Oxidative stress indicators in human and bottlenose dolphin leukocytes in response to a pro-inflammatory challenge

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Key words: Antioxidant defenses, Marine mammals, Oxidative stress, Reactive oxygen species, White blood cells

Abstract: Marine mammals undergo cycles of tissue ischemia and reperfusion during the dive response. Reperfusion injury can result in oxidative tissue damage and the activation of a pro-inflammatory immune response. The risk of oxidative damage is reduced by antioxidants. Our hypothesis is that the reported higher antioxidant defenses within marine mammal tissues provide additional protection in situations that produce oxidative stress, like in inflammation, in comparison to terrestrial mammal tissues. Leukocytes were isolated from the whole blood of Pacific bottlenose dolphins (Tursiops truncatus gilli) and humans (Homo sapiens) and were exposed to lipopolysaccharides (LPS, 10 µg/mL) in vitro to simulate a pro-inflammatory challenge. Oxidative stress indicators, including superoxide radical (O2−) production, activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST), as well as oxidative protein damage, were quantified by spectrophotometry. Following 48 h under experimental conditions, bottlenose dolphin leukocytes produced 1.9 times more O2− but displayed 2.0 times lower protein carbonyl concentrations compared to human leukocytes. Following 48 h under experimental conditions, bottlenose dolphin leukocytes displayed 7.9, 2.0, 11.1, and 3.3 times more activities of CAT, GPx, GR, and GST, respectively, compared to human leukocytes. These results suggest that, under cell culture conditions, the antioxidant defenses in bottlenose dolphin leukocytes provide additional protection against pro-inflammatory challenges in comparison to human leukocytes, likely as an adaptive advantage.

Introduction

In contrast to terrestrial mammals, cetaceans such as bottlenose dolphins (Tursiops truncatus gilli) frequently engage in the dive response as part of a completely aquatic lifestyle. The dive response of mammals is characterized by apnea, bradycardia, and peripheral vasoconstriction (Elsner et al., 1969). Therefore, during a dive, tissues, except for those within the central nervous system, experience ischemia (Elsner et al., 1969; Ridgway, 1986). Reactive oxygen species (ROS), such as superoxide radical (O2−) and hydrogen peroxide (H2O2), are produced throughout the dive response, during reserve oxygen (O2) metabolism, adenosine triphosphate (ATP) catabolism, and tissue reperfusion (Kevin et al., 2005; Granger and Kvitets, 2015). Antioxidants reduce the likelihood of oxidative tissue damage resulting from ROS overproduction and accumulation (Fridovich and Freeman, 1986; Halliwell and Gutteridge, 1990). However, the accumulation of oxidative products in tissues can initiate processes of cellular apoptosis or necrosis, leading to the activation of the pro-inflammatory response as part of the innate immune system (Kevin et al., 2005; Hussain et al., 2016).

Activation of a pro-inflammatory response also occurs following exposure to pathogens, including bacterial products (Ulevitch and Tobias, 1999). For defense, all living organisms possess an innate immune system (Keogh et al., 2011; Miller et al., 2005). Lipopolysaccharides (LPS) are structural elements in the external membrane of gram-negative bacteria, including Escherichia coli (Guha and Mackman, 2001; Ohishi et al., 2011). Through interactions with host receptors, the innate immune system recognizes LPS as a foreign body and initiates a pro-inflammatory response within hours to days (Ulevitch and Tobias, 1999; Davis et al., 2008; de Vos et al., 2009; Keogh et al., 2011). The activation of innate immunity results in the recruitment...
of inflammatory cells, including phagocytic leukocytes, and the liberation of pro-inflammatory mediators, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Repine et al., 1997; Miller et al., 2005). NADPH oxidase catalyzes the oxidative burst, the rapid production of ROS, as a mechanism to destroy foreign bodies and damaged tissues (Halliwell and Gutteridge, 1990; Kevin et al., 2005; Sokolova, 2005; Birben et al., 2012).

