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Doctoral Thesis

CELL-BASED BIOASSAYS FOR TESTING BIOACTIVE COMPOUNDS IN FARM ANIMALS

(SSD AGR/18)

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1. **Summary**

Cell-based assays can be adopted as *in vitro* method to evaluate the bioavailability and functionality of different nutraceutical and bioactive compounds, particularly in view of the need to use alternatives to animal studies. The interest in these bioactive compounds in animal sciences is not only related to medical research. It also represents an enormous benefit for health food companies and the animal produce sector in general.

The general aim of my PhD study was to evaluate nutraceutical effects at a cellular level in response to different stress challenges.

In the first section of my thesis, the protective role of α-tocopherol in counteracting the cytotoxicity and DNA damage induced by Ochratoxin A (OTA) in primary porcine fibroblast cell cultures (ear and embryo), was determined by using the MTT assay, LDH release, DNA fragmentation, and TUNEL stain.

The aim of the second section was to evaluate the protective role of bovine Lactoferrin (bLf), added to the culture medium, against lipopolysaccharide (LPS) cytotoxicity using the established bovine mammary epithelial cell line BME-UV1 as an *in vitro* model of the bovine mammary epithelium. In addition, we assessed whether BME-UV1 cells were able to express endogenous bLf after *in vitro* exposure to LPS.

A further objective of my thesis work was to use cell-based bioassays to investigate the plasmin-plasminogen system. This system plays a key role in cellular responses, and is involved both in physiological and in pathological conditions in the mammary gland. The aim of the third section was to determine the effect of growth factors (IGF-1 and EGF) and three hormones (insulin, dexamethasone, and prolactin) on the expression of plasminogen activator (PA)-related genes (u-PA, u-PAR, PAI-1, PAI-2) and BME-UV1 cell

proliferation. In addition we investigated the effects of *E. coli* LPS on cell viability, the modulation of cell-associated u-PA activity and the regulation of u-PA and u-PAR RNA expression in BME-UV1 cells.

Below are more details on what each section covers:

The first section reports how the role of α-tocopherol in counteracting OTA toxicity was evaluated in various experimental conditions using primary porcine fibroblasts. Cells showed a dose-, time- and origin-dependent (ear vs. embryo) sensitivity to ochratoxin A. Pre-incubation for 3 h with 1 nM α-tocopherol significantly ($P < 0.01$) reduced OTA cytotoxicity, lactate dehydrogenase release and DNA damage in both fibroblast cultures. These findings indicate that α-tocopherol administration may counteract short-term OTA toxicity, thus supporting its defensive role at a cell membrane level.

The second section describes how BME-UV1 was used as an *in vitro* model to evaluate the protective role of exogenous bovine Lf (bLf) against the cytotoxic damage induced by bacterial lipopolysaccharides (LPS). Exogenous bLf showed a protective effect against endotoxin cytotoxicity, which could be mediated by the LPS-neutralizing capability of bLf. In addition, in BME-UV1 cells the response to LPS exposure did not involve endogenous bLf mRNA expression, suggesting that this cell line lacks functional LPS-responsive elements.

The third section details how cell proliferation was measured using the MTT assay and direct cell enumeration on BME-UV1 treated with physiological stimuli. Results showed that both IGF-1 and EGF increased cell proliferation. Neither of the growth factors had any effect on the expression of PAI-1 and PAI-2. In line with changes in gene expression, EGF and IGF-1 upregulated total cell-associated, membrane-bound and secreted u-PA activity. Dexamethasone alone and when combined with insulin or prolactin upregulated the gene expression of both PAI- and PAI-2, but not that of u-

PA and u-PAR without affecting cell proliferation. Total decreased cell-associated, membrane-bound and secreted u-PA activity was detected in cells cultured in the presence of dexamethasone when combined with insulin or prolactin. However no such effect was observed in the presence of dexamethasone alone. This thus suggests that when dexamethasone acts synergistically with prolactin or insulin it inhibits the activation of the plasmin-plasminogen system, but this inhibition is not correlated with any changes in cell proliferation. In addition, the plasmin-plasminogen system was examined, using the BME-UV1 cell model, in order to evaluate the effects of *Escherichia coli* LPS on cell viability, the modulation of cell-associated u-PA activity, and the regulation of u-PA and u-PA receptor (u-PAR) RNA expression. LPS did not affect cell viability, but led to an increase in u-PA activity, with the maximum response after 6 h of incubation. In addition, u-PA and u-PAR mRNA expression were both up-regulated in BME-UV1 cells after 3 h of incubation with LPS. These data indicated that *E. coli* LPS increased u-PA activity and RNA expression of u-PA and u-PAR in BME-UV1 cells, thus strengthening the role of the PA system during pathological processes.

In conclusion, the application of appropriate *in vitro* models represents a fundamental requirement for the study of cellular responses to different stimuli as in the case covered in my thesis regarding nutraceutical compounds. Cell-based assays are a valuable tool for assessing fundamental regulatory mechanisms at a cellular level, such as the plasmin-plasminogen system. Cell-based assays allow both the functionality of nutraceutical and cellular response mechanisms to be evaluated reducing use animal models in the preliminary study phase. Obviously, data obtained in cell culture models must be interpreted carefully, since this system represents a simplification of the intricacies of the numerous reactions and interactions that occur *in vivo*.

2. Introduction

Over the past few years, food and feed sciences have evolved as a result of different factors, such as improved safety, changes in the European Union agriculture policy, the need to recover consumer confidence and the functional aspects of foods, taking into account the changes in the alimentary habits of the population. It is well known that food can have biological activities that are beyond classical nutritional values. This aspect has gained increasing attention in the food industry but also in animal nutrition, and so-called nutraceuticals are offered both for food and for feed applications.

Nutraceutical is defined as "a food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease". This term was coined by DeFelice, in 1989, from "nutrition" and "pharmaceutical" (DeFelice, 1995).

Some years later, the U.S. Institute of Medicine's Food and Nutrition Board defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Rodriguez et al., 2006).

Nutraceutical differs slightly from functional foods; in fact it refers equally to nutraceuticals and functional foods, however some authors (Gulati and Ottaway, 2006) differentiate the former from the latter on the basis of its formulation.

Kalra (2003) proposed redefining nutraceutical and functional foods: when functional food aids in the prevention and/or treatment of diseases and/or disorders other than anemia, it is called a nutraceutical". The term nutraceutical is used to describe substances that range from isolated nutrients to processed products. More broadly, nutraceutical refers to all those

substances that have positive physiology effects on the human/animal body (Gulati and Ottaway, 2006).

The interest in nutraceutical products has increased over the last decade in Europe and reflects the fact that epidemiological surveys, animal research, clinical trials and research in nutritional biochemistry indicate that a specific diet or dietary supplements are associated with lower risks for a wide range of diseases such as cancer, osteoporosis and heart disease.

From a regulatory point of view, the European Union defines a nutraceutical on the basis of its accepted effects on the body: if the substance only maintains healthy tissues and organs, it can be considered a food ingredient. On the other hand, if it has been proved to modify the effects on the body's physiological processes, then it may be considered as a medicinal substance (Fig.1).

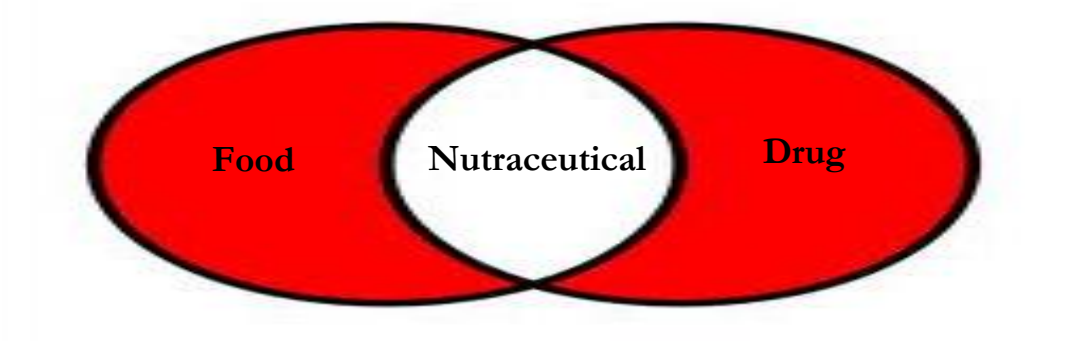

Fig. 1: Where nutraceutical stand between food and drug; (modified by Gulati and Ottaway, 2006)

If a vitamin is fed at levels close to the daily requirement (Recommended Daily Allowance), it is considered as a food ingredient because it maintains the function of healthy organs and tissues. However, if the same vitamin can be demonstrated to modify, restore or correct the functions, it, or the product containing it, may be considered as medicinal (Gulati and Ottaway, 2006).

If food and feed are brought onto the market with "nutritional and health claims", these claims must be objective, scientifically supported, and verifiable by the competent authorities (Regulation (EC) No 1924/2006 of the European Parliament and of the Council; Regulation (EC) 767/2009 of the European Parliament and the Council) (Cheli and Baldi, 2011).

The use of nutraceuticals in human nutrition, as described above, has been the focus of many studies and economic interests. Nutraceuticals have also taken up a key role in animal dietary studies. Since 2000 following the publication of the European Commission's White Paper, the relationship between health and nutrition in European animal production has become increasingly recognized. This White Paper stipulated that animal feeds must be subject to the same conditions as food for human consumption, and that animal health and welfare should be considered in animal production (Baldi, 2005a).

2.1 Bioactive compounds in food

Many nutraceuticals bioactive compounds show strong antioxidant activity. Packer et al. (2004) highlighted the crucial role that antioxidants play in preventing oxidative stress and maintaining the physiological redox status of cellular constituents: "Antioxidants may quench free radicals, change their redox state, be targeted for destruction, regulate oxidative processes involved in signal transduction, affect gene expression and pathways of cell proliferation, differentiation and death". This is achieved at various subcellular and molecular levels including antioxidants that interact with the redox antioxidant network, such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E) (Mandel et al., 2005).

Vitamin E is one of the most important components of cellular antioxidant systems. In nature it is present under eight different forms, four tocopherols (α-, β-, γ-, δ) and four tocotrienols (α-, β-, γ-, δ), of which α-tocopherol is the

most bioactive. As an antioxidant, α-tocopherol can prevent free-radical mediated tissue damage, and thus can prevent or delay the development of degenerative and inflammatory diseases, thus it has been extensively investigated in many species, including humans. According to several surveys, vitamin E supplementation helps to enhance animal health and production and, when dietary selenium is adequate, it significantly reduces the incidence of intramammary infections (IMI) and clinical mastitis (Moyo et al., 2005). Vitamin E also seems to be crucially involved in the immune system so that supplementation with supra-nutritional levels, at least in some instances, results in improved immune responses (Baldi, 2005a). Vitamin E supplementation is a common practice in animal nutrition, and increasing the feed content of synthetic or natural vitamins is currently considered the best way to maintain adequate plasma levels for animal health.

An important functional food containing a large numbers of bioactive components is milk. Milk is a polyphasic secretion of the mammalian gland, and is an excellent source of well-balanced nutrients. Its benefits are mainly due to its peptides and proteins, which range from facilitating the digestive system to the growth and development of specific organs. Some of the biological activities of milk protein components are released only upon proteolytic action during the digestion of milk in the gastrointestinal tract and also during fermentation and food processing (Singh and Sachan, 2011). Milk contains many bioactive components such as: vitamin D and vitamin K which promote strong bones; vitamin B12 and riboflavin, which are necessary for cardiovascular health and energy production; and vitamin A, which is essential for the immune function (Singh and Sachan, 2011). In addition, milk produced by grass-fed cows contains Conjugated Linoleic Acid (CLA), which can play a role in the prevention of some types of cancer such as breast cancer and help to prevent atherosclerosis (Midau et al., 2010).

	Concentration (g/l)		
Protein	Cow	Human	Function
Total Caseins	26.0	2.7	Ion carrier (Ca, PO_4, Fe, Zn, Cu) precursors of bioactive peptides
α-Casein	13.0		
β-Casein	9.3		
ϰ-Casein	3.3		
Total whey protein	6.3	67.3	
β-Lactoglobulin	3.2		Retinol carrier, binding fatty acids, possible antioxidant
α-Lactoalbumin	1.2	1.9	Lactose-synthesis in mammary gland, Ca carrier, immunomodulation, anticarcinogenic
Immunoglobulins (A, M and G)	0.7	1.3	Immune protection
Serum albumin	0.4	0.4	
Lactoferrin	0.1	1.5	Antimicrobial, antioxidative, immunomodulation, iron absorption, anticarcinogenic
Lacoperoxidasetoferrin	0.03		Antimicrobial
Lysozyme	0.0004	0.1	Antimicrobial, synergistic effec with immunoglobulins and lactoferrin
Miscellaneous	0.8	1.1	
Proteose-peptone	1.2		Not characterized
Glycomacropeptide	1.2		Antiviral, bifidogenic

Table. 1: Concentration and biological functions of major milk proteins (Séverin and Wenshui, 2005)

The protein component of milk consists of caseins and whey proteins; in bovine milk, caseins comprise about 80 per cent of the total protein content and are divided into α-, β- and ϰ-caseins. Whey proteins consist of β-lactoglobulin, α-lactoalbumin, immunoglobulins (IgGs), glycomacropeptides, bovine serum albumin and minor proteins such as lactoperoxidase, lysozyme and lactoferrin (Table.1) (Singh and Sachan, 2011).

Lactoferrin is a non-haem iron-binding glycoprotein with a molecular weight of about 80 kDa, synthesized by glandular epithelial cells. It is found in many biological secretions, such as colostrum and milk (Legrand et al., 2008), and is stored in the secondary granules of neutrophils (Masson et al, 1966).

Several physiological roles have been attributed to Lf, including antibacterial, antiviral and anticancer activities, the regulation of iron homeostasis, an anti-

inflammatory role, antioxidant properties, and the modulation of immune responses (Ward et al., 2005; Legrand et al., 2008).

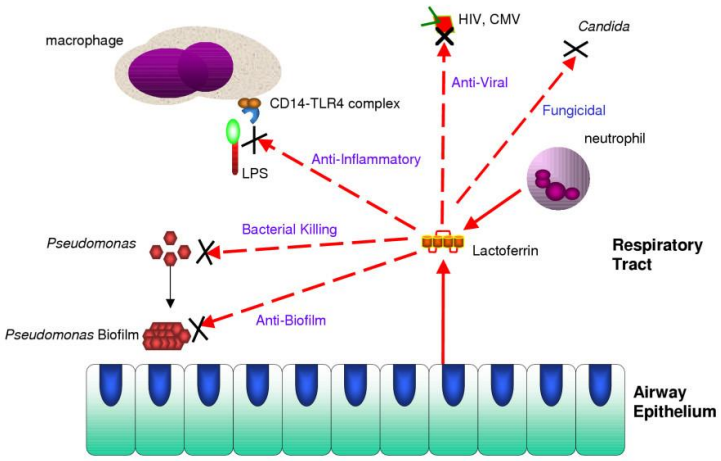

Fig. 2: Multifunctional properties of Lf (Rogan et al 2006).

The polyfunctionality of Lf is related to its ability to interact with several targets (Fig. 2). The function of the iron-chelating activity of the protein is to sequester the free element, thus controlling the growth of pathogenic bacteria, and limiting the production of reactive oxygen species that can damage cellular components (Legrand et al., 2006; Paesano et al., 2009). In addition, a growing number of studies have revealed that some of the physiological effects of Lf are mediated by the interaction with cellular structures, such as lipopolysaccharides (Elass-Rochard et al., 1995), proteoglycans (Legrand et al., 1997) and cell-type specific receptors (Suzuki et al., 2005).

Lf is an innate factor of the mammalian immune system and displays a broad-spectrum antimicrobial activity against both Gram-negative bacteria, such as *E. coli* and *Klebsiella sp.,* and Gram-positive bacteria, including *Staphylococcus aureus* and *Streptococcus uberis* (Valenti and Antonini, 2005; Rainard and Riollet, 2006; Jenssen and Hancock, 2009).

2.2 In vitro models for the study of bioactive components in food/feed

2.2.1 General introduction

In order to use nutraceuticals in both human and animal nutrition, it is important to develop protocols and models to evaluate the bioaccessibility, bioavailability and functionality of the bioactive components. The evolution of food analysis over the past few years has led to improvements in chemical analysis and instrumental tools with significant enhancements in analytical accuracy, precision, detection limits, and sample throughput (Mc Gorrin, 2009). The transition from a chemical analysis approach to cell-based bioassays supports the new need for food/feed analysis in terms of bioactivity and functional properties. The need for reliable *in vitro* cellular models as alternatives to animal studies has became an important issue and is considered in European legislation (http://www.euractiv.com/en/science/eu-wants-fewer-animals-used-research-news-497607).

The interest of the food and pharmaceutical industry in cell-based bioassays focuses above all on toxicological and bioavailability tests of newly-developed food ingredients and drugs, which are key to bringing products to the market. In the food/feed industry, research regarding the safety and efficacy of additives and new functional food and feed could take benefit from the development and validation of specific cell-based functional bioassays.

Obviously the use of cell cultures has both advantages and disadvantages.

Advantages:

- Control of chemical, physical and physiological conditions
- Characterization and homogeneity of the samples
- Low price and speed of response

- Possible reduction of animal testing.

Disadvantages:

- Depending on the model selected, loss of architecture and three-dimensional tissue-specific interactions between cells
- Difficulty to maintain cell differentiation
- Loss of control systems that depend on the endocrine and nervous systems
- Instability and duration
- Need for experience and organization

2.2.2 Types of cell cultures

A cell culture is a generic term used to indicate the process by which cells, arising from the breakdown of tissues or isolated from biological fluids such as blood, are grown and maintained under controlled conditions.

In vitro systems have been designed to simulate what occurs *in vivo* so as to preserve both the characteristics of the original tissue and the cell-cell interactions (Zucco et al., 2004). A wide range of *in vitro* models are currently available including organ cultures, primary cultures, immortalized cells, stem cells, barrier systems and three-dimensional models (Zucco et al., 2004).

Organ cultures

This type of culture maintains both the composition and architecture of original tissue *in vitro*. However, cells remain in culture for a limited period of time and vary individually which can lead to problems in replicating the experiment.

Primary cultures

In this type of cell culture, cells derive from the mechanical and enzymatic dissociation from the tissue of origin. Cell population of a primary culture is

not homogeneous and can be propagated to a limited number of steps. The physiological and metabolic properties of this *in vitro* model are very similar to those found *in vivo*.

Immortalized cell lines

Through the introduction of sequences of DNA tumor viruses, such as SV40, Papilloma virus and Epstain-Barr virus, it is possible to obtain immortalized cells which undergo cycles of indeterminate replication. Cells retain some characteristics of differentiated tissue, and a relative homogeneity; their use facilitates the comparison of results from different laboratories.

Barrier systems

This culture system enables not only the interactions established *in vivo* between cells to be reproduced *in vitro*, but also their functional characteristics. Barrier systems are used to study epithelia, which are focal points of interaction with xenobiotics and nutrients (Fig. 3).

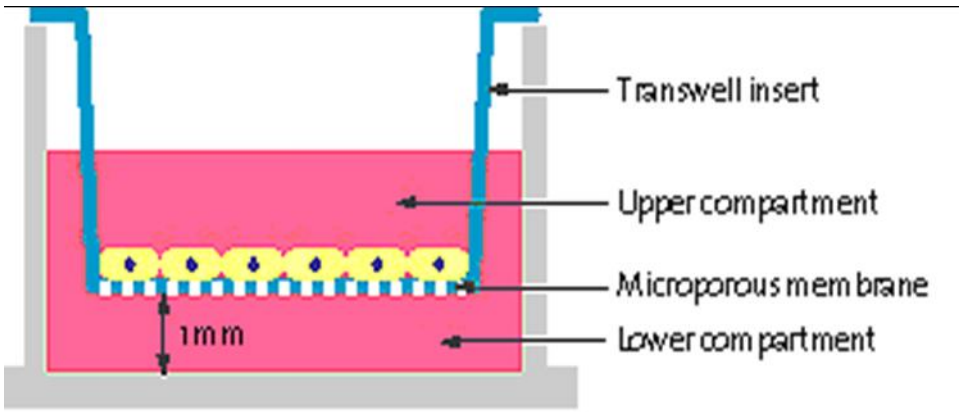

Fig 3: Bicameral system; (http:// www.corning.com/lifesciences/cell_culture/transwell_permeable_supports.asp)

Three-dimensional models

This type of cell culture maintains cellular interactions and the relationship between structure and function. Three-dimensional models make use of different substrates and the culture can be homotypic or heterotypic. This

system enables the main cellular functions to be maintained for a limited period of time.

2.2.3 In vitro models to study antioxidants

The antioxidant activity of pure compounds, food, feed, and dietary supplements has been extensively studied and numerous analytical approaches and assay models have been developed, involving various systems from simple chemical assays to animal models and human studies. There is evidence that food/feed antioxidants and antioxidant supplementation may protect against oxidative stress-induced diseases.

The properties and sensitivity of the cells and their growth status are critical factors that affect the evaluation of antioxidant activity. In addition, cellular response to antioxidant components is quite complex, depending on the component, exposure dose, and time. Several cell types have been used to set up cell-based bioassays for assessing the antioxidant activity of foods and dietary supplements with wide concentration ranges.

Primary isolated rat hepatocytes have been used to evaluate the protective effects of melanoidins in adriamycin-induced oxidative stress (Valls-Belles et al., 2004). Fusi et al. (2010) evaluated the protective effects of α-tocopherol in ochratoxin A (OTA) induced oxidative damage in primary porcine fibroblasts. These researchers found a different sensitivity of ear and embryonic fibroblasts both to oxidative damage and antioxidant treatment. Primary cells, isolated from human or animal tissue, could be a good cell-based model as they retain the majority of the *in vivo* functionality; however they survive and maintain their differentiation for only a few days in cell cultures. If the primary cells are chosen, it is important to consider that they are usually derived from different individuals in each test; consequently, the reproducibility of results may differ significantly from one test to another.

When phenotypic screens using primary cells are included in cell-based bioassays, a constant supply of biologically homogenous cells is thus necessary to support a large scale study. Moreover, the isolation procedure for primary cell culture preparation can reduce the survival of the cells and cause changes in gene expression, metabolic activity, and the levels of enzymes involved in the oxidant and antioxidant systems.

Halliwell (2003) discusses in detail how "culture shock" can affect cell survival and metabolic activity, and concludes that the cells which do survive appear to be those that have adapted rapidly, and are probably not representative of the original cells harvested from a tissue.

Other types of cells that can be used in antioxidant testing are immortalized cell lines. It is fundamental that the cell lines show no altered functional response to oxidative stress. A cell line basically comprises a phenotypically and genetically uniform population of individual cells that have been derived from a single tissue. Cell lines are robust, grow, and divide easily in culture, are simple to handle and may provide a good platform for cell-based assays. The availability of a wide number of human and animal cell lines with diverse genotypes and tissue-origins provides broad potential models for the study of various biological processes.

Baldi et al. (2004) evaluated the protective effects of α-tocopherol in OTA induced oxidative damage. A panel of five well characterized human and animal cell lines, SK-N-MC (human neuroblastoma), MDCK (Madin Darby canine kidney), AML-12 (mouse liver hepatocytes), LLC-PK1 (pig kidney), and BME-UV1 (bovine mammary epithelium), were successfully used to investigate the effect of α-tocopherol on ROS production on OTA-treated cells. A significant difference in cell sensitivity was also found, with BME-UV1 and MDCK being the most sensitive cell lines. Although the choice of the most suitable cell-based assays is key in the correct evaluation of the

antioxidant activity of food/feed compounds, there are other important elements to be considered: choice of biomarkers, endpoint, effect of cell culture medium and environment, and the cell culture system selected. The transition from research models to test models for cell-based bioassays still needs the optimization, standardization and validation of analytical protocols. Thus, despite the fact that antioxidant activity has been demonstrated *in vitro*, it has not always been demonstrated *in vivo*. This reflects the limitation and complexity of human clinical studies where there are several confounding factors present such as sex, age and total antioxidant status (Cheli and Baldi, 2011).

