PREVENTION OF THE NEUTROPHIL-INDUCED MAMMARY EPITHELIAL DAMAGE DURING BOVINE MASTITIS

BY

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ABSTRACT

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Reduction of milk production following acute bovine mastitis causes important economic losses. In this study, two experiments were conducted to asses the ability of different antioxidants to prevent neutrophil (**PMN**)-induced mammary damage in acute bovine mastitis. First, a co-culture model composed of bovine mammary epithelial cell line (**MAC-T cells**) and bovine PMN activated by phorbol myristate acetate was used. Activated PMN release reactive oxygen species that are cytotoxic for bovine epithelial cells. Addition of dimethylthiourea or bathocuproinic acid did not induce any protective effect. On the other hand, addition of catechin, deferoxamine or glutathione ethyl ester (**GEE**) significantly reduced PMN-induced cytotoxicity in a dose-dependent manner as demonstrated by lower levels of released lactate dehydrogenase (**LDH**). The second experiment was undertaken with the last three antioxidants to evaluate their protective effects *in vivo*. A model of LPS-induced mastitis on dairy cows was used. The extent of cell damages was evaluated by measuring quarter milk levels of LDH and 4-methylumbelliferyl N-acetyl ß-D-glucosaminidase (**NAGase**) at varying intervals before and after intramammary infusions of LPS, with or without antioxidants. Milk levels of haptoglobin and bovine serum albumin were also analysed. Catechin and GEE did not induce any protective effect whereas infusions of deferoxamine, a chelator of iron, decreased milk levels of LDH, NAGase and haptoglobin hence suggesting a protective effect against PMN-induced damage. Deferoxamine did not interfere with PMN migration into the mammary gland. Additionally, deferoxamine inhibited bacterial growth *in vitro* but did not affect PMN's ability to phagocytize live *Escherichia coli*. Overall, our results suggest that local infusion of deferoxamine may be an effective tool to protect mammary tissue against PMN-induced oxidative stress during bovine mastitis.

RÉSUMÉ

Maîtrise en Sciences (Mammite) Sciences Animales

Karoline Lauzon

PRÉVENTION DES DOMMAGES À L'ÉPITHÉLIUM MAMMAIRE LORS D'UNE MAMMITE

La mammite bovine endommage les cellules de la glande mammaire et cause une baisse de production laitière. Deux expériences ont été entreprises afin d'évaluer l'habilité de différents antioxydants à prévenir le dommage cellulaire causé par les neutrophiles lors des mammites aigues. Premièrement, un modèle *in vitro* de co-culture composé de cellules épithéliales mammaires (**MAC-T**) et de neutrophiles bovins activés par le phorbol myristate acétate a été utilisé. Les neutrophiles activés relâchent des composés oxygénés toxiques essentiels à l'élimination des pathogènes, qui endommagent les cellules mammaires. L'addition de dimethylthiourea ou d'acide bathocuproine disulfonique n'a pas diminué le niveau de dommage, mais la présence de catéchine, glutathion éthyl ester (**GEE**) ou déferoxamine (**DFO**) a protégé les co-cultures des dommages oxydatifs, tel que mesuré par la relâche de lactate désygrogénase (**LDH**). Ces trois antioxydants ont ensuite été sélectionnés afin d'évaluer leur effet protecteur *in vivo* en utilisant un modèle de mammite induite par l'infusion intramammaire de lipopolysaccharides (**LPS**). Des échantillons de lait ont été prélevés à différents intervalles avant et après l'injection des LPS, avec ou sans antioxydant afin d'y quantifier les niveaux de dommages cellulaires via des marqueurs tels la LDH et la NAGase. Les niveaux d'haptoglobine, d'albumine bovine et l'activité protéolytique totale ont également été mesurés. L'injection de catéchine ou de GEE n'a pas protégé les cellules mammaires tandis que l'infusion de DFO a diminué les taux de LDH, NAGase et haptoglobine mesurés. *In* vitro, la DFO a inhibé la croissance bactérienne sans affecter l'habilité des neutrophiles à phagocyter *Escherichia coli*. Nos résultats suggèrent qu'une injection intramammaire de DFO pourrait être efficace pour prévenir les dommages oxydatifs causés à l'épithélium mammaire par les neutrophiles lors d'une mammite bovine.

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I dedicate this thesis to my father...

Daddy, I sincerely hope that you can read it from where you are...

CONTRIBUTION OF AUTHORS

In accordance to McGill thesis submission guidelines, this manuscript-based thesis includes a table of contents, a brief abstract both in English and French, an introduction, a comprehensive review of literature, a final conclusion and summary, a thorough bibliography and appendices when appropriate.

Parts of this thesis have already been or will be submitted for publication and were reproduced with permission of all co-authors. These manuscripts were co-authored by Anthony Bouetard who, under my supervision, did the phagocytosis assay and bacterial growth assay reported in the first sets of experiments; Dr Jalil Mehrzad who contributed to the experimental design of experiment 2 and contributed to proof-reading of the manuscripts; Dr Benoît Paquette who contributed to the initial selection of antioxidants and proof-reading of the related manuscript; Dr Louis Delbecchi who contributed to proof-reading of the manuscripts; and finally Dr Pierre Lacasse and Dr Xin Zhao who both contributed to experimental designs of the two projects, provided all materials required and contributed to proof-reading of the manuscripts.

TABLE OF CONTENT

SECTIONS

LIST OF ABBREVIATIONS

APP	=	Acute phase proteins
APR	=	Acute phase response
BCS	=	Bathocuproine disulfonic acid
BSA	=	Bovine serum albumin
CMB	=	Culture medium background
DFO	=	Deferoxamine
DMTU	=	Dimethylthiourea
E. coli	=	*Escherichia coli*
GSH	=	Glutathione
GEE	=	Glutathione ethyl ester
H_2O_2	=	Hydrogen peroxide
HOCl	=	Hypochlorous acid
Hp	=	Haptoglobin
KDO	=	2-keto-3-deoxyoctonic acid
LBP	=	LPS-binding protein
LDH	=	Lactate dehydrogenase
LPS	=	Lipopolysaccharide
MMP	=	Matrix metalloproteinases
MPO	=	Myeloperoxidase
NADPH	=	Nicotinamide adenine dinucleotide phosphate (reduced)
NAGase	=	4-methylumbelliferyl N-acetyl ß-D-glucosaminidase
OD	=	Optical density
O_2^-	=	Superoxide
$OH^\bullet$	=	Hydroxyl radical
PCH	=	Post-challenge hours
PMA	=	Phorbol 12-myristate 13-acetate
PMN	=	Polymorphonuclear neutrophils
ROS	=	Reactive oxygen species
SAA	=	Serum amyloid A
SCC	=	Somatic cell count
sCD14	=	Soluble CD14
SOD	=	Superoxide dismutase
S. aureus	=	Staphylococcus aureus
SV	=	Simian Virus-40
TNFα	=	Tumour necrosis factor alpha

LIST OF FIGURES

SECTION I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Milk has always been considered as a highly nutritive food and this has led to the development of an important dairy industry. In Canada, the dairy industry is the 3rd biggest industry in the agricultural sector and Quebec alone produces 46% of the Canadian milk supply. As the dairy industry was developing, considerable efforts have been made to increase milk production resulting in today's high-yielding cows.

To be able to reach her full production potential, a cow must be healthy. Any factor that has a negative impact on a cow's general health will most likely affect milk yield and/or quality. This is the case with bovine mastitis. Mastitis is defined as an inflammation of the mammary gland and it can affect all lactating mammals. In the dairy sector, interests are focused on pathogen-induced mastitis since they are, by far, the most frequently occurring type of mastitis. Bovine mastitis is one of the most depressing and costly diseases for the world dairy industry. According to recent reports, each year approximately 38% of the cows in the United States have an intramammary infection causing an annual economic loss of approximately 185$US per cow (Yancey, 1999). Furthermore, approximately 70-80% of these losses are due to decreased milk production (Lightner et al., 1988). Even if there has been tremendous progress in the field of mastitis prevention, these facts clearly highlight the need for further research in the fields of mastitis control and prevention of milk losses.

Resolution of mastitis is a complex process that requires a cow to trigger different mechanisms to eliminate pathogens, including the inflammatory response. The inflammatory response is an important component of immunity and it has evolved as a protective response against injury and infection. Under certain circumstances, this

inflammatory response will lead to the production of substances that are necessary for the cow's defence but harmful to the host tissue. Benjamini (2000) has defined inflammation as an acute response to tissue injury or infection involving accumulation of leukocytes, plasma proteins, and fluid. Accordingly, it is generally accepted that one main characteristic of bovine mastitis is the accumulation of neutrophils (**PMN**) into the mammary gland (Sordillo *et al.*, 1997). When a local inflammatory response happens in the mammary gland, bovine PMN rapidly leave the blood circulation to reach the infected mammary tissue where they engulf intruders and release a large spectrum of inflammatory mediators and bactericidal substances such as oxygen-derived radicals. It is also believed that PMN play an important role in the damage to the tissue (Smith, 1994) via their premature activation during migration and the extracellular release of oxidative molecules and proteases. Indeed, it has been demonstrated that the inflammatory response activated by the cow to fight mastitis causes permanent scarring to the secretory tissue of the bovine mammary gland (Oliver and Calvinho, 1995; Ledbetter *et al.,* 2001) and, consequently, decreases milk production. Several studies have tried to identify inflammatory mediators involved in mastitis in order to find specific targets that would help to lower the inflammation process and thus prevent tissue damage (Boulanger *et al.*, 2002; Ginsburg, 1998; Shuster *et al.*, 1993). However, mastitis remains a complex event and further studies are necessary to understand host defence mechanisms and factors involved in mammary tissue damage.

In vivo, there is a constant production of reactive oxygen species (**ROS**), a collective term used for oxygen radicals and non-radical derivatives of oxygen. These ROS can lead to lipid peroxidation, DNA damage as well as protein oxidation and degradation. Usually, cells possess several biological systems, defined as 'scavengers', to protect themselves from the radical-mediated damage. When a severe

inflammation occurs, immune cells such as PMN may discharge their arsenal of toxic agents near host tissues, resulting in oxidative damage. Production of free radicals can modulate the expression of diverse immune and inflammatory molecules that both exacerbate inflammation and cause tissue damage. Consequently, it has been previously proposed that antioxidants could modify these deleterious effects by interfering with the release of oxidative products (Oishi and Machida, 1997).

The main objective of the present work was to show that a better control of the inflammation could prevent tissue damage during mastitis. The first part of the study consisted of evaluating the protective potential of different substances (antioxidants, chelators, enzymes) on cell survival using an *in vitro* system. Then, some promising substances selected *in vitro* were used to asses their protective effects in an *in vivo* lipopolysaccharide (**LPS**)-induced mastitis model.

SECTION II

LITERATURE REVIEW

LITERATURE REVIEW

1. Immunobiology of the Bovine Mammary Gland

Native defences of bovine mammary gland are continuously challenged by environmental exposure to bacteria. The immunobiology of the mammary gland is composed of three defence mechanisms that must interact with each other to be efficient: 1) the anatomical or physical defences mainly composed of the sphincter muscle and the keratin layer; 2) the soluble defences mostly constituted of immunoglobulins; 3) the cellular defences essentially composed of granulocytes, lymphocytes and macrophages (Sordillo et al., 1997).

For a long time, cows' conformation and especially anatomical characteristics of the udder and teats have been used as selection criteria in dairy cows breeding mainly because of milking ease, but also because they are associated with udder health and mastitis resistance (Seykora and McDaniel, 1985). Within the anatomical defences, the teat canal is especially important since it is the portal of entry by which pathogens penetrate into the mammary gland. It is also generally accepted that the teat end is the first line of defence against intramammary infections by providing a barrier against entry of mastitis-causing bacteria into the teat cistern. The teat end includes a sphincter muscle, which is a ring of smooth muscle surrounding the teat canal. Its function is to keep the teat canal tightly closed between milking and thus prevent milk leakage as well as bacterial incursion. The epithelial cells lining the teat canal produce keratin, a waxy material composed of fibrous protein with lipid components (long chain fatty acids). This keratin has bacteriostatic properties (Treece et al., 1966) and forms a barrier against bacteria by trapping invading pathogens (Hibbitt et al., 1969).

When pathogens succeed in bypassing the anatomical defences, cellular defences are the next host defence mechanism that pathogens meet. Pathogens will first encounter resident leukocytes (mostly macrophages) before the "alarm" is sent to recruit fresh leukocytes. Pathogens release metabolic by-products, enterotoxins, or cell-wall components that can act directly or indirectly as chemoattractants (Sordillo et al., 1997). In response to these stimuli, new leukocytes migrate from the bloodstream toward the infected site (mammary gland) to phagocytize bacteria. Freshly recruited leukocytes are composed of PMN, macrophages, lymphocytes, and natural killer lymphocytes (Sordillo et al., 1997). When pathogens are eliminated, recruitment of fresh leukocytes is stopped and the mammary gland recovers its healthy status. If leukocytes fail to eliminate pathogens, cellular recruitment is enhanced and inflammation is augmented. Therefore, the cascade of events leading to mastitis can be divided into three stages: invasion, infection and inflammation. This emphasizes the fact that cellular defences are of particular importance during the early stages of infection and that the rapidity of recruitment is a key-factor for determining the outcome of the invasion.

The soluble defences of the mammary gland function in concert with cellular defences found in milk and tissue. Bovine soluble defences in the mammary gland are mainly composed of cytokines, immunoglobulins, and some non-specific bacteriostatic components such as lactoferrin, complement, lysozyme and the lactoperoxidase-thiocyanate-hydrogen peroxide system (Sordillo et al., 1997). Immunoglobulins mainly penetrate into the udder after mastitis has developed, but they also can be produced locally (Pyorala, 2002). They play an important role in the local defence of the mammary gland against pathogens. Therefore, soluble and cellular defences are of crucial importance because once bacteria have

penetrated the teat end opening, it is the efficacy of these defence mechanisms that determine the resistance of the mammary gland to the infection.

2. Factors Affecting Mammary Gland Defences

A large number of studies have been performed on various environmental, nutritional, chemical and genetic factors that modulate host responses to bacterial invasion in bovine mammary gland. One of these factors is the physiological state of the cow. For example, the mammary gland of high yielding cows is more susceptible to infections during the periparturient period because of the physiological stress of lactogenesis and decreased number of circulating PMN (Vandeputte-Van Messom, 1993). Additionally, older cows are generally more susceptible to Gram-negative bacteria and have a higher rate of clinical mastitis than primiparous cows.

Similarly, general welfare and comfort of the cows including the housing and the environment of dairy cows play an important role in helping the cows to maintain a healthy udder. Furthermore, environmental factors such as the type of milking machines, the season and the weather can also influence mammary gland susceptibility. Defective milking equipments can result in problems such as overmilking which can increase mastitis incidence. Additionally, it has been reported that teat duct colonization by bacteria and new infection risks are significantly linked to machine-induced changes in teat thickness after milking (Pyorala, 2002).

The discovery that vitamin E and selenium deficiencies in dairy cows were related to decreased host defences has resulted in increased supplementation of these micronutrients to dairy cows. Accordingly, it was

reported that deficiencies of some trace elements (such as selenium, copper and zinc) and vitamins such as vitamin E were predisposing factors for mastitis (Sordillo et al., 1997). Furthermore, lack of selenium suppresses phagocytosis and thus compromises the function of PMN, which are primary effector cells in the initial elimination of infections. Therefore, nutrition is an important factor in resistance against disease.

Another important factor is the genetic differences existing between cows not only for diverse immune functions, but also for some physical traits such as the udder conformation (Burvenich *et al.*, 2000) and the teat sphincter ability to be leakproof. These criteria have been used for selection in breeding programs for long and are known to play a role in the ability of a cow to resist bacterial invasion. Based on the heritability of somatic cell count (**SCC**; Shook, 1989), selection against mastitis has also been targeted towards low milk SCC. This term (SCC), approved by the National Mastitis Council, refers to the whole combination of cells found in bovine milk and includes the PMN, the lymphocytes, the eosinophils, the macrophages and the epithelial cells (Paape *et al.*, 2000). As SCC is part of the defence system of the udder against mastitis, it was also suggested that very low SCC could be associated with a higher incidence of environmental mastitis (Suriyasathaporn, et al., 2000). Even if it is not likely that dairy cows will reach "too low" levels of SCC in the near future due to selective breeding, attention must be paid not to compromise mammary gland defences in cows.

Since the rapid genetic increase in milk yield is associated with the increased genetic susceptibility to mastitis, it is more than likely that genetic improvement could be able to reduce need for treatment and culling. However, proper sanitation and good management practices will always be necessary.

3. Acute Phase Response

During an infection, a cow will trigger different mechanisms such as induction of fever, increase in blood white cells production and emission of cytokines to fight off the intruder. The whole combination of the different actions taken by the host is generally called the inflammatory response. More specifically, the early and non-specific defence mechanisms occurring shortly after any insult and before the specific immunity are known as the *acute phase response* (**APR**; Baumann and Gauldie, 1994). This process involves important metabolic and systemic changes that are induced by cytokines secreted mainly by activated monocytes. The initiation of APR occurs at the site of injury, its purpose being to prevent further injury, to isolate and remove harmful molecules, debris or invading pathogens and to finally activate reparation processes (Baumann and Gauldie, 1994).

During the acute phase, hepatocytes change their pattern of protein synthesis and release in bloodstream some important proteins identified as "acute phase proteins" or **APP**. These proteins are produced within a few hours after tissue injury and usually reach their peak values within one day after initiation of APR (Boosman et al., 1989). Haptoglobin (**Hp**), serum amyloid A (**SAA**) and C-reactive proteins are known to increase their plasma concentration following infection. Haptoglobin and SAA can be found in milk during mastitis because they pass through the milk barrier but also because the mammary gland has the ability to synthesize APP locally (Jacobsen, 2004).

The possible biological role of APP is protecting the host. The primary function of Hp is to prevent iron loss by forming a very stable complex with free haemoglobin released in blood by damaged erythrocytes. Therefore, Hp exerts a bacteriostatic effect by restraining

access to this free iron pool, which is necessary for bacterial growth (Petersen *et al.*, 2004). Indeed, it has been reported that Hp fully protected rats from mortality when simultaneously injected intraperitoneally with *Escherichia coli* compared to rats inoculated only with the bacteria (Eaton et al., 1982).

4. Mastitis

Mastitis is the name of the inflammation state of the mammary gland. In dairy cows, mastitis is generally associated with an intramammary infection usually caused by bacteria. In this case, bacterial invasion and growth within the mammary gland is the main cause of mastitis. There are two main types of mastitis-causing agents: the contagious pathogens (that can be transmitted from one cow to another) such as *Staphylococcus aureus,* and the opportunistic environmental pathogens such as *Escherichia coli.* The contagious pathogens can be described as microorganisms adapted to survive and establish themselves within the mammary gland whereas environmental pathogens are considered as opportunistic invaders of the mammary gland not adapted to long-term survival within the host. Typically, they invade, multiply, trigger a host immune response and are then rapidly eliminated (Bradley, 2002). The incidence of mastitis caused by contagious pathogens has decreased considerably due to new management systems and widespread adoption of post-milking teat disinfection.

Based on the severity of the infection, there are three different forms of mastitis: sub-clinical, clinical and chronic. The sub-clinical state is the most common form of mastitis. This state of infection elicits a discrete inflammatory reaction with no observable clinical signs. Together with the fact that pathogens can be found in milk, the slightly higher SCC will lead

to important modifications of milk physical and chemical properties. The sub-clinical mastitis-causing agents have the capacity to damage the secretory mammary cells. Any stress experienced by the animal can make the sub-clinical mastitis evolves into a clinical mastitis that can eventually becomes chronic.

Obvious signs of mammary inflammation that are visible with the eye characterize the clinical form of mastitis. There are three sub-forms of clinical mastitis.

1- The peracute mastitis is characterized by a sudden onset. The udder is extremely inflamed and milk is serous if not reduced to a bloody or brownish secretion. Even if only one quarter is infected, the whole mammary gland often becomes non-functional (agalactia). Many factors such as bacteria, toxins (endotoxins or exotoxins) and leukocytes products can initiate this systemic inflammation. The cow will suffer of fever, anorexia, depression, decreased rumen motility, high breathing rate and dehydration. Death occurs in very severe cases.

2- The acute mastitis is also characterized by a sudden onset in one or more quarters. However, the inflammation of the udder is moderate to severe and leads to a decreased milk production. Signs of redness, swelling, hardness and heat are observable on the mammary gland. Milk has a thick consistency due to the presence of flakes or serous milk/fibrin clots. Systemic signs are similar to the peracute ones but in a less severe way.

3- The sub-acute mastitis is characterized by a mild inflammation without systemic symptoms. The udder does not always show

obvious signs of inflammation but the milk generally has a curdled appearance.

The last form of mastitis is called the chronic mastitis and is an infection of long duration, sometimes years, often characterized by a persistent sub-clinical state with occasional clinical episodes. Because available treatments fail to fully eliminate pathogens, the secretory tissue becomes severely damaged.

In general, when a mastitis-causing agent infects a quarter, a decrease in the milk yield is observed. These losses result mainly from the reduced milk production because all the milk is being discarded and also because a cow affected by mastitis can never recover her full production yield within the same lactation (Rajala-Schultz *et al.*, 1999). The other factors that contribute to the losses are the cost of veterinary services and medication along with the higher culling and mortality rate (Shuster *et al.*, 1993).

4.1 Diagnosis

During mastitis, the hallmark signs of local inflammation are usually detected. These are swelling, redness, heat, pain and a loss of function of the mammary gland (Burvenich *et al.*, 1994). Immediately after the entry of the pathogen, the small blood vessels of the infected region are dilating themselves to allow an increase in the blood flow reaching the area but also to increase their permeability. This results in a massive influx of fluid from blood into tissues where it accumulates and causes oedema and swelling. Concomitantly, there is an increase in cell migration from blood to the impaired tissue.

When a mastitis-causing agent has infected a quarter, several changes can be observed in the constituents of the milk produced by this

quarter, directly or indirectly caused by the multiplication of the pathogen. One of these changes is the increased SCC, which refers to the whole combination of cells found in bovine milk (Paape *et al.*, 2000). Macrophages are the main cell type found in normal milk where they account for 30% to 74% of the SCC. However, the number of PMN in milk increases drastically with the severity of mastitis and can represent 90% of the SCC. Because it was demonstrated that SCC is a good mastitis indicator (Kitchen *et al.*, 1980), many studies use SCC as an indicator of an animal's ability to fight the pathogen. It is known that quarters with elevated SCC are more resistant to mastitis than quarters with low SCC (Pyorala, 2002).

Bovine serum albumin (**BSA**) in milk was the first APP measured and used as an indicator of inflammation such as mastitis. However, milk BSA has a low discriminatory capacity. Additionally, it has been suggested that APP such as Hp and SAA could be more rapid and sensitive markers of acute inflammation than SCC and therefore could be used in the herd to detect subclinical infections. Haptoglobin is closely related to the evolution of the disease and can be used as an alternative and accurate method to monitor animal health. Higher Hp concentrations have been found in milk from infected quarters compared to milk from the opposite non-infected quarters and to milk from cows without mastitis (Petersen et al., 2004). Furthermore, milk Hp levels are unaffected by extramammary inflammation. As of milk SAA levels, it has been reported to differ between mild and moderate mastitis (Pyorala, 2003).