Endotoxin A, a constituent of LPS, is associated with illness, including sepsis and septic shock, in humans and other mammals (Ulevitch and Tobias, 1999; Guha and Mackman, 2001). Endotoxin sensitivity in mammals occurs over a wide spectrum (Lehmann et al., 1987). Basic investigations indicate that humans are highly sensitive models, as physiological alterations occur rapidly following endotoxin exposure (Wolff, 1973; Copeland et al., 2005; de Vos et al., 2009). Current knowledge of the immune system and immune response mechanisms in marine mammals, especially cetaceans, is limited (de Guise et al., 2009). Previous studies have analyzed the immune response of marine mammals following in vitro exposure, including the response of leukocytes from bottlenose dolphins exposed to bacteria (Sokolova, 2005; Keogh et al., 2011) and bacterial products (Shiraishi et al., 2002; Ohishi et al., 2011) under laboratory conditions. Of these studies, Ohishi et al. (2011) report that the exposure of bottlenose dolphin leukocytes to LPS concentrations as low as 5 µg/mL LPS is sufficient to induce an immune response.

Few comparative immunotoxicity studies have been conducted analyzing the immune response between terrestrial and marine mammals (Shiraishi et al., 2001), largely in relation to legal, ethical, and financial constraints, as well as limitations in immune function assay availability (Copeland et al., 2005; Levin et al., 2007; Ruiz et al., 2009). Under physiological conditions, the tissues of marine mammals generally display greater antioxidant defenses, compared to terrestrial mammals, likely as an adaptive advantage to decrease oxidative damage resulting from the dive response (Wilhelm Filho et al., 2002; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2007). Few studies have analyzed the indicators of oxidative stress in the tissues of marine mammals following immune activation (Walsh et al., 2015; Del-Águila-Vargas et al., 2019).

The objective of this study was to compare the indicators of oxidative stress, including the production of superoxide radical (O$_2^-$), antioxidant enzyme defenses, and oxidative damage to proteins in leukocytes isolated from marine and terrestrial mammals in response to a pro-inflammatory challenge. Our hypothesis is that the greater antioxidant defenses in the tissues of marine mammals provide additional protection against situations that induce oxidative stress, such as inflammation, compared to the tissues of terrestrial mammals.

Materials and Methods

Unless otherwise stated, all materials and solutions were obtained or prepared from reactants acquired from Sigma (St. Louis, MO, USA). All analyses were performed in triplicate.

Sample collection

Residual whole blood samples (20 mL) were obtained from 12 healthy human (Homo sapiens) volunteers of both genders between the ages of 18–65, from the Instituto Mexicano del Seguro Social (IMSS) blood bank in La Paz, Baja California Sur, Mexico. Human blood samples were collected in blood collection bags containing the anticoagulant citrate phosphate dextrose (CPD). All donors were deemed eligible to donate blood following the donor’s guidelines established by IMSS; donor identities, including name, age, and gender, were maintained anonymous. All blood samples were collected with permission from the Comisión Nacional de Bioética (CONBIOÉTICA-C23-CEI-009-20160601; registration number 2018-785-010). Whole blood samples (8–15 mL) were collected from 12 healthy Pacific bottlenose dolphins of both sexes between the ages of 4 to 16 (average 9.3 years) maintained under human care by Cabo Dolphins, Cabo San Lucas, Baja California Sur, Mexico. Blood samples from bottlenose dolphins were collected from the ventral caudal fluke by veterinarians using established sanitary protocols. Bottlenose dolphin blood samples were collected in plastic tubes (BD Vacutainer, Franklin Lakes, NJ, USA) containing the anticoagulant dipotassium ethylenediaminetetraacetic acid (EDTA-K2). Samples from humans and bottlenose dolphins were maintained at 4°C during transport to the Oxidative Stress Laboratory within the Centro de Investigaciones Biológicas del Noroeste (CIBNOR), where they were maintained refrigerated at 4°C overnight.