2.2.4 In vitro models to study milk bioactive compounds

When assessing the bioactive compounds of milk, it is important to know the physiology of the organ that produces the milk and how this organ and its cells respond to both physiological and pathological stimuli. If the object of the study is Lactoferrin this aspect is important as mammary epithelium cells produce Lf and, at the same time, are sensitive to its action. Understanding the origin of bioactive compounds and their role in nature may influence the research into how this nutraceutical can improve the health of consumers.

Studies on cellular responses to physiological and pathological conditions generally focus on the plasmin-plasminogen system since it is one of the main cellular response systems that play a pivotal role in the mammary gland, both in development and defense mechanisms (Baldi et al., 1996; Politis 1996; Cheli et al., 1999, Cheli et al., 2003).

The mammary gland is a complex organ that undergoes continuous changes. Thus the choice of the most efficient model to study mechanisms *in vitro* that regulate the production of milk and bioactive compounds is very important, because the type of cell and the culture system adopted need to enable the suitable phenotype to be maintained. In addition, the cells need to be able to synthesize the various milk constituents and respond to lactogenic stimuli.

The transition from undifferentiated epithelial cells in the active growth phase, isolated from pregnant or not yet mature animals, to differentiated cells that are able to synthesize milk protein has been achieved in differentiated cultures. Epithelial cells are characterized by a high degree of polarization and compartmentalization of milk protein. These features are essential requisites for the mammary gland to function properly. Established bovine mammary epithelial cell lines, well characterized and with the ability to synthesize milk proteins efficiently, represent a good *in vitro* model to study the physiology of the mammary gland.

The ability of cells to proliferate or maintain a phenotype *in vitro* is closely related with the culture conditions and characteristics of the substrate. Finally, the possibility to use a porous insert in a bicameral culture system to grow epithelial cells represents an interesting *in vitro* system. Primary cultures of epithelial cells of the mammary gland cultured from inserts maintain a high degree of functional differentiation, presenting a polarized secretion of milk proteins (Claudon et al., 1997).

MAC-T cell lines, BME-UV and BME-UV1 are some of the *in vitro* models used to study the biology of the mammary gland of ruminants.

MAC-T cells maintain the morphological characteristics of the mammary epithelium *in vitro*, and are able to secrete milk proteins (Huynh et al., 1991). This cell model has been widely used to characterize the role of various growth factors or inhibitors in the growth of bovine mammary epithelial cells

or to study the interaction of pathogenic bacteria with bovine mammary epithelial cells.

Studies on this cell model have helped to clarify the role of 'IGF-I as a mitogen factor (Zavizion et al., 1995). The growth of these cells, however, seems to be inhibited in the presence of molecules such as Lf (Hurley et al., 1994) and α-lactalbumin (Rejman et al., 1992).

These studies also found that the cells of the line MAC-T when grown in the absence of both serum and proteins were able to produce uPA, uPA inhibitors and receptors uPA.

In addition to the obvious advantages of choosing this cell line in studies on the metabolism and response mechanisms of the epithelial cells of the bovine mammary gland, there are also disadvantages: it is not a homogeneous line and, if the aim is to clarify the mechanisms that involve EGF as a factor that influences cell growth, this cell line is not sensitive to the EGF action (Woodward, 1994).

Zavizion et al. (1996b) from the University of Vermont obtained two cell lines, BME-UV1 and BME-UV2, which are able to synthesize low levels of α-lactalbumin and as1-casein. Cells of the BME-UV line express not only the typical functional and biochemical markers of mammary epithelial cells, but also the morphology "cobblestone" of such cells.

Such cells form desmosomes and show cytoplasmic cytokeratin fibrils. When grown on plastic, they take on a polygonal morphology, while on collagen they exhibit morphology at Column and a dome structure, typical of this type of epithelial cells *in vivo*. Furthermore they form long cytoplasmic projections, which allow contact between cells to be established.

The BME-UV line was obtained by the microinjection of a plasmid, of clones of epithelial cells containing antigen SV-40 large, derived from the enzymatic dissociation of the breast tissue (Zavizion et al., 1996b). These authors were

the first to report the use of microinjection to immortalize bovine epithelial cells for. In fact previously this method had been used to immortalize mammary cells derived from humans (Heim et al., 1995).

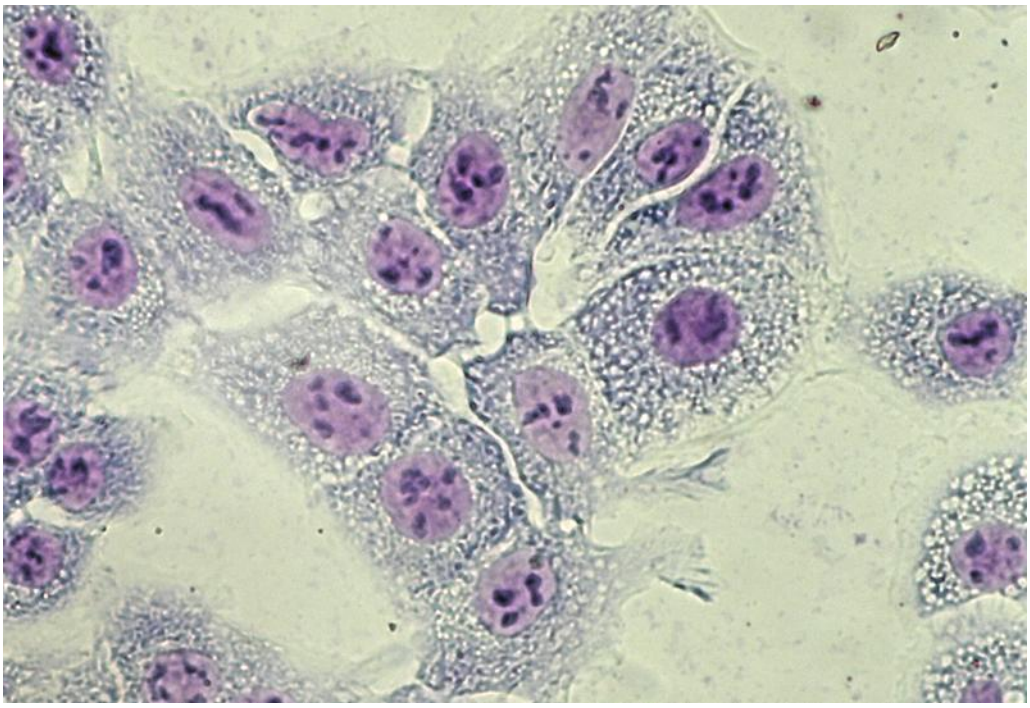

Fig. 4: BME-UV1 cells aquired by Image Vision (Media Cybernetics, OM 40X)

Thus the BME-UV1 cell line (Fig. 4) is a good *in vitro* model to study both tissue remodeling events and Lf action. In fact BME-UV1 is sensitive to Lf action and is able to synthesize α-lactoalbumin and αs1-casein as well as express the components of the plasmin-plasminogen system.

Naturally, the immortalized cell lines are only one of the models available for *in vitro* studies on ruminants; for studies to assess the synthesis of milk fat the primary cell lines of mammary gland are the most widely used.

For example, bovine and goat mammary gland cell cultures were used in studies to estimate the possibility of changing the content and the fatty acid

profile of milk fat, proving that this cell model is able to maintain *in vitro* the ability to synthesize fatty acids ex novo (Cifrian, 1993).

3. Aim of the study

The general aim of my PhD study was to evaluate nutraceutical effects at a cellular level in response to different stress challenges.

In the first section of my thesis, the protective role of α-tocopherol in counteracting the cytotoxicity and DNA damage induced by Ochratoxin A (OTA) in primary porcine fibroblast cell cultures (ear and embryo), was determined by using the MTT assay, LDH release, DNA fragmentation, and TUNEL stain (Chapter 4).

The aim of the second section was to evaluate the protective role of bovine Lactoferrin (bLf), added to the culture medium, against lipopolysaccharide (LPS) cytotoxicity using the established bovine mammary epithelial cell line BME-UV1 as an *in vitro* model of the bovine mammary epithelium. In addition, we assessed whether BME-UV1 cells were able to express endogenous bLf after *in vitro* exposure to LPS (Chapter 5).

A further objective of my thesis work was to use cell-based bioassays to investigate the plasmin-plasminogen system. This system plays a key role in cellular responses, and is involved both in physiological and in pathological conditions in the mammary gland. The aim of the third section was to determine the effect of growth factors (IGF-1 and EGF) and three hormones (insulin, dexamethasone, and prolactin) on the expression of plasminogen activator (PA)-related genes (u-PA, u-PAR, PAI-1, PAI-2) and BME-UV1 cell proliferation. In addition we investigated the effects of *E. coli* LPS on cell viability, the modulation of cell-associated u-PA activity and the regulation of u-PA and u-PAR RNA expression in BME-UV1 cells (Chapter 6).

4. α-Tocopherol counteracts the cytotoxicity induced by Ochratoxin A in primary porcine fibroblasts

4.1 Introduction

Oxidative stress can be defined as an imbalance between oxidants and antioxidants (Cheli and Baldi, 2011). Normally the body is protected from oxidative damage by a wide range of antioxidants, forming an integrated defence system (Miller et al., 1993). As such, antioxidants can be broadly defined as any substance that delays, prevents or removes oxidative damage to target molecules (Halliwell and Gutteridge, 2007). Cell antioxidant system includes preventive and scavenging molecules and enzymes that remove superoxides and peroxides before they react, and chain breaking antioxidants as vitamin E, C, carotenoids, that interrupt the free radical chain reaction cascades initiated by ROS. Some of these antioxidants share both the activities. Under physiological conditions, the presence of antioxidants that function at cytosolic, extracellular or lipophilic (membrane lipids) phases can handle the ROS produced during oxygen metabolism by effectively neutralizing and eliminating them and play an essential role in maintaining tissue integrity (Sordillo and Aitken, 2007). When ROS are generated in excess or cellular level of some antioxidant decreases, as typically occurs in ageing or during diseases, the free radicals produced can overwhelm the protection system and become toxic to the cell reacting with and damage many biological molecules, such as proteins, lipids and DNA (Cheli and Baldi, 2011).

Nutrition can have a major influence on pro-oxidant/antioxidant balance, since several components of the antioxidant system are micronutrients

(vitamin C, E, carotenoids) or require dietary micronutrients (Se and Zn). A deficiency of any of these nutrients may affect the antioxidant system efficiency, impairing immunity status and health.

In particular, Vitamin E plays a crucial role in protecting cell membranes from oxidative stress damages.

4.1.1 Vitamin E structure

Vitamin E was discovered in 1922 by Evans and Bishop (1922); it was identified as a micronutrient essential for reproduction in rats. In 1936 the same researcher isolated the active substance of vitamin E and called it tocopherol (Wang et al., 1999). On the same year two compounds with vitamin E activity have been identified and were designated α- and β-tocopherol (Evans et al., 1936). Two other forms of tocopherol were also discovered γ and δ (Emerson et al., 1937; Stern et al., 1947) in addition to the tocotrienols (Pennock et al., 1964).

The prefixes α, β, γ and δ are used to distinguish the form of both tocopherols and tocotrienols. Number and position of the methyl substituent attached to the chromanol ring allow to characterise α-, β-, γ- and δ-isomers (Wang et al., 1999).

When α-tocopherol is synthesised chemically, a racemic mixture of all 8 possible isomers of α-tocopherol in equimolar concentrations is produced (synthetic form, also referred as all-rac α-tocopherol and historically labeled dl-α-tocopherol), with four stereoisomers showing a 2R configuration (RRR, RRS, RSS, RSR, having R configuration at position 2' of the phytyl tail) and four stereoisomers possessing a 2S configuration (SRR, SSR, SRS, and SSS, having S configuration at position 2' of the phytyl tail, Figure 5). Among possible different forms of α-tocopherol, RRR α-tocopherol is the only isomeric form of vitamin E produced by plants (natural form, historically

labeled d-α-tocopherol) and is therefore the only one naturally present in feedstuffs.

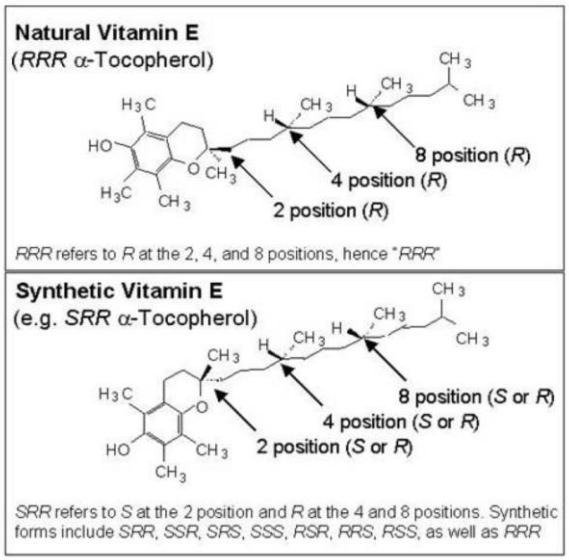

Fig 5. Natural and synthetic vitamin E**.**

The different stereoisomeric forms of vitamin E have different bioavailability and different biological activity in humans and animals, with RRR isomer having a greater biopotency than the synthetic form on a molar or mass basis. Several factors such as structural diversity of compounds with antioxidant activity, food matrix structure and antioxidants relative proportions in standard diets influenced antioxidant bioavailability.

4.1.2 Vitamin E absorption and metabolism

Hydrophobic nature of vitamin E requires special transport mechanisms to go through aqueous environment of body and cells. Vitamin E is absorbed in small intestine where it is emulsified with fat-soluble components of the food. Following action of bile salts and pancreatic juices vitamin E is incorporated

into mixed micelles which are absorbed by brush border membrane of enterocytes by passive diffusion.

Into Golgi of enterocyte, α-tocopherol is packaged into chylomicrons together with triglycerides, phospholipids, cholesterol and apolipoproteins; chylomicrons are excreted by exocytosis and they reach blood stream via lymphatic system (Azzi et al., 2000; Brigelius-Flohé et al., 1999; Rimbach et al., 2002).

In blood stream, chylomicrons gain apolipoproteins C and E from HDL; apolipoprotein C actives endothelial lipoprotein lipase (LPL) in target tissue such as adipose tissue and muscle. LPL takes part in catabolism of chylomicrons by hydrolyzation of triglyceride forming remnants that can be absorbed by hepatic cells by receptor-mediated endocytotic processes.

While uptake of vitamin E from food in small intestine involves an unspecific mechanism, in liver hepatic cells own a specific transport protein (α-TTP) that allow they to uptake α-tocopherol excluding both other form of natural and sintetic tocopherol.

α-TTP preference for α form of tocopherol and its proportional enrichment in plasma lipoproteins has been observed in rats, guinea pigs, salmons, monkeys and in humans (Traber et al., 2004).

Since α-TTP is a specific transport protein for alpha form of tocopherol, it is recognized to be the most important determinant of plasma tocopherol level and mutation of gene for α-TTP could reduce plasma level of α-tocopherol. Although Vitamin E is generally studied in order to its antioxidant function, it seems that it can influences various signaling cascades at the cellular level by inhibiting protein kinase C (PKC) that has an important role in the cell signal transmission. Some studies (Azzi et al., 2000; Brigelius-Flohé et al., 2002) suggest that α-tocopherol may control many cellular events by the regulation of PKC at the gene level.

4.1.3 Antioxidant activity of vitamin E

Vitamin E is the most important lipid-soluble antioxidant and is an integral component of all lipid membranes. It acts as a radical scavenger and serves to protect lipid membranes from attack by ROS. In particular polyunsaturated fatty acids (PUFA) of membranes are vulnerable to attack by ROS, which can initiate a chain reaction of lipid peroxidation that destroy cell membrane.

Due to its hydrophobicity, vitamin E tends to position itself either into tissue lipids or in hydrophobic domains of molecules like lipoproteins, in fact, in cellular environment; vitamin E is localized in cell membranes and lipoproteins. In these sites vitamin E can explicate its antioxidant role against free radical action.

Vitamin E peroxyl radical scavenging function allows cell to maintain both integrity and bioactivity of long-chain polyunsaturated fatty acids. These bioactive lipids are important for membrane plasticity and are signaling molecules. Change in their amounts or in their oxidation products can modify cell response and balance between cell and environment (Traber et al., 2007).

Free radicals damages can be reversible or irreversible and they are addressed to target molecules such as DNA, lipids and proteins. High speed generation of ROS is due to their ability to propagate the reaction started with a single initiating radical species to a wide number of target molecules so antioxidant action speed is very important to stop ROS generation and its damages.

Cells own antioxidant defense mechanisms that are normally able to detoxify the reactive intermediates or to repair damages. Imbalance between ROS production and biological antioxidant system implies oxidative stress. Differently from other cellular antioxidants, α-tocopherol activity is nonenzimatic and fast.

Oxidation of lipid is a process that involves a free radical chain reaction. Chain propagation of lipid oxidation is allowed by reaction between a lipid

peroxyl radical and a lipid target. Lipid peroxyl radical takes away a hydrogen atom from the lipid target producing a lipid hydroperoxide and a carbon-centred lipid radical. The latter one reacts with molecular oxygen to generate another lipid peroxyl radical starting the chain reaction (Wang et al., 1999).
Vitamin E does its antioxidant role by scavenging the peroxyl radical and blocking free radical propagation. Reaction between lipid peroxyl radical and α-tocopherol results in α-tocopheroxyl radical and lipid peroxide; following ascorbic acid can reduce α-tocopheroxyl radical back to α-tocopherol by donating electrons (Wang et al., 1999; Azzi et al., 2000).
In vitro studies have shown that scavenging action of α-tocopherol to peroxyl radical is faster than action of this one to lipid target (Wang et al., 1999).

4.1.4 Antioxidant activities in vitro measured

Measuring production of ROS following exposure to oxidative stimuli, it is possible to evaluate the ability of Vitamin E to scavenge free radicals and, consequently, protects the membrane of cell.
Of the many environmental oxidative stressors, xenobiotics, like Ochratoxin A (OTA), represents a cause of ROS generation that may compromise antioxidant defense mechanisms. OTA is a mycotoxin produced by *Aspergillus* and *Penicillum* species, it is present in food and feedstuffs and it is responsible for ROS formation. International Agency for Research on Cancer considered it as a possible carcinogenic (group 2B) to humans. The scientific panel on contaminants in the food chain of the European Food Safety Authority released an opinion related to OTA in food (EFSA 2006), in which it summarized the major information on OTA related to human health but also indicated the susceptibility of pigs and other animals to OTA.

In porcine species OTA contamination is responsible for acute, subchronic and chronic intoxications determining financial losses in the agriculture and food industry (O'Brien et al. 2005).

At the cellular level OTA can act directly or by indirect mechanisms and it involves various mechanisms of action: lipid peroxidation, disruption of calcium homeostasis, inhibition of protein synthesis, mitochoindrial respiration and DNA damage (Ringot et al., 2006). At the cellular level α-tocopherol acts as an antioxidant terminating the chain of events of oxidative processes by donation of its phenolic hydrogen to chain propagating lipid peroxyl radicals, resulting in the enhanced formation of the less reactive α-tocopheroxyl radical (Zhang and Omaye, 2001).

In vitro studies (Baldi et al., 2004; Fusi et al., 2008) have investigated that α-tocopherol has protective activities against OTA reducing ROS production.

Particularly, Baldi et al. (2004), using BME-UV1 cell line susceptible to OTA cytotoxicity (LC_{50}=0.8 μg/ml at 24 h), have tested a protective effect of α-tocopherol against OTA stimulus. Three hours pre-incubation with antioxidant significantly ($P < 0.05$) ameliorated the OTA-induced reduction in cell viability and significantly ($P< 0.05$) decreased ROS production concluding that: (1) oxidative stress is an important factor in OTA cytotoxicity and (2) supplementation with α-tocopherol is able to counteract the short-term toxicity of OTA.

In the same study researchers have used other cell lines to test α-tocopherol ability to protect cells against OTA action finding that α-tocopherol significantly inhibited OTA-induced ROS production and this inhibition was concentration-dependent.

4.2 Aim

Under the light of the effects of OTA on porcine species, as described above, and considering primary cell culture as a good cell-based model since primary cells retain most of the *in vivo* functionalities, the aim of this part of my PhD was to determine the toxic effects of OTA in primary porcine fibroblast cell cultures by using the MTT assay, LDH release, DNA fragmentation and TUNEL stain. At the same time another purpose of the study was to determine the contribution of α -tocopherol in counteracting the cytotoxicity and DNA damage induced by OTA in the same *in vitro* model.

4.3 Materials and methods

4.3.1 Chemicals

Ochratoxin A, racemic α-tocopherol, penicillin, streptomycin and 3-(4,5-imethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis MO, USA). Dulbecco's Minimum Essential Medium (DMEM) and glutaMax were obtained from Gibco (Invitrogen); and FBS (South America) from Bio Whittaker. Ochratoxin A was dissolved in methanol to obtain a stock solution of 5000 μg/mL. According to the data obtained in preliminary studies (Baldi et al., 2002; Cheli et al., 2003), racemic α-tocopherol was dissolved in absolute ethanol to prepare a stock solution of 10 mM.

These stock solutions were used for further dilutions in DMEM containing 0.6% FBS to obtain the final concentrations of each compound.

4.3.2 Cell culture

In this study, primary porcine fibroblasts isolated from embryo and from ear were used. Fibroblasts were grown in monolayers in 75-cm2 plastic culture

flasks (Nunclon, Nunc Denmark) in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 1% glutaMax, 2500 I.U./mL penicillin, 2.5 mg/mL streptomycin and 10% FBS. Primary fibroblasts were cultivated in a humidified atmosphere with 5% CO_2 at 37 °C and split 1:4 once a week. In all experiments in this study, we used embryonic porcine fibroblasts between passages 5 and 10 and ear between 5 and 8. All experiments were conducted in media containing 0.6% serum, the minimum useful concentration to maintain the selected *in vitro* models.

4.3.3 Determination of the half-lethal concentration and LDH release induced by ochratoxin A

Cells were seeded in 96-well culture plates (3000 cells/well, in 200 μL of complete medium) and were cultured for 24 h. A dose–response experiment was set up. Fibroblast cultures were exposed to increasing concentrations of OTA (0–10 μg/mL) for the following 24 or 48 h. The effects of OTA treatments on fibroblast viability were determined using the MTT test. Cellular viability was determined using a colorimetric assay based on the production of the chromophore formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT). Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase. At the end of the incubation, the media were removed, the monolayers were washed with PBS twice, 150 μL MTT stock solution (5 mg/mL) in PBS was added to each well, and the plates were incubated for 3 h at 37 °C in a humidified chamber. To dissolve the formazan, 150 μL dimethyl sulfoxide was added to each well, after discharging the MTT solution. Absorbance at 540 nm was determined on a Biorad 680 microplate reader (Bio-rad, Veenendaal, The Netherlands). Cells incubated with culture medium alone, representing 100% viability, were

included as negative controls in all experiments. The percentage cytotoxicity was calculated as follows: Percentage cytotoxicity = (1 – mean optical density in presence of OTA/mean optical density of negative control) × 100. From these data the half-lethal concentration (LC_{50}) of OTA for each primary cell culture was calculated. Cell membrane damage induced by OTA was detected by LDH release using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) as instructed by the manufacturer. Primary porcine fibroblasts were seeded in 96-well plates at the density as described above and cultured for 24 h. Afterward, the cells were exposed to OTA solutions (concentration range: 0–10 μg/mL) for 24 or 48 h. At the end of the incubation period the media were removed and cells washed with PBS twice.

