



Short communication

Simultaneous measurement of phagocytosis and respiratory burst of leukocytes in whole blood from bottlenose dolphins (*Tursiops truncatus*) utilizing flow cytometry

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ABSTRACT

Phagocytic and respiratory burst activity was simultaneously measured by flow cytometry in polymorphonuclear leukocytes (PMN) and monocytes in whole blood from bottlenose dolphins (*Tursiops truncatus*). Blood was collected from 16 adult dolphins, 12 males (6–34 years of age) and 4 females (11–30 years) and subsequently incubated with a bacteria-to-leukocyte ratio of 25:1 and 10 μ l of 500 μ M 2',7'-dichlorofluorescein diacetate for 70 min at 37 °C. PMN (44.5 \pm 3.2%) and monocytes (33.5 \pm 3.0%) were positive for propidium iodide-labeled *Staphylococcus aureus*, indicating phagocytosis. Respiratory burst activity after 70 min as measured by the mean fluorescence intensity (MFI) was 68.0 \pm 14.4 in PMN and 47.0 \pm 10.3 in monocytes. There were no significant differences in MFI or percentage of phagocytizing PMN ($p > 0.094$) or monocytes ($p > 0.275$) after storage at 4 °C for 24 h when compared to activity measured in fresh blood. Nor was there an effect of storage on respiratory burst activity (MFI or percentage) in PMN ($p > 0.420$) or monocytes ($p > 0.301$). This assay may be particularly useful to assess the ability of dolphins to effectively combat bacterial pathogen challenges with minimal amounts of blood.

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1. Introduction

Bottlenose dolphins (*Tursiops truncatus*) inhabit coastal and pelagic waters in tropical and temperate oceans throughout the world. As long-lived, top predators in the

marine environment, bottlenose dolphins bioaccumulate organochlorines (Ridgway and Reddy, 1995; Reddy et al., 1998; Parsons and Chan, 2001), heavy metals (Roditi-Elasar et al., 2003), polychlorinated biphenyl (Litz et al., 2007), and polybrominated diphenyl ethers (Johnson-Restrepo et al., 2005; Fair et al., 2007; Litz et al., 2007; Johnson-Restrepo et al., 2008) contributing to their proposed status as sentinels of the marine environment (Wells et al., 2004), and leading to increased interest in the immune function in coastal populations of bottlenose dolphins. Given the potential immuno-suppressive effects of contaminants, the prevalence of bacterial and fungal infections associated with morbidity and mortality events (Buck et al., 1991; Ewalt et al., 1994; Higgins, 2000; Parsons and Jefferson, 2000; Durden et al., 2009), and presence of terrestrial

Abbreviations: PI-staph, propidium iodide labeled *Staphylococcus aureus*; DCFH-DA, 2',7'-dichlorofluorescein diacetate; FC, flow cytometry; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; MFI, mean fluorescence intensity; NEM, N-ethylmaleimide.

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pathogens including sewage-borne bacteria in dolphins (Parsons and Jefferson, 2000), further research is needed on the innate immune function of bottlenose dolphins.

Innate immunity is a nonspecific host defense that exists prior to exposure to an antigen and includes phagocytic and respiratory burst activity. The innate immune response includes professional phagocytes including neutrophils and monocytes which respectively comprise 22–72% and 0–11% of the circulating white blood cells in adult bottlenose dolphins (Goldstein et al., 2006; Hall et al., 2007; Venn-Watson et al., 2007; Schwacke et al., 2009). Phagocytosis and subsequent respiratory burst activity, which results in the generation of reactive oxygen species (ROS), are integral components of the immune response to bacterial and fungal infections. ROS can directly kill microbial pathogens or act as signaling molecules modulating phagocytosis in PMN (Fialkow et al., 2007).