Leukocyte isolation

Leukocytes were isolated from whole-blood samples following the methods of Corredor (1994). Briefly, whole blood samples obtained from humans and bottlenose dolphins were individually mixed with Hanks balanced saline solution (HBSS; Gibco, Paisley, RFW, UK) or Dulbecco phosphate buffer solution (PBS; Gibco, Paisley, RFW, UK) in a Nest centrifugation tube (Fisher Franklin Lakes, NJ, USA) containing the anticoagulant. Bottlenose dolphin blood samples were collected in plastic tubes (BD Vacutainer, Franklin Lakes, NJ, USA) containing the anticoagulant dipotassium ethylenediaminetetraacetic acid (EDTA-K2). Samples from humans and bottlenose dolphins were maintained at 4°C under human care by Cabo Dolphins, Cabo San Lucas, Baja California Sur, Mexico. Blood samples from bottlenose dolphins were collected from the ventral caudal fluke by veterinarians using established sanitary protocols. Bottlenose dolphin blood samples were collected in plastic tubes (BD Vacutainer, Franklin Lakes, NJ, USA) containing the anticoagulant dipotassium ethylenediaminetetraacetic acid (EDTA-K2). Samples from humans and bottlenose dolphins were maintained at 4°C during transport to the Oxidative Stress Laboratory within the Centro de Investigaciones Biológicas del Noroeste (CIBNOR), where they were maintained refrigerated at 4°C overnight.

Leukocyte viability

Leukocytes were isolated from whole-blood samples following the methods of Corredor (1994). Briefly, whole blood samples obtained from humans and bottlenose dolphins were individually mixed with Hanks balanced saline solution (HBSS; Gibco, Paisley, RFW, UK) or Dulbecco phosphate buffer solution (PBS; Gibco, Paisley, RFW, UK) in a Nest centrifugation tube (Fisher Franklin Lakes, NJ, USA) containing the anticoagulant dipotassium ethylenediaminetetraacetic acid (EDTA-K2). Samples from humans and bottlenose dolphins were maintained at 4°C during transport to the Oxidative Stress Laboratory within the Centro de Investigaciones Biológicas del Noroeste (CIBNOR), where they were maintained refrigerated at 4°C overnight.

Leukocyte viability and total count

Leukocyte viability was assessed using a Neubauer chamber (Hauser Scientif, Horsham, PA, USA) following the methods of Colotta et al. (1992). To be utilized in this investigation, aliquots of leukocytes isolated from whole blood (0 h) were required to present at least 90% viability. The average cell count of each aliquot was assessed, and the estimated total leukocyte count was determined.

Exposure of leukocytes to lipopolysaccharides (LPS)

A solution of LPS isolated from E. coli O55:B5 (028M4138V) was prepared following the manufacturer’s instructions.
Approximately $1 \times 10^6$ leukocytes from each sample were separated into individual cell culture flasks. Human and bottlenose dolphin leukocytes were exposed to control (LPS, 0 µg/mL) or experimental (LPS, 10 µg/mL) conditions and incubated (Thermo Scientific, Waltham, MA, USA) at 37°C with 5% CO₂ for 24 and 48 h, in duplicate. Following the designated incubation time, the cell culture flasks were gently mixed, and the samples were centrifuged at $360 \times g$ at ambient temperature. The supernatant was eliminated, and PBS was added to the leukocyte packets. Samples were stored in labeled cryogenic vials (Corning Incorporated, Corning, NY, USA) and maintained at −80°C until analyzed.

**Production of superoxide radical ($O_2^{•−}$)**

The production of $O_2^{•−}$ was determined in human and bottlenose dolphin leukocytes following methods adapted for microplate (Costar assay plate, Corning Incorporated, Corning, NY, USA), from Markert et al. (1984) and Drossos et al. (1995). Samples were thawed on ice, and aliquots were separated into individual 1.5 mL tubes (Neptune Scientific, San Diego, CA, USA). Samples were mixed with 18 mM Krebs buffer, and 15 µM cytochrome C was added. Samples were transferred to a 37°C water bath (Polyscience, Niles, IL, USA) for 15 min, then placed in ice water for 5 min while 3 mM N-ethylmaleimide was added. Samples were centrifuged at 500 × g and 4°C, and the absorbance of the supernatant was determined at 550 nm using a spectrophotometer (Multiskan FC, Thermo Scientific, Waltham, MA, USA). The production of $O_2^{•−}$ was determined following 24 and 48 h of incubation. Results are expressed in nanomoles $O_2^{•−}$ per milligram of protein per minute.