LDH is a stable cytosolic enzyme released upon cell lysis. The amount of LDH was measured with an enzymatic assay using tetrazolium salts in conjunction with diaphorase. Briefly, after treatments supernatants were removed and centrifuged for 5 min at 1500 × g at 4 °C. 50 μL of each supernatant was transferred to a 96 well plate. Cells were lysed by adding 15 μL of 9% Triton X-100 solution in water per 100 μL of culture medium containing 0.6% of serum, followed by incubation for 1 h at 37 °C. Cells debris were removed by centrifugation for 5 min at 1500 × g at 4 °C and 50 μL of each sample was transferred to 96 well plate. Then, 50 μL of LDH substrate was added to the supernatants and cell lysates. After incubation for 30 min at room temperature in the dark, the enzymatic assay was stopped by adding 50 mL of 1 M acetic acid and the plate was read at 490 nm using a microplate reader. The percentage of LDH release was calculated as the amount of LDH in the supernatant over total LDH from both supernatant and cell lysate.

4.3.4 Detection and quantification of DNA damage induced by ochratoxin A

DNA fragmentation was measured with the diphenylamine method as described by Sandau et al. (1997). Primary porcine fibroblasts were seeded in 75-cm2 flasks (density: 0.55 × 10^5 cells/mL) and grown at 37°C in 5% CO_2 for 5 days. At sub-confluence, fibroblasts were incubated with OTA (0–10 μg/mL) for 24 h. After incubation, the media were removed and centrifuged at 1,800 × g for 20 min to collect the detached cells (fraction S). The fraction S was subsequently lysed in 5 mL of ice-cold lysis buffer [10 mM Tris, 1 mM EDTA (pH 8.0), 0.5% Triton X-100] for 30 min at 4 °C. The remaining adherent cells were scraped off the plastic and lysed in 5 mL of ice-cold lysis buffer as well.

After cell lysis, the intact chromatin (fraction B) was separated from DNA fragments (fraction T) by centrifugation for 20 min at 13,000 × g. Samples were treated with one volume of 25% trichloroacetic acid (TCA), precipitated overnight at 4 °C, and recentrifuged 20 min a 13,000 × g at 4 °C, and then the supernatants were removed. DNA was hydrolysed by adding one volume of 5% TCA to each pellet and heating 15 min at 90 °C in a heating block. DNA content was quantitated using the diphenylamine reagent. Afterward, 1600 μL of DPA solution (in 10 mL glacial acetic acid: 150 mg diphenylamine, 150 μL H_2SO_4 and 50 μL acetaldehyde 16 mg/mL solution) were added to each fraction and incubated for 4 h at 37 °C in the dark. The OD600 of each fraction (S, B, and T) was determined. The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant (T) to that in the pellet (B), considering also the quantity released by cells undergoing apoptosis and lysis during the experiment.

Percentage of fragmented DNA = [(S + T)/(S + T + B)] × 100

4.3.5 Detection of DNA damage induced by ochratoxin A by TUNEL (TdT-mediated dUTP nick end labeling) assay

Primary porcine fibroblasts were seeded at a density of 0.4 ×105 cells/mL in chamber slides, which had two chambers (Nunc Lab-Tek, Nunc, Denmark), which were previously coated with a thin layer of poly-L-lysine 0.01% (Sigma) to support cellular adhesion. Cells were cultured for 24 h in complete medium. Afterward, the media were removed and the fibroblast monolayers washed twice with PBS. Based on results obtained from previous assays, 0.6 μg/mL OTA and the appropriate LC_{50} (embryonic LC_{50}: 5 μg/mL OTA; ear LC_{50}: 1.2 μg/mL OTA) were added to each chamber slide for the following 24 h. At the end of the incubation, the media were removed and the cells fixed with 4% paraformaldehyde at room temperature for 25 min. The TUNEL assay was performed using the DeadEnd Colorimetric Apoptosis Detection kit (Promega, Madison, WI, USA). The monolayers were washed twice with PBS and permeabilized by immersing the slides in 0.2% Triton X-100 solution in PBS for 5 min at room temperature. After washing with PBS, cells were incubated with biotinylated nucleotide mixture together with terminal deoxynucleotidyl transferase enzyme. Horseradish peroxidase–labeled streptavidin (streptavidin HRP) was then added to bind to these biotinylated nucleotides, which are detected using the peroxidase substrate hydrogen peroxide and the stable chromogen diaminobenzidine (DAB). Afterward, to visualize and estimate the apoptotic and normal cells, haematoxylin staining was performed. Images (20X and 40X) were captured under an Olympus BX51 microscope. For each experiment, ~500 cells were counted in randomly selected fields, and the percent of TUNEL-positive cells was calculated.

4.3.6 Determination of the effect of a-tocopherol against OTA-induced toxicity

To evaluate the most suitable concentrations of α-tocopherol for interaction experiments with OTA, a dose–response curve for this compound was established using serial concentrations from the nanomolar to micromolar range (data not shown). Cell culture setup and conditions were as detailed above. Primary porcine fibroblasts were cultured with LC_{50} doses of OTA in the presence or absence of α-tocopherol (1 nM or 1 μM). Cell viability and LDH release after antioxidant treatment were assessed as previously described. To determine cell viability, primary fibroblasts were first pre-incubated for 3 h with α-tocopherol and then exposed to increasing concentrations of OTA for 24 or 48 h. Cells were also exposed to antioxidant alone or ethanol (the α-tocopherol solvent) alone to evaluate any non-specific effects. Inhibition of cytotoxicity was determined by MTT, LDH release, DNA fragmentation and TUNEL assays and calculated as the percentage inhibition (percentage cytotoxicity OTA–percentage cytotoxicity of (OTA + antioxidant)).

4.4. Statistical analysis

The data are expressed as means ± standard errors (SE). At least three replicates at each incubation time were performed, and all the experiments were performed twice. Obtained data were analyzed by one-way ANOVA (General Linear Models Procedure); Duncan's post-hoc multiple range test was used, with $P \leq 0.05$ considered statistically significant.

4.5. Results

4.5.1 Cytotoxic effect and LDH release induced by ochratoxin A

We first investigated the LC_{50} of OTA after 24 h and 48 h of treatment in ear and embryo porcine fibroblasts and found that the LC_{50} differed between the two cell types. At all incubation times, the fibroblasts derived from ear were the most sensitive to OTA cytotoxicity (LC_{50} = 0.93 µg/mL after 24 h; LC_{50} = 0.92 µg/mL after 48 h), while fibroblasts isolated from the embryo showed a time-dependent sensitivity (LC_{50} = 4.24 µg/mL after 24 h, 2.34 µg/mL after 48 h). Figure 6 shows the data on LDH release by primary porcine ear and embryo fibroblasts in the presence of several concentrations of OTA at 24 and 48 h of incubation. In both cell types, LDH release increased significantly ($P < 0.01$) at OTA concentrations above 2.5 µg/mL after 24 and 48 h of incubation.

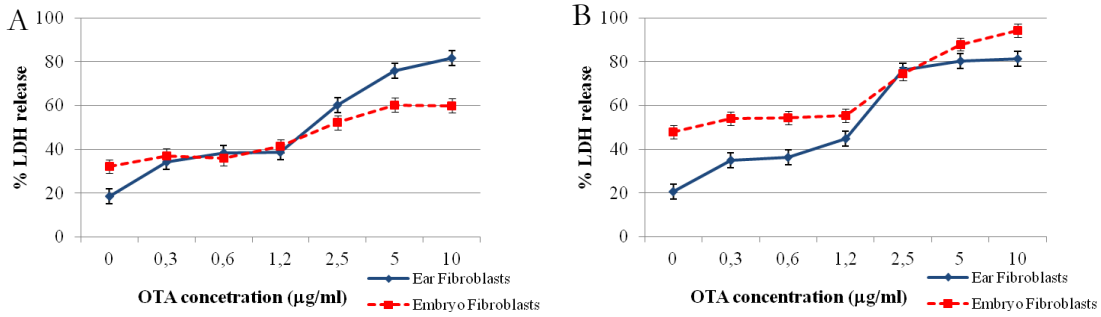

Fig. 6: Concentration-dependent release of LDH into culture media by primary porcine ear and embryo fibroblasts after 24 h (A) and 48 h (B) exposure to OTA concentrations.

4.5.2 Detection and quantification of DNA damage induced by ochratoxin A

OTA was able to induce DNA fragmentation in both primary porcine fibroblast cultures, as measured by a diphenylamine assay. After 24 h of incubation, in the absence of OTA (control), 14% DNA fragmentation in

both primary cultures occurred. A dose-dependent fragmentation in fibroblast cultures, due to the exposure to increasing concentrations of OTA, was found (Figure 7). At 0.6 μg/mL of OTA, the DNA fragmentation percentages of both types of fibroblasts were similar to control levels. Exposure to high OTA concentrations (2.5, 5, or 10 μg/mL) led to increasing percentages of DNA fragmentation in the two types of cells. At 10 μg/mL of OTA, DNA fragmentation was 92% in fibroblasts isolated from ear and 66% in embryonic fibroblasts.

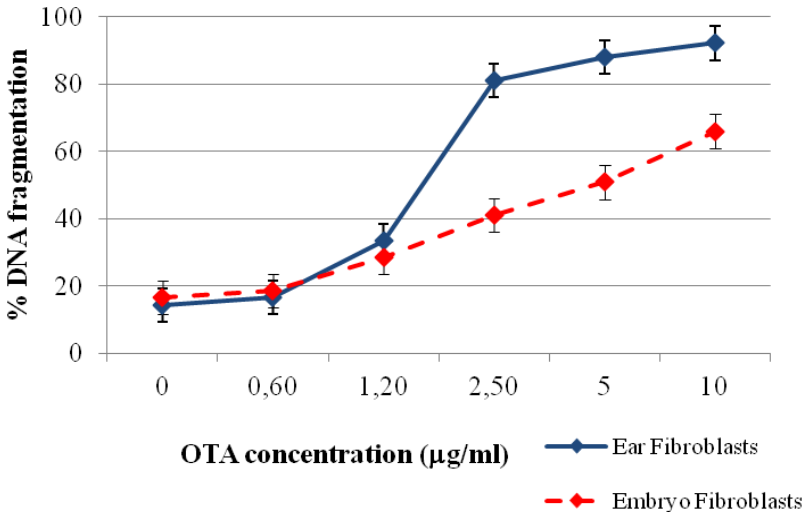

Fig 7: DNA fragmentation in primary fibroblasts 24 h post–OTA stimulation.

Cell death may occur by several mechanisms. It is well known that LDH release is a marker of cellular membrane damage, while DNA fragmentation measurements give an indication of the percentage of fragmented DNA out of the total nuclear DNA of cultured cells. A comparison of DNA fragmentation with LDH release after 24 h of OTA exposure is shown in Figure 8. After 24 h exposure to 0.6, 5, or 10 μg/mL OTA in both fibroblast

cultures, we observed that the changes in DNA fragmentation and LDH release were almost proportional.

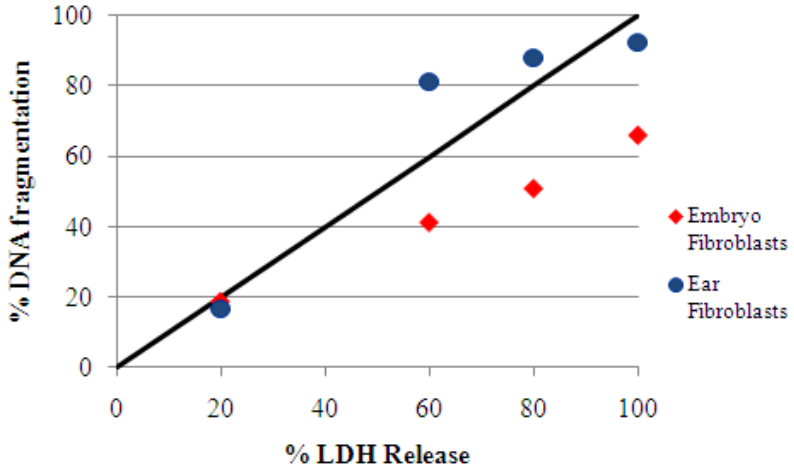

Fig 8: Comparison of DNA fragmentation and LDH release in fibroblasts cultures after 24 h of exposure to 0.6, 2.5, 5, or 10 µg/mL OTA. The solid line indicates an equal percentage of DNA fragmentation and LDH release. During necrosis, the experimental values should be close to this line (Gekle et al., 2000). In the presence of the selected OTA concentrations, in both cell cultures the experimental values indicated proportional changes in DNA fragmentation and LDH release.

4.5.3 Detection of DNA damage by TUNEL assay

In a series of experiments, we examined the effect of OTA exposure on fibroblast cultures using the TUNEL assay. Figure 9 shows representative photos of ear fibroblast morphology and nuclear stains in cells maintained in culture and exposed to OTA for 24 h. In the absence of OTA, the uniformity of monolayers, the typical fibroblast shape and cell-cell interactions in cultures were evident in fibroblasts from both sources tested. Only 4% and 6% of nuclei were apoptotic in cultures of primary ear and embryonic fibroblasts, respectively. The percentage of dark-brown apoptotic nuclei in primary fibroblasts, isolated from ear and embryo, co-incubated for 24 h with 0.6 µg/mL of OTA, were 32% and 16%, respectively. In both ear and embryo

fibroblast cultures after 24 of OTA incubation above LC_{50}, monolayers were completely destroyed and cell debris were evident in all the microscopic fields.

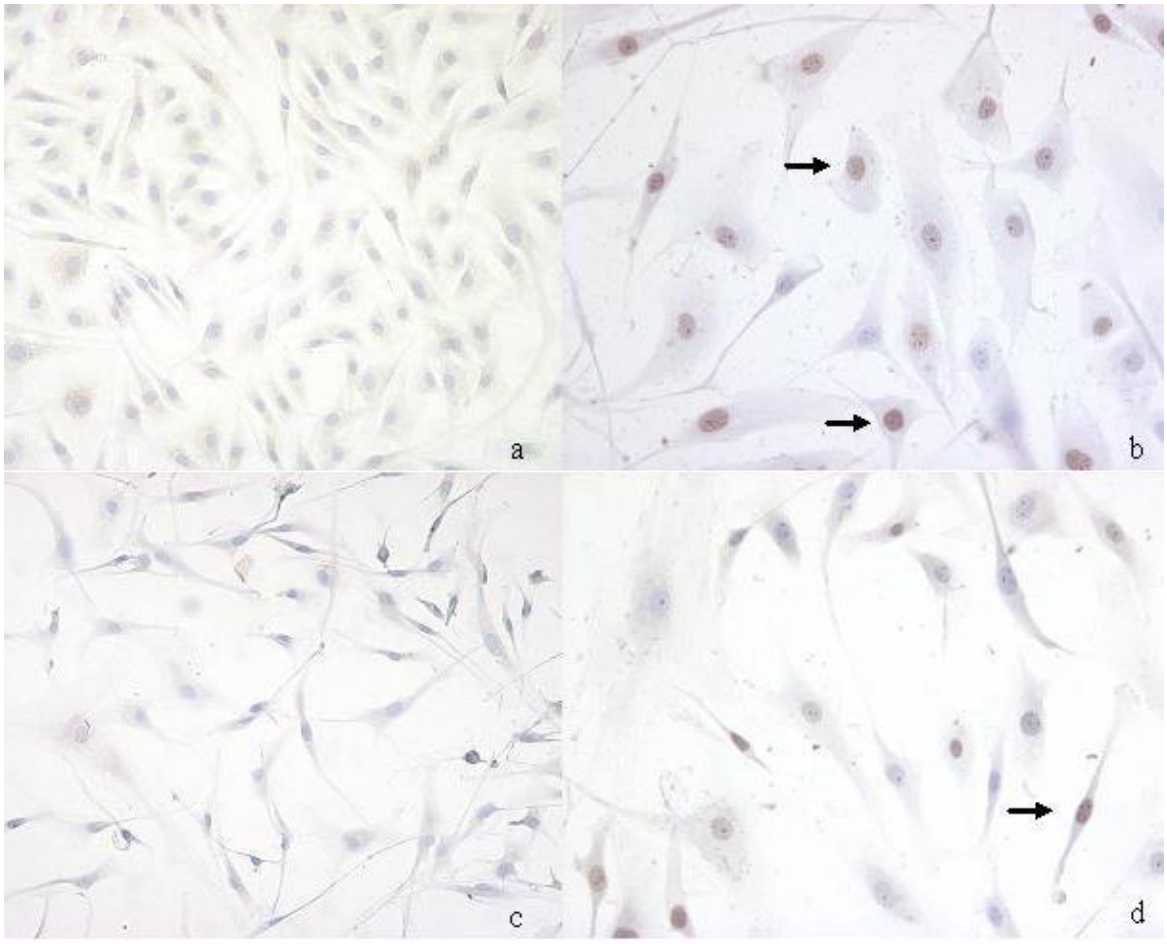

Fig 9: Representative photographs of selected fields of TUNEL-stained ear fibroblasts are shown. The TUNEL-positive nuclei, indicating apoptotic cells, are stained brown (black arrows), while the vital nuclei are stained violet (haematoxylin). (a) Ear fibroblasts in culture for 24 h (no OTA). (b) Ear fibroblasts treated with 0.6 µg/mL OTA for 24 h. (c) Ear fibroblasts treated with 1.25 µg/mL OTA for 24 h. (d) Ear fibroblasts treated with 1 nM α-tocopherol and 0.6 µg/mL OTA for 24 h. Bars = 200 µm.

4.5.4 Effect of a-tocopherol on ochratoxin A–induced toxicity

The inhibitory effects of α-tocopherol on OTA cytotoxicity were evaluated using several toxicity assays: MTT test, LDH release, DNA fragmentation and TUNEL assays. Pre-treatment with α-tocopherol (1 nM) for 3 h, followed by OTA exposure for 24 h, significantly ($P < 0.01$) reduced the loss of cell

viability induced by OTA in both *in vitro* models, as determined by the MTT test: by 17.5% in ear fibroblasts exposed to 0.3 µg/mL OTA and by 14.7% in embryonic fibroblasts exposed to 0.6 µg/mL OTA. Treatment with 1 µM of antioxidant solution did not affect OTA toxicity. After 48 h, α-tocopherol at both concentrations was unable to counteract the OTA-induced cytotoxicity in either cell type. The data on LDH release induced by OTA after 3 h pre-incubation with 1 nM or 1 µM α-tocopherol in fibroblast cultures are shown in Figure 10. At 24 h of incubation, in ear fibroblasts, α-tocopherol at both concentrations significantly ($P < 0.01$) decreased LDH release when OTA was present at 0.3–0.6 µg/mL. In embryonic fibroblasts at both concentrations of antioxidant, LDH release in the presence of all OTA concentrations used was significantly reduced ($P < 0.05$) compared to control cultures (OTA only). After 48 h of co-incubation with tocopherol and OTA, no difference in LDH release in either cell type was detected. These data confirm that the origin of the cells can not only explain the response to OTA stimuli, but also the sensitivity to antioxidant response.

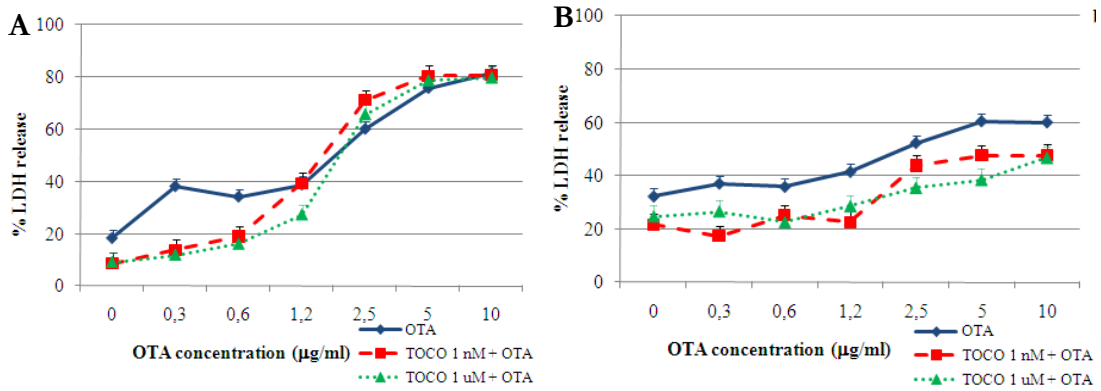

Fig 10: Effect of α-tocopherol on Ochratoxin -A-induced LDH release in primary porcine fibroblasts at 24 h. Values are means, with standard errors of the mean represented by vertical bars. A: Ear fibroblasts; B: Embryonic fibroblasts

The effects of 3 h pre-incubation with 1 nM or 1 μM α-tocopherol on OTA-induced DNA fragmentation in primary fibroblasts is shown in Figure 11. Treatment with 1 nM α-tocopherol resulted in a significant ($P < 0.05$) decrease in DNA fragmentation compared with control incubation (OTA only). This decrease was 15% in the presence of 5 μg/mL of OTA in ear fibroblasts and 16% in the presence of 10 μg/mL of OTA in embryonic fibroblasts.

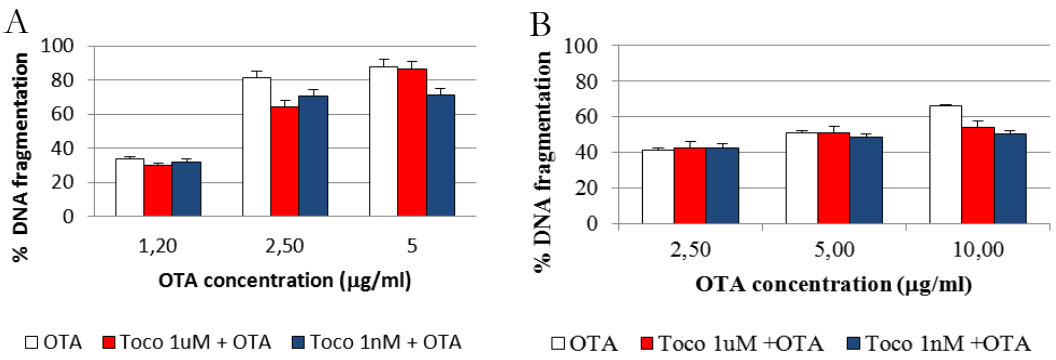

Fig 11: Effect of α-tocopherol on OTA-induced DNA fragmentation in primary porcine fibroblasts at 24 h. Values are means, with standard errors of the means represented by vertical bars. A: Ear fibroblasts; B: Embryonic fibroblasts

The effects of α-tocopherol pre-treatment on DNA fragmentation induced by OTA were evaluated by TUNEL assay. In ear fibroblasts α-tocopherol pre-treatment (1 nM or 1 μM) did not significantly reduce the percentage of apoptotic nuclei by 12% and 9%, respectively. A representative photograph of ear fibroblasts pre-incubated with α-tocopherol and co-incubated with 0.6 μg/mL OTA for 24 h is shown in Figure 9(d). In embryonic fibroblasts, α-tocopherol pre-treatment (1 nM or 1 μM) insignificantly decreased the percentage of apoptotic nuclei in the presence of 0.6 μg/mL OTA by 11% and 8%, respectively. α-tocopherol was not able to reduce the damage to nuclei when OTA concentrations were above LC_{50} (embryonic: 5 μg/mL

OTA; ear: 1.2 μg/mL OTA), since the cells of treated monolayers detached, and cellular debris floated in the observed fields (data not shown).

4.6 Discussion

The present work has shown an origin-dependent sensitivity to OTA. Previous studies have shown different cytotoxic responses to *in vitro* OTA challenges in different cell lines (Baldi et al., 2004). Our results confirm that the origin of the cells could explain the response to OTA stimuli, as reported by several groups (Schwerdt et al., 2007; Gekle et al., 2005). To date, most of the studies have been conducted using epithelial cells originated from several mammalian species, but only human fibroblasts were used in some studies (Russo et al., 2005; Schwerdt et al., 2007). In order to evaluate membrane damage, we have used LDH release as memabrane damage marker. In our study, after only 24 h of incubation, the fibroblast cultures showed considerable LDH release when OTA was present at concentrations similar to the doses used by Russo et al. (2005), indicating early cellular membrane damage. Schwerdt et al. (Schwerdt et al., 2007), in their studies on the long-term effects of OTA on primary fibroblasts, indicated that LDH release in the media increased only after five days of exposure to OTA, while Russo et al. (2005) reported a significant LDH release after 72 h of OTA treatment.

