

Lactoferrin as an Adjunctive Agent in the Treatment of Bacterial Infections Associated with Diabetic Foot Ulcers

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1. Introduction

Lactoferrin is a protein of mammalian origin secreted in the milk of several animals, including human beings, cows, horses, pigs, goats and mice (Masson & Heremans, 1971). From the moment that bovine (Tomita et al., 2009) and human lactoferrin (Weinberg, 2001) were isolated from the milk of their respective species, they began to receive great attention due to their multifunctional properties that are distinctive from lactoferrin from other mammals. In general, lactoferrin is regarded as a modulator of humoral and cellular components involved in inflammatory and immune responses (Actor et al., 2009; Legrand et al., 2004), which has broad implications. For instance, lactoferrin displays antimicrobial activity against a wide range of pathogens, including virus, bacteria, fungi and parasites (Jenssen & Hancock, 2009). It is also able to promote skin integrity by regulating the generation of humoral components of the inflammatory and immune responses, including such cytokines as the tumor necrosis factor (TNF) alpha and interleukin (IL) 1beta, as well as the migration of Langerhans cells (Kimber et al., 2002). Moreover, lactoferrin enhances collagen gel contractile activity of fibroblasts, leading to skin wound healing (Takayama & Takezawa, 2006).

The broad scope of activities of lactoferrin with potential clinical applications, depicted in the Table 1, has led to large scale production of native and recombinant preparations of this glycoprotein for commercial and/or clinical applications (Drago-Serrano, 2007). Among these products are Talactoferrin alfa®, a recombinant human lactoferrin (Agennix, Houston TX, USA), and Bioferrin®, a native lactoferrin from bovine origin (Glanbia, Nutritionals Inc, Monroe, WI, USA). Through the modulation of inflammation, Talactoferrin alfa has proven effective as a wound healing factor in the experimental model of diabetic mice (Engelmayer et al., 2008). Indeed, the promising healing properties of Talactoferrin alfa have been tested in a phase 1/2 clinical study for the treatment of ulcers in patients with diabetic

ORIGIN	ACTIVITY	REFERENCE
Antibacterial effect		
Bovine Lf	↓ <i>Pseudomonas aeruginosa</i> anti-biofilm formation in combination with xylitol (a sugar derivative alcohol) (<i>in vitro</i> test)	Ammons et al., 2011A Ammons et al, 2009
Phagocytic cell function		
Neutrophil human Lf	↑ Bacterial killing by enhancing reactive oxygen species (ROS) generation (<i>in vitro</i> test)	Ambruso & Johnston, 1981
Neutrophil human Lf	↓ oxidative effect of ROS released by degranulated neutrophils (<i>in vitro</i> test)	Britigan et al, 1991
Anti-Inflammatory effect		
Bovine Lf	↓ Tumor necrosis factor (TNF) production induced during bacteremia in mice	Zimecki et al., 2004
Human Lf	↓ Lipopolysaccharide (LPS)-induced expression of endothelial adhesion molecules (E-selectin, ICAM) (<i>in vitro</i> test)	Baveye et al., 2000A
Pro-inflammatory effect		
bLf/hLf	↑ human neutrophil derived interleukin (IL)-8 production (<i>in vitro</i> test)	Shinoda et al., 1996
recombinant human Lf (rHLf)	↑ human dendritic cell derived IL-8 production (<i>in vitro</i> test)	Spadaro et al., 2008
Granulopoiesis		
Bovine Lf	↑ output of neutrophil precursors in human volunteers	Zimecki et al., 1999B
Mouse Lf	↑ colony stimulating factor (CSF) in mice	Sawatzki & Rich, 1989
Human Lf	↓ output of granulocyte macrophage progenitor cells from spleen and femur of mice	Gentile & Broxmeyer, 1983
Effects on skin		
Human Lf	anti-allergen effect by decreasing Langerhans cell migration induced by allergens in humans	Griffiths et al., 2001
rHLf	↑ re-epithelialization (<i>in vitro</i> test)	Tang et al., 2010A Tang et al., 2010B
Bovine Lf	↑ gel contractile activity in human fibroblasts (<i>in vitro</i> test)	Takayama et al., 2003
Bovine Lf	↑ nerve growth factor synthesis/secretion in mouse fibroblasts (<i>in vitro</i> test)	Shinoda et al., 1994
Bovine Lf	↑ hyaluronan synthesis in human dermal fibroblast (<i>in vitro</i> test)	Saito et al., 2011
Wound healing		
(rHLf)	Healing enhancement of foot ulcers in diabetic mouse model	Engelmayer et al., 2008
(rHLf)	Healing enhancement of diabetic foot ulcers in human patients	Lyons et al., 2007

Table 1. Overview of multifunctional properties of lactoferrin

foot (Lyons et al., 2007). Bioferrin, on the other hand, has been found to have antibacterial activity, for which reason it has been used in the control of bacterial infections associated with diabetic foot ulcers (Ammons et al., 2011A; Ammons et al., 2009). Such infections represent a serious challenge, since they can lead to devastating consequences and possibly to limb amputations (Powlson & Coll, 2010). Hence, this glycoprotein is endowed with natural antibacterial and wound healing properties that can be beneficial in the treatment of either infected or uninfected diabetic foot ulcers. However, given the complex convergence of pathologies which encompass diabetic foot syndrome, lactoferrin should not be considered as a *panacea* able by itself to cure this disorder.

The aim of this chapter is to describe the applications of lactoferrin as an antibacterial and wound healing agent. We explore its use as an adjunctive agent to enhance the antibacterial activity of antibiotics and/or improve the efficacy of other adjunctive methods of antibiotic treatment, the latter of which include growth factors, dressings and other topical applications, debridement, and hyperbaric oxygen therapy (O'Meara et al., 2000). In parallel we give an overview of the biological and modulating properties of lactoferrin in relation to the inflammatory and immune responses, as these properties underlie the reestablishment of homeostasis at the dermal level and thus contribute to ulcer skin healing. Finally, we discuss the precautions that should be taken into account for the clinical applications of this glycoprotein.

2. Lactoferrin: An overview

2.1 Structural features

Lactoferrin is a member of transferrins, a family of proteins with iron chelating properties. This monomeric glycoprotein has a molecular weight of 80 kDa, and is highly cationic with an isoelectric point of 9.6 (Baker & Baker, 2009). Its tertiary structure consists of two main N and C lobes, within which are found the N1/N2 and C1/C2 domains, respectively. The N and C lobes are linked at the N1 and C1 domains by a three turn alpha chain (Baker & Baker, 2009). Human milk lactoferrin contains two sites of glycosylation (Anderson et al., 1987) with N-acetylneuraminic acid, N-acetylglucosamine, galactose and mannose (Spik et al., 1982). In spite of the fact that bovine milk lactoferrin has five glycosylation sites, only four of them are actually glycosylated (Moore et al., 1997) with N-acetylneuraminic acid, galactose, manose, fucose, N-acetylglucosamine and N-acetylgalactosamine (Coddeville et al., 1992). Between the N1/N2 and C1/C2 regions, a cleft is formed by R amino acid groups of an aspartic, a histidine and two tyrosine residues. At this cleft, iron in ferric form (Fe^{3+}) along with the bicarbonate (HCO_3^{1-}) ion can be bound reversibly to lactoferrin (Anderson et al., 1987; Moore et al., 1997). The iron loaded state of lactoferrin is called hololactoferrin, while its iron free form is known as apolactoferrin. On the other hand, the N1 domain, devoid of any iron chelating activity, has a region known as lactoferricin, which is characterized by its strong cationic charge. Lactoferricin can be obtained as a free peptide by the enzymatic proteolysis of parental lactoferrin with pepsine. The iron independent properties of lactoferrin, such as some forms of antibacterial activity as well as various modulating properties of the immune and inflammatory responses, arise from this lactoferricin domain (Gifford et al., 2005).

