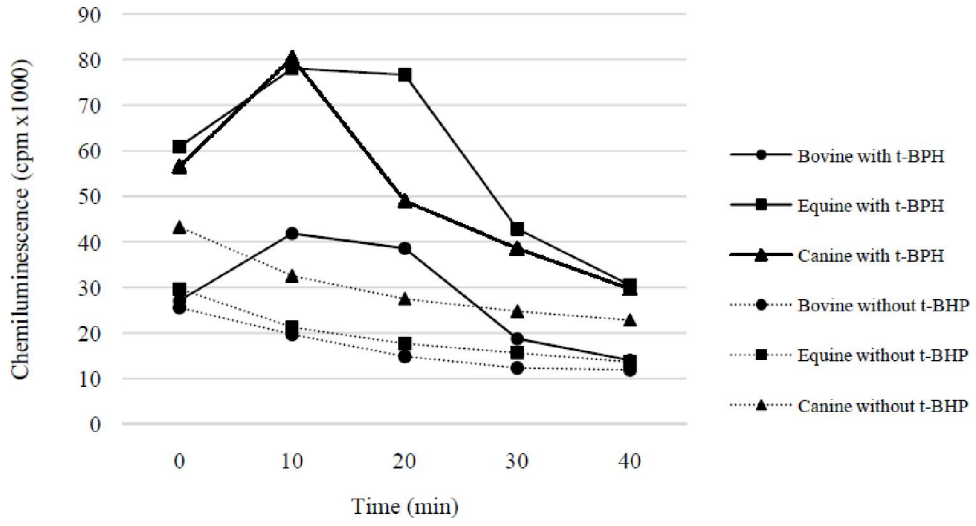


**Figure 1 : Time course of light emission of bovine, equine and canine suspension of lysates erythrocyte incubated with or without t-BHP**

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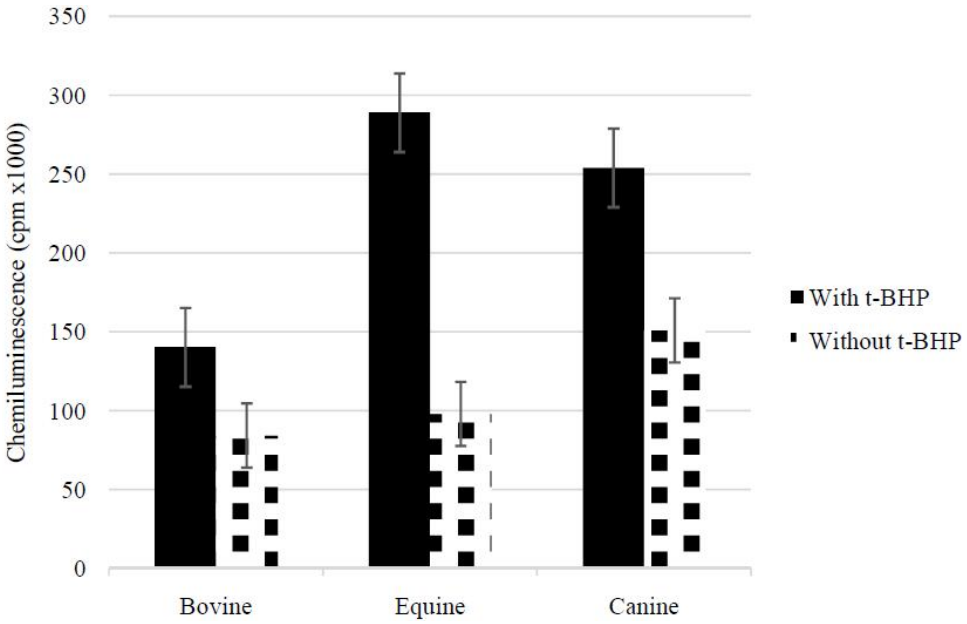


**Figure 2 : Total chemiluminescence during incubation with or without t-BHP of suspension of lysates erythrocyte of bovine, equine and canine. Data are given as the mean ±SD of six experiments**

## DISCUSSION

The erythrocyte is an early model for studies of oxidative stress and has been used as a simple model to study the cellular effects of various compounds[38]. Because of relatively high oxygen tensions, the presence of haemoglobin, and a plasmatic membrane rich in polyunsaturated lipids, they should be prone to oxidative reactions. Erythrocytes have many scavenger systems, and can be used to examine the balance between pro-oxidants and antioxidants since they are representative cells where superoxide radicals are being continuously generated by autooxidation of haemoglobin.

The determination of t-BHP initiated chemiluminescence appears as a sensitive assay that has been applied to detect the existence of oxidative stress associated to experimental pathological situations. Although it is possible to have chemiluminescence without lipid peroxidation in cell-free systems, it is established that an increase in lipid peroxidation rate in organs and isolated cells produces a parallel increase in photoemission. In the present study, suspension of lysates erythrocyte from canine, equine and bovine were exposed to a prooxidant (t-BHP). We used lysed red cells because we believe it is a relatively simple model, since in these cells the presence of redox-active hemoglobin residues, with peroxidative activity, potentially catalyzes the oxidation of membrane components including polyunsaturated lipids[11, 3, 36, 20, 4]. The extent of lipid peroxidation to cell membranes was assessed by measuring light emission in native and peroxidized membranes. Although it is possible to have chemiluminescence without lipid peroxidation in cell-free systems, it is established that an increase in lipid peroxidation rate in organs and isolated cells produces a parallel increase in photoemission. Since oxygen radicals are produced continually in erythrocytes by hemoglobin autooxidation[26], and the erythrocytes possess redundant and overlapping mechanisms for protection of their cytoplasmic contents against oxidative damage, including catalase[1], superoxide dismutase[12], and low molecular weight antioxidants such as ascorbate[24]. Defenses against oxidative damage in the plasma membrane are poorly understood but relate primarily to prevention and reversal of lipid peroxidation of polyunsaturated fatty acids in the lipid bilayer[28].

The main conclusion of this work is that the methodology used in this study, can be a suitable model for future studies in order to understand factors linked to the lipid peroxidation process from different animal species.

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