Phagocytosis in bottlenose dolphins has been measured utilizing microscopy (Noda et al., 2003, 2007; Sokolova, 2005) or flow cytometry (FC) (Levin et al., 2004; Pellisso et al., 2008; Reif et al., 2009). Respiratory burst activity has been assessed by the nitroblue tetrazolium reduction assay (Noda et al., 2003; Reif et al., 2009), chemiluminescence (Itou et al., 2001, 2002, 2005; Shiraishi et al., 2002), or FC (Levin et al., 2007). While these studies have added to the limited knowledge of the innate immune response in dolphins, they assessed phagocytic and respiratory burst activity separately, primarily in PMN. Further, these assays employed time consuming cell isolation procedures requiring large volumes of blood and utilized artificial targets such as latex beads. Simultaneous measures of phagocytic and respiratory burst activity utilizing FC have been developed for use in humans (Hasui et al., 1989; Cinco et al., 1994; Prodan et al., 1995), cows (Smits et al., 1997), horses (Massoco and Palermo-Neto, 2003), pigs (Busque et al., 1998), and sheep (Whist et al., 2002). An assay that measures phagocytosis and respiratory burst simultaneously utilizing minimal amounts of blood is highly desirable in dolphins given field logistical and sampling constraints.

To this end, an immune functional assay was developed in order to: (1) simultaneously measure phagocytic and respiratory burst activity in bottlenose dolphin PMN and monocytes; (2) utilize a minimal amount of whole blood; (3) utilize a natural immune stimulus; and (4) determine the effects of storage at 4 °C for 24 h for shipment from field sites to the laboratory.

2. Materials and methods

2.1. Animals

Blood was collected from 16 bottlenose dolphins including 12 males (6–34 years of age) and 4 females (11–30 years) cared for by the U.S. Navy Marine Mammal Program. Blood samples were collected under behavioral control from the ventral aspect of the tail stock directly into Vacutainer® tubes (BD Diagnostic systems, Sparks MD) with sodium heparin. One tube was analyzed within 4 h of collection while the second tube was stored at 4 °C for 24 h after which it was brought to 37 °C and assayed under the same conditions. In addition, treatment with

staurosporine, a protein kinase C inhibitor demonstrated to suppress neutrophil respiratory burst activity in other species (Dewald et al., 1989; Garland, 1992; Smits et al., 1997), was used as an additional validation of this assay in dolphins. Heparinized blood obtained from an additional 13 wild dolphins was used to test blocking of respiratory burst with staurosporine. Blood used for staurosporine testing was shipped on wet ice and assayed within 24 h of collection.

2.2. Phagocytosis and respiratory burst assay

Propidium iodide labeled *Staphylococcus aureus* (PI-staph) was used to measure phagocytosis while 2',7'-dichlorofluorescein diacetate (DCFH-DA) quantified respiratory burst activity. DCFH-DA is a non-fluorescent cell permeable dye which upon oxidation becomes fluorescent (Bass et al., 1983). *Staphylococcus aureus*, strain ATCC 25923, was cultured for 18 h in trypticase soy broth (BD Diagnostic systems, Sparks, MD) at 37 °C. Bacteria were washed three times in PBS without calcium and magnesium (pH 7.2) (Invitrogen Corp., Carlsbad, CA), heat-killed at 70 °C for 1 h and washed three times in PBS. Bacteria were labeled with a 5% solution of propidium iodide (Molecular Probes, Eugene, OR) in PBS for 1 h at room temperature in the dark, washed three times and re-suspended in PBS. Bacterial density was adjusted to an absorbance value of 2.50 at 620 nm with a spectrophotometer (Ultra-spect 1000, Pharmacia Biotech) for an approximate density of 2.4×10^9 CFU/ml (Hasui et al., 1989). PI-staph was stored in the dark at –70 °C and thawed immediately before use. DCFH-DA (Molecular Probes, Eugene, OR) was dissolved in DMSO (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 25 mM and stored in the dark at –70 °C. DCFH-DA was thawed and diluted in PBS for a working concentration of 500 μM.

Methods previously established for simultaneously measuring the phagocytic and respiratory burst activity in PMN and monocytes (Hasui et al., 1989; Smits et al., 1997; Whist et al., 2002; Kampen et al., 2004) were validated for use with dolphins. Briefly, 100 μl of whole blood was added to sterile 12 mm × 75 mm polystyrene tubes for each time point (0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 min) in triplicate. All controls were performed in duplicate and consisted of: (1) whole blood; (2) whole blood and PI-staph; (3) whole blood with 10 μl of 500 μM DCFH-DA; and (4) whole blood with unlabeled *S. aureus* and 10 μl of 500 μM DCFH-DA. For all relevant control and non-control tubes, blood was pre-incubated with DCFH-DA for 10 min in a shaking water bath at 37 °C. Total leukocyte counts were performed with a hemacytometer using trypan blue (Sigma–Aldrich, St. Louis, MO) exclusion as a measure of viability (>95%). PI-staph was added for final ratio of 25:1 bacteria to leukocyte to all relevant control and non-control tubes.