2.2 Biological properties

2.2.1 Distribution

Lactoferrin is secreted in the milk of several mammals and is regarded as an essential component of innate mechanisms of host defense. Evidence of this role is the strategic location of lactoferrin at mucosal surfaces, which are frequently colonized and/or invaded by pathogenic micro-organisms (Russell et al., 2005). Lactoferrin is also stored in secondary (specific) granules of neutrophils, which upon degranulation normally release this glycoprotein in serum (Levay & Viljoen, 1995). The extent of neutrophil degranulation and the level of lactoferrin in serum increase during an inflammatory response from infectious origin (Legrand et al., 2004).

2.2.2 Effects on cellular components of the adaptive immune response

As aforementioned, lactoferrin is a modulator of humoral and cellular components involved in inflammatory and immune responses. Among its effects on the cellular components of the adaptive host defense, lactoferrin positive or negatively modifies the activity of B lymphocytes, T cells, macrophages, dendritic cells, neutrophils, and Langerhans cells involved in specific immune responses (Actor et al., 2009). For instance, it is a factor in B lymphocyte maturation, a process that defines the phenotype and function of these cells (Zimecki et al., 1995). On the other hand, T cell proliferation (Bi et al., 1997) and maturation (Zimecki et al., 1991) are enhanced by lactoferrin, evidenced by an increased expression of CD4 antigen in the presence of this glycoprotein (Bi et al., 1997; Zimecki et al., 1991; Dhennin-Duthille et al., 2000). Other studies show that lactoferrin abrogates the inhibitory effect of the iron saturated transferrin on T cell proliferation (Djeha & Brock, 1992), and inhibits the proliferative response and cytokine production of Th1 but not Th2, which logically affects the activity of Th1/Th2 subpopulations (Zimecki et al., 1996).

Lactoferrin is also able to modulate macrophages, as antigen presenting cells, to induce a Th1 response. That is, in the presence of this glycoprotein, there is an increase of IL 12, which in turn is required to combat intracellular pathogens (Wilk et al., 2007). It has been reported that lactoferrin expressed on the surface of neutrophils of healthy patients interacts with TCD4+ cells to up-regulate the production of IL 10 and down-regulate the production of interferon (IFN) gamma. In contrast, in patients with systemic lupus erythematosus, lactoferrin expressed on the surface of neutrophils negatively modulated the production of IL 10 and IFN gamma by TCD4+ cells. These results suggest that endogenous lactoferrin is able to regulate the expression of interleukins by interacting with TCD4+ lymphocytes (Li et al., 2006). Moreover, Lactoferrin acts as a maturation factor of antigen presenting cells, such as dendritic cells (De la Rosa et al., 2008; Spadaro et al., 2008) and macrophages (Sorimachi et al., 1997), evidenced by an up-regulated expression of CD80/86, the increased priming of T cells, and the enhanced secretion of pro-inflammatory mediators such TNF and IL 8.

The modulatory effects of lactoferrin on cellular components (neutrophils, keratinocytes and Langerhans cells) as well as humoral components (cytokines) of the inflammatory response involved in the innate mechanisms of bacterial control or dermal homeostasis are addressed in section 4 of this chapter.

3. Anti-bacterial activity of lactoferrin

3.1 Importance of iron in infections

Human beings as well as microorganisms that infect them require iron for living, since this metal is necessary for all redox reactions. This transition element is part of the heme group in hemoglobin and is a cofactor of enzymes such as ribonucleotide-reductase, indispensable for DNA synthesis (Andrews et al., 2003).

In the body, iron is usually bound to proteins. It is important for the body to maintain a low free-iron concentration, as otherwise free iron would lead to the overproduction of reactive oxygen species by the Fenton reaction, and these molecules are toxic for proteins, DNA and lipids. Moreover, an excessive free-iron concentration would easily be employed by invading pathogens for their survival and proliferation. The extracellular iron concentration in humans is approximately 10^{-18} M, a level too low to sustain the growth of microbes trying to colonize and invade a host (Bullen et al., 2005; Nairz et al., 2010; Weinberg, 2009).

Mammals have evolved a general strategy of decreasing the free-iron concentration in order to protect themselves against invading microbes. Complex iron-withholding systems are based on iron-binding proteins that chelate this ion, such as transferrin that transports and delivers iron to all cells, ferritin that stores iron inside the cells in order to avoid its toxicity and to maintain cell iron homeostasis (Chiancone et al., 2004), and lactoferrin that, as a cationic glycoprotein of the innate immune system, sequesters iron in mucosae and infection sites, precisely to avoid the accessibility of iron to all intruders (Bullen et al., 1972, 2005; Arnold et al., 1977; Jenssen & Hancock, 2009). Indeed, this was one of the first properties of this glycoprotein to be discovered.

3.2 Bacterial species that infect the diabetic foot

One major problem caused by pathogenic bacteria to a human host is the constant infection suffered by diabetic patients in their inferior extremities. Infected foot ulcers are complex interactions of microorganisms that often progress to deeper spaces and tissues. These infections can become chronic and incurable, and may even degenerate into septic gangrene, which could require foot amputation. Although chronic infections are frequently polymicrobial, it has been reported that acute infections in patients not previously treated with antibiotics are mainly caused by a single pathogen, usually a Gram-positive coccus (Edmonds, 2006; Armstrong & Lipsky, 2004; Powlson & Coll, 2010). Accordingly, early surface infections of skin ulcers on the feet of untreated diabetic patients are associated with aerobic Gram positive cocci, including *Staphylococcus aureus* and beta haemolytic streptococci group A and B. On the other hand, for diabetic patients with chronic lesions, infections are characterized by a polymicrobial mixture of aerobic Gram positive and Gram negative bacteria (*Escherichia coli*, *Proteus spp.*, *Klebsiella spp.*), anaerobic bacteria (*Bacteroides spp.*, *Clostridium spp.*, *Peptococcus spp.* and *Peptostreptococcus spp.*) and *Pseudomonas aeruginosa* (Citron et al., 2007; Dowd et al., 2008).

Staphylococcus aureus methicillin-resistance strains (MRSA) and *Pseudomonas aeruginosa* are the most prominent components of biofilms, and bacterial biofilm is a major contributor to non-healing chronic lesions of diabetic patients. For instance, *Pseudomonas aeruginosa* is an opportunistic pathogen in chronic lesions that is refractory to the killing activity of

phagocytic cells (e.g., neutrophils), and it is an underlying factor promoting bacterial resistance to antibiotics (Guo & DiPietro, 2010).

