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**Intramammary administration of oxygen/ozone
mix in oil vehicle blended platelet concentrate as
an unconventional therapy in bovine mastitis.
First clinical application**

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CHAPTER 1

Foreword

1. Foreword

Biological systems show a high level of complexity.

For years single-target drugs have been successful and many pathologies have been considered cured and eradicated. As a matter of fact, the biological complex ecosystem was using the single-target chemical drugs information to evolve and find its own shortcuts.

This is the case of antibiotic resistance. Planktonic bacteria evolve generating biofilm communities that are able to gambol antibiotic aggression and possibly evade the host immune system mechanisms.

Synergistic solutions are needed to reactivate a virtuous balance in host's favor: these solutions must be able to cooperate and eventually boost the host immune system, which has a naturally-embedded synergistic modus operandi; they must produce a reduced number of inconvenient metabolites, and they should exert a multifunctional activity.

Antimicrobial peptides seem to convey the features required as they deploy a multifunctional antimicrobial set of measures, complying with the host immune system and offering regenerative effects on tissues.

On the other end, ozone has been investigated for its bactericidal capacity and for its potential to reactivate depressed immune systems.

As this experimental study accounts for, the administration to bovine mastitis of a blending of platelet concentrate, rich in antimicrobial peptides, with oxygen/ozone mix in oil vehicle suggests the importance of further investigation and evidence of a possible new generation of treatments.

CHAPTER 2

Objectives

2. Objectives

The aim of this study was to evaluate the potential of some treatments that are typically used in regenerative medicine and apply them to some veterinary relevant states.

Both stem cells and platelet concentrate were investigated.

As for the first, an application to horse injuries led to the paper reported in Chapter 9.

The second was administered to bovine mastitis and its enhancement with ozonated oil showed very interesting results.

Chapters 3 to 8 report the fascinating research of a new viable interpretation of bovine mastitis, in relation to antibiotic resistance, and, hopefully, a new type of effective treatment seems to be discovered.

Due to the complexity of the problem and to the many variables involved, these results express a hint for further experimental trials.

Chapter 3 outlines the strict relation between tissue damage and mastitis worsening, the demonstrated dependence between antibiotic resistance and the malicious presence of bacterial biofilm, the regenerating and antimicrobial activity of platelet concentrate and the beneficial properties of ozone and ozonated oil.

Chapters 4 and 5 describe the experimental trials, which took place between December 2011 and May 2014.

Chapter 6 discusses the most recent scientific evidence trying to find a significance for the collected results.

In addition, Chapter 10 presents a work on bovine reproduction, driven by my main scientific passion.

CHAPTER 3

State of the Art

3. State of the Art

3.1 Mastitis

Mastitis has a complex aethiology and is the most costly disease in dairy cattle. Decreased milk production, loss of lactating cows or quarters, spreading of microbial agents throughout the livestock and consequent proliferation of the disease are the main problems related to the phenomenon.

Mastitis is defined as an inflammatory reaction of the mammary gland that is usually caused by a microbial infection (Zhao, 2008). It is reported that mammary tissue damage reduces the number and activity of epithelial cells and consequently contributes to decreased milk production (Zhao, 2008).

Mastitis usually occurs primarily in response to intramammary bacterial infection, but also to intramammary mycoplasmal, fungal, or algal infections. Mechanical trauma, thermal trauma, and chemical insult predispose the gland to intramammary infection. Occurrence of mastitis depends on the interaction of host, agent, and environmental factors and, once occurred, worsening of microbial aggression, arising of mammary tissue damage and decreasing milk production set up a negative loop, hard to reverse.

For this reason, early diagnosis and fast effectiveness of therapy are fundamental to control losses.

Bovine mammary glands are exposed to diverse bacteria throughout lactation and in non-lactating periods. Pathogens commonly isolated from mastitic milk can be classified as noncontagious (most are environmental) and contagious microorganisms. The former include *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, and coagulase-negative staphylococcus species, whereas the latter include *Staphylococcus aureus* and *Streptococcus agalactiae*. The teat and streak canal are the first line of defense of the mammary gland. The keratin lining in the streak canal provides a physical and chemical barrier against bacterial penetration (Capuco, 1992). Bacteria may escape the natural defense mechanisms by multiplication along the streak canal (especially after milking), or by propulsion into the teat cistern by vacuum fluctuations at the teat end during milking. The infection occurs after bacteria gain entrance to the mammary gland via the teat canal. After bacteria overcome the anatomical defense, they must evade the cellular and humoral defense mechanisms of the mammary gland to establish disease (Sordillo, 2002). If the infection is not eliminated, bacterial levels in the mammary gland eventually rise to a level at which they begin to damage the

mammary epithelium. As infection persists, the number of somatic cells in milk continues to increase and, concomitantly, tissue damage is worsened. The alveoli in the gland start to lose structural integrity and the blood-milk barrier is breached. This allows extracellular fluid to enter the gland and mix with the milk. Visible changes in the milk and the udder start to occur. These can include external swelling, reddening of the gland, and clotting and wateriness of the milk. By definition, this is the start of clinical symptoms (Zhao, 2008).

In brief, bovine mammary epithelial cells can be damaged during mammary inflammation by:

- 1) release of a range of cellular and extracellular products from bacterial pathogens;
- 2) lysosomal enzymes and oxidative products released from phagocytes during phagocytosis of invading organisms, and
- 3) proteases from blood and cytokines released during the immune response.

Particular attention must be paid to tissue damage, which occurs through two distinct types of cell death, apoptosis and necrosis: these may be distinguished by morphological, biochemical, and molecular changes in dying cells and are both reported in association to the presence of microorganisms, such as *E. coli* and *S. aureus* (Zhao, 2008).

In very severe cases of *Escherichia coli* inflammation, the infection progressed via the ductile system to produce a limited inflammatory reaction but with an extensive involvement of the secretory tissue. In its most severe form with uncontrolled bacterial multiplication, all lactiferous sinus epithelia were lost, interstitial tissue became hemorrhagic, and often the animal died of toxemia within a few hours of infection (Zhao, 2008).

On the other hand, it is also reported that *Staph. aureus*. exhibited less milk synthesis and secretion, due to necrosis of the secretory tissues, as evidenced by more interalveolar stroma and involuting alveolar epithelium and less alveolar luminal space compared with uninfected contralateral controls. Moreover, these changes were associated with replacement of secretory tissue with non-secretory tissue (Zhao, 2008).

3.1.1. Involvement of neutrophils in tissue damage

Following detection of pathogen invasion into the mammary gland, macrophages and epithelial cells release chemoattractants. These agents trigger the migration of leukocytes, mainly PMN, from the blood toward the mammary gland and increase their proportions from a basal level of 5 to 25% to approximately 90% of total cells in the milk. These PMN are considered as the second line of defense of the

mammary gland. The presence of functional PMN is crucial to the host defense against bacterial pathogens (Zhao, 2008).

The main functions of PMN are to engulf pathogens and destroy them via oxygen-dependent and oxygen-independent systems. At the same time, PMN can potentially harm the mammary gland. The exact mechanism by which PMN damage bovine epithelial cells during mastitis is still not fully understood. Neutrophils may promote tissue injury and disturb mammary function, via reactive oxygen metabolite generation (i.e., the respiratory burst) and granular enzyme release (i.e., degranulation) (Zhao, 2008).

3.1.2. Involvement of bacteria in tissue damage

There is increasing evidence that pathogens use various mechanisms to impinge upon cell death pathways. A number of pathogens are armed with an array of virulence determinants, which interact with key components of a host cell's death pathways or interfere with regulation of transcription factors monitoring cell survival. These virulence factors induce cell death by a variety of mechanisms, which include:

- 1) pore-forming toxins, which interact with the host cell membrane and permit the leakage of cellular components;
- 2) toxins that express their enzymatic activity in the host cytosol;
- 3) effector proteins delivered directly into host cells by a highly specialized type-III secretory system;
- 4) super-antigens that target immune cells, and
- 5) other modulators of host cell death. Much progress has been made in understanding the role of apoptosis and necrosis in response to bacterial infection (Zhao, 2008).

3.1.3. Involvement of plasma proteins and cytokines in tissue damage

At the onset of mastitis, increased permeability of the blood-milk mammary epithelial barrier leads first to an influx of serum constituents, such as plasminogen and numerous other enzymes, and second to a massive recruitment of somatic cells, in particular, PMN. Milk contains two proteinase systems derived from blood, one of which is involved in dissolving blood clots (i.e., plasmin) and the other in defense against invasive microorganisms (i.e., lysosomal proteinases of somatic cells). Whereas plasmin is the principal proteinase in good-quality milk, other proteinases, including cathepsins and elastase, are probably also active, particularly as the somatic cell count of milk increases. This is supported by the observation that the protease activities of plasmin and mastitic milk differ. In addition, mammary epithelial cells also express certain matrix metalloproteases and serine proteases, which are involved in the activation of plasminogen to plasmin. How

much plasmin contributes to tissue damage during mastitis is debatable. Mammary epithelial cell viability depends on attachment to the extra cellular matrix, so it is reasonable to postulate that extra cellular matrix degradation is involved in tissue damage and cell death during mastitis (Zhao, 2008).

Very little work has been conducted to determine the role of cytokines in the regulation of tissue damage during mastitis. Cytokines recruit PMN that function as phagocytes at the site of infection and sometimes induce apoptosis in a variety of cell types, including bovine endothelial cells and human mammary epithelial cells. Levels of these cytokines increase during *E. coli* mastitis, and it is tempting to assume that they also induce apoptosis in bovine mammary epithelial cells. A range of cytokines are also known to promote a wide variety of functions of PMN, including adhesion, surface receptor expression, free radical production, and release of lysosomal constituents. Therefore, the effects of cytokines on tissue damage are more likely to be mediated through recruitment and activation of PMN (Zhao, 2008).

3.1.4. Protective role of cathelicidins in mammary tissue (Whelehan, 2014)

Recently, it is also shown that constitutive cathelicidin expression in the mammary gland suggests a possible role for these host defence peptides its protection.

Cathelicidins comprise a major group of host-defence peptides. Conserved across a wide range of species, they have several functions related to host defence. Only one cathelicidin has been found in humans but several cathelicidin genes occur in the bovine genome. It has been proposed that these molecules may have a protective role against mastitis. The study characterized the cathelicidin gene-cluster in the bovine genome and identified sites of expression in the bovine mammary gland.

Results showed that the seven known protein-coding cathelicidin genes are constitutively expressed in a broad range of tissue types, including the mammary gland. Cathelicidin proteins are a major component of the neutrophil secondary granule and increased levels of these proteins are observed in a range of inflammatory conditions. Extensive neutrophil recruitment from the circulation to the lumen of the mammary gland is a hallmark of the early immune response to mammary infection.

The study also suggested that other cells in addition to immune cells are capable of secreting these potent immunomodulatory molecules. Cathelicidins are constitutively expressed in the healthy mammary gland, while transcripts of several other protein-coding genes can be detected in leukocytes extracted from the milk of animals with a high somatic cell count.

3.2 Antibiotic resistance

During the last decades infections have lowered their sensibility to antibiotics.

Antimicrobial agents that are effective *in vitro* have failed or have been less powerful at the same concentration when used *in vivo* (Melchior, 2006-2). Actually, this difference of efficacy is related to planktonic bacteria versus sessile ones: *in vitro* studies regarding sessile communities of bacteria, attached to a surface and organized in their slurry matrix to form biofilm, have shown a loss of sensitivity to virtually all classes of antibiotics (Melchior, 2006-1).

For this reason, the lack of efficacy of antibiotics in killing pathogens, commonly called antibiotic resistance, is considered caused by bacterial biofilm (Melchior, 2006-1).

In addition, biofilm-associated bacteria shows an innate resistance not only to antibiotics and disinfectants but also to clearance by host defense mechanisms (Melchior, 2006-1).

An understanding of biofilm architecture and chemistry is needed to assess a valid eradication strategy.

3.2.1. *Biofilm*

The current definition of a bacterial biofilm is a well organized population of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton, 1999).

In most natural environments, microorganisms try to adhere to available surfaces. Hence, the free-swimming (or planktonic) phase can be viewed as a mechanism of dispersal from one surface to another (Melchior, 2006-1).

Biofilm formation process can be described in three basic stages.

During phase 1, there is attachment of cells to a surface. The exopolysaccharides slime produced by cells in this initial phase is effective in facilitate clumping: the presence of adhesins, which are products of various genes by the living surface, actually promotes the adhesion of the first group of bacterial cells (Mack, 1999; Melchior, 2006-1). In addition, the presence of proteins such as fibronectin and fibrinogen speeds up the process (Melchior, 2006-1).

During phase 2, cell multiplication and formation of a mature structure consisting of many layers of cells, connected to each by extracellular hydrated polysaccharides, occur. This slime layers creates on the external surface a diffusion barrier that further protect the biofilm bacteria (Melchior, 2006-1).

In a final third phase, there is detachment and dispersal of planktonic bacteria from biofilm. These new bacteria are now free to find new surfaces to colonize and set up new populations.

In particular this matrix seems able to protect bacteria both from components of the host immune system and from antibiotic aggression, enhancing the bacteria survival rate.

The mechanisms involved in bacterial survival to both host immune system and antimicrobial agents are commonly synthesized in three ways (Melchior, 2006-1; Lewis, 2001):

- delayed and/or restricted penetration of the antimicrobial agents and of components of the host immune system through the biofilm matrix,
- reduced growth rate of biofilm organisms,
- physiological changes into the biofilm and expression of possible biofilm-specific resistance genes.

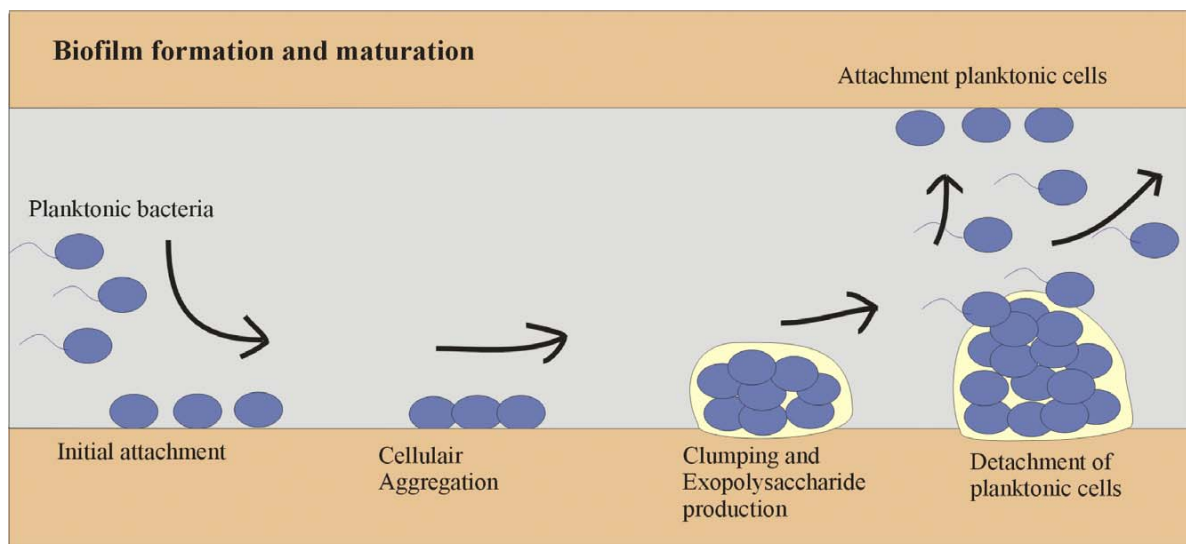


Figure 3.2.1 Biofilm formation and maturation: attachment of planktonic bacteria, cellular aggregation, clumping and exopolysaccharide slime production, mature biofilm with detachment of planktonic cells (Melchior, 2006-1).

Limited penetration

The biofilm matrix acts as a diffusion barrier that protects the external surface of the biofilm: it regulates the crossing of different substances through it and it is able to bind antimicrobials (Lewis, 2001). The barrier effect is competent to avoid the intrusion of large molecules, like antimicrobial proteins lysozyme and complement, as well as smaller antimicrobial peptides, like defensins and their analogs (Lewis, 2001).

As for large positively charged antibiotics molecules, these are possibly neutralized or bound by the negatively charged exopolysaccharide protecting cells (Lewis, 2001) and are effectively 'diluted' to sublethal concentrations before they can reach all of the individual bacterial cells within the biofilm (Hall-Stoodley, 2004). The barrier

properties of the exopolysaccharide hydrogel might also protect against UV light and dehydration, and might localize enzymatic activity (Hall-Stoodley, 2004).

The penetration rate varies depending on the type of aggression. In most cases involving small antimicrobial molecules, the barrier of the polysaccharide matrix operates a delayed penetration, postponing the killing effect of antimicrobials on bacteria rather than provide full immunity (Lewis, 2001).

In the meanwhile, within the biofilm, bacteria can operate an antimicrobial degradation, taking advantage of low concentration of antimicrobials that infiltrated the biofilm. Biofilm showed a synergistic effect between retarded diffusion and enzyme degradation. From this perspective an analogy between the single bacteria with its own outer membrane versus the biofilm community with the slime protection has been outlined (Lewis, 2001).

Decreased growth rate

Many antibiotics require at least some degree of cellular activity to produce their effect. In fact the mechanism of action of most antibiotics involves disruption of a microbial process (Hall-Stoodley, 2004).

Within a biofilm, bacteria grow at different rates and some of them are present in a starving mode. This fact enhances the survival rate of the population, both modulating the nutrients intake and availability within the matrix (Costerton, 1999) and binding their existence to antimicrobials (Lewis, 2001).

For instance, oxygen limitation is thought to contribute to the antibiotic resistance of biofilms since the efficacy of some antibiotics is reduced in its absence: in fact reduced oxygen levels may cause physiological and phenotypic changes that caused increased biofilm resistance to antibiotics (Clutterbuck, 2007).

Gene expression

Biofilms are described as bacteria microcolonies attached on a surface, well developed in organized communities with functional heterogeneity, specifically aimed to survive in a hostile environment (Costerton, 1999).

Studies show how biofilms display significant convergent survival strategies, which mainly come from structural specialization. These attributes about specialization within the biofilm and biofilm development tactics seem to be conserved among an wide range of prokaryotes (Hall-Stoodley, 2004).

Many authors report that biofilm formation is accompanied by notable genetic and physiological variations in the microorganisms (Melchior, 2006-1).

Within the biofilm, cells in different regions display a different gene expression (Costerton, 1999). Genetics can be considered as a survival biofilm active response, while adaptation to environmental condition is commonly appraised as passive

response (Hall-Stoodley, 2004). It has been proposed that the open water channels or voids that surround the structures within biofilms may act as pools of genes allowing for genetic acquisition and exchange by horizontal transfer (Wuertz, 2004; Clutterbuck, 2007).