Following each period of incubation in a shaking water bath at 37 °C, tubes were placed on ice and 10 μl 1 mM N-ethylmaleimide (NEM) was added to stop phagocytosis (Smits et al., 1997). Erythrocytes were subsequently lysed with 2 ml lysing buffer (0.01 M Tris; 0.001 M EDTA; 0.17 M NH₄Cl, pH 7.4) followed by washing 2 × with PBS

and re-suspension in 500 μ l cold 1% paraformaldehyde. Samples were placed on ice in the dark and read on an LSR flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

2.3. Quenching of external fluorescence

Attached PI-staph were differentiated from those internalized by quenching the fluorescence of attached bacteria with 20 μ l 0.4% trypan blue solution (Flaminio et al., 2000; Raidal et al., 2000; Flaminio et al., 2002) and repeating the FC analysis.

2.4. Staurosporine

Following the pre-incubation with DCFH-DA, 10 μ l of staurosporine (S5921, Sigma–Aldrich, St. Louis, MO, USA) at 5 μ g/ml was added prior to the addition of bacteria. Staurosporine was dissolved in DMSO at 50 μ g/ml then diluted to 5 μ g/ml with PBS immediately prior to use. DMSO control tubes consisting of blood incubated with 10 μ l DMSO diluted 1:10 in PBS were included. All tubes were assayed in triplicate and were incubated at 37 °C for 75 min at which point the tubes were treated with NEM and placed on ice as described above.

2.5. Flow cytometry

Samples were analyzed on a LSR BD flow cytometer with linear amplification of the forward scatter (FSC) and side scatter (SSC) signals and logarithmic amplification of the fluorescence signals (Fig. 1A). Fifty thousand events were collected; PMN and monocytes were gated based on size and complexity. An example of FC analysis for phagocytosis of PI-staph and the resulting respiratory burst from PMN are shown in Fig. 1B. Respiratory burst activity as quantified by DCFDA conversion to DCF was measured at 530 \pm 30 nm (FL1 detector) while phagocytosis was measured at 585 \pm 42 nm (FL2). Both fluorochromes were analyzed following fluorescence compensation to correct for overlap between FL1 and FL2 signals. Respiratory burst activity and phagocytosis of gated PMN and monocytes were quantified as the mean fluorescence intensity (MFI) and percentage of positive cells in FL1 or FL2, respectively. MFI is correlated to the mean number of bacteria ingested per phagocyte (Menge et al., 1998; Moya et al., 2008).

2.6. Statistical analysis

Data were analyzed with Systat 10™ (Systat Software, Inc, Point Richmond, CA, USA). Repeated measures analysis of variance (ANOVA) was used to evaluate the phagocytic and respiratory burst activity over time. Data are expressed as means plus or minus standard error (\pm SE). The effect of storage, staurosporine, and quenching with trypan blue were assessed by paired *t*-tests. Results were considered statistically significant at $p \leq 0.05$.