Some anaerobes are also responsible for infection in foot ulcers, and even skin commensal bacteria may cause damage to tissue (Edmonds, 2006). For mild and severe infections, some antibacterials have been shown to be effective in clinical trials, but have numerous side effects for patients. Both specific antibiotics as well as those of a wide spectrum have been reported to produce distinct side effects, such as nephro- and hepato-toxicity, blood dysplasias, diarrhea, and disorders in the commensal flora equilibrium that lead to other syndromes by opportunistic microbes (López-Novoa et al., 2011). A very serious problem with antibiotic treatments is the emergence of multiresistant strains, generally in long-standing infections, a result of the fact that the rate of development of new antimicrobial drugs is slower than the evolution of resistance. In the case of anaerobes, metronidazole is the drug of choice, used together with antibiotics. However, metronidazole is toxic, causing nausea, vomiting, abdominal pain and other effects. In addition, it might be carcinogenic to patients when used at high doses and/or as a long-term treatment, evidenced by DNA breakages and chromosome aberrations caused by this drug in cultured cells, and by its carcinogenic effects in animal tests (Roe, 1983; Dobias et al, 1994; El-Nahas & El-Ashmawy, 2004; Kapoor et al., 1999; Mudry et al., 1994).

3.3 Lactoferrin as an option in therapy against microbial infections

Due to the aforementioned problems with antibiotics and other drugs, alternative therapies against microbial pathogenic populations have been explored in the last few years. One such alternative is lactoferrin, mainly prescribed when infections are chronic and when bacteria are found to be resistant to all treatments. Lactoferrin is abundant in colostrum and milk as a defense mechanism in newborns. Its iron-free form (apolactoferrin) is secreted by the acinar glands to mucosae or by neutrophils in infection sites. This form can be microbistatic, depriving pathogens of iron and thus leaving them in a latent state, or microbicidal, leading to the death of pathogens (Jenssen & Hancock, 2009; Ling & Schryvers, 2006; Yamauchi et al., 2006).

3.4 Lactoferrin is able to synergize with other proteins and antimicrobials

3.4.1 Natural synergy of lactoferrin with innate immune-system proteins to combat pathogenic microorganisms

It has been demonstrated that lactoferrin synergizes with other proteins of the innate immune system, such as lysozyme and IgA, in order to eliminate microbial infections. Indeed, these three proteins are secreted together to mucosae (Leitch & Willcox, 1998; Vaerman, 1984). Lactoferrin has large cationic patches on its surface, facilitating direct interaction with anionic lipid A from the lipopolysaccharide (LPS) in Gram-negative bacteria. This interaction leads to a greater permeability of the outer membrane of such bacteria (Jenssen & Hancock, 2009). In the case of *Staphylococcus epidermidis*, a Gram-positive bacterium, it has been proposed that lactoferrin interacts with lipoteichoic acid on the bacterial surface, leading to a decrease of the negative charge on the membrane, which in turn allows lysozyme to reach the cell wall-associated peptidoglycan where this enzyme acts (Leitch & Willcox, 1999A).

3.4.2 Lactoferrin also synergizes with drugs and antibiotics, enhancing their microbicidal action

Lactoferrin has the great advantage of synergizing with antiviral and antiparasitic drugs as well as with antibiotics, intensifying the antimicrobial activity. In some cases an antibiotic dose can be decreased if administered with lactoferrin, which diminishes the side effects to patients (Jenssen & Hancock, 2009; Bullen et al., 1972; Arnold et al., 1977; Farnaud & Evans, 2003; Leon-Sicairos *et al.*, 2006; Sanchez & Watts, 1999; Diarra et al., 2002; Viani et al., 1999). Another adjunctive effect of lactoferrin is that biofilms of *Staphylococcus epidermidis* become more susceptible to lysozyme and vancomycin treatment when used in combination with this glycoprotein (Leitch & Willcox, 1999B). Interestingly, it was found that lactoferrin synergized with bacteriophages (bacterial viruses) in a patient suffering a prolonged antibiotic-resistant post-influenza otitis media infection due to *Staphylococcus epidermidis*, and the infection was resolved (Weber-Dabrowsca et al., 2006).

3.5 Lactoferrin and lactoferricins are able to prevent the formation of biofilms

Biofilms are a matrix-encased colonial organization of cells that more readily become resistant to drugs, antibiotics and antibodies than their free-living, planktonic counterparts. This matrix is composed of exopolysaccharide, and its formation is under the control of quorum sensing, a bacterial mechanism for assessing population density and altering gene expression in order to carry out processes that require the cooperation of a large number of cells (Miller & Bassler, 2001). Lactoferrin and its derivative peptides have been used against bacteria in biofilms. Furthermore, the synthetic peptides derived from the N-terminal of bovine lactoferrin, lactoferricin and lactoferrampin, and a fusion peptide of both, lactoferrin chimera, have been assayed against a wide range of pathogens, showing a very high probability of success against multiresistant bacteria as well as those accustomed to organizing and forming biofilms (Bolscher et al., 2009; Xu et al., 2010; Flores-Villaseñor et al., 2010). Furthermore, synergistic activity of bovine lactoferricin (11 amino acids) with antibiotics was demonstrated against multiresistant clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro* and in a model of corneal infection in mice. Lactoferricin also had an anti-inflammatory effect in cornea, suggesting that it leads to a decrease of cytokines and mediators involved in inflammation (Oo et al., 2010). Hence, lactoferrin as well as its derived peptides could be potential adjuncts to conventional antibiotics in combating these pathogens, which also infect diabetic foot ulcers.

Singh et al. (2002) evaluated the effect of lactoferrin on biofilm development by *Pseudomonas aeruginosa*. In this study, the use of a green fluorescent protein, GFP, as a marker of the bacterial cells in continuous-culture allowed for the tracking of biofilm development over time. Interestingly, they demonstrated that lactoferrin disrupted biofilm pattern development, even at concentrations lower than those needed to kill bacteria or prevent their regular growth. By chelating iron, lactoferrin stimulated bacterial twitching, which is a specialized surface motility in which bacteria wander across the surface instead of forming cell clusters and biofilm. As was hypothesized, this lactoferrin effect was iron-dependent and only the iron-free form, apolactoferrin, showed this antimicrobial action. However, bacteria in an established biofilm were resistant to lactoferrin.

Neutrophil derived lactoferrin also was able to prevent bacteria biofilm development of *Pseudomonas aeruginosa* (Leid et al., 2009). Other assays show that lactoferrin in combination

with xylitol (a sugar derived penta-hydroxy alcohol) decreases the viability of established *Pseudomonas aeruginosa*, a consequence of both the disruption of the biofilm structure and the permeabilization of the bacterial membrane (Ammons et al., 2009).