Another change that results from mastitis is the increased concentration of an enzyme called N-Acetyl-Glucosaminidase (**NAGase**). This enzyme originates mainly from mammary tissue cells and can therefore be a simple way of estimating the degree of tissue damage in the udder. It was shown that bovine milk NAGase is located mainly in the

soluble whey protein fraction and that quite low levels are found in normal milk due to normal process of secretion (Kitchen *et al.* 1978). However, during udder infection such as mastitis, tissue damage to the secretory epithelium combined with a modified permeability of its plasma membrane will result in an intense increase of cytoplasmic material such as NAGase shed into milk. Fox (1988) demonstrated that NAGase activities of peripheral PMN and milk PMN were not significantly different. Additionally, the results from that study also showed that milk somatic cells were contributing to less than 15% of the total milk NAGase activity. Therefore, the major source of milk NAGase is the mammary cells.

Lactate dehydrogenase (**LDH**) is a cytosolic enzyme present in all cells that is released upon cell lysis. Therefore, the concentration of LDH in mastitic milk is significantly higher compared to normal milk. There is evidence that the elevated LDH level in mastitis milk originates from both leukocytes and udders epithelial and interstitial cells that are damaged during inflammation (Bogin *et al.*, 1977). However, both LDH and NAGase in milk are generally considered as sensitive indicators of epithelial cell damage (Kitchen *et al.*, 1978 and 1980).

Mastitis obviously causes tissue damage and leads to a decreased ability of the secretory cells to exert their biochemical activity such as lactose biosynthesis. Consequently, lactose concentration of milk significantly declines during inflammation and can also be used as an indicator of mastitis. While some may contest the usefulness of lactose to detect mastitis since the percent decrease is relatively small, others suggest that lactose is one of the most useful markers of mastitis (Pyorala, 2003).

4.2 *Escherichia coli* Mastitis

Escherichia coli are Gram-negative bacteria, non-spore-forming rod, which belongs to the family *Enterobacteriaceae* and to the coliform group. They are usually found in the normal environment of the cow, principally in her faeces and bedding. This pathogen is a frequent mastitis-causing agent that often leads to acute forms of mastitis (Hogan and Smith, 2003). The average length of intramammary infections is less than ten days. The portal of entry of this mastitis-causing agent is the teat canal but the manner how it succeeds in crossing the teat canal to reach the inside of the mammary gland still remains unknown. Indeed, demonstration has been made that bovine teat canal is not susceptible to colonization by coliforms (Hogan and Smith, 2003). These bacteria are therefore multiplying themselves in the mammary gland secretion without colonizing the tissues of the mammary gland.

Following infection, coliform bacteria such as *E. coli* rapidly increase in number, often reaching peak concentrations in milk within 5 to 16 hrs (Erskine et al., 1989). As a result, a viscous and yellowish milk secretion is observed, which confirms the negative effects of mastitis on milk composition and quality. Alterations, such as clots and flakes, are also often seen in milk (Burvenich *et al.*, 1994).

Coliforms are able to metabolize lactose as an energy source and to survive in almost anaerobic conditions. Since lactose is the principal milk carbohydrate and the oxygen tension is very low in the mammary gland, *E.* coli is able to grow easily, reaching populations exceeding 10^8 colony-forming units per milliliter of milk.

The primary pathogenic factor of Gram-negative bacteria is their ability to release endotoxins at the time of cell death, which is an aggravating factor of the inflammation (Rietschel et al., 1993). *In vivo*,

Gram-negative bacteria probably release minute amounts of endotoxin while growing. It is known that small amounts of endotoxin may be released in a soluble form, especially by young cultures (Ishiguro et al., 1986). Endotoxins refer to the lipopolysaccharide portion of the Gram-negative bacterial wall. They are responsible for triggering an intense inflammatory response. In fact, much of the inflammatory and systemic changes observed during the course of acute coliform mastitis are due to release of LPS from the bacteria following phagocytosis and killing by neutrophils (Carroll et al., 1964). Consequently, severe inflammation usually leads to permanent damage to the secretory epithelium (Oliver and Calvinho, 1995). Signs of anorexia and fever are typically observed and both bacteraemia and septicaemia can occur because of the disrupted blood-milk barrier. According to Wenz *et al.* (2001), the odds that a cow suffering of severe *E. coli* mastitis would die or be culled are of 52%.

4.3 Milk Composition Changes

The changes induced by mastitis not only affect bovine milk production yield but also milk biochemical composition. During the inflammation, there are three major mechanisms responsible for all the observed changes: 1) a decreased milk synthesis, 2) an increase in the milk barrier permeability, and 3) an increase in milk proteolytic activity (Le Roux et al., 2003).

It is known that the inevitable increase of SCC is responsible for modifications in milk composition that are also correlated to the type of pathogen involved. Therefore, a SCC exceeding 10^5 cells/ml will be accompanied by a decrease in milk lactose content, which is the major osmoregulator found in milk. As a result, milk production typically diminishes as well as casein and ß-lactoglobulin content (Oliver and Calvinho, 1995). Additionally, milk produced after the onset of mastitis will

have a lower fat content and increased protein content (Houben *et al.*, 1993).

Compared to normal milk, mastitic milk contains high levels of immunoglobulins, BSA, matrix metalloproteinases (**MMPs**) and plasmin (E.C. 3.4.21.36). Plasmin is a highly active serine protease that originates from blood and contributes to casein proteolysis (Moussaoui *et al.*, 2003). The plasmin system has been widely studied in both normal (Heegaard et al., 1994) and mastitic milk (Zachos et al., 1992) and it has been proposed that plasmin acts as the main source of proteolytic activity in bovine mastitic milk (Verdi and Barbano, 1991). Nonetheless, it was recently demonstrated that MMPs are more active than plasmin when proteolytic activity is measured using gelatine instead of casein (Mehrzad et al., 2005).

4.4 Control and Prevention of Mastitis

Because of the great economic losses associated with this inflammation, mastitis control programs have became a necessity. In the United-States, the generally adopted approach for mastitis management consists of a ten-point plan. This mastitis control plan is recommended by the National Mastitis Council. It includes 1) the establishment of goals for udder health, 2) the maintenance of a clean and comfortable environment, 3) proper milking procedures, 4) proper maintenance and use of milking equipment, 5) good record keeping, 6) rapid identification and treatment of clinical mastitis, 7) effective dry cow management, 8) maintenance of biosecurity for contagious pathogens and marketing of chronically infected cows, 9) regular monitoring of udder health status, and 10) periodic review of mastitis control program. Hence, several factors must be well managed to get a functional program of control.

Environmental factors and management

The general will to diminish the number of mastitis cases has led to large advances regarding milking hygiene and reduced exposure to environmental pathogens. These are very important features in preventing the disease. Indeed, according to Kehrli and Shuster (1994), the mammary gland of a dairy cow might experience a sub-clinical response several times a week following milking. These episodes would be caused by a small number of microorganisms that succeed to penetrate the teat canal before the teat sphincter tightly seals.

Environmental pathogens are believed to be more difficult to control than the contagious pathogens since they can be anywhere in cows' normal environment. However, Gram-negative bacteria are unable to survive and multiply on teat skin. Therefore, the number of bacteria found on teat skin is directly related to the cow's exposure to contaminated environment. Coliforms are commonly found in feedstuffs, manure, water and soil, but the primary source of bacterial contamination remains the bedding. Accordingly, it has been demonstrated that the incidence of mastitis caused by Gram-negative bacteria is usually reduced by lowering the number of bacteria present in bedding (Hogan and Smith, 2003).

Post-milking teat dipping is crucial in good milking management and is usually practised in most dairy herds. It has been reported that a properly used teat dip could reduce by at least 50% the incidence of new udder infections. However, post-milking practices as a way to control environmental mastitis have a limited success since contamination of teats by coliforms such as *E. coli* can occur between milking. Hence, efficient control of environmental mastitis requires a plan of action that includes a clean environment, culling of chronically infected cows, good milking practices and milking machines.

Antibiotic Therapy

In 1945, the use of penicillin became a major treatment breakthrough for bacterial infection. Later, many other antibiotics were discovered (Miles *et al.*, 1992). Intramammary injection of antibiotics is generally used to help the cows to cure mastitis (Hoeben *et al.*, 1998). However, usage of antibiotics to treat *E. coli* mastitis is often useless because of the short duration of infections and high spontaneous cure rates. Moreover, antibiotics approved for mastitis treatment are inefficient against Gram-negative bacteria.

Antibiotic therapy on dairy cows at drying-off has been successfully performed for decades and represents one of the cornerstones of mastitis control. There are two methods of dry cow therapy. The blanket dry cow treatment treats all quarters of all cows immediately following the last milking and is usually the preferred method in Canada. This prophylactic dry-cow treatment has demonstrated substantial benefit in preventing new intramammary infections and clinical mastitis (Hillerton and Berry, 2003). The alternative procedure is the selective dry cow therapy in which only infected cows are treated. Nevertheless, the concerns of antibiotic residues in milk and apparition of new antibiotic-resistant pathogens that could enter the food chain always remain.

Vaccination

Vaccination against mastitis is a relatively new field and only a few vaccines against some mastitis-causing agents are available. Most of the research on vaccination against mastitis has been performed on *E. coli* and *S. aureus*. However, mastitis is problematic because of the high number of potential pathogens and their heterogeneity. Indeed, Watts (1988) identified 137 different organisms (bacteria, mycoplasma, yeasts and algae) capable of causing mastitis. Therefore, good immunity against a specific pathogen does not protect cows from others. For these reasons,

most vaccines are unsuccessful in reducing the number of new cases and the overall success of vaccination is minimal (Philpot and Nickerson, 2000).

The core antigen J5 vaccine is commercially available to prevent coliform mastitis. Gonzales et al. (1989) were the first to report that administration of the E. coli J5 vaccine significantly reduced the incidence of clinical cases of Gram-negative mastitis under conditions of natural exposure during the first three months of lactation of dairy cows. Since then, other studies have showed that J5 vaccine reduces incidence of coliform mastitis (Hogan et al., 1992a). On the other hand, reports on its efficacy are still controversial as two experimental challenge trials have demonstrated that J5 failed to show protection against the establishment of infection and all challenged cows developed clinical mastitis (Hogan et al., 1992b ; Hill, 1991) though Hogan et al. (1992b) reported less severe infections in vaccinated cows.

The exact mode of action of J5 vaccine is still unknown. Protection offered by J5 vaccine is thought to be achieved by immunoglobulins that are specific to the core portion of the lipopolysaccharide, which is structurally and antigenically conserved among Gram-negative bacteria (Hogan and Smith, 2003). Different protective mechanisms have been suggested: 1) neutralisation of LPS toxic effects by core LPS antibodies; 2) increased complement-mediated bacteriolysis; and 3) core LPS antibodies promote elimination of LPS and/or bacteria through opsonization and enhanced phagocytosis. Recently, a new hypothetical mechanism of action of the J5 has been suggested. The proposed mechanism is based on the enhancement of PMN diapedesis upon intramammary infection (Pyorala, 2002).

Interestingly, an enhanced opsonization by serum originating from vaccinated cows was reported by Hogan et al. (1992c). Furthermore, this enhanced opsonization coincided with high serum IgM titers against *E. coli* J5 bacteria. Consequently, colostrums and milk collected 21 days post calving from vaccinated cows also showed higher IgM titers against *E. coli* J5. These results led the authors to suggest the hypothesis that an improved opsonization capacity leads to a reduced bacterial numbers, and thus to a diminution of clinical mastitis cases.

In the United States, there are currently three core antigens vaccines that are commercially available. Two of them are based on *E. coli* J5, while the third one is based on a Re-17 mutant of *Salmonella typhimurium* (McClure et al., 1994). According to the National Mastitis Council website, approximately 54% of the cows in the USA are being immunized with one of the three available vaccines.

5. Cellular Defences

The first line of defence against mammary infection such as mastitis is the teat canal. When bacteria pass this barrier and enter the teat cistern, they will meet the second line of defence constituted by the phagocytic leukocytes that are part of the innate immunity. In the mammary gland, these phagocytic cells include macrophages and PMN that are both specialized in ingesting and killing pathogens. Mastitis first involves milk macrophages that locate and engulf the invading bacteria. Once macrophages have performed the phagocytosis, they release chemoattractants that rapidly and massively recruit PMN to the infected gland (Sordillo *et al.*, 1997).

5.1 Macrophages

Macrophages are large phagocytic leukocytes found in tissues. They are derived from blood monocytes (Benjamini, 2000). Monocytes originate from bone marrow and are small, spherical, with abundant cytoplasm and many granules but little endoplasmic reticulum. They also have a defined bean-shaped nucleus that makes migration between endothelial cells laborious. Following migration of monocytes from the blood to various tissues of the body, they undergo a differentiation into a variety of histological forms. For instance, in the liver, they become Kuppfer cells and in the lung, alveolar macrophages. All these forms play a role in phagocytosis. In general, macrophages have two main functions : 1) engulf and break down trapped material into simple amino acids, sugars and other substances for excretion or reutilization; and 2) take up antigens and process them for presentation on their surface to specific T cells (Politis et al., 1991). These cells are the first ones to make contact with the pathogens and/or antigens because they normally patrol host tissues (Baumann and Gauldie, 1994). Thus, they play a crucial role in the success of both innate and acquired immunity.

In the healthy mammary gland, macrophages are the predominant cell type found in milk and tissues. They are able to ingest bacteria, cellular debris and some milk components (Sordillo et al., 1997), which slightly lowers their efficiency. Together with epithelial cells, macrophages are responsible for the initiation of the inflammatory response. Macrophages play an essential role since they release chemoattractants (e.g. tumor necrosis factor, interferons, and interleukins) to rapidly attract PMN to the foci of infections (Baumann and Gauldie, 1994; Paape et al., 1995).

5.2 Polymorphonuclear Neutrophils

Polymorphonuclear neutrophils or PMN have a multi-lobed nucleus. This nucleus shape is perfectly adapted to the PMN function because the nuclear lobes must be aligned in a thin line in order to facilitate the PMN migration between endothelial cells (Paape *et al.*, 2000). Influx of PMN into the healthy mammary gland occurs at low level for immune surveillance but increases rapidly in response to bacterial invasion. They play an important role in the mammary gland defence because they are the first new phagocytic cells to reach the infection site. Therefore, they rapidly become the predominant cell type found in the mammary tissues and secretions during early inflammation, sometimes accounting for over 90% of the total mammary gland leukocytes. Since PMN are very effective phagocytes, their ability to reach the infected area rapidly is critical for the outcome of the disease.

The general life-cycle of a PMN is relatively short. Neutrophils originate from bone marrow and then migrate into bloodstream where they circulate briefly (half-life of 8.9 h) (Carlson and Kaneko, 1975). They then leave the blood circulation by diapedesis between endothelial cells to enter healthy tissues where they will act as phagocytes for 1-2 days before undergoing apoptosis and being removed by macrophages (Paape et al., 2002). Neutrophils exist in various state of activation, from dormant, primed to fully activated. Activation induces the immediate expression of PMN bactericidal activity whereas priming stimuli serve to amplify the magnitude of the response when the PMN is later activated. Accordingly, priming with LPS was reported to significantly increase superoxide generation by human PMN upon exposure to N-formylmethionylleucylphenyl-alanine (FMLP), a bacteria-derived peptide (Kobayashi et al., 2003). However, bovine PMN do not respond to FMLP. Priming is closely related to neutrophil activation, which is an integral component of the systemic host response. Additionally, intracellular

calcium ions and protein kinase C (PKC) are two intracellular signals that are believed to trigger PMN activation (Karlsson *et al.*, 2000).

5.2.1 Diapedesis

To be able to phagocytose pathogens in the host tissue, PMN must leave the blood circulation to reach the site of inflammation. Neutrophils rapidly leave the bloodstream by diapedesis between endothelial cells to enter tissues where they exert their phagocytic function for 1-2 days (Paape *et al.*, 2000). This diapedesis process occurs in response to chemical messengers released during the early step of the inflammatory reaction. These chemoattractants diffuse from the injured area to form a concentration gradient that guide the PMN via a directed migration toward the highest concentration (Burvenich *et al.* 1994). Several molecules are able to act as chemical messengers or chemoattractants for bovine PMN, notably the C5a complement component, LPS, interleukin (**IL**)-1, Il-2, Il-8 and leukotriene B4.

In *E. coli* mastitis, the released endotoxins or **LPS** are bound by LPS-binding protein (**LBP**). This complex is recognised by the membrane CD14 receptor of monocytes, macrophages and PMN that are present in milk. This will cause the release of tumour necrosis factor alpha (**TNFα**) (Kielian and Blcha, 1995; Paape et al., 2003). The soluble form of CD14 (**sCD14**) may also bind endotoxins, either directly or by binding the endotoxin-LBP complex (Paape et al., 2003). The endotoxin-LBP-sCD14 complex is then recognised by the Toll-like receptors on the epithelial and endothelial cells of the mammary tissue, instigating the secretion of different chemoattractants, such as cytokines and interleukins, that will in turn act both locally and systemically (Paape et al., 2003). When this type of chemoattractants bind to specific receptors on the PMN plasma membrane, PMN become activated and are now able to adhere to endothelial surface through specific PMN adhesion molecules

(CD11/CD18 family and LeuCAMs). Once a PMN has bound to the endothelium, it can leave the blood circulation and migrate to the extracellular matrix near the mammary epithelial cells (Burvenich *et al.*, 1994).

According to Harmon and Heald (1982), the diapedesis of PMN implies the crossing of up to five structural barriers: 1) the endothelial cells lining the capillary lumen, 2) the basal lamina around the capillaries, 3) the periendothelial cells, 4) the basal lamina around the alveoli, and 5) the single layered epithelium facing the mammary alveoli. It has been reported that cows affected with mild mastitis exhibited a higher rate of PMN influx that cows suffering from severe mastitis (Burvenich et al., 1994). Therefore, the rapidity of PMN to cross structural barriers and reach the infected site is crucial in determining the outcome of the disease.

5.2.2 Phagocytosis

The highly convoluted surface of PMN allows them to neutralize targets by ingesting them. This engulfment process is called phagocytosis and it is enhanced by opsonization of bacteria with serum proteins such as complement and antibodies. The first step of phagocytosis is recognition and binding of invading bacteria by either antibody-bacteria or antibody-complement-bacteria complexes (Paape et al., 2002). This attachment is made through the Fc receptors located on the PMN surface and it initiates the phagocytosis process by making the PMN membrane invaginate itself around the target to form a phagosome. Neutrophils have little if no ability to distinguish between foreign and host antigens and thus rely on other components of the immune system (e.g., antibodies, complement, and cytokines) to select their targets. In milk, PMN have a lower phagocytic rate and a decreased bactericidal activity due to ingestion of milk fat globules and casein (Paape *et al.*, 2003). This can be problematic since it

is well known that the ability of PMN to phagocytose the bacteria is critical to the development of mastitis (Burvenich et al., 2003).

5.2.3 Microbial killing

Once the phagosome is formed, azurophilic granules will merge with the phagosome to release their content both into the extracellular medium and into the phagosome to create a highly bactericidal environment (Weiss, 1989). The phagosome becomes the phagolysosome, where the killing and degradation of bacteria take place via a combination of oxidative (respiratory burst) and non-oxidative processes (lysosomal enzymes) via degranulation (Smith, 1994). The complex microbial killing ability of PMN will be further discussed in the next section.

6. Microbial Killing and Bacterial Degradation by Neutrophils

There is no doubt that the functionality of PMN at killing and degrading microbial intruders is crucial for the outcome of mammary infection. To insure the maximum harmful potential toward intruders, PMN combine two independent but complementary mechanisms. One is oxygen-dependant while the other mechanism is non-oxidative.

6.1 Respiratory Burst

Neutrophils oxidative or respiratory burst is triggered upon phagocytosis (*in vivo*) or when the pathway is appropriately activated by a stimulus (*in vivo* and *in vitro*). In all cases, this process firstly needs the enzyme NADPH oxidase to be functional and activated. For instance, in human, it is well known that the PMN oxidative burst plays a crucial role in microbial killing since people with a deficient oxidative burst pathway are likely to suffer from repeated infections that eventually lead to early death

(Smith, 1994). Correspondingly, in early lactating cows, respiratory burst activity of both blood and milk PMN was found to be impaired when compared with that of mid and late lactating cows (Kehrli et al., 1989; Mehrzad et al., 2001b). Additionally, Shuster et al. (1996) reported that cows within 10 days of lactation showed higher bacterial (*E. coli*) growth in their mammary gland and became more severely diseased compared to those in mid lactation. Taken together, these findings suggest that an impairment of bovine PMN respiratory burst activity can be associated with an increased susceptibility to bacterial infections (Burvenich et al., 1996).

The respiratory burst process is so-called because of its high oxygen consumption that serves to generate ROS that, in turn, act as bacterial oxidizing agents. Reactive oxygen species or ROS is a collective term used in to refer to oxygen radicals such as superoxide ion (O_2^-) and some non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). However, because neither O_2^- nor H_2O_2 is extremely reactive in aqueous solutions, some authors prefer to use the term "oxygen-derived species" or "oxygen-derived metabolites".

6.1.1 The Neutrophil NADPH oxidase and ROS generation

The enzyme nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)-oxidase is a multicomponent flavoprotein not only found in PMN but also in oesinophils and mononuclear phagocytes. This enzyme remains on a latent (disassembled) form until the cells are properly activated. The first pool of NADPH-oxidase to be identified was plasma membrane localized.

The neutrophil NADPH oxidase complex is composed of several soluble components that are essential to its activity: (1) the heterodimeric membrane-associated flavocytochrome b588 protein (itself composed of the sub-units $gp91^{phox}$ and $p22^{phox}$ for phagocyte oxidase); (2) the cytosolic

components $p47^{phox}$ and $p67^{phox}$; (3) the small GTPase(s) Rac 1 or Rac 2. An additional cytosolic protein, named $p40^{phox}$ is also associated with the oxidase but its functional role remains unclear (Babior et al., 2002).

There is growing evidence that a second pool of NADPH oxidase may be present inside human PMN, more specifically in granule membranes. Knowing that 80-85% of the b-cytochrome is found in granule membranes, it is easy to assume that NADPH-oxidase can also be assembled and activated in the membrane of this organelle (Borregaard et al., 1983). However, little is known about this pool and most of the existent studies have been done on the plasma membrane one.

Upon cell activation, a stimulus induces a signal transduction cascade that in turn causes the oxidase components to rapidly assemble from the cytosol to the plasma membrane making both bovine and human NADPH oxidase a hydrophobic membrane-bound enzyme. It is generally accepted that cell stimulation only occurs upon binding and further phagocytosis of bacteria, opsonized latex or zymosan. However, in human PMN, it was demonstrated that the complement component C5a was able to stimulate the respiratory burst via cell receptor mechanisms. Furthermore, activation of Nicotinamide adenine dinucleotide phosphate reduced (**NADPH**)-oxidase has been found to occur without phagosome formation or extracellular release of generated ROS (Lundqvist et al., 1995).