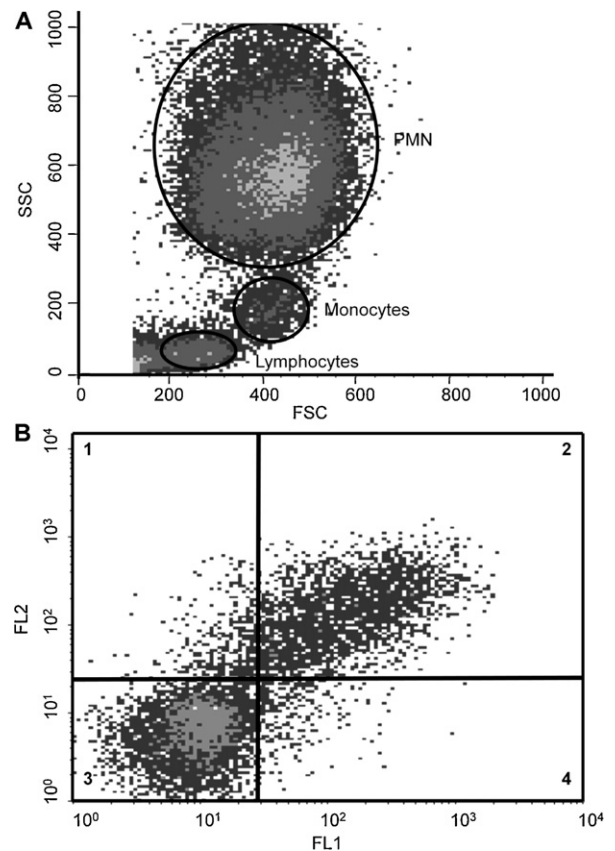


Fig. 1. An example of FC analysis of the phagocytosis of PI-staph and the resulting stimulations of respiratory burst activity from one bottlenose dolphin. (A) The distribution of leukocytes was displayed according to their size (FSC) and granularity (SSC) enabling the identification of the leukocyte populations (lymphocytes, monocytes, PMN). (B) Individual cells within each gated population of PMN or monocytes were analyzed for respiratory burst (FL1) and phagocytic (FL2) activity. Cells with phagocytic activity are present in quadrants 1 and 2 while cells with respiratory burst activity are found in quadrants 2 and 4. Cells with both phagocytic and respiratory burst activity appear in quadrant 2 while quadrant 3 contains cells with no phagocytic or respiratory burst activity detected.

3. Results and discussion

FC methods developed for human and veterinary medicine were applied to simultaneously quantify phagocytic and respiratory burst activity in whole blood from bottlenose dolphins. The present assay, including all controls and a 70 min incubation period requires only 1.1 ml of blood, allowing for assessment of individuals in which there is a limited amount of blood collected such as with calves, sick or wild dolphins. Further, additional incubation periods can be added without dramatically increasing the required volume of blood allowing for studies on the kinetics of phagocytic and respiratory burst activity in dolphins. Compared to previous studies in cetaceans, the current procedure: (1) reduced blood volume; (2) eliminated leukocyte isolation prior to assay; (3) reduced time and labor in the lab; (4) used autologous plasma for opsonization of bacteria; (5) provided independent and comparative values for phagocytic and respiratory

