Antioxidants to Prevent Bovine Neutrophil-Induced Mammary Epithelial Cell Damage*

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ABSTRACT

Activated neutrophils are able to produce a large quantity of bactericidal molecules such as reactive oxygen species that have been associated with tissue damage in several inflammation models. The protective effects of antioxidants in a context of neutrophil-induced damage to mammary epithelial cells were first evaluated in vitro using a coculture model of activated bovine neutrophils and a bovine mammary epithelial cell line (MAC-T cells). Cell damage was determined by quantifying the release of lactate dehydrogenase by MAC-T cells in culture medium. Morphological observation of cells stained with acridine orange was used to visualize the extent of cell damage. When incubated with neutrophils activated by lipopolysaccharides and phorbol 12-myristate 13-acetate, MAC-T cells released large amounts of lactate dehydrogenase indicating significant cell damage. The addition of dimethylthiourea or bathocuproine disulfonic acid did not reduce the damage whereas catechin, deferoxamine or glutathione ethyl ester significantly reduced neutrophil-induced cytotoxicity in a dose-dependent manner. The effect of deferoxamine, an iron chelator, on the growth of *Escherichia coli* and the ability of bovine neutrophils to phagocytose these bacteria were then assessed in vitro. Our data showed that deferoxamine did not interfere with the phagocytic activity of neutrophils but inhibited growth of the bacteria. Overall, our results suggest that antioxidants may be effective tools for protecting mammary tissue against neutrophil-induced oxidative stress during bovine mastitis.

(Key words: mastitis, inflammation, antioxidant, reactive oxygen species)

Abbreviation key: BDA = bathocuproine disulfonic acid, CAT = catechin, DFO = deferoxamine, DMEM =

Dulbecco's modified Eagle medium, DMTU = dimethylthiourea, FBS = fetal bovine serum, GEE = glutathione ethyl ester, GSH = glutathione, LDH = lactate dehydrogenase, PMA = phorbol 12-myristate 13-acetate, PMN = polymorphonuclear neutrophils, ROS = reactive oxygen species.

INTRODUCTION

The economic losses caused by mastitis are well known in the dairy industry. Mastitis is an inflammatory reaction that usually occurs following an IMI. Local recruitment of immune cells such as macrophages and neutrophils (PMN) is a crucial step in the development of an acute inflammatory response. Neutrophils are essential for effective host defense but they are also known to be involved in various inflammatory diseases (Weiss, 1989; Smith, 1994). Activated PMN reach the infected site and release toxic reactive oxygen species (ROS) via an oxygen-dependent pathway. The cornerstone of this process is the generation of superoxide anion (O_2^-) via the enzyme NADPH-oxidase, which leads to the production of other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), and hypochlorous acid (HOCl). Although these ROS are harmful to bacteria, they also have the potential to destroy host cells. Several studies on the inflammation process have shown that oxidants released by PMN induce tissue damage (Weiss, 1989; van Asbeck, 1990). Oxidants are known to be cytotoxic through many different mechanisms such as protein and amino acid oxidation, lipid peroxidation, and DNA damage. Some ROS can play a role in the transmission of intracellular signals.

Recently, it was demonstrated in vitro that activated blood PMN have a cytotoxic effect on mammary epithelial cells (Ledbetter et al., 2001) potentially through the release of ROS such as hydroxyl radicals (Boulanger et al., 2002). In cases of acute mastitis such as those caused by *Escherichia coli*, the amount of ROS released by PMN may overwhelm the cow's endogenous antioxidant protection mechanisms and therefore add to the inflammation causing extensive tissue damage. There-

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fore, it is plausible that supplementation with exogenous antioxidants may facilitate the cow's recovery from mastitis and protect the secretory epithelial cells.