In vitro, activation of PMN's oxidative metabolism can be achieved using soluble chemicals such as LPS or phorbol-12-myristate-13-acetate (**PMA**), a direct activator of the protein kinase C cascade (Ginsburg and Kohen, 1995). Furthermore, during PMA stimulation, the NADPH oxidase components have been shown to assemble and cause a high increase in ROS both intracellularly and extracellularly (Karlsson et al., 2000). This

process has been observed for NADPH-oxidases from both pools, notably plasma membrane and granules membrane. Therefore, phagocytosis process is not a necessary step for activation of NADPH oxidase and subsequent production of O_2^- and other ROS.

Babior et al. (1973) showed that this enzyme was able to produce superoxide ($\mathbf{O_2^-}$) at the expense of NADPH upon phagocytosis of bacteria and provided an explanation for the increase of oxygen consumption associated with phagocytosis. It is now accepted that the function of NADPH oxidase is to catalyze generation of O_2^- from oxygen (O_2) and NADPH:

$$NADPH + O_2 \rightarrow NADP^+ + H^+ + O_2^-.$$

By reducing oxygen to superoxide, NADPH oxidase is probably the only enzyme in a body that produces O_2^- on purpose rather than by accident to release it into the extracellular space or into a preformed phagosome (McCord, 1995) .

The enzyme NADPH oxidase is an essential part of the host defence against bacterial infections since it has been demonstrated that cells from patients with chronic granulomatous disease were deficient in O_2^- generation and thus unable to kill bacteria. In fact, production of large amounts of O_2^- by PMN is the starting point for a cascade of other ROS implicated in bacterial killing and degradation.

6.1.2 Production of ROS by neutrophils

Superoxide generated by NADPH oxidase is rapidly transformed into H_2O_2 by spontaneous dismutation or by the catalytic action of

superoxide dismutase (**SOD**). The latter reaction is 4 times faster than the spontaneous one:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2.$$

However, a study performed on human PMN ingesting opsonized bacteria showed that approximately 80% of the H_2O_2 generated was obtained by spontaneous dismutation of O_2^- (Weiss and LoBuglio, 1982). Hydrogen peroxide can be reduced via three general mechanisms:

1- H_2O_2 can be the substrate for the detoxifying enzymes catalase and glutathione peroxidase. Catalase directly catalyzes decomposition of H_2O_2 to molecular O_2:

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2,$$

whereas glutathione peroxidase removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione (**GSH**):

$$H_2O_2 + 2\,GSH \rightarrow GSSG + 2\,H_2O\,.$$

2- H_2O_2 can be converted by myeloperoxidase (**MPO**) into hypochlorous acid (HOCl), which is the major end product of neutrophils oxidative metabolism. Neutrophils are known to secrete MPO into the extracellular medium where oxidation of Cl^- takes place:

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O\,.$$

3- H_2O_2 and O_2^- can interact with low molecular weight iron (Fe) to yield the highly reactive hydroxyl radical (**$OH^\bullet$**) via the Haber-Weiss reaction expressed as follows:

(1) $O_2^- + Fe^{+3} \rightarrow O_2 + Fe^{+2}$,

(2) $H_2O_2 + Fe^{+2} \rightarrow OH^- + OH^\bullet + Fe^{+3}$.

(3) $H_2O_2 + O_2^- \rightarrow O_2 + OH^\bullet + OH^-$

In the equation 1, O_2^- reacts with oxidized iron, which corresponds to the oxidation state in which the metal is stored in the tissues. The equation 2 is also known as the Fenton reaction whereas the equation 3 represents the classical Haber-Weiss reaction, which has no biological significance without a metal catalyst

At this point, it is important to differentiate the hydroxyl radical ($OH^\bullet$) from the hydroxyl ion (OH^-) because they are often confused in the literature. The hydroxyl radical is made of 9 protons and 9 electrons and therefore has no charge. The fact that 1 electron is unpaired makes $OH^\bullet$ highly reactive. The hydroxyl ion has a negative charge because it is composed of 9 protons and 10 electrons. It is not considered as a free radical because it does not have a non-coupled electron and is therefore not as reactive as hydroxyl radical. By definition, a free radical is any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge, 1999). Therefore, the main metabolites derived from oxygen can be classified into two groups: 1) Radicals with an unpaired electron on the oxygen (e.g., $OH^\bullet$ and O_2^-) and 2) products containing molecular oxygen (e.g., H_2O_2).

6.2 Non-Oxidative Mechanisms

Neutrophils also exert their bactericidal activity through many granules contained in their cytoplasm. Bovine PMN are known to contain three different types of intracellular granules: azurophilic, specific, and tertiary granules (Paape et al., 2003). As soon as NADPH oxidase is activated, these granules will merge with the plasma membrane of PMN or with the phagosomal membrane to release a wide spectrum bactericidal weapons that are non-oxidative into the phagocytic vacuole or into the extracellular compartment. The biologically active molecules released at degranulation (mainly proteases, antimicrobial proteins, and peroxidases) will participate to the digestion of dead microbes and damaged host cells (Smith, 1994). Therefore, PMN granule enzymes are the cornerstone of innate immunity in host defence.

Azurophilic Granules

One type of granules known to PMN is identified as "azurophilic" or "primary" granules. They are the first granules to appear during the granulopoiesis process. They exhibit a great heterogeneity in their size and shape and several subpopulations exist with distinct physical, cytochemical and morphological features (Faurschou and Borregaard, 2003). The content of the azurophilic granules is mainly neutral proteolytic enzymes and acidic proteases able to digest microbial structural proteins as well as glycosaminoglycans. Contrary to other species, bovine azurophilic granules do not contain lysozyme but they do contain MPO, a microbicidal hemoprotein released into the phagosome or to the extracellular space upon PMN activation. As discussed before, MPO reacts with H_2O_2 to yield HOCl, which increases the toxic potential of PMN. Primary granules also contain structurally related serine proteases known for their antimicrobial potential: cathepsin G and elastase (Burg and Pillinger, 2001). They display proteolytic activities against several extracellular matrix components such as elastin, fibronectin and type IV

collagen. They are also known to induce activation of endothelial and epithelial cells, macrophages and lymphocytes. Azurophilic granules have low levels of exocytosis or degranulation and thus contribute primarily to the killing and degradation of internalized microorganisms within the phagolysosome (Klebanoff, 1970).

In milk, the activity of PMN is different from that of blood PMN, especially during inflammation. It has been reported that the predominant cytoplasmic granules found in milk PMN are dense azurophilic ones due to a reduced number of secondary and tertiary granules (Paape et al., 2003). For that reason, the MPO-hydrogen peroxide halide system found in azurophilic granules can be seen as a major bactericidal mechanism for milk PMN (Klebanoff, 1970).

Specific Granules

When formation of specific granules begins, formation of azurophilic ones stops. Bovine specific granules (also known as secondary granules) rapidly outnumber the azurophilic granules. They are devoid of MPO but rich in antimicrobial substances such as lactoferrin, which sequesters iron and deprives microorganisms of an essential nutrient (Sordillo, 1997), and lysozyme, a bactericidal protein that cleaves peptidoglycans (Reiter, 1978).

Gelatinase Granules

Recently, a third "novel" peroxidase-negative large type of granules was highlighted in sheep, cows and goats. The tertiary type of granules has been found to be larger than the azurophilic and specific ones in human PMN (Faurschou and Borregaard, 2003) whereas the inverse was reported for bovine PMN (Paape et al. 2003). Since they are rich in gelatinase, this third type of granules is also known as "gelatinase

granules". In bovine PMN, gelatinase granules are more numerous than the other types of granules and contain the majority of the antimicrobial protein activity attributed to PMN (Gennaro et al., 1983). They contain lactoferrin, but they do not possess the usual components associated with human azurophilic or specific granules. Instead, they include a group of highly cationic proteins known as "bactenecins" that permeabilize the outer and inner membrane of *Escherichia coli*. Up to now, two bactenecins were identified: Bac7 and Bac5 (Frank et al., 1990). The tertiary granules also store small peptides called ß-defensins that are cytotoxic to a broad range of Gram-positive and Gram-negative bacteria, enveloped viruses, protozoa and fungi by making numerous transmembranous pores (Savoini et al., 1984).

Matrix Metalloproteinases

In humans, the specific and tertiary granules also contain matrix metalloproteinases (MMPs) that are released at degranulation. The MMPs are calcium-requiring enzymes released in inactive proenzyme forms. They are considered as relevant mediators of remodelling and degradation of extracellular matrix and basal membrane components. They may also be required to allow PMN migration through the basal membrane. The MMP family includes interstitial collagenase, gelatinases (collagenase type IV), stromelysins and other MMPs. During migration of human PMN toward the inflammation site and their subsequent degranulation, the main proteolytic enzymes released are elastase (E.C. 3.4.21.36) and cathepsin G (E.C. 3.4.21.20) (Weiss, 1989). Recently, another MMP was discovered in human PMN, namely leukolysin (MMP-25) (Faurschou and Borregaard, 2003).

Bovine blood PMN have been shown to secrete MMPs but there are only few studies on MMPs related to milk or bovine mastitis. In a murine mammary gland, MMP-2 and MMP-3 as well as a 120 kD

gelatinase have been found to be expressed. Recently, a study showed that bovine gelatinase A (MMP-2) and gelatinase B (MMP-9) levels were increased in milk following both naturally occurring *E.coli* mastitis and LPS-induced mastitis (Raulo et al., 2002). The most relevant MMPs for potential tissue damage of bovine secretory epithelium are collagenase (MMP-8), gelatinase A (MMP-2), and gelatinase B (MMP-9).

It is known that MMP-9 can be produced by bovine blood PMN (Li et al., 1999). The established bovine mammary epithelial cell line MAC-T also secretes MMP-9 and gelatinase B is expressed at very low levels in healthy bovine mammary gland (Long et al., 2001). Therefore, the authors suggest that the mammary epithelial cell could play a role in the early increase in expression levels of MMP-9 that follows *E. coli* infections. However, this augmentation can also be attributed to the rise in the number of PMN recruited to the mammary gland since both increases are concomitant. Milk PMN isolated from cows affected by mastitis also has been shown to contain collagenase (Mehrzad et al., 2005).

Neutrophils are specially designed to use both the NADPH oxidase system and the granule-based chemicals in a complementary manner. This cooperation of the two mechanisms insures the maximum efficacy of the microbial killing step. Indeed, Gram-negative bacteria have been reported to be resistant to lysozyme unless simultaneously subjected to oxidants and/or complement factors (Smith, 1994). Unfortunately, this combination also increases the potential for host tissue damage.

7. Relationship between Neutrophils and Tissue Damage

Mechanisms that normally protect the host from infection and eliminate bacteria can also cause extensive tissue injury (Weiss, 1989). Therefore, there is a close relationship between SCC, milk composition

changes and mammary tissue damage. Under normal circumstances, PMN can migrate to the site of infection without damaging host tissues. During LPS-induced mastitis, it is generally accepted that massive migration of PMN across luminal cells causes local disruption of epithelial tight junctions that lead to leakage of plasma proteins such as BSA into milk (Kitchen, 1981). It is also believed that this PMN massive diapedesis contributes to tissue damage by injuring epithelial cells leading them to necrosis (Frost et al., 1980; Harmon and Heald, 1982). However, one *in vitro* study conducted by Lin et al. (1995) on an epithelial cell line did not evidence any morphological damage to the epithelial cells following PMN diapedesis. Nonetheless, premature activation of PMN during migration and/or failure to successfully terminate the acute inflammatory response can lead to secretory cell scarring.

The mechanisms underlying tissue damage during *E. coli* mastitis are still not well understood. Using healthy mammary gland tissue explants, Capuco et al. (1986) showed epithelial cell damage by treating the explants with phagocytosing PMN isolated from LPS-treated mammary gland. Tissue damage potentially caused by trafficking PMN may originate from two sites. Firstly, tissue scarring may occur from the inside of the parenchyma (intratissular) starting at the basal side of epithelium but it may also occur on the intraluminal side of the udder, especially when PMN are recruited massively. Secondly, intraluminal-induced damage mainly originates from the luminal part of the mammary cistern and ducts where cells and chemical mediators of the host are locally released. As inflammation progresses and extends itself, the alveoli will become full of potentially harmful exudates released by PMN.

The rapid influx of PMN following an infection constitutes a double-edged sword (Smith, 1994). While PMN are essential for the host defences, they are also highly destructive cells programmed to destroy in

a non-specific manner. They have been shown to contribute to various inflammatory conditions and tissue damage by releasing cytotoxic molecules into the extracellular environment (van Asbeck, 1990). Recently, Ledbetter *et al.* (2001) demonstrated *in vitro* that damage to the mammary epithelium could be caused by as few as 20 000 PMN/ml.

In the bovine mammary gland, PMN may also damage the mammary epithelium through release of lysosomal enzymes and ROS such as O_2^-, H_2O_2, $OH^\bullet$, singlet oxygen, and halide derivatives generated by the reaction of H_2O_2 and myeloperoxidase (Capuco et al. 1986). Damage to tissue can be limited by induction of programmed cell death in PMN and by their engulfment by macrophages.

7.1 Cytotoxicity of Important Radicals and Non-Radicals Derived from Neutrophils

It was demonstrated that production of large amounts of ROS by PMN was able to cause red blood cell damage and destruction *in vivo* (Smith, 1994). This is achieved by creating an environment that is highly destructive and harmful to nearby tissues. Furthermore, Poch *et al.* (1999) reported that ROS produced by neutrophils during acute pancreatitis increase local tissue damage and act as PMN attractants. There are four principal mechanisms by which radicals are able to induce cell injury and tissue damage: 1) ROS can directly cause damage through direct attack on essential and sensitive biological targets (e.g., protein oxidation), 2) the damage can be mediated by destruction of the lipid bilayer via lipid peroxidation, 3) ROS can inhibit cellular metabolic pathways and, 4) secondary reactions of lipid degradation products with critical targets (e.g., enzymes, DNA). These pathways have been widely studied (Miller and Brzezinska-Slebodzinska, 1993; Emerit *et al.* 2001) and there is a wide consensus that ROS generated by activated phagocytes might constitute

the major injurious agents responsible for cellular damage in inflammatory and in ischemic conditions (Ginsburg, 1998; Varani and Ward, 1994).

Hydroxyl radical

Of the three intermediates (O_2^-, H_2O_2, $OH^\bullet$) of oxygen reduction, hydroxyl radical ($OH^\bullet$) is particularly reactive and noxious reacting, as soon as it is formed, with virtually all known biomolecules (lipids, proteins, DNA, sugars and all organic molecules). Even if its estimated half-life in cells is only 10^{-9} seconds, $OH^\bullet$ has a diffusion radius of 2 to 3 nm (van Asbeck, 1990). Therefore, if the site of production is near DNA or membranes, $OH^\bullet$ can have a devastating effect. For example, $OH^\bullet$ can add itself to the double bond in the pyrimidine base thymine resulting in the generation of a thymine radical and therefore DNA strand breakage. The hydroxyl radical also has the ability to initiate lipid peroxidation that will eventually lead to accumulation of lipid hydroperoxides. These ones will allow iron to directly initiate additional lipid peroxidation adding to membrane structure destruction and loss of function (Emerit *et al.*, 2001). It was also reported that an excessive production of $OH^\bullet$ is capable of inducing a membrane lesion and a secondary phagocytosis defect (Van Asbeck et al., 1984). For all these reasons, $OH^\bullet$ is believed to be the most reactive oxygen radical known.

Superoxide and hydroperoxyl radical

Superoxide is far less reactive than $OH^\bullet$ with non-radicals molecules in aqueous solution. Its rate of reaction with DNA, lipids, amino acids and other biomolecules is very slow and may approach zero. However, it does react easily with some other radicals, for instance nitric oxide (NO). Therefore, the direct biological damage caused by O_2^- is itself minimal since the reaction of O_2^- with other radicals to form less damaging molecules happens very quickly. Indeed, the enzymatic reaction catalyzed by SOD is 4 times faster than the spontaneous reactions, which tends to

demonstrates that O_2^- is more toxic for a cell than H_2O_2 and that its fast removal is crucial to limit damage (Rosen *et al.*, 1995). It is also believed that superoxide can decrease the activity of some antioxidant defense enzymes such as catalase and glutathione peroxidase.

The interest of considering this radical as a potential target to lower cell damage lies in the major fact that O_2^- is the starting point for generation of other more toxic species as discussed above. Furthermore, protonation of O_2^- will yield a more powerful oxidant, for instance perhydroxyl radical ($HO_2^\bullet$). This one has the ability to directly initiate lipid peroxidation of fatty acids and to easily cross membrane since it does not possess the negative charge of O_2^- (Halliwell and Gutteridge, 1999).

Despite the fact that only few studies have been able to demonstrate that O_2^- alone was able to produce a toxic effect, the ability of O_2^- to reduce or oxidize transitions metal ion complexes, to form ligands with metals, to oxidize organic substrates, and to be protonated into $HO_2^\bullet$ could play an important role in PMN-mediated toxicity (Weiss et al., 1982).

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is water-soluble and is very diffusible within and between cells *in vivo*. It has been shown to be toxic to most cells at levels between 10-100 µM. It is also believed that sustained production of H_2O_2 during inflammation process can damage host tissue and activate inflammatory cascades (Smith, 1994). Hydrogen peroxide is a strong oxidant but it reacts slowly with organic substances although it can attack some enzymes such as glyceraldehyde-3-phosphate dehydrogenase and inhibit glycolysis (Halliwell and Gutteridge, 1999). It also has been reported to induce mitochondrial swelling (a precursor sign of necrosis), to cause membrane damage and to alter signal transduction in mammalian cells (Ginsburg and Kohen, 1995). Additionally, H_2O_2 reacts

easily with transition metal ions and their complexes to yield other highly reactive oxygen metabolites. The MPO released by activated PMN will exaggerate the oxidizing potential of H_2O_2 by using it as a substrate in the presence of physiological concentrations of chloride to produce HOCl. Additionally, H_2O_2 is able to inactivate some antiproteases and activate latent metalloproteinases such as collagenases and gelatinases that may in turn contribute to the degradation of host tissue.

Hypochlorous acid

Hypochlorous acid is predominantly produced by activated PMN (Fliss, 1988). It is an extremely powerful oxidant that kills bacteria by oxidizing and chlorinating their proteins. However, HOCl remains a non-specific oxidizing agent that can be damaging to every biological molecule encountered (e.g., amines, amino acids, thiols, nucleotides) making its fast removal crucial (Smith, 1994).

Even if HOCl is the major end product of neutrophils' oxidative metabolism, it does not accumulate in biological systems because it instantly disappears in multiple reactions. For example, HOCl can react with primary amines to produce chloramines (R-NCl), a less reactive group of oxidants. Evidences suggest that HOCl exerts its toxic effect by a direct attack to membrane-associated targets whereas chloramines diffuse across the plasma membrane to attack cytosolic components.

HOCl is approximately 100-1000 times more toxic than either superoxide or hydrogen peroxide (Conner and Grisham, 1996). *In vitro*, HOCl at the concentration of as low as 5% has been shown to solubilize skin or liver tissues. It was also demonstrated that 10^6 maximally triggered PMN were able to generate approximately 2×10^{-7} mol of HOCl. Furthermore, the amount of HOCl detected accounted for nearly all the H_2O_2 yielded by the cells (Weiss, 1989). Knowing that PMN will generate

HOCl as long as H_2O_2 is supplied and that human PMN are known to produce H_2O_2 for up to 3 hours after triggering, one can consider the prolonged exposure of cells and tissue to HOCl as an extremely damaging process.

A study performed by Weiss (1989) investigated the effect of ROS on activation of latent MMPs. It was demonstrated that HOCl was able to activate latent (and thus inactive) collagenases and gelatinases. Additionally, HOCl has been reported to inactivate the major circulating inhibitor of serine proteinases, namely the α-1 antiproteinase (Burg and Pillinger, 2001), confirming that HOCl has damaging effects on host tissues.

Even if the implication of all these ROS in mammary gland tissue scarring is more than probable, no study has been performed to specifically determine the effect of each of these ROS on the bovine mammary epithelium. Therefore, the exact mechanism of tissue damage remains uncertain.

7.2 Cytotoxicity Associated with Neutrophil-Derived Proteases

Neutrophils are known to release a variety of both acidic (cathepsin G) and neutral proteases (elastase, collagenase, gelatinase) at degranulation. They are released both into preformed vacuoles and extracellular milieu and are able to depolymerise a wide variety of substrates, including extracellular matrix proteins, which make them potential mediators of tissue destruction in inflammatory conditions. Matrix metalloproteinases have been associated with tissue injury in both PMN and macrophage-dependant models of lung injury (Gibbs et al., 1999). Damage to the epithelium is also often associated with an extensive infiltration of PMN. In inflammatory diseases, it is suggested that

proteases released by transmigrating PMN may be responsible for disruption of tight junctions (Del Maschio et al., 1996).

Neutrophil-derived elastase is believed to play an important role in degradation of connective tissue matrix (Ginsburg and Kohen, 1995). In a human colonic epithelial cell line (T84 cells), PMN-derived proteases, likely elastase, have been related with focal epithelial cell loss and creation of circular defects within the monolayer (Ginzberg et al., 2001). Collagenases have a strong ability to degrade naïve collagen whereas gelatinases degrade denatured collagen as well as types IV and V collagen (Burg and Pillinger, 2001). Proteases and ROS might also interact to trigger apoptosis whereas proteases alone are believed to facilitate arachidonate release, which may act in concert with ROS to favour membrane damage (Ginsburg, 1998). In bovine mastitic tissues, it was demonstrated that *E. coli* mammary infection leads to an increase in the apoptotic activity and degradation of the extracellular matrix (Long *et al.*, 2001). Additionally, it was recently demonstrated by *in situ* zymography that proteolytic activity was significantly higher in bovine mastitic tissue compared to healthy udder tissue (Mehrzad et al., 2005). Accordingly, PMN isolated from cows with mastitis have been reported to contain and release both collagenase (Li et al., 1999) and elastase (Moussaoui et al., 2003).

Compared to normal milk, mastitic milk has a much higher content of proteases leading to a proteases-antiproteases imbalance that cause extensive tissue damage. During udder inflammatory response, mastitic milk proteases are largely associated with milk PMN (Mehrzad et al., 2005). Additionally, a study done by Verdi and Barbano (1991) showed that the proteolytic activity was much higher in somatic cells isolated from milk compared to somatic cells isolated from blood.

It is now obvious that PMN are extremely potent cells that have little capacity to distinguish between foreign and host antigens because their bactericidal weapons are of the broadest possible spectrum. Therefore, host tissue cells are killed together with the bacteria making tissue damage inevitable (Boulanger et al., 2002). However, mechanisms of cellular damage caused by infections and inflammatory processes are complex and are still not fully understood.