As is well known OTA was able to induce DNA fragmentation, our results confirm the different sensitivities of fibroblasts to OTA-induced cell damage. Russo et al. (2005) report that human fibroblast cultures show DNA damage after 72 h of OTA exposure at high concentration. However, OTA concentrations lower than the dose used by Russo et al. (2005) revealed that these damages occurred earlier and were present after 24 h of OTA exposure. The different origins of the cells (human vs. porcine) could explain the difference in sensitivity. Moreover, as indicated by O'Brien et al. (2001) the

cellular response to OTA toxicity related not only to the amount of the mycotoxin that gained access to the cells but also to the individual cell tolerance to OTA load. Cell death may occur by several mechanisms. It is well known that LDH release is a marker of cellular membrane damage, while DNA fragmentation measurements give an indication of the percentage of fragmented DNA out of the total nuclear DNA of cultured cells. Based on our data, we suggest that the cell death process induced by OTA involved both cellular and DNA damage. Gekle et al. (2000), showed that OTA exposure induces apoptosis and necrosis in the MDCK-C7 and MDCK-C11 cells, respectively, as indicated by comparing DNA fragmentation with LDH release.

At the cellular level, OTA toxicity involves various mechanisms of action including DNA damage. Although studies conducted by several groups (Petrik et al., 2003; Schwerdt et al., 2007; Gekle et al., 2000) indicate the apoptotic pathway as the means by which OTA induces toxicity *in vivo* and *in vitro*, the determination of predominant cell death pathways depends on several conditions, such as the experimental model, the dose of toxin and the duration of the exposure. The mechanisms and morphologies of apoptosis and necrosis are different, but there is an overlap between these two processes, in what has been called "necrosis–apoptosis continuum" (Zeiss et al., 2003). In fact, it is not simple to distinguish the predominant process, as apoptosis and necrosis can occur simultaneously (Elmore et al., 2007). Understanding the evolution of cell death in cellular models is critical. Therefore, once again, it is important not only to consider the concentration and duration of toxin exposure and the cell type, but also to employ multiple endpoint assays (O'Brien et al., 2001).

Taken together, our results indicate that α-tocopherol counteracts the toxicity of the mycotoxin OTA. In particular, the pre-incubation with α-tocopherol

reduced LDH release and DNA damage in both fibroblast cultures, thereby preserving the integrity of the cells. It is well known that α-tocopherol is fundamental to cellular defence mechanisms against endogenous and exogenous oxidant agents (Wang et al., 1999). This compound is a peroxyl radical scavenger that terminates the free radical chain reaction (Traber et al., 2007), and its hydrophobic nature stabilises its position in the phospholipid bilayer. In fact, in the core of the cellular membrane, as described by Traber and Atkinson (Traber et al., 2007), the antioxidant characteristics of α-tocopherol are due to its H atom donating ability, its position and movements in the cellular membrane, and its activity in cytosolic reduction reactions that recycle tocopheroxyl radicals, thus preventing lipid peroxidation. As demonstrated by Baldi et al. (2004), in MDCK and BME-UV1 cell lines, the cytotoxicity and ROS production induced by OTA are reduced by antioxidant pre-treatments. In particular, α-tocopherol significantly reduces OTA-induced ROS production. This inhibition is concentration-dependent: treatment with a higher concentration (10 mM) results in a significantly greater degree of protection against ROS production. OTA causes lipid peroxidation and free radical formation in mammalian cells. The oxidative metabolism sustained by enzymes such as cytochrome P450 enzymes and enzymes with peroxidase activities, is responsible for OTA biotransformation and subsequent ROS production (Manderville et al., 2008). The OTA toxicity reduction is due to the scavenging of lipid hydroperoxil radicals by vitamin E. This is due to the increase in the activity of the glutathione peroxidase, which utilizes GSH for catalyzing the reduction of hydroperoxides (Grosse et al., 1997).

Moreover, in BME-UV1 cells, α-tocopherol at two different concentrations (1 nM and 10 μM) reduces DNA fragmentation (Fusi et al., 2008). In this study, antioxidant pretreatment resulted in a reduction of DNA fragmentation by 2–5% in the presence of 0.6–2.5 μg/mL OTA. Schaaf and co-workers (Grosse

et al., 1997), in their studies on oxidative damage and free radical generation induced by OTA, observed that α-tocopherol at micromolar concentrations does not prevent the loss of cell viability in cultured renal cells. As described by Azzi (2007), the effect of α-tocopherol depends on the oxidative environment in which it is active. This interaction allows α-tocopherol to act as a sensor, monitoring the cellular environment through concentration changes and transferring the information from the membrane to the nucleus. The effects of α- tocopherol on protein kinase C and the other molecular signaling pathways, as suggested by Fazzio et al. (1997), could vary, considering the cell-specific pathways of cellular proliferation in which vitamin E can act.

Primary porcine fibroblast cultures offer new *in vitro* opportunities to study OTA cytotoxicity and the role of α-tocopherol in counteracting the several types of damage induced by this mycotoxin. OTA cytotoxicity developed through several mechanisms of action. The cellular inhibition associated with the LDH release and DNA fragmentation induced by OTA showed different sensitivities in the two fibroblast cultures. α-Tocopherol treatments could reduce the damage induced by OTA at different cellular levels. Our results point to the conclusion that the use of α-tocopherol offers new strategies to reduce OTA cytotoxicity, supporting its defensive role in the cell membrane and its multiple functions in cellular metabolism.

5. Evaluation of the protective effect of bovine lactoferrin in a bovine mammary epithelial cell line

5.1 Introduction

Milk has a composition that would be able to give the newborn everything he needs to grow and develop properly in the first months of life. Milk, in fact, provide not only proteins, fats and nutrients needed for growth, but also a number of biologically active proteins, provided also by colostrum, involved in ensuring the development of the neonate and his state of health. Among these active proteins lies lactoferrin (Lf) an 80 kDa iron-binding glycoprotein that is a member of transferrin protein family and shows high homology among species.

Lf is a multifunctional protein that is involved in a wide variety of physiological functions ranging from regulation of iron absorption to antimicrobial activity.

Because of the multiple beneficial functions of Lf, it is considered as a "nutraceutical protein" (Vogel, 2012). Lf has been used for clinical trials and industrial applications, whose first application was in infant formula but currently it is added to immune system-enhancing nutraceuticals, cosmetics, pet care supplements, drinks, fermented milks (Garcìa-Montoya et al., 2012).

The first to isolate Lf from bovine milk were Sorensen and Sorensen in 1939, afterwards three independent laboratories (Groves et al., 1960; Johanson et al., 1960; Montreuil et al., 1960) concluded that this protein was the main iron binding protein in human milk.

As a bioactive component of the mammary secretions, Lf is endowed with modulator roles and protective functions for the newborn, and its biological activity affects the maternal mammary gland as well (Baldi et al., 2005b; Pecorini et al., 2005; Pecorini et al., 2009).

Lf is produced in mammalian species such as human and cow, by glandular epithelial cells. This glycoprotein is abundant in human colostrum with an amount major of 7 g/L and approximately 1-2 g/L in mature milk (Hu et al., 2012). It was also found in several external mucosal secretions, including saliva, nasal and bronchial secretions as well as in bodily fluids such as blood and plasma. Several studies have suggested that Lf can be classified as an acute-phase protein because of high concentration in the blood during most inflammatory reactions, infection, and excessive intake of iron and tumor growth (Adlerova et al., 2008). In fact it has been shown that Lf is able to bind iron ions even at low pH, this characteristic is important on the site of infection and inflammatory where pH may decrease fewer than 4.5. In this situation Lf can prevents bacterial proliferation binding iron release from transferrin. Several studies have shown that some of the Lf' functions are not connected with its ability to bind iron (Adlerova et al., 2008) but are mediated by the interaction with different cellular structure such as lipopolysaccharides , proteoglycans and cell-type receptors (Puddu et al., 2010).

5.1.1 Structure of Lactoferrin

Metz-Boutique et al. (1984) have discovered the molecular structure and amino acid sequence of human Lf. They have classified it as a member of the transferrin family owing to its identity of 60% with serum transferrin.

Baker and Baker (2005) have analysed three-dimensional configuration of Lf by crystallographic analysis revealing a highly conserved structure with some

differences between species. In spite of Lf is considered a member of transferrin family differs from it for some of its important properties. One of the differences is the biological location: transferrin is mainly located in bloodstream while Lf in exocrine secretions. Lf is also able, contrary to transferrin, to retain iron binding capacity at low pH (Valenti and Antonini, 2005). In the intestinal epithelial cells transferrin is located only in cytoplasm upon internalization while Lf can be found both in cytoplasm and into the nucleus (Ashida et al., 2004).

Another important feature of Lf that differentiates it from transferrin is its bactericidal ability that is linked to its positive charge concentrated in N-terminus region (1-7 amino acids), in the first helix (13-30 amino acids) and in the region that connects the two lobes (Puddu et al., 2010).

Lf is a polypeptide chain of ca 700 amino acids folded into two symmetrical lobes (N and C lobes) that are connected by a hinge region containing parts of a α-helix between amino acids 333 and 343 in human Lf (hLf), which provides additional flexibility to the molecule (Öztaş Yeşim et al., 2005).

N and C lobes are composed of α-helix and β-pleated sheet structures that create two domains for each lobe called C1, C2, N1 and N2. These domains create one iron binding site on each lobe that can bind two ferric ions in synergy with the carbonate ion (CO_3^{2-}) (Vogel, 2012). Amino acids involved in iron-binding ability are Asp, Tyr and His, whilst Arg is involved in the bond with the CO_3^{2-} ion (Fig.12).

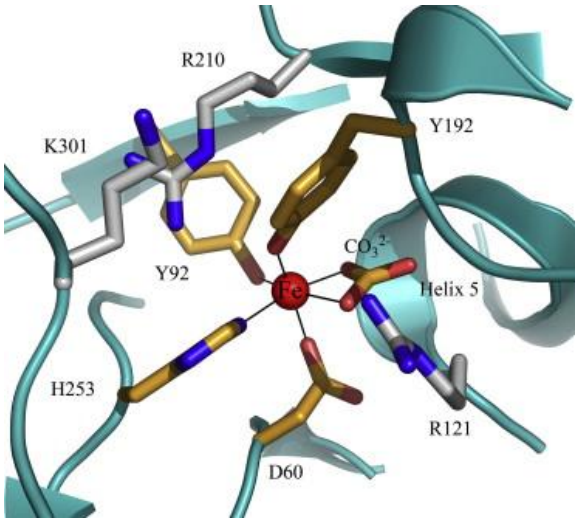

Fig. 12: Binding between Lf and Fe (Baker and Beker, 2009)

Although Lf binds Fe^{2+} or Fe^{3+} ions, it has also been observed bind other ions, such as Cu^{2+}, Zn^{2+} and Mn^{2+} and molecules such as lipopolysaccharides, heparin, DNA (Adlerova et al., 2008). The primary structure of Lf shows the number and position of Cys residues that allow the formation of intramolecular disulphide bridges; Asn residues in the N- and C- terminal lobes provide several potential N-glycosylation sites. The bound between Lf and Fe^{3+} is reversibly so Lf can exist in three different forms: apo-Lf (iron free), holo-Lf (binds two Fe^{3+} ions) (Fig.13) and monoferric form (one ferric ion) (Adlerova et al., 2008). The last one shows greater resistance to proteolysis (Öztaş Yeşim et al., 2005; Drago, 2006).

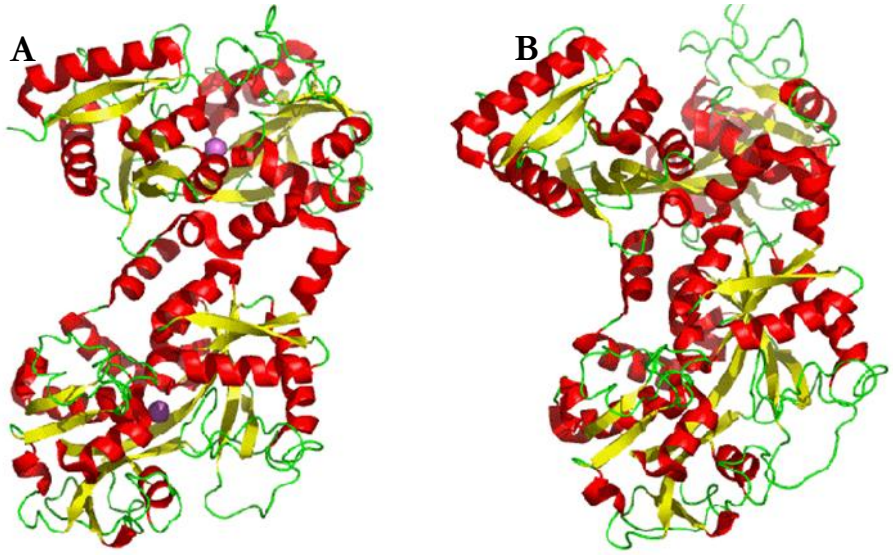

Fig 13: apo-Lf (A)and holo-Lf(B) (http://chemistry.umeche.maine.edu/CHY431/Conformation3.html)

According to the species, Lf contains a variable numbers of sites for potential glycosylation; the degrees of glycosylation determine the resistance to proteases or to low pH.

5.1.2 Regulation of Lactoferrin synthesis and its receptor

Lf is expressed in several tissues in human and in animals. It is expressed both constitutively, such as in mucosal surfaces, and inducibly, such as in some tissues by external agents (Garcìa-Montoya et al., 2012).

Kang et al. (2008) have analyzed 60 sequences of Lf genes with full coding regions finding that the length of the gene varies from species to species, from 2055 to 2190 residues, owing to detections, insertions and mutations in the stop codon.

Regulation of Lf synthesis depends of the type of cells that produce it. In the case of reproductive tissues the amount of Lf production is under the control of estrogens while its production in mammary gland is controlled by prolactin (González-Chávez et al., 2009). Endometrium production of Lf is determined

by both estrogens and epidermal growth factor. In neutrophils, the Lf synthesis happens during differentiation phase and is subsequently stored in specific granules. When neutrophils are mature they stopped production of Lf that is stored in the secondary granules (Adlerova et al., 2008).

Synthesis' regulation of the Lf varies, not only based on the type of cell that produces it, but also according to age, gender and in particular changes of life of the individual as for example the pregnancy during that there is a progressive increase up to 29th week, afterwards concentration of Lf settles at a constant level (Adlerova et al., 2008).

5.1.3 Antibacterial activity of Lactoferrin

Lf has shown anti-bacterial activity against Gram negative, Gram positive and some acid-alcohol-resistant bacteria both *in vivo* and *in vitro* studies. Lf can support the growth of bacteria considered as beneficial with lower iron demands such as *Lactobacillus spp*. and *Bifidobacterium spp*. (Chih-Ching et al., 2011)

Antibacterial function is divided into bacteriostatic and bactericidal activities; the first one is linked to the ability of Lf to bind and sequester the Fe^{3+} ion limiting its use by bacteria and consequently inhibiting the growth of them. Of course, bacteria have developed mechanisms to counteract iron sequestering action from Lf releasing iron chelating compounds called siderophores that are able to compete with Lf for Fe^{3+} ions. Other types of bacteria express specific receptors able to of bind Lf changing in its tertiary structure and consequently leading to iron dissociation (Adlerova et al., 2008).

Lf exhibits Gram negative bactericidal activity. Lf, interacting with lipopolysaccharides (LPS), is able to damage external membrane of Gram negative bacteria. Lf prevent the interaction between LPS and the bacterial

cations (Ca^{2+} and Mg^{2+}) by positive charge of its N-terminal region; this interaction causes release of LPS from the cell wall increasing membrane permeability damaging bacteria (González-Chávez et al., 2009).

LPS is the major constituent of the outer membrane of Gram-negative bacteria and is considered one of the most potent stimuli of the innate immune response. LPS consists of three parts: a core oligosaccharide, a distal hydrophilic O side chain and a highly conserved lipid A portion (Puddu et al., 2010). The last one is responsible for LPS toxic pro-inflammatory properties.

The interaction between N-terminus of Lf and LPS in addition to damaging bacteria is able to potentiate the action of other antibacterial such as lysozyme (Garcìa-Montoya et al., 2012).

Bactericidal action against Gram positive bacterial is based on binding among positive charge of Lf structure and anionic molecules present on the bacterial surface (for example lipoteichoic acid); this bond reduces negative charge on the cell wall favoring antibacterial action of lysozyme (González-Chávez et al., 2009). Lf has shown ability to prevent the attachment of some bacteria to the host cell; probably by binding between oligomannoside glycans of Lf and adhesins of bacteria although the mechanism of action is not yet known (Drago, 2006).

5.1.4 Lactoferrin in iron metabolism

The ability of an organism to regulate iron homeostasis is of primary importance for its proper function. Whether this micro-nutrient, that is required in many metabolic functions, is present in excess, it is toxic, promoting microbial growth and cell damages.

Bindings between lactoferrin and enterocytes are mediated by specific receptors and determine Lf degradation with consequent release of Fe^{3+} ions.

A deficiency of intracellular iron may determine an increase in expression of specific receptors on the surface of enterocytes and, consequently, an elevated absorption of lactoferrin-bound iron (Suzuki et al., 2005). The high concentration of Lf in milk, associated with high bioavailability of iron in milk, suggests that this glycoprotein is involved in iron absorption in the intestine of the newborn. In breast-fed infants there was a better iron accessibility than infants on formula; conversely, other researches, have observed a higher iron absorption in infants fed lactoferrin-free human milk (Adlerova et al., 2008). It seems that Lf property to bound Fe^{3+} ions is not linked mainly to its role in regulating iron homeostasis but to other functions of it.

5.1.5 Immunomodulatory and anti-inflammary activity

Wakabayashi et al. (2006) have conducted a study on the expression of 20 immune-related genes in the small intestine of mice fed bovine Lf (bLf) (2.5 g/Kg). They have observed that it can modulate the expression of these genes by both specifically and non-specifically mechanisms.

Thanks to its ability to bind iron and interact with target cells and molecules, Lf is considered a modulator of the innate and acquires immune system (Legrand et al., 2006). On the one hand the bound between positive charge of Lf and negatively charge of molecules on the surface of some immune system' cells strengthen the immune response supporting proliferation, differentiation and activation of immune system cells. On the other hand Lf has shown an anti-inflammatory action, in fact its ability to bind components of bacteria cell walls such as LPS allow it to prevent the development of inflammation (Adlerova et al,. 2008).

Lf has shown anti-inflammatory activity by the inhibition of pro-inflammatory cytokines such as interferon-gamma, tumor necrosis factor-alpha and

interleukin (IL)-1β, IL-2 and IL-6. At cellular level, Lf increases number of natural killer cells, boost the recruitment of polymorphonuclear cells in the blood, induces phagocytosis and can modulate the myelopoietic process (González-Chávez et al., 2009).

Some studies (Legrand et al., 2004; Öztaş Yeşim et al., 2005) have shown that Lf can activates different signaling pathways by be transported into the nucleus and bind DNA, activating different signaling pathways.

Furthermore, since iron is a catalyst for the production of reactive oxygen species (ROS), Lf binding iron action can reduce the harmful influence of ROS produced by leukocytes during inflammation (Adlerova et al., 2008; Ward et al., 2005).

In addition to Lf antimicrobial activity, is well-known its antiviral activity against wide-range of RNA and DNA viruses infected both humans and animals and its antifungal activity against for example *Candida spp.*

5.1.6 Enzymatic activity

Lf has been shown an enzyme-like activity. Among milk proteins, Lf has shown the highest levels of amylase, DNAse, RNAse and ATPase activities (Garcìa-Montoya et al., 2012). Lf enzyme-like activity seems to be attributed to: the multiple isoforms of this protein, degrees of glycosylation; tertiary structure; and the degree of oligomerisation (González-Chávez et al., 2009).

5.1.7 Bioactive peptides derived from lactoferrin

By digestion, fermentation and enzymatic digestion Lf generates bioactive peptides which can exhibit different properties respect native Lf.

In 1992 it has been identified a region, called lactoferricin B (LFc B), that shows a greater antimicrobial activity than the full molecule. Subsequently

(Rodríguez-Franco et al., 2005) was observed that region corresponding to LFc B in bovine (residues 17-41) corresponds to residues 12-48 in other mammalian species with highly homologous sequences. Of course during the hydrolysis of bLf many peptides are generated, characterizing them it was found that minimal variations in amino acid sequence have effect on antimicrobial activity of peptide. For example LFampin 268-284 and LFampin 265-284 that differs in only three amino acids presented different antimicrobial activity (van der Kraan et al., 2006).

The bactericidal action of Lf is mediated by its lactoferricin (Lfcin) domain. Several studies have demonstrated that Lfcin is critical also for many binding properties to cell-surface molecules such as glycosaminoglycans, to bacterial surface molecules and LPS (Senkovich et al., 2007; Valenti and Antonini, 2005; Puddu et al., 2010).

5.1.8 In vitro evaluation of Lf protective role against cytotoxic stimuli

Lf is an innate factor of the mammalian immune system; in particular in the bovine mammary gland bLf concentration dramatically increases in response to infection. Several studies have been conducted in order to evaluate the contribution of mammary epithelial cells in secreting immunological components after exposure to endotoxins using primary bovine mammary cell culture systems (Okada et al., 1997; Wellnitz and Kerr, 2004), the bovine mammary epithelial cell line MAC-T (Boudjellab et al., 1998), the murine mammary epithelial cell line HC11 (Zheng et al., 2006; Li et al., 2009) and bovine mammary explants (Rabot et al., 2007a).

The established bovine mammary epithelial cell line BME-UV1 is an *in vitro* model of the bovine mammary epithelium that is able to synthesize α-lactoalbumin and αs1-casein as well as express the components of the

plasmin-plasminogen system, that play a pivotal role in mammary gland development and defense mechanisms (Baldi et al., 1996; Politis, 1996; Cheli et al., 1999, Cheli et al., 2003).

As describe above Lf could contribute to neutralize the effect of endotoxins through binding to their lipid A portion (Appelmelk et al, 1994; Baker and Baker, 2005).

5.2 Aim

With regard to this part of my PhD concerning Lf study, the aim was to evaluate the protective role of bLf, added to the culture medium, against a cytotoxicity stimuli identified in LPS. Furthermore I assessed if BME-UV1 cells are able to express endogenous bLf after *in vitro* exposure to LPS.

5.3 Materials and methods

5.3.1 Cell line and cell culture

The BME-UV1 cell line was created at the University of Vermont from primary bovine mammary epithelial cells in culture by stable transfection with SV-40 large T-antigen (Zavizion et al., 1996b) and is maintained in the Laboratory of Cell Culture – Department VSA (University of Milan; Italy). Cells are routinely cultivated into 75 cm2 tissue culture flasks (Costar, Corning, NY, USA), in a mixture of 50% DMEM-F12, 30% RPMI-1640 and 20% NCTC-135 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Cambrex, Belgium), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1 μg/ml insulin, 5 μg/ml transferrin, 1 μg/ml hydrocortisone, 0.5 μg/ml progesterone, 10 μg/ml L-ascorbic acid and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml). All medium supplements are from Sigma-Aldrich.

The cells are maintained at 37°C in a humidified 5% CO_2 incubator until confluence. Cells used in the present work were at passage numbers between 37 and 40.

5.3.2 Determination of Inhibitory Concentration 50 of LPS

Cell viability after incubation with LPS was determined by measuring the production of the chromophore formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma-Aldrich), as previously described (Baldi et al., 2004). Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase.