The small pool of cellular iron that is loosely bound to cellular constituents is believed to participate in the generation of highly reactive oxygen species (Dehne et al., 2001). When deferoxamine (**DFO**) chelates free iron, it forms ferrioxamine, which is a very stable complex distributed in the extracellular space and unable to penetrate cells (Emerit et al., 2001). Gannon et al. (1987) demonstrated that pretreating cultured bovine pulmonary artery endothelial cells with DFO decreases PMN-mediated injury. Thus, the use of this chelator may prevent the Fenton reaction and generation of hydroxyl radicals. Dimethylthiourea (**DMTU**) is a hydroxyl radical and hydrogen peroxide scavenger that is able to inactivate ROS (Sprong et al., 1997; Visseren et al., 2002). Protective effects of DMTU were found in several models of acute lung injury induced by phorbol 12-myristate 13-acetate (**PMA**). Cuprous ions are also thought to be strong pro-oxidants that form radicals by a Fenton-type reaction or by transition complexes with molecular oxygen. Therefore, elimination of these ions by chelators such as bathocuproine disulfonic acid (**BDA**) could lower the oxidizing effects of various ROS. Oikawa and Kawanishi (1996) showed that BDA completely prevented DNA damage that was likely mediated by H_2O_2 . Furthermore, using a human breast carcinoma cell line, Verhaegh et al. (1997) demonstrated in vitro that BDA prevented hydroxyl radical formation. Catechin (**CAT**) is a flavonoid polyphenol extracted from plants. It has oxygen-scavenging properties and anti-inflammatory effects (Yang et al., 1998). Glutathione (**GSH**) is a key component of the cellular defense cascade against injury caused by ROS (Kennedy and Lane, 1994). It is the key substrate of the endogenous H_2O_2 -removing enzyme GSH peroxidase. It is also a scavenger of hydroxyl radicals and singlet oxygen. Reduced glutathione ethyl ester (**GEE**) is a cellular antioxidant that can easily cross membranes and help to maintain the intracellular GSH concentration.

In this study, a coculture model composed of MAC-T cells (Huynh et al., 1991) and bovine PMN was used to evaluate the effects of selected antioxidants and chelators on PMN-induced damage of mammary epithelial cells. The effect of DFO on bacterial growth and on the ability of PMN to ingest *E. coli* was also investigated.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise specified, all the reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). Lipopolysaccharide (*Escherichia coli* O55:B5), DFO,

GEE, DMTU, and BDA were dissolved in Hanks' balanced salt solution. Catechin was dissolved in 100% ethanol before being further diluted in Hanks' balanced salt solution. Phorbol 12-myristate 13-acetate was dissolved in dimethyl sulfoxide.

Cell Culture and Coculture

The bovine mammary epithelial cell line MAC-T was maintained in culture by serial passages in 75-cm² flasks at 37°C in a humidified atmosphere containing 5% CO₂. The routine culture medium was composed of complete Dulbecco's modified Eagle's medium (**DMEM**) supplemented with 10% fetal bovine serum (**FBS**; HyClone, Logan, UT) and 1% (vol/vol) antibiotic/antimycotic (Sigma).

Neutrophils were isolated from healthy midlactating Holstein cows using a method described by Carlson and Kaneko (1973) with some modifications. Blood samples were collected from the caudal vein into EDTA-coated Vacutainer tubes (Becton Dickinson Co., Franklin Lakes, NJ). Whole blood was diluted 1:1 with prewarmed (37°C) Hanks' balanced salt solution and carefully layered over 20 mL of Ficoll-Paque Plus (Amersham Pharmacia, Montreal, QC, Canada) in 50-mL Falcon tubes. The tubes were then centrifuged at 1000 × *g* for 40 min at 4°C, after which the plasma and Ficoll were gently removed and discarded. The PMN were then isolated by 2 hypotonic lyses of the erythrocyte pellet using 15 mL of Tris-buffered 0.15 *M* ammonium chloride. The cells were washed twice in Hanks' balanced salt solution using centrifugation at 350 × *g* for 10 min before being resuspended at a concentration of 5 × 10⁶ PMN/mL in the cytotoxicity medium [47.5% phenol red-free RPMI 1640 medium, 47.5% phenol red-free DMEM, 2.5% very-low endotoxin FBS (HyClone), 1% antibiotic/antimycotic solution, 5 µg/mL of bovine insulin, 2 mM L-glutamine, and 1.4 mg of D-(+)-glucose/mL]. Cell viability was evaluated using trypan blue dye exclusion and normally exceeded 95% in this study. More than 95% of the cells isolated were neutrophils.