7.3 Neutrophils Endogenous Antioxidants and Oxidative Stress

Under normal conditions, a balance is maintained between the amount of ROS produced (oxidants) and the amount scavenged by a cell (endogenous antioxidants). Generally, cells only have enough antioxidant defences to cope with their physiological exposure to ROS. Oxidative stress arises when this equilibrium is disturbed, especially when the cellular scavenging systems (SOD, catalase, GSH) are overwhelmed by increase in ROS (Sharma and Agarwal, 1996; Warren *et al.* 2000). Therefore, oxidative stress can result from a diminished antioxidant levels and/or by an increased production of ROS.

A cell undergoing oxidative stress has two possible fates: adaptation or cell injury. Halliwell and Gutteridge (1999) defined "cell injury" as the result of a chemical or physical stimulus that transiently or permanently alters cell homeostasis. Cells are usually able to tolerate mild oxidative stress by up-regulating synthesis of antioxidants to achieve an appropriate oxidant/antioxidant balance. If oxidative stress persists and/or increases, cells may be injured and even committed to die.

Neutrophils contain large reserves of endogenous antioxidants that can be classified into two groups, namely enzymatic antioxidants and non-enzymatic antioxidants. The enzymatic group represents the first line of defence and includes SOD, catalase and GSH whereas the non-

enzymatic one consists of antioxidants such as α-tocopherol and ascorbate. All these compounds are involved in preventing neutrophils death by self-destruction (Smith, 1994). However, mammary cells do not have such large reserves and are thus extremely susceptible to oxidative damages.

7.4 Antioxidants Therapy in Inflammatory Conditions

Since the presence of PMN-released oxidative products is toxic to for epithelial cells, it was proposed that antioxidants could modify these noxious effects by interfering with the release of oxidative products (Oishi and Machida, 1997; Ginsburg and Kohen, 1995 ; Chen and Tappel, 1995). Free radicals are known to be very difficult to eliminate because the reaction of a free radical with a non-radical species invariably produces another (but different) free radical that could be more or less than or equally reactive to the original radical. The presence of effective free radicals scavengers is therefore crucial. To be able to prevent biomolecules from hydroxyl attack, it was demonstrated that the scavenger had to be present in extremely high non-physiological concentrations to efficiently compete with biomolecules, which is impossible to achieve *in vivo* because of the self-toxicity induced by high amounts of scavenger (Eberhardt, 2001). Therefore, it is impossible to protect cells against $OH^{\bullet}$ attack. However, it is possible to prevent $OH^{\bullet}$ formation by eliminating the precursors, namely H_2O_2 and Fe^{2+} by using specific antioxidants.

Antioxidants are defined as substances that significantly delay or inhibit oxidation of a substrate when present in low concentrations. They exert their effect by scavenging free radical (ex: α-tocopherol), by converting highly oxidizing molecules to less or not dangerous substances (ex: catalase), by chelating metals (ex: deferoxamine), or by activating a

battery of detoxifying/defensive proteins (Conner and Grisham,1996 ; Matés, 2000).

It is known that a good status of nutritional antioxidants helps to diminish tissue damage in a wide range of diseases (Eberhardt, 2001) such as bovine coliform mastitis where antioxidant nutrition plays a critical role in mammary resistance to this inflammation (Chaiyotwittayakun et al., 2002). However, supplementing antioxidants in food does not guarantee that enough antioxidant will reach the target organ *in vivo* and that those antioxidants still will be active. Therefore, the idea of supplementing antioxidants directly to the place where needed becomes attractive.

Ginsburg and colleagues (1997) also showed in an *in vitro* system that scavenging either peroxide with catalase or other ROS by appropriate antioxidants could inhibit cell damage. Another study carried out by Boulanger *et al.* (2002) using a bovine co-culture system of LPS-induced-PMN and MAC-T cells showed *in vitro* that antioxidants such as catalase (scavenger of H_2O_2) and melatonin (scavenger of hydroxyl radical and peroxinitrite) were able to prevent disruption of the mammary epithelial cells monolayer.

In cows, it was demonstrated that intravenous injections of ascorbic acid (an antioxidant) increased milk production recovery following a LPS-induced mastitis (Chaiyotwittayakun et al., 2002). However, to the best of our knowledge, no study has been performed to evaluate *in vivo* the effect of local mammary supplementation of antioxidants in mastitis-induced tissue damage.

8. Cell Death

Cell death is a necessary mechanism that contributes to maintain general homeostasis. It is generally accepted that cell death naturally occurs via two principal distinct mechanisms: necrosis or apoptosis. However, several recent studies have suggested existence of intermediates cell death forms, such as apoptosis-like and necrosis-like programmed cell death, paraptosis, autophagy an many other examples of cell death (Leist, M and M. Jaattela, 2001; Zhivotovsky, 2004). Nevertheless, only the features associated with classical apoptosis and necrosis will be discussed here as they are well characterised and widely accepted as the main pathways of cellular death.

Some compounds (e.g., ROS) can have the ability to cause cell death. For example, some mammalian cells exposed to millimolar levels of H_2O_2 were shown to die by necrosis whereas lower levels of H_2O_2 were reported to trigger apoptosis (Halliwell and Gutteridge, 1999). These compounds are said to be cytotoxic to the cell. Cytotoxicity is the cell-killing property of a chemical compound or a mediator cell such as a neutrophil.

8.1 Necrosis

Necrosis (Figure 2.1) is the pathological process, which occurs when cells are exposed to a serious physical or chemical insult (e.g. LPS, ROS). It is an unplanned cell death resulting either from exposure of a cell to extreme conditions or to a damaged plasma membrane. The necrosis process begins with the inability of a cell to maintain its homeostasis, leading to an influx of water and extracellular ions. As a result, intracellular organelles are swelling and flocculent densities appear in the mitochondrial matrix. Consequently, the entire cell begins to swell and the plasma membrane is ruptured leading to total cell disintegration

(Boehringer, 1998). However, the pattern of the chromatin is conserved. The ultimate breakdown of the plasma membrane releases the cytoplasmic content (including lysosomal enzymes) into the extracellular environment.

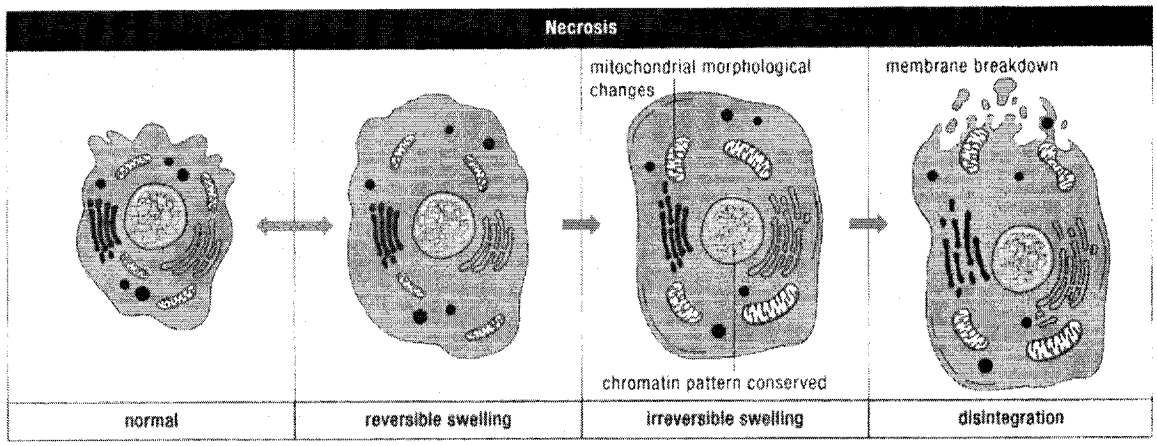

Figure 2.1: Illustration of the morphological features of necrosis. (Boehringer, 1998)

8.2 Apoptosis

Apoptosis (Figure 2.2) is the physiological process by which unwanted or useless cells are eliminated. This mechanism of cell death is also known as "programmed cell death" and usually occurs under normal physiological conditions (e.g. cell turnover and tissue homeostasis, nervous system development, etc.). In the early stages of apoptosis, changes occur on cell morphology, mainly at the cell surface and plasma membrane. Unlike necrosis, apoptosis lead to chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and fragmented nucleus into membrane-bound vesicles (known as apoptotic bodies), which contain ribosomes and morphologically intact mitochondria (Kerr et al., 1972). Apoptotic cells do not release their content into the extracellular milieu so apoptosis does not affect surrounding cells.

In vivo, these apoptotic bodies are rapidly recognized and phagocytized by macrophages without eliciting an inflammatory response. Several recent reports show that apoptosis plays a crucial role in

resolution of several inflammatory conditions. Apoptotic PMN are recognised and ingested by macrophages in heifers' mammary gland (Sladek and Rysanek, 2000).

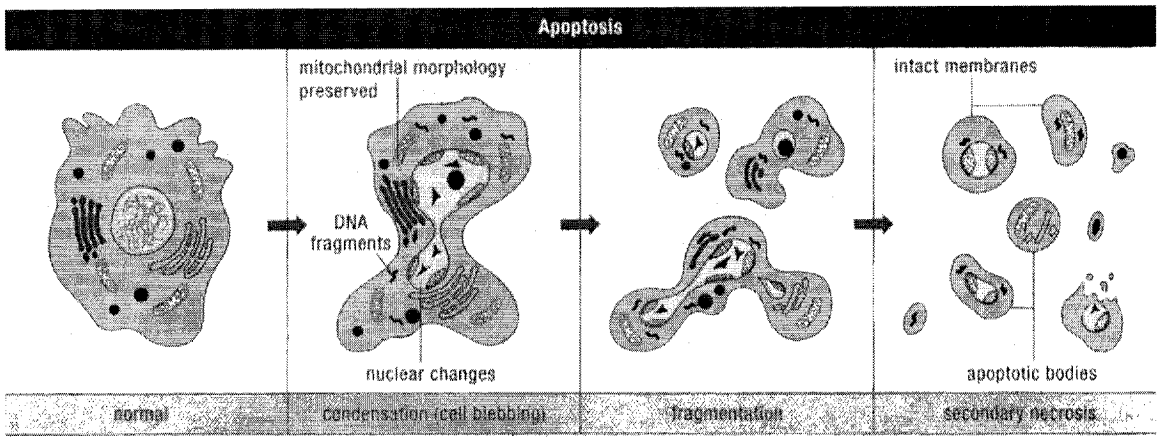

Figure 2.2: Illustration of the morphological features of apoptosis. (Boehringer, 1998)

During apoptosis, genomic DNA is irreversibly fragmented predetermining the cell to death. It is also known that oxidative stress is one of the many conditions that trigger apoptosis (McCord, 1995). Apoptosis of PMN is a key factor to insure that an appropriate number of neutrophils is maintain under physiological conditions (Simon, 2003). Additionally, neutrophil apoptosis is believed to be a new field in mastitis research.

8.3 Relationship between Cytotoxicity, Apoptosis and Necrosis

In contrast to apoptosis and necrosis, cytotoxicity does not define a specific cell death mechanism. Cytotoxicity is the cell-killing property of a chemical compound or a mediator cell such as PMN and macrophages. Cytotoxicity is independent of the pathway that ultimately leads the cell to die. For example, the cell-mediated cytotoxicity caused by immune system cells recognising and/or destroying damaged or infected host cells

illustrates well the concept of cytotoxicity. Then, the infected cell will disappear via an "induced" apoptosis or via a lytic mechanism due to toxic molecules secreted by PMN or macrophages.

8.4 Methods to study Cytotoxicity and Cell Integrity

Because of the features discussed above, assays to measure cytotoxicity can be based on the alteration of plasma membrane permeability and the resulting leakage of components. Therefore, the existing assays to measure cell cytotoxicity are based on at least one of the following principles:

1) Uptake of dyes normally excluded by viable cells such as trypan blue that color non-viable cells in dark blue.

2) Release of artificial labels such as ^{3}H (e.g. proliferating cells that have previously incorporated ^{3}H are put in presence of a cytotoxic agent and the amount of ^{3}H released is then measured);

3) Release of cytoplasmic enzymes such as LDH.

Although simple, these permeability assays have one main disadvantage in that the initial site of damage of most cytotoxic agents is intracellular. Therefore, even if the cell is dying, an intact cell plasma membrane will lead to an underestimation of the real levels of cellular damage. It may then be of interest to countercheck the obtained results using a second method, for example a visual observation of the cells using microscopy techniques. The general cell morphology can be observed and cells with an irregular shape spotted. This step can also be combined with the use of a specific staining agent that stains cells committed to die. Acridin orange is a fluorochrome used to evidence condensed DNA in apoptotic cells. Upon Acridin orange staining, DNA appears yellow-green

and is diffuse in normal cells, while it appears bright green and very condensed in apoptotic cells (Van Cruchten and Van den Broeck, 2002).

9. Endotoxin-Induced Mastitis as a Model to study Mastitis

A lot of studies on mastitis use lipopolysaccharides (LPS) to simulate the effects of a natural occurring mastitis. As discussed above, LPS, also known as endotoxin, is a glycolipid which is an important part of the outer membrane of Gram negative bacteria such as *E. coli*. Minimal structure of LPS consists of a lipid component (termed lipid A), a hydrophilic lipolysaccharide core, and repeated polysaccharide units called O-antigens or O-specific chain (Figure 2.3) (Holst et al., 1996).

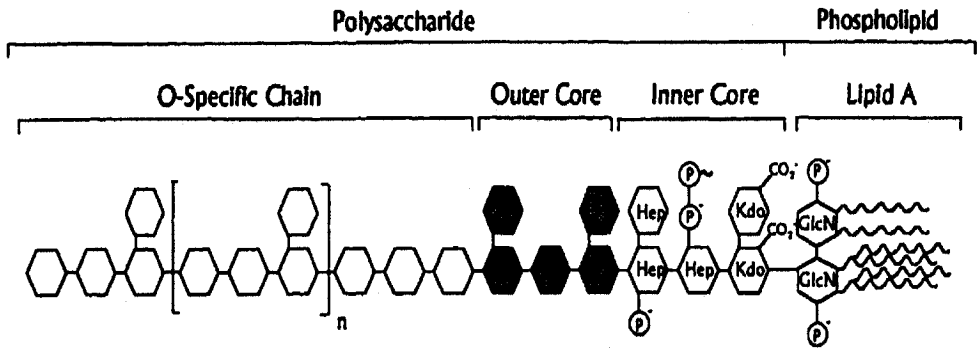

Figure 2.3: Chemical structure of lipopolysaccharide. (Holst et al., 1996)

The lipid A part is the inner part of LPS and it is lipophilic. Lipid A is a phosphoglycolipid that, together with the KDO-containing inner core, is the most conserved structure of LPS. Additionally, most of the toxic effects of LPS on animals or cultured cells are attributable to lipid A (Hogan and Smith, 2003; Holst et al., 1996). The core region is minimally formed of at least one or two residues of 2-keto-3-deoxyoctonic acid (**KDO**) or KDO derivatives (Holst et al., 1996) and provides an attachment site for O-antigens. The O-specific chain is a polymer of oligosaccharides, each

containing between two and eight sugar monomers. This region functions as an important surface antigen.

Lipopolysaccharides may directly affect PMN migratory function via a variety of direct and indirect effects (Wagner and Roth, 1999). The interest of using LPS lies in the fact that it is able to induce an inflammation that closely mimics the symptoms of a natural mastitis without the complications that result from bacterial development. Exposure of PMN to LPS (both *in vivo* and *in vitro*) has been shown to lead to release of biologically active substances that act as endogenous inflammatory mediators without direct toxicity (Oliver and Calvinho, 1995; Ginsburg and Kohen, 1995). Biological effects of LPS include non-specific activation of the immune system and activation of the complement cascade (Paape *et al.*, 1996). Intramammary injection of *E.coli* endotoxin was shown to induce both local and systemic responses (Oliver and Calvinho, 1995).

It was demonstrated that LPS does not cause direct damage to the mammary secretory epithelium. Therefore, the effect of LPS on lactational performance is only due to the induced inflammatory response (Kehrli and Shuster, 1994). Cows infused with LPS recover by themselves and do not represent a danger for the herd. Furthermore, the drop in milk production is short lasting and completely recovered. Thus, the LPS-induced mastitis constitutes a practical, useful and pertinent model to study mammary inflammation (Lohuis *et al.*, 1988a).

10. MAC-T Cell Line

The MAC-T cell line originates from primary bovine mammary alveolar cells that were immortalised with Simian virus-40 (**SV**-40) large T antigen under the transcriptional control of the ß-actin promotor (Huynh *et*

al., 1991). Cellular immortalization is defined experimentally by acquisition of unlimited growth capacity *in vitro*. MAC-T cells retain the phenotypic characteristics of bovine mammary epithelial cells. They are able to form a confluent monolayer with characteristic tight junctions, basal-apical polarity, and functional barriers to the trypan blue dye (Lin *et al.* 1995). MAC-T cells are thus often used as an *in vitro* model to perform researches related to mammary epithelial cells. The main advantage of such easy-to-use models is the reproducibility of the results. Unlike PMN, which are sensitive to PMA, MAC-T cells are resistant to the cytotoxic effects of PMA (Ledbetter *et al.*, 2001).

SECTION III

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

The excessive amount of ROS and proteases released by PMN during mastitis disturbs mammary functions and causes damage. Consequently, we postulated that a better control of the inflammation may accelerate cows' recovery and contribute to maintain the integrity of the mammary epithelial cells. More specifically, knowing that ROS are involved in cellular damage, we proposed that a local supplementation of antioxidants may prevent deleterious effects caused by ROS in a context of acute inflammation such as mastitis.

The main objective of our researches was to verify the protective potential of different substances with known antioxidant properties on cell survival. To assess this issue, we first looked at the protective effects of several compounds using an *in vitro* co-culture system of bovine mammary epithelial cell and polymorphonuclear leukocytes developed by Boulanger *et al.* (2002). We then selected the most promising compounds to assess their protective effects *in vivo* on both cellular damage and milk production during LPS-induced mastitis.

SECTION IV

ANTIOXIDANTS TO PREVENT BOVINE NEUTROPHIL-INDUCED MAMMARY EPITHELIAL CELL DAMAGE

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

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* Dairy and Swine Research and Development Centre contribution.

ABSTRACT

Activated neutrophils are able to produce large quantities 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 co-culture 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 media. Morphological observation of cells stained with acridine orange was also 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 phagocytize 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, antioxidants, reactive oxygen species)

Abbreviation key: BCS = bathocuproine disulfonic acid, **CAT** = catechin, **CFU** = colony forming units, **CMB** = culture medium background, **DFO** = deferoxamine, **DMTU** = dimethylthiourea, **GSH** = glutathione, **GEE** =

glutathione ethyl ester, **LDH** = lactate dehydrogenase, **LPS** = lipopolysaccharide, H_2O_2 = hydrogen peroxide, $OH^{\bullet}$ = hydroxyl radical, **OD** = optical density, **PMA** = phorbol 12-myristate 13-acetate, **PMN**= polymorphonuclear neutrophils, **ROS** = reactive oxygen species, O_2^- = superoxide.

INTRODUCTION

The economic losses caused by mastitis are well known in the dairy industry. Mastitis is an inflammatory reaction that usually occurs following an intramammary infection. Local recruitment of immune cells such as macrophages and neutrophils (**PMN**) is a crucial step in development of an acute inflammatory response. Neutrophils are essential for effective host defence but they are also known to be involved in various inflammatory diseases (Smith, 1994; Weiss, 1989). 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 (O_2^-) via the enzyme NADPH-oxidase, which leads to production of other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and hypochlorous acid. While 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 (van Asbeck, 1990; Weiss, 1989). 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 also play a role in the transmission of intracellular signals (Smith, 1994).

Recently, it was demonstrated *in vitro* that activated blood PMN had a cytotoxic effect on mammary epithelial cells (Ledbetter et al., 2001) potentially through release of ROS such as hydroxyl radicals (Boulanger et

al.; 2002). In cases of acute mastitis like those caused by *Escherichia coli*, the amount of ROS released by PMN may overwhelm the cow's endogenous antioxidant protection mechanisms and therefore and cause extensive tissue damage. Therefore, 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 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). It has been demonstrated that PMN-mediated injury was decreased by pretreating cultured bovine pulmonary artery endothelial cells with DFO (Gannon *et al.*, 1987). 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 (Visseren et al., 2002; Sprong et al., 1997). Protective effects of DMTU were found in several models of acute lung injury induced by phorbol 12-myristate 13-acetate (**PMA**; Chu et al., 2004). Cuprous ions are also thought to be strong pro-oxidants that form radicals by a Fenton-type reaction and/or by transition complexes with molecular oxygen. Therefore, elimination of these ions by chelators such as bathocuproine disulfonic acid (**BCS**) could lower the oxidizing effects of various ROS. Oikawa and Kawanishi (1996) showed that BCS 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 BCS prevented $OH^{\bullet}$ formation. Catechin (**CAT**) is a flavanoid 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 defence 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 co-culture 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 to 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 (**LPS**; *Escherichia coli* O55:B5), DFO, GEE, DMTU and BCS were all dissolved in Hank's Balanced Salts Solution (HBSS). Catechin was dissolved in 100% ethanol before being further diluted in HBSS. Phorbol 12-myristate 13-acetate was dissolved in dimethyl sulfoxide before being further diluted in HBSS.

Cell Culture and Co-culture

The bovine mammary epithelial cell line MAC-T was maintained in culture by serial passages in 75-cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2. The routine culture medium was composed of complete Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (HyClone, Logan, UT) and 1% (v/v) antibiotic/antimycotic (Sigma).

Neutrophils were isolated from healthy mid-lactating 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. Whole blood was diluted 1:1 with pre-warmed (37°C) HBSS and carefully layered over 20 ml of Ficoll-Paque Plus (Amersham Pharmacia, Montreal, QC) in 50 ml Falcons tubes. The tubes were then centrifuged at 1000 x g for 40 min at 4°C, after which the plasma and Ficoll were gently removed and discarded. The PMN were then isolated by two hypotonic lyses of the erythrocytes pellet using 15 ml of Tris-buffered 0.15 M ammonium chloride. The cells were washed twice in HBSS using centrifugation at 350 x g for 10 min before being suspended at a concentration of 5 x 10^6 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, Logan, UT), 1% antibiotic/antimycotic solution, 5µg/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%. More than 95% of the cells isolated were neutrophils.

Co-culturing of bovine PMN and MAC-T cells was performed as described by Boulanger et al. (2002). Briefly, epithelial cells were grown to 80% confluence in 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 x 10^6 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 membrane and granule membranes) and induces

extracellular release of oxygen metabolites (Karlsson et al., 2000). The plates were returned in the incubator for 24 hours. The substances evaluated in this study were DFO (0-300 µM), CAT (0-75 µM), BCS (0-1000 µM), DMTU (0-10,000 µM), and GEE (0-100 µM).