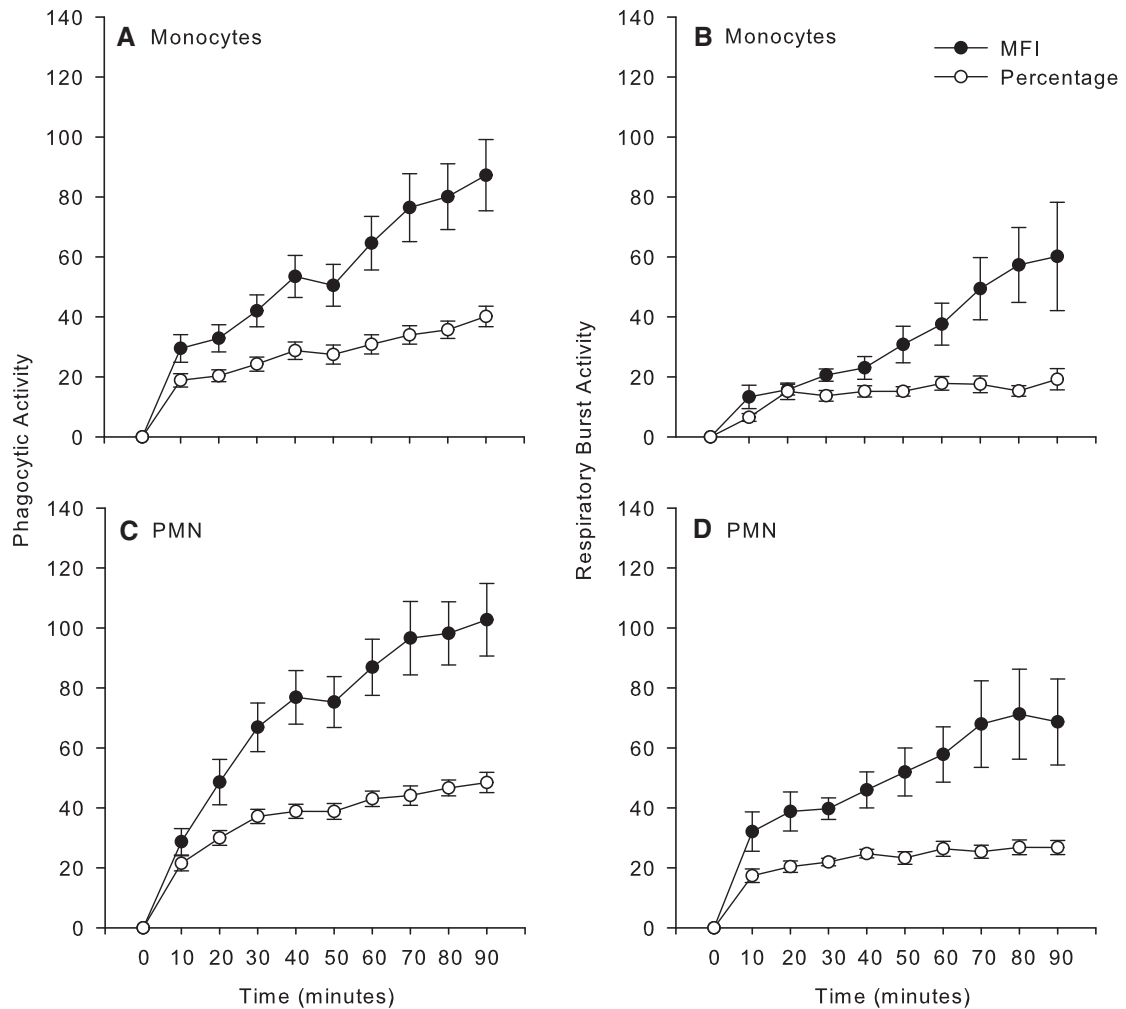


Fig. 2. Time dependent phagocytic activity measured as percentage of positive cells for PI-staph and mean fluorescence intensity (MFI) in (A) monocytes and (C) PMN. Time dependent respiratory burst activity as measured by percentage of positive cells for DCF and mean fluorescence intensity (MFI) in (B) monocytes and (D) PMN. Results are mean \pm SEM of triplicate measures.

burst activity induced by a natural pathogen in PMN and monocytes; and (6) allowed processing 24 h after blood collection.

Dolphin PMN and monocytes were able to phagocytize PI-staph inducing the production of ROS as indicated by the oxidation of DCFH-DA (Fig. 2). Phagocytic and respiratory burst activity was measurable in PMN and monocytes following 10 min incubation with PI-staph (Fig. 2). There was not a significant difference in phagocytic or respiratory burst activity (%) following the 70 min incubation period ($p > 0.111$); therefore only results from the 70 min incubation period were used for further analysis.

Following 70 min incubation with PI-staph, 44.1% (± 13.0) of the PMN and 33.5% (± 12.2) of monocytes were positive for phagocytosis. Phagocytosis quantified as MFI was 96.6 (± 49.0) for PMN and 74.6 (± 45.3) for monocytes. Phagocytosis in PMN and monocytes was strongly correlated ($r > 0.557$, $p < 0.025$) whether reported as MFI or percentage indicating dolphins with higher phagocytosis in PMN had higher phagocytic activity in monocytes. PMN in

the present study reached phagocytic activity (%) similar to those previously reported for bottlenose dolphins following 18 h incubation with *S. aureus* strain 25293 (Noda et al., 2003, 2007) or latex beads (Levin et al., 2004). In these previous studies, leukocytes or PMN were isolated and erythrocytes lysed prior to assessment of phagocytosis with or without opsonization of targets. Phagocytosis assessed with the use of opsonins is based upon the innate cellular function of the phagocyte and opsonic activity in serum or plasma. Opsonin independent phagocytosis has been shown to increase with increasing target to cell ratio and/or incubation period (Dunn and Tyrer, 1981; Lehmann et al., 1998). As suggested by de Guise et al. (1998) this likely contributed to the required incubation of 18 h and the 100:1 ratio of latex bead to leukocyte employed in these previous studies.