Coculturing of bovine PMN and MAC-T cells was performed as described by Boulanger et al. (2002). Briefly, epithelial cells were grown in the cytotoxicity medium on 24-well plates (~50,000 cells/well) precoated with a collagen matrix (see below). Once the epithelial cells were 80% confluent, the medium was removed and freshly isolated PMN were put into each well (2.5 × 10⁶ PMN/well). Lipopolysaccharide (1 µg/mL) and PMA (1 µg/mL) were then added to each well in the presence or absence of various concentrations of tested antioxidants. All treatments were done in triplicate. Phorbol 12-myristate 13-acetate was used because it activates both pools of NADPH oxidase in PMN (plasma mem-

brane and granule membranes) and induces extracellular release of oxygen metabolites (Karlsson et al., 2000). The plates were returned in the incubator for 24 h. The substances evaluated in this study were DFO (0 to 300 μM), CAT (0 to 75 μM), BDA (0 to 1000 μM), DMTU (0 to 10,000 μM), and GEE (0 to 100 μM).

Cytotoxicity Assay

After an incubation of 24 h, the coculture supernatants were transferred into 1.5-mL Eppendorf tubes and then centrifuged at $500 \times g$ for 5 min in a microcentrifuge. The lactate dehydrogenase (LDH) assay was performed using the CytoTox96 Nonradioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions and Fischer et al. (1998). The following controls were included in each assay: MAC-T cell spontaneous LDH release, PMN spontaneous LDH release, culture medium background, and MAC-T maximal release of LDH (15-min incubation of cells with lysis solution). For each experimental well, percentage cytotoxicity was calculated using the manufacturer's equations:

- Eq. 1: Experimental well optical density – culture medium background optical density = A
- Eq. 2: MAC-T cell spontaneous LDH release optical density – culture medium background optical density = B
- Eq. 3: PMN spontaneous LDH release optical density – culture medium background optical density = C
- Eq. 4: MAC-T cell maximum LDH release optical density – volume correction optical density = D
- Eq. 5: % cytotoxicity = $(A - B - C)/(D - B) \times 100$.

Acridine Orange Staining of Cocultures

Once supernatants were carefully collected for the LDH assay, the remaining cells were gently washed twice with Hanks' balanced salt solution before acridine orange staining solution (8 $\mu g/mL$ in PBS) was added (200 μL /well). The 24-well plates were incubated for 10 min at room temperature. The acridine orange solution was then removed and the cells were fixed by adding 500 μL of 4% (wt/vol) formaldehyde solution in PBS. Fluorescence was visualized using a fluorescence microscope (Hund Wilovert S, Wetzlar, Germany) and photographs were taken with a digital camera (Nikon, Coolpix 4500, Tokyo, Japan) coupled to the microscope.

Preparation of Calf Collagen

All procedures were conducted at 4 to 8°C as described by Miller and Rhodes (1982) with some modifications. Briefly, a skinless tail was immersed in 95% ethanol for 15 min to facilitate tendon detachment. All tendons were excised with a scalpel and placed in 95% ethanol. The tendons were dried under UV light overnight. The next morning, they were cut into 1-cm-long pieces and placed into 500 mL of 0.5 N acetic acid to extract collagen. The extraction was carried out for 3 to 4 d at 4°C with continuous vigorous shaking. The mixture was then filtered through several layers of cheesecloth to remove insoluble residues. Finally, the solution was centrifuged at $50,000 \times g$ for 45 min at 4°C to be clarified. The collagen solution was kept at 4°C.

Bacterial Growth Assay

First, *E. coli* P4:O32 (H37, β -glucuronidase+, hemolysin-) were grown in 50 mL of brain-heart infusion medium for 2 h at 37°C. An aliquot was then serially diluted to perform a bacteriological count (cfu) on tryptic soy agar plates that were incubated overnight at 37°C. The 50-mL culture was kept at 4°C overnight. The cells were then centrifuged at $50,000 \times g$ for 10 min, suspended in DMEM/F12 medium without phenol red at 2×10^7 cfu/mL, and kept on ice until needed. In a 96-well plate containing DMEM/F12, DFO was added to obtain final concentrations of 0, 50, 200, or 800 μM (in triplicate). Inactivated FBS was then added (5% final concentration), and 125 μL of the solution of bacteria was transferred into the wells (final volume of 250 μL). Absorbance of the plate was then immediately read at 600 nm using a plate-reader; this was used as the time-zero reading. The plate was put in a shaking incubator at 37°C and absorbance was read every 15 min for 2 h. The blank was composed of phenol red-free DMEM/F12 supplemented with 5% FBS. The assay was repeated 3 times.