Cytotoxicity Assay

After an incubation of 24 hours, the co-culture supernatants were transferred into 1.5 ml Eppendorf tubes and then centrifuged at 500 x g for 5 min in a microcentrifuge. Lactate dehydrogenase (**LDH**) activity was measured using the Cytotox96® Non-Radioactive Cytotoxicity assay (Promega Corporation, Madison, WI), a colorimetric assay utilizing the conversion of tetrazolium salt into a red formazan product to quantitatively measure released LDH (Nachlas et al., 1960). The LDH assay was performed according to the manufacturer's instructions. The following controls were included in each assay: MAC-T cell spontaneous LDH release, PMN spontaneous LDH release, cytotoxicity medium background, MAC-T maximal release of LDH (15-min incubation of cells with lysis solution). Measurement of optical density (**OD**) was performed at 492 nm. For each experimental well, percent cytotoxicity was calculated using the manufacturer's equations:

Eq.1 Experimental well OD – Culture media background (**CMB**) OD = **A**

Eq.2 MAC-T cell spontaneous LDH release OD – CMB OD = **B**

Eq.3 PMN spontaneous LDH release OD – CMB OD = **C**

Eq.4 MAC-T cell maximum LDH release OD – volume correction OD = **D**

Eq.5 $$\% \text{ cytotoxicity} = \frac{(A - B - C)}{(D - B)} \times 100$$

Acridine orange staining of co-cultures

Once supernatants were carefully collected for the LDH assay, the remaining cells were gently washed twice with HBSS before acridine orange staining solution (8 µg/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% (w/v) formaldehyde solution in PBS. Fluorescence was visualized using a fluorescence microscope (Hund Wilovert S, Germany) and pictures were taken with a digital camera (Nikon, Coolpix 4500) coupled to the microscope.

Preparation of calf collagen

All procedures were conducted at 4°C - 8°C as described by Miller and Rhodes (1982) with some modifications. Briefly, a skinless calf's tail was immersed in 95% ethanol for 15 min to facilitate tendon detachment. All tendons were excised with a scalpel and put in 95% ethanol. The tendons were dried under UV overnight. The next morning, they were cut into 1 cm-long pieces and put into 500 ml of 0.5 N acetic acid to extract collagen. The extraction was carried out for 3-4 days at 4°C with continuous vigorous shaking using an orbital shaker (500 rpm). The mixture was then filtered through several layers of cheesecloth to remove the insoluble residues. Finally, the solution was centrifuged at 50 000 x 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-glucoronidase +, haemolysin -) were grown in 50 ml of the brain heart infusion (BHI) medium for 2 hours at 37°C. An aliquot was then serially diluted to perform a bacteriological

count (colony forming units, **CFU**) on tryptic soy agar plates, which were incubated overnight at 37°C after inoculation. The 50-ml culture was cooled on ice before being kept at 4°C overnight. The next day, cells were centrifuged at 50,000 x g for 10 min, suspended in DMEM/F12 medium without phenol red at 2 x 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 triplicates). 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 and this was used as the time-zero reading. The plate was put in a shaking incubator (150 rpm) at 37°C and absorbance was read every 15 min for 2 hours. The blank was composed of phenol red-free DMEM/F12 supplemented with 5% FBS. The assay was repeated three times.

Phagocytosis Assay

Bacterial suspension (*E. coli* P4) was prepared as described above to a concentration of 4 x 10^7 CFU/ml. One ml of bacteria was then added to a tube already containing 1 ml of freshly isolated PMN (2 x 10^6) and DFO (final concentration in the tubes where phagocytosis occurs: 0, 50, 200, or 800 µM) in DMEM/F12 containing 5% inactivated FBS. These test tubes were then transferred to a 37°C shaking bath (80 rpm) and phagocytosis was allowed to proceed for 30 minutes. During incubation time, a viable plate counts was performed on the initially prepared bacterial suspension in order to determine the exact amount of bacteria added to the test tubes. At the end of the 30 minutes incubation, 100 µl of suspension was taken from each test tube and was serially diluted in triplicate to perform a viable plate counts. This count determines the number of bacteria remaining in the supernatant and hence the quantity of non-phagocytized bacteria. By

subtracting this number from the number of bacteria initially added, the number of ingested bacteria was determined. The assay was repeated three times.

Statistical Analysis

All data from the cytotoxicity assay and the phagocytosis assay were analysed for statistical significance using the GLM procedure of SAS. The linear and quadratic effects of each antioxidant on cell cytotoxicity were assessed and the effects were considered to be significant at $P \leq 0.05$ and to tend to be significant at $0.05 < P \leq 0.10$. When relevant, data are expressed as mean ± 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:

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

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, Ak is the assay effect and ε_{ijk} the residual error. Effects were considered to be significant at $P \leq 0.05$ and to tend to be significant at $0.05 < P \leq 0.10$

RESULTS

Effect of PMN, Antioxidants, LPS and PMA on viability of MAC-T cells

Incubation of MAC-T cells with non-activated bovine PMN (5×10^6 /ml) or LPS and PMA was not damaging to MAC-T cells after 24 hours ($P > 0.1$). When MAC-T cells were incubated with 25 μM CAT, 200 μM DFO

or 50 µM 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% ± 2.9%). These results were consistent with those obtained by the acridine orange staining: MAC-T cells incubated with non-activated PMN (Figure 4.1A) did not show any sign of cell death and monolayer integrity was conserved whereas nuclear condensation, abnormal cellular shapes and disrupted monolayer were observed when the MAC-T cells were incubated with activated PMN (Figure 4.1B).

Effect of Antioxidants on Neutrophil-induced Mammary Cell Damage

The effect of various antioxidants and chelators was evaluated using the co-culture model of bovine PMN and MAC-T cells described above. When added to the co-cultures of MAC-T cells and activated PMN, DMTU did not show any protective effect on PMN-induced cytotoxicity (Figure 4.2 A and B). Addition of BCS reduced cytotoxicity levels by 33% ($P < 0.001$) at the highest concentration of 1000 µM (Figure 4.3A).

On the other hand, CAT, DFO and GEE decreased PMN-induced cytotoxicity in a dose-dependent manner. Addition of 25 and 50 µM of CAT reduced cell cytotoxicity by 77% and 100%, respectively ($P < 0.001$; Figure 4.3B). Similarly, addition of DFO at 100, 200, and 300 µM has lowered cytotoxicity by 58%, 88% and 98%, respectively ($P < 0.001$; Figure 4.3C). Addition of GEE at 50 and 100 µM decreased cytotoxicity levels by 82% and 83%, respectively ($P < 0.001$; Figure 4.3D). Protective effects of CAT, GEE and DFO were also confirmed by visual observation of cells stained with acridine orange under fluorescence microscopy (Figure 1B-E).

Bacterial growth assay

The effect of DFO on *E. coli* growth was evaluated in an *in vitro* assay. A significant reduction of bacterial growth ($P < 0.05$) was observed after an incubation of 60 minutes with DFO (Figure 4.4). 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 (without PMN) only in DMEM/F12 medium for 30 minutes did not induce cell death. Additionally, seventy eight % of the bacteria were ingested by PMN in control wells containing medium only. Deferoxamine did not affect ($P > 0.4$) the PMN' phagocytic ability as shown on Figure 4.5.

DISCUSSION

In the first part of the study, an *in vitro* model of co-culture of MAC-T cells and activated bovine PMN was used to investigate whether some antioxidants and ions chelators were able to prevent PMN-induced mammary epithelial damages. Our results showed that BCS 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 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 a decreased milk production (Sordillo et al., 1997). In the present study, activated PMN induced a considerable cytotoxicity in MAC-T cells whereas incubation of these cells with non-activated PMN did not cause any cytotoxicity. In our assay, activation of the PMN' oxidative metabolism was achieved by supplementing culture media with PMA (1 µg/µl) while LPS (1 µg/µl) was added to prevent PMN from undergoing spontaneous apoptosis and to extend their life span (Lee et al., 1993). Unlike PMN, MAC-T cells are not affected by the presence of LPS and PMA as evidenced 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 BCS to the co-culture only induced a slight level of protection at very high concentration. However, this concentration would hardly be achievable *in vivo* since incubation of 1000 µM of BCS 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 (Visseren et al., 2002). However, it has also been reported that DMTU could act as a copper chelator (Hanna and Mason, 1992) like BCS.

Therefore, the relative ineffectiveness of both DMTU and BCS in preventing cell cytotoxicity suggests that free copper is not an important mediator in PMN-induced bovine epithelial cell damage. These data can also suggest that the distribution 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 flavanoid polyphenol found in tea that has been reported to be 20 times more potent antioxidant than vitamin C (Craig, 1999). Our results show that CAT reduced LDH release and thus cell cytotoxicity by 77% at 25μM and by 100% at 50 μM when added to the co-culture of activated PMN and MAC-T cells. This protective effect was also 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 significantly increased cell viability and decreased lipid peroxidation levels in lead-exposed HepG2 cells where 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 since 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 co-cultures were supplemented with 50 or 100 µM GEE. This protective effect was also confirmed by visual observation of cells stained with acridine orange. Furthermore, no abnormality in cellular morphology was observed. The protective effect of GEE has also been reported by others authors. 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 co-culture model, it is likely that GEE was able to increase the endogenous pool of GSH in MAC-T cells and, thus, enhance 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 hours, 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 hours (Fratti et al., 1998). Furthermore, DFO has been shown to be protective in various models of ROS-induced cellular injury (e.g. heart ischemia reperfusion, paraquat toxicity in mice) 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 reaction 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). Since 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 bovine pulmonary artery 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 show a 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 (50-800 μM). Iron is known to be essential to all living organisms, including *E. coli* (Chart and Griffiths, 1985). The DFO-induced inhibition of bacterial growth observed in our experiment can be compared to what was observed in an *in vivo* study with cows, where the addition of DFO to milk from LPS-infused quarters depressed *E. coli*

growth (Lohuis et al., 1988b). The same study also demonstrated that *E. coli* growth was promoted by adding iron to milk. Although some pathogens such as iron-restricted *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 seven iron-acquisition systems and as they can increase their capacity to assimilate iron when an iron deficiency occurs, it is possible that the observed inhibition may only be transient and last only while *E. coli* modifies its metabolism to activate other mechanisms of iron uptake.

In our assay, phagocytosis was demonstrated by the decreased number of bacteria remaining in the supernatant of control wells as compared to the number of bacteria initially added. Our results did not show a significant effect of DFO on PMN' ability to ingest *E. coli* since 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 as 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 human with DFO induced progressive alteration of phagocytosis performance (Cantinieaux et al., 1990), while 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 also by the use of different bacteria (*Staphylococcus aureus*, *Yersinia enterolitica* 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 to catalyze Fenton reaction. As a consequence, 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, deferoxamine or glutathione ethyl ester), a finding that suggests that ROS are a major cause of PMN-mediated tissue damage during mastitis. In addition, DFO inhibited bacterial growth but did not interfere with the ability of bovine PMN to phagocytize *E. coli*. Therefore, the potential 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.

ACKNOWLEDGEMENTS

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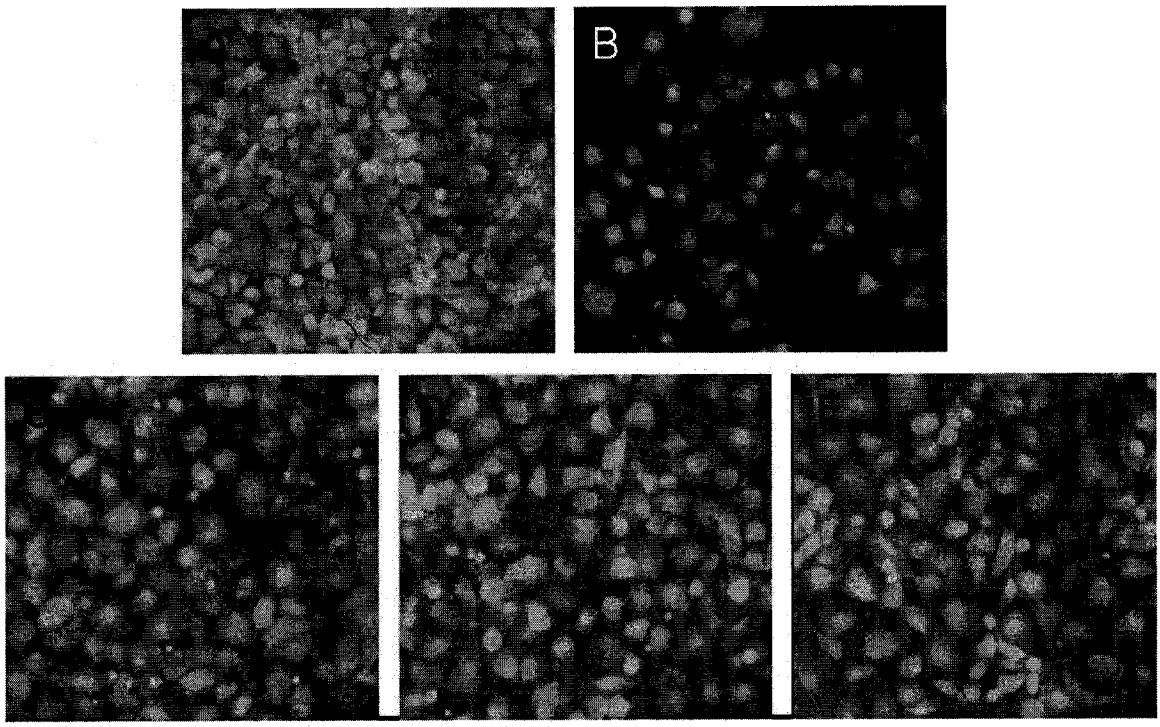

Figure 4.1. Typical acridine orange staining of MAC-T cells following incubation for 24 hours with (A) non-activated neutrophils (PMN) (5 x 10^6/ml), (B) PMN activated with lipopolysaccharides (1 μg/ml) and phorbol 12-myristate 13-acetate (1 μg/ml), (C) activated PMN and catechin (25 μM), (D) activated PMN and deferoxamine (200 μM), or (E) activated PMN with glutathione ethyl ester (50 μM).

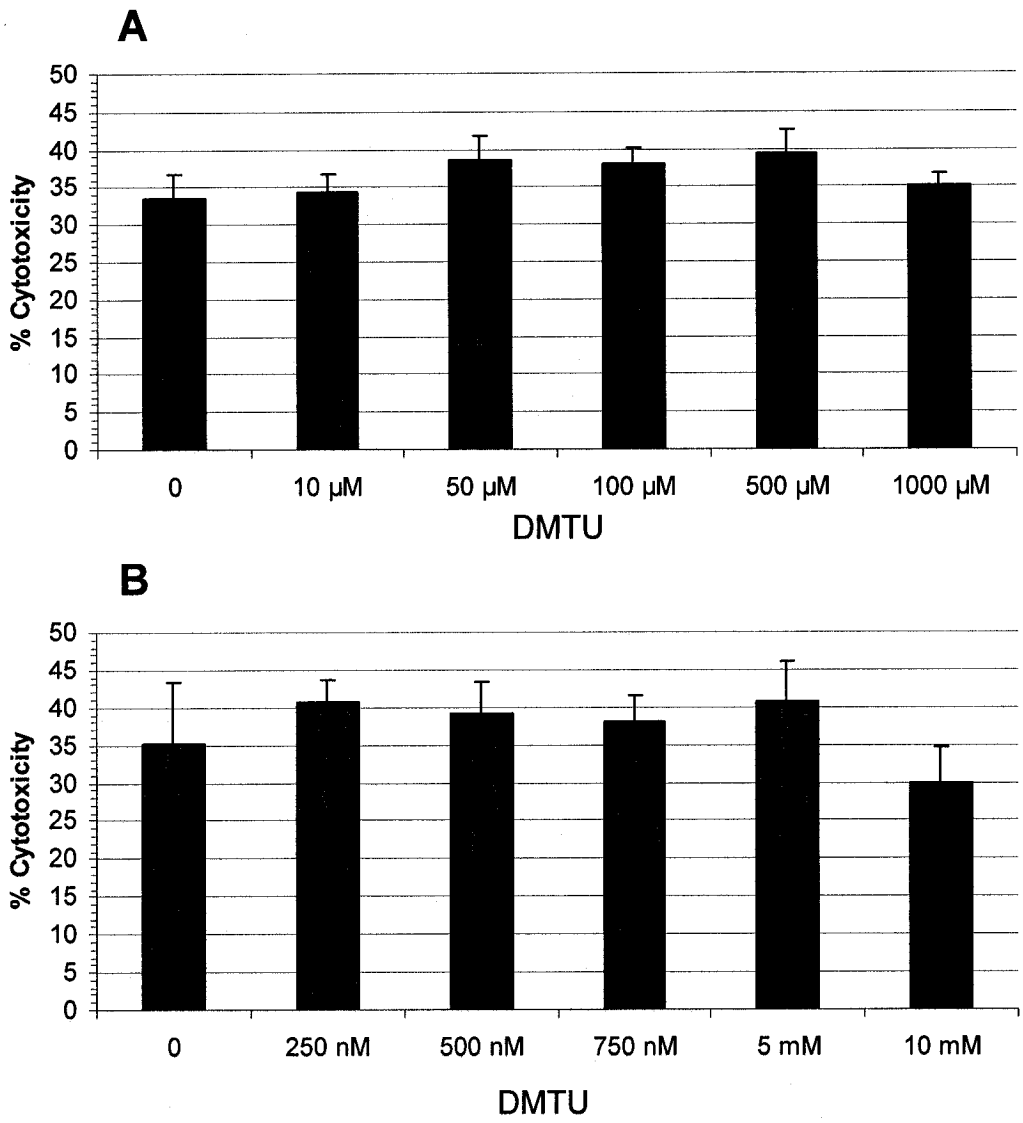

Figure 4.2. Levels of cellular damage in MAC-T cells co-incubated with activated neutrophils (PMN; 5 x 10^6/ml) in the presence of dimethylthiourea (DMTU). Upper panel (A) represents the first concentrations tested, whereas lower panel (B) represents concentrations under and above the initial concentrations tested. After 24 hours of co-incubation, cytotoxicity was determined by measuring the released lactate dehydrogenase. Data are expressed as % of cytotoxicity and represent means ± SEM.

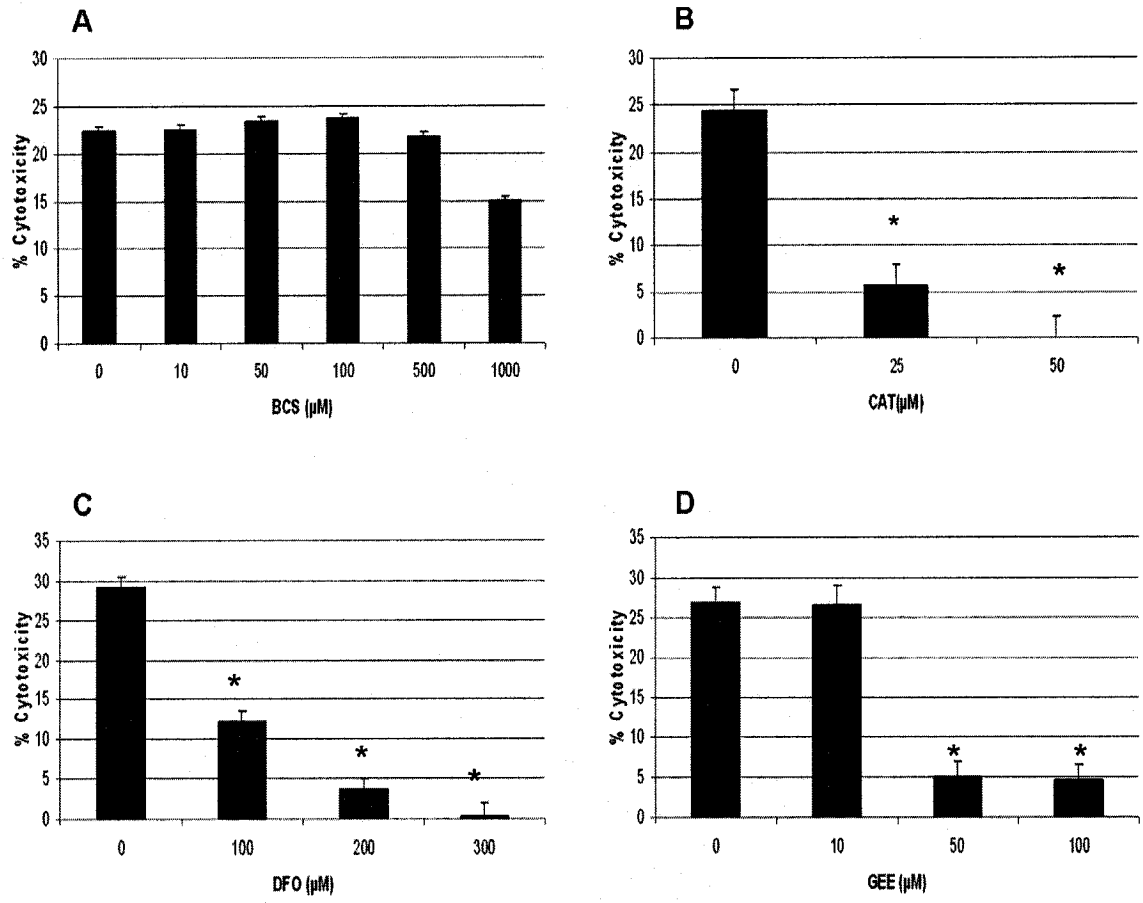

Figure 4.3. Levels of cellular damage in MAC-T cells co-incubated with activated neutrophils (PMN; 5 x 10^6/ml) in the presence of (A) bathocuproine disulfonic acid (BCS), (B) catechin (CAT), (C) deferoxamine (DFO) or (D) glutathione ethyl ester (GEE). After 24 hours of co-incubation, cytotoxicity was determined by measuring of released lactate dehydrogenase. Data are expressed as % of cytotoxicity and represent LS means ± SEM. *The value is significantly different ($P < 0.05$) from the control (0).

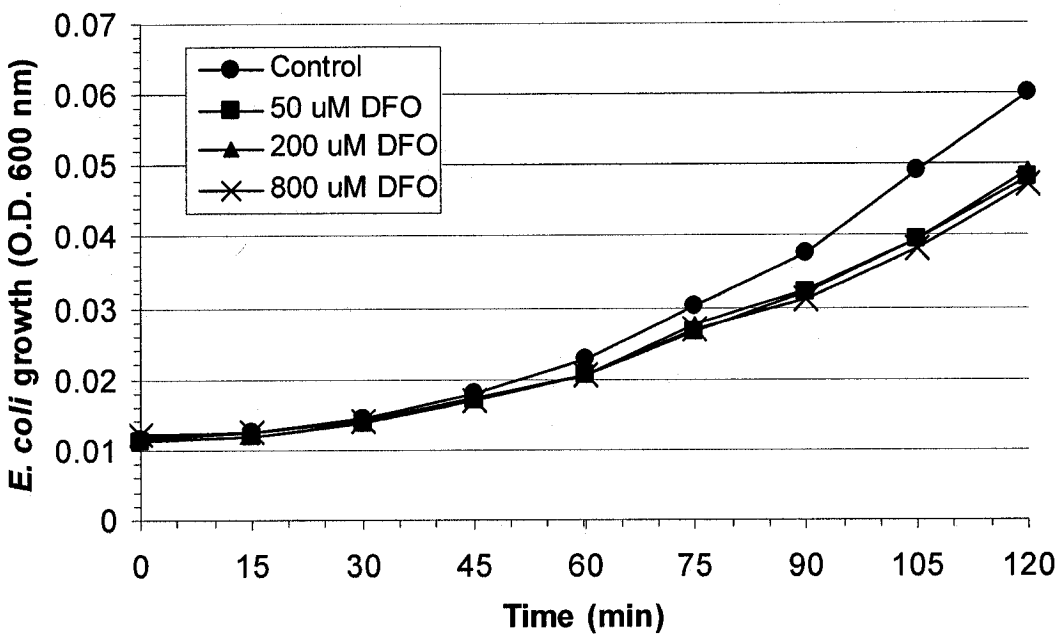

Figure 4.4. Effect of different concentrations of deferoxamine (DFO) on *E. coli* growth. Bacterial growth was determined by measurements of optical density at 600 nm. The values represent the means of three independent experiments in which each DFO concentration was tested in triplicates. Bacterial growth in the presence of DFO was significantly lowered ($P < 0.05$) from time point 60 min for all concentrations tested.