3.5.1 Lactoferrin and lactoferricins in the battle against infections associated with diabetic foot

Reproducing diabetic foot in animal models with the aim of studying ulcer infections has not proved easy. Nevertheless, an *in vitro* model of chronic lesion biofilms used to evaluate antimicrobial susceptibilities was recently reported. This model imitates the lesion environment and allows researchers to study various aspects of the physiology of biofilms, as well as the effect of drugs and antibiotics on the same. In this model, pretreatment with lactoferrin was employed to test for the inhibition of biofilm development by clinical isolates of diverse bacteria species. Results showed that this pretreatment did not affect biofilm formation or the number of bacteria, leading the authors to conclude that a continuous exposure of biofilm to lactoferrin is necessary in order to achieve this effect, as has been suggested in reports by other researchers (Hill et al., 2010).

In relation to foot ulcers of diabetic patients, there are scant reports about the use of lactoferrin. The anti-biofilm efficacy of adjunctive methods, such as silver wound dressings, for the therapy of infections associated with chronic ulcers is significantly increased when combined with lactoferrin-xylitol. The mechanisms underlying this cooperative antimicrobial effect are the iron chelating property of lactoferrin, which destabilizes the bacterial membrane, and the inhibitory effect of xylitol on the ability of the bacteria to produce siderophores under conditions of iron restriction (Ammons et al., 2011A, 2011B). As is well-known, iron deprivation promotes siderophore secretion for acquisition of iron essential for bacterial growth (Skaar, 2010). These studies point to the great potential of lactoferrin and lactoferricins as an adjunct in the treatment of diabetic foot infections. Further studies are needed to explore this potential.

4. Modulatory activities of lactoferrin on inflammation

The pleiotropic property of lactoferrin spans a wide array of regulatory activities on cellular and humoral components of the inflammatory response. The capacity of lactoferrin to modulate the inflammatory response has been explored *in vitro* and *in vivo*. The animal models include the use of exogenous lactoferrin from bovine and human origin (Legrand et al., 2004), as well as the observation of untreated animals in order to explore the role of endogenous lactoferrin (Legrand & Mazurier, 2010). Here we focus on the regulatory effects of lactoferrin on mielopoiesis, the production of cytokines and the generation of reactive oxygen species (ROS), and in particular on the inflammatory response in skin allergy and skin healing in non-infected lesions.

4.1 Modulatory effects on ROS generation

4.1.1 Up-regulation

Lactoferrin is a key component of immune homeostasis, able to positively or negatively regulate the function of cellular components of innate mechanisms of defense involved in

inflammation, such as mononuclear cells (monocytes and macrophages) and neutrophils. According to *in vitro* assays with neutrophils treated with bovine lactoferrin, the ability of this glycoprotein to enhance phagocytosis may be a result of its direct contact along with an opsonin-like action on phagocytic cells (Miyachi et al., 1998). However, the role of lactoferrin in phagocytosis has been linked with its ability to positively regulate the generation of ROS with bactericidal activity. Evidence of this up-regulating action is the fact that lactoferrin in secondary granules of neutrophils enhances the intracellular killing of *Pseudomonas aeruginosa* by promoting the generation of ROS during phagocytosis (Bullen & Armstrong, 1979). It has been reported that exogenous lactoferrin enhances the activity of ROS production in neutrophils (Ambruso & Johnston, 1981; Gahr et al., 1991) and macrophages (Lima & Kierszenbaum, 1987), and that the presence of iron is necessary for this effect to take place (Gahr et al., 1991; Lima & Kierszenbaum, 1987). Hololactoferrin (the iron-loaded form) seems to deliver the iron that can act as an enzymatic catalyst of Haber-Weiss reactions, which in turn generate various types of ROS, including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and the hydroxyl radical ($OH\cdot$) (Actor et al., 2009). Reactive oxygen species are substrates for the enzymatic generation of antibacterial molecules. For instance, H_2O_2 is a precursor of hypochlorous (HClO). The latter is a strong bactericidal molecule generated enzymatically by myeloperoxidase (MPO) (Hampton et al., 1998), a prominent component of azurophil granules in neutrophils (Leffell & Spitznagel, 1974).

4.1.2 Down-regulation

Lactoferrin can also have a down-regulatory effect on ROS production, such as is the case with phagocytes. The mechanism of this effect is the reduction of oxidative stress and the control of an excessive inflammatory response (Actor et al., 2009). During the oxidative burst, intracellular generation of ROS is essential for the control of bacterial growth (Hampton et al., 1998). However, under conditions of oxidative stress the excessive release of ROS to the extracellular milieu has devastating effects on the structure of macromolecules and cell functions (Actor et al., 2009). Unlike azurophil granules that store MPO, usually associated with phagosomes during bacteria phagocytosis, secondary (specific) granules that contain lactoferrin usually become degranulated (Leffell & Spitznagel, 1974). Evidence from *in vitro* assays shows that lactoferrin released during neutrophil degranulation decreases the overproduction of ROS (Britigan et al., 1989). Moreover, the uptake of exogenous lactoferrin by mononuclear phagocytes that do not contain endogenous lactoferrin inhibits their ability to form the hydroxyl radical and protects them from membrane auto-peroxidation (Britigan et al., 1991). This effect may be linked to the property of lactoferrin to inhibit lipid peroxidation (Gutteridge et al., 1981). In the mouse model of pollen induced allergic airway, lactoferrin has been effective in inhibiting the generation of the superoxide anion. Pollen grains contain reduced nicotinamide adenine dinucleotide phosphate (NADH) oxidases. Their product, O_2^- , is a precursor of H_2O_2 and $OH\cdot$, which increase pollen induced airway inflammation (Kruzel et al., 2006).

Apart from its role in inhibiting the overproduction of ROS by phagocytes, lactoferrin can also down-regulate ROS production by neutrophils that are stimulated by LPS. Lipopolysaccharide interacts with the soluble receptor lipopolysaccharide binding protein (LPB), which in turn enables the binding of LPS to CD14, a membrane receptor of phagocytes (Van Amersfoort et al., 2003). After binding to the surface membrane via the

LBP/CD14 receptor complex, LPS released by dead or growing bacteria causes neutrophil activation, as evidenced by the oxidative burst accompanied with ROS generation. Moreover, when LPS is massively released, such as in septic shock, it binds to L-selectin, an integral glycoprotein of membrane leukocytes, and this complex also results in the production of ROS (Malhotra et al., 1996). Both of these mechanisms of ROS production are impeded by the capacity of lactoferrin to bind to LPS (Wang et al., 1995). Additionally, lactoferrin blocks the interaction of LPS with the L-selectin of neutrophils, which also avoids the production of ROS (Baveye et al., 2000B). It has been shown that either endogenous lactoferrin secreted by neutrophils (Wang et al., 1995) or exogenous lactoferrin (Cohen et al., 1992) can inhibit the ability of LPS to prime neutrophils for enhanced superoxide production.

Interestingly, the IL 10 receptor (IL-10R), a component of secondary granules, also has an anti-inflammatory effect, like lactoferrin, in the presence of LPS. This receptor is exported from secondary granules to the membrane of neutrophils primed with LPS. When interacting with its respective ligand, these IL 10 receptors reduce the production of ROS stimulated by LPS (Elbim et al., 2001).