Phagocytosis Assay

Bacteria (*E. coli* P4) were prepared as described above at 4×10^7 cfu/mL. One milliliter of bacteria was added to a tube containing 1 mL of freshly isolated PMN (2×10^6) and DFO (final concentration: 0, 50, 200, or 800 μM) in DMEM/F12 containing 5% inactivated FBS. The tubes were then transferred to a 37°C shaking bath and phagocytosis was allowed to proceed for 30 min. Another bacterial count was performed on an aliquot of the added solution of bacteria to determine the exact amount of bacteria added. At the end of the incubation, 100 μL of suspension was taken from each tube and serially diluted in triplicate to perform a count

(cfu). This count determines the number of bacteria remaining in the supernatant and hence the quantity of nonphagocytosed bacteria. By subtracting this number from the number of bacteria initially added, the number of ingested bacteria was determined. The assay was repeated 3 times.

Statistical Analyses

All data from the cytotoxicity assay and the phagocytosis assay were analyzed for statistical significance using the GLM procedure of SAS (version 9.1, 2002; SAS Institute, Inc., Cary, NC). The linear and quadratic effects of each antioxidant on cell cytotoxicity were assessed and the effects were considered significant at $P \leq 0.05$ and tended to be significant at $0.05 < P \leq 0.10$. When relevant, data are expressed as mean $\pm$ SEM.

The effect of DFO on bacterial growth was subjected to repeated-measures ANOVA using the MIXED procedure of SAS. The covariance structure used was the spatial power and the model was:

$$Y_{ijk} = \mu + D_i + T_j + A_k + (D_i \times A_k) + (D_i \times T_j) + (D_i \times T_j \times A_k) + \varepsilon_{ijk}$$

where Y_{ijk} is the variable studied (bacterial growth), μ is the overall mean, D_i is the DFO-treatment effect, T_j is the time effect, A_k is the assay effect, and ε_{ijk} the residual error.

RESULTS

Effect of PMN, Antioxidants, LPS, and PMA on Viability of MAC-T Cells

Incubation of MAC-T cells with nonactivated bovine PMN (5×10^6 /mL) or LPS and PMA was not damaging to MAC-T cells after 24 h ($P > 0.1$). When MAC-T cells were incubated with 25 μ M CAT, 200 μ M DFO, or 50 μ M GEE in the presence of LPS and PMA but without PMN, the cytotoxicity levels were not significantly different from zero ($P > 0.1$), indicating that these antioxidants were not impairing cellular integrity. On the other hand, exposure of MAC-T cells to PMN activated by LPS and PMA resulted in a significant decrease of cell viability as revealed by the high levels of LDH released in supernatants (average cytotoxicity = $25.7 \pm 2.9\%$). These results are consistent with those obtained by the acridine orange staining; MAC-T cells incubated with nonactivated PMN (Figure 1A) did not show any sign of cell death, and monolayer integrity was conserved, whereas nuclear condensation, abnormal cellular shapes, and disrupted monolayers were observed

when the MAC-T cells were incubated with activated PMN (Figure 1B).

Effect of Antioxidants on Neutrophil-Induced Mammary Cell Damage

The effects of various antioxidants and chelators were evaluated using the coculture model of bovine PMN and MAC-T cells described above. When added to the cocultures of MAC-T cells and activated PMN, DMTU did not show any protective effect on PMN-induced cytotoxicity (data not shown). Addition of BDA reduced cytotoxicity levels by 33% ($P < 0.001$) at the highest concentration (1000 μ M; Figure 2A). On the other hand, CAT, DFO, and GEE decreased PMN-induced cytotoxicity in a dose-dependent manner. The addition of 25 and 50 μ M of CAT reduced cell cytotoxicity by 77 and 100%, respectively ($P < 0.001$; Figure 2B). Similarly, the addition of DFO at 100, 200, and 300 μ M lowered cytotoxicity by 58, 88, and 98%, respectively ($P < 0.001$; Figure 2C). Addition of GEE at 50 and 100 μ M decreased cytotoxicity levels by 82 and 83%, respectively ($P < 0.001$; Figure 2D). Protective effects of CAT, GEE, and DFO were confirmed by visual observation of cells stained with acridine orange under fluorescence microscopy (Figure 1, panels B through E).

Bacterial Growth Assay

The siderophore DFO can be used as a source of iron and promote growth of some bacteria (Diarra et al., 2002). Therefore, the effect of DFO on *E. coli* growth was evaluated using an in vitro assay. A significant reduction of bacterial growth ($P < 0.05$) was observed after a 60-min incubation with DFO (Figure 3). Indeed, for all concentrations tested, the optical density of wells containing DFO was significantly lower than that of control for time points 60, 75, 90, 105, and 120 min ($P < 0.05$).