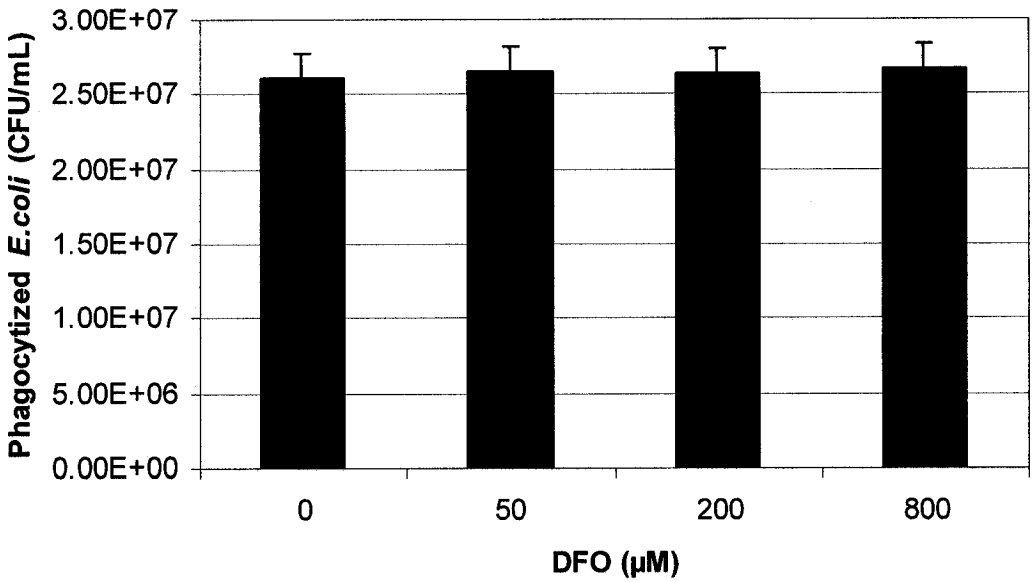

Figure 4.5. Effect of deferoxamine (DFO) on neutrophils' ability to phagocytize *E. coli*. The results represent the number of ingested bacteria and are expressed as means of CFU/ml ± SEM.

CONNECTIVE STATEMENT

The first study presented here showed *in vitro* that some compounds were able to attenuate oxidative damage caused to mammary epithelial cells by activated PMN. More specifically, deferoxamine, catechin and glutathione ethyl ester exerted a significant protective effect i*n vitro*. The next chapter presents a study where these same three compounds were used to assess *in vivo* their effects on both cellular damage and milk production during LPS-induced bovine mastitis.

SECTION V

DEFEROXAMINE PREVENTS TISSUE DAMAGE DURING ENDOTOXIN-INDUCED MASTITIS IN DAIRY COWS

Deferoxamine Prevents Tissue Damage during Endotoxin-Induced Mastitis in Dairy Cows

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ABSTRACT

Protective effects of three antioxidants on PMN-induced damages on mammary cells were evaluated *in vivo* using a model of endotoxin-induced mastitis. Fifteen healthy mid-lactating cows with no history of clinical *E. coli* mastitis were randomly assigned to one of the three treatment group corresponding to each modulator evaluated, that is, deferoxamine, catechin, and glutathione ethyl ester. Each cow had one quarter infused with saline (control quarter) and one quarter infused with the selected modulator; a third quarter was infused with LPS while the fourth quarter received a combination of LPS and modulator. Quarter milk samples were collected regularly. Rectal temperature, visual observations of udder inflammation and milk appearance was recorded. Infusion of lipopolysaccharides caused an acute mastitis as demonstrated by different mastitis markers. The extent of cell damage was evaluated by measuring milk levels of lactate dehydrogenase and NAGase activity at varying intervals before and after intramammary infusions of lipopolysaccharides, with or without antioxidants. Milk levels of haptoglobin, BSA and total proteolytic activity were also assessed. For all measured parameters, intramammary infusions of catechin or glutathione ethyl ester did not exert any protective effect, whereas infusion of deferoxamine, a chelator of iron, decreased milk levels of lactate dehydrogenase, NAGase suggesting a protective effect against neutrophil-induced damage. Additionally, milk level of haptoglobin was significantly lower in deferoxamine-treated quarter while proteolytic activity of mastitic milk was not influenced by the presence of deferoxamine. Our data also demonstrate that deferoxamine does not interfere with influx of neutrophils into the mammary gland and that injections of deferoxamine alone is not damaging to the mammary gland. Overall, our results suggest that local infusion of deferoxamine may be an effective tool to protect mammary tissue against neutrophil-induced oxidative stress during bovine mastitis.

(Key words: neutrophil-induced damage, bovine epithelial cell, antioxidants, reactive oxygen species, proteases)

Abbreviation key:, **BSA** = bovine serum albumin, **DFO** = deferoxamine, **GEE** = glutathione ethyl ester, **GSH** = glutathione, **Hp** = haptoglobin, **LDH** = lactate dehydrogenase, **LPS** = lipopolysaccharide, **NAGase** = 4-methylumbelliferyl-N-acetyl-ß-D-glucosaminidase, **PCH** = post-challenge hours, **PMN** = polymorphonuclear neutrophils, **ROS** = reactive oxygen species, **SCC** = somatic cell count.

INTRODUCTION

Mastitis is an inflammatory reaction that usually occurs following an intramammary infection. The inflammatory response involves massive trans-migration of polymorphonuclear leukocytes (**PMN**) from blood into the mammary gland (Paape et al., 2000). Presence of functional neutrophils is known to be crucial to host defence against bacterial pathogens (Kehrli et al., 1990). This has also been previously demonstrated by Schalm et al. (1976) who showed that treating cows with an anti-bovine leukocyte serum could turn chronic *Staphylococcus aureus* mastitis into a gangrenous disease. The main functions of PMN are to engulf pathogens and destroy them via a variety of bactericidal mechanisms. Indeed, PMN contain intracellular granules that contain bactericidal peptides, proteins and enzymes such as elastase and other proteinases and myeloperoxidase that are released into phagocytic vacuoles or extracellular environment (Borregaard et al., 1993). Additionally, activated PMN have been recently found to release granule proteins and chromatin that together forms extracellular fibers. These extracellular traps would bind microorganisms and ensure a high local concentration of antimicrobial agents to degrade virulence factors and kill

bacteria (Brinkmann et al., 2004). The other mechanism by which PMN eliminates bacteria is oxygen-dependant and produce toxic reactive oxygen species (**ROS**). The cornerstone of this process is generation of superoxide (O_2^-) via the enzyme NADPH-oxidase. The superoxide further reacts to yield other toxic ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and hypochlorous acid (HOCl).

In several studies on inflammation, oxidants and proteases released by PMN have been associated with tissue damage (Weiss 1989; van Asbeck, 1990; Mehrzad et al., 2005). Oxidative stress can cause damage to all types of biomolecules (DNA, proteins, lipids and carbohydrates) and therefore induce tissue injury. In cases of acute coliform mastitis, 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. For that reason, antioxidants could be therapeutic agents that could be used to neutralize the effect of an overproduction of ROS. Protective effects of various antioxidants against cytotoxic effect of ROS have been demonstrated in diverse human diseases both *in vitro* (Richter-Landsberg and Vollgraf, 1998) and *in vivo* as reviewed by Halliwell and Gutteridge (1999).

Involvement of PMN's extracellular ROS (Capuco et al., 1986) and pro-ROS cytokines (Shuster et al., 1997) in mammary tissue damage during mastitis have been demonstrated. *In vitro,* activated blood PMN have been shown to be cytotoxic for mammary epithelial cells (Lauzon et al., in press; Ledbetter et al., 2001) possibly via release of extracellular ROS such as hydroxyl radicals (Boulanger et al., 2002). Additionally, we demonstrated that addition of exogenous deferoxamine (**DFO**), catechin or GEE was able to prevent damage caused by phorbol 12-myristate 13-acetate (**PMA**)-activated PMN to mammary cells in culture (Lauzon et al.,

in press). Therefore, the use of these antioxidants in our *in vivo* study may lower oxidative stress experienced by the mammary cells and accelerate cellular recovery.

Intramammary infusion of LPS is often used to study events occurring during *E. coli* mastitis because it mimics the symptoms of a natural-occurring mastitis without development of microorganisms and toxin production that could cause direct damaging effects to the mammary epithelial cells (Oliver and Calvinho, 1995). In lesions associated with acute inflammation, bacterial endotoxins damage tissue either directly or by attracting PMN that, in turn, release damaging substances (Birkedal-Hansen, 1993). In this study, an endotoxin induced model of mastitis was used to evaluate protective effects of intramammary infusion of catechin, DFO or glutathione ethyl ester (**GEE**) on PMN-induced epithelial mammary damages.

MATERIALS AND METHODS

Chemicals and Reagents

Unless specified, all the reagents used were purchased from Sigma Chemical Co. (St-Louis, MO). Lipopolysaccharides (*E. coli* O55:B5), DFO, and GEE were dissolved in sterile saline. Catechin was dissolved in 100% ethanol before being further diluted in saline. The acrylamide-bis-acrylamide solution, ammonium persulfate, TEMED (N,N,N',N'-tetra-methylethylenediamide), kaleidoscope prestained standards, and Coomassie brilliant blue R-250 were from BIO-RAD Laboratories (Hercules, CA).

Animals and Experimental Procedures

Experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Fifteen healthy high-yielding Holstein in mid-lactation with no history of clinical *E. coli* mastitis were used. Only cows with bacteriological negative milk samples and a milk somatic cell count (**SCC**) of less than 2 x 10^5 cells/ml milk per individual quarter were used in the study. Cows were randomly assigned to one of the three treatment groups corresponding to each modulator evaluated, that is, DFO, catechin, and GEE. Each individual mammary quarter was designated as an experimental unit. For each modulator selected, 5 cows had their left front quarter injected with 20 ml saline (saline group) whereas the right front quarter was injected with 20 ml of the selected modulator (modulator group). Both front quarters served as controls for the rear quarters. Hence, the left hind quarter of all cows was injected with 500 µg of LPS (*E. coli*, 055:B5) in 20 ml saline (LPS group) whereas the right hind quarter was injected with 500 µg of LPS in 10 ml saline plus 10 ml of the modulator (LPS+modulator group). These injections were performed immediately after morning milking. Intramammary doses of DFO, catechin and GEE were 500 mg, 50 mg, and 50 mg per injection, respectively. All solutions to be injected were prepared aseptically fresh. Injection of modulators was carried out immediately after the LPS challenge and was repeated at post-challenge hours (**PCH**) 4, 12 and 24 immediately after milking. Using a specially designed milking machine, quarter milk samples were collected on day -7, -4 -1 and day 0 (immediately before LPS challenge) and at PCH 3, 6, 12, 24 36, 48, 60 and 72. Rectal temperature, visual observations of udder inflammation (redness and swelling) and milk appearance were also performed following the same schedule by two observers unaware of treatments given. For a reason non-related to the experiment, one cow from the DFO

group had to be discarded during the experimental period leaving only 4 cows in this treatment group.

Milk Processing

Following milking, aliquots of quarter milk samples were sent to a commercial laboratory (PATLQ, Ste-Anne-de-Bellevue, Canada) for determination of somatic cell count and infrared evaluation of lactose content and protein content. The remaining quarter milk samples were centrifuged 15 minutes at 1000 x g (4°C) to be defatted and frozen in small aliquots. To obtain whey, a part of defatted milk was ultra-centrifuged (100,000 x g) at 4°C for 20 minutes and aliquots were stored at -20°C. Milk samples from each quarter was analysed for various parameters as described below to evaluate the effect of each antioxidant on various inflammation and mastitis markers.

Evaluation of Milk BSA content

Bovine serum albumin (**BSA**) concentrations in quarter milk samples were determined in triplicates as described by Bouchard et al. (1999) with some modifications. Briefly, 10 µl of skim milk or BSA standards was mixed with 500 µl of water and 500 µl of bromocresol green working solution (mixture of one volume of 1.2 mM bromocresol green dissolved in 5 mM NaOH with 3 volumes of 0.2 M succinic acid pH 4.0 with Brij-35 added to a final concentration of 0.8% vol/vol). Tubes were then centrifuged at room temperature for 15 minutes at 2500 x g to pellet caseins. One hundred fifty µl of supernatant was transferred in 96-well plates and absorbance was read at 640 nm. BSA concentrations in milk samples were determined by comparison with a standard BSA curve (0-64 mg/ml BSA in reconstituted powdered milk).

Evaluation of Milk N-acetyl-ß-D-glucosaminidase content (NAGase) activity

The NAGase activity was determined in triplicates as described by Kitchen et al. (1978). Briefly, 50 µl of diluted skim milk samples or standards were mixed with 200 µl of substrate (2 mM 4-methylumbelliferyl-N-acetyl-ß-D-glucosaminide in 0.25 M sodium citrate buffer pH 4.6) and incubated in the dark for 15 minutes at room temperature before 1.0 ml of stop solution (0.1 M sodium carbonate, pH 10.0) was added. The amount of 4-methylumbelliferone was measured in The NAGase activity or concentration in milk samples were obtained by comparing the released product with 4-methylumbelliferone standards (0 to 75 µM). One unit of NAGase activity corresponds to 1 µmol of 4-methylumbelliferone produced in 1 ml of milk in 1 minute.

Evaluation of Milk LDH activity

Milk lactate dehydrogenase (**LDH**) activity was measured using the Cytotox96® Non-Radioactive Cytotoxicity assay kit (Promega Corporation, Madison, WI), a colorimetric assay utilizing the conversion of tetrazolium salt into a red formazan product to quantitatively measure released LDH (Nachlas et al., 1960). In order to reduce the background, whey samples were diluted 1/5 in PBS with 1% BSA and measured. One hundred fifty µl of diluted samples or standard was mixed with 150 µl of reconstituted substrate before being incubated 15 minutes in darkness. Reactions were stopped by adding 300 µl of the stop solution and then centrifuged 5 minutes at 10,000 x g at room temperature in a micro-centrifuge. One hundred fifty µl of supernatant was transferred into a 96-well plate and absorbance was measured at 492 nm. Milk LDH content was calculated using the standard curve (0-1000 U/µl) made by diluting the LDH positive control supplied with the kit in PBS with 1% (w/vol) BSA. Initial

concentration of the LDH control provided with the kit was 1000 U/µl LDH (bovine heart LDH) in PBS + 1% (w/vol) BSA. Each tested sample was done in triplicates.

Evaluation of Milk Haptoglobin content

Milk haptoglobin (**Hp**) content was measured using an ELISA kit (Tridelta Development Ltd., Bray, Ireland) according to the manufacturer's instructions. Briefly, 100 µl of diluted skim milk or standards of known Hp content were added into wells coated with haemoglobin. Incubation was allowed to proceed for 1 hour at 37°C so that any Hp present in the well was captured by haemoglobin. Wells were washed three times with the provided wash buffer and 100 µl of a Horseradish peroxidase (HRP)-labelled anti-Hp monoclonal antibody was added to each well. Incubation was allowed to proceed for 1 hour at 37°C. After three washes, 100 µl of TMB (Tetra Methyl Benzidine) substrate was added and incubation was performed at room temperature for 15 minutes before 100 µl of the stop solution was added. The absorbance of each well was read at 450nm using 630nm value as a blank. Each sample was evaluated in duplicates and milk Hp content was obtained using a standard curve drawn from the standards.

Zymography for Milk Proteolytic Analysis

Zymography was performed according to Raser et al. (1995) in the Mini-Protean II systems from BIO-RAD. Briefly, the zymogram gels (0.75 mm) were made of 0.2% (w/v) gelatine, 10% (w/v) acrylamide, 0.32% (w/v) bis-acrylamide (N'N'-bis-methylene-acrylamide) in 375 mM Tris-HCl buffer, pH 8.8. Polymerisation was initiated by adding 0.4% (v/v) ammonium persulfate (10% solution) and 0.05% (v/v) TEMED. Once polymerised, a stacking gel (4% acrylamide and 0.11% bis-acrylamide in

330 mM Tris-HCl buffer, pH 6.8) with the same polymerising agents was poured over the zymogram gel. Gels were pre-run at 4°C for 15 minutes at 150 V to remove all non-polymerised particles. Ten microliters of skim milk were mixed with 10 µl of 2X non-denaturing loading buffer (150 mM Tris-HCl, pH 6.8, 20% glycerol and 0.0004% (w/v) bromophenol blue before being loaded on the zymograms. Samples were run over-night at 4°C at 45 V in a running buffer composed of 25 mM Tris-base, 192 mM glycine, 0.1% SDS, pH 8.3. Gels were run until the lower bands (6.5 kDa, 12.5 kDa, 24.7 kDa) of the Kaleidoscope prestained standards had gone out of the gels. They were then removed from the casts and soaked at room temperature with gentle agitation into a solution of 2.5% Triton X-100 for 30 minutes to wash out SDS and renature the proteases. Following that, the renaturation buffer was replaced by the developing buffer (50 mM Tris pH 7.6, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% Brij35) for 30 minutes. The developing buffer was changed and gels were put at 37°C for 10 hours to allow development of enzymatic activity. Finally, zymograms were stained at room temperature for 30 minutes in a 0.5% (w/v) Coomassie blue R-250 solution dissolved in 40% (v/v) methanol and 10% (v/v) acetic acid. Gels were destained several times with a solution of 50% methanol and 40% acetic acid until clear proteolysis bands appeared over a dark-blue background. Gels were then rehydrated into water for at least 30 minutes before being scanned. The molecular weight was calculated using molecular weight standards ranging from 30.3 kDa to 194.6 kDa (Kaleidoscope prestained standards).

Total proteolytic activity was calculated after videodensitometry analysis of the negative images of the gelatine zymograms. Intensity of each proteolysis band was quantified and total proteolytic activity was obtained by adding together the optical densities measured in each lane and was arbitrarily expressed as optical density units.

Statistical analysis

Data were analyzed as repeated measurements using the MIXED procedure of SAS by groups. The covariance structure used was the spatial power. Effects of antioxidant infusion on milk parameters were evaluated by comparing saline-infused quarters with modulator-infused quarters whereas effects of LPS+modulator combination were obtained by comparison with solely LPS-infused quarters. Differences among groups were determined at each level of time using the least squares means statement with the slice option of SAS. Because values after LPS challenge were not normally distributed, statistical analyses of results were performed on the Log10 transformed values for the following parameters: SCC, LDH, NAGase, BSA, Hp and Zymography (proteolytic activity). For all parameters studied, statistical analysis was performed according to the following model:

$$Y_{iJk} = \mu + C_i + M_J + T_k + (M_J * T_k) + \varepsilon_{ijk}$$

Where $\mathbf{Y_{ijk}}$ is the studied variable, $\boldsymbol{\mu}$ is the overall mean, $\mathbf{C_i}$ is the cow effect, $\mathbf{M_J}$ is the effect of modulator (DFO or GEE or Catechin), $\mathbf{T_k}$ is the time effect, and $\boldsymbol{\varepsilon_{ijk}}$ the residual error. Effects were considered to be significant at $p \leq 0.05$ and to tend to be significant at $0.05 < P \leq 0.10$

RESULTS

Intramammary infusion of LPS resulted in the expected inflammatory reactions as measured by standard diagnostic markers of mastitis. For all cows, intramammary infusion of LPS caused a significant increase in rectal temperature ($P < 0.001$; Figure 5.1). Additionally, all quarters injected with LPS developed mastitis as indicated by existence of

udder inflammation symptoms (swelling and redness), whereas control quarters infused with saline or modulator remained normal. Two cows enrolled in the catechin group suffered from severe acute mastitis throughout trial duration. Endotoxin infusion also significantly reduced quarter milk production ($P < 0.001$; Figure 5.2), both in quarters that received an infusion of LPS and in quarters that did not receive LPS infusion (control quarters). Milk production tended to return to preinfusion values within 72 hours. However, one of the cows experiencing severe mastitis (catechin group) suffered from agalactia in her two LPS-infused quarters from 36 hours to 72 hours post-infusion.

As early as three hours following LPS infusions, milk lactose content of all quarters significantly dropped ($P < 0.001$; Figure 5.3). The decrease was more pronounced in quarters infused with LPS or LPS+modulator and lactose content remained low without returning to preinfusion levels throughout trial duration. On the other hand, decrease in milk lactose of both saline and modulator control quarters was not as marked and lactose content tended to return to preinfusion value within 48 hours for catechin ($P = 0.07$; Figure 5.3A) and within 12 hours for DFO ($P = 0.06$; Figure 5.3B). All LPS-infused quarters exhibited a similar and significant increase in SCC ($P < 0.001$; Figure 5.4), which remained high during the time of experiment with a peak at 60 hours. Intramammary injections of DFO, catechin or GEE alone did not increase SCC ($P > 0.1$). Furthermore, LPS-elicited PMN influx towards the mammary gland was not affected ($P > 0.1$) by catechin, DFO or GEE (Figure 5.4 A, B, and C respectively).

For all animals, concentrations of BSA increased ($P < 0.001$; Figure 5.5) in milk from LPS and LPS+modulator quarters, reaching a peak at 3 hours post-infusion, whereas it remained low in control quarters (saline

and modulator). Intramammary injections of DFO, catechin or GEE alone did not increase BSA concentrations ($P > 0.1$). Additionally, levels of BSA in milk originating from LPS-infused quarters were not modified ($P > 0.1$) by the infusion of catechin, DFO or GEE (Figure 5.5 A, B, and C respectively).

The NAGase and LDH activity increased ($P < 0.001$) in milk from all LPS and LPS+modulator quarters, whereas they remained low in saline and modulator quarters as represented on figure 5.6. For both parameters, a peak of activity was reached 36 hours after LPS injection before a gradual decline. Intramammary injections of GEE or catechin did not result in lower levels of LDH or NAGase activity when compared to LPS ($P > 0.1$; Figure 5.6A and C). Interestingly, injections of DFO into LPS-infused quarters resulted in a decrease ($P < 0.05$) in milk NAGase activity at PCH 36 whereas only a tendency (Figure 5.6B; $P = 0.07$) was observed at PCH 48. Additionally, milk from LPS+DFO-infused quarters exhibited a lower LDH activity than milk from the LPS-infused group of quarters but the effect only tended to be significant at PCH 36 ($P = 0.1$) and 48 ($P = 0.1$; Figure 5.6B).