At confluence cells were detached from the flasks by trypsinisation (Trypsin-EDTA 0.05%, Sigma-Aldrich) and re-suspended in complete culture medium to a concentration of 1.5×10^5 cells/ml. Then, cells were dispensed into wells of a 96-well tissue culture plate. After 24 h of incubation at 37°C in an atmosphere of 5% CO_2, medium was removed and cell monolayer was washed three times with Hanks' Balanced Salt solution (HBSS, Sigma-Aldrich). BMEUV1 cells were then exposed to increasing concentrations of *E. coli* LPS O111:B4 (0-0.75- 1.5-3.12-6.25-12.5-25-50-100 μg/ml) (Sigma-Aldrich) in DME-F12 medium without FBS.

Cells incubated with DME-F12 medium alone, representing 100% viability, were included as negative controls. After 24 h media were removed, 150 μl MTT stock solution (5 mg/ ml) in PBS were added to each well and the plates were incubated for 3 h at 37°C in a humidified chamber. The reaction was terminated by removing the incubation solution and adding 150 μl dimethyl sulfoxide to dissolve the formazan. The optical density of the dimethyl sulfoxide solution at 540 nm was determined on a Biorad 680 micro plate reader (Biorad, Hercules, CA, USA). The percentage cytotoxicity was

calculated as follows: Percentage cytotoxicity = 1 x (mean optical density in presence of LPS / mean optical density of negative control) x 100.
From these data the Inhibitory Concentration 50 (IC_{50}) of LPS was calculated. At least three replicates were performed and the experiment was performed at least twice.

5.3.3 Evaluation of the protective effect of bLf against LPS cytotoxicity

In order to examine the protective role of bLf against LPS cytotoxicity, BME-UV1 cells were re-suspended in complete culture medium to a concentration of 1.5×10^5 cells/ml and dispensed into wells of a 96-well tissue culture plate. After 24 h of incubation at 37°C in an atmosphere of 5% CO_2, medium was removed and cells were washed three times with HBSS. They were subsequently treated with DMEM-F12 medium containing 30 and 50 μg/ ml of LPS O111:B4 in the presence or absence of various concentrations of bLF (6.25-12.5- 25-50 μg/ml). bLf was from bovine milk and was purchased from Sigma-Aldrich. LPS doses were selected according to the IC_{50}, while bLf concentrations were chosen according to previous experiments (Pecorini et al., 2006). Negative control consisted of DMEM-F12 alone. After 24 h of treatment, the inhibition of LPS cytotoxicity by bLf was assessed by MTT test, as described earlier. The experiment included at least three replicates per treatment and was repeated at least twice.

5.3.4 Isolation of total RNA after LPS exposure and evaluation of its quality

The isolation of total RNA from BME-UV1 cells after LPS treatments was performed after assessing the effect on BME-UV1 viability of sub toxic levels of endotoxins, selected according to the cytotoxicity test carried out previously. Cells were seeded at the concentration of 1.5×10^5 cells/ml into

wells of a 96-well tissue culture plastic plate in complete culture medium containing 10% FBS. After 24 h, media were removed and cells were washed three times with HBSS. They were then treated with DME-F12 medium containing sub toxic concentrations (1-10-20 μg/ml) of LPS O111:B4. Cells incubated with DME-F12 medium alone, representing 100% viability, were included as negative controls.

After 3, 6, 12 and 24 h of incubation in the presence of LPS, cell viability was assessed by MTT test, as described earlier. There were at least three replicates per treatment and the experiment was repeated at least twice. In order to isolate total RNA, BME-UV1 cells were seeded in 60-mm Petri dishes at the concentration of $0.2–0.4\times10^6$ cells/ml in complete medium containing 10% FBS.

After 24 h, media were removed, cells were washed three times with HBSS and complete medium containing 1, 10 or 20 μg/ml of LPS was added. Control consisted in complete medium with omission of LPS. After 3, 6, 12 and 24 h, total RNA was extracted by TRI Reagent (Sigma-Aldrich) following manufacturers' instructions and stored at −80°C.

The quality and the integrity of total RNA samples from LPS treated and control BMEUV1 cells were confirmed by the following approaches. Firstly, the optical density OD260 nm/OD280 nm absorption ratio was measured by a NanoDrop ND-1000 UV-Vis Spectrophotometer. Secondly, RNA samples were separated on 1.6% agarose gel containing formaldehyde and stained with etidium bromide. The presence of intact 28S and 18S rRNAs was checked under ultraviolet light. Finally, cDNA of β-actin was detected by reverse transcription-polymerase chain reaction (RT-PCR). First-strand cDNA was synthesized by reverse transcription with M-MuLV Reverse Transcriptase (Fermentas, Burlington, Canada) and random nonamers primers (Sigma-Aldrich) at 37°C for 60 min.

Negative controls were prepared under the same conditions without reverse transcriptase. cDNA (1/10 of reverse mixture) of β-actin was amplified by using the forward primer ACT-for and the reverse primer ACT-rev (Table 2). Amplification reactions were performed in Gene Amp PCR System 9700 (Perkin Elmer, Waltham, MA, USA) with the enzyme AccuTaq LA DNA Polymerase (Sigma-Aldrich) by initial denaturation at 96°C for 30 s, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 68°C for 30 s and final extension at 68°C for 10 min. PCR products were separated on 2% agarose gel in 1% TBE buffer and stained with etidium bromide. Their expected size was 178 bp.

Gene	GenBank Accession Number	Primer name	Primer sequence (5'→3')	Product length
β-actin	NM_173979	ACT-for	ATCGTCCGTGACATCAAGGAG	178 bp
		ACT-rev	CTCTTCCAGCCTTCCTTCCT	
bLf	L08604	bLf-for 1*	GGCCTTTGCCTTGGAATGTATC	338 bp
		bLf-rev 1*	ATTTAGCCACAGCTCCCTGGAG	
		bLf-for 2	CCACCAGCAGGCTCTGTTTG	132 bp
		bLf-rev 2	CGTTGGTCTGCCTCCAAGT	
		bLf-for 3	CGACAGTGTTTGAGAACTTGCC	131 bp
		bLf-rev 3	GACAGCATGAGAAGGGACCTG	
*** The sequence of these primers was from Pfaffl et al. (2003)**				

Table 2: Sequences of PCR primers for β-actin and bLf. Primer names, GenBank Accession number of the used published bovine nucleic acid sequences and products length are indicated

5.3.5 Evaluation of bLf mRNA expression by Real-Time PCR

In order to evaluate whether BME-UV1 cells are able to express endogenous bLf after *in vitro* exposure to the different LPS treatments, a Sybr Green Real-

Time PCR approach was used. First-strand cDNA was synthesized by reverse transcription with M-MuLV Reverse Transcriptase (Fermentas) and random nonamers primers (Sigma-Aldrich) at 37°C for 60 min. Three primer pairs for the amplification of a fragment of the bLf cDNA were tested (Table 2). The primer pair bLf-for1 and bLf-rev1 was designed by Pfaffl et al (2003), while the other two bLf primer pairs were designed for this study using Primer3 v 0.4.0 (Rozen and Skaletsky, 2000). β-actin was chosen as the housekeeping gene (Fusi et al., 2002) and the primer pair ACT-for, ACT-rev was used (Table 2). All Real-Time PCR reactions were performed on Biorad IQ5 (Biorad) using 400 nm of each primer, 12.5 μl of BIORAD IQ Sybr-Green Supermix (Biorad) and various cDNA quantities were tested, ranging from 25 ng to 1 μg. 25 ng of cDNA obtained from bovine mammary gland tissue was included as a positive control in all reactions. The thermal profile used for all experiments was as follows: 95°C for 2 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final melt curve from 55°C to 95°C with increment of 0.5°C per cycle.

5.4 Statistical analysis

Results are presented as mean and SD. Effects of various treatments were evaluated by oneway analysis of variance using the GLM procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Mean comparisons were done using Duncan's multiple range test with $P<0.05$ as the level of significance.

5.5 Results

5.5.1 Protective role of bLf against LPS cytotoxicity

The cytotoxicity of LPS on BME-UV1 cell line was evaluated by MTT test. After 24 h, the IC_{50} was 47.44 µg/ml. According to the IC_{50}, BME-UV1 cells were treated for 24 h with two concentrations of LPS, 30 and 50 µg/ml, in order to examine the protective effect of exogenous bLf against endotoxins cytotoxicity. In the presence of 30 µg/ml of LPS, bLf treatments did not limit significantly endotoxins cytotoxicity, whereas in the presence of 50 µg/ml of LPS, bLf contributed to reduce significantly ($P<0.05$) LPS cytotoxicity (Fig. 14).

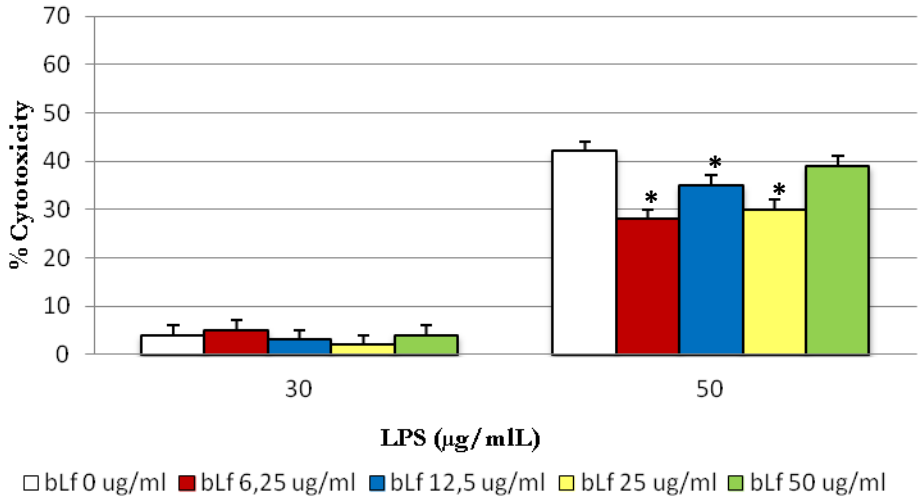

Fig. 14: Evaluation of the protective effect of exogenous bLf against LPS cytotoxicity. BME-UV1 cells have been treated with both LPS and bLf for 24 h, and the defensive role of bLf has been evaluated by MTT test.
*P:<0.05

5.5.2 Investigation of bLf mRNA expression after LPS exposure

The effect of sub toxic concentrations of LPS, chosen according to the cytotoxicity test, was evaluated by MTT test. As shown in Fig. 15, sub toxic levels of LPS did not evidenced cytotoxic effects on BME-UV1 cells.

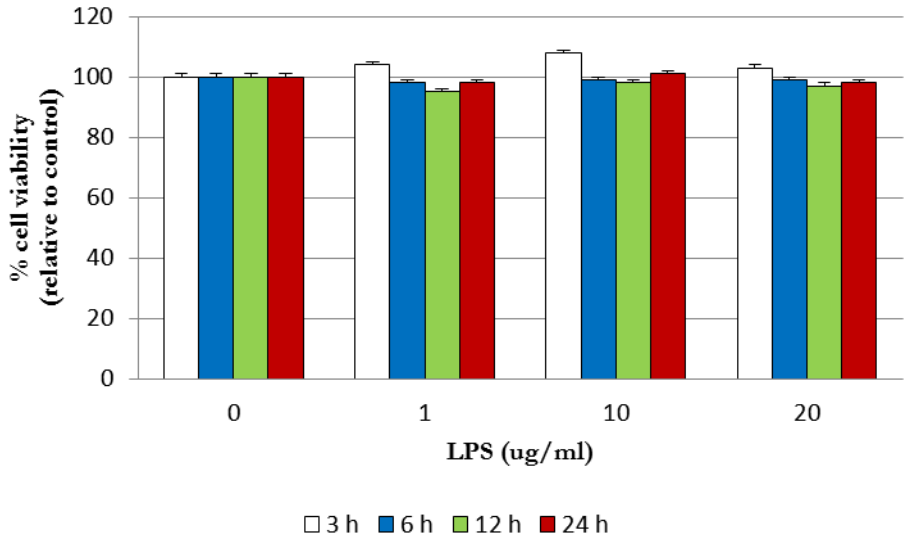

Fig. 15: Effect of sub toxic levels of LPS on the viability of BME-UV1 cells. Cells have been treated with sub toxic concentrations of LPS, as described in the Materials and Methods section, for 3, 6, 12 and 24 h. The effect of the treatments on cell viability has been evaluated by MTT test

The total RNA samples extracted from BME-UV1 cells after LPS treatments revealed optical density OD260nm/OD280nm absorption ratios comprised between 1.98 and 2.00, thus documenting the good quality of the nucleic acid. The total RNA integrity was confirmed by the presence of intact 28S and 18S rRNAs under ultraviolet light after denaturated agarose gel (Fig. 16a).

Moreover, the expected band of 178 bp, corresponding to the β-actin, was detected in all samples, whereas no amplification product was found in control samples without reverse transcriptase, thus confirming the quality and the integrity of the total RNA extracted (Fig. 16b). Real-Time PCR on bLf was performed on all cDNA samples, testing different cDNA quantities, and did not show any amplification of bLf in any of the cDNA examined, neither from LPS treated cells nor from control cells. Successful amplification of bLf from mammary gland tissue cDNA (data not shown) indicates the

effectiveness of the three primer pairs used, while successful amplification of β-actin from cell line cDNAs confirms the quality of the templates.

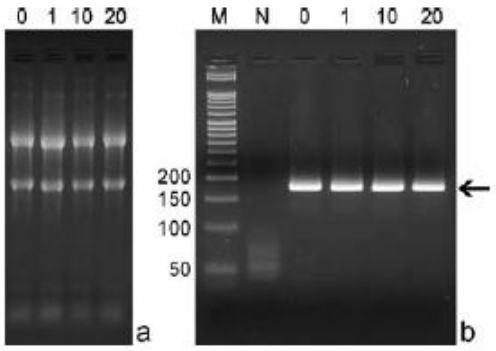

Fig. 16: Representative image of the integrity and quality of total RNA extracted from BME-UV1 cells after 24 h of LPS treatments by denaturing gel electrophoresis (a) and β-actin amplification (b). The amplification of β-actin produced a band of 178 bp (arrow), whereas no amplification product was found in control samples without reverse transcriptase (N). 0, 1, 10 and 20 refer to the LPS treatments. M=50 bp DNA ladder

5.6 Discussion

The present work has shown that exogenous bLf has a protective effect against LPS cytotoxicity in the BME-UV1 *in vitro* model. Depending on the dose, LPS not only are toxic to a number of target cells, but also significantly alter normal cell functions (Buommino et al., 1999). Since cells were incubated in presence of both bLf and LPS, the defensive role of bLf could be related to its cationic nature, which allows the neutralization of LPS by binding to their anionic lipid A portion (Appelmelk et al., 1994; Puddu et al., 2010). Several studies have documented the protective effect of Lf against endotoxin mediated damage. Hirotani et al. (2008) have reported the defensive role of Lf against LPS mediated intestinal mucosal damage in human intestinal epithelial Caco-2 cell line and *in vivo* studies have provided evidence that orally administered bLf has a beneficial effect against a lethal dose of LPS, thus preventing septic shock (Lee et al., 1998; Kruzel et al., 2000). Tian et al. (2010)

have reported the cytoprotective effect of bLf against endotoxin LPS in both immune cells and colon epithelial cells. bLf is also a protective factor against LPS-induced diarrhea in mice, by modulating the cyclooxygenase pathway in the gut (Talukder and Harada, 2007). In the mammary gland LPS neutralisation by Lf may contribute to counteracting tissue damages induced by oxidative stress at the sites of inflammation during mastitis (Nuijens et al., 1996). In support to this, accumulating evidence has pointed out to a role for Lf as a potent antioxidant molecule. Lf is involved in the inhibition of reactive oxygen species production mediated by the binding of LPS to L-selectin on neutrophils (Baveye et al., 2000). In addition, the protein serves as a scavenger of free iron, which accumulates in inflamed tissues and provokes the release of hydroxyl radicals (Legrand et al., 2006). It has been reported that in neurodegenerative diseases, in which iron accumulation leads to oxidative stress and neuronal death, Lf is overexpressed in specific areas of the brain, thus supporting the role of Lf as a protective factor against oxidative damage (Fillebeen et al., 2001). In addition, Kruzel et al. (2009) have shown that Lf attenuates oxidative insult at the mitochondrial level after LPS exposure. In the present work we have also examined whether BME-UV1 cells are able to express endogenous bLf mRNA after LPS exposure. *In vivo*, it has been shown that injection of bovine mammary gland with *E. coli* LPS caused a significant increase within hours of bLf mRNA expression (Pfaffl et al., 2003; Schmitz et al., 2004). In addition, a rise in bLf mRNA expression after LPS challenge has been observed in a primary bovine mammary cell culture system (Wellnitz and Kerr, 2004) and the murine mammary epithelial cell line HC11 (Li et al., 2009). However, in BME-UV1 cells Real-Time PCR analysis carried out on total RNA from control and LPS treated cells did not allow the detection of bLf mRNA expression, whereas Lf mRNA expression was detected in the positive control from bovine mammary gland tissue (data not shown). On one

hand, the discrepancy in terms of bLf mRNA expression observed between BME-UV1 and other *in vitro* models of the bovine mammary epithelium used in previous studies could be explained by the lack of LPS responsive elements in BME-UV1 cell line and are in line with outcomes of others. Zheng et al. (2005) have provided evidence that Lf promoter contains LPS-responsive regions and that the response to infection is mediated by the NF-kappaB pathway. It is tempting to consider that inBME-UV1 cells these regions are not functional. On the other hand, the fact that bLf mRNA expression has not been detected in BME-UV1 cells could be explained by the lack of Lf expression observed in immortalized mammary cell lines (Panella et al., 1991). In support of this, the bovine mammary epithelial cell line MAC-T cells do not appear to produce Lf (Zheng et al., 2005). Interestingly, Baumrucker et al. (2003) have found that BME-UV cells show faint expression of bLf after treatment with retinoic acid. In conclusion, our results showed that in BME-UV1 cells exogenous bLf acts as a protective factor against LPS cytotoxicity, while the response to LPS treatments does not involve endogenous bLf mRNA expression.

6. Plasmin-plasminogen system in bovine mammary gland

6.1 Introduction

The plasmin-plasmnogen system plays a crucial role in a variety of extracellular proteolytic events, such as fibrinolysis, cell migration and tissue remodelling events (Schaller and Gerber, 2010). The cascade of reactions that leads to plasminogen activation is controlled by a complex network of molecular interactions between plasminogen activators (urokinase-type, u-PA and tissue-type, t-PA) and at least two types of plasminogen activator inhibitors (PAI-1 and PAI-2) (Lampidonis et al., 2011). The inactive plasminogen is present in most body fluids, including milk, and it can be defined as a "proteolytic reservoir" due to its unlimited supply of proteolytic activity (Politis, 1996). During tissue remodeling events, plasmin plays its role by acting on proteins located both at the extracellular matrix and basement membrane level of cell leading to its proteolysis and, consequently, detachment from the basal lamina.

At the same time, plasmin initiates a cascade of reactions that cause the activation of other degradative enzymes called matrix metalloproteinases (MMP) (Deryugina and Quigley, 2012).

MMPs are a family of extracellular soluble or membrane-bound proteases that can degrade or modify all major components of the matrix including extracellular collagen, laminin and proteoglycans (Cuadrado et al., 2008). As described in literature, the involvement of plasmin-plasminogen system in invasive and metastatic tumors, in the invasiveness and metastatic potential of cancer seems to be correlated with the activity of u-PA localized on the cell membrane. As a consequence the system plays a crucial role in leading local

destruction of the extracellular matrix and it is involved in cancerous cells migration and metastases generation. The role of u-PA in development of malignant tumours has been studied and highlighted in different types of cancers including breast and liver cancers (Tang and Han, 2012).

A prime example of tissue that undergoes extensive tissue remodelling throughout its growth and its development is the mammary gland (Theodorou et al., 2010). In ruminants, after weaning or following cessation of milking, major well-controlled degenerative events occur as the mammary gland is remodelled in preparation for the next lactation (Lampidonis et al., 2011). There is evidence proving its role in mammary gland both in physiological and pathological conditions, such as during mastitis.

6.1.1. Plasminogen and plasmin in bovine milk

Bovine plasminogen is a single-chain glycoprotein of 786 amino acids that consists of five characteristic loop structures called kringles that are responsible for binding between plasminogen and fibrin in blood system and casein in milk. Plasminogen is converted in the active and proteolytic form, called plasmin, by the action of plasminogen activators (PAs), which are able to cleave plasminogen at the Arg 557-Ile558 peptide bond [L. Berglund, M.D. Andersen, and T.E. Petersen, 1995, unpublished data](Schaller et al., 1985). Plasmin, formed by this process, is a serin protease able to degrading some matrix components and activates other matrix-degrading enzymes (Silanikove et al., 2006).

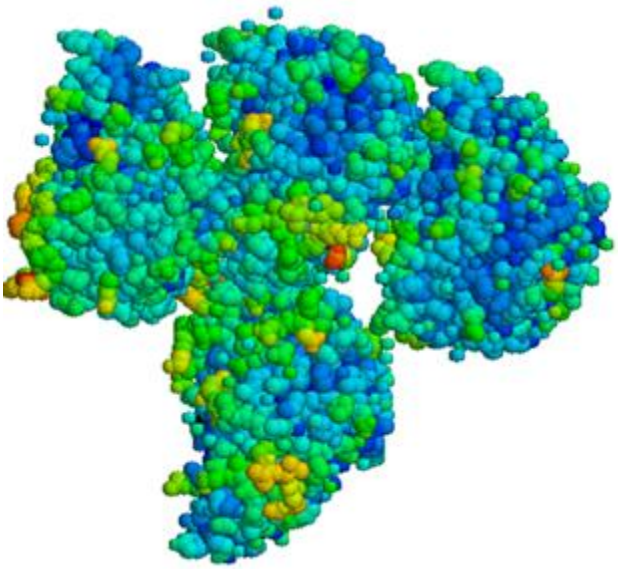

Fig 17: Three dimentional structure of plasmin .
(http:// upload.wikimedia.org/wikipedia/it/thumb/6/6d/Plasmina.png/199px-Plasmina.png)

The resulting activated plasmin (Fig.17) consists of two chain held together by disulfide bonds. It is involved in a wide variety of biological function ranging from thrombolysis to tissue remodeling (Alfano et al., 2005). This serine protease is a heat-stable enzyme and its optimal pH is of 7.4. Plasmin shows a trypsin-like specificity cleaving peptide bonds adjacent to Lys or Arg. In milk both αs- and β-casein are hydrolyzed by plasmin, which, however, does not seem to be able to act on κ-casein (Politis, 1996).

6.1.2. Plasminogen activators and Plasminogen activator inhibitors

In mammals exists two types of PA enzymes: urokinase-PA (u-PA) and tissue-PA (t-PA). Although both of these two enzymes cleave the same peptide bond of their substrate, plasminogen, they are antigenically distinct and derived from different genes.

Bovine u-PA is a single-chain consisting of 413 amino acids, it can be converted in two chain u-PA held together by disulfide bonds, this form is enzymatically more active than single-chain (Politis, 1996).

u-PA is composed of (Politis, 1996):

- Epidermal growth factor (EGF)-like domain that is structurally similar to the receptor-binding region of EGF;
- One kringle structure
- Carboxyl terminal region that contains the active site of the enzyme.

EGF-like domain and kringle structure form the amino-terminal fragment (ATF) that mediates binding between u-PA and specific cellular receptor presents on numerous cell types.

t-PA is composed of (Danø et al., 1985; Saksela and Rifkin, 1988):

- EGF-like domain
- Two kringles structures
- Carboxyl terminal region
- Finger-like domain that is highly homologous with the fibrin-binding fingers present in fibronectin.