Phagocytosis Assay

The release of free radicals is important for the functionality of PMN. Therefore, the effect of DFO on the ability of PMN to ingest *E. coli* was evaluated in vitro. A known amount of bacteria was added to different wells containing PMN (2×10^6 PMN) with or without DFO. Incubating *E. coli* in the DMEM/F12 medium for 30 min did not induce cell death (data not shown). Seventy-eight percent of the bacteria were ingested by PMN in control wells containing medium only. Deferoxamine did not affect ($P > 0.4$) the phagocytic ability of PMN, as shown in Figure 4.

Figure 1. Typical acridine orange staining of MAC-T cells following incubation for 24 h with (A) nonactivated neutrophils (PMN; $5 \times 10^6/\text{mL}$), (B) PMN activated with LPS ($1 \mu\text{g}/\text{mL}$) and phorbol 12-myristate 13-acetate ($1 \mu\text{g}/\text{mL}$), (C) activated PMN and catechin ($25 \mu\text{M}$), (D) activated PMN and deferoxamine ($200 \mu\text{M}$), and (E) activated PMN with glutathione ethyl ester ($50 \mu\text{M}$).

Figure 2. Levels of cellular damage in MAC-T cells coincubated with activated neutrophils (PMN; $5 \times 10^6/\text{mL}$) in the presence of (A) bathocuproine disulfonic acid (BDA), (B) catechin (CAT), (C) deferoxamine (DFO), and (D) glutathione ethyl ester (GEE). After 24 h of coincubation, cytotoxicity was determined by measuring of released lactate dehydrogenase. Data are expressed as percentage of cytotoxicity and represent LS means $\pm$ SEM. *Value is significantly different ($P < 0.05$) from the control (no antioxidant).

Figure 3. Effect of different concentrations of deferoxamine (DFO) on *Escherichia coli* growth. Bacterial growth was determined by measurements of optical density at 600 nm. The values represent the means of 3 independent experiments in which each DFO concentration was tested in triplicate. Bacterial growth in the presence of DFO was significantly slower ($P < 0.05$) from time point 60 min for all concentrations tested compared with the control.

DISCUSSION

In the first part of the study, an in vitro model of coculture of MAC-T cells and activated bovine PMN was used to investigate whether some antioxidants and ion chelators were able to prevent PMN-induced mammary epithelial damages. Our results showed that BDA and DMTU did not protect MAC-T cells against oxidative damages at the doses used. On the other hand, addition of CAT, DFO, and GEE led to a dose-dependent reduction of PMN-induced cell cytotoxicity, as demonstrated by the significant decrease in released LDH and by visual observation of the integrity of MAC-T cells under the microscope.

The exact mechanism by which bovine epithelial cells are damaged in mastitis is still not fully understood. It is well known that PMN, when activated, release

bactericidal molecules such as ROS that eliminate microbes but are also harmful toward host cells. Using inflammation models, PMN have been shown to induce tissue damage through the release of ROS, proteases, and lysozymes (Varani and Ward, 1994; Ginsburg, 1998; Poch et al., 1999). In the mammary gland, prolonged diapedesis of PMN induces damage to mammary parenchyma tissue, which results in decreased milk production (Sordillo et al., 1997). In the present study, activated PMN induced considerable cytotoxicity in MAC-T cells, whereas incubation of these cells with nonactivated PMN did not cause any cytotoxicity. In our assay, activation of the oxidative metabolism of PMN was achieved by supplementing culture medium with PMA ($1 \mu\text{g}/\mu\text{L}$), whereas LPS ($1 \mu\text{g}/\mu\text{L}$) was added to prevent PMN from undergoing spontaneous apoptosis and to extend their life span (Lee et al., 1993). Unlike PMN, MAC-T cells were not affected by the presence of LPS and PMA as demonstrated by the absence of significant LDH release. These results are in agreement with previous observations (Ledbetter et al., 2001; Boulanger et al., 2002).