The ultimate aim of biofilm active gene reaction seems to ensure survival to a subpopulations of resistant phenotypes within the colony (Hall-Stoodley, 2004).

This type of gene regulation has been termed quorum sensing and response and it is responsible to facilitate the cell-to-cell signaling required for biofilm development. These quorum sensing molecules differ between bacteria, with most Gram-negative organisms utilizing acylhomoserine lactones, and most Gram-positive organisms using secreted peptides. The coordination of behavior that is facilitated by quorum sensing is thought to play an important role in the pathogenicity of certain organisms by regulating the expression of virulence determinants so that high cell densities of bacteria are reached before these determinants are expressed, thereby increasing the likelihood of a successful attack on the hosts defenses (Clutterbuck, 2007).

In addition, quorum sensing is also thought to be involved in determining biofilm thickness, being then an actual regulator of biofilm formation and maturation. In staphylococci, QS is established by the accessory gene regulator (Agr) system, which produces a secreted, post-translationally modified peptide that interacts with a two-component system in an auto feedback loop, ultimately resulting in a considerable shift in gene expression patterns during early stationary growth phase. In general, Agr up-regulates toxins and other acute virulence factors and down-regulates surface proteins such as MSCRAMMs. Recent reports provided important insight into the QS-controlled factors that structure biofilms and cause detachment (Joo, 2012).

This small fraction of persister cells seems basically responsible for the very high level of resistance of the biofilm to killing (Lewis, 2001). The majority of bacteria within the biofilm is not necessarily resistant to antibiotics. However growth related antibiotics aggression forces part of biofilm bacteria to perform a slow growth rate. This way persisters survive and are indeed preserved by the presence of an antibiotic that inhibits their growth. Paradoxically, the antibiotic helps persisters persevere (Lewis, 2001).

Persisters could represent cells with disabled the programmed cells death process, a safety mechanism producing cells that will survive if an antibiotic reaches the entire population. Similarly, cells would need to discriminate between an unreparable defect and starvation. Development of tolerance to antibiotics in starved cells might result from inhibition of the programmed cells death process, and might be aimed at preventing suicide when nutrients are limiting (Lewis, 2001).

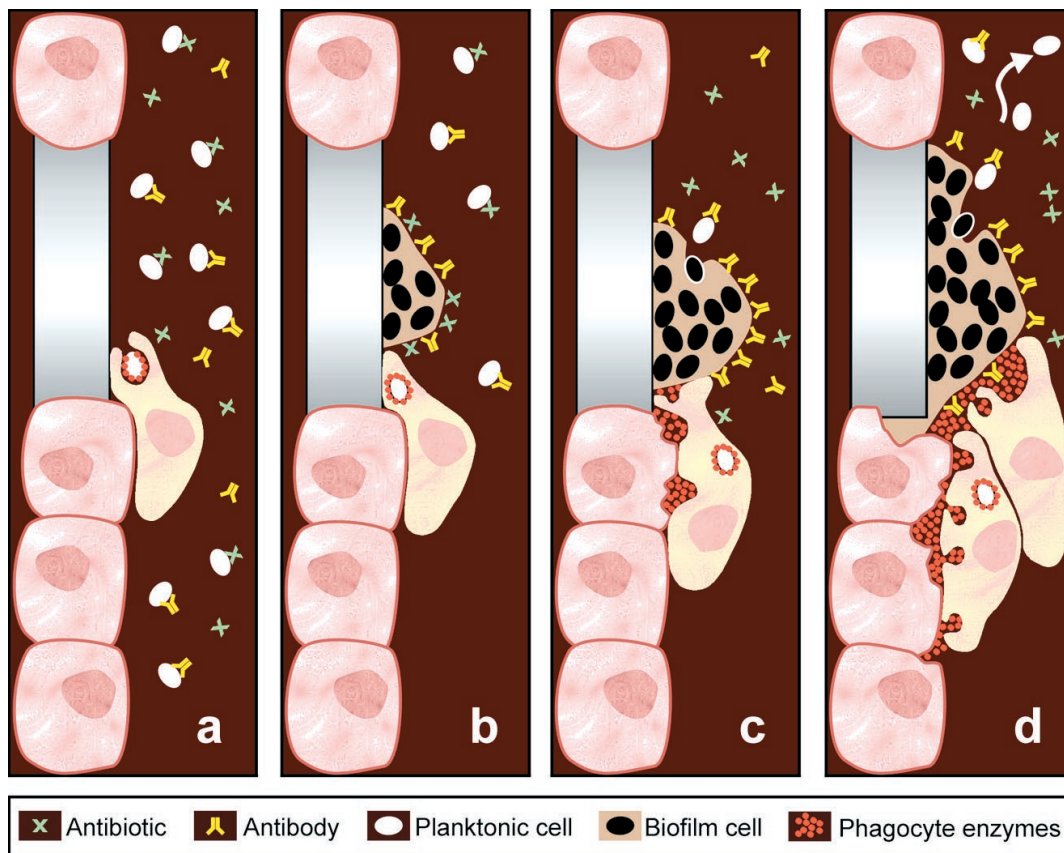


Figure 3.2.2. Diagram of a medical biofilm. (A) Planktonic bacteria can be cleared by antibodies and phagocytes, and are susceptible to antibiotics. (B) Adherent bacterial cells form biofilms preferentially on inert surfaces, and these sessile communities are resistant to antibodies, phagocytes, and antibiotics. (C) Phagocytes are attracted to the biofilms. Phagocytosis is frustrated but phagocytic enzymes are released. (D) Phagocytic enzymes damage tissue around the biofilm, and planktonic bacteria are released from the biofilm. Release may cause dissemination and acute infection in neighboring tissue (Costerton, 1999).

In conclusion, biofilms are hard to eradicate and are the cause of many persistent infections (Lewis, 2001), since they show resistance to bacterial agents 10-1000 times more than planktonic bacteria of the same strain (Melchior, 2006-1). Some authors hypothesize that biofilm formation is likely to be an ancient adaptation of prokaryotic life (Hall-Stoodley, 2004).

Increasing evidence suggests that antibiotics are at the present time not only less effective against bacterial biofilms, but also may stimulate the biofilm formation. (Melchior, 2006-1).

3.2.2. Biofilm and possible deactivation of host immune system

It has been also proposed that biofilm infections are in a sense very similar to planktonic infections in the absence of an immune response. The biofilm exopolymer physically protects the cells from the components of the immune system. In fact, usual therapies are based on the assumption that killing a majority of pathogens in many cases is just as good as killing them all. This is often the case, because the immune system collaborates with the antibiotics and probably “mops up” the remaining persisters. Persisters do become a problem when the immune system is not operating.

The presence of persisters that can rebound when the antibiotic concentration drops would explain the necessity of therapy with a combination of unrelated drugs that together probably eradicate persisters.

If the concentration of the antibiotic temporarily drops or if symptoms disappear due to the eradication of planktonic cells and therapy is discontinued, the persisters will reform the biofilm, which will begin to shed off new planktonic cells. This dynamic explains the relapsing nature of biofilm infections and the need for a lengthy antibiotic therapy or a definitive effective therapy. This view of a biofilm infection suggests, somewhat counterintuitively, that the recalcitrance of biofilms does not necessarily rely on their higher levels of intrinsic resistance to killing by antibiotics than the level of intrinsic resistance of planktonic cells. Indeed, if a biofilm of a particular species under given conditions in vivo happens to be just as sensitive or even more sensitive to killing by antibiotics than a planktonic population (say, that this biofilm produces fewer persisters than a planktonic population), it will still survive better than planktonic cells, since it is invulnerable to immune attack (Lewis, 2001).

3.2.3. Biofilm and mastitis

The relationship between the ability to produce chronic bovine mastitis and biofilm formation has been demonstrated (Cucarella, 2004).

Studies of *Staphylococcus aureus* in mammary tissue in acute and chronic infections revealed that the bacteria are typically located in clusters within the alveoli and lactiferous ducts in association with the epithelial cells and invaded in the interstitial tissue (Hensen, 2000; Melchior, 2006-1). These bacterial clusters appear approximately 24 h after exposure to the pathogen along with the establishment of the intramammary infection (Melchior, 2006-1).

According to a recent study, the increase in the somatic cell count in *S. aureus* infections is markedly higher than in coagulase-negative staphylococci mastitis, since the species capable of coagulating blood plasma are considered potentially

more pathogenic due to the production of an enzyme that enables the microorganism to persist in the presence of a host immune response.

Moreover, some milk samples analyzed were negative on Californian Mastitis Test but positive for *Staphylococcus* spp. This result reinforces the fact that even when triage tests are used on the dairy farms, there are animals that may harbor mastitis-causing agents. (da Costa Krewer, 2014).

It is in fact reported (Cucarella, 2004) that this situation has important consequences in milk production at the farm level, as mastitis control inadvertently allowed the misclassification of cows as uninfected when they were in fact infected with ica- and bap-positive bacteria. These microorganisms remained undetected in the udder and the infected cows were not submitted to antibiotic treatment, so that a further spread and persistence of mastitis in the farm with consequent production losses could take place. The low SCC associated with a high mammary gland colonization by some bap-positive ica-positive isolates also suggests a reduced toxin production during these infections.

On one hand, bacteria may form biofilms with little or no obvious harmful effect on the host which are therefore frequently undetected. Alternatively, non-biofilm-forming bacteria may produce toxins, become more pathogenic in the short term, and cause damage to the host tissues. This may result in an exposure of host adhesins which enable the bacteria to adhere by interacting with host receptor proteins and to spread to other body sites.

Antibiotics to control chronic bovine mastitis are usually chosen on the basis of conventional in vitro diffusion and dilution evaluation methods and without taking into account the role of biofilm formation on resistance. As high concentrations of many known antibiotics are required to kill biofilm bacteria and lead only to a partial killing effect, there is a need to find antimicrobials that are efficient against biofilm bacteria.

It is likely that the presence of Bap reduced infectivity in the short term by blocking early adherence dependent on host- MSCRAMM interaction but had the opposite effect in late adherence, allowing longer bacterial persistence in the mammary gland (Cucarella, 2004).

In addition other studies outline that skim milk and slime production are two factors that have a positive effect on biofilm production by bovine isolates of *S. aureus*. In fact, biofilm production is increased in a skim-milk medium (Fabres-Klein, 2015). Lactose and milk whey contribute to capsule polysaccharide and biofilm formation in *S. aureus*, because the presence of slime and milk seems able to promote a change in the bacterial lifestyle that increases the chances of survival in the extracellular medium. For this reason, mastitic altered milk boosts biofilm production and hence promotes bacterial survival, setting up a vicious cycle. Biofilm formation has thus an important role in the virulence of *S. aureus* isolated from bovine intramammary infections (Clutterbuck, 2007).

3.3 Antimicrobial peptides

3.3.1. Multifunctional role of AMPs

Antimicrobial peptides (AMPs) have an essential role in the mammals immune defense system complexity, where they exert a multifunctional activity.

The extreme diversity of molecules referred to as AMPs embraces a list of 1200 AMPs and a variety of classification has been proposed, based on their being small, having positive charge, amphiphatic structure, α -helix structures, β -sheet structures stabilized by disulfide bridges or peptides with extended or loop structures.

The expression of AMPs differs depending on the cell and tissue type, but in most cases AMPs are co-expressed as groups that act together.

Relatively little information is available on the immunological function for most of these peptides, and it is unlikely that the immunomodulatory actions of the mammalian AMPs are also a component of the biology of the hundreds of AMPs produced by most prokaryotes and invertebrates. Thus, the first important concept to confer in a discussion of the AMPs is that they are similar only by their capacity to directly kill or inhibit the growth of microbes.

Some similarities in their structure are possibly responsible for their ability to influence lipid membrane structures. Cathelicidins and defensins are the most studied AMPs in mammals.

Cathelicidins.

Cathelicidin peptides are a highly heterogeneous group of molecules both within and between species: they are named together because, despite their structural diversity, they encode a protein that acts as a cysteine protease inhibitor and also possesses some antimicrobial activity.

In human neutrophils and other cell types hCAP18 is processed to release from the carboxy (C-) terminus of the precursor protein an antimicrobial peptide of 37 amino acids. As a mature peptide, LL37 is well known for its rapid, potent and broad-spectrum antimicrobial activity when released from the C-terminus of hCAP18. Its processing is essential for activation of its antimicrobial activity in vivo.

Defensins.

In mammalian species, around 50 α -defensins and 90 β -defensins and also θ -defensins have been identified.

All defensins are cationic, microbicidal, lack glycosyl- or acyl- side-chain modifications and contain six highly conserved cysteine residues which form three pairs of intramolecular disulfide bonds. Although mammalian defensin peptides exhibit a large variability in their sequences, the six cysteines are retained.

Similar to cathelicidins, defensins are activated by proteolytic processing from an inactive precursor.

3.3.1.1. Expression and regulation of AMPs

Because AMPs are expressed as pro-peptides followed by proteolytic processing to release the biologically active peptides, regulation of function is as dependent on the expression of appropriate proteases for processing as it is on the expression of the AMP gene product itself. These are accomplished differentially depending on the specific peptide and the tissue or cell type.

Recent observations have shown that the constitutive expression of AMPs is under strict developmental control and influenced by age and sexual maturation.

The down-regulation of AMPs occurs when expression is turned off and is often associated with increased susceptibility to infection by pathogens otherwise sensitive to the AMPs. For example, psychological stress has been shown to have adverse effects on cathelicidin expression through the induction of endogenous glucocorticoids (GCs). Indeed, psychological stress and systemic or topical GC administration has been observed to down-regulate epidermal expression of murine cathelicidin and α -defensin 3, leading to increased severity of group A *Streptococcus pyogenes* skin infection. In addition, the capacity to decrease AMP expression is also a microbial virulence factor.

3.3.1.2. AMPs selective antimicrobial activity

Usually, the expression of these AMPs is increased at the onset of infection in response to stimuli. In vitro, most AMPs act against many different types of microbes including gram-negative and gram-positive bacteria, protozoa, fungi and some viruses, due to their capacity to directly disrupt the microbial cell membrane and thereby result in killing. Disruption of lipid bilayers by AMPs occurs through a variety of mechanisms.

In general, cationic AMPs are attracted by electrostatic forces to the negative phospholipid headgroups on the membrane surface provided by capsular polysaccharides, which include lipopolysaccharides (LPS) in Gram-negative bacteria and teichoic acids (TA), lipoteichoic acids (LTA) and lysylphosphatidylglycerol in Gram-positive bacteria. Once AMPs gain access to the cytoplasmic membrane they interact with the lipid bilayer, followed by displacement of lipids, alteration of membrane structure and the creation of a physical hole causing cellular contents to leak out. Collectively, all these are well established models representative of the mechanisms for AMPs, and each model provides a different view of peptide activity, but none of these models is capable of adequately explaining their effectiveness in vivo.

The selective toxicity of AMPs against microbes and not against host cells is possibly due to the cholesterol content of eukaryotic membranes. Unlike eukaryotic cell membranes, bacterial cell membranes are free of cholesterol. For example, the AMP sarcotoxin IA was shown to be less disruptive to liposomes containing cholesterol than cholesterol-free liposomes. Because cholesterol is known to cause condensation of phospholipid bilayers, it might prevent AMPs from penetrating into the cytoplasmic membrane of eukaryotic cells.

In addition, the asymmetric distribution of phospholipids in the cytoplasmic membrane of eukaryotic cells might also contribute to the insensitivity of eukaryotic cells to AMPs.

Moreover, the cytotoxic effects of AMPs to eukaryotic host cells *in vivo* are also substantially attenuated by serum components such as apolipoprotein A-I and B, or other lipoproteins, which have been shown to bind to LL37.

Despite the selective activity of many AMPs for microbial membranes, several studies have also shown that these AMPs at high concentrations will also destroy eukaryotic cells *in vitro*, such as T lymphocytes, leading to the hypothesis that they might also have a role in tumor surveillance. In spite of this, the cytotoxic concentrations of AMPs are, in general, higher than the concentrations required for elimination of microbes, revealing a cell-selective killing mechanism.

3.3.1.3. AMPs modulation of host inflammatory responses

The minimal inhibitory concentrations (MIC) of AMPs against microbes *in vitro* are typically much higher than the physiological concentrations of peptides found *in vivo* under resting conditions.

It has been suggested that they can either:

- act synergistically with other classes of AMPs to exert their desired effect,
- accumulate, in situations of inflammation, at a high local concentration sufficiently above the MIC, thus acting alone as a classic AMP,
- function as potent immune regulators, altering host gene expression, acting as chemokines and/or inducing chemokine production, inhibiting LPS- or hyaluronan-induced pro-inflammatory cytokine production, promoting wound healing and modulating the responses of dendritic cells or T cells of the adaptive immune response.

In this way they act as a bridge between innate and adaptive immunity.

AMPs influence the outcome of infection in at least three ways:

- they act as direct antimicrobials,
- they are chemotactic and they induce the release of multiple cytokines and chemokines that further refine and amplify the innate immune response. Upon stimulation by microbial pathogens, local cells release AMPs at the site of the infection or injury. In addition to inhibiting microbial growth, an additional function of some of these AMPs is that they act to directly recruit leukocytes or

induce the expression of chemokines or cytokines, indirectly promoting recruitment of effector cells such as neutrophils, monocytes, macrophages, immature dendritic cells and T cells. Both defensins and cathelicidins have been shown to be chemotactic.

- they modify inflammation: mammalian AMPs have a crucial role in regulation of TLR-dependent inflammatory response. In this scenario, the AMP acts as a scavenger for the activating ligand, removing it before it can trigger inflammation. Moreover, it is suggested that AMPs might have a role in preventing sepsis.
- promote wound healing, by many mechanisms, such as stimulation of migration, proliferation and tube formation of endothelial cells in wounds, leading to accelerated wound closure.

AMPs have been shown to participate in alerting, mobilizing and amplifying innate and adaptive immune responses of the host, and will confer protection against microbial infections.

Decreased expression of AMPs can increase susceptibility to infectious diseases.

3.3.1.4. Microbial resistance to AMPs

A particular attention must be focused on microbes evolution and their attempt to conquer AMPs resistance. AMPs exert a multidimensional strategy to respond to microbial invasion. On the contrary, by acting through a single approach, antibiotic function can be completely evaded by a single resistance system. It is apparent today that many pathogenic microbes have evolved mechanisms to avoid the antimicrobial action of different AMPs, yet the expression of AMPs still protects the host.