**Antioxidant activity**

To quantify antioxidant enzyme activities, 50 mM homogenization solution (0.1 M potassium phosphate buffer and 60 mM EDTA (Fermont, Monterrey, SA, MX), and 1 mM phenylmethylsulfonyl fluoride (PMSF)) was added to each sample. The activity of superoxide dismutase (SOD; E.C. 1.15.1.1) was determined following methods, adapted for microplate, from Suzuki (2000). Samples were mixed with working solution (50 mM sodium carbonate buffer, 100 µM xanthine, 0.25 mM nitroblue tetrazolium (NBT), 1 mM EDTA, and xanthine oxidase (0.1 units)). The absorbance was determined at 500 nm every minute over 3–5 min. The activity of SOD was determined following 24 and 48 h of incubation. The activity of SOD is expressed as U per milligram of protein. One unit of activity is defined as the quantity of SOD necessary to inhibit the reaction between $O_2^{•−}$ and NBT by 50%.

The activity of catalase (CAT; E.C. 1.11.1.6) was determined following methods adapted from Aebi (1984). Each sample was placed into a crystal cuvette (VWR Spectrophotometry Cell, Radnor, PA, USA), and working solution (0.1 M phosphate buffer solution, 20 mM H₂O₂) was added. The absorbance was determined at 240 nm every 1 s over 120–180 s (DU 800, Beckman Coulter, Brea, CA, USA). The activity of CAT was determined following 24 and 48 h of incubation. The activity of CAT is expressed as U per milligram of protein. One unit of activity is defined as the quantity of CAT necessary to reduce 1 µmol of H₂O₂ per minute.

The activity of glutathione peroxidase (GPx; E.C. 1.11.1.9) was determined following methods, adapted for microplate, from Flohé and Günzler (1984). Samples were mixed with 500 mM potassium phosphate buffer solution, 50 mM EDTA, 20 mM sodium azide, and 10 U/mL glutathione reductase (GR), 1.5 mM NADPH, 250 mM glutathione (GSH), and 10 mM H₂O₂. The absorbance was determined at 340 nm every minute over 3–5 min. The activity of GPx was determined following 24 and 48 h of incubation. The activity of GPx is expressed as U per milligram of protein. One unit of activity is defined as the activity of GPx necessary to cause the oxidation of 1.0 µmol GSH to form glutathione disulfide (GSSG) per minute.

The activity of glutathione reductase (GR; E.C. 1.8.1.7) was determined following methods, adapted for microplate, from Goldberg and Spooner (1987). Samples were mixed with 500 mM potassium phosphate buffer solution, 50 mM EDTA, 2 mM NADPH, and 10 mM GSSG. The absorbance was determined at 340 nm every minute over 3–5 min. The activity of GR was determined following 24 and 48 h of incubation. The activity of GR is expressed as U per milligram of protein. One unit of activity is defined as the activity of GR necessary to reduce 1.0 µmol GSSG to GSH per minute.

The activity of glutathione S-transferase (GST; E.C. 4.4.1.34) was determined following methods, adapted for microplate, from Habig and Jakoby (1981). Samples were mixed with 0.1 M phosphate buffer solution, 10 mM GSH, 60 mM EDTA, and 10 mM 1-chloro-2,4-dinitrobenzene (CDNB). The absorbance was determined at 340 nm every minute over 3–5 min. The activity of GST was determined following 24 and 48 h of incubation. The activity of GST is expressed as U per milligram of protein. One unit of activity is defined as the quantity of GST necessary to catalyze the conjugation of 1.0 µmol of CDNB per minute.

**Oxidative damage to proteins**

Oxidative damage to proteins was determined following methods, adapted for microplate, from Levine et al. (1990). Each sample was mixed with 5% sulfosalicylic acid. Following centrifugation at 350 × g and 4°C, the supernatant was removed. Dinitrophenyl hydrazine (10 mM) or hydrochloric acid (HCl, 2.5 M; Fermont, Monterrey, MX; blanks) was added. Vials were maintained in a dark area for 1 h, with mixing every 15 min. Following this period, 20% trichloroacetic acid was added, and the tubes were incubated in 4°C water for 10 min. Following centrifugation at 350 × g at ambient temperature, the supernatant was removed, and each tube was rinsed with 1:1 ethanol:acetic acid (J.T. Baker, Phillipsburg, NJ, USA). Following centrifugation using the aforementioned conditions, 6 M guanidine chloride was added, and all tubes were incubated in a 37°C water bath for 15 min. Each tube was centrifuged, and the absorbance of the supernatant was analyzed in the range between 340 and 405 nm. The concentration of protein carbonyls was determined using the maximum absorbance registered for each sample. The concentration of protein carbonyls was determined following 24 and 48 h of incubation. The concentration of protein carbonyls is expressed as micromoles per milligram of protein.
**Total protein**