At the concentrations tested, DMTU was unable to reduce cell death. Adding BDA to the coculture only induced a slight level of protection at a very high concentration. However, this concentration would not be achievable in vivo because incubation of $1000 \mu\text{M}$ of BDA with MAC-T cells alone was toxic to MAC-T cells as shown by an increase in cytotoxicity levels. In general, DMTU is regarded as a free-radical scavenger. However, it has also been reported that DMTU could act as a copper chelator (Hanna and Mason, 1992) like BDA. Therefore, the relative ineffectiveness of both DMTU and BDA in preventing cell cytotoxicity suggests that free copper is not an important mediator in PMN-induced bovine epithelial cell damage. These data might suggest that the biodistribution of DMTU in our cell system was not appropriate to react with the hydroxyl radicals.

In the past decades, it has been demonstrated both in vitro and in vivo that tea and tea polyphenols exhibit strong antioxidant properties, including scavenging of oxygen radicals (hydrogen peroxide, hydroxyl radicals) and lipid radicals (Salah et al., 1995). Catechin is a flavonoid polyphenol found in tea that has been reported to be 20 times more potent than vitamin C as an antioxidant (Craig, 1999). Our results show that CAT reduced LDH release and thus cell cytotoxicity by 77% at $25 \mu\text{M}$ and by 100% at $50 \mu\text{M}$ when added to the coculture of activated PMN and MAC-T cells. This protective effect was confirmed by visual observation of acridine orange-stained MAC-T cells. This is consistent with the findings of an in vitro study conducted by Chen et al. (2002), who observed that tea catechin treatment

Figure 4. Effect of deferoxamine (DFO) on neutrophils' ability to phagocytose *Escherichia coli*. The results represent the number of ingested bacteria (cfu/mL) and are expressed as means $\pm$ SEM.

significantly increased cell viability and decreased lipid peroxidation levels in lead-exposed cells in which the damage was mediated by ROS. Furthermore, green tea polyphenols were found to block LPS-induced lethality when given orally to BALB/c mice before an intraperitoneal injection of LPS (Yang et al., 1998). Taken together, these results suggest that catechin may be useful as a therapy in various inflammatory processes such as mastitis.

Under physiological conditions, a small amount of ROS is produced due to the metabolic processes of all aerobic cells. These ROS are neutralized by endogenous antioxidants such as GSH. Several studies have linked GSH to the ability of a cell to resist the deleterious effect of ROS (Singhal and Jain, 2000), and more specifically H_2O_2 (Seo et al., 2004). Indeed, reduced GSH is the substrate of the H_2O_2 -removing enzyme glutathione peroxidase. In our model, GEE was used to increase the endogenous pool of GSH in MAC-T cells because GEE can easily enter cells (unlike GSH). Once inside the cells, GEE is rapidly hydrolyzed to yield reduced glutathione (Anderson et al., 1985). Our results show that GEE was able to prevent PMN-induced damage in MAC-T cells, with an 83% decrease in cell cytotoxicity when cocultures were supplemented with 50 or 100 μM GEE. This protective effect was also confirmed by observation of cells stained with acridine orange. Furthermore, no abnormality in cellular morphology was observed. The protective effect of GEE has been reported by others; for example, Morris et al. (1995) demonstrated that GEE was able to attenuate LPS-induced injury of cultured bovine pulmonary artery endothelial cells. Therefore, in our coculture model, it is likely that GEE was able to increase the endogenous pool of GSH in MAC-T cells and, thus, enhanced the ability of these cells to detoxify H_2O_2 . This explanation is supported by a study performed by Michiels et al. (1994) in which the rate of H_2O_2 elimination was shown to increase linearly with GSH concentration when GSH was unable to saturate glutathione peroxidase. Knowing that H_2O_2 is the central component from which the majority of ROS are produced, it is not surprising to observe a decrease in cell cytotoxicity caused by PMN oxidative burst. These data suggest that H_2O_2 is an important mediator of tissue damage in bovine mammary epithelial cells and that an efficient scavenging of H_2O_2 could significantly reduce cellular damage when oxidative stress arises from massive PMN activation.