4.2 Modulatory effects of lactoferrin on pro-inflammatory cytokines

Lactoferrin is a modulatory agent whose positive or negative effects on cytokine production and phagocytes involved in the inflammatory response is highly dependent on the type of inducer recognized by the immune system and also on environmental conditions (Actor et al., 2009). The modulatory activity of lactoferrin in inflammation has been addressed in animal models, such as that of colitis induced by dextran sulfate (Håversen et al., 2003A; Togawa et al., 2002) and that of hind-foot inflammation by carrageenan (Zimecki et al., 1998). This modulatory activity has also been assessed under *in vitro* and *in vivo* conditions by using LPS as an inducer (Puddu et al., 2010). The proven substantive regulatory effects of lactoferrin on inflammation induced by LPS are not fully understood. However, it is known that the intrinsic binding properties of lactoferrin to LPS and/or LPS receptors results in an interaction with toll like receptor (TLR)-4 and possibly with other unknown receptors, leading to the increase (Na et al., 2004) or decrease (Håversen et al., 2002) of cytokine production.

4.2.1 Down-regulation

The effects of lactoferrin on inflammation have been correlated with a decrease in three pro-inflammatory mediators: TNF (Håversen et al., 2003A; Togawa et al., 2002; Zimecki et al., 1998), IL 6 (Togawa et al., 2002; Zimecki et al., 1998) and IL 1 (Togawa et al., 2002). These effects have also been associated, in some cases, with an increase in two anti-inflammatory interleukins: IL 4 and IL 10 (Togawa et al., 2002). In animal models of infection with *Shigella flexneri* (Gómez et al., 2002), *Salmonella typhimurium* (Mosquito et al., 2010) and *Lysteria monocytogenes* (Lee et al., 2005), lactoferrin from bovine or human origin has been effective in reducing inflammatory necrosis in liver (Lee et al., 2005) and intestine (Mosquito et al., 2010), as well as in intestinal edema (Gomez et al., 2002). Owing to its anti-inflammatory effect, as evidenced by the reduced levels of TNF (Lee et al., 2005; Komine, et al., 2006; Zimecki et al., 2004) and IL 6 (Håversen et al., 2003B), lactoferrin contributes to a decrease in

tissue damage caused by the inflammation induced by bacterial pathogens. Other assays also have shown that lactoferrin released by phagocytes acts as a negative feedback factor by decreasing the TNF released from these cells, which in turn prevents their excessive recruitment and activation of phagocytes. In this way, lactoferrin modulates the recruitment to sites of inflammation (Crouch et al., 1992).

There is also evidence that lactoferrin increases the expression of LFA-1, an adhesion molecule of blood mononuclear cells (Zimecki et al., 1999A). This is another molecule involved in the migration of phagocytes to inflammatory sites. On the other hand, lactoferrin inhibits LPS-induced expression of two adhesion molecules, E-selectin and ICAM-1, by endothelial cells (Baveye et al., 2000A). Furthermore, it decreases the IL 8 production induced by LPS and competes with this chemokine for its binding to proteoglycans of endothelial cells (Elass et al., 2002).

4.2.2 Up-regulation

In addition to its role as a down-regulator of some pro-inflammatory cytokines, lactoferrin has also been found to stimulate the production of other pro-inflammatory cytokines associated with resistance to bacteria. For instance, the activation by lactoferrin of mononuclear cells and polymorphonuclear leukocytes has been correlated with its ability to stimulate the release of IL 8 (Spadaro et al., 2008; Sorimachi et al., 1997; Shinoda et al., 1996). Up-regulatory properties of lactoferrin on TNF levels increase the efficacy of the BCG vaccine by inducing the increase of pro-inflammatory cytokines like IL 6 and IFN gamma, involved in the resistance against *Mycobacterium tuberculosis* (Hwang et al., 2007). In transgenic mice carrying a functional human *Lf* gene and infected with *Staphylococcus aureus*, causing septic arthritis, the increase of a pro-inflammatory Th1 response due to greater TNF and IFN gamma levels (and a decreased Th2 response of anti-inflammatory mediators IL 5 and IL 10) was associated with a reduced incidence of arthritis and mortality (Guillén et al., 2002). Thus, the pro-inflammatory effects of lactoferrin contribute to resistance to infections caused by the aforementioned pathogens.

4.3 Modulatory effects on mielopoiesis

Another protective effect of bovine lactoferrin in the protection against *Escherichia coli* bacteremia in mice has been associated with an increased turnover of neutrophils (Zimecki et al., 2004). In this regard, the demand for neutrophils greatly increases in the inflammatory response caused by infections. Thus, the effect of lactoferrin on bacterial resistance may be linked to its regulatory properties on cytokines involved in mielopoiesis, including IL 1, IL 6, IL 8 and TNF, as well as on mielopoietic growth factors, such as granulocyte macrophage colony stimulating factor (GM-CSF), G-CSF and M-CSF (Artym & Zimecki, 2007). These soluble factors favor mielopoiesis, i.e., the process of the generation of granulocyte and monocyte leukocytes from a bone marrow pluripotent cell.

The modulatory activity on mielopoiesis has been described in relation to lactoferrin released by human neutrophils and exogenous lactoferrin tested with culture cells or administered to animals or human volunteers (Artym & Zimecki, 2007). The modulatory role of lactoferrin upon progenitor cells of the granulocyte/macrophage lineage has been controversial (Artym & Zimecki, 2007). In healthy humans orally treated with bovine

lactoferrin, the up-regulatory action of lactoferrin on granulopoiesis was linked to the increased output of neutrophil precursors along with an attenuated release of TNF and IL-6 (Zimecki et al., 1999B). The effect of lactoferrin in promoting granulopoiesis apparently results from its ability to stimulate the increase in CSF production, as was shown under *in vitro* and *in vivo* conditions (Sawatzki & Rich, 1989). With assays of mouse bone marrow and peritoneal cells, both cultured with or without indomethacin (a non-steroidal anti-inflammatory drug used as an inhibitor of prostaglandin synthesis), mouse or human lactoferrin promoted an increase in CSF. On the other hand, injection of mice with endotoxin free mouse lactoferrin stimulated an early increase in CSF production, and a more delayed increase in bone marrow granulocyte macrophage progenitor cells (GM-CFC) (Sawatzki & Rich, 1989).

Lactoferrin also can have a negative effect on mielopoiesis, as evidenced by the iron dependent capacity of human lactoferrin to suppress the number of granulocyte macrophage progenitor cells from femur and spleen of mice (Gentile & Broxmeyer, 1983). This iron dependent suppressor effect of human lactoferrin, when tested in human bone marrow derived cells and blood monocytes, was linked to the decrease of GM-CSF (Broxmeyer et al., 1980). However, the role of lactoferrin as a modulating agent on granulopoiesis was not at all confirmed by some other studies (DeForge et al., 1985; Winton et al., 1981).