The concentration of total soluble proteins in each sample was determined following methods, adapted for microplate, from Bradford (1976), to standardize the results for the oxidative stress indicators. A standard curve was prepared using albumin dilutions between 0.005 to 0.2 mg/mL. Each sample and the standard curve dilutions were mixed with homogenization solution, PMSF, distilled water, and Coomassie blue (Bio-Rad, Hercules, CA, USA). After 15 min incubation under ambient conditions, the absorbance of the supernatant was registered at 590 nm. The total concentration of soluble proteins in each sample was determined using the equation of the standard curve following 24 and 48 h of incubation. The concentration of total proteins is expressed in micrograms per milliliter.

**Statistical analysis**

Statistical analyses were conducted using IBM SPSS Statistics Viewer software. Normality and homogeneity were determined using Kolmogorov-Smirnov and Levene’s tests, respectively (Zar, 1999; Garson, 2012). Parametric results were analyzed with independent t-tests and dependent t-tests to determine if differences existed in the indicators of oxidative stress between species and between treatments within each species, respectively (Zar, 1999). Non-parametric results were analyzed with Mann-Whitney and Wilcoxon tests for two related samples to determine if differences existed in the indicators of oxidative stress between species and between treatments within each species, respectively (Zar, 1999). Statistical significance was assumed when $P < 0.050$ ($\alpha$ of 5%).

**Results**

Production of superoxide radical ($\text{O}_2^{\cdot-}$)

Results of the $\text{O}_2^{\cdot-}$ production in human (N = 12) and bottlenose dolphin (N = 12) leukocytes in response to LPS are shown in Fig. 1. Significant differences between species were observed. Under control conditions (LPS, 0 µg/mL) following 24 h, $\text{O}_2^{\cdot-}$ production in human leukocytes was 1.7 times higher than in bottlenose dolphin leukocytes ($P = 0.001$). Under experimental conditions (LPS, 10 µg/mL) following 48 h, $\text{O}_2^{\cdot-}$ production in bottlenose dolphin leukocytes was 1.9 times higher than in human leukocytes ($P = 0.004$).

An effect of exposure to LPS was observed in bottlenose dolphin leukocytes (Fig. 1). Under control conditions (LPS, 10 µg/mL) following 48 h, bottlenose dolphin leukocytes produced 2.1 times more $\text{O}_2^{\cdot-}$ compared to those cultured under control conditions (LPS, 0 mg/mL) for 48 h ($P = 0.005$).

An effect of incubation time was observed in bottlenose dolphin leukocytes (Fig. 1). Under control conditions (LPS, 0 µg/mL) following 48 h, bottlenose dolphin leukocytes produced 1.5 times more $\text{O}_2^{\cdot-}$ compared to leukocytes cultured for 24 h ($P = 0.019$). Under experimental conditions (LPS, 10 µg/mL) following 48 h, bottlenose dolphin leukocytes produced 2.5 times more $\text{O}_2^{\cdot-}$ compared to those exposed for 24 h ($P = 0.010$).