Several mechanisms have been described for microbial resistance to AMPs. Some microbes use advanced strategies to down-regulate the expression of AMPs or produce proteases to degrade AMPs. Microbes seem able to:

- release plasmid DNA to turn off the expression of antimicrobial peptides in epithelial cells or monocytes, and this helps the bacteria evade AMP function,
- for the microbe to enzymatically inactivate the AMP before it can act,
- to change the composition of their cell membrane, resulting in the reduction of the net negative charge of the bacterial membrane so as to repel cationic AMPs.

Notwithstanding the AMPs antimicrobial activity against planktonic bacteria, also some experiments about AMPs efficacy against biofilm have been reported. In particular, RNA III inhibiting peptide (RIP) is known to inhibit *S. aureus* pathogenesis by disrupting the quorum-sensing mechanisms that characterizes bacteria sessile growth.

However, RIP was not able to totally eradicate biofilm formation when it was used alone. It is reported that the activity of RIP was synergistic with those of antibiotics for the complete prevention of drug-resistant *S. aureus* infections (Giacometti, 2003).

In conclusion, along with the capacity of AMPs to directly kill microbes, AMPs also boost specific innate immune responses and exert selective immunomodulatory effects on the host. Upon exposure to danger, AMPs create an overall balance by inhibiting microbial growth, attenuating exacerbated inflammatory responses and stimulating certain beneficial aspects of inflammation. This combined functional profile highlights the unique temporal niche that AMPs occupy in microbial defense (i.e. they are ideally suited for rapid but transient

In-depth analysis: Membrane action of AMPs (Lai, Gallo, 2009)

There are several mechanisms by which different classes of AMPs could act on the microbial target membrane. In the 'carpet model', peptides accumulate on and orient parallel to the membrane surface. At a certain high concentration of peptides, AMPs disrupt the bilayer membrane in a detergent-like manner, resulting in the formation of micelles and leakage of cellular contents. In the 'toroidal-hole model', the polar heads of the peptides face the polar head groups of the lipids, inducing the lipids to form a continuous bend from the top to the bottom in the fashion of a toroid which is lined by both the peptides and the lipid head groups. Some cationic AMPs adopt an α -helical configuration, attach to, aggregate and insert into oriented bilayers that are hydrated with water vapor, leading to the formation of 'barrel-stave' holes in the membrane. For this model, the hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore. Notably, the differences between the toroidal model from the barrel-stave model is that peptides in the former are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer. Finally, although most AMPs have been shown to disrupt cell membranes and induce microbial killing, a few AMPs kill bacteria without any detectable lysis. These AMPs can penetrate the cell membrane and bind to different targets, such as DNA, to inhibit bacterial growth.

In-depth analysis: AMPs conquer microbial resistance (Lai, Gallo, 2009)

Microbes have evolved several mechanisms to degrade AMPs, yet the expression of AMPs continues to provide protection against infection. Why? Several theories can be advanced to explain their effectiveness in mammalian immunity. First, even though most pathogens have developed some countermeasures against AMPs, strict regulation of the expression of AMPs at the infection site leads to extremely high concentrations of AMPs in vivo that cannot be completely overcome by the microbe. This combined with simultaneous production of a variety of different AMPs, impedes microbial colonization and growth. In addition, the high costs for microbes to develop and express resistance genes preclude modifications that allow total resistance. In particular, it is difficult to evade the membrane-disturbing activity of AMPs while preserving the functional and structural integrity of the microbial cell wall and membrane. Furthermore, a strategy of destruction of the AMPs also poses several problems for the microbe. Most peptides are created from sequences of amino acids lacking unique epitopes that could serve as the recognition site of a specifically evolved protease that would be required for selective destruction of the AMP. Thus, it is difficult to design a degradation mechanism that would not also degrade necessary host proteins for attachment or microbial proteins necessary for survival. Finally, we have seen that all multicellular organisms attack microbes with multiple peptides of different structural classes, hence destruction of one peptide might not suffice to avoid the lethal assault of a diverse group of AMPs.

responses). This overall presentation of AMPs is owed to Lai, Gallo, 2009, where not differently reported.

3.3.2. AMPs modulating activity during inflammation and tumorigenesis

The strict relation between inflammation and cancer imply the need of a deeper understanding of the complex role of AMPs during tumorigenesis.

A number of studies have addressed the role of AMP during carcinogenesis and cancer progression, arriving at contradictory conclusions with regard to anti-tumor or tumor promoting activities. It has been recently reported that AMPs seem to exert a cytotoxic effect against tumor cells (Al-Rayahi, 2015).

During inflammation, some complement components are generated leading to chemoattraction of immune cells such as T lymphocytes, monocytes, and eosinophils. Subsequently, the interaction of these components with their receptors leads to release of cytokines and reactive oxygen species resulting in tissue damage. Persistent inflammation leads to the establishment of a microenvironment supportive for tumor growth. Tumor-mediated complement activation provides a continuous source of complement activation bioactive products creating an inflammatory environment supporting tumor growth. It was found that activation of C3aR and C5aR lead to an increase in expression of IL-6 mRNA, which is a potent cytokine capable of stimulating angiogenesis and inhibiting apoptosis in tumor cells (Al-Rayahi, 2015).

In addition to complement components, there is good evidence that certain AMP such as defensins and Cathelicidins influence the mobilization from bone marrow and recruitment of cells in the tumor microenvironment (Al-Rayahi, 2015).

Taken together, both complement components and some antimicrobial peptides of the innate immune defense arm have a role in regulating and trafficking of immune cells during tumorigenesis as well as in modulating the adaptive anti-tumor immune response (Al-Rayahi, 2015).

3.3.3. Endogenous AMPs activity during bovine mastitis infection

The role of defensins and cathelicidins in protecting from bovine mastitis infection has been demonstrated (Roosen, 2004; Tomasinsig, 2010). In addition, it is reported that milk cathelicidin levels increase following intramammary infection (Smolenski, 2011). Increased expression of genes encoding β -defensins in the infected udder confirms their crucial role in the defense of the cow mammary gland against mastitis. On the other hand, the elevated cathelicidin transcripts in non-infected tissues indicate their role in the maintenance of healthy mammary tissues. The expression levels of investigated genes are likely to depend on the duration of the infection and type of bacteria (Kosciuczuk, 2014).

It is likely that the simultaneous presence of a wide range of these peptides at an inflammatory site results in mutual potentiation of the antimicrobial activity. This may be achieved, for instance, through faster cellular delivery of AMPs acting on intracellular targets as a result of perturbation of bacterial membranes by membrane-active AMPs. The occurrence of multiple targets would also decrease the likelihood of microorganisms acquiring resistance to the AMPs (Tomasinsig, 2010).

By extension, the overall antimicrobial activity is expected to increase dramatically when the entire arsenal of antimicrobial peptides, including cathelicidins, defensins, and other AMPs, is released from neutrophils, a circumstance that likely takes place in vivo in mastitic milk following neutrophil recruitment and activation at sites of udder infection (Tomasinsig, 2010).

In addition, it has been demonstrated that some AMPs are largely inhibited in milk and fully active in whey (Tomasinsig, 2010). The strong influence of the milk composition on the antimicrobial activities of cathelicidins becomes particularly relevant when the physical and chemical properties of healthy and mastitic milk are compared. Mastitis causes alterations that turn milk into a whey-like fluid with a reduced content of different milk components, including caseins and fat, and this provides a more suitable environment for efficient activity of AMPs, as confirmed by the finding that cathelicidins in general maintained efficient activity in mastitic milk.

Moreover, AMPs triggered the expression of proinflammatory mediator tumor necrosis factor alpha (TNF- α) in mammary epithelial cells at low micromolar concentrations, thus providing an important mechanism for the stimulation of other immune responses. The relevance of TNF- α to mastitis is suggested by its increased expression in milk somatic cells after *E. coli* intramammary infection and by the finding that it improves phagocytosis and killing of *S. aureus* by neutrophils (Tomasinsig, 2010).

Besides, the cytokine promotes the recruitment of neutrophils to infected mammary glands by inducing expression of adhesion molecules in endothelial cells and stimulates AMP production, setting up a positive loop (Tomasinsig, 2010).

3.3.4. Experimental data about exogenous AMPs efficacy during bovine mastitis infection

Experimental studies investigated AMPs activity in different animal pathologies, such as promoting wound healing in mice, dogs (Kim, 2009) and horses (Carmona, 2006), resolution of equine joint (Broeckx, 2014) and tendon (Carter, 2003) diseases, and bovine udder regeneration (Lange Consiglio, 2014).

Among these studies, the intramammary administration of platelet concentrate (PC) as an unconventional therapy in bovine mastitis (Lange Consiglio, 2014) shows a higher efficacy of the associated treatment PC-antibiotic versus the PC-alone and antibiotic-alone treatments.

The experimental approach is based on the regenerative properties of the platelet concentrate, obtained as stated in literature (Anitua, 2004; Zimmermann, 2003).

Comparing the antibiotic-alone versus the PC-alone treatments, the rate of improvement and relapse were not statistically different regarding acute mastitis, but it is noteworthy that the treatment with PC alone was better than antibiotic alone for chronic mastitis. Particularly in the case of chronic mastitis, these findings suggested a possible role of growth factors in tissue protection or repair processes, or both. Platelet concentrate, in fact, contains chemokines, some of which act in synergy with white blood cells, whereas others are chemotactic and able to recall monocytes and macrophages at the site of infection. The chemotactic action of PC is not limited to mobilize macrophages to the site but also to activate them. The macrophages, attracted by the chemotactic action of growth factors, would be able to resolve the inflammation phase, promoting tissue remodeling due to the secretion of factors that stimulate the production of fibroblasts (such as PDGF), factors that regulate the synthesis of tissue connective components (such as TGF- β), and angiogenic factors (such as vascular endothelial growth factor). In summary, the therapy is believed to be based on the action of both growth factors and chemokines released by platelets that act synergistically to increase the infiltration of neutrophils and macrophages, promote angiogenesis, fibroplasia, matrix deposition, and re-epithelialization (Lange Consiglio, 2014).

The study leads to the belief that, even missing a complete list of AMPs present in bovine PC, it is possible to speculate that PC comprises an uncertain amount of AMPs and is also able to exert an antibacterial effect.

The promising results of AMPs and GFs in different clinical pathologies is leading to a standardization and optimization of platelet-rich plasma preparation, aimed to a better quantification of cytokines and growth factors platelet derived.

As far as known, PC contains different AMPs and GFs and for human blood growth factors, cytokines and chemokines secreted by the concentrated platelets has been quantified: high concentrations of platelet-derived growth factor, endothelial growth factor and transforming growth factor (TGF) were secreted, together with the anti-inflammatory and a various range of proinflammatory cytokines interleukin, tumor necrosis factor and interferon (Amable, 2013).

3.4 Ozone

Ozone is a three oxygen molecule, and has interesting physical properties. It is an unstable gas that has an half-life of about 40 minutes at 20°C (Bocci, 2011) and it decomposes generating a diatomic oxygen molecule and very active atomic oxygen (Bialoszewski, 2011).

Ozone is well known for being very useful in the stratosphere because it absorbs dangerous UV radiations. However, its characteristic of being toxic in the troposphere for the pulmonary tract makes its use controversial (Bocci, 2006).

Ozone is 1.6-fold denser and 10-fold more soluble in water (49.0 mL in 100 mL water at 0°C) than oxygen. It is the third most potent oxidant after fluorine and persulfate and because of this oxidative feature it exhibits antimicrobial activity (Bocci, 2006).

As any residual ozone naturally decomposes into non-toxic oxygen, ozone presents interesting aspects for its use in the food industry or in medicine (Bialoszewski, 2011).

It is an excellent disinfectant, able to eliminate most of pathogens contaminating surfaces and aqueous solutions: its effectiveness in destroying bacteria, fungi and viruses has been largely demonstrated, but when in contact with corporal fluids it displays complex mechanisms of action and it loses its direct killing power, as further described (Bocci, 2012).

Ozone is used for medical purpose in a gaseous oxygen/ozone mixture, where oxygen rate is minimum 95%. Depending on therapeutical purposes, ways of administration and ozone concentrations, it is possible to obtain very different effects. For internal use its versatility is impressive because if properly used it can be proficient in vascular diseases (chronic limb ischemia, diabetic foot, heart infarction and stroke), besides in orthopedics and odontoiatry (Bocci, 2011). A deeper understanding of its mechanism of action, where known, is necessary to properly link specific concentrations range and applications to beneficial effects.

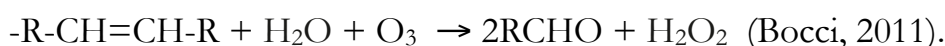
Ozone has therapeutical effects on metabolism in inflamed tissues, activates the body's immune response and shows antimicrobial and regenerating activity for topical use. The chemical properties of ozone are utilized in ozone therapy to treat infected wounds, decubitus ulcers, burns, ulcerations, inflammation of skin and bone tissue. Ozone therapy is also used to treat inflammation and infections of certain internal organs, especially when antibiotic therapy has failed to control multidrug-resistant bacteria (Bialoszewski, 2011).

3.4.1. *Ex vivo administration - AHT*

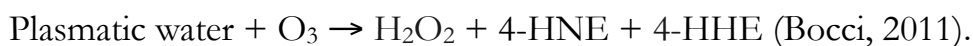
One of the most studied ozone way of administration is auto-hemo-transfusion (AHT). Its understanding is useful to clarify the ozone multiple mechanisms of action and thereby it is here reported.

AHT consists in a blood collection of about 200 mL, thus ozonized through blending with a gaseous oxygen/ozone mixture and then re-infused into the same donor patient.

When in contact with blood, ozone reacts in a few seconds, producing a cascade of effects. Owing to the potent antioxidant capacity of blood due to its hydrophilic, lipophilic antioxidants and cellular enzymes, some of the ozone dose dissolved in the water of plasma is instantly quenched by free antioxidants (mainly uric acid, ascorbic acid, reduced glutathione - GSH, cysteine and albumin), while the remaining ozone reacts with polyunsaturated fatty acid (PUFA) mostly present in the three hydrophobic tasks of albumin.



Thus, the potential energy of ozone is finally transferred into two fundamental messengers such as H_2O_2 as a reactive oxygen species (ROS) and aldehydic molecules of which 4-hydroxynonenal (4-HNE) and trans-4- hydroxyhexenal (4-HHE) are the relevant lipid oxidation products (LOP):



Both ROS and LOPs ozone metabolites acts as messenger and cause a mild oxidative stress that boost the host immune system, activating a cascade of beneficial effects, such as:

- (1) Improvement in blood circulation and oxygen delivery to ischemic tissues as a result of the concerted effect of NO and CO and an increase in intraerythrocytic 2,3-diphosphoglycerate (2,3-DPG) level.
- (2) Enhancement of the general metabolism through improved oxygen delivery.
- (3) Up-regulation of a number of antioxidant enzymes as well of heme-oxygenase-1 and heat stress protein-70.
- (4) Induction of a mild activation of the immune system and enhanced release of growth factors.
- (5) Absence of acute or late adverse effects.
- (6) A feeling of wellness, most likely due to the stimulation of the neuroendocrine system (Bocci, 2012).

Due to the high ozone reactivity, these biochemical reactions occur in a few seconds and in fact, within the canonical five minutes of mixing an average 200 mL of human blood *ex vivo* in a sterile glass bottle with the 200 mL corresponding volume of the gas mixture oxygen/ozone, ozone is totally exhausted while about 95% oxygen, dissolved in the plasma water, fully saturates hemoglobin (Bocci, 2011).

During the initial fast and multiple reactions of ozone with the plasmatic components, a variable amount of the ozone dose is neutralized by the wealth of the hydrophilic antioxidants. It is noteworthy that, with the exception of uric acid oxidized to allantoin, dehydroascorbate and GSH disulfide are reduced back to their normal value in less than twenty minutes due to the exceptional efficiency of the recycling system based on a multitude of reducing molecules such as alpha-lipoate, Vitamin E, thioredoxin, and last but not least NADPH acting in a well-coordinated sequence of electron donations (Bocci, 2011).

Most importantly it is reported (Bocci, 2011) that, H_2O_2 , being unionized, rapidly enters into all blood cells and the chemical gradient between plasma-cells has been measured to be about 10% of the extracellular concentration. In other words, when the highest ozone concentration is mixed with blood, depending upon the inter-individual variability of antioxidant potency (1.28-1.83 mmol/L plasma), the highest H_2O_2 concentration measured in plasma is about 40 μ M and therefore inside the cells is at most 4 μ M. This sudden inflow of this small amount of H_2O_2 inside blood cells is the indispensable stimulus to activate a series of biochemical reactions as follows:

- in the erythrocytes: activation of glycolysis with increase of ATP and 2,3-diphosphoglycerate. Functionally, the oxyhemoglobin sigmoid curve shifts to the right and increases the release of oxygen at the tissue level. The erythrocytes mop up most of the H_2O_2 and promptly reduce it to water by GSH. The sudden formation of GSSG (oxidized glutathione) alters the GSH/ GSSG ratio but the cell quickly correct it by either extruding some glutathione-disulphide or by reducing it via GSH reductase at the expense of either ascorbic acid or thioredoxin, which has two-SH groups. Moreover, the activation of glucose-6-phosphate dehydrogenase (G6PDH) provides reducing power and activate glycolysis;
- in the leukocytes: neutrophil phagocytic activity is enhanced. Inside monocytes and lymphocytes, H_2O_2 activates a tyrosin-kinase with consequent phosphorylation of I κ B, one of the trimeric components at rest of the NF- κ B. The phosphorylated I κ B detaches from the trimer and it is broken down in the proteasome. The remaining heterodimer p50-p65 is transferred into the nucleus where it can activate about 100 genes. Of great significance it is the final release of some cytokines (IFN γ and IL-8) and of some acute-phase proteins;

- in the platelets. In relation to the ozone concentration, we have measured release of PDGF-AB, TGFb-1 and IL-8. Growth factors have a specific relevance in enhancing ulcer's healing in peripheral arterial disease (PAD).

It must be said that the H₂O₂ concentration in the cells (4 μM) is essential for switching on cellular responses and it probably lasts few seconds as GSH-Pxs, peroxiredoxin and catalase promptly reduce it to H₂O. In plasma, the H₂O₂ half-life is less than 1 minute and it is absent during blood re-infusion (Bocci, 2011).

3.4.2. Use of ozone as antimicrobial agent

In the last decades, various in vitro and in vivo experimental and clinical studies have demonstrated the bactericidal effect of the topical application of ozone in different situations, including the management of infected wounds. Ozone is a potent oxidant and an important disinfectant, acting on microorganisms by means of oxidation of their membrane. Ozone reacts with lipid double bonds, thus leading to bacterial wall lysis and bacterial cell content extravasation. By entering the cell, ozone promotes oxidation of nucleic and amino acids. Cell lysis depends on the extent of these reactions disinfectant effect of ozone.