Phagocytosis in the present study was higher in PMN (44.1 \pm 13.0%; 96.6 \pm 12.2 MFI) than in monocytes (33.5 \pm 12.2%; 74.62 \pm 11.3 MFI). These results differ from studies where phagocytosis (%) in monocytes were similar

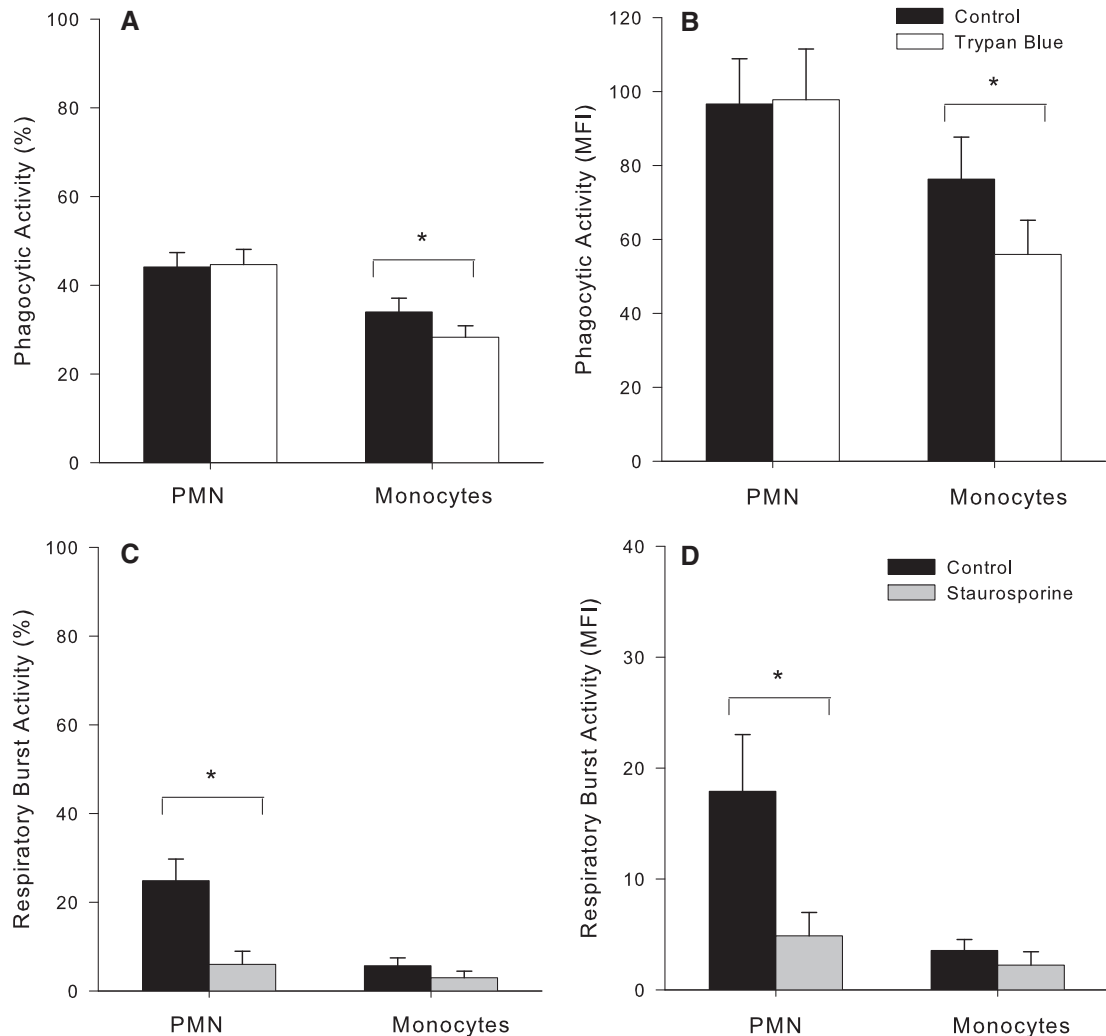


Fig. 3. Effect of blocking experiments. Quenching of fluorescence with trypan blue on phagocytic activity in PMN and monocytes as measured as (A) percentage of positive cells for PI-staph and (B) mean fluorescence intensity (MFI). Respiratory burst activity as measured by (C) percentage of positive cells for DCF and (D) mean fluorescence intensity (MFI) of DCF in monocytes and PMN leukocytes following incubation with DMSO (control) or staurosporine. Mean \pm SEM following an incubation period of 70 min. * $p < 0.05$.