**Antioxidant activity**

The results for the activities of SOD, CAT, GPx, GR, and GST in human (N = 12) and bottlenose dolphin (N = 12) leukocytes in response to LPS are shown in Fig. 2. Between species, significant differences in antioxidant enzyme activities were found. Following 24 h under control conditions (LPS, 0 µg/mL), bottlenose dolphin leukocytes displayed 2.4 times less SOD activity ($P = 0.002$), 7.6 times more CAT activity ($P = 0.038$), 6.1 times more GR activity ($P = 0.001$), and 2.2 times more GST activity ($P = 0.002$) compared to human leukocytes. Following 24 h under experimental conditions (LPS, 10 µg/mL), bottlenose dolphin leukocytes displayed 2.3 times less SOD activity ($P = 0.015$), 12.9 times more GR activity ($P = 0.000$), and...
2.4 times more GST activity ($P = 0.005$) compared to human leukocytes. Following 48 h under control conditions, bottlenose dolphin leukocytes displayed 4.4 times more CAT activity ($P = 0.050$), 7.2 times more GR activity ($P = 0.000$), and 1.9 times more GST activity ($P = 0.007$) compared to human leukocytes. Following 48 h under experimental conditions, bottlenose dolphin leukocytes displayed 7.9 times more CAT activity ($P = 0.001$), 2.0 times more GPx activity ($P = 0.038$), 11.1 times more GR activity ($P = 0.000$), and 3.3 times more GST activity ($P = 0.000$) compared to human leukocytes.

Within each species, effects of LPS exposure were found in relation to antioxidant enzyme activities (Fig. 2). Following 24 h under control conditions (LPS, 0 µg/mL), human leukocytes displayed 1.9 times more SOD activity ($P = 0.008$), 2.9 times more GR activity ($P = 0.028$), and 1.4 times more GST activity ($P = 0.034$) compared to those exposed to experimental conditions (LPS, 10 µg/mL) for 24 h. Following 24 h under control conditions, bottlenose dolphin leukocytes displayed 1.8 times more SOD activity ($P = 0.019$) compared to those exposed to experimental conditions (LPS, 10 µg/mL) for 24 h.
Discussion

Reactive oxygen species, including \( \text{O}_2^- \), are produced during normal \( \text{O}_2 \) metabolism, phagocytosis, and reperfusion (Fridovich and Freeman, 1986; Halliwell and Gutteridge, 1990; Kevin et al., 2005; Birben et al., 2012). Approximately 1–4% of \( \text{O}_2 \) is reduced to ROS during mitochondrial \( \text{O}_2 \) metabolism (Kevin et al., 2005). Around one dozen mitochondria are distributed throughout the cytoplasm in human leukocytes (Anderson, 1966). ROS production, observed in this study, in human and bottlenose dolphin leukocytes under control conditions, at both 24 and 48 h, was likely associated with normal cellular processes, including ATP metabolism and apoptosis (Geering et al., 2013; Granger and Kvietys, 2015).

In this study, differences in oxidative stress indicators in bottlenose dolphin and human leukocytes were found at different incubation times. Differences in phagocyte quantity and phagocytic capacity influence ROS production and, thus, oxidative stress indicators (Sokolova, 2005; Keogh et al., 2011; Geering et al., 2013). Reports indicate that bottlenose dolphins have 42 to 75% neutrophils, 15 to 34% lymphocytes, 2 to 40% eosinophils, 0 to 4% monocytes, and <1% basophils in circulation, depending on the pathophysiological conditions (Medway and Geraci, 1964; Ridgway et al., 1970; Engelhardt, 1979; Shirai and Sakai, 1997). A similar leukogram has been reported in humans, except for a lower eosinophil count ranging from 1 to 4% (Shirai and Sakai, 1997; Francischetti et al., 2010; Kobayashi et al., 2017; Nouri-Shirazi et al., 2017). Neutrophils act as the first line of defense against bacteria and bacterial products (Kobayashi et al., 2017) and have been reported to demonstrate greater phagocytic capacity compared to eosinophils and basophils (Geering et al., 2013). Differences in half-life between leukocyte cell types, related to function, may also contribute to explain the observed differences, even in the absence of LPS. In humans, unstimulated neutrophils have a half-life of approximately 7.6 to 90 h \textit{in vivo} (Dancey et al., 1976; Pillay et al., 2010) and approximately 5 to 10 h \textit{in vitro} (Dancey et al., 1976; Lee et al., 1993; Francischetti et al., 2010). \textit{In vivo} studies in humans indicate that unstimulated T-lymphocytes have an approximate half-life of 77 to 87 days (Hellerstein et al., 1999), basophils have a half-life of up to a few days, eosinophils have a half-life of approximately 8 to 18 h