It has been reported that ozone can be employed as a bactericidal agent under various forms, such as ozonized saline solution, ozonized water, ozonized oil, ozone associated with other substances and it is valuable in all cutaneous and mucosal infections and ulcers. The topical use of ozone has also been reported for environment decontamination in diverse situations, since low doses of gaseous oxygen/ozone mixture completely prevented the in vitro growth of all bacterial strains (Fontes, 2012).

With the spreading microbial lack of sensitivity to killing agents, studies have been performed to evaluate the efficacy of ozone on specific microbial strains. Results showed that a gaseous oxygen/ozone mixture effectively inhibited the growth of all potentially pathogenic bacterial strains with known antimicrobial resistance, by a single topical application by nebulization of a low ozone dose. (Fontes, 2012).

3.4.3. Ozone as active agent against microbial biofilm

Recently, in vitro experiments with ozonated saline showed its efficacy against both planktonic and biofilm growing *Staphylococcus aureus* (Al-Saadi, 2015) and other bacterial biofilm (Bialoszewski, 2011).

The study (Al-Saadi, 2015) showed rapid clearance of planktonic bacteria, and a delayed, but complete, eradication of biofilm bacteria.

Ozonated saline was found to be effective in rapidly killing planktonic *S. aureus* at a very high initial density.

Although planktonic bacteria are found in clinical infections, bacterial biofilms represent a more challenging treatment target in comparison with planktonic bacteria. The ability of ozone to eradicate *S. aureus* biofilms is, therefore, crucial to the suitability of this technique for the eventual clinical application. In comparison with the effect on planktonic bacteria, biofilm growth rendered the *S. aureus* significantly more resistant to ozone, despite the fact that the total number of bacterial cells in the planktonic experiment exceeded the number of bacteria in the biofilm experiment by a factor of approximately 100. After 60 minutes, all the planktonic bacteria were entirely eradicated; however, after the same period, the adherent biofilm was relatively unaffected by ozone with only minor loss of viability or structural damage. Complete eradication of the biofilm from the surface of the TAN discs was achieved after 360 minutes of ozone exposure.

Also a cytotoxicity test was performed. Cell cytotoxicity measurements showed no persistent effect on primary ovine osteoblasts and no effect after short exposure time.

Ozone antimicrobial broad spectrum effectiveness, its lack of cytotoxicity and its rapid degradation into its well known metabolites are a significant advantage over other antiseptics, which would not be so rapidly degraded in human tissue and represent a greater toxicity risk (Al-Saadi, 2015).

3.4.4. Ozone treatment against bovine mastitis

As for bovine mastitis, the therapeutical use of ozone reviewed the infusion of gaseous ozone into the inflamed quarter and the mammary irrigation with ozonated water have been investigated.

In the first study, ozone gas insufflation into the udder showed an interesting efficacy: 60% of mastitic cows did not require any antibiotics for recovery (Ogata, 2000).

However, due to technical difficulties, this practice didn't receive attention from the farmers.

The second study clearly demonstrated that cows receiving mammary irrigation had higher cure rates than those receiving an antibiotic treatment alone.

Although it is well recognized that ozone has sterilization capabilities, it was reported that physical elimination of both microorganisms and endotoxins from the mammary glands by flushing might be essentially important, rather than the sterilization effect of irrigation fluids.

In addition, it has been reported that treatment with ozone released fewer endotoxins in the milk than treatment with antibiotics. Moreover ozone may result in the down-regulation of the cytotoxic activity of immunocytes.

At the time, the study did not confirm the efficacy of ozone water in the in vivo evaluation, since mammary irrigation with normal saline was as effective as ozone water for acute coliform mastitis in dairy cattle (Shinozuka, 2009).

3.5 Ozonated oil

Ozonated oil is a vegetable oil bubbled for a precise amount of time with a oxygen/ozone mixture, where oxygen rate is at least 95%.

During the last decades, the usage of vegetable matrices in the form of ozonated oils has been proposed and several researchers have shown a positive effect in the wound, based on their bactericidal, antiviral, and antifungal properties.

Thus, several experiments have been performed to evaluate the MIC antimicrobial activity of ozonated oil and to optimize its efficacy in relation to properties of different vegetable oils.

The direct ozonation of vegetable oils with unsaturated fatty acids leads to the formation of the 1,2,4-trioxolane moiety, which represents the active form of ozone in these substrates. The trioxolane ring within the vegetable ozonated matrices quickly generates some compounds responsible for the healing process when applied in either a humid wound or an ulcer. Moreover, it is accountable for antimicrobial and antimycotic treatments. All these effects occur in the absence of cutaneous adverse reactions (Zanardi, 2013).

During the ozonation process, ozone reacts with olefins by adding on the carbon-carbon double bonds therefore the longer the reaction, the more unsaturations are consumed inducing a decrease in II. As regard for instance to sunflower oil, because it contains more unsaturations than other vegetable oils, it takes more time to consume them all (Moureu, 2015).

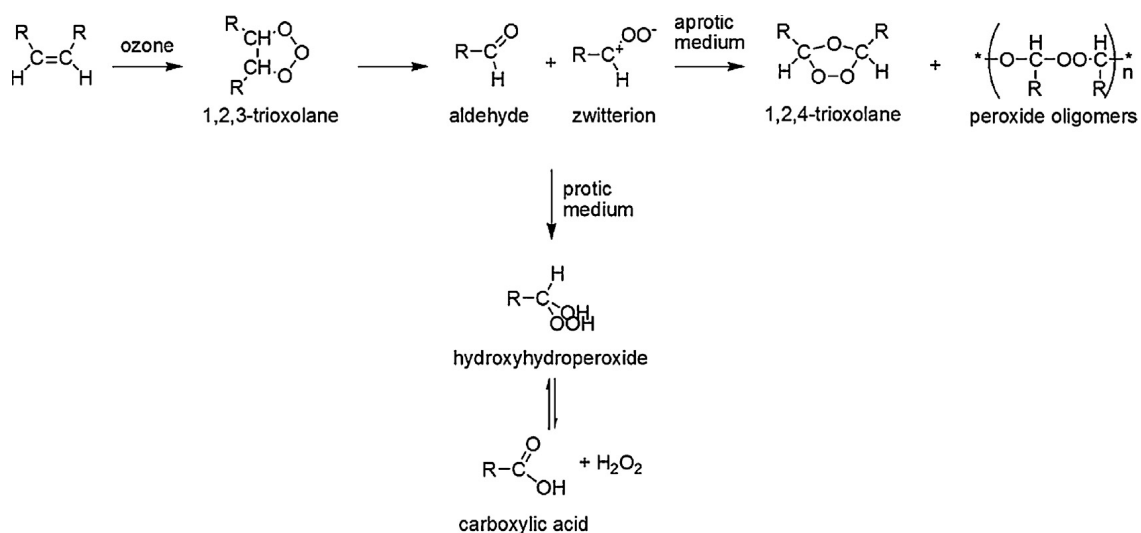


Figure 3.5.1. Criegee mechanism during oil ozonation (Moureu, 2015).

3.5.1. Antimicrobial activity of sunflower ozonated oil

Among different oils, sunflower oil is so far the best known, and at least it was the best investigated at the beginning of the experiment further described.

The remarkable sunflower ozonated oil (SOO) bactericidal properties and its direct action on the pathogenic microorganism without damaging the human epithelium have been reported (Sechi, 2001).

The stability of SOO at different temperatures was also determined stable for up to 1 year in the temperature range -10 to $+8^{\circ}\text{C}$. Moreover, it is stable for up to 6 months at room temperature ($27\pm 30^{\circ}\text{C}$); after this period, the antimicrobial properties diminish. The pH is also stable for up to 1 year in the temperature range -10 to $+8^{\circ}\text{C}$. At 30°C , the pH is stable for up to 6 months. The MIC values after this period increase from 2,37 to 19 mg/ml (Sechi, 2001).

For a deeper understanding of its properties, SOO that shown antibacterial and antifungal activity was characterized by:

Peroxide Index (IP), which indicates the quantity of peroxide within the SOO. It is defined as the quantity of active oxygen per kilogram of SOO (mmol/kg). A range of IP between 500 and 800 (mmol/kg) was considered. The best antimicrobial activity was seen with an IP of 650 (mmol/kg).

Acidity Index, which indicates the free fatty acid in the SOO. It is defined as the number of milligrams of potassium hydroxide that are necessary to neutralize the free fatty acid in 1 milligram of SOO. In SO, the value must range between 6 and 8 units whereas in SOO, it is not above to 25 units.

Aldehyde concentration. The aldehyde concentration is measured by adding free hydroxylamine to the aldehyde carboxylic group. The results are expressed in mmol/g of SOO; the interval must range between 0,4 and 0,9 mmol/g.

Iodine Index, which is a measure of the unsaturation rate of sunflower oil expressed as the number of grams of iodine that react with 100 grams of SO. In SO, the rate varies between 125 and 135 units whereas in SOO, the value is between 50 and 90 units.

Viscosity, which is a measure of the polymerization by condensation of the peroxides forming in the SO ozonization process. The values are expressed in mPa.s (centipoise or cP value; Institute of Standards and Technology). In order to obtain SOO with an IP between 500 and 800, the viscosity must be between 100 and 450 mPa.s.

The activity of SOO against all tested bacteria, including b-lactam-, vancomycin- and gentamicin-resistant strains, is expressed in mg/ml. These concentrations may seem high if compared with the amount of antibiotics, expressed in $\mu\text{g/ml}$,

necessary to inhibit bacterial growth. This is due to the dilution of the active compounds in the SO that has not been altered in the ozonization process.

It seems from these preliminary results that Mycobacteria are even more susceptible to SOO than the other bacteria tested. This may be explained in part by the composition of their cell wall and the high lipid content, which may facilitate the passage of SOO-active compounds into the bacteria (Sechi, 2001).

Susceptibility to SOO of different pathogens is shown in the following tables.

Species (no. of strains)	MIC (mg ml ⁻¹)
<i>M. tuberculosis</i> H37 Rv (1)	0.95
<i>M. smegmatis</i> mc155 (1)	0.95
<i>M. abscessus</i> (1)	2.37
<i>M. aurum</i> (1)	0.95
<i>M. avium</i> (2)	2.37
<i>M. fortuitum</i> (2)	0.95–2.37
<i>M. chelonae</i> (2)	2.37
<i>M. tuberculosis</i> (1)	0.95
<i>M. tuberculosis</i> MDR (2)	0.95–2.37

Table 1.5.1
Susceptibility to Oleozon (SOO) of different species of Mycobacteria (Sechi, 2000)

ATCC strains	MIC (mg ml ⁻¹)
<i>Ent. hirae</i> ATCC 9790	9.5
<i>Ent. faecalis</i> ATCC 35038	9.5
<i>Ent. faecium</i> ATCC 19474	9.5
<i>Ent. gallinarum</i> ATCC 49573	2.37
<i>Ent. casseliflavus</i> ATCC 25778	9.5
<i>Ent. maleodoratus</i> ATCC 43197	4.75
<i>Ent. durans</i> ATCC 19432	9.5
<i>Ent. solitarius</i> ATCC 49428	9.5
<i>Ent. pseudoavium</i> ATCC 49372	9.5
<i>Ent. avium</i> ATCC 14025	9.5
<i>Ent. saccharolyticus</i> ATCC 43076	9.5
<i>Ent. mundtii</i> ATCC 43186	2.37
<i>Staph. aureus</i> ATCC 29213	9.5
<i>Staph. epidermidis</i> ATCC 14990	2.37
<i>E. coli</i> ATCC 25922	4.75
<i>E. coli</i> XL1	1.18
<i>Ps. aeruginosa</i> ATCC 27853	4.75

Table 1.5.2
Susceptibility to Oleozon (SOO) of different enterococci, staphylococci, Escherichia coli and Pseudomonas aeruginosa ATCC strains (Sechi, 2000)

Strains (no. of micro-organisms)	MIC (mg ml ⁻¹)		
	Range	50%	90%
<i>Ent. faecium</i> (21)	1.18–9.5	4.75	9.5
<i>Ent. faecalis</i> (10)	4.75–9.5	4.75	9.5
<i>Strep. pyogenes</i> (40)	2.37–9.5	4.75	9.5
<i>Staph. aureus</i> (50)	2.37–9.5	4.75	9.5
<i>Staph. epidermidis</i> (19)	2.37–9.5	4.75	9.5
<i>E. coli</i> (40)	1.18–9.5	4.75	9.5
<i>Ps. aeruginosa</i> (40)	4.75–9.5	4.75	4.75

Table 1.5.3
Susceptibility of different clinical isolates of Enterococcus faecium, Ent. faecalis, Streptococcus pyogenes, Staphylococcus aureus, Staph. epidermidis, Escherichia coli and Pseudomonas aeruginosa (Sechi, 2000)

There is thus a general conviction that ozone is one of the best compounds for killing bacteria, viruses, and parasites present as well as against biofilms: antibacterial effectiveness of ozonated oils in mucosal and cutaneous infected wounds and ulcers has been successfully proven (Zanardi, 2013). SOO is effective against herpes, mycobacteria, staphylococci and streptococci and it is considered a competitive antimicrobial agent (Sechi, 2001).

As it is possible to observe in figure 1.5.2, experiments showed how both bacterial and *C. albicans* cells maintained intact shapes and size, just after ozonated oil exposition. Also the surface morphology of the cells was unaltered with respect to untreated ones, as well as the number of damaged cells. The only exception occurred at cellular surface of *P. aeruginosa* where the cells showed a rough outside with the appearance of tiny bumps similar to small vesicles, after contact with ozonated oil (Zanardi, 2013).

The believe is that ulcers with scarce tendency to heal are due to a local hypoxic situation, presence of bacteria, minimal cell proliferation, and a reduced production of extracellular matrix. In reported experiments, by using the ozonated oil in vivo, the “restitutio ad integrum” including the final healing and scar tissue remodeling takes much less time in elderly and/or diabetic patients without any generalized or local side effects (Zanardi, 2013).

How ozonated oil precisely acts remains a debatable question. According to the SEM results, the anti-infective activity is not dependent on structural alterations at the level of microorganisms. However, it seems likely that 1,2,4-trioxolane present in the ozonated oil, when added to the warm exudates film of the ulcer, slowly decomposes generating local oxygen, H_2O_2 as reactive oxygen species (ROS), and a trace of lipid oxidation products (4-HNE). Such a cascade can explain the prolonged disinfectant action and stimulation of proliferative activity of fibroblasts and keratinoblasts (Zanardi, 2013).

Type	Treatment time	25 mg OSO/5 mL of microorganism suspension			50 mg OSO/5 mL of microorganism suspension		
		l-OSO	m-OSO	h-OSO	l-OSO	m-OSO	h-OSO
<i>S. aureus</i>	1 h	65	58	58	57	47	47
	3 h	20	21	0.2	1	3.6	0.2
	6 h	0	0	0	0	0	0
<i>P. aeruginosa</i>	1 h	100	100	100	100	100	25
	3 h	100	100	5.7	13.7	15.1	2
	6 h	5	5	0	1.8	3.8	0
<i>E. faecalis</i>	1 h	100	100	100	100	100	100
	3 h	100	100	100	100	27	23
	6 h	20	15	0.1	7	0.1	0
<i>E. coli</i>	1 h	100	100	100	100	100	100
	3 h	0.8	0.8	0.5	0.4	0.4	0.4
	6 h	0.4	0.4	0	0.3	0.2	0
<i>C. albicans</i>	1 h	100	100	100	100	100	100
	3 h	39	38	18	13	13	0.2
	6 h	1.6	0	0	0	0	0

Table 3.5.4 Viability (%) of the different strains as obtained with respect to control (microbial count in the presence of the corresponding amount of SO (Zanardi, 2013).

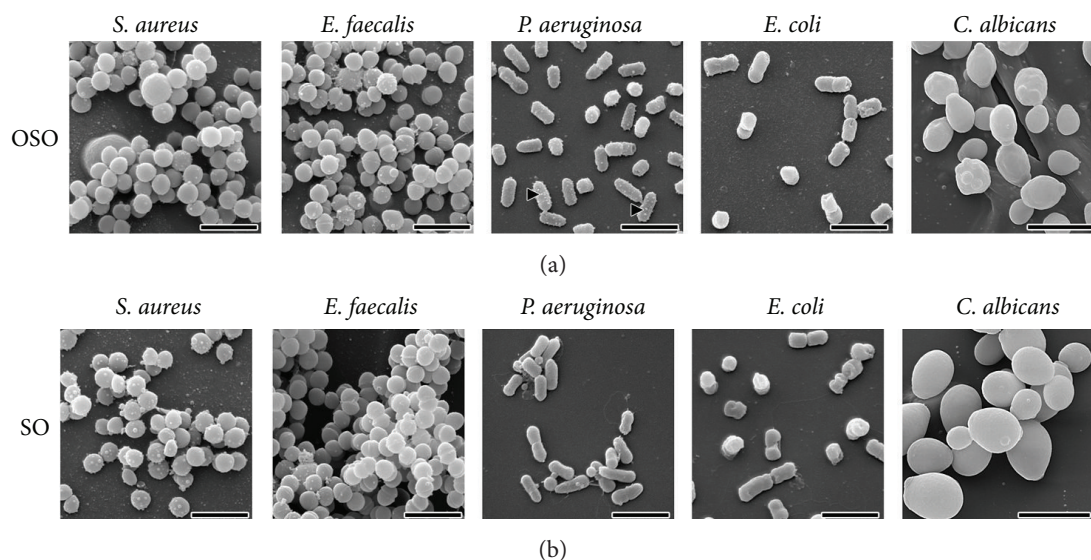


Figure 3.5.2 Scanning electron micrographs of the surface morphology of the cells after contact with either ozonated sesame oil (a) or sesame oil as control (b). Scale bars correspond to 2 μ m, except for *Candida albicans* (5 μ m). Arrowheads show small vesicles on cellular surface of *Pseudomonas aeruginosa* (Zanardi, 2013).

3.5.2. Wound healing mechanisms of ozonated oils

The effect of different ozonated oils (olive, sesame and linseed) with the same level of ozonation have on wound healing rate has been studied and showed that the factors that seems to be involved in the wound healing process are the peroxidation levels of the ozonated oil and their different composition in PUFA (Valacchi, 2013).

It has been demonstrated that ozone easily reacts with PUFA of the vegetable oils firstly forming the trioxolane moiety that when an ozonated oil is applied on the wound containing exudates it decomposes releasing its derivative products such as aldehydes and H₂O₂, that act as previously described enhancing tissue oxygenation and regeneration.