or higher to PMN (Cinco et al., 1994; Prodan et al., 1995; Menge et al., 1998; Riber and Lind, 1999; Levin et al., 2004; Reif et al., 2009). These findings may be due to methodological differences including whether targets were opsonized or as a result of the different pathways used by PMN and monocytes. Monocytes utilize C3b generated via the alternative pathway (Leijh et al., 1979; Jensen-Waern et al., 1994) while PMN can generate C3b either through the alternative or classical pathways (Leijh et al., 1981) during an innate immune response. Similar to the present study, Levin et al. (2004) reported phagocytosis higher in PMN than monocytes in dolphins. However, while phagocytosis in PMN was similar to our study, phagocytosis in monocytes was approximately half as observed in the present study and may in part be due to methodological differences. Cinco et al. (1994) reported a difference in phagocytosis of PMN and monocytes incubated with opsonized versus unopsonized bacteria. While phagocytosis was higher for

opsonized bacteria in both leukocyte populations, the effect of opsonization was greater in monocytes. Therefore, the higher phagocytosis in monocytes in our study compared to Levin et al. (2004) may be due to our use of whole blood which utilizes the Ig and complement components present for opsonization of bacteria (Guidry et al., 1974; Jensen-Waern et al., 1994).

External fluorescence was quenched by trypan blue allowing differentiation between attached and engulfed PI-staph. There was a significant number of attached PI-staph to monocytes (MFI $p = 0.001$; percentage $p = 0.001$) but not PMN (MFI $p = 0.687$; percentage $p = 0.435$) (Fig. 3A and B). Attached PI-staph accounted for 9.1% of monocytes positive for phagocytosis and 15.4% of the MFI. PMN did not have a significant number of PI-staph attached indicating phagocytosis was based only upon ingested PI-staph. The differences observed in attached PI-staph between PMN and monocytes further highlights the differences in innate

immune response between PMN and monocytes in dolphins.

Large individual variation in phagocytosis in PMN (range; 23.3–66.1%, 26.3–191.8 MFI) and monocytes (range: 15.1–50.5%; 23.0–192.4 MFI) was observed in the present study. Phagocytosis is regulated by surface receptors and the response to various stimuli is governed by prior exposure to priming agents such as bacteria, cytokines, and hormones. Differences in phagocytosis are likely due to the type of circulating PMN since the gated PMN in the present study is comprised of neutrophils, eosinophils and basophils. Bottlenose dolphin eosinophils exhibit phagocytosis (Sokolova, 2005) and elevated eosinophil counts were associated with higher phagocytosis in dolphins (Schwacke et al., 2010). Monoclonal antibody specific for bottlenose dolphin neutrophils (Kato et al., 2009) can be combined with the present assay to quantify the phagocytic and respiratory burst activity of neutrophils which are not easily discernible from eosinophils by forward/side scatter.

PMN and monocytes undergo an increase in oxygen consumption associated with phagocytosis resulting in the generation of ROS which is integral to the immune response to bacterial and fungal infections. Previous studies found respiratory burst activity in dolphin PMN induced by PMA, ConA, LPS, and bacteria vary with dose, incubation time and temperature (Itou et al., 2001; Shiraishi et al., 2002). Further, respiratory burst activity was stimulated when dolphin neutrophils were incubated solely with heated plasma and increased when zymosans were opsonized compared to unopsonized zymosan (Shiraishi et al., 2002) supporting the role of heat labile and heat stable components of the complement system in respiratory burst in dolphin PMN. Therefore, the use of whole blood in the present study ensured the presence of heat labile and stable components of the complement system employed during respiratory burst activity.

Following the 70 min incubation period with PI-staph, 25.4% (± 2.2) of PMN and 20.4% (± 2.0) of monocytes exhibited respiratory burst activity. Respiratory burst activity quantified by MFI was 68.0 (± 14.4) for PMN and 47.0 (± 10.3) for monocytes. Respiratory burst activity also had large individual variation in PMN (range 8.2–41.6%; 19.7–216.3 MFI) and monocytes (5.2–36.9%; 5.2–181.4 MFI). Similar to phagocytosis, respiratory burst activity (MFI) in PMN and monocytes were strongly correlated ($r = 0.719$, $p = 0.002$); however this relationship was not significant when activity was measured as a percentage of positive cells ($r = 0.464$, $p = 0.070$).