![FIGURE 3. The concentration of protein carbonyls (\( \mu \text{mol milligram}^{-1}\) of protein) in human, \textit{Homo sapiens} (N = 12), and bottlenose dolphin, \textit{Tursiops truncatus gilli} (N = 12) leukocytes, maintained under primary culture in control (LPS, 0 \( \mu \text{g/mL} \)) and experimental (LPS, 10 \( \mu \text{g/mL} \)) conditions following 24 and 48 h incubation. Data are presented as mean ± standard error. Significant differences (\( P < 0.050 \)) between species, \( * \) within the same species and time of incubation, but different lipopolysaccharide concentrations, \( \dagger \) within the same species and lipopolysaccharide concentrations, but different incubation times.](image)
Differences in oxidative stress indicators in bottlenose dolphin and human leukocytes between treatments (control and experimental groups) were found. During the respiratory burst, phagocytes increase their O2 metabolism, resulting in ROS production from both NADPH oxidase and mitochondrial metabolism (Babior, 1999; Shiraishi et al., 2002; Keogh et al., 2011). In humans, rapid immune responses are initiated by small LPS concentrations (Guha and Mackman, 2001; de Vos et al., 2009). Modifications in antioxidant enzyme expression occur during periods of increased ROS production, including during immune responses (Davies, 2000; Marikovsky et al., 2003), contributing to decreased oxidative stress (Fridovich and Freeman, 1986; Halliwell and Gutteridge, 1990; Kevin et al., 2005). Following exposure to LPS isolated from E. coli, SOD, GR, and GPx activities significantly increased in the livers of Balb/c mice (Mus musculus) in comparison with controls (Kurhaluk et al., 2018). In this study, the significant increases observed in GPx, CAT, GST, and GR activities in bottlenose dolphin leukocytes and of GST and GR in human leukocytes, under experimental conditions between 24 and 48 h, likely relate to immune and antioxidant responses resulting from altered leukocyte metabolism in the presence of LPS.

Differences in oxidative stress indicators between bottlenose dolphin and human leukocytes were found in this study; greater ROS production was observed in bottlenose dolphin leukocytes following 48 h under experimental conditions as compared to human leukocytes. In this study, bottlenose dolphin leukocytes generally presented greater antioxidant defenses, which likely contributed to the lower oxidative damage observed as compared to human leukocytes. Previous reports suggest that H. sapiens are highly sensitive endotoxin models, compared to other mammals (Wolff, 1973; Guha and Mackman, 2001; Shiraishi et al., 2002; Copeland et al., 2005; de Vos et al., 2009). Marine mammals experience drastic fluctuations in O2 availability during the dive response. These species generally have greater concentrations of O2-binding proteins (hemoglobin, myoglobin) and greater antioxidant defenses in comparison with terrestrial mammals (Kooyman and Ponganis, 1998; Wilhelm Filho et al., 2002; Ponganis et al., 2003). Wilhelm Filho et al. (2002) reported that under physiological conditions, in the erythrocytes of various marine mammal species, GPx, CAT, GST, and GR activities were significantly greater, as compared to the erythrocytes of various terrestrial mammal species. Vázquez-Medina et al. (2007) reported significantly greater GR activity and GSH concentrations in the tissues of ringed seals (Phoca hispida) in comparison with the tissues of domestic pigs (Sus scrofa domesticus), indicating a greater capacity to recycle GSH in marine mammals. Both GPx and CAT act to reduce H2O2 produced during phagocytosis (Fridovich and Freeman, 1986). CAT primarily acts during periods of escalated H2O2 production, such as during inflammation (Fridovich and Freeman, 1986; Burdon, 1995). GR catalyzes the formation of GSH from GSSG (Sies, 1997), while GST acts to eliminate reactive electrophiles, such as carbonyl groups, through their conjugation with GSH (Sies, 1997). Based on the results obtained in this study, it is likely that bottlenose dolphin leukocytes are better equipped at clearing H2O2 during a pro-inflammatory response, have a greater capacity to eliminate reactive groups, and demonstrate a greater capacity to recycle GSH, in comparison with the leukocytes of humans.