In our study, use of a highly sensitive ELISA allowed us to detect Hp levels as low as 300 ng/mL. Milk Hp started to increase sharply at PCH 12 and peaked at PCH 36. Haptoglobin content started to decline at PCH 48 in quarters infused with LPS+DFO and at PCH 60 in solely LPS-infused quarters. Overall, Hp content of milk from LPS+DFO-infused quarters was lower than that of milk from LPS-infused quarter but the effect only tend to be significant at PCH 48 (Figure 5.7; $P = 0.08$).

Proteolytic activity of mastitic milk produced 3 different bands on gelatin zymograms. Bands were typically found at 72 kDa, 92 kDa and 120 kDa. Milk from non-challenged quarters (saline or modulator) expressed

only low levels of gelatinolytic activity. Kinetics of milk proteolysis capacity on gelatin zymograms showed that, in LPS-challenged quarters, proteolytic activity increased ($P < 0.05$; Figure 5.8) as early as 3 hours following LPS infusion, remaining high during the whole sampling period. None of the antioxidants tested significantly influenced total proteolytic activity ($P > 0.1$; Figure 5.8A, B, and C).

DISCUSSION

The effect of selected modulators on mastitis markers and PMN-induced tissue damage was investigated. Injection of LPS induced mastitis in all challenged quarters. The response profiles for rectal temperature, clinical signs of mastitis, milk production, lactose content, and BSA leakage in milk were in agreement with previous reports (Bouchard et al., 1999; Bannerman et al., 2003). Additionally, SCC increased drastically in LPS-challenged quarters 6 hours post-injections and remained high for at least 72 hours whereas control quarters did not show any significant increase in somatic cells. This sharp increase is in agreement with previous studies that detected peak concentration of SCC in early inflammation (Lee et al., 2003; Shuster and Harmon, 1991). However, these studies also report a gradual but continual decline in SCC after 24 hours whereas in our study SCC remained high after 12 hours in all of the three treatments groups. This is probably due to the fact that cows were injected with a massive dose of LPS (500 μg LPS/quarter) in our study whereas others usually work with lower doses. Indeed, since the amount of LPS produced is directly related to the number of *E. coli* bacteria (Cross et al., 1993), our observations are similar to those made by Vangroenweghe et al. (2004) who reported a sustained high level of SCC for at least 72 hours following intramammary injection of high dose of live *E. coli* (1×10^4 - 1×10^6 cfu).

Levels of milk NAGase increased sharply 24 hours after LPS infusion, reaching values slightly higher than what was reported by others (Bouchard et al., 1999) and suggesting that mammary cells were damaged by the LPS-elicited PMN influx into the mammary gland. This is in agreement with previous studies that reported a high production of ROS and pro-ROS cytokines throughout day 1 of endotoxin mastitis (Hagiwara et al., 2001; Mehrzad et al., 2001a), which potentially boost mammary tissue damage. Reports regarding the usefulness of NAGase as an indicator of tissue damage are controversial. While some studies demonstrate that the major source of milk NAGase is the mammary epithelial cells (Fox et al., 1988; Kitchen et al., 1978), some suggest that PMN can contribute for up to 22% of the amount of NAGase detected (Capuco et al., 1986). Therefore, NAGase alone cannot be used as the sole indicator of tissue damage. In our assay, the increased levels of LDH measured in milk following LPS infusion confirm the results suggested by the measurement of milk NAGase activity. Indeed, the elevated level of LDH in mastitic milk was previously found to originate mainly from damaged parenchyma cells of the udder (Bogin et al. 1977). Taken together, these data suggest that LPS-elicited influx was damaging to mammary cells.

The use of acute phase proteins such as Hp as markers for diagnosis of mastitis has been previously suggested (Eckersall et al., 2001). In bovine, concentration of acute phase proteins correlates well with the severity of an infection and with tissue damage (Nielsen et al., 2004). In our study, levels of milk Hp started to increase as soon as 3 hours after LPS infusion reaching maximal levels near 180 µg/mL. These results are in agreement with previous findings (Hiss et al., 2004). Our results also show that maximal Hp concentrations are reached between 24 and 48 hours after LPS infusion. Since the only study to report milk Hp

levels before and after intramammary infusion of LPS did not measure milk concentrations of Hp at time points above 12 hours post-infusion (Hiss et al., 2004), we cannot compare our results with those from other studies. However, since our LDH and NAGase results also demonstrated that the extent of tissue damage was higher between 24 and 48 hours after LPS infusion, it is reasonably consistent with our own results to see high levels of Hp at these time points.

Recently, it was demonstrated that mammary gland itself was able to synthesize Hp, confirming that levels found in milk are not only due to increased permeability of blood milk barrier (Hiss et al., 2004). In our study, BSA leakage was maximal in early inflammation whereas no drastic increase in Hp levels was detected suggesting that Hp found in our milk samples was from local origin. Additionally, peak values of milk Hp were observed when BSA values (cows enrolled in DFO group) were declining. Therefore, our results support the view that Hp may be synthesised locally by bovine mammary glands and that its presence in milk is not only due to leakage of blood milk barrier as initially suggested by Eckersall et al. (2001).

In our study, the apparition of 92 kDa and 72 kDa gelatinases in milk from LPS-infused quarters is in agreement with other *in vivo* studies where an endotoxin-induced model of bovine mastitis was used (Raulo et al., 2002; Mehrzad et al., 2005). These enzymes have been identified as matrix metalloproteinase-2 (72 kDa) and matrix metalloproteinase-9 (92 kDA). Matrix metalloproteinases are generally considered as relevant mediators of the remodelling and degradation of extracellular matrix and basal membrane components (Birkedal-Hansen et al., 1993). They also induce tissue damage by cleaving type IV collagen (Woessner, 1991), which constitutes the backbone onto which other basal membrane components are attached (Timpl, 1989). Additionally, proteases have been

demonstrated to be actively involved in udder tissue damage during mastitis (Mehrzad et al., 2005). To the best of the author's knowledge, the presence of a 120 kDa gelatinase has not been reported during bovine mastitis. However, it has been previously reported to be expressed by a murine mammary epithelial cell line, SCp2 (Desprez et al., 1998). The same study also demonstrated that the 120 kDa gelatinase had the characteristics of an MMP. Additionally, they reported that this gelatinase was expressed in the early stages of murine mammary gland involution, a period associated with extensive tissue remodelling. Therefore, we can speculate that this 120 kDa gelatinase might be secreted by bovine mammary epithelial cells in response to mastitis in order to accelerate the resolution of tissue damage caused by bacterial growth and/or PMN metabolism.

Taken together, all these results suggest that intramammary infusion of LPS resulted in the expected inflammatory response. Moreover, results from all markers of tissue damage used in this study seem to indicate that epithelial cells of LPS-infused quarters experienced significant tissue damage. The use of LPS instead of live bacteria allows us to attribute the observed tissue damage directly to the metabolism of PMN since it was demonstrated that LPS does not cause direct damage to the mammary secretory epithelium (Kehrli and Shuster, 1994). Therefore, it confirms that the use of such a model of LPS-induced mastitis was pertinent to study protective effect of some modulators on PMN-induced mammary damage.

Infusion of catechin, DFO or GEE did not affect immune cells diapedesis towards milk as demonstrated by low SCC and low milk BSA content in quarters infused with each modulator alone. Furthermore, for all three modulator tested, migration of somatic cells was almost identical in milk from LPS-challenged quarters and milk from LPS+modulator-infused

quarters. For all three modulators evaluated in this study, BSA levels between LPS and LPS+modulator quarters were similar, indicating that the extent of opening of blood-milk barrier was equivalent. However, since two cows enrolled in the catechin group of treatment had severe acute mastitis in LPS and LPS+catechin-infused quarters, the average measured milk BSA levels were higher than those typically measured in inflamed quarters in this study, explaining the high variation observable on the graph. Overall, the SCC and BSA results suggest that intramammary infusions of catechin, DFO or GEE do not interfere with migration of phagocytic cells from blood to infection site, a crucial step in efficient bacterial killing.

Infusion of catechin, DFO or GEE alone was not damaging for epithelial cells, as demonstrated by low activity of LDH and NAGase (two cytosolic enzymes) measured in milk from quarters infused with each modulator alone. These results are in agreement with what was observed *in vitro* using a coculture model of bovine epithelial cells and freshly isolated bovine PMN (Lauzon et al., in press). Additionally, infusion of catechin or GEE in LPS-challenged quarters did not decrease levels of LDH and NAGase activity subsequently measured in milk, suggesting that these two modulators are not able to prevent tissue damage in a context of acute mammary inflammation such as bovine mastitis. On the other hand, the amount of NAGase released by epithelial cells was lower in milk from LPS+DFO-treated quarters compared to only LPS-infused quarters, suggesting a potential effect of DFO in preventing PMN-induced damage. As mentioned above, some studies have raised the question of the usefulness of NAGase as an indicator of tissue damage by suggesting that PMN can contribute for up to 22% of the amount of NAGase detected (Capuco et al., 1986). Nevertheless, considering the fact that in our study there was no difference of SCC between LPS and LPS+DFO-infused quarters, it is plausible that the difference in milk NAGase activity

observed in this study between LPS and LPS+DFO quarters is mostly due to the degree of mammary cell damage. This standpoint is reinforced by the fact that the amount of LDH released by epithelial cells was also lower in inflamed quarters that received DFO, suggesting a protective effect of DFO against PMN-induced damage. For both NAGase and LDH, effect of DFO became more obvious 36-48 hours after LPS infusion, which also corresponds to the point where release of NAGase and LDH in milk was maximal. Consequently, it is reasonable to suggest that release of ROS was also maximal near these time points. Therefore, protective effect induced by DFO may become significant only when levels of ROS and thus tissue damage are quite high.

Though only a tendency was observed, our results also showed that the amount of Hp in milk from LPS+DFO quarters was lower than that of LPS quarters. This result seems to be in agreement with our LDH and NAGase results since the extent of tissue damage always tended to be lower in LPS+DFO-infused quarters. Furthermore, the fact that Hp levels measured in LPS quarters is lower than that measured in LPS+DFO quarters when no difference in BSA levels were observed suggest that the mechanism of regulation for Hp local synthesis is, at least partially, independent of the systemic response.

Neither infusion of catechin, DFO nor GEE modified total proteolytic activity since the proteolysis pattern was identical in milk from LPS-challenged quarters and milk from LPS+modulator-treated quarters at all time points. Knowing that proteases are mainly released by somatic cells (Mehrzad et al., 2005) and that somatic cells migration was not affected by the addition of any of the three modulators assessed, it is not surprising to observe a similar pattern in all LPS-challenged quarters. Therefore, our results suggest that catechin, DFO or GEE do not have any inhibitory or

activatory effects on proteases activated following mastitis since total proteolytic activity remains the same in LPS and LPS+modulator quarters.

The use of antioxidants to prevent oxidative stress arising in several inflammation states has been widely studied (Cuzzocrea et al., 2004). The total ineffectiveness of catechin, a flavanoid polyphenol extracted from plants, to prevent tissue damage in mastitis is surprising since a lot of studies have reported a protective effect of catechin in different models of ROS-induced damage, such as in myocardial damage induced by ischemia-reperfusion (Aneja et al., 2004) and in LPS-induced lethality in BABL/c mice (Yang et al., 1998). Additionally, it was reported that catechin had the ability to repress MMP-2 and MMP-9 in cancer and angiogenesis (Garbisa et al., 2001), which differs from what was observed in our study. It was recently suggested in models of angiogenesis and pulmonary fibrosis that the principal mode of action of catechin was the inhibition of PMN recruitment, hence attenuating the deleterious effect caused by PMN (Donà et al., 2003; Aneja et al., 2004). As mentioned above, catechin did not modify PMN influx to the mammary gland in inflamed quarters, maybe explaining why no effect of catechin on tissue damage was observed in our study. Hence, it may be suggested that the dose of catechin reached intramammary was not high enough to exert an inhibitory effect on PMN influx. It is also possible that the LPS-elicited PMN influx is too strong and overwhelms the ability of catechin to inhibit the migration of PMN. Additionally, it may be proposed that milk is not an optimal environment for catechin to exert its anti-inflammatory effect. Indeed, a study performed by Krul et al. (2001) using an *in vitro* gastrointestinal model, which simulated the conditions in the human digestive tract, demonstrated that the antimutagenic activity of green tea (catechin) was reduced by more than 90% when whole milk was added.

Hydrogen peroxide is an important factor in ROS-induced tissue damage since it is a precursor of other species that have a higher reactivity (e.g. hydroxyl radical). Additionally, H_2O_2 controls the process of inflammation by acting on the synthesis of vasoactive and chemotactic compounds (van Asbeck, 1990). Therefore, it seemed a logical approach to evaluate the effect of GEE on tissue PMN-induced damage in our study. Glutathione (**GSH**) is a key component of the cellular defence cascade against injury caused by ROS (Kennedy and Lane, 1994). Indeed, GSH is the key substrate of the endogenous H_2O_2-removing enzyme GSH peroxidase whereas reduced glutathione ethyl ester (GEE) is a cellular antioxidant that can easily cross membranes and help to maintain the intracellular GSH concentration. Supplementation of GEE has been demonstrated to prevent hyperoxia-induced mortality in newborn rats (Singhal and Jain, 2000) and to attenuate oxidant stress occurring after exposure of bovine pulmonary artery endothelial cell to LPS (Morris et al., 1995). Additionally, using a co-culture model of activated bovine PMN and a bovine mammary epithelial cell line (MAC-T cells), we previously demonstrated *in vitro* that PMN-induced damage to mammary epithelial cells was prevented by addition of GEE to the cocultures (Lauzon et al., in press). However, no preventive effect of GEE was observed in the present *in vivo* study. As GEE supplements GSH pool, the inability of GEE to efficiently prevent tissue damage may suggest that H_2O_2 is not a major ROS involved in PMN-induced tissue damage in the mammary gland during LPS mastitis. This finding would be surprising as H_2O_2 is known to play a central role in tissue damage induced by ROS (van Asbeck, 1990). Therefore, it may be more plausible to suggest that elevating intracellular GSH concentration in mammary epithelial cell does not increase the cells' overall ability to withstand or recover from oxidative stress. This would be in agreement with a study performed by Spector et al. (1987) which reported that elevated GSH levels were deleterious to lens epithelial cells causing a decreased ability to recover from oxidative stress.

It is generally agreed that tissue injury induced by H_2O_2 is often, if not always, dependent on the presence of a metal catalyst. Additionally, because the concentration of iron in animal tissue exceeds by far that of other transition metals, iron is considered as the most effective catalyst for oxidative reactions such as the Haber-Weiss reaction by which hydroxyl radicals are generated (van Asbeck, 1990). Therefore, prevention of injury by ROS may be achievable by using iron chelator in order to directly inhibit the generation of hydroxyl radical. When DFO chelates free iron, it forms ferrioxamine that is a very stable complex distributed in the extracellular space and unable to penetrate cells (Emerit *et al.*, 2001). Deferoxamine has been shown to be protective in various models of ROS-induced cellular injury, as reviewed by van Asbeck (1990). For example, it has been demonstrated that PMN-mediated injury was decreased by pretreating cultured bovine pulmonary artery endothelial cells with DFO (Gannon *et al.*, 1987). Additionally, we also previously demonstrated *in vitro* that PMN-induced damage to mammary epithelial cells was prevented by addition of DFO to the cocultures (Lauzon et al., in press). The results of these studies are in agreement with the results obtained with the present study since we demonstrated that intramammary supplementation of DFO tends to prevent tissue damage, as revealed by lower levels of milk LDH and NAGase measured in milk from LPS+DFO treated quarters when compared to those of LPS treated quarters.

Because DFO is an iron chelator, protective effect of DFO is probably achieved by preventing the iron to be used in the Haber-Weiss reaction ($H_2O_2 + O_2^{-} \rightarrow O_2 + OH^{\bullet} + OH^{-}$), more specifically in the Fenton reaction ($H_2O_2 + Fe^{+2} \rightarrow OH^{-} + OH^{\bullet} + Fe^{+3}$) that is included in the general Haber-Weiss reaction (Gutteridge et al., 1979). Additionally, since hydroxyl radicals are the most noxious intermediates of oxygen reduction because they can oxidize most organic compounds (Wrigglesworth and Baum, 1980), it is coherent to observe a lower level of tissue damage in

inflamed quarters treated with DFO. Hence, our study may suggest that hydroxyl radicals are specifically involved in mammary tissue damage induced by PMN-generated ROS in cases of acute bovine mastitis.

The exact mechanism by which DFO succeds to lower the extent of mammary epithelial damage was not assessed in this study. In a clinical trial, Menasche et al. (1988) demonstrated that PMN exposed to DFO had a decreased free radical production during cardiopulmonary bypass in human patients. The authors also suggested that it is possible that DFO, by protecting the vascular endothelium against injury, prevents the release of inflammatory substances that prime the PMN for increased responsiveness and/or act as chemoattractants. Varani et al. (1996) also reported that DFO inhibited PMN adhesion to lung epithelial cells and vascular endothelial cells, thus preventing PMN-mediated killing of the same target cells. The authors concluded that this ability of DFO to interfere with the binding of PMN onto target cells contributed to the anti-inflammatory activity attributed to DFO. Thus, in our study, it is possible that the protective effect of DFO was not only mediated through iron chelation but also through a certain local anti-inflammatory effect.

Nonetheless, in regard of the results presented above, it appears that intramammary supplementation with DFO is not sufficient to counter all damaging effects caused by PMN. Furthermore, the use of DFO alone to prevent PMN-induced damage in the context of naturally occurring mastitis would not be an effective tool since some bacteria such as *Staphylococcus aureus* are able to use DFO as an exogenous iron source (Diarra et al., 2002). Therefore, the use of another chelator of iron which remains unavailable for mastitis-causing bacteria may be a more promising approach. In addition, synergistic interactions between DFO (or another chelator of iron), other antioxidants and/or proteases inhibitors

could be an interesting thing to assess in the future, in order to increase the protective effect induced by intramammary infusions.

In summary, LPS-induced mastitis seems to be a good model to assess the protective effect of different antioxidants (namely catechin, DFO and GEE) on PMN-induced mammary epithelial cell damage. The deleterious effects induced by ROS released by PMN in the context of LPS induced mastitis tended to be lower in inflamed quarters infused with DFO as demonstrated by lower levels of LDH, NAGase and Hp measured in milk originating from these quarters. Neither catechin nor GEE showed such protective effects. Additionally, our results suggest that DFO does not interfere with PMN functions, such as migration towards mammary gland. No effect of catechin, DFO or GEE on PMN-released proteases has been observed. The presence of a 120-kDa gelatinase in mastitic milk is also reported. Our results also support the view of a local production of Hp by the bovine mammary gland in response to LPS-infusion. Our study also suggests that hydroxyl radicals are specifically involved in mammary tissue damage induced by PMN-generated ROS in cases of acute bovine mastitis. The use of antioxidants and iron-chelator such as DFO in mastitis treatments to prevent mammary tissue damage is worth investigating as they may aid to lower damage induced to secretory cells and thus prevent subsequent milk loss.

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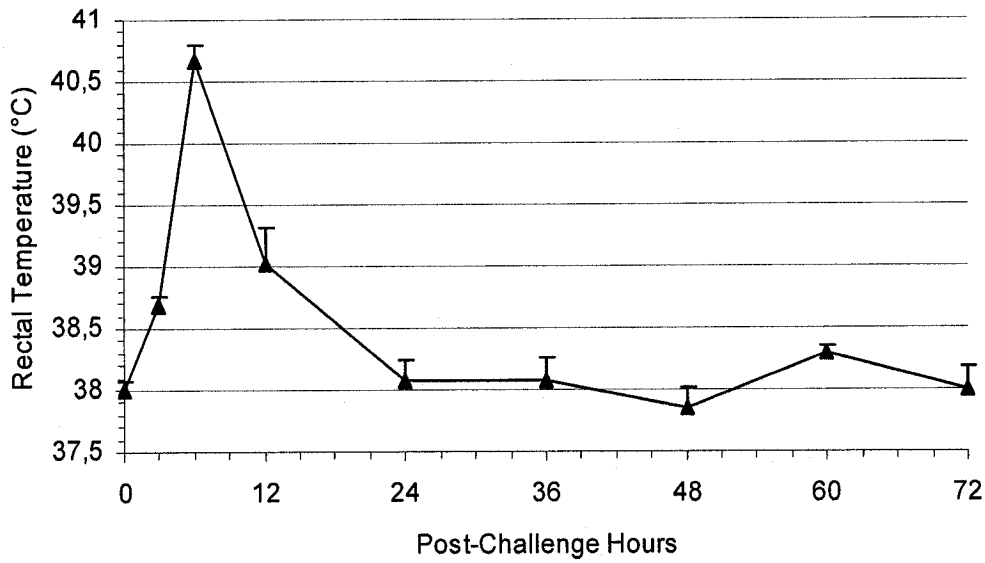

Figure 5.1. Effect of intramammary infusion of LPS on rectal temperature (n = 14). Data are expressed as means ± SEM. The time scale refers to 0 h as the time of intramammary infusions.

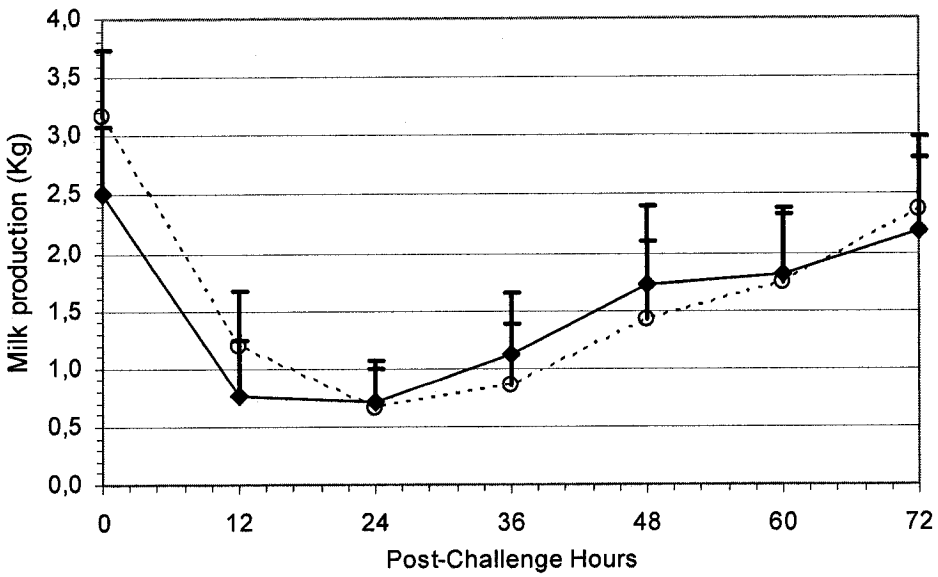

Figure 5.2. Quarter milk production of control quarters (not infused with LPS; n = 28) (♦) compared to that of quarters infused with 500 µg of lipopolysaccharide (--o--) (n = 28). Milk production refers to the amount of milk produced for one milking. Data are expressed as means ± SEM. The time scale refers to 0 h as the time of intramammary infusions.