These divergent results in regard to the modulatory effects of lactoferrin on leukocyte generation have probably arisen from variations in experimental design. After all, the inflammatory response is a very complex spatio-temporal process of multiple steps. The effect of lactoferrin on mielopoiesis must therefore be dependent on the stage of the inflammatory response. Lactoferrin as an immunoregulatory protein should presumably act first by enhancing the inflammatory response, and later by suppressing the same (Artym & Zimecki, 2007).

4.4 Modulatory effects of lactoferrin on inflammation in models of skin allergy and skin wound healing

The skin is the largest organ of the human body, made up of three main layers: epidermis, dermis and the hypodermis (Kanitakis, 2002). The epidermis layer contains no blood vessels and is made up of 90-95 % keratinocytes and 5% Langerhans cells, melanocytes and Merkel cells. Keratinocytes, upon dying due to a lack of blood supply, are responsible for skin keratinization by releasing keratin to extracellular surroundings. Langerhans cells are mobile dendritic presenting cells derived from CD34+ bone marrow precursor cells. Melanocytes produce melanin, a natural dye to protect skin from ultraviolet radiation. Merkel cells display neuroendocrine and epithelial features and function as mechanoreceptors by their contact through synaptic junctions with dermal sensory axons.

The dermis, the second layer of the skin, is tightly connected to epidermis by a basement membrane which protects the latter layer from stress. The dermis is a vascularized layer formed by an elastic and compressible connective tissue that contains fibroblasts, dermal dendrocytes (mesenchymal dendritic cells), mast cells and a dense network of collagen fibers.

The hypodermis represents the inner layer of skin and is made up of loose connective tissue and elastin. The main types of cells present in this layer are fibroblasts, macrophages and adipocytes. This layer contains 50% body fat and plays a relevant role in thermoregulation, insulation and supply of energy. It also acts as a shield against mechanical injuries (Kanitakis, 2002).

Lactoferrin displays a role in skin homeostasis by its ability to positively or negatively modulate the generation cytokines derived from keratinocytes and Langerhans cells, with an essential role in immune and inflammatory responses (Wang et al., 1999).

4.4.1 Modulation of the inflammatory response associated with skin allergy

Trials with human volunteers and assays on mice have addressed down-regulatory properties of bovine or human lactoferrin on pro-inflammatory cytokines generated during dermal allergic responses induced by diphenylcyclopropenone (DPC) (Griffiths et al., 2001; Kimber et al., 2002) and oxazolone (Cumberbatch et al., 2000).

In models of skin allergy induced by DPC or oxazolone, epidermal Langerhans cells migrate to skin-draining lymph nodes. As a consequence, there is an accumulation and maturation of Langerhans cells as dendritic cells able to perform their antigen presentation role to naïve T cells (Wang et al., 1999). In the aforementioned models of skin allergy, the migration of Langerhans cells depends on the local production by skin keratinocytes of IL 1beta, which in turn is dependent on *de novo* production of TNF alpha. In humans, TNF alpha stimulates the activation and migration of Langerhans cells from the dermis, and their subsequent accumulation in human skin-draining lymph nodes (Cumberbatch et al., 1999). This cytokine also promotes the migration of immature Langerhans cells to human lymph nodes involved in chronic skin inflammation (Geissmann et al., 2002). In this regard, the anti-inflammatory property of lactoferrin applied topically to humans and mice has been linked to a decrease in the migration of skin Langerhans cells induced by DPC or oxazolone (Griffiths et al., 2001; Kimber et al., 2002; Cumberbatch et al., 2000). As in the case of DPC or oxazolone, homolog lactoferrin inhibits the mobilization of Langerhans cells induced by intradermic IL 1beta in humans (Cumberbatch et al., 2003) and mice (Cumberbatch et al., 2000). However, in mice, homolog lactoferrin fails to reduce the migration of Langerhans cells induced by the application of exogenous TNF alpha (Cumberbatch et al., 2000). It seems that the down-regulatory effect of lactoferrin on Langerhans cell migration induced by DPC or oxazolone allergens, occurs after inhibiting *de novo* synthesis of TNF alpha by epidermal keratinocytes.

Another down-regulatory effect of bovine lactoferrin has also been described in the mouse model of zymosan-ear skin inflammation (Hartog et al., 2007). In this model, bovine lactoferrin had an anti-inflammatory effect by suppressing local production of TNF alpha. Furthermore, a combination of lactoferrin and glycine generated a synergistic anti-inflammatory effect (Hartog et al., 2007).

In patients with eczematous dermatitis caused by contacting nickel, recombinant human lactoferrin decreased the inflammatory response by inhibiting type 1 and type 2 T cell responses. In that study lactoferrin suppressed T cell proliferation and inhibited the expression of chemokine receptors CCR10, CXCR3 (expressed on Th1) and CCR4 (expressed

on Th2 cells). However, the production of IFN gamma by Th1 and IL 5 by Th2 was partially suppressed by lactoferrin (Moed et al., 2004).

4.4.2 Effect of lactoferrin on cutaneous homeostasis and skin wound healing

4.4.2.1 Stages of skin wound healing

Some of the humoral and cellular components of the inflammatory and immune responses that are modulated by lactoferrin in turn regulate mechanisms with an essential role in cutaneous homeostasis and wound healing. As is known, dermal wound healing is a normal process of repair that involves four continuous, overlapping and precisely programmed stages: i) hemostasis ii) inflammation, iii) fibroplasia, and iv) remodeling (Hardy, 1989; Guo & DiPietro, 2010).

Hemostasis results from vasoconstriction and clotting by fibrin deposition to control bleeding caused by the vascular injury. The clot along with injured surrounding tissue release pro-inflammatory mediators and growth cell factors, such as transforming growth factor (TGF) beta1, fibroblast growth factor (FGF) and epidermal growth factor (EGF) (Guo & DiPietro, 2010).

In the inflammatory phase, sequential infiltration of neutrophils, macrophages and lymphocytes occurs in damaged tissue. The presence of phagocytes is mandatory for bacterial and cellular debris clearance (Hardy, 1989). The pro-inflammatory contribution of lactoferrin in this stage may be linked to its ability to up-regulate the bactericidal activity of neutrophils (Ambruso & Johnston, 1981; Gahr et al., 1991) and macrophages (Lima & Kierszenbaum, 1987) aimed at the control of bacterial growth. During this stage, lactoferrin also modulates the overproduction of ROS released during neutrophil degranulation in order to protect the organism from toxic effects of the same (Britigan et al., 1989).

In the early inflammatory phase, TNF alpha has an essential role in wound healing. However, since its overproduction can cause tissue damage, the fact that lactoferrin decreases TNF released from phagocytes may contribute to preventing the excessive recruitment and activation of phagocytes to sites of inflammation (Crouch et al., 1992). Evidence from *in vitro* cultures of peripheral and bone marrow monocytes of human origin suggest that the down-regulating effects of lactoferrin on inflammation may also be linked to its suppression of the release of IL 1 from monocytes. This in turn decreases GM-CSF production by fibroblasts (Zucali et al., 1989), which may contribute to controlling the mobilization of peripheral blood cells to inflammatory sites.