Several histological parameters and the level of key proteins such as VEGF and PCNA have been analyzed. Only treatment with ozonated sesame oil showed a faster wound closure in the first 7 days. This effect paralleled with the increased VEGF and PCNA levels, NFκB nuclear translocation and 4-HNE formation. The study showed that not only the ozonation grade is of importance for the improvement of wound healing process but also the typical composition of the oil. NFκB activation has been also associated with VEGF and PCNA induction and this explains the increased levels of both proteins in the tissue treated with ozonated sesame oil. In general, the events that characterize the wound healing process can be summarized as the inflammation stage (Phase I); proliferation with synthesis of extracellular matrix (Phase II) and the remodeling stage (Phase III) in which the scar is transformed to the healed wound. During this well coordinated process there is the appearance of several cell types that follow a specific order (platelets, neutrophils, macrophages, lymphocytes, fibroblasts and angiogenesis) and it has been shown that VEGF plays a critical role the regulation of all these events. In addition, it has been shown a direct correlation between NFκB activation and PCNA regulation: in fact an increased NFκB nuclear translocation in the tissue treated with ozonated sesame oil were detected. NFκB is a redox transcription factor, modulated by oxidative stress and peroxidation products and the role of 4-HNE in the activation of NFκB has been studied in several cells type. Studies show that ozonated sesame oil induced a clear activation of NFκB in cutaneous tissues (Valacchi, 2013).

3.5.3. Use of ozonated oil for bovine mastitis therapy

The use of ozonated oil has been recently suggested for dairy mastitis treatment (Moureu, 2015). Ozonized vegetable oils could be a new way to prevent and/or to treat mastitis. It has been shown that ozonized sunflower oil has an antibacterial activity against *S. aureus* and *E. coli* but the effect of this product on *S. uberis* has never been studied (Moureu, 2015).

Previous reported cases (Marusi, 2003) showed a beneficial use of ozonated oil as bovine mastitis treatment, describing an interesting cure rate without antibiotics (60-70%), and overall a significant reduction in swelling and redness.

Despite these promising results, ozonated oils don't have diffusion among dairy herds yet.

CHAPTER 4

Preliminary Trial:
**Intramammary administration of oxygen/
ozone mix in oil vehicle blended platelet
concentrate in bovine mastitis.**
Control group: ATB

4.1 Abstract

A preliminary study was performed between December 2011 and May 2012. A MIX made up of PC and OO was compared to ATB to test acute mastitis resolution efficacy. MIX showed promising results and, in the single concentration tested, was able to kill *S.aureus* in 2 cases on 4.

4.2 Introduction

Herds characterized by the presence of contagious and non contagious pathogens were enrolled in this preliminary study. MIX was administrated in 5 mL filled-up syringes (a half PC dose and a half OO dose), within three days from infection detecting, for a total of six doses (2 after morning milking and 2 after evening milking). MIX results are at least comparable to ATB ones, and MIX could also have a potential in killing contagious pathogens.

4.3 Materials and methods

4.3.1. *Experimental design*

A preliminary study was performed between December 2011 and May 2012.

Farms. The preliminary experiment was carried out in 2 farms managed in a cubicle yard and divided into two different lactation groups. Herd size varied from 80 to 100 cows in lactation, following a twice-daily milking regimen. Cows suffering from mastitis were identified at milking time by the farmer. Mastitis diagnosis was subsequently confirmed by the veterinarian enrolled in the study for examining the cows for inclusion in the trial and for data gathering.

Mastitis Diagnosis. The careful diagnosis of mastitis took into account the changes in the milk (chunks, flakes, clots or blood), the changes in the condition of the affected quarter (swelling, redness, hardening, heat or pain), and the general condition of the animals. Due to well known difficulties of chronic mastitis diagnosis, this study took into consideration acute mastitis only for its purpose.

Acute mastitis is characterized by visible signs, such as abnormal milk (flakes, clots, decrease in milk production, and sometimes milk discoloration) and hot, swollen, and painful quarters. Milk production decreases and the milk composition is considerably altered (Harmon, 1994).

Animals. A total of 40 animals were enrolled in this preliminary study. On each farm, cows that fulfilled the inclusion criteria were randomly allocated into 2 groups: (1) antibiotic (control) group (20 animals), which was treated with intramammary antibiotic; (2) MIX group (20 animals), which was treated with a

blending of platelet concentrate with ozonated oil, as further described. Each animal was enrolled only once.

4.3.2. Preparation of therapies

4.3.2.1. Preparation of platelet concentrate (PC)

Collection of Blood. Because it takes time for PC preparation and to have the prescribed amount of PC immediately available for treatment as soon as a mastitis case was diagnosed, a stock of PC was prepared and kept separated for each one of the farms involved in the experiment. In this way, the PC obtained from blood donor cows, in good health, free from infections and without any medication during the previous 2 mo, was used in a heterologous way for treating only the mastitic cows belonging to the same farm of the blood donor cows.

In addition, all herds followed a national control program against Tbc, Laeukosis and Brucellosis, along with a voluntary control plan on other contagious pathologies, such as Bvd and Ibr. Blood donor cows were controlled and enrollment admitted immune certified cows only.

After surgical scrub preparation of a few centimeters of skin around the subcutaneous jugular vein, 450 mL of blood was collected in ad hoc Terumo blood bags (Terumo Srl, Rome, Italy) containing citrate-phosphate-dextrose-adenine (CPDA-1) using the 16-gauge needle provided with the bags. The bags were transported at +4°C to the laboratory within 2 h of collection and immediately processed.

Double Centrifugation Method. All separation steps were performed under a horizontal laminar flow hood in aseptic conditions. To prepare the PC, the blood was drawn into sterile Falcon tubes of 50 mL each (EuroClone SpA, Milan, Italy). The tubes were centrifuged at $100 \times g$ for 30 min at 4°C. This caused separation of the blood into 3 components: red blood cells at the lowest level, “buffy coat” in the middle layer, and platelet-rich plasma (PRP) in the upper layer. Afterward, the PRP was carefully aspirated and distributed in new 50-mL tubes and centrifuged again at $1,500 \times g$ for 10 min at 4°C to obtain the platelet pellet and the poor platelet plasma (PPP) at the upper layer. Afterward, two-thirds of the volume of PPP was aspirated for later use and the pellet mixed in the residual PPP volume to allow for platelet count before the final dilution with PPP to obtain PC at a standard concentration of 1×10^9 platelet/mL. All platelet counts on peripheral blood, PRP, and PC were performed using a HeCo Vet automatic hematology analyzer (SEAC, Florence, Italy).

The total amount of PC obtained for each donor was aliquoted in 10 mL ready-to-use doses that were stored in syringes. Each syringe has been filled up with 2,5 mL of PC and kept under the horizontal laminar flow hood in aseptic conditions for MIX preparation, as further described.

4.3.2.2. Preparation of ozonated oil

Edible raw squeezed sunflower oil was poured into a sterile glass beaker and bubbled with O₂/O₃ mixture for different times in order to obtain a O₂/O₃ mixture saturation in the oil at the desired proportion.

The O₂/O₃ mixture was prepared by Dr.Hansler GmbH-Ozonosan ozone generator and settled to have a O₃ concentration in the mixture of 30 mg/L. A bubble time of 15 minutes every 100ml of oil led the oil to saturation, as controlled samples declared.

4.3.2.3. Preparation of mix

The blending of PC platelet concentrate with OO ozonated oil was prepared using the PC syringes and the OO, both previously prepared and kept under the horizontal laminar flow hood in aseptic conditions.

Each syringe of 2,5 mL of PC has been added with 2,5 mL of OO. The MIX syringes were then frozen at -196°C in liquid nitrogen and thawed at 25°C to allow the release of platelet-derived factors, through thermal shock. Platelet count on a sample of each preparation has been performed to guarantee the total platelet breakdown and release of platelet-derived factors. In addition, the MIX was subjected to bacteriological examination to verify its sterility.

Syringes containing the MIX dose were kept frozen at -20°C until use.

4.3.3. Indicators of udder inflammation

SCC. Somatic cell count was performed starting at d 0, and then at d 7, and 14 of treatment. Somatic cell counts were assessed by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna.

Bacteriological Analysis. Quarter milk samples for bacteriological assays were collected before milking using an aseptic procedure at the start of the treatment (d 0) from each affected quarter. Bacteriological analyses were performed by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna.

4.3.4. Intramammary administration of therapies

All herd milking procedures included pre-milking cleaning of the teats with detergent and single-use paper towels and teat dipping at the end of milking with an approved teat disinfectant. All cows were treated after milking as hereafter described. After thawing, the syringe in which MIX was stored was connected to a sterile disposable teat cannula to help the intramammary inoculation of MIX through the teat canal. The antibiotic for local use, chosen on the basis of an antibiogram test, was inoculated using the same procedure, according to

manufacturer indications. Following inoculation, the udder was gently rubbed with upward movement of the hands to distribute the product.

As for the preliminary study, five milliliters of MIX or antibiotic alone was administered for 3 consecutive days, depending on the treatment group.

After these treatments, teat dipping was performed.

4.3.5. Classification of outcomes

Treatment outcomes were assessed by clinical and laboratory evaluations. Clinical improvement was defined as the return of quarter and milk secretion to normal. It was assessed by visual observation of milk composition and palpation of the udder. Good improvement of an udder quarter was defined as having clinically progressed and showing, at the time of last examination, an SCC value decreasing at least under a value of 300.000 cells/mL.

Bacteriological assays were performed at day 0 and at day 14. Resolution of the infection was besides defined as showing no bacterial growth at day 14.

4.3.6. Statistical analysis

The collection efficiency of platelets for each PC obtained by double centrifugation tube methods was analyzed using the following formula (Weibrich, 2005):

efficiency for platelet collection = [platelet count μL in PC \times volume of PC] / [platelet count in whole blood μL \times volume of whole blood].

Data were collected in a database and analyzed by the general linear model for repeated measures on GraphPad InStat 3.00 for Windows software (Graph-Pad Software Inc., La Jolla, CA). The between-subjects factor was represented by treatments (3 levels) and the within-subjects factor was represented by sampling time (4 levels) and the model applied was a full factorial, with polynomial contrasts for within-subjects factor. The statistical analysis of bacteriological data was conducted using the Fisher exact test. For all tests, differences were considered statistically significant at $P \leq 0.05$.

4.4 Results

4.4.1. Platelet collection efficiency

The platelet collection efficiency for the PC obtained by the double centrifugation protocol was (mean \pm SD) $10^9 \pm 2.3\%$, indicating that $10^9 \pm 2.3$ mL of PC at the concentration of 1×10^9 platelet/mL was obtained from 10 mL of blood.

4.4.2. SCC and bacteriological assay

At time 0, the average value of SCC was $7,4 \times 10^6$ cells/ mL with a standard deviation of $6,2 \times 10^6$ cells/mL.

The allocation of the enrolled quarters into the 2 different experimental groups was performed randomly at the treatment start before the results of the bacteriological assays. The distribution of the enrolled quarters positive for bacterial growth was random and not significantly different among the treatment groups, except for 4 cases of Staphylococcus Aureus in the mix group.

Bacteriological detection at day 0 is reported in the following Table 4.1.

Bacteriological assay at day 0	Total	ATB %	ATB n.	MIX %	MIX n.
No bacterial growth	7	57,14%	4	42,86%	3
Enterococcus	4	75,00%	3	25,00%	1
Enterococcus faecalis	4	50,00%	2	50,00%	2
Escherichia coli	2	50,00%	1	50,00%	1
Haemolytic Escherichia coli	2	50,00%	1	50,00%	1
Polymorphic Bacterial Flora	6	50,00%	3	50,00%	3
Gen: Staphylococcus	5	60,00%	3	40,00%	2
Streptococcus Uberis	6	50,00%	3	50,00%	3
Staphylococcus aureus (contagious)	4	0,00%	0	100,00%	4
	40		20		20

Table 4.2 - Allocation of enrolled quarters with acute mastitis positive to bacterial growth into 2 experimental groups.

4.4.3. Treatments

Results of the treatments with ATB and MIX are reported in Table 4.2. Statistical analysis showed that the MIX treatment had significantly superior performance compared with antibiotic relative to the improvement of mammary mastitic quarters.

The MIX treatment decreased the mean value of SCC more significantly than the antibiotic treatment.

	SCC MED S0	SCC SD± S0	SCC MED S7	SCC SD± S7	SCC MED S14	SCC SD± S14
MIX	8,30E+06	6,31E+06	1,09E+06	1,55E+06	6,71E+05	1,16E+06
ATB	6,53E+06	6,22E+06	5,20E+05	5,12E+05	2,19E+06	2,71E+06

Table 4.2 - Preliminary Study Results. Mean values and SD at day 0,7,14 for each group MIX and ATB.

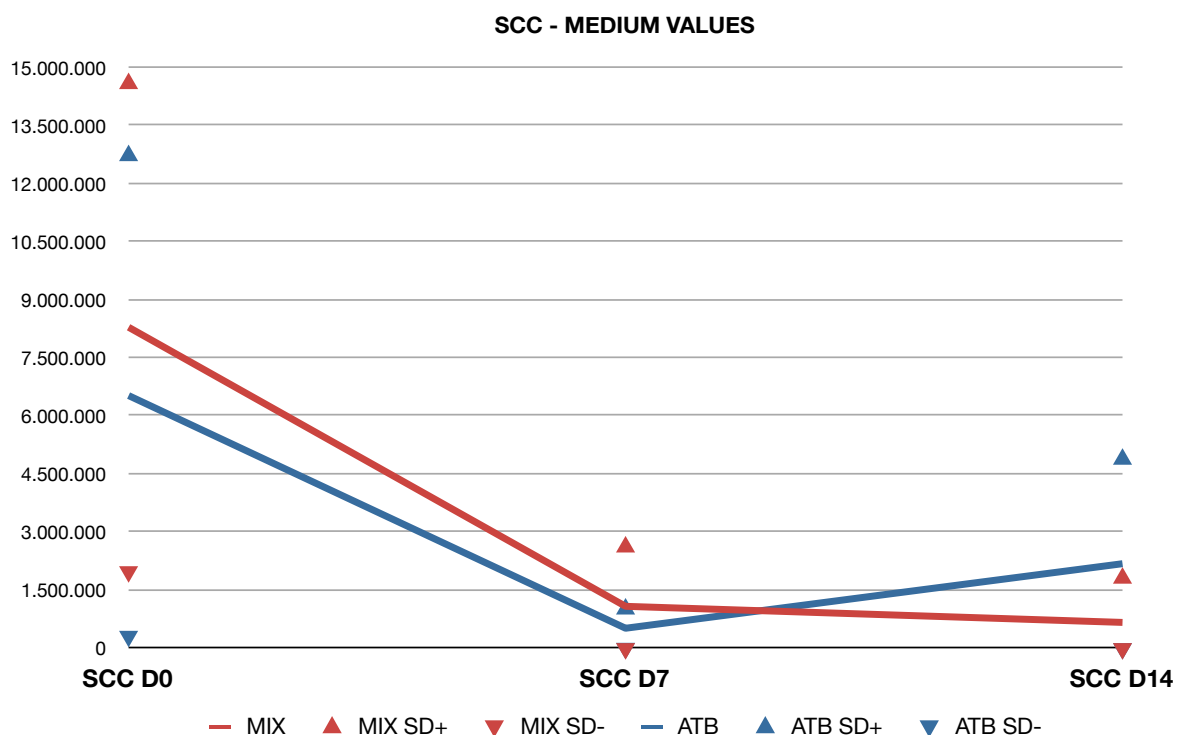


Figure 4.1 - Preliminary Study Results. Overall mean values and SD at day 0,7,14.

As for bacteriological assay, no bacterial growth was detected at day 14 in 60% of quarters. Two out of four cases of S.aureus treated with MIX showed a complete resolution of the disease, while one case suggested the presence of biofilm formation without host immune response.

Results are shown in Table 4.

Case	SCC day 0	SCC day 7	SCC day 14	Bacteriological assay day 0	Bacteriological assay day 14
1	12.741.000	395.000	5.010.000	Staphylococcus aureus	Staphylococcus aureus
2	100.000	25.000	10.000	Staphylococcus aureus	Staphylococcus aureus
3	2.524.000	63.000	60.000	Staphylococcus aureus	No bacterial growth
4	650.000	577.000	599.000	Staphylococcus aureus	No bacterial growth

Table 4.2 - Preliminary Trial Results. Bacteriological assay at day 0 and day 14 for the four cases of S.aureus infection.

4.5 Discussion

Interesting results collected in the preliminary study suggested to try to evaluate the eventual synergistic mechanism of action by separating the different active components. For this reason an advanced study was performed, in order to estimate the single effect of OO and PC, both in comparison with MIX, under an antibiotic control.

In addition, the partial efficacy of MIX against S.aureus suggested to investigate different MIX concentrations and longer duration treatment. This point is postponed to further research.

CHAPTER 5

Advanced Trial:

**Intramammary administration of oxygen/
ozone mix in oil vehicle blended platelet
concentrate in bovine mastitis.**

Control groups: ATB, PC, OO

5.1 Abstract

An advanced study was performed between October 2013 and May 2014, and total of 80 animals were enrolled. The ATB, PC, OO and MIX performances were assessed. MIX showed the best results, suggesting a possible synergistic rapid activity operated by PC and OO, and between therapy and host immune system.

5.2 Introduction

Results collected from the preliminary trial suggested an evaluation of single MIX components. In order to get comparable results, only one concentration of PC and only one concentration of OO were prepared. PC, OO and MIX were administrated in 5 mL filled-up syringes each, within two days from infection detecting, for a total of four doses each (2 after morning milking and 2 after evening milking). MIX was prepared with a half PC dose and a half OO dose, as further described. Results showed a MIX activity far beyond the sum of PC and OO activities, leading to the belief of a synergistic action of two component.

5.3 Materials and methods

5.3.1. *Experimental design*

After data computation from the preliminary study and collection of promising results, an advanced study was performed between October 2013 and May 2014.

Farm. The advanced experiment was carried out in a farm managed in a cubicle yard and divided into three different lactation groups. Herd size was of 900 cows in lactation, following a twice-daily milking regimen. This herd was characterized by high efficiency in the overall sanitary cattle management and usually free of contagious pathogens. As in the previous study, cows suffering from mastitis were identified at milking time by the farmer. Mastitis diagnosis was subsequently confirmed by the veterinarian enrolled in the study for examining the cows for inclusion in the trial and for data gathering.

Mastitis Diagnosis. In this second study, in addition to what established in the previous experiment about mastitis diagnosis, clinical and instrumental data have been collected.

Animals. A total of 80 animals were enrolled in this advanced study. Cows that fulfilled the inclusion criteria were randomly allocated into 4 groups: (1) ATB (control) group (20 animals), which was treated with intramammary antibiotic, chosen as further described; (2) PC group (20 animals), which was treated with platelet concentrate, prepared as further described; (3) OO group (20 animals),

which was treated with ozonated oil, prepared as further described; (4) MIX group (20 animals), which was treated with a blending of platelet concentrate with ozonated oil, prepared as further described.