Conversely to u-PA, t-PA has high affinity for fibrin. High affinity is due to the presence, in t-PA structure, of the finger domain and the two kringles structure. When t-PA binds fibrin occurs a conformational change that decreases the Michaelis constant of t-PA for plasminogen enhancing the enzyme activity, but this not occurs when fibrin binds u-PA (Zamarron et al., 1984). The different response of this binding with fibrin is the bases for the general belief that the physiological function of t-PA is in thrombolysis while that of u-PA is in tissue remodeling events (Danø et al., 1985; Saksela and Rifkin, 1988).

The interaction between PAs activity and its specific inhibitors (PAIs) controls the rate of plasminogen conversion (Fig.18). Two fast-acting PAIs

have been identified and designated PAI-1 and PAI-2; while the third inhibitor, PAI-3, isolated from urine, shows an inactivating ability slower than other two PAIs (Andreasen et al., 1990; Danø et al., 1985; Saksela and Rifkin, 1988).

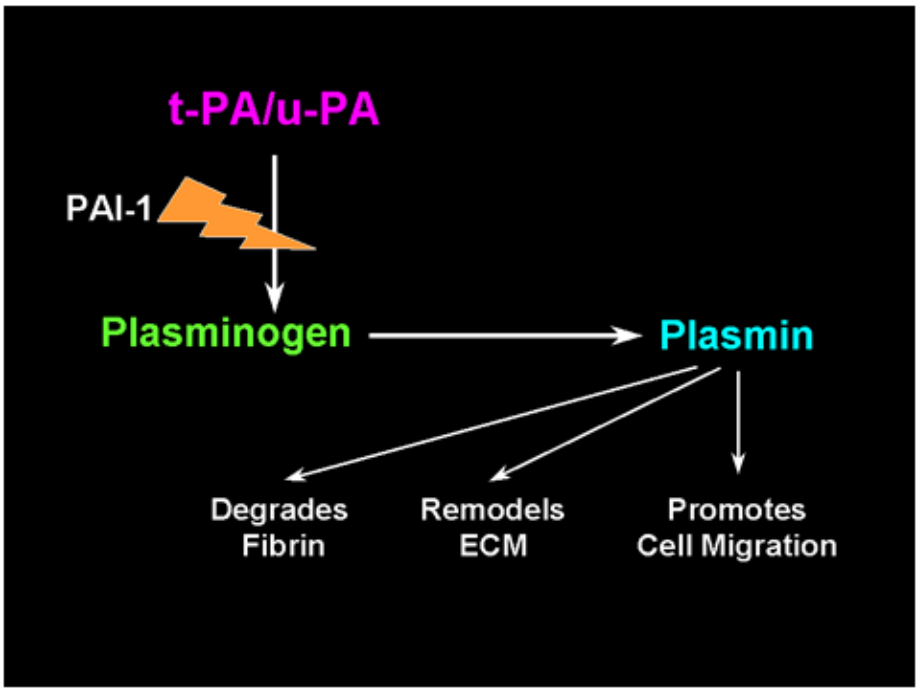

Fig 18: PAI-1 action (Brown NJet al., 2000)

The rapidity of interaction between inhibitors and activators is an important parameter to evaluate the efficacy inhibitors so PAI-1 and PAI-2 inactivate rapidly PA forming equimolar complexes with u-PA and t-PA. In addition to PA inhibitors, there are inhibitors that act directly on plasmin; the two most important of which are α2-antiplasmin and α2-macroglobulin. The last one is a nonspecific inhibitor that reacts with the most of proteases while α2-antiplasmin is a specific and rapidly plasmin inhibitor (Politis, 1996).

6.1.3. u-PA receptor

u-PA receptor (u-PAR) is a membrane protein able to bind u-PA with high affinity (dissociation constant= 0.1 to 1 mmol/l) (Politis, 1996).

Bovine u-PAR is a protein of 310 amino acids highly glycosylated with six N-linked glycosylation sites (Kratzschmar et al., 1993; Reuning et al., 1993). u-PAR is composed of three structurally homologous domains each of which is characterized by a high cysteine content (9%).

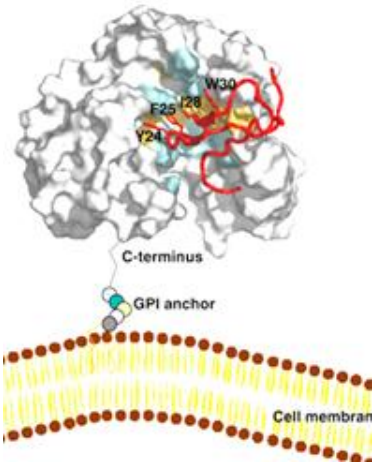

Fig.19: uPAR structure (http://www.nature.com/emboj/journal/v24/n9/fig_tab/7600635f6.html)

The amino acid sequence of each repeat is distantly related with a homology lower than 20%. u-PAR is attached to cell surface by an anchor of glycosyl, phophatidyl and inositol (GPI) (Fig.19) whose biological significance may lie in the greater lateral mobility than other transmembrane proteins (Politis, 1996). At NH_2 terminal region level of u-PAR there is the repeat appointed to bind the EGF-like domain, which is part of ATF region of u-PA (Saksela and Rifkin, 1988).

The primary function of u-PAR is to localize plasminogen activation at the cell surface because u-PA bound to its receptor retains its proteolytic activity (Fig.20) (Theodorou et al., 2011).

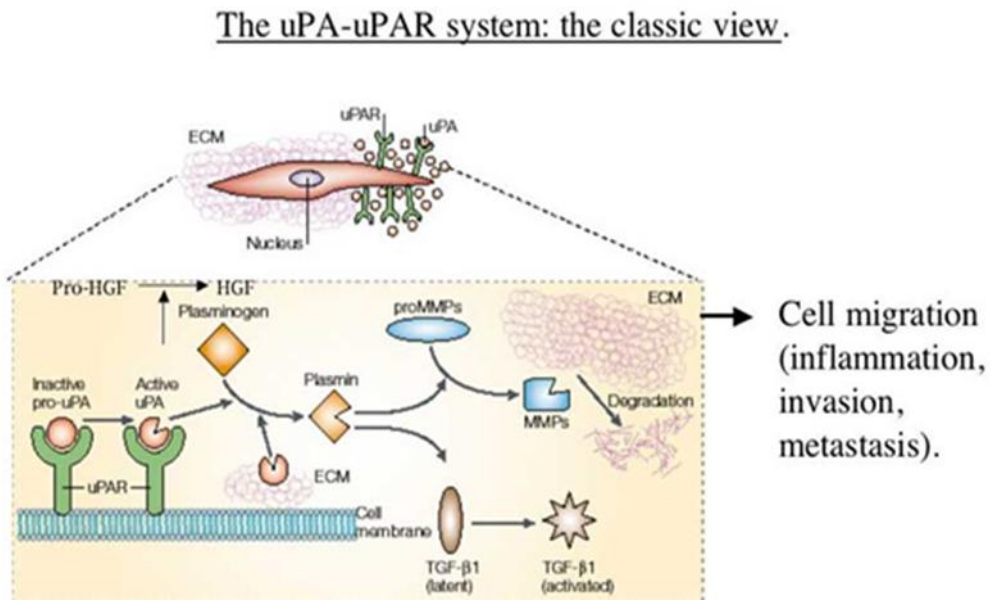

Fig 20: uPA-uPAR system (Blasi&Carmeliet,2002)

Bound between u-PA and u-PAR, leading to accelerate production of plasmin and initiates a cascade of reactions determining degradation of extracellular matrix proteins during tissue remodeling events (Politis, 1996). Plasmin so formed owns different behaviour from that in solution; in fact, α2-antiplasmin inactivates plasmin on the membrane more slowly than that in solution (Ellis and Dano, 1991; Ploug et al., 1991). On the contrary, PAI-1 is able to inhibit both types of plasmin. When u-PA is bound to u-PAR, forming a complex with PAI-1 is internalized by the cell. This complex is degraded into the cell and u-PAR is recycled on the cell membrane (Ellis and Dano, 1991; Nguyen et al., 1992; Nusrat and Chapman Jr, 1991); thus bound between PAI-1 and receptor-bound u-PA provides a mechanism that continuously change the

proteolytic activity on cell surface (Politis, 1996). Without this binding, receptor-bound u-PA would be equally internalized but more slowly than the complex (Ellis and Dano, 1991; Nguyen et al., 1992; Nusrat and Chapman Jr, 1991).

6.1.4 Role of plasmin-plasminogen in cell growth

Already in the early 1990s was known a correlation between cell proliferation and expression of u-PA. Politis (1996) has shown that the most powerful growth regulators of cultured cells are among the main factors regulating u-PA expression *in vitro*. Comparing several studies, Alfano et al. (2005) have concluded that u-PA may support cell proliferation and the matrix remodeling associated with tissue regeneration by plasmin-mediated activation of other proteinase classes and growth factors as well as through u-PAR-dependent signaling.

Despite, at the molecular level, u-PA and PAI-1 have been extensively analyzed in human and rodents, there is limited evidence regarding the role of genes implicated in the plasmin-plasminogen system in the bovine mammary gland.

Politis et al. (1989) have shown that the conversion of plasminogen to plasmin is related to the gradual decline faced by the gland breast. Baldi et al. (2002) have observed that during late lactation both plasmin and PAs increase and the rate between plasminogen and plasmin decreases indicating an increase in the conversion of plasminogen to plasmin during this phase. Rabot et al (2007b) have detected that, in dairy cow, during late lactation, u-PA, u-PAR and PAI-1 expression increased suggesting that high levels of expression of these enzymes might be considered a characteristic of the non-lactating gland. In contrast with what is known in other species, in the same study, were also

observed high levels of expression of all three genes in mammary tissue obtained from lactating dairy cows.

With respect to cell growth, the evidence regarding the role of u-PA/u-PAR/PAIs is limited and conflicting as well. Politis et al. (1995) have revealed that PA synthesis is controlled by lactogenic hormones and growth factors and is, also, influenced by extracellular support on which epithelial cells are cultured. In the same study, it has been reported that in MAC-T cell line, treated with insulin and insulin growth factor-1 (IGF-1), there had been an enhanced cell proliferation accompanied by increased both u-PA gene expression and u-PA activity.

Cheli et al (2003), conversely, have showed that retinoids treatments were able to inhibit BME-UV1 cell proliferation but not u-PA gene expression or activity.

6.1.5 Correlation between plasminogen-plasmin system and pathological conditions

Plasminogen-plasmin system can be activated not only by physiological stimuli but also in pathological conditions such as during the course of mastitis. Mastitis is an inflammation of the mammary gland and represents the most costly disease in the dairy industry (Zhao and Lacasse, 2008). The main cause of mastitis is intramammary bacterial infection, generally due to the gram-negative bacteria. Among them, *Escherichia coli* cause mainly acute and severe infections in high-producing cows, in particular around parturition or during early lactation (Burvenich et al., 2003; Hogan and Larry Smith, 2003).

During clinical and subclinical infections of the mammary gland, has been observed an increase both in proteolytic activity in milk and in plasmin plasminogen activator and in somatic cell count (Baldi et al., 1996; Politis et

al., 2004). In neutrophils and monocytes of dairy ewes has been observed an up-regulation of expression of u-PA, u-PAR and PAI during mastitis (Theodorou et al., 2010). Furthermore, Long et al. (2001) have detected, in bovine mammary tissue during *E.coli*-induced mastitis, an increased expression of u-PA mRNA.

Important components of *E. coli* and other gram negative bacterial wall, namely LPS, are able to induce a significant increase within hours in mRNA expression and release of inflammatory mediators and protective factors, such as cytokines and antimicrobial proteins (reviewed in Rainard and Riollet, 2006). Several surveys (Ohta et al., 2000; Long et al., 2001; Cunningham et al., 2009) have shown that LPS can upregulate the expression of the component of the plasmin-plasminogen system in several tissues, such as the microglia and human prostatic cancer cells. Even though we know that LPS is able to stimulate plasmin-plasminogen system and that *E. coli* is the most common aethiological agent causing acute and severe intrammary infection. Investigations in this direction can contribute to provide more insights into the correlation between mammary gland defense mechanisms against the most common agents isolated from acute cases of mastitis.

6.2 Aim

This section of my work is divided in two studies, in the first of these, the aim was to determine the effect of growth factors (IGF-1 and EGF-1) and three hormones (insulin, dexamethasone and prolactine) on expression of plasminogen activator (PA)-related genes (u-PA, u-PAR, PAI-1 and PAI-2) and BME-UV1 cell proliferation. I used BME-UV1 cell line that represents an *in vitro* model of bovine mammary epithelia and, at the same time, is able to

produce itself the components of the plasminogen activator system (Cheli et al., 1999).

In the second study the aim was to investigate the effects of *E. coli* LPS, used in order to simulate *E. coli* infection of the mammary epithelium *in vitro*, on cell viability, modulation of cell-associated u-PA activity and the regulation of u-PA and u-PAR RNA expression on mammary gland I used BME-UV1 cells.

1st study

6.3 Material and methods

6.3.1 Cell culture

The BME-UV1 cell line was established from primary bovine mammary epithelial cells by stable transfection with a plasmid, carrying the sequence of the simian virus 40 early region mutant tsA58, encoding the thermolabile large T antigen (Zavizion et al. 1996b) and is maintained in the Laboratory of Cell Culture, Department of Veterinary Science and Technologies for Food Safety of University of Milan. Cells are routinely cultivated into 75-cm^2 tissue culture flasks (Corning Life Sciences, Corning NY, USA), in normal growth medium which consisted of 50% DMEMF12, 30% RPMI-1640 and 20% NCTC-135 (Sigma-Aldrich, St. Louis MO, USA), supplemented with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM-glutathione, 1 μg/ml insulin, 5 μg/ml transferrin, 1 μg/ml hydrocortisone, 0.5 μg/ml progesterone, 10 μg/ml L-ascorbic acid and antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml). All medium supplements are from Sigma-Aldrich. Cells are maintained at 37 °C in a humidified 5%-CO_2 incubator until confluence.

6.3.2 Cell proliferation

The first experiment examined the effect of the various hormonal and growth factor treatments on cell proliferation using two methodologies. The first method included the use of the MTT assay, which measures the production of the chromophore formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in cultured cells (Sigma-Aldrich), as previously described (Baldi et al., 2004). Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase. The second methodology used was direct cell enumeration. All details of this method have been described by Politis et al. (1995). Results paralleled those of the MTT assay and therefore are not presented in detail in the Results section. Cells were seeded (2.5×10^4 cells) in each well of 96-well tissue culture plastic plates (Corning Life Sciences) in normal growth medium. After 24 h media was removed, cells were washed twice with Hank's balanced salt solution (HBSS) and test medium was added. Test medium consisted of serum-free DMEM alone, used as control, and serum-free DMEM containing insulin (bovine; 0·1 and 1 μg/ml), EGF (human; 50 ng/ml), IGF-1 (mouse; 50 ng/ml), prolactin (ovine; 0.01 and 1 μg/ml), dexamethasone (100 nM), dexamethasone (100 nM) plus insulin (1 μg/ml) and dexamethasone (100 nM) plus prolactin (1 μg/ml). All hormones and growth factors were purchased from Sigma-Aldrich. Preliminary time-course experiments showed that both IGF-1 and EGF affected cell proliferation at 24, 36 and 48 h but not at 12 h of incubation. Therefore, incubation for 24 h was selected in all subsequent experiments. Furthermore, other preliminary experiments with various doses of the growth factors were performed to detect the optimum concentrations of IGF-1 and EGF. With respect to IGF-1, three concentrations were tested (1, 10 or 50 ng/ml) and the optimal concentration was found to be the 50 ng/ml which is consistent with the concentration used by Zavizion et al.

(1996b) in the BME-UV cells. With respect to EGF, two concentrations were tested (10 or 50 ng/ml) and they were equally effective but the concentration of 50 ng/ml gave more consistent results. This concentration is higher than the 10 ng/ml utilized by Zavizion et al. (1996b) and Accornero et al. (2009) in the BME-UV cells. The concentrations of all three hormones (insulin, dexamethasone and prolactin) were similar to those utilized by Politis et al. (1995) in the MAC-T cell line which was derived in a similar manner with BME-UV cell line. After 24 h of incubation, treatment test medium was removed, then 150 µl MTT stock solution (5 mg/ml) in PBS was added to each well and finally the plates were incubated for 3 h at 37 °C in a humidified chamber. The reaction was terminated by removing the incubation solution and adding 150 µl of dimethyl sulphoxide to dissolve the formazan. The optical density of the dimethyl sulphoxide solution at 540 nm was determined on a Biorad 680 microplate reader (Biorad, USA). The percentage of cell proliferation was calculated as follows: Percentage cell proliferation=(mean optical density in presence of treatment/mean optical density of control)×100. For each treatment three biological replicates were used and the experiment was performed twice.

Target Gene	Name	Oligonucleotide sequences (5'→3')	Primer length	Amplicon size	Corresponding GenBank sequence
b-actin	F_b-actin	CCAAAGCCAACCGTGAGA	18 nt	114 bp	AF481159.1(*C.Hircu*)
	R_b-actin	CCAGAGTCCATGACAATGC	19 nt		AY141970.1(*B. aurus*)
u-PA	F_u-PA	GGCCAGAAGGCTCTGAGGC	19 nt	133 bp	BT030737.1(*B.Tauru*)
	R_u-PA	GGCTGCCACCACACAAGTAGG	21 nt		
u-PAR	F_u-PAR	GGGACAGGACCTCTGCAGG	19 nt	117 bp	NM_001005377.2(*H. Sapiens*)
	R_u-PAR	CGATAGCTCAGGGTCCTGTTG	21 nt		NM_174423.3(*B.Taurus*)
					NM_011113.3 (M. Musculus)
PAI-1	F_PAI-1	CTACACTGAGTTTACCACCC	20 nt	234 bp	BT025406 (*B. Taurus*)
	R_PAI-1	GACATTCCCAAGTTCTCCAG	20 nt		
PAI-2	F_PAI-2	CTTGGAGTTGCTGGAGAG	18 nt	229 bp	NM_001192051.1 (*B. Taurus*)
	R_PAI-2	ACACTTCAGACAGAAACAGG	20 nt		

Table 3. Nomenclature, nucleotide sequences, length of the primers used and the size of the resulting amplicons for all Real time PCR reactions

6.3.3 Gene expression of PA-related genes

The second experiment examined the effects of insulin, EGF, IGF-1, prolactin and dexamethasone (Sigma-Aldrich) on expression of four PA-related genes. Cells were seeded in 6-cm culture dishes (10^6 cells) in normal growth medium. After 24 h media were removed, cells were washed twice with Hank's balanced salt solution (HBSS) and test medium was added for another 24 h. Test medium used was the same as described in the first experiment. After 24 h of incubation, cells were removed by complete trypsinization and RNA was extracted using TRI reagent (Sigma-Aldrich). Relative levels of mRNA were quantified with real-time, quantitative RT-PCR. A pair of primers for each of the target genes (u-PA, u-PAR, PAI-1 and PAI-

2) and the housekeeping gene (β-actin) was constructed using PERL primer software (Marshall, 2004). Primers for u-PA, PAI-1 and PAI-2 were designed based on Bos taurus sequences, while β-actin and u-PAR primers were designed in highly homologous regions, between different species, of their respective cDNAs. PCR products from all primer pairs used were verified by sequencing. All primer pairs are presented in Table 3. The amount of sample RNA was normalized by using β-actin as a housekeeping gene. Equal amounts of total RNA were reverse transcribed with the iScript™cDNA Synthesis Kit (Biorad, Hercules CA, USA) according to the manufacturer's instructions using a mix of random hexamers and oligo-dT primers. Real time PCR was performed in the MyiQ2 cycler (BioRad) using the SsoFast™ EvaGreen® Supermix (BioRad) according to the manufacturer's protocol. Each reaction (total volume 20 μl) for the quantification of the housekeeping gene and the target genes contained 50 ng RNA equivalents as well as 450 nM of forward and reverse primers for b-actin and PAI-1 and 350 nM of forward and reverse primers for u-PA, u-PAR and PAI-2. The reactions were incubated at 95 °C for 30 s followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C. This was followed by a melt curve analysis to determine the reaction specificity. For each treatment two biological replicates were used, each sample was measured in duplicate and the experiment was performed twice. The comparative Ct method was used for relative quantification. The amount of target normalized to b-actin and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$.

6.3.4 Determination of PA activity

The third experiment examined the effect of the insulin, EGF, IGF-1, prolactin and dexamethasone on cell-associated, membrane-bound and secreted u-PA activity by BME-UV cells. Cells were seeded (1.5×10^5 cells) in

each well of 24-well tissue culture plastic plates (Corning Life Sciences) in normal growth medium. After 24 h media were removed, cells were washed twice with Hank's balanced salt solution (HBSS) and test medium was added for another 24 h. Test medium used was the same as described in the first experiment. After hormonal treatment the medium was recovered and stored for the determination of secreted PA activity. The protocol used for recovery of cell-associated and membrane-bound PA has been described previously (Stoppelli et al., 1986). Briefly, membrane-bound u-PA, was recovered by a 3-min treatment at room temperature with 50mM-glycine–HCl buffer, pH 3·0, containing 0·1 M-NaCl, then quickly neutralized with 0.5 M-Hepes buffer, pH 7.5 containing 0.1 M NaCl. Cell-associated u-PA was obtained from cells after lysis with 1% Triton X-100, 20mM Hepes buffer, pH 7.5, containing 10% glycerol. PA activity in all three fractions was quantified using the method described by Politis et al. (1995) and Politis (1996). This method is based on the conversion of exogenously provided plasminogen to plasmin from the PA present in the fraction. Plasmin so produced, is subsequently allowed to attack the chromogenic substrate Valine-Leucine-Lysine-p-nitroaniline adjacent to Lysine and liberate the free chromophore p-nitroaniline (Sigma Aldrich). In this system, changes in colour are directly related to plasmin levels and therefore indirectly to PA activity. All forms of PA activity were normalized by the number of cells. Cell numbers were quantified using a haemocytometer after trypsinization. For each treatment three biological replicates were used, each sample was measured in triplicate and the experiment was performed twice.

6.4 Statistical analysis

All data are presented as means and SEM. The effect of the various treatments was assessed by ANOVA. Fischer's LSD test was used post hoc, with a 95% confidence interval. All analyses were performed using the PASW Statistics 18 release 18.01 (SPSS Inc., USA) program.

6.5 Results

The effect of growth factors and lactogenic hormones on cell proliferation following 24 h incubation was determined and the results are presented in Fig. 21. Both IGF-1 and EGF caused significant increases in cell proliferation. However, EGF was a more effective ($P<0.01$) mitogen than IGF-1. No effect ($P>0.05$) was observed when cells were cultured in the presence of insulin, prolactin, dexamethasone. The combinations of dexamethasone with insulin or prolactin showed a trend for increased cell proliferation, but it was not significant.

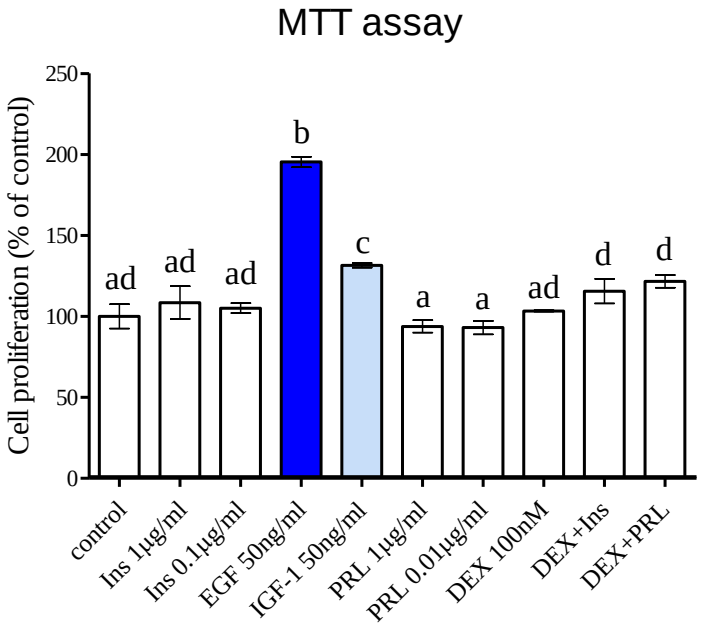

Fig. 21. Effect of lactogenic hormones [insulin (Ins), prolactin (PRL) and dexamethasone (DEX)] and growth factors (EGF and IGF-1) on cell proliferation of BME-UV1 mammary epithelial cells after 24-h treatment. Treatment combinations were made using the highest concentration for each compound. All data are presented as means±SEM (n=6). Means without a common letter differ *P: <0.05.