The present study also shows that the iron chelator DFO reduced the release of LDH by MAC-T cells in a concentration-dependent manner. Acridine orange staining revealed an intact monolayer of MAC-T cells in contrast with cells incubated with activated PMN alone. Furthermore, when MAC-T cells were incubated

with DFO alone, no significant increase in LDH release was observed after 24 h, showing that DFO was not intrinsically toxic. This is consistent with another in vitro study performed on human endothelial cells, which reported that DFO did not increase cell susceptibility to exogenous oxidants after 48 h (Fratti et al., 1998). Furthermore, DFO has been shown to be protective in various models of ROS-induced cellular injury as reviewed by van Asbeck (1990). Additionally, DFO was found to react with oxygen products released by PMN following PMA activation (Soriani et al., 1993). In our model, the protective effect of DFO is probably accomplished by chelation of free iron, which then becomes unavailable for the Fenton reactions and therefore hydroxyl radical generation. This standpoint is reinforced by a study indicating that DFO completely prevented iron from catalyzing Haber-Weiss and Fenton reactions (Gutteridge et al., 1979). Because hydroxyl radical is thought to be the most harmful radical for tissue damage, it is not surprising to see a lower level of cytotoxicity in DFO-treated cells. Additionally, it has been reported that pretreating endothelial cells with DFO is sufficient to decrease PMN-induced damage; this also suggests that target cells and not PMN provide the necessary iron (Gannon et al., 1987). Therefore, DFO may be a useful chemical for preventing PMN-induced cell damage.

The second part of the study evaluated the in vitro effect of DFO on bacterial growth (*E. coli*) and the ability of PMN to ingest *E. coli*. Our results showed significant inhibition of the bacterial growth rate when DFO was added to the culture medium. The difference between the control and the DFO-treated *E. coli* became significant after 60 min of incubation for all concentrations tested (50 to 800 μM). Iron is known to be essential to all living organisms, including *E. coli*. The DFO-induced inhibition of bacterial growth observed in our experiment can be compared with the findings in an in vivo study with cows, in which addition of DFO to milk from LPS-infused quarters depressed *E. coli* growth (Lohuis et al., 1988). The same study also demonstrated that *E. coli* growth was promoted by adding iron to milk. Although some pathogens such as *Staphylococcus aureus* can use DFO as an iron source (Diarra et al., 2002), it appears that iron chelated by DFO is unavailable for *E. coli*. However, as this bacterium possesses at least 7 iron-acquisition systems and they can increase their capacity to assimilate iron when an iron deficiency occurs, it is possible that the observed inhibition may be transient and last only as long as it takes *E. coli* to modify its metabolism to activate other mechanisms of iron uptake.

In our assay, phagocytosis was demonstrated by the decreased number of bacteria remaining in the super-

nant of control wells compared with the number of bacteria initially added. Our results did not show a significant effect of DFO on PMN ability to ingest *E. coli* because the ability of PMN to ingest bacteria was the same with or without DFO. This suggests that iron chelation by DFO did not induce an iron deficiency in PMN because phagocytosis requires energy, and production of energy through oxygen metabolism requires iron as a transition metal (Macdonald et al., 1985). There are contradictory reports in the literature regarding the effect of DFO on phagocytosis. Indeed, some studies have reported that incubating PMN (from healthy humans) with DFO induced progressive alteration of phagocytosis performance (Cantinieux et al., 1990), whereas others have concluded that DFO enhanced phagocytic function (van Asbeck et al., 1984; Ewald et al., 1994). These differences are probably caused by different experimental procedures that include or exclude serum, and by the use of different bacteria (*Staphylococcus aureus*, *Yersinia enterocolitica*, and *E. coli*).

The effect of DFO on the internal bactericidal activity of bovine PMN was not specifically investigated in the present study but plating of PMN lysate showed no significant bacterial growth, suggesting that killing ability of PMN was not affected. Using human PMN, some studies have demonstrated in vitro that DFO might reduce the microbicidal activity of phagocytes by completely preventing iron from catalyzing the Haber-Weiss reaction. Consequently, the amount of ROS produced by PMN may not be sufficient to complete the intracellular killing (Ewald et al., 1994). Whether DFO interferes with the ability of bovine PMN to kill ingested bacteria still needs to be evaluated.

In conclusion, activated bovine PMN caused severe damage to MAC-T cells. These deleterious effects could be inhibited partially or totally through supplementation with antioxidants (catechin, deferrioxamine, or glutathione ethyl ester), a finding that suggests that ROS are a major cause of PMN-mediated tissue damage during mastitis. In addition, the protective effect of these antioxidants was observed at concentrations that would be reachable by intramammary infusion. Therefore, the use of antioxidants in treating bovine mastitis is worth investigating as they may aid in preventing mammary tissue damage. Additionally, some antioxidants such as DFO may mitigate the impact of *E. coli* invasion by inhibiting bacterial growth.

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