The fibroplastic phase is characterized by three processes: i) re-epithelialization, ii) wound contraction and iii) production of extracellular matrix components such as collagen (Hardy, 1989). Numerous subtypes of keratinocytes (Patel et al., 2006) and fibroblasts (El Ghalbzouri et al., 2004) play an essential role in scar tissue formation by contributing to wound re-epithelialization. In the early response to injury, re-epithelialization occurs when fibroblasts in the neighborhood of the wound proliferate and then migrate to the wound bed, where they are induced by chemotactic mediators to produce extracellular matrix components (Hardy, 1989). Among these components are proteoglycans (heparan-, chondroitin- and keratan-sulfate), non-proteoglycan polysaccharide (hyaluronic acid) and fibers (collagen, elastin) (Labat-Robert et al., 1990). Dermal healing is accompanied by a process of granulation, which

results from the accumulation of fibroblasts and extracellular matrix components along with the formation of new blood vessels to constitute granular tissue (Hardy, 1989).

Generation of granular tissue is a complex process regulated by cytokines such as TGF beta1 produced by keratinocytes, macrophages and fibroblasts (Werner et al., 2007). Transforming growth factor beta1 stimulates the expression of integrins in keratinocytes, which facilitates their migration to perform the re-epithelialization during wound healing (Gailit et al., 1994). Granulation is also dependent on growth factors such as EGF, platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Werner et al., 2007). *In vitro* assays show that TGF beta1 and PDGF regulate fibroblast attachment, which represents the step between cell migration and proliferation *in vivo* during wound healing (Ihn et al., 1995). Additionally, TGF beta1, bFGF and other blood derived factors promote cell migration and proliferation leading to the increase of cells in the denuded area, or wound space (Schreier et al., 1993). Other agents that exercise healing effects, by enhancing migration and proliferation of keratinocytes for re-epithelializing of the wound bed, include keratinocyte growth factor 2 (Soler et al., 1999), heparin binding EGF like growth factor (Shirakata et al., 2005), skin beta-defensins (Niyonsaba et al., 2007) and insulin (Liu et al., 2009).

4.4.2.2 Effects of lactoferrin on wound healing

The role of lactoferrin in the fibroplastic phase has been addressed in some *in vitro* cultures of human skin fibroblasts (Tang et al., 2010A) and keratinocytes (Tang et al., 2010B). These assays show that recombinant human lactoferrin sustains the survival of fibroblasts and keratinocytes by protecting them from apoptosis, and in this way enhances re-epithelialization by stimulating the proliferation and migration of these cells.

It has been reported that bovine lactoferrin is also able to enhance collagen gel-contractile activity in human fibroblasts, which is the *in vitro* model for the reorganization of the collagen matrix during the wound healing process. Gel contractile activity induced by bovine lactoferrin is accompanied by the activation of extracellular regulated kinase (ERK) 1/2 and myosin light chain kinase (MLCK), and the subsequent elevation of myosin light chain phosphorylation. The effects of lactoferrin on gel contractile activity and its signaling pathway were mediated by the low density lipoprotein receptor related protein (LRP) (Takayama et al., 2003).

Lactoferrin may contribute to wound healing in the fibroplastic stage by its promotion of the synthesis and secretion of the nerve growth factor, which was observed in one study during the contact of bovine lactoferrin with mouse fibroblasts (Shinoda et al., 1994). The nerve growth factor (NGF) is an angiogenic agent with healing activity on diabetic skin ulcers in humans (Aloe, 2004) and mice (Graiani et al., 2004). Also in the fibroplastic stage, lactoferrin promotes the synthesis of components of extracellular matrix, such as type 1 collagen and hyaluronan, the latter through the activation of the TGF beta1 signaling pathway. This has been shown *in vitro* with assays on human fibroblasts (Saito et al., 2011).

5. Applications of lactoferrin as an adjunctive agent in the treatment of infections associated with diabetic foot ulcers

Whereas the previous discussion demonstrates that lactoferrin possesses pleiotropic properties on cell components involved in the inflammation associated with skin allergy or

skin reparation of non-infected surface wounds, what follows is intended to describe potential applications of lactoferrin as an adjunctive agent to control bacterial growth in infected foot ulcers, which can threaten a limb or even the life of diabetic patients. Diabetic foot syndrome encompasses a complex convergence of pathologies, including diabetic neuropathy, peripheral vascular disease, Charcot's neuroarthropathy, and osteomyelitis and foot ulceration. Diabetic foot ulcers are usually detected at the plantar surface and result from neuropathy, inadequate blood supply (ischemia) (Khanolkar et al., 2008), hypoxia (Tamir, 2007) and disturbances in neutrophil phagocytic function (Bader, 2008). Neuropathy plays a major role in foot ulceration since it underlies the loss of limb sensibility to skin pain caused by dermal abrasions and blisters, owing to disturbances of sensory, motor and autonomic functions (Khanolkar et al., 2008).

At present, the evaluation of lactoferrin as an agent with wound healing properties has been limited to only two studies: an experimental study on diabetic mice (Engelmayer et al., 2008) and a clinical study on patients (Lyons et al., 2007). In the former, topically applied Talactoferrin, versus the vehicle or becaplermin (a recombinant human platelet-derived growth factor), increased the closure rate. *In vitro* culture assays suggest that the underlying mechanism of the healing effect of Talactoferrin on mouse diabetic ulcers could be linked with its ability to enhance the migration of dermal fibroblasts, THP-1 macrophages, Jurkat T cells and mouse granulocytes. Other activities of Talactoferrin associated with its healing properties include its ability to enhance the production of IL-8, IL-6, MIP-1 alpha and TNF α , all of which have an essential role in the early inflammatory phase of wound healing (Engelmayer et al., 2008).

In the clinical study, Talactoferrin was delivered topically in gel to the non-healing chronic ulcers from fifty five diabetic patients (patients with any sign of osteomyelitis, gangrene or deep tissue infection were excluded). The first phase, an open label, sequential, dose-escalation study, evaluated the safety of Talactoferrin. Groups of patients were treated with 1% (n=3), 2.5% (n=3) or 8.5% (n=3) of Talactoferrin gel applied topically at the ulcer twice daily for 30 days in combination with standard wound care. No adverse effects were detected. The second phase assessed the efficacy of two distinct doses of Talactoferrin. This was a single blind randomized, stratified placebo controlled pilot study of three groups of patients treated with gel containing 2.5% (n=15) and 8.5% (n=15) of Talactoferrin gel or placebo gel (n=16). In combination with standard wound care, Talactoferrin gel was administered topically twice daily to the ulcers for 12 weeks. The results from the second phase showed that Talactoferrin was effective as a healing agent, evidenced by a significant difference ($P < 0.01$) between the results in the Talactoferrin-treated and control groups. The incidence in the reduction of the size of ulcers in the groups treated with 2.5% and 8.5 % Talactoferrin gel was $\geq 75\%$ compared with 45%-25% in the groups treated with placebo gel. Accordingly, Talactoferrin has been shown to be an effective agent with healing properties and seems to be pharmacologically safe and well tolerated. Thus, it may be useful in the treatment of foot ulcers in diabetic patients (Lyons et al., 2007).