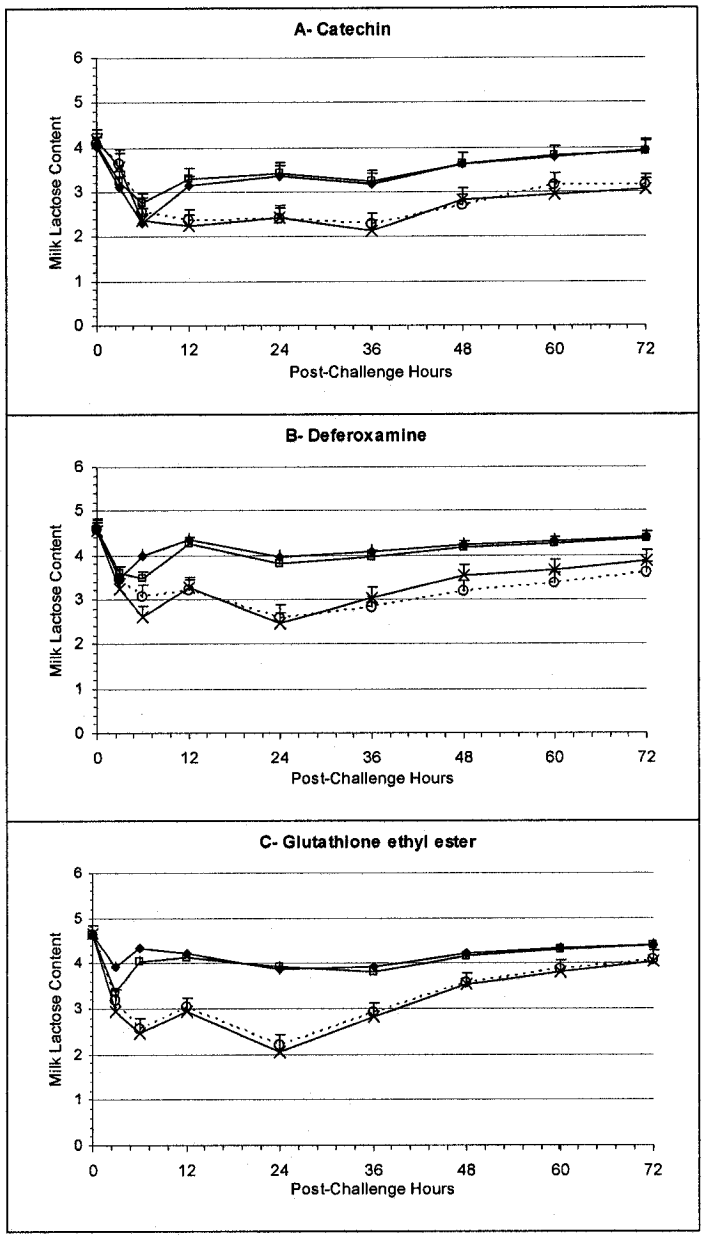

Figure 5.3. Milk lactose content in milk from quarters infused with saline (♦), modulator (□), lipopolysaccharide (LPS) (o), or LPS and modulator (--x--), where modulator corresponds to (A) catechin (n = 5), (B) deferoxamine (n = 4) or (C) glutathione ethyl ester (n = 5). Data are expressed as least square means ± SEM. The time scale refers to 0 h as the time of intramammary infusions.

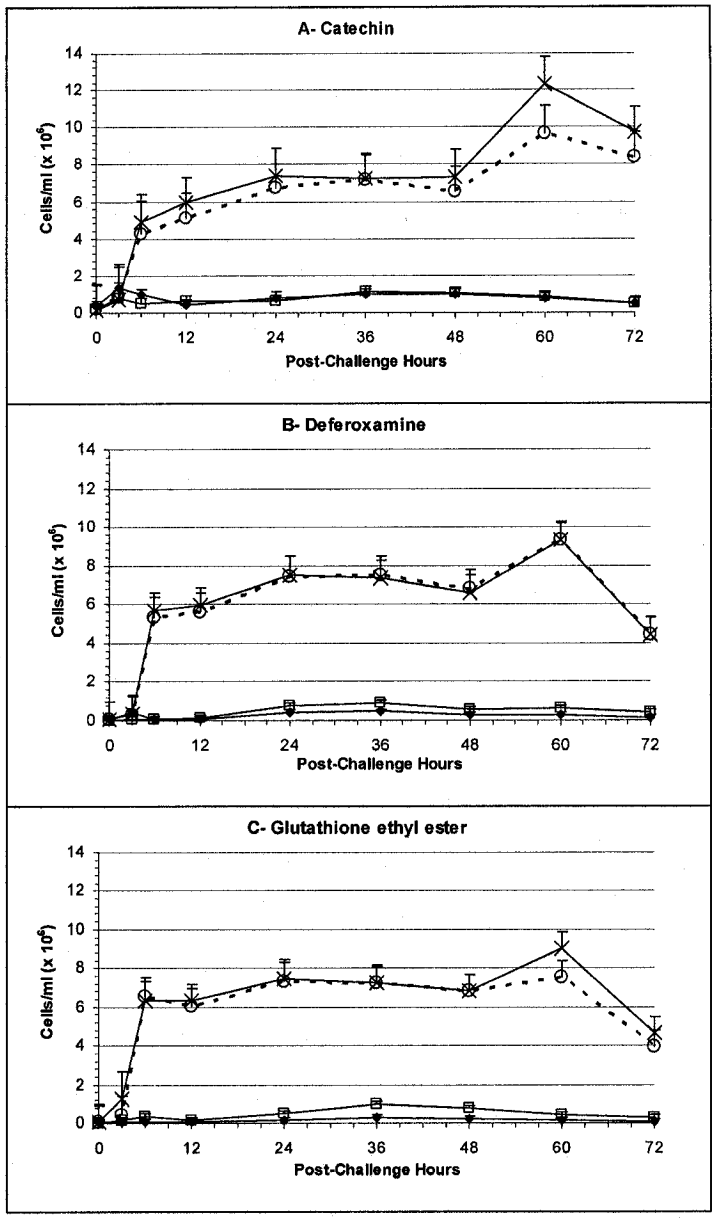

Figure 5.4. Somatic cell counts in milk from quarters infused with saline (♦), modulator (□), lipopolysaccharide (LPS) (o), or LPS and modulator (--x--), where modulator corresponds to (A) catechin (n = 5), (B) deferoxamine (n = 4) or (C) glutathione ethyl ester (n = 5). Data are expressed as least square means ± SEM. The time scale refers to 0 h as the time of intramammary infusions.

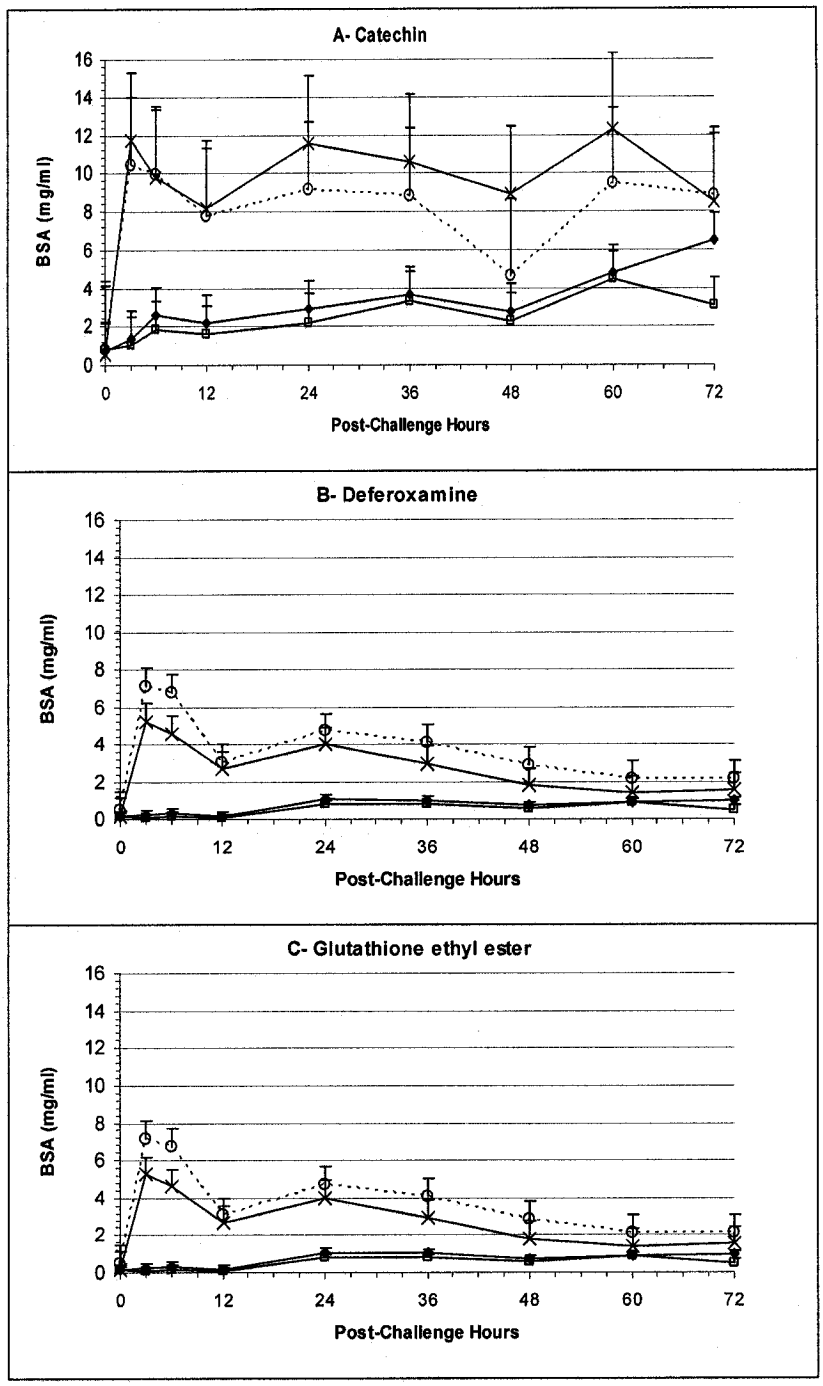

Figure 5.5. Concentration of BSA in milk from quarters infused with saline (♦), modulator (□), lipopolysaccharide (LPS) (o), or LPS and modulator (--x--), where modulator corresponds to (A) catechin (n = 5), (B) deferoxamine (n = 4) or (C) glutathione ethyl ester (n = 5). Data are expressed as least square means ± SEM. The time scale refers to 0 h as the time of intramammary infusions.

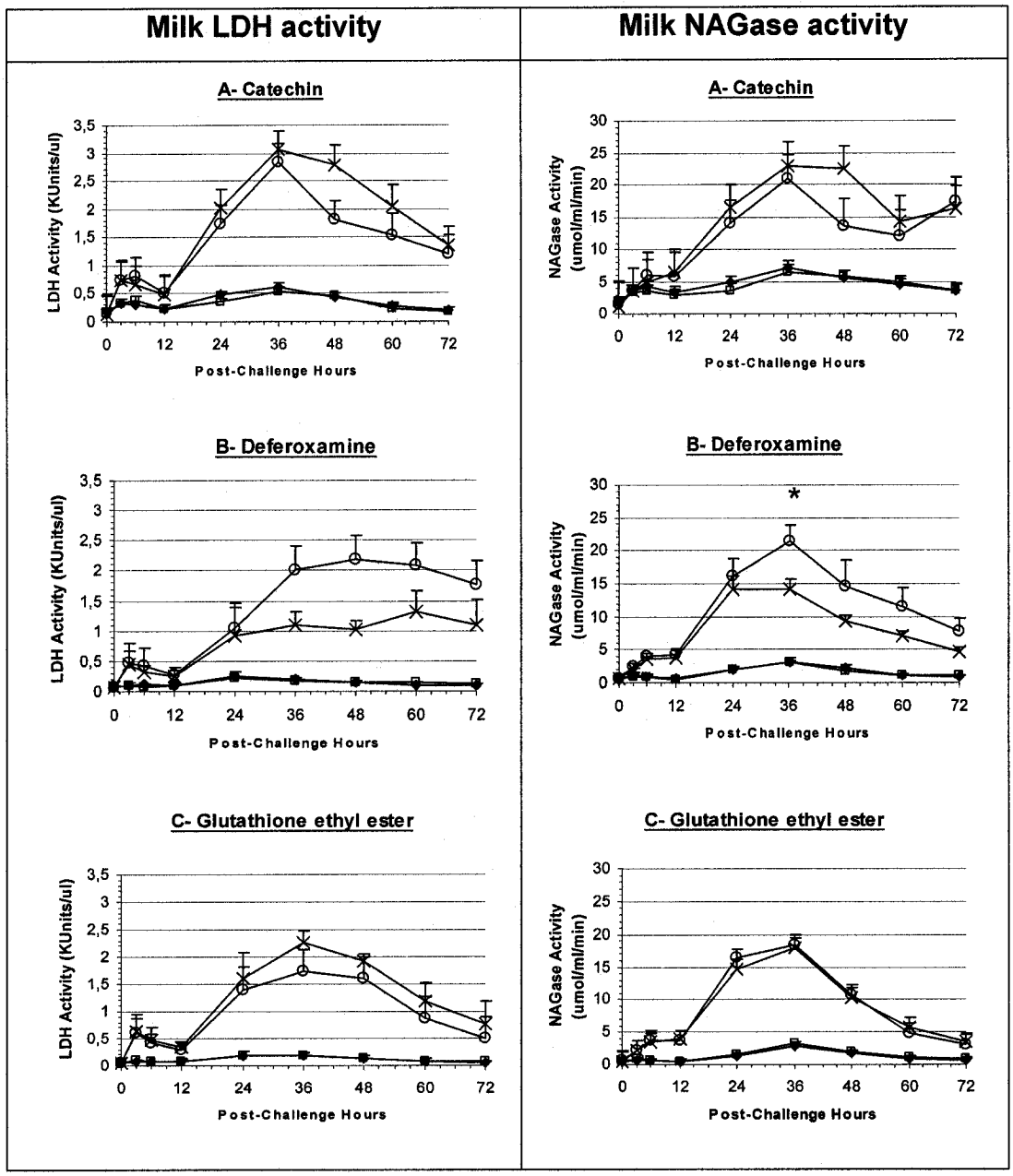

Figure 5.6. Activity of LDH (left) or NAGase (right) in milk from quarters infused with saline (♦), modulator (□), lipopolysaccharide (LPS) (o), or LPS and modulator (x), where modulator corresponds to (A) catechin (n = 5), (B) deferoxamine (n = 4) or (C) glutathione ethyl ester (n = 5). Data are expressed as means ± SEM (*P < 0.05). The time scale refers to 0 h as the time of intramammary infusions.

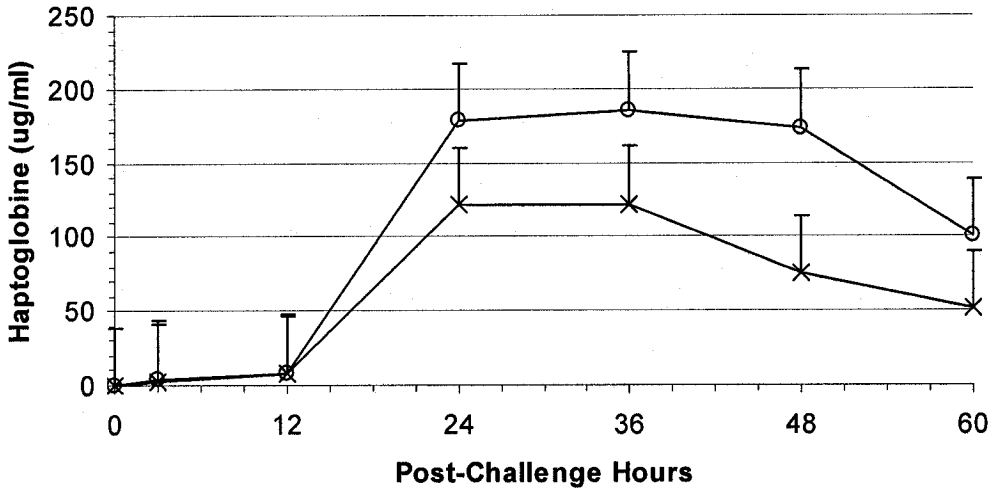

Figure 5.7. Concentration of haptoglobin in milk from quarters infused with lipopolysaccharides (o), or lipopolysaccharides and deferoxamine (x) (n = 4). Data are expressed as least square means ± SEM. The time scale refers to 0 h as the time of intramammary infusions.

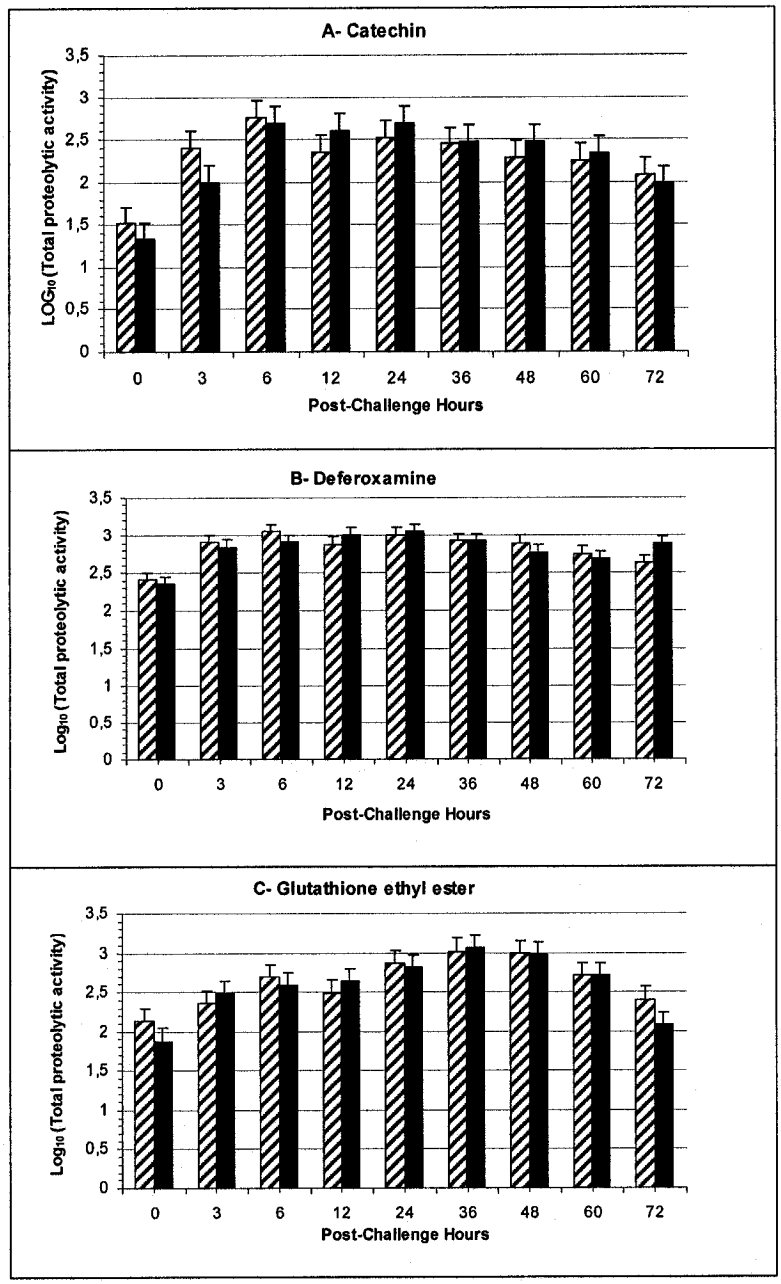

Figure 5.8. Total proteolytic activity in milk from quarters infused with lipopolysaccharide (hatched) or lipopolysaccharide and modulator (black), where modulator corresponds to (A) catechin (n = 5), (B) deferoxamine (n = 4) or (C) glutathione ethyl ester (n = 5). Proteolytic activity was measured from densitometric analysis of zymogram using gelatin as substrate. Values represent the LOG_{10} of least square means ± SEM. Time scale refers to 0 h as the time of intramammary infusions.

SECTION VI

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

Mastitis caused by *Escherichia coli* is common in dairy cows, resulting in decreased milk production. Pathogenesis of *E. coli* mastitis differs from Gram positive mastitis because of the intense host response that is elicited by the infection. Neutrophils are the first line of cellular defence against invading pathogens since they engulf and eliminate infectious agents. However, since this process is made through release of non-specific toxic oxygen radicals and proteases, a prolonged exposure of mammary tissue to PMN cause injury to the secretory epithelium and permanent diminution of milk production (Sordillo et al., 1989).

The first experiment reported here investigated the ability of different antioxidants to prevent these PMN-induced damage towards mammary cells in an *in vitro* system. Results obtained suggest that some antioxidants such as catechin and DFO are able to protect mammary cells against toxic molecules released by activated neutrophils. Additionally, we demonstrated that DFO inhibits bacterial growth without interfering with phagocytic activity of PMN. These observations suggest that some antioxidants may be effective tools to protect mammary tissue against neutrophil-induced oxidative stress during acute bovine mastitis.

The second experiment was undertaken to assess *in vivo* the ability of the antioxidants identified in the first experiment to protect mammary tissue against oxidative stress resulting from mastitis. In our trial, we used a split-quarter design and intramammary injections of LPS were utilized to induce mastitis as a way to confirm that resulting damages would be caused by PMN not bacteria. Interestingly, our results showed that quarters treated with intramammary infusion of DFO tend to exhibit a lower extent of tissue damage as measured by indicator of cellular integrity in milk. Moreover, intramammary injections of DFO did not interfere with

influx of neutrophils and was not damaging to the mammary gland. This protective effect was not observed with catechin and glutathione ethyl ester. These observations suggest that intramammary injection of DFO or another iron chelator may be a helpful tool to protect mammary tissue against neutrophil-induced oxidative stress during acute bovine mastitis and consequently minimize permanent tissue scarring and subsequent drop in milk production. Additionally, there is a possibility to find an iron chelator that would combine these protective effects to a certain antibacterial activity which would help to resolve the infection more efficiently.

Results from the present studies provide important information about mechanisms involved in mammary tissue damage by PMN during acute mastitis. Indeed, our results showed both *in vitro* and *in vivo* that oxidants released by PMN are important players in this mechanism. Additionally, since different antioxidants with different properties have been tested here, these studies can be used as a start point towards a better understanding of how mammary cells are affected by ROS and to find out which ROS are more cytotoxic. Information on the mechanisms activated by the mammary gland to repair tissue damage occurring during acute mastitis is also provided by our study on milk gelatinases appearing following LPS infusion, more precisely by the finding of a 120 kDa gelatinase. Very few studies have reported the use of an intramammary antioxidant to prevent neutrophil-induced damages to the secretory epithelium during mastitis. Although the results presented in this study are interesting and promising, additional studies are needed to understand the exact kinetics of tissue damage in acute mastitis. Furthermore, synergistic interactions involving different antioxidants and/or proteases inhibitors could be a very interesting to assess.

SECTION VII

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REFERENCES

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SECTION VIII

APPENDIX