The effect of growth factors and lactogenic hormones on expression of u-PA and u-PAR, by bovine mammary epithelial cells was examined and the results are presented in Fig. 22. IGF-1 caused 2-fold increases (P<0.05) in expression of u-PA (Fig. 22A) and u-PAR (Fig. 22B). EGF caused a 3·8-fold increase (P<0.01) in expression of u-PAR (Fig. 22B).

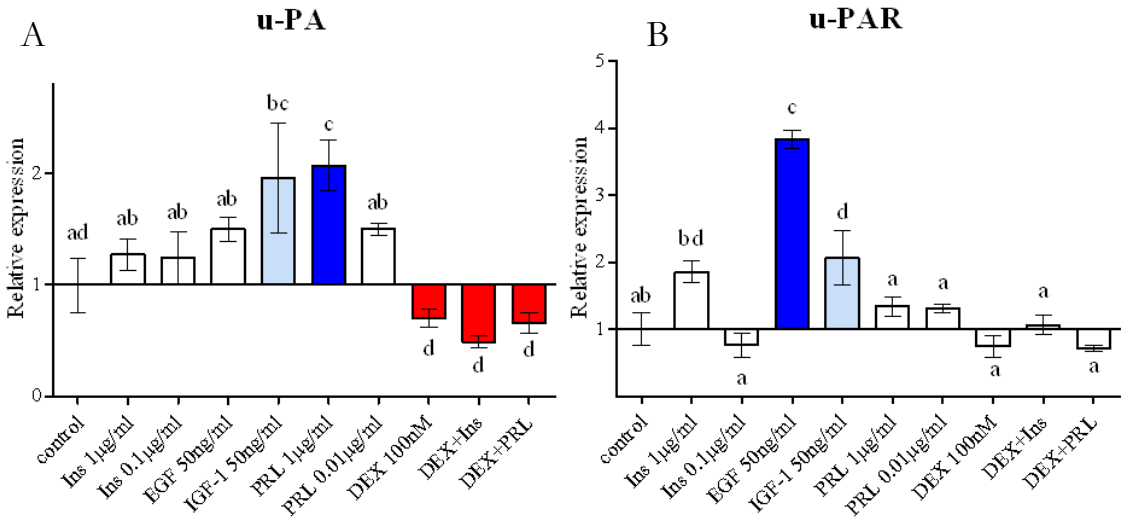

Fig. 22: Effect of lactogenic hormones [insulin (Ins), prolactin (PRL) and dexamethasone (DEX)] and growth factors (EGF and IGF-1) on gene expression of (A) urokinase plasminogen activator (u-PA) and (B) u-PA receptor (u-PAR) in BME-UV1 mammary epithelial cells after 24-h treatment. Treatment combinations were made using the highest concentration for each compound. All data are presented as means±SEM (n=4). Means without a common letter differ *P: <0·05.

Treatment of cells with prolactin at the high concentration (1 µg/ml) caused a 2-fold increase (P<0.05) in expression of u-PA. Dexamethasone alone or in combination with insulin or prolactin showed a tendency to decrease expression of u-PA but this decrease did not reach the designated level of significance (P>0.05). The effect of growth factors and lactogenic hormones on mRNA expression of PAI-1 and PAI-2 by bovine mammary epithelial cells was examined and the results are presented in Fig. 23. Dexamethasone alone

and when combined with insulin or prolactin increased by 2–3·5-fold expression of PAI-1 (Fig. 23A) and by 3–4-fold that of PAI-2 (Fig. 23B).

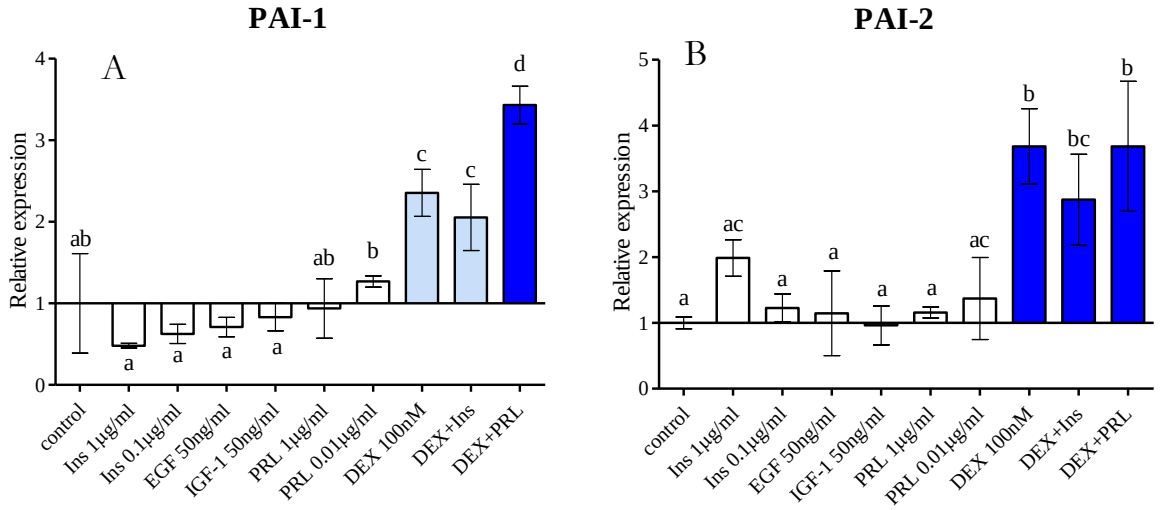

Fig.23: Effect of lactogenic hormones [insulin (Ins), prolactin (PRL) and dexamethasone (DEX)] and growth factors (EGF and IGF-1) on gene expression of (A) plasminogen activator inhibitor type 1 (PAI-1) and (B) plasminogen activator inhibitor type 2 (PAI-2) in BME-UV1 mammary epithelial cells after 24-h treatment. Treatment combinations were made using the highest concentration for each compound. All data are presented as means±SEM (n=4). Means without a common letter differ *P: <0.05.

Treatment of cells with insulin had no effect (P>0.05) on expression of all four PA-related genes (u-PA, u-PAR, PAI-1 and PAI-2). However, treatment of cells with the high concentration of insulin (1 µg/ml) showed a trend for increasing expression of PAI-2, but was not significant (Fig. 23B). IGF-1 and EGF had no effect on PAI-1 and PAI-2 gene expression. The effect of growth factors and lactogenic hormones on total cell-associated, membrane-bound and secreted u-PA activity was examined and the results are presented in Fig. 4. IGF-1 and EGF increased (P<0.001) total, membrane-bound and secreted u-PA activity on a per cell basis (Fig. 24); EGF increased total cell-associated and membrane-bound activity more (P<0.01) than did IGF-1. Treatment of cells with prolactin at both concentrations (0.01 or 1 µg/ml) had no effect on all forms of u-PA activity. Insulin did not cause any significant

(P>0.05) change in any form of u-PA activity when compared with control values (Fig. 24).

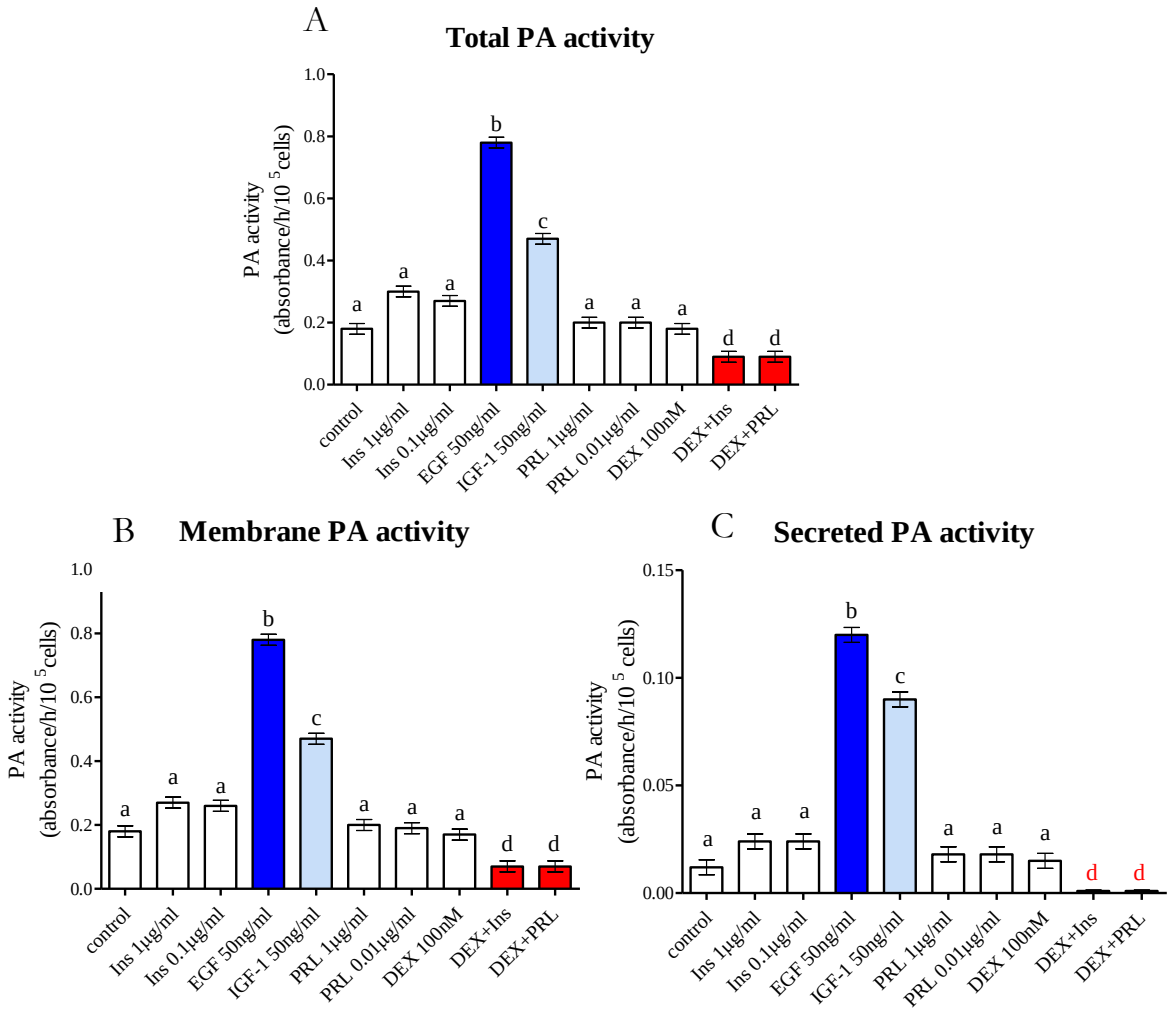

Fig. 24: Effect of lactogenic hormones [insulin (Ins), prolactin (PRL) and dexamethasone (DEX)] and growth factors (EGF and IGF-1) on (A) total, (B) membrane-bound and (C) secreted plasminogen activator (PA) activity in BME-UV1 mammary epithelial cells after 24-h treatment. Treatment combinations were made using the highest concentration for each compound. All data are presented as means±SEM (n=6). Means without a common letter differ *P: <0.05.

Consistent with the main effect concerning up-regulation of PAI-1 gene expression, dexamethasone when combined with insulin or prolactin decreased ($P<0.05$) all three forms of u-PA activity (Fig. 24) but surprisingly treatment with dexamethasone alone had no effect on u-PA activity.

6.6 Discussion

We investigated whether IGF-1 and EGF regulated the expression of genes implicated in activating (u-PA, u-PAR) or blocking (PAIs) the plasminogen activating cascade in the BME-UV1 non-invasive bovine mammary epithelial cell line. Our working hypothesis was that both growth factors enhance cell growth and up-regulate genes implicated in activating the system (u-PA, u-PAR) and/or down-regulate genes implicated in blocking the activation of the system. We showed that both EGF and IGF-1 increased cell proliferation. With respect to gene expression, we found some subtle differences in the mode of action of these two growth factors. For example, EGF increased expression of u-PAR while IGF-1 up-regulated expression of both u-PA and u-PAR. Both growth factors affected neither the expression of PAI-1 nor the expression of PAI-2 by BME-UV1 cells. In a manner consistent with the expression data, both EGF and IGF-1 increased all forms of measured u-PA activity (total cell-associated, membrane-bound and secreted). These data taken together suggest that a relationship exists between cell proliferation and expression of the two genes implicated in the activation of the plasmin-plasminogen system (u-PA, u-PAR). Our data suggest that EGF was a more effective mitogen than IGF-1. Furthermore, EGF increased the amount of u-PA present in cell membranes more than that caused by IGF-1 presumably because EGF induced more u-PAR expression than IGF-1. These data are consistent with those of others (Alfano et al., 2005) who indicated that what

truly matters is the physical binding of u-PA to u-PAR, in addition to increased expression of u-PAR. In fact, Jo et al. (2007) proposed that both conditions are necessary for increased cell proliferation. Previous studies have provided conflicting results concerning whether activation of u-PA gene or activity is implicated in the mechanism through which IGF-1 affects cell proliferation. The present data suggesting that IGF-1 upregulated u-PA and u-PAR without affecting any of the PAIs agree with those of Politis et al. (1995) who similarly reported up-regulation of u-PA together with elevated u-PA activity on a per cell basis in response to IGF-1. Similar results suggesting that IGF-1 is acting through induction of the u-PA/u-PAR system were provided by Dunn et al. (2000; 2001) using the highly invasive MDA-MB-231 breast cancer cell line. However, against this notion, Cheli et al. (2003) showed that retinoids inhibit insulin/IGF-1 mediated BMEUV cell proliferation but have no effect on u-PA mRNA levels and activity. Future u-PA/u-PAR gene silencing experiments will solve the discrepancy between these studies. With respect to the lactogenic hormones, we observed that dexamethasone alone and when combined with insulin or prolactin up-regulated expression of PAI-1 and PAI-2 by BME-UV1 cells. The combination of dexamethasone and prolactin was more effective than dexamethasone alone or the combination of dexamethasone and insulin indicating a synergistic effect of dexamethasone and prolactin in upregulating PAI-1 but not PAI-2 synthesis. On the other hand, dexamethasone alone had no effect on u-PA expression but when used in combination with prolactin down-regulated the prolactin-induced increase in u-PA synthetic ability of the BME-UV1 cells. With respect to u-PA activity, dexamethasone alone had no effect but when combined with insulin or prolactin reduced all forms of measured u-PA activity (total cell-associated, membrane-bound and secreted). Thus, the prominent effect of dexamethasone in the BME-UV cells is its ability to down-regulate the

plasminogen activating cascade. None of the lactogenic hormones when used alone or combined had any effect on cell proliferation. This finding taken together with the expression data leads to two conclusions. First, the induction of PAI-1 and PAI-2 synthesis caused by dexamethasone and the overall down-regulation of the plasminogen activating cascade at the molecular and the protein levels are not related to cell growth. Second, the ability of prolactin to induce u-PA expression without an effect on u-PA activity suggests that changes in the u-PA system at the protein level together with physical binding of u-PA to u-PAR might be necessary for induction of cell growth. Our findings suggest that all modulators of mammary function studied regulate both plasminogen activator inhibitors (PAI-1 and PAI-2) in a similar manner, thus creating an apparently functionally redundant mechanism for inhibition of the system in bovine mammary epithelial cells. Both PAIs share a similar manner of action in blocking the PAs. Therefore, one of them could easily block the system, thus, making the second one redundant. The functional role of PAIs may no longer be simply to inhibit over-expressed PAs but they may serve some additional role in regulating mammary cell functions. Results from various mammary cell systems suggest that glucocorticoids exercise mainly an anti-proliferative effect on breast cancer cells (Lipka et al., 2004; Rubis et al., 2004). Furthermore, glucocorticoids suppress u-PA gene expression and activity (Henderson and Kefford, 1993; Politis et al., 1995b; Sasaki et al., 1999). Busso et al. (1987) reported that the glucocorticoid-induced inhibition of the plasminogen activating cascade was related to both a decrease in the synthesis of u-PA and a concomitant increase in the production of PAI-1 in the highly invasive MDAMB- 231 mammary carcinoma cell line. We showed that dexamethasone in BME-UV cells down-regulated the plasminogen activating cascade at the molecular level by causing an increase in expression of both PAIs and by inhibiting the prolactin-induced

increase in expression of u-PA. Our data concerning the effect of prolactin and the lack of an insulin effect on expression of u-PA related genes and cell proliferation deserve some comments. With respect to prolactin, Politis et al. (1995) showed that prolactin had no effect on proliferation of MAC-T bovine mammary epithelial cells. In contrast, others have shown that prolactin enhanced proliferation of bovine mammary epithelial cells (Olazabal et al. 2000) and that of the invasive MCF7 cells (Doll et al., 2007). Our data indicate that prolactin up-regulated expression of u-PA but had no effect on all forms of u-PA activity by BME-UV1 cells. Furthermore, as expected, prolactin had no effect on proliferation of BME-UV1 cells. These data taken together indicate that the induction of the u-PA gene alone does not support mammary epithelial cell proliferation but binding of u-PA to u-PAR might be necessary to prime mammary epithelial cells for enhanced proliferation. With respect to the lack of the insulin effect on u-PA expression, it was very surprising to us that insulin did not act in a similar manner to IGF-1. This is because the concentration of insulin used should have activated the IGF-1 receptor. The similarity of the effectiveness between insulin and IGF-1 in increasing both gene expression and activity of u-PA was demonstrated in the MAC-T cell line by Politis et al. (1995), which was derived in a similar manner to the BME-UV1 cell line. This discrepancy between the earlier and the present study cannot be explained, but we believe that the lack of the effect is not an artifact or a problem related to biological activity of the insulin used because the combination of insulin and dexamethasone was more effective in reducing all forms of PA activity than dexamethasone alone. In conclusion, IGF-1 and EGF increased cell proliferation and in parallel caused induction of the u-PA/u-PAR system using the non-invasive bovine mammary epithelial BME-UV1 cells as a model system. On the other hand, the prominent effect of dexamethasone was to induce PAI-1 and PAI-2 expression and overall to

down-regulate the plasminogen activating cascade at the molecular and protein levels. Alterations in the expression of PA-related genes and those observed at the protein level following treatment of BME-UV1 cells with dexamethasone or prolactin (induction of u-PA) are not related to bovine mammary epithelial cell growth. Further studies using various techniques of gene silencing will provide the ultimate answer as to whether u- PA and u-PAR play a role in bovine mammary epithelial cell proliferation.

2nd study

6.7 Materials and methods

6.7.1 Cell line and cell culture

The origin of the BME-UV1 established bovine mammary epithelial cell line has been described previously (Zavizion et al., 1996b). Cells were routinely cultivated in plastic Petri dishes (Corning Glass, Corning, NY, USA) in complete medium, which was a mixture of 50% DME-F12, 30% RPMI-1640 and 20% NCTC- 135 supplemented with 10% foetal bovine serum (FBS) (BioWhittaker, Cambrex, Belgium), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 1.0 μg/ml insulin, 5.0 μg/ml transferrin, 1.0 μg/ml hydrocortisone, 0.5 μg/ml progesterone, 10.0 μg/ml $_L$-ascorbic acid and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). All medium supplements were obtained from Sigma– Aldrich. Cells were maintained at 37°C in an incubator in a humidified atmosphere containing 5% CO_2.

6.7.2 Cell viability assay

Cell viability after incubation with LPS was determined by measuring the production of the chromophore formazan from 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma– Aldrich), as previously described (Baldi et al., 2004). Formazan is produced in viable cells by the mitochondrial enzyme, succinate dehydrogenase. Briefly, BME-UV1 cells were exposed to increasing concentrations of *E. coli* LPS O111:B4 (0.1, 1.0 and 10 μg/ml) (Sigma–Aldrich) in complete medium for 3, 6 and 12 h of incubation time. The concentrations were selected from a larger range of concentrations that were previously tested to correspond to subtoxic levels of LPS as described by Pecorini et al. (2010). Cells incubated with complete medium alone, representing 100% viability, were included as negative controls. At least three replicates were performed in each experiment, and each experiment was performed at least twice. The percentage of cell viability was calculated as follows:

percentage viability = (mean optical density in the presence of LPS/mean optical density of negative control) x 100.

6.7.3 Effect of LPS treatment on total BME-UV1 cell-associated u-PA

To determine the effect of LPS on u-PA activity, total cell-associated u-PA activity was evaluated using a colorimetric assay (Politis et al., 1995a; Cheli et al., 2003). Briefly, cells were seeded in a 24-well tissue culture plate (0.5 x 10^6 cells/ml) in complete culture medium containing 10% FBS. After 24 h, media were removed, cells were washed three times with HBSS and test medium was added. Test medium consisted of complete culture medium alone (control) or complete medium containing various concentrations (0.1, 1.0 and 10 μg/ml) of LPS. After incubation for 3, 6 and 12 h, cell monolayers were washed three

times with HBSS and treated with a mildly acidic solution (50 mM glycine–HCl buffer and 0.1 M NaCl, pH 3.0) for 3 min to liberate membrane-bound u-PA. After adding the neutralizing solution (0.5 M Hepes buffer and 0.1 mM NaCl, pH 7.5), the fraction corresponding to membrane bound u-PA was collected and stored at -80°C for further analysis. Cells were then trypsinised by incubation at 37°C with 0.05% Trypsin– EDTA for approximately 5 min and counted in a haemocytometer. Finally, cells were lysed using lysis solution (1% Triton X-100, 20 mM Hepes buffer, and 10% glycerol, pH 7.5), and the lysates were cleared using centrifugation at 12,700g at 4°C for 30 min. The cleared lysates, containing intracellular u-PA, were stored at -80°C. At least three replicates were evaluated per treatment, and the experiment was performed at least twice. Total cell-associated (intracellular plus membrane bound) u-PA activity was measured using a colorimetric assay (Politis et al., 1995a). This system utilises the enzymatically active u-PA present in the medium to convert exogenously supplied plasminogen to active plasmin. Plasmin then attacks the chromogenic substrate Val-Leu-Lys-p-nitroanilide (Sigma–Aldrich), thus liberating the free chromophore p-nitroanilide. In this system, changes in colour are directly related to plasmin amounts and, consequently, indirectly related to u-PA activity. Assays were performed in 250 μl of 100 mM Tris buffer (pH 8.0) containing plasminogen (50 pg/ ml; Sigma–Aldrich), chromogenic substrate (6 mM) and 5 μl of medium from LPS-treated and non-treated cells. A sample without plasminogen served as a control. The reaction mixture was incubated for 3 h, and the absorbance was determined at 405 nm every 30 min using a Biorad 680 microplate reader (Biorad). The rate of p-nitroanilide formation was calculated from the linear part of the curve of absorbance versus time. Previous studies have demonstrated that u-PA activity is linear for up to 3 h of incubation (Politis, 1996).

6.7.4 Determination of free u-PA-binding sites on the cell membrane

It has been previously shown that the u-PA-binding sites on the cell membrane may be fully saturated (Politis et al., 2004). The u- PA-binding sites that are unoccupied can be fully saturated by the addition of purified u-PA. Therefore, the difference in membrane bound u-PA before and after this incubation correlates to the presence of free u-PA-binding sites on the cellular surface. Cells were seeded in a 24-well tissue culture plate in complete culture medium containing 10% FBS. After 24 h, media were removed, cells were washed three times with HBSS and test medium was added. Test medium consisted of complete culture medium either without (control) or with various concentrations (0.1, 1.0 and 10 μg/ml) of LPS. After 3, 6 and 12 h, media were removed, and cells were washed four times with HBSS. Then, cells were incubated in HBSS containing purified u-PA (10 units/ml; Sigma–Aldrich) to fully saturate all u-PA-binding sites on the cell membrane (Politis et al., 2002). After incubation for another 30 min at 37 °C, cells were washed three times with HBSS and treated with a mildly acidic solution to liberate u-PA from the cell surface, as mentioned previously. Membrane-bound u-PA activity was determined using the colorimetric assay described above.