Each animal was enrolled only once.

5.3.2. Preparation of therapies

5.3.2.1. Preparation of platelet concentrate (PC)

Collection of Blood and Double Centrifugation Method for PC collection was performed as described in the preliminary study.

Half the amount of PC obtained for each donor was aliquoted in 10 mL ready-to-use doses that were stored in syringes. Each syringe has been filled up with 5 mL of PC. The second half of total amount of PC was kept under the horizontal laminar flow hood in aseptic conditions for MIX preparation, as further described. The PC syringes were then frozen at -196°C in liquid nitrogen and thawed at 25°C to allow the release of platelet-derived factors, through thermal shock. Platelet count on a sample of each preparation has been performed to guarantee the total platelet breakdown and release of platelet-derived factors. In addition, the PC was subjected to bacteriological examination to verify its sterility.

Syringes containing the PC dose were kept frozen at -20°C until use.

5.3.2.2. Preparation of ozonated oil

OO was prepared as described in the preliminary study.

Half the amount of OO was aliquoted in 10 mL ready-to-use doses that were stored in syringes. Each syringe has been filled up with 5 mL of OO. Syringes containing the OO dose were then frozen and kept at -20°C until use.

The second half of total amount of OO was kept under the horizontal laminar flow hood in aseptic conditions for MIX preparation, as further described.

5.3.2.3. Preparation of mix

The blending of PC platelet concentrate with OO ozonated oil was prepared using the second half of total amount of PC kept under the horizontal laminar flow hood in aseptic conditions. The PC obtained for each donor was aliquoted in 10 mL ready-to-use doses that were stored in syringes. Each syringe has been filled up with 2,5 mL of PC and then added with 2,5 mL of OO, previously prepared and kept in sterile environment. The MIX syringes were then frozen at -196°C in liquid nitrogen and thawed at 25°C to allow the release of platelet-derived factors. The MIX was subjected to bacteriological examination to verify its sterility. Moreover,

platelet count on a sample of each preparation has been performed to guarantee the total platelet breakdown and release of platelet-derived factors.

Syringes containing the MIX dose were kept frozen at -20°C until use.

5.3.3. Indicators of udder inflammation

Clinical data were collected at d 0, d 1, d 2, d 3 from day of inflammation and the following parameters were recorded:

S - SWELLING of inflamed area, expressed on a clinical scale of evaluation based on: S=0 no swelling, S=1 localized swelling at the teat area of the inflamed quarter, S=2 localized swelling at the inflamed quarter, S=3 spreading out of the swelling into the whole udder.

PT - PAIN TO TOUCH of inflamed area, expressed on a clinical scale of evaluation based on: S=0 no pain, S=1 moderate animal reaction to touch, S=2 visible animal anxiety and hostility to touch, presence of complaints during the milking, S=3 important animal repulsion, opposition to milking, kicking.

RT - RECTAL TEMPERATURE expressed in $^{\circ}\text{C}$.

MQ - MILK QUALITY, expressed on a scale of evaluation based on: MQ=0 not altered milk, MQ=1 presence of chunks, flakes or clots in the milk, MQ=2 milk is visibly skimmed or similar to whey, MQ=3 presence of blood or color alteration.

MDP - MILK DAILY PRODUCTION, as indicated by the measurement instrument applied to the milking system and summarized for a daily value.

SCC - Somatic cell count was performed starting at d 0, and then at d 3, d 7 and d 10 of treatment. Somatic cell counts were assessed by ISZLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna).

Bacteriological Analysis. Quarter milk samples for bacteriological assays were collected at d 0 and performed as described in the preliminary study.

5.3.4. Intramammary administration of therapies

All herd milking procedures included pre-milking cleaning of the teats with detergent and single-use paper towels and teat dipping at the end of milking with an approved teat disinfectant. All cows were treated after milking as hereafter described. After thawing, the syringe in which PC, or OO, or MIX was stored was

connected to a sterile disposable teat cannula to help the intramammary inoculation of the contents through the teat canal. The antibiotic for local use, chosen on the basis of an antibiogram test, was inoculated using the same procedure, according to manufacturer indications. Following inoculation, the udder was gently rubbed with upward movement of the hands to distribute the product.

As for the advanced study, five milliliters of PC alone or five milliliters of OO or five milliliters of MIX was administrated for 2 consecutive days, depending on the treatment group. Antibiotic only was administrated for 3 or 5 consecutive days, according to manufacturer indications.

After these treatments, teat dipping was performed.

5.3.5. Classification of outcomes

Treatment outcomes were assessed by clinical and laboratory evaluations. Clinical improvement was defined as the return of quarter and milk secretion to normal. It was assessed by visual observation of milk composition and palpation of the udder. Good improvement of an udder quarter was defined as having clinically progressed and showing, at the time of last examination, an SCC value decreasing under 300.000 cells/mL, in addition to S=0, PT=0, MQ=0, RT<39°C.

5.3.6. Statistical analysis

The collection efficiency of platelets for each PC and subsequently for each MIX was performed as in the preliminary trial.

5.4 Results

5.4.1. Platelet collection efficiency

The platelet collection efficiency for the PC obtained by the double centrifugation protocol was examined as shown in the preliminary trial.

5.4.2. SCC and bacteriological assay

At time 0, the average value of SCC was above $1,5 \times 10^6$ cells/ mL. For the purpose, single value detection above $1,5 \times 10^6$ cells/mL is here considered not meaningful. In fact, such value is itself predisposing to a self-instigating tissue damage. Prompt SCC fall under such a value is considered indicative of negative loop arrest and inversion, and necessary to reach the required improvement.

The allocation of the enrolled quarters into the 4 different experimental groups was performed randomly at the treatment start before the results of the bacteriological assays. The distribution of the enrolled quarters positive for

bacterial growth was random and not significantly different among the treatment groups.

5.4.3. Treatments

Results of the treatments with ATB, PC, OO and MIX are reported in Table , which shows

Different clinical and instrumental parameters are described separately.

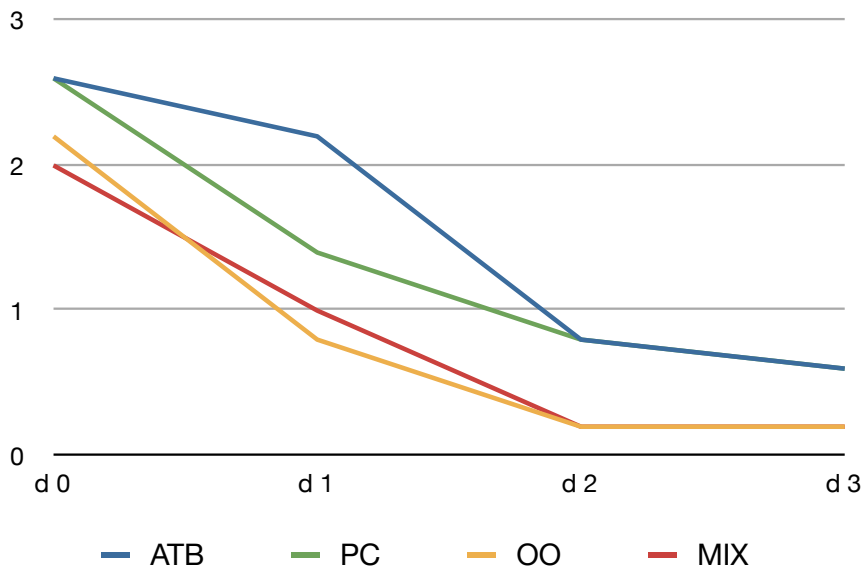
5.4.3.1. Swelling

Data are reported in Figure 5.4.1.

The initial medium range was comprised between 2 and 2.5 for all groups.

PC, OO and MIX showed a faster decrease of swelling at day 1, being assessed under S=1 from day 2.

ATB showed a slower swelling reduction, but from day 2 reached the beneath 1 value, becoming comparable to PC.



SWELLING Evaluation scale	
S=0	no swelling
S=1	localized swelling at the teat area of the inflamed quarter
S=2	localized swelling at the inflamed quarter
S=3	spreading out of the swelling into the whole udder

Figure 5.4.1. Mean Swelling values at day 0-1-2-3 for each congruous group ATB, PC, OO, MIX.

5.4.3.2. Pain to Touch

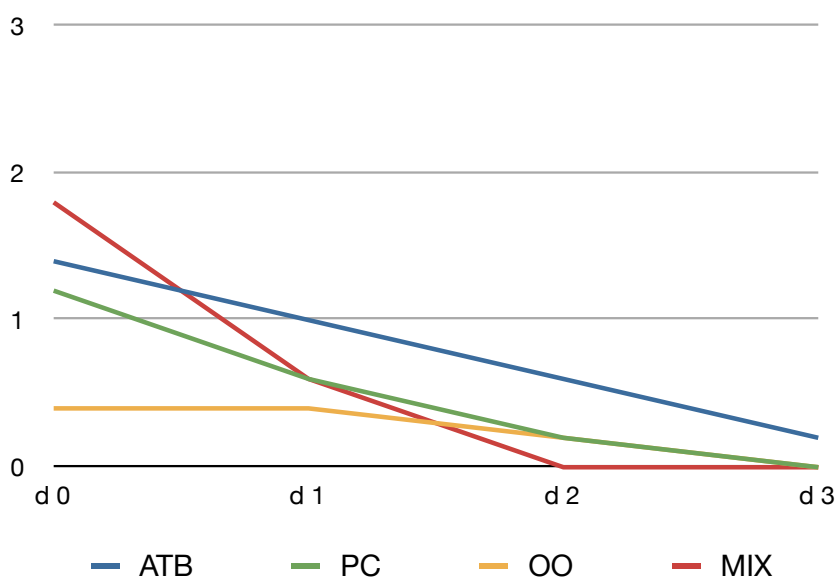
Data are reported in Figure 5.4.2.

The initial medium range was comprised between 1.2 and 1.8 for ATB, PC, and MIX groups, being MIX the worse value. OO group started from a lower mean value (0.4).

MIX showed the faster decreasing of pain to touch at day 1, being assessed on S=0 from day 2.

ATB and PC showed a slower linear swelling reduction, but PC alone reached S=0 at day 3.

OO started decreasing linearly only at day 1 and reached S=0 at day 3.



PAIN TO TOUCH Evaluation scale

P=0	no pain
P=1	moderate animal reaction to touch
P=2	visible animal anxiety and hostility to touch, presence of complaints during the milking
P=3	important animal repulsion, opposition to milking, kicking

Figure 5.4.2. Mean Pain to Touch values at day 0-1-2-3 for each congruous group ATB, PC, OO, MIX.

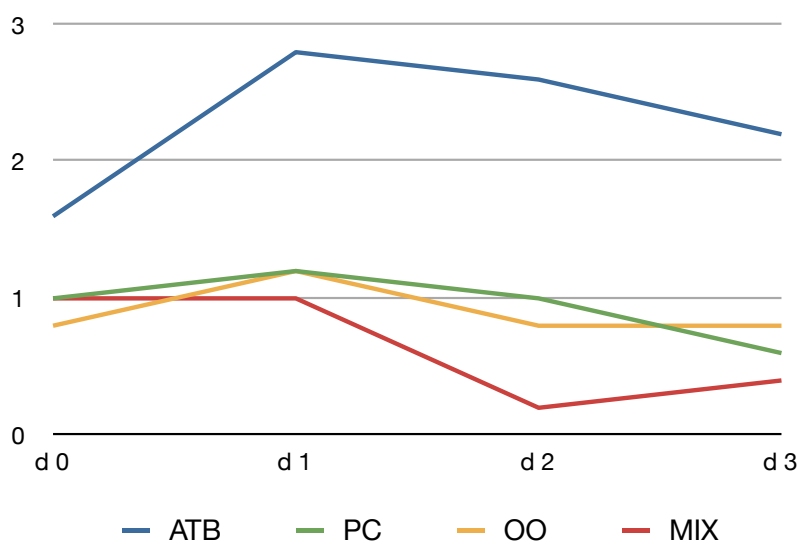
5.4.3.3. Milk Quality

Data are reported in Figure 5.4.3.

The initial medium range was similar for PC, OO and MIX groups, being comprised between 0.8 and 1.0. ATB group started from a higher mean value (1.6). MIX showed the best performance, staying unchanged during day 0 and starting a fast decreasing at day 1. MIX showed a modest worsening from day 2, remaining clearly under the other groups yet.

PC and OO showed a similar progression, with a slight worsening during day 0 and a slow recovering from day 1. Final value at day 3 was between 0.6 and 0.8.

ATB showed the worst performance, with a significant regress during day 0, a 2.8 value at day 1 and a following progressive slow reduction: it reached at day 3 a value (2.2) above the initial one.



MILK QUALITY Evaluation scale

MQ=0	not altered milk
MQ=1	presence of chunks, flakes or clots in the milk
MQ=2	milk is visibly skimmed or similar to whey
MQ=3	presence of blood or color alteration

Figure 5.4.3. Mean Milk Quality values at day 0-1-2-3 for each congruous group ATB, PC, OO, MIX.

5.4.3.4. Rectal Temperature

Data are reported in Figure 5.4.4.

The initial medium range was similar for all groups, being comprised between 39.0°C and 40.0°C. MIX showed the worse initial value (39.9°C) and OO the best (39.0°C).

MIX showed the best performance, with a significant improvement during day 0 (from 39.9°C to 38.9°C) and a linear progression towards 38.3°C at day 3.

ATB and PC showed a similar progression, with an interesting improvement during day 0, but staying assessed over 39.0°C at day 1, and a slight progression towards day 3. Final value at day 3 was 38.9°C for both.

OO showed the worst performance, staying mainly unchanged on 39.0°C value.

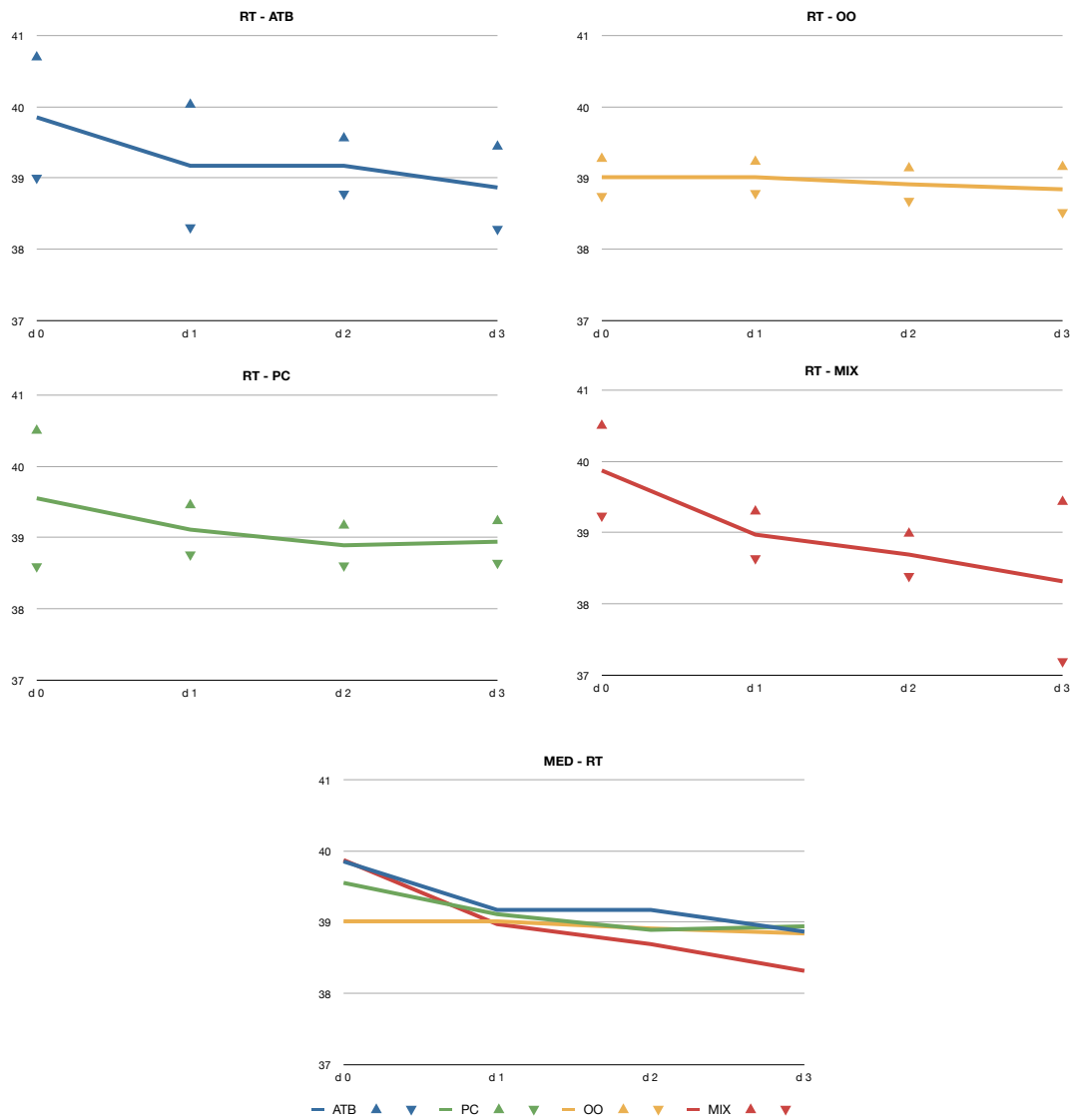


Figure 5.4.4. Mean Rectal Temperature values at day 0-1-2-3 for each congruous group ATB, PC, OO, MIX. Arrows show SD.

5.4.3.5. Milk Daily Production

Data are reported in Figure 5.4.5.

The initial medium range was similar for all groups, being comprised between 18 L and 25 L. All groups showed an initial decrease in milk production.

PC, OO and MIX showed a similar progression, with an interesting improvement from day 1, and reaching final values at day 3 between 24 L and 32 L.

ATB showed the worst performance, with a critical decrease at day 1. Improvement was no significant for ATB.