Due to the multifactorial characteristic of the diabetic foot syndrome, lactoferrin does not fulfill the therapeutic requirements for use as a single healing agent. However, some standard techniques of un-infected and un-ischemic diabetic ulcers like debridement (i.e., removal of unhealthy tissue from the wound bed) (Tamir, 2007) may be improved by including lactoferrin as an adjunctive therapeutic agent.

Some pharmaceutical formulations of lactoferrin, including liposomes administered intra-articularly in joints (Trif et al., 2001) or by oral route (Ishikado et al., 2005) to control the chronic inflammation of arthritis or other pathologies, as well as bioadhesive tablets administered to treat inflammation associated with oral ulcers (Takahashi et al., 2007; Takeda et al., 2007), may represent systemic or oral delivery formulations for adjunctive treatment of chronic wounds. As is known, a prolonged inflammatory response is a contributing factor of chronic wounds, since it abrogates the normal process of ulcer healing (Pierce, 2001).

Diabetic patients usually suffer skin dryness, which prompts injuries that are highly susceptible to bacterial infections. When foot skin of the plantar surface is injured, microorganisms that are normally retained gain access to underlying layers where bacterial replication leads to local and/or systemic infections (Guo & DiPietro, 2010). Bacterial infections associated with chronic non-healing foot ulcerations, apart from being a common cause of limb amputation, put the life of the diabetic patient at risk (Bader, 2008).

The effectiveness of standard methods of care, including off-loading, debridement, systemic and local antibiotics and topical antiseptics, can be substantially improved when combined with adjunctive techniques. In some cases, adjuncts like wound dressings (Hilton et al., 2004), hyperbaric oxygen and growth factors are needed to provide better conditions for closure. Such conditions include a moist environment to enhance an optimal inflammatory milieu, as well as an adequate supply of oxygen and nutritional factors (Hopf et al., 2001).

Silver wound dressings have been applied to control the formation of biofilms associated with acute and chronic ulcers in diabetic patients (Hilton et al., 2004). However, as the treatment progresses, the single antibacterial effect of the silver is surpassed by the generation of antibacterial resistance caused by the establishment of bacterial biofilms which in turn impede healing of chronic ulcers in diabetic patients. Lactoferrin may be included in the list of adjuncts with a role in the establishment of the proper conditions for the healing of the wound bed, owing to its aforementioned natural antibacterial and wound healing properties. In this regard, a pharmaceutical presentation of lactoferrin and xylitol in hydrogel proved effective in enhancing the antibiofilm efficacy of a commercial silver wound dressing (Ammons et al., 2011B). That study showed that the combination of lactoferrin, xylitol and silver provides significant antimicrobial efficacy against established biofilms formed by methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. Consequently, lactoferrin in combination with xylitol would be recommended to increase the antibacterial effect of silver wound dressings applied to infected wounds of diabetic patients.

Thus, wound healing and anti-biofilm properties enable lactoferrin, acting as an adjunctive agent, to enhance the efficacy of anti-biofilm targeted techniques for the treatment of infected chronic ulcers like silver wound dressings.

6. Safety measures in the use of Lactoferrin

Toxicological assays on rats chronically treated by the oral route with human recombinant (Cerven et al., 2008; Appel et al., 2006) or native bovine (Yamauchi et al., 2000) lactoferrin show that regardless of their origin, these proteins are pharmacological safe products. In spite of their free toxicity status, some concerns have been raised, including bacterial resistance to human lactoferrin, as in the case of *Streptococcus pneumoniae* (Hammerschmidt

et al., 1999), and the autoimmune potential of cross reactive anti-lactoferrin antibodies which recognize bacterial antigens like the 65 kDa protein of *Mycobacterium tuberculosis* (Esaguy et al., 1991). Although neither *Streptococcus pneumoniae* or *Mycobacterium tuberculosis* are associated with infections in diabetic foot ulcers, bacterial resistance and the autoimmune potential of cross reactive anti-lactoferrin antibodies warn of the need to be alert to possible problems in the application of lactoferrin as an antibacterial agent. While this requires attention to side effects that could present themselves, it does not in any way undermine all of the positive results of lactoferrin found to date.

The pathological relevance of anti-lactoferrin autoantibodies remains uncertain (Audrain et al., 1996). However, it is possible that in some chronic degenerative diseases such as rheumatoid arthritis (Esaguy et al., 1993), ulcerative colitis, primary sclerosing cholangitis and Crohn's disease, autoantibodies to lactoferrin may aggravate and sustain the inflammatory condition (Peen et al., 1993).

Host-produced lactoferrin has been associated with some degenerative diseases and therefore the administration of exogenous lactoferrin may aggravate and contribute to the multifunctional pathology of diabetic foot syndrome. In this regard, endogenous lactoferrin has been reported as a major component of amyloid depots in patients with gelatinous drop-like corneal dystrophy (Klinworth et al., 1997), and it has been detected in pathological lesions in a variety of neurodegenerative disorders as Alzheimer's disease, amyotrophic lateral sclerosis/parkinsonism dementia complex of Guam and sporadic amyotrophic lateral sclerosis, or Pick's disease (Leveugle et al., 1994).

On the other hand, in some sensitized individuals, lactoferrin from bovine milk can be a strong milk allergen (Gaudin et al., 2008). Therefore in such individuals with diabetic foot, the allergenic potential of bovine lactoferrin should be taken into account before prescribing its oral administration. Additionally, the undesirable pro-inflammatory effects of iron loaded lactoferrin on vascular permeability, as evidenced in the dermis of rats by the increase of albumin extravasation (Erga et al., 2001), could negatively affect the process of skin healing.

7. Conclusion

The antibacterial and wound healing properties of lactoferrin may contribute to enhancing the effectiveness of standard techniques for treatment of infections of diabetic foot ulcers. Futures studies are needed to address the substantive therapeutic contribution of lactoferrin as an adjunctive agent in the treatment of infections associated with diabetic ulcer, including the concerns and limitations of its application.

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9. References

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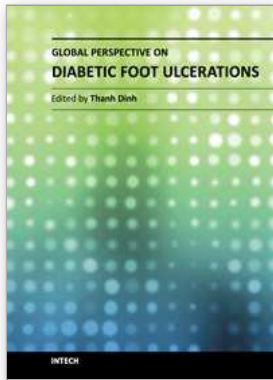
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Over the last decade, it is becoming increasingly clear that diabetes mellitus is a global epidemic. The influence of diabetes is most readily apparent in its manifestation in foot complications across cultures and continents. In this unique collaboration of global specialists, we examine the explosion of foot disease in locations that must quickly grapple with both mobilizing medical expertise and shaping public policy to best prevent and treat these serious complications. In other areas of the world where diabetic foot complications have unfortunately been all too common, diagnostic testing and advanced treatments have been developed in response. The bulk of this book is devoted to examining the newest developments in basic and clinical research on the diabetic foot. It is hoped that as our understanding of the pathophysiologic process expands, the devastating impact of diabetic foot complications can be minimized on a global scale.

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