6.7.5 Effect of LPS exposure on u-PA and u-PAR mRNA expression

6.7.5.1 LPS treatment

BME-UV1 cells (0.2–0.4 x 10^6 cells/ml) were seeded in 60-mm Petri dishes in complete medium containing 10% FBS. After 24 h, media were removed, cells were washed three times with HBSS and test medium consisting of complete medium containing 0.1, 1.0, or 10 μg/ml of LPS were added. After 3, 6, and 12 h, cells were harvested using trypsinisation, and total RNA was extracted using TRI Reagent (Sigma–Aldrich) and stored at -80°C until further analysis.

6.7.5.2 u-PA and u-PAR mRNA expression

The reverse transcription (RT) reaction was performed on 1 μg RNA using an iScript™ cDNA Synthesis Kit (Biorad) using cycling conditions suggested by the manufacturer's protocol. Quantitative reactions were performed in 25 μl of SYBR® Green mix (Biorad) using 350 nM b-actin and u-PA primers and 450 nM u-PAR primers. The sequences of the primers used in the quantitative reactions are indicated in Table 4. Each sample was tested in duplicate. To evaluate the qRT-PCR efficiency, dilution series were prepared by performing fourfold serial dilutions starting from the reference samples. The thermal profile used (95°C for 90 s and 50 cycles of 95°C for 15 s and 62°C for 60 s; for melting curve construction, 55°C for 60 s and 80 cycles starting at 55°C and increasing 0.5°C each 10 s) was the same for each target gene. The results obtained were compared using the delta–delta Cq method (Giulietti et al., 2001) after normalisation of the sample using the b-actin gene as the reference gene.

Target Gene	Primer sequence (5'→3')	Product length
β- actin	Sense CCAAAGCCAACCGTGAGA	133 bp
	Antisense CCAGAGTCCATGACAATGC	
u-PA	Sense GGCCAGAAGGCTCTGAGGC	133 bp
	Antisense GGCTGCCACCACACAAGTAGG	
u-PAR	Sense GGGACAGGACCTCTGCAGG	117 bp
	Antisense CGATAGCTCAGGGTCCTGTTG	

Table 4: Sequences of the primers used in the quantitative PCR reactions.

6.8 Statistical analysis

Results are presented as the mean and SD. The effects of incubation time and various treatments were evaluated using a one-way analysis of variance using the GLM procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Mean comparisons were per performed using Duncan's multiple range test, with $P < 0.05$ as the level of significance.

6.9 Results

6.9.1 LPS had no effects on cell viability

Endotoxin had no significant effect on cell viability, confirming the results previously published by Pecorini et al. (2010). Moreover, when observed under the microscope, treated cells did not show visible morphological differences compared to control cells (data not shown).

6.9.2 LPS modulated total BME-UV1 cell-associated u-PA activity

LPS stimulated total cell-associated u-PA activity at 6 h of treatment in the presence of 1.0 and 10 μg/ml LPS ($P < 0.05$ and $P = 0.07$, respectively; Fig. 25). When LPS was used, u-PA activity increased after 6 h of incubation, but at 12 h, u-PA activity decreased to control levels, showing a time-dependent effect ($P < 0.01$). u-PA activity in control cells did not show any significant variation up to 12 h of treatment.

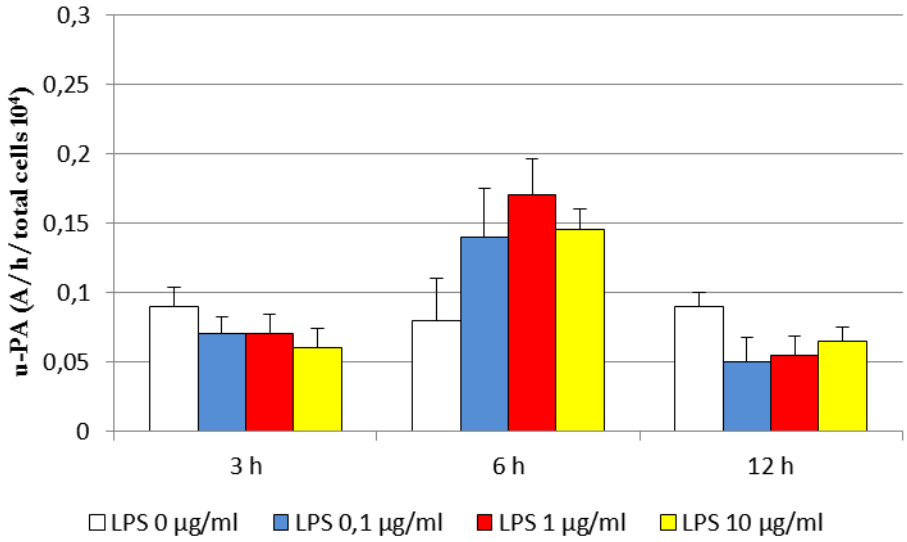

Fig. 25: Effect of LPS on total cell-associated u-PA activity in BME-UV1 mammary epithelial cells after 3, 6 and 12 h of treatment. All data are presented as means and SD.

Membrane-bound u-PA levels were also measured. The results indicated that in both treated and control BME-UV1 cells, u-PA binding sites on the cell membrane were fully saturated (data not shown). Therefore, no free u-PA-binding sites were available at the cellular surface, indicating that once produced at the cellular level, u-PA is immediately bound to its receptor.

6.9.3 LPS up-regulated u-PA and u-PAR mRNA expression

To assess whether LPS could modulate u-PA mRNA levels, the relative quantification of u-PA mRNA was performed using qRT– PCR. The gene expression levels of u-PAR were also investigated. Complementary DNA was relatively quantified using the delta– delta Cq method after normalisation of the sample using b-actin as the reference gene. Results are presented in Fig. 26. Both u-PA and u-PAR mRNA expression were up-regulated in BME-UV1 cells after 3 h of incubation with LPS compared to control cells ($P < 0.01$; Fig. 26). The LPS effect was not dose dependent, but it was time dependent. In

fact, the peak induction was observed 3 h after stimulation, after which u-PA and u-PAR levels returned to control levels, and no statistically significant regulatory activity was detected after 6 h of stimulation.

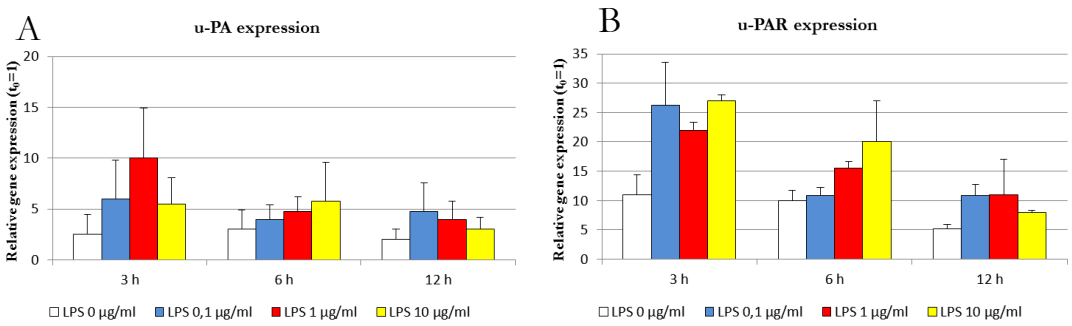

Fig. 26: Effect of subtoxic doses of LPS on the gene expression of (A) urokinase plasminogen activator (u-PA) and (B) u-PA receptor (u-PAR) in BME-UV1 mammary epithelial cells after 3, 6 and 12 h of treatment. All data are presented as means and SD.

6.10 Discussion

The available evidence indicates that bovine mammary epithelial cells respond to bacterial LPS and other microbial products through the activation of signaling pathways necessary to mount defensive mechanisms against pathogens (Bruckmaier, 2005; Bannerman, 2009; Pecorini et al., 2010). The present study has shown that exposure to LPS induced an up-regulation of gene expression levels of u-PA and u-PAR in a bovine mammary epithelial cell line. u-PA enzymatic activity was also increased. Several studies have shown that the PA system plays a role in inflammatory responses during mastitis (Long et al., 2001; Politis et al., 2002; Zhao and Lacasse, 2008; Theodorou et al., 2010). To the best of the authors' knowledge, this is the first study providing evidence that the plasminogen activator system is involved in cellular responses to LPS in bovine mammary epithelial cells *in vitro*. These results are consistent with previous reports that demonstrated the induction

of u-PA expression in response to pathogens (Cheng et al., 2009; Ohta et al., 2000; Oikawa et al., 1993). Previous results by Zavizion et al. (1997) have shown that u-PA activity is increased in BME-UV1 cells after stimulation with *Staphylococcus aureus*, whereas our findings indicate that the PA system is enhanced in response to LPS challenge. Together, these data suggest that the PA system plays a key role in cellular responses to both Gram-positive and Gram-negative bacterial infections in the mammary gland. The increase in u-PA activity is probably induced by an up-regulation of the expression of the genes within the PA system, because quantitative gene expression data demonstrated that both u-PA and u-PAR mRNA were overexpressed after 3 h. The early response of u-PA and u-PAR gene expression suggests that the PA system may be activated in mammary epithelial cells during the initial phase of the inflammatory response. The role of u-PAR is to restrict u-PA proteolytic activity to the cell surface. BME-UV1 cells produce and secrete u-PA, which binds to the u-PAR present on the cell surface (Politis, 1996). The results of this study represent evidence that u-PA-binding sites on the cell membrane of BME-UV1 cells are fully saturated because no difference in u-PA activity before and after saturation with purified u-PA was observed. It is therefore plausible that the harmonised expression of both of these components of the PA system in BME-UV1 cells maximises cellular responses to invading pathogens. In this context, the activation of the u-PA system might not only favour the recruitment of neutrophils to the site of inflammation but also activate the release of pro-inflammatory mediators, thus enhancing the acute inflammatory reaction (Mondino and Blasi, 2004). In fact, the involvement of the u-PA/u-PAR system in the regulation of inflammation is not confined within the activation of the fibrinolysis system. There is growing evidence that both u-PA and u-PAR fulfill important roles during innate immunity reactions, such as the upregulation of LPS-induced

neutrophil activation, chemotaxis and reactive oxygen species (ROS) (Abraham et al., 2003; Boyle et al., 1987; Cao et al., 1995). The short lag between stimulation with LPS and the increase in u-PA/u-PAR mRNA expression (3 h) suggests that the u-PA system belongs to the network of molecules that are overexpressed during the first phases of the innate immune reaction against invading pathogens, with the aim to potentiate the clearance of bacteria by neutrophils. An additional finding of this study was that LPS stimulation was followed by a peak of u-PA activity after 6 h of LPS exposure, whereas gene expression was statistically significant after 3 h. The discrepancy between the u-PA protein peak (6 h) and the u- PA mRNA peak (3 h) can be explained by the timeframe necessary for the protein to be synthesised and post-translationally modified within the cells. Interestingly, u-PA enzymatic activity returned to baseline after 12 h of stimulation. A possible explanation of this finding suggests the involvement of PAIs during the latter part of the inflammatory response, thus limiting the plasminogen-activating cascade (Theodorou et al., 2010). It has been shown that the binding of PAI-1 facilitates internalisation of the u-PA/u-PAR complex within the cell, therefore providing a strategy to modulate the proteolytic activity on the cell membrane (Politis, 1996). Zavizion et al. (1996a) reported the synthesis of PAI-1 by BME-UV1 cells, and recent studies performed in our laboratory showed that some modulators of mammary function regulated both PAI-1 and PAI-2 in a similar manner (Theodorou et al., 2011). It is also noteworthy that there was a decrease in both u-PA and u-PAR mRNA expression after 6 h of stimulation with LPS. Therefore, the reduction in u-PA activity is likely to be induced by a combination of down regulation of gene expression and protease inhibition by PAI. Future work will be necessary to investigate the functional significance of PAIs in the plasminogen cascade in mammary epithelial cells during inflammation. u-PA may serve multiple functions at the

cellular level under both physiological and pathological conditions, including cell proliferation and migration, tissue remodeling and inflammation (Mondino and Blasi, 2004). In the present study, BME-UV1 cells exposed to LPS showed increased u-PA expression and activity, which was independent from cell viability and cell appearance. This result indicates an effect of LPS without any impact on mammary epithelial cell proliferation. In conclusion, the present study revealed that *E. coli* LPS induced an increase in u-PA expression and activity in association with an increase in u-PAR mRNA levels. These novel findings support the biological significance of the PA system during pathological processes and strengthen the role of mammary epithelial cells in mounting an inflammatory response against invading pathogens.

7. Conclusion

My PhD was focused on the evaluation of nutraceutical bioactive compounds using cell-based assay, particularly in view of the need to use alternatives to animal studies.

Data described in the first section of my thesis showed the cellular inhibition, associated with the LDH release and DNA fragmentation, induced by *in vitro* OTA challenges in ear and embryo porcine fibroblasts cultures. Both primary porcine fibroblast cultures offer new *in vitro* opportunities to study Ochratoxin A (OTA) cytotoxicity. Furthermore obtained results demonstrated that α-tocopherol supplementation may counteract short-term OTA toxicity at different cellular levels, supporting its defensive role in the cell membrane.

The results of the second part of my study showed that in BME-UV1 cells, exogenous bovine Lactoferrin (bLF) acts as a protective factor, confirming the defensive role of bLf probably related to its cationic nature, which allows the neutralization of LPS. In addition our data showed that the response to LPS treatments does not involve endogenous bLf mRNA expression, in BME-UV1 mammary cell line probably due to the lack of functional LPS-responsive elements bacterial lipopolysaccharides (LPS).

The third part, focalized on the cellular response of Plasmin-plasminogen system to both physiological and cytotoxic stimuli in bovine mammary epithelial BME-UV cells as a model system, showed that IGF-1 and EGF increased cell proliferation and in parallel caused induction of the u-PA/u-PAR system. On the other hand, the prominent effect of dexamethasone was to induce PAI-1 and PAI-2 expression and overall to down-regulate the plasminogen activating cascade at the molecular and protein levels. Alterations in the expression of PA-related genes and those observed at the protein level following treatment of BME-UV1cells with dexamethasone or prolactin

(induction of u-PA) are not related to bovine mammary epithelial cell growth. Furthermore the present study revealed that *E. coli* LPS induced an increase in u-PA expression and activity in association with an increase in u-PAR mRNA levels. These novel findings support the biological significance of the PA system strengthening the role of mammary epithelial cells in mounting an inflammatory response against invading pathogens. Plasmin-plasminogen system has a key role during mammary gland development and pathological processes.

In conclusion in order to use nutraceuticals in both human and animal nutrition, it is important to develop protocols and models to evaluate the bioaccessibility, bioavailability and functionality of the bioactive components. The approach to cell-based bioassays supports the new need for food/feed analysis in terms of bioactivity and functional properties.

According to our results cell-based assays are a valuable tool for assessing fundamental regulatory mechanisms at a cellular level. Cell-based assays are a valuable tool for assessing fundamental regulatory mechanisms at a cellular level, such as the plasmin-plasminogen system. Cell-based assays allow both the functionality of nutraceutical and cellular response mechanisms to be evaluated reducing use animal models in the preliminary study phase. Obviously, data obtained in cell culture models must be interpreted carefully, since this system represents a simplification of the intricacies of the numerous reactions and interactions that occur *in vivo*.

8. References

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9. Publications

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Scientific Research

Vitamin E Bioavailability: Past and Present Insights

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ABSTRACT

Over the past decades several studies have investigated the importance of an adequate vitamin E status to sustain both animal health and production in dairy cows. Cow milk is considered as a remarkable source of bioactive components promoting human health, which has renewed interest in the effects of vitamin E supplementation on its nutritional value, sensory quality and shelf life. Thus, defining relative bioavailability, utilisation and transfer into milk of different vitamin E formulations is particularly important to assess the adequate levels of supplementation for animal health and milk quality. In nature vitamin E is present under one isomeric form, RRR α-tocopherol; when α-tocopherol is synthesized chemically, a racemic mixture of 8 possible isomers of α-tocopherol in equimolar concentrations is produced (all-rac α-tocopherol). The different stereoisomers have different biopotencies in humans and livestock; the conversion factor between RRR and all-rac vitamin E was estimated by early studies on the basis of the rat foetal resorption bioassay, and then extended to other species. Recent advances on the distribution of vitamin E stereoisomers in plasma and tissues have highlighted the need to formulate new conversion factors in dairy cows as well as in humans. On account of this, the present article aims to consider past and recent data related to vitamin E in dairy cow nutrition.

Keywords: *Vitamin E, Dairy Cows, Bioavailability, Milk*

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Article

Alpha-Tocopherol Counteracts the Cytotoxicity Induced by Ochratoxin A in Primary Porcine Fibroblasts

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Abstract: The aims of the current study were to determine the half-lethal concentration of ochratoxin A (OTA) as well as the levels of lactate dehydrogenase release and DNA fragmentation induced by OTA in primary porcine fibroblasts, and to examine the role of α-tocopherol in counteracting its toxicity. Cells showed a dose-, time- and origin-dependent (ear *vs.* embryo) sensitivity to ochratoxin A. Pre-incubation for 3 h with 1 nM α-tocopherol significantly ($P < 0.01$) reduced OTA cytotoxicity, lactate dehydrogenase release and DNA damage in both fibroblast cultures. These findings indicate that α-tocopherol supplementation may counteract short-term OTA toxicity, supporting its defensive role in the cell membrane.

Keywords: ochratoxin A; α-tocopherol; DNA damage; fibroblasts; swine

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ORIGINAL ARTICLE

Evaluation of the protective effect of bovine lactoferrin against lipopolysaccharides in a bovine mammary epithelial cell line

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Abstract Lactoferrin (Lf) is a non-haem iron-binding glycoprotein with a molecular weight of about 80 kDa, synthesized by glandular epithelial cells and stored in the secondary granules of neutrophils. The physiological significance of Lf is related to non-specific immune defence against pathogens, immunomodulatory activity, iron homeostasis, antioxidant properties and regulation of cell growth. Lf is a bioactive component of the mammary secretions and its modulatory and defensive functions do affect the newborn and the mammary gland as well. In this work a bovine mammary epithelial cell line (BME-UV1) was used as an in vitro model of the bovine mammary epithelium to examine the protective role of exogenous bovine Lf (bLf) against the cytotoxic damage induced by bacterial lipopolysaccharides (LPS) and the endogenous bLf mRNA expression after LPS exposure. In the in vitro model used, exogenous bLf exerts a protective effect against endotoxin cytotoxicity, which could be mediated by the LPS-neutralizing capability of bLf. In addition, in BME-UV1 cells the response to LPS exposure does not involve bLf mRNA expression, suggesting that this cell line lack of functional LPS-responsive elements.

Keywords Lactoferrin · BME-UV1 · Lipopolysaccharides · MTT test · Real-Time PCR

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Effect of growth factors and lactogenic hormones on expression of plasminogen activator-related genes and cell proliferation in a bovine mammary epithelial cell line

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There is conflicting evidence in the literature as to whether up-regulation of urokinase plasminogen activator (u-PA) expression is related to bovine mammary epithelial cell growth. The role of u-PA receptor (u-PAR) and that of the plasminogen activator inhibitors type 1 and type 2 (PAI-1 and PAI-2) in bovine mammary epithelial cell proliferation is not known. The effect of growth factors and various hormones known to affect mammary function on expression of u-PA, u-PAR, PAI-1, PAI-2 and cell proliferation using the BME-UV1 bovine mammary epithelial cell line was examined. Cell proliferation was measured using the MTT assay and direct cell enumeration. Results showed that both IGF-1 and EGF increased cell proliferation but EGF was a more potent mitogen than IGF-1. Furthermore, IGF-1 increased by 2-fold expression of both u-PA and u-PAR while EGF increased by 3·8-fold the expression of only u-PAR. Both growth factors had no effect on expression of PAI-1 and PAI-2. In a manner consistent with changes in gene expression, EGF and to a lesser extent IGF-1 up-regulated total cell associated, membrane-bound and secreted u-PA activity. Thus, a strong correlation exists between u-PAR gene expression along with the activity of u-PA present on cell membranes and cell proliferation. Dexamethasone, prolactin and surprisingly insulin had no effect on cell proliferation. Dexamethasone alone and when combined with insulin or prolactin up-regulated gene expression of both PAI- and PAI-2 but not that of u-PA and u-PAR. Decreased total cell-associated, membrane-bound and secreted u-PA activity was detected in cells cultured in the presence of dexamethasone when combined with insulin or prolactin. However no such effect was observed in the presence of dexamethasone alone. Thus, dexamethasone acting synergistically with prolactin or insulin inhibits the activation of the plasmin-plasminogen system but this inhibition is not correlated with any changes in cell proliferation.

Keywords: Growth factors, lactogenic hormones, plasminogen activator-related genes, mammary proliferation.

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Effect of *Escherichia coli* lipopolysaccharide on u-PA activity and u-PA and u-PAR RNA expression in a bovine mammary epithelial cell line

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ABSTRACT

It is well known that the plasminogen-activating (PA) system plays a key role in the bovine mammary gland during tissue remodelling. However, the modulation of the PA cascade after bacterial infections needs to be elucidated. This study examined the effects of *Escherichia coli* lipopolysaccharide (LPS) on cell viability, the modulation of cell-associated u-PA activity, and the regulation of u-PA and u-PA receptor (u-PAR) RNA expression using the BME-UV1 bovine mammary epithelial cell line. LPS did not affect cell viability, but induced an increase in u-PA activity, with the maximum response after 6 h of incubation. Moreover, u-PA and u-PAR mRNA expression were both up-regulated in BME-UV1 cells after 3 h of incubation with LPS. These data indicated that *E. coli* LPS led to an increase in u-PA activity and RNA expression of u-PA and u-PAR in BME-UV1 cells, thus strengthening the role of the PA system during pathological processes.

Conference paper

- Effect of hormones and growth factors on cell proliferation and expression of urokinase related genes in a bovine mammary epithelial cell line / G. Theodorou, C. Pecorini, R. Rebucci, F. Saccone, C. Lecchi, E. Fusi, I. Politis, L. Pinotti. - In: Italian journal of animal science. - ISSN 1594-4077. - ISSN 1828-051X. - 10:Suppl. 1(2011), pp. 42-42. (19° ASPA congress, Cremona 2011).
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Oral presentation

National Congress•

- Valutazione dei profili metabolici in suinetti alimentati con una dieta contenente panello di seme di tabacco. / L. Rossi, F. Saccone, G. Selmini, A. Baldi, V. Dell'Orto, C. Fogher. - In: Atti della Società italiana delle scienze veterinarie 64(2010), pp. 515-517. (64°. SISVet, Asti 2010).

International Congress•

- Evaluation of the cytotoxic effects induced by ochratoxin A in a bovine mammary cell line / A. Baldi, E. Fusi, R. Rebucci, C. Pecorini, L. Pinotti, F. Saccone, F. Cheli (EAAP Book of abstracts ; 16). - In: Book of Abstracts of the 61th Annual Meeting of the European Association for Animal ProductionWageningen : Wageningen Academic Publishers, 2010.

- ISBN 978-90-8686-152-1. - pp. 7-7 (61°. Annual Meeting of the European Association for Animal Production, Heraklion 2010).

- Oral administration and evaluation in piglets of tobacco seeds expressing antigenic proteins against Oedema disease / L. Rossi, S.Vagni, F. Saccone, S. Reggi, A. Baldi, C. Fogher, V. Dell'Orto. (PBVA, Porto 2011).

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