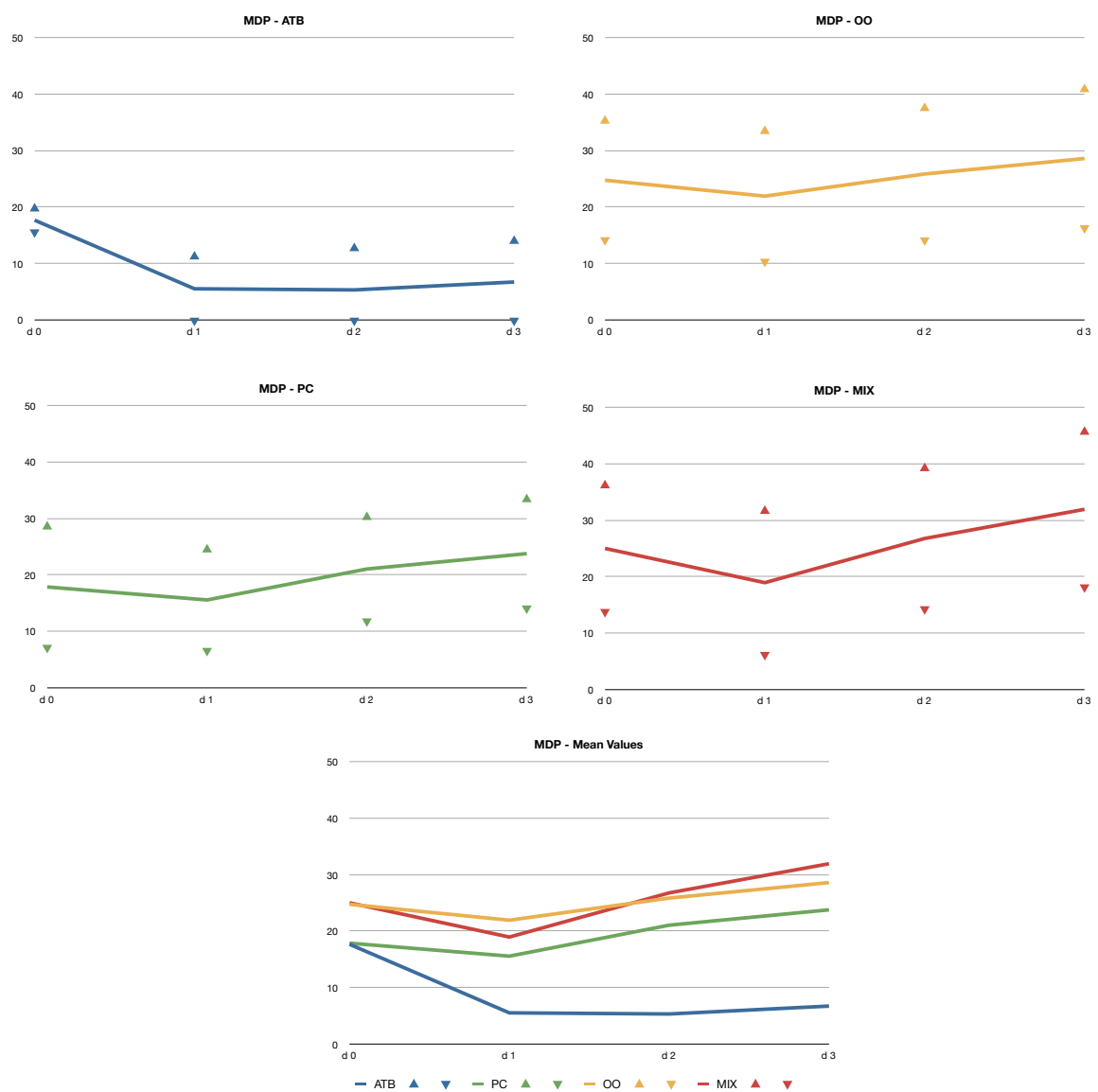


Figure 5.4.5. Mean Milk Daily Production values at day 0-1-2-3 for each congruous group ATB, PC, OO, MIX. Arrows show SD.

5.4.3.6. Somatic Cells Count

Data are reported in Figure 5.4.6. All data equal or above $1,5 \times 10^6$ cells/mL were shown at $1,5 \times 10^6$ cells/mL.

The initial value was then the same for all groups, being over $1,5 \times 10^6$ cells/mL.

MIX showed the best performance, with a significant improvement during the first three days (from $>1,5 \times 10^6$ cells/mL to $1,1 \times 10^6$ cells/mL) and a linear progression towards $2,8 \times 10^5$ cells/mL at day 10.

ATB, PC and OO showed no improvement during day 0. PC showed a clear recovery between day 3 and day 10, assessing the final value under $4,0 \times 10^5$ cells/mL at day 10. ATM showed no improvement regarding the mean values. A slight improvement is reported by its SD value. OO performed in the middle between PC and ATB, with a final value at day 10 of $1,15 \times 10^6$ cells/mL.

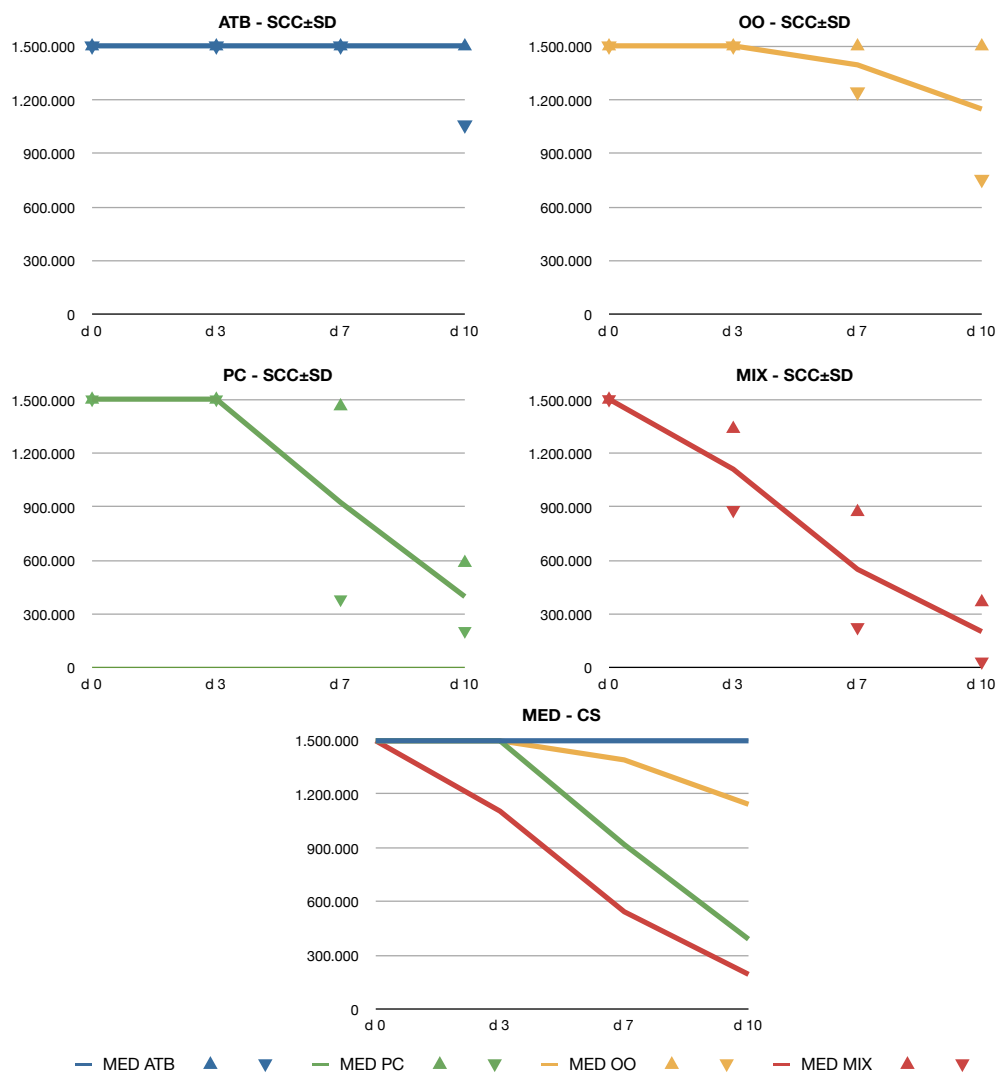


Figure 5.4.6. Mean SCC values at day 0-1-2-3 for each congruous group ATB, PC, OO, MIX. Arrows show SD.

5.4.3.7. Bacteriological assay

The distribution of the enrolled quarters positive for bacterial growth was random and not significantly different among the four treatment groups. Data are reported in Table 5.4.7.

	Total n.	ATB %	ATB n.	PC %	PC n.	OO %	OO n.	MIX %	MIX n.
Escherichia coli	15	33,33%	5	33,33%	5	0,00%	0	33,33%	5
Haemolytic Escherichia coli	25	20,00%	5	20,00%	5	20,00%	5	40,00%	10
Polymorphic Bacterial Flora	25	20,00%	5	40,00%	10	36,00%	9	4,00%	1
Gen. Staphylococcus	2	0,00%	0	0,00%	0	100,00%	2	0,00%	0
Gen. Streptococcus	13	40,00%	5	0,00%	0	30,00%	4	30,00%	4

Table 5.4.7. Bacterial assay distribution for each congruous group ATB, PC, OO, MIX. Percentages and total amount of cases within the same groups.

Pathogens were essentially environmental noncontagious microorganisms, according to the choice of this second trial herd, which is characterized by high efficiency in the overall sanitary cattle management.

The regular pathogens distribution among different groups allows a consistent analysis of the other data collected.

CHAPTER 6

Discussion

Bovine mastitis is a public health issue and its control is fundamental to avoid serious consequences in the sanitary field and in food industry.

Mastitis usually occurs primarily in response to intramammary bacterial infection, but also to intramammary mycoplasmal, fungal, or algal infections. Mechanical trauma, thermal trauma, and chemical insult lead the gland to intramammary infection. Occurrence of mastitis depends on the interaction of host, agent, and environmental factors.

The worsening of tissue damage operated by the presence of a high congestion of neutrophils, cytokines and bacteria establishes a negative cycle, leading to the increasing of microbial aggression, decreasing milk production and additionally arising of mammary tissue damage.

These interacting mechanisms are very complex to understand and represent. The breakdown of this intricate negative loop is very hard and its reversing requires a great and synergistic effort.

For these reasons, prevention should be the first approach to the problem: new predictive protocols and containment means, based on the updated evidence of interaction among environmental factors, host immune system and evolution of bacterial aggression, need to be deployed and shared.

In occurrence, early diagnosis and therapy prompt effectiveness are fundamental to control losses.

Tissue damage is here considered a key factor in understanding the great complexity of this problem and, as far as known, tissue protection and regeneration operated by cathelicidins and other AMPs has been accounted for.

On the other end, bacteria behavior and their evolution in biofilm structures have become an issue for further consideration of the relevance of clinical and instrumental parameters usually considered to measure the state of inflammation. In the matter, SCC data alone has become an unreliable variable, considering the nasty occurrence of sessile bacteria that are out of sight for the host immune system, and are responsible for undetected infections and recurrent relapses. It is reported that the presence of biofilm associated genes likely reduced infectivity in the short term but had the opposite effect in late adherence, allowing longer bacterial persistence in the mammary gland (Cucarella, 2004).

This first approach takes into consideration evident acute clinical mastitis and gathers new findings from the literature with experimental results.

As might be expected considering the size of the trial, only a certain concentration was designed, administered and evaluated. A deeper comprehension of the complementary activity of all mix components could be assessed after gauging a broad range of different concentrations and shot volumes.

6.1. Significance of collected data

The collected data have been assessed a conceivable significance.

Somatic Cells Count, Rectal Temperature, and Swelling are considered as parameters related to a general activation of host immune system, subsequent to bacteria aggression.

On the other side, Pain to Touch, Milk Quality, and Milk Daily Production are considered as tissue damage indicators.

According to the declared relation between immune cells congestion and tissue worsening, the two parameter groups are not completely independent, as might be evident from collected results. In order to reach a final assay, and a better results comparison, a unique scale of evaluation is proposed, assigning a value comprised between [-2,+2] according to the inclination changes of the correlated graph line.

Advanced Trial	HOST IMMUNE RESPONSE / BACTERIA AGGRESSION PARAMETERS									TISSUE DAMAGE PARAMETERS						IMMUNE RESPONSE SUBTOTAL	TISSUE DAMAGE SUBTOTAL	TOTAL SCORE			
	SCC Somatic Cells Count			RT Rectal Temperature			S Swelling			PT Pain to Touch			MQ Milk Quality						MDP Milk Daily Production		
	d1	d7	d10	d1	d2	d3	d1	d2	d3	d1	d2	d3	d1	d2	d3				d1	d2	d3
ATB	0	0	0	2	0	1	1	2	1	1	1	1	-2	1	0	-2	-2	-2	7	-4	3
PC	0	2	2	2	1	0	2	1	1	1	1	1	-1	1	1	-1	1	1	11	5	16
OO	0	1	1	0	0	0	2	1	1	0	1	1	-1	1	0	-1	1	1	6	3	9
MIX	2	2	2	2	2	2	2	2	1	2	2	2	0	2	1	-1	2	2	17	12	29

Table 6.1. Functional activity global evaluation chart for each congruous group ATB, PC, OO, MIX.

Treatment - Advanced Trial	ATB			PC (5ml × 4 doses)			OO (5ml × 4 doses)			MIX (5ml × 4 doses)		
TIME (Day)	d1	d2	d3	d1	d2	d3	d1	d2	d3	d1	d2	d3
SCC - Somatic Cells Count (d3, d7, d10)	0	0	0	0	2	2	0	1	1	2	2	2
RT - Rectal Temperature	2	0	1	2	1	0	0	0	0	2	2	2
S - Swelling	1	2	1	2	1	1	2	1	1	2	2	1
PT - Pain to Touch	1	1	1	1	1	1	0	1	1	2	2	2
MQ - Milk Quality	-2	1	0	-1	1	1	-1	1	0	0	2	1
MDP - Milk Daily Production	-2	-2	-2	-1	1	1	-1	1	1	-1	2	2
Total score per day	0	2	1	3	7	6	0	5	4	7	12	10

Table 6.2. Time depending global evaluation chart for each congruous group ATB, PC, OO, MIX. Promptness is estimated equalizing SCC days of detection (0-3-7-10) to other parameters days of detection (0-1-2-3).

In addition, the same data is represented in an inverted chart, in order to endorse time-depending effectiveness of results.

According to these charts, a possible role for different components on tissue regeneration and pathogens aggression is further evaluated.

6.2. Antibiotic results (ATB): lack of tissue protection

In the study, antibiotic results sum to an overall score of 3 points. In regards to bacteria aggression and consequential host immune response, the subtotal reached by antibiotic is 7 points, while the tissue damage scores a negative -4 points. This is in accordance to literature (Cucarella, 2004): antibiotic is single-target drug and doesn't provide any protection to the tissue involved in the inflammatory process. Unfortunately, tissue damage plays an active role in the general worsening of the inflammation and it can't be underestimated. This is the first reason for ATB lack of effectiveness.

In addition, as for usual MICs, ATB is mainly reliable against planktonic bacteria and it is too often ineffective against sessile communities of bacteria, which can display a decreased growth and establish persisters communities, allowing further subclinical consequences.

Considering the importance of the time variable, ATB showed 3/6 positive results on day 1 and 3/6 positive results on day 2. However, the total score was 0 (zero) on day 1 and 2 (two) on day 2, giving evidence of a general lack of synergy of the remedy, mostly due to the lack of tissue protection.

During the trial, ATB was not able to arrest the negative loop bacteria aggression-host immune response-tissue damage, nor to reverse it.

6.3. Platelet concentrate results (PC): tissue regeneration and host immune response

PC showed interesting positive results both in its synergistic contribution to host immune response and for its regenerative value for the tissues. As explained, time is a key point on overall problem resolution. PC showed 3/6 positive results on day 1 and 6/6 positive results on day 2, enhancing a complete restoration of the tissue integrity.

Total score of PC was 3 on day 1 and 7 on day 2: PC showed a prompt effectiveness and a synergistic approach, gaining a total score of 16 points, thus performing the best result after MIX.

Through both tissue regeneration and host immune response support, PC seemed able to stop and reverse the inflammation negative loop and its dangerous consequences on the secreting gland.

PC seems to show a synergistic action deploying at the same time its antimicrobial activity, its immunomodulatory role and its wound healing contribution. This synergy is particularly made possible by PC ability to exploit different features in milk or in whey, hence only when these are really needed, thus avoiding superfluous and counter-productive efforts.

Likely, as previously reported, PC alone is not able to face biofilm persisters.

4.4. Ozonated oil results (OO): biofilm breakdown, mild host immune response enhancement, possible tissue protection

OO positive results could be mainly related to the effectiveness of its assault to both planktonic and biofilm bacteria. In addition, its mild host immune response enhancement and its regenerative activity on tissues led to a total score of 9 points, better than ATB. OO showed only 1/6 positive results on day 1 but 5/6 positive results on day 2, leading to the belief that the positive cycle was settled.

As might be expected, OO shows a synergistic activity, but without the required promptness. Total score of OO was 0 on day 1 and 5 on day 2.

We hypothesize that OO plays a role in tissue protection: possibly OO upholsters the damaged tissue, adhering to epithelial cells and protecting them from host immune cells congestion.

Moreover, if the tissue is already covered by bacterial biofilm, OO covers the biofilm itself and within the typical tissue temperatures range, it releases gaseous ozone. Thus, ozone, being in contact with biological fluids, degenerates into its usual metabolites. Among these, H_2O_2 enters the biofilm protection, assaults and decomposes the bacterial membrane, killing the pathogen community.

Demonstrated OO efficacy against both planktonic and biofilm growing *Staphylococcus aureus* (Al-Saadi, 2015) and other bacterial biofilm (Bialoszewski, 2011) conveys a possible specific role of OO into the MIX.

4.5. MIX synergistic results: possible biofilm aggression and functional integrity restoration

MIX showed 4/6 positive results on day 1 and 6/6 positive results on day 2, enhancing a complete restoration of the tissue integrity.

Total score of MIX was 7 on day 1 and 12 on day 2: MIX showed a prompt effectiveness and a synergistic approach, gaining a total score of 29 points, thus performing the best result of all.

Through both tissue regeneration and host immune response support, MIX was able to stop and reverse the inflammation negative loop, besides its dangerous consequences on the secreting gland. Moreover it boosted a positive cycle restoring the udder functional integrity and enhancing the natural protective mechanisms.

The better MIX performance root might lie in the promptness of the beginning of the healing process: it seems that MIX is able to induce valuable and fast-paced synergistic activities together with host immune system and tissue protection mechanisms. Our understanding is that if the above mentioned positive cycle gets generated within the first 12 hours (time lapse between two consecutive milkings) since the early infection diagnosis, the recovery chances are exponentially higher.

Accordingly, a chronic mastitis detection protocol must be in place in such a way that the chronicities are eliminated (not only contained), acute occurrences are prevented, while, at the same time, the contagious pathogens are eradicated, as proven possible in the second trial herd.