Respiratory burst activity was assessed after treatment with staurosporine, a potent protein kinase C inhibitor, or DMSO as a vehicle control (Fig. 3C and D). Previous studies have demonstrated that staurosporine suppresses neutrophil respiratory burst activity (Dewald et al., 1989; Garland, 1992; Smits et al., 1997); however, the effect on monocytes has not previously been examined. Dolphin PMN treated with staurosporine exhibited reduced respiratory burst activity (MFI $p = 0.018$; percentage $p = 0.003$) with no observed effect in monocytes (MFI $p = 0.360$; percentage $p = 0.261$). These results warrant further investigation of the production of ROS in PMN and monocytes in dolphins.

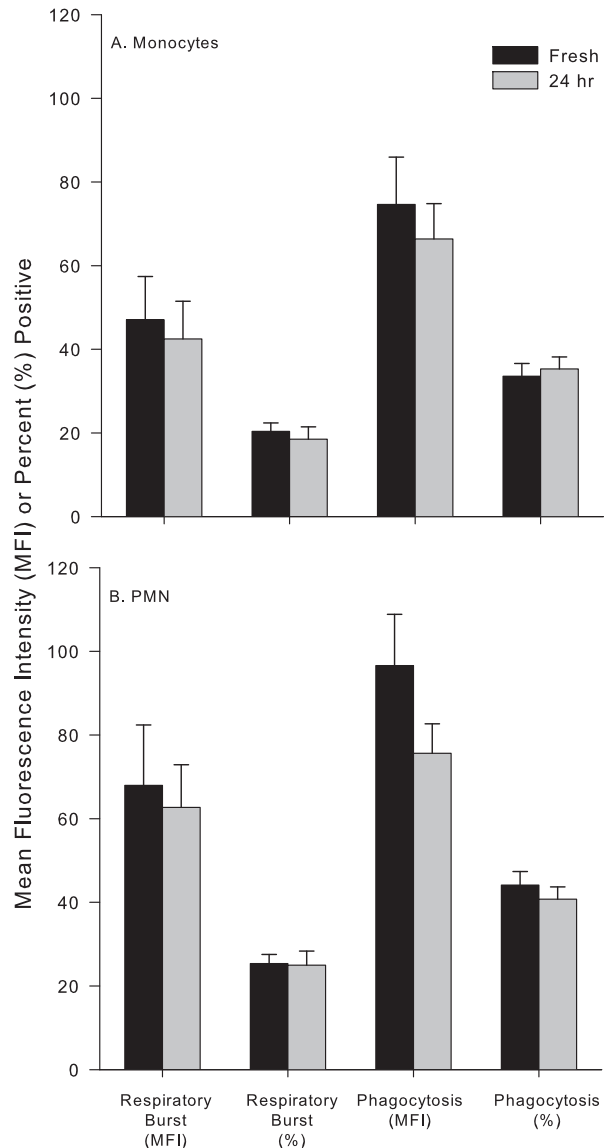


Fig. 4. Effect of storage at 4 °C for 24 h on phagocytic and oxidative burst activity in (A) monocytes and (B) PMN. Results are mean \pm SEM following an incubation period of 70 min.

Finally, we assessed the effects of storage at 4 °C for 24 h which is needed for future studies involving shipment from field sites. No difference was observed in phagocytosis of PMN (MFI $p = 0.094$; percentage $p = 0.377$) or monocytes (MFI $p = 0.275$; percentage $p = 0.606$) (Fig. 4). Nor was there an effect of storage on respiratory burst activity measured in PMN (MFI $p = 0.420$; percentage $p = 0.983$) or monocytes (MFI $p = 0.301$; percentage $p = 0.502$). Similarly, storage of blood for 24 h did not alter either the phagocytic or respiratory burst activity in dogs (Eickhoff et al., 2004). These findings demonstrate that samples from remote field sites or facilities that do not have FC capabilities can be processed up to 24 h following collection without negative effects. Therefore, the present assay is ideal for simultaneously quantifying phagocytosis and respiratory burst in

PMN and monocytes from dolphins while utilizing minimal amounts of blood. Further, the assay can utilize different bacteria and fungi enabling comparison between strains and species and does not require species-specific reagents allowing for its application to other cetacean species with minimal effort.

Conflict of interest

The authors have no conflict of interest.

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