Leukocytes and other immune cells incorporate free unsaturated fatty acids into their cellular membranes, increasing their susceptibility to oxidative damage (Rustan and Drevon, 2005; Poggi et al., 2015). Aldehyde formation following lipid peroxidation can damage membrane-bound proteins, leading to the loss of membrane integrity, structure, and function (Birben et al., 2012). In this study, following 24 and 48 h under both control and experimental conditions, greater protein carbonyl concentrations were observed in human leukocytes compared to bottlenose dolphin leukocytes. These results were similar to those reported by Del-Aguila-Vargas et al. (2019) in that the skeletal muscle cells from northern elephant seals (Mirounga angustirostris) incubated under control conditions demonstrated lower (non-significant) protein carbonyl concentrations compared to the muscles of humans.

In summary, the results from this study suggest that in response to LPS and compared to human leukocytes, bottlenose dolphin leukocytes may increase their O2 metabolism to a higher degree as a means to quickly and efficiently terminate the pro-inflammatory response, resulting in increased ROS production. Greater antioxidant defenses were found in bottlenose dolphin leukocytes compared to human leukocytes. This likely contributed to the reduced oxidative damage observed in bottlenose dolphin leukocytes. Host immune responses can vary between individuals, between species in relation to habitat, diet, metabolic rate, sex, age, and in relation to the presenting pathogen, dose, and administration method (Lehmann et al., 1987; Shiraishi et al., 2002; Copeland et al., 2005; Miller et al., 2005; Venn-Watson et al., 2011; Kurhaluk et al., 2018). Thus, discrepancies with other reports likely relate to the tissue analyzed, the phagocytic capacity of each leukocyte group, and species-specific evolutionary histories. How diet, sex, age, and habitat affect the indicators of oxidative stress during a pro-inflammatory response in bottlenose dolphins leukocytes are not known. Further studies should aim to compare immune sensitivity to LPS and the production of immune factors, such as cytokines, between marine and terrestrial mammals. Studies addressing the effects of pro-inflammatory factors in other marine mammal species, including comparisons between cetaceans and pinnipeds, are needed.

Conclusion

Our knowledge of the immune response in marine mammals remains fragmentary. In this study, exposure to LPS induced...
an immune response in the leukocytes of both *H. sapiens* and *T. truncatus gilli*. Following 48 h under experimental conditions, greater ROS production and lower oxidative damage were observed in bottlenose dolphin leukocytes, likely as a result of greater antioxidant defenses compared to human leukocytes. These results provide support to the hypothesis that greater antioxidant defenses in the tissues of marine mammals provide additional protection against situations that induce oxidative stress, such as inflammation, compared to the tissues of terrestrial mammals. The results of this study suggest that the immune system of marine mammals is better equipped for the efficient termination of pro-inflammatory responses, likely as an evolutionary advantage, in comparison with their terrestrial counterparts.

**Acknowledgement:** The authors wish to thank O. Lugo Lugo, P. Hernández Almaraz, and C. Reyes Ramos for their assistance in the laboratory. The authors thank the management, training, and veterinary teams (especially L. Ibarra Vargas, M. C. Lopez, M. F. Ramírez, and R. Santos Camacho) from Cabo Dolphins for generously providing blood samples (*Tursiops truncatus gilli*) for this study. Finally, the authors thank the staff from IMSS for the generous donation of residual blood (*Homo sapiens*) for this investigation.

**Availability of Data and Materials:** Requests for the datasets analyzed during the current study can be requested from the corresponding author.

**Author Contribution:** The authors confirm contribution to the paper as follows: study conception and design: T. Zenteno-Savín; data collection: T. E. Symon; analysis and interpretation of results: T. E. Symon, R. Gaxiola-Robles, T. Zenteno-Savín; draft manuscript and preparation: T. E. Symon, T. Zenteno-Savín, R. Gaxiola-Robles, C. J. Hernández-Camacho. All authors reviewed the results and approved the final version of the manuscript.

**Ethics Approval:** All blood samples were collected with permission from the Comisión Nacional de Bioética (CONBIOÉTICA-09-CEI-009-20160601; registration No. 2018-785-010). Bottlenose dolphin blood samples were obtained with informed consent from Cabo Dolphins.

**Funding Statement:** Funding for this project was received from CONACyT [Project CB-2016-01-283669] and CIBNOR (Línea Estratégica II. Estrés Oxidativo). TES received a CONACYT graduate studies scholarship (CVU No. 922584).

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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