In addition, as it occurred in the preliminary trial, in two out of four cases of *S.aureus* treated with MIX no pathogen growth was detected at day 14. A gauge of the broad range of different concentrations and shot volumes should be tested.

4.6. Possible further research

Possible further research could explore:

- administration of different AMPs concentrations and different amounts of PC for each MIX dose;
- administration of different ozone concentrations and different amounts of OO for each MIX dose;
- administration of different MIX treatment durations;
- study of the results in relation to specific pathogens typically growing in biofilm;
- study of the results in relation to the relapse rate and a possible persisters detection;
- study of the combined components teratogenicity, already tested separately;
- study of a possible preventive usage of MIX before non lactating period.

CHAPTER 7

Conclusion

Bovine mastitis is a public health issue and its control is fundamental to avoid serious consequences. Prevention should be the first approach to the problem: new predictive protocols and containment means need to be deployed and shared.

However feasible effective treatments need to be available.

The results of this clinical application point to a promising direction of investigation.

In comparison with other therapies based on either a single component or a control group with antibiotic, the blending of platelet concentrate with oxygen/ozone mix in oil vehicle accomplished the best outcomes in resolving the mastitic occurrence, downgrading and fixing the symptoms, and enhancing animal general conditions.

Conceivable explanations for its efficacy have been suggested. Further and deeper research is required.

CHAPTER 8

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CHAPTER 9

Appendix I

Cellule staminali mesenchimali dell'amnios equino per il trattamento delle tendinopatie del cavallo sportivo: prima segnalazione

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9. Cellule staminali mesenchimali dell'amnios equino per il trattamento delle tendinopatie del cavallo sportivo: prima segnalazione

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9.1 Abstract

Regenerative medicine is an emerging and multidisciplinary field which draws on biology, medicine and genetic manipulation for the development of strategies aimed at maintaining, enhancing or restoring the function of tissues or organs that have been compromised by disease or injury. Because of their ability to differentiate into different cell lines, stem cells undoubtedly play a key role in developing such strategies. The embryonic cells are totipotent, but their isolation involves the destruction of the embryo and the application is limited from their oncogenic potential. Adult stem cells (i.e. from bone marrow, BM) have a limited potential compared to embryonic stem cells in terms of both *in vitro* proliferation ability and differentiation capacity, and do not appear to noticeably improve long-term functionality. Stem/progenitor cells derived from extra-fetal sources may represent attractive alternative candidates with the potential to circumvent many of these limitations, opening new perspectives for developmental biology and regenerative medicine. The aims of this work were to provide, for the first time, an isolation protocol for horse amnion-derived cells, to investigate the biological properties of these cells and to assess whether horse amnion-derived cells can be tolerated and exert beneficial effects *in vivo* when allogeneically transplanted into horses with tendon injuries.

Our results have shown that these cells have high prolificacy and plasticity, differentiating *in vitro* toward mesodermic and ectodermic lineages, and own ability to be frozen without loss of their characteristics.

Through cell transplantation studies *in vivo*, we found that the transplanted equine amnion-derived cells were well-tolerated by horses, and all of the clinical findings reported provided compelling evidence to support the exertion of beneficial effects by the injected cells. The possibility of administering an immediate intralesional treatment which is available before any ultrastructural change is observed within the injured tendon, together with the plasticity effect of amniotic MSCs, represent the major features of interest for this novel biotechnological approach to equine tendinopathies.

9.2 Introduction

Il cavallo atleta è continuamente sottoposto a sollecitazioni meccaniche che spesso possono determinare lesioni all'apparato muscolo-scheletrico, in particolare tendini e legamenti, che costituiscono la più comune causa di ritiro dei soggetti sportivi dall'attività agonistica (Lam *et al.*, 2007). Per queste lesioni muscolo-tendinee le terapie tradizionali conservative mediante, per esempio, iniezioni intralesionali di steroidi (Kapetanios 1982), di acido ialuronico (Foland *et al.*, 1992) o di glicosaminoglicani polisolfati (PSGAG) (Marr *et al.*, 1993), nonché terapie chirurgiche quali lo splitting transcutaneo accompagnato da desmotomia della briglia radiale, associate a riposo dell'animale per diversi mesi, conseguono nella formazione di tessuto cicatriziale con matrice connettivale povera e fibrille collagene con diametri ridotti che ne compromettono la guarigione (Woo *et al.*, 1999). Infatti, in seguito a lesioni tendinee, il tessuto cicatriziale che si forma nel focolaio di rottura rappresenta una causa di calo di prestazioni e di aumento del rischio di recidiva che ammonta al 56% per i cavalli da salto e al 66% per quelli da corsa (Dyson 2004).

Poiché è noto che il tessuto cicatriziale non consente il ripristino di una condizione anatomo-funzionale prossima a quella precedente il trauma, nel corso di questi ultimi anni sono state sviluppate numerose strategie terapeutiche finalizzate ad accelerare i tempi e, soprattutto, la qualità dei processi riparativi tendinei. In quest'ottica, del tutto innovativo e promettente è il recente ricorso, anche in medicina veterinaria, alla terapia rigenerativa, nuovo emergente campo multidisciplinare, che sfrutta la biologia e la manipolazione cellulare per lo sviluppo di strategie atte a ripristinare la funzione di organi o tessuti compromessi da eventi patologici.

La terapia cellulare è basata sulla capacità delle cellule staminali mesenchimali (MSCs) ottenute da midollo osseo (BM) o tessuto adiposo, di differenziare in diverse linee cellulari. In medicina veterinaria, approcci terapeutici per trattare danni ai tessuti muscolo-scheletrici sono stati sviluppati usando MSCs multipotenti

(Smith *et al.*, 2003; Pacini *et al.*, 2007; Crovace *et al.*, 2007; Fortier e Smith, 2008; Guest 2008).

Si ipotizza che le MSCs impiantate in una lesione tendinea possano influire sulla rigenerazione tissutale secondo due meccanismi: differenziandosi in tenociti e secernendo matrice extracellulare tendinea, oppure, secernendo fattori di crescita che inducano le cellule impiantate o quelle residenti a produrre tale matrice (Murphy *et al.*, 2003; Caplan e Dennis 2006). I risultati ottenuti da diverse prove di applicazione delle MSCs per la rigenerazione di tessuti danneggiati di varia natura sono positivi, sia dal punto di vista della ricostituzione della struttura sia del recupero della funzionalità. Diversi studi hanno dimostrato che, in seguito ad inoculazione di MSC da BM in lesioni tendinee del tendine flessore superficiale delle falangi, in tempi relativamente brevi (40-100 giorni) si è verificata la completa ricostituzione dell'architettura e della struttura del tessuto, valutata mediante tecnica ultrasonografica, istologica ed immunoistochimica (Smith *et al.*, 2003; Koch *et al.*, 2008; Lacitignola *et al.*, 2008; Crovace *et al.*, 2010).

Nonostante l'incremento della terapia cellulare per trattare problemi di natura ortopedica nell'equino, molte problematiche rimangono senza risposta relativamente alla fonte ottimale di cellule mesenchimali, ai tempi ed alle modalità di trattamento in base al tipo di lesione, alla valutazione della sicurezza delle terapie in termini di potenziale tumorigenico ed all'efficacia del trattamento (Berg *et al.*, 2009). Il BM è stato considerato, per lungo tempo, la principale fonte di MSCs adulte. In numerosi studi è stato dimostrato come l'inoculo di cellule provenienti da BM in tendini anche gravemente danneggiati producesse una significativa rigenerazione in luogo di una riparazione cicatriziale (Caplan 2007). Per queste loro attitudini, l'uso di cellule staminali mesenchimali isolate da BM o talvolta, in modo più empirico, l'impiego di BM tal quale, è stato introdotto tra le terapie delle tendinopatie e delle desmopatie del cavallo (Smith *et al.*, 2003; Crovace *et al.*, 2007; Pacini *et al.*, 2007; Wilke *et al.*, 2007; Fortier e Smith 2008; Guest 2008). Il BM tal quale contiene, però, anche derivati e precursori ossei e cellule adipose che potrebbero essere deleteri per la guarigione innescando la formazione di mineralizzazioni distrofiche o metaplasie nella sede di inoculo. Inoltre, è riportato in letteratura che le cellule derivanti da BM mostrano una limitata capacità di proliferazione (circa 32 giorni per l'espansione fra l'isolamento e l'impianto) e differenziamento (Guest *et al.*, 2010) che aumenta con l'età del donatore ed il numero di passaggi *in vitro* (Digirolamo *et al.*, 1999; Guillot *et al.*, 2007) e non danno miglioramenti funzionali a lungo termine (Paris e Stout, 2010). Infine, la raccolta di BM richiede una procedura invasiva che è stata associata occasionalmente nell'equino a pneumopericarditi (Durando *et al.*, 2006). Altra fonte di MSCs è rappresentata dal tessuto adiposo, ma alcuni ricercatori hanno evidenziato che queste cellule posseggono un minor potenziale osteogenico e condrogenico (Winter *et al.*, 2003; Muschler *et al.*, 2004; Im *et al.*, 2005).

Le cellule provenienti dagli annessi fetali potrebbero rappresentare una valida alternativa per superare alcune di queste limitazioni, aprendo nuove prospettive per lo sviluppo della medicina rigenerativa. In medicina umana, la membrana amniotica isolata dalla placenta a termine è una fonte particolarmente attraente di cellule staminali mesenchimali perché è solitamente eliminata alla nascita, il suo utilizzo non comporta conflitti etici e permette un recupero molto efficiente di MSCs senza richiedere procedure invasive. Il fatto che la placenta sia fondamentale per il mantenimento della tolleranza materno-fetale durante la gravidanza suggerisce, inoltre, che le cellule presenti nel tessuto placentare possano avere caratteristiche immuno-modulatorie che riducono i rischi del rigetto immunologico delle cellule staminali trapiantate da parte del ricevente (Evangelista *et al.*, 2008). Infatti, la membrana amniotica umana e le cellule epiteliali amniotiche isolate da essa hanno mostrato la capacità di sopravvivere per lungo tempo in animali immunocompetenti quali conigli (Avila *et al.*, 2001), ratti (Kubo *et al.*, 2001), porcellini d'India (Yuge *et al.*, 2004) e scimmie (Sankar e Muthusamy, 2003). Recenti studi hanno anche dimostrato che cellule mesenchimali di amnios umano possono fortemente inibire la proliferazione dei linfociti T (Magatti *et al.*, 2008) e mostrare bassa immunogenicità (Bailo *et al.*, 2004). Inoltre, è stato riportato che tali cellule posseggono l'abilità di differenziarsi in più linee cellulari (Ilancheran *et al.*, 2007; Evangelista *et al.*, 2008) ed avere funzioni anti-infiammatorie (Sarugaser *et al.*, 2005).

In medicina veterinaria, la membrana amniotica *in toto* è stata usata in terapie cliniche per la ricostruzione della superficie oculare in cavalli (Ollivier *et al.*, 2006; Plummer 2009) e cani (Arcelli *et al.*, 2009) poiché è una struttura avascolare, promuove la ri-epitelizzazione, decresce infiammazione e fibrosi (Solomon *et al.*, 2005) e modula l'angiogenesi (Dua *et al.*, 2004). Considerando che il processo di guarigione richiede fattori di crescita che stimolano l'angiogenesi, la mitogenesi e la formazione di matrice, le cellule derivanti dall'amnios potrebbero rappresentare un prezioso strumento per la terapia cellulare in ambito veterinario.

Lo scopo di questa sperimentazione è stato la messa a punto di un protocollo per l'isolamento di cellule mesenchimali dalla membrana amniotica e la caratterizzazione delle presunte cellule staminali sulla base della loro capacità proliferativa e differenziativa in senso osteogenico, adipogenico, condrogenico e neurogenico, anche in seguito a crioconservazione. Inoltre, per la prima volta, un ulteriore obiettivo di questo studio è stato l'impiego di cellule mesenchimali allogeniche di derivazione amniotica per il trattamento delle patologie dell'apparato muscolo-scheletrico nel cavallo.

9.3 Materials and methods

Tutti i reagenti impiegati in questa sperimentazione, se non diversamente indicato, sono stati acquistati dalla Sigma Aldrich, Milano, Italia.

Raccolta della membrana allanto-amniotica Per questo studio sono stati lavorati cinque campioni di membrana amniotica prelevati al momento del parto. I campioni sono stati posti a 4°C in tampone fosfato salino privo di calcio e magnesio (PBS, Euroclone, Milano, Italia), supplementato con 100 U/ml penicillina, 100 µg/ml streptomina e 0,25 µg/ml amfotericina B e processati entro 12 ore dal prelievo. La membrana amniotica, sottile e trasparente, è stata separata meccanicamente dall'allantoide vascolarizzata (Fig. 1) e sottoposta a digestione enzimatica.

Isolamento di cellule amniotiche mesenchimali I frammenti di ogni campione di amnios sono stati incubati per 9 minuti a 37°C in PBS contenente 2,4 U/ml dispasi (Becton Dickinson and Company, Milano, Italia). Dopo un periodo di 5-10 minuti a temperatura ambiente in High Glucose - Dulbecco's Modified Eagle's Medium (HG-DMEM; Euroclone), addizionato con 10% di siero fetale bovino (FBS, Fetal Bovine Serum) e 2 mM L-glutamina, i frammenti sono stati digeriti con 0,93 mg/ml collagenasi tipo I e 20 µg/ml DNAsi (Roche, Mannheim, Germany) per circa 3 ore a 37°C. Il prodotto della digestione è stato filtrato utilizzando un filtro da 100 µm. Il filtrato è stato centrifugato a 200xg per 10 minuti per raccogliere le cellule mesenchimali isolate. Queste cellule sono state denominate cellule mesenchimali amniotiche (AMCs). Il numero delle cellule vitali isolate è stato contato mediante saggio di esclusione con il colorante Trypan blue, usando la camera di Bürker.

Coltura, espansione e conta cellulare Successivamente all'isolamento, le AMCs sono state seminate alla densità di 1x10⁵ cell/cm² e le colture cellulari sono state mantenute con HG-DMEM addizionato con 10% FBS, 10 ng/ml epidermal growth factor (EGF), 100 U/ml penicillina, 100 µg/ml streptomina, 0,25 µg/ml amfotericina B e 2 mM L-glutamina. Le colture cellulari sono state mantenute in incubatore ad un'atmosfera al 5% CO₂ e 90% umidità, ed alla temperatura di 38,5°C per gli esperimenti descritti in seguito. Il terreno di coltura è stato cambiato per la prima volta dopo 72 ore dalla semina per eliminare le cellule non aderenti. Il mantenimento delle colture cellulari ha previsto la rimozione del terreno di coltura due volte la settimana. Al raggiungimento di una confluenza pari a circa l'80% della superficie della fiasca a passaggio 0 (P0) la coltura primaria è stata espansa, staccando le cellule dal fondo della fiasca con 0,05% tripsina/0,02% EDTA in

tampone fosfato (Euroclone) a 37°C per 2-3 minuti. Dopo la conta, la sospensione cellulare è stata ridistribuita in fiasche di coltura più ampie in rapporto 1:2 (P1) seminando circa 1×10^4 cell/cm² al fine di mantenere ed espandere la coltura fino al decimo passaggio.

Analisi della proliferazione cellulare *Doubling Time (DT)* Per l'analisi del DT è stata utilizzata la metodica della conta seriale, cioè le cellule sono state contate a passaggi successivi da P1 a P10. Per questo scopo, le AMC's sono state seminate alla densità di 3×10^3 cell/cm² ed il cambio di terreno è stato eseguito ogni tre giorni sino al raggiungimento di circa l'80% di confluenza, momento in cui le cellule sono state tripsinizzate, contate e seminate alla stessa densità.

Il DT è stato ottenuto a ciascun passaggio accordandosi alle formule $CD = \log(N_f/N_i)/\log 2$, e $DT = CT/CD$, dove CD rappresenta il fattore di duplicazione cellulare, N_f è il numero finale di cellule a confluenza, N_i è il numero iniziale di cellule seminate, CT rappresenta il tempo di mantenimento della coltura.

Analisi delle unità formanti colonie fibroblastoidi (CFU-F) Per il test delle CFU-F, le AMC's sono state seminate, subito dopo il loro isolamento (P0) a densità differenti (100, 250, 500 e 1000 cell/cm²) e mantenute in incubatore a 38,5°C, in un'atmosfera con il 5% CO₂ e 90% umidità, per 2 settimane in terreno HG-DMEM arricchito come descritto precedentemente. Le colonie sono state fissate in formalina al 4%, colorate con 1% di Blu di Metilene (Serva, Heidelberg, Germania) in tampone borato 10mM (pH 8,8; Fluka, BioChemika, Buchs, Svizzera) a temperatura ambiente e lavate con acqua distillata per due volte. Le colonie comprendenti un numero di cellule nucleate maggiore di 16-20 sono state contate utilizzando un microscopio rovesciato Olympus BX71.

Differenziamenti in vitro

Lo studio prevedeva di analizzare il potenziale plastico delle cellule isolate, mediante induzione del differenziamento osteogenico, adipogenico, condrogenico e neurogenico.

Per tale studio, le cellule isolate da amnios sono state espanse e a P3 sono state seminate alla densità di 3×10^3 cellule/cm² per il trattamento differenziativo e ad una densità di 1×10^3 cellule/cm² per il controllo. Per i primi 3-4 giorni, le cellule sono state incubate con il terreno utilizzato per la loro espansione (HG-DMEM arricchito) in modo da permetterne l'adesione ed il raggiungimento della confluenza, momento in cui sono stati avviati i trattamenti differenziativi.

Differenziamento osteogenico

Per il differenziamento osteogenico, le colture cellulari sono state incubate in HG-DMEM addizionato con 10% FBS, 100 U/ml penicillina, 100 µg/ml streptomina,

0,25 µg/ml amfotericina B, 2 mM L- glutammina, 10 mM β-glicerofosfato, 0,1 µM desa- metasone, 250 µM acido ascorbico.

Il differenziamento osteogenico è stato condotto incubando le cellule per 3 settimane a 38,5°C in un'atmosfera con il 5% CO₂. Per il controllo è stato utilizzato il terreno di espansione cellulare di base per il mantenimento delle cellule amniotiche in coltura. Al 21° giorno, l'osteogenesi è stata valutata con la convenzionale colorazione von Kossa usando 1% di nitrato d'argento e 5% di tiosolfato di sodio per la determinazione dei depositi di calcio.

Differenziamento adipogenico

Per indurre il differenziamento adipogenico, le cellule sono state stimulate con tre cicli di induzione/mantenimento. Ogni ciclo prevedeva una coltura di 3 giorni con un terreno di induzione specifico per l'adipogenesi ed un altro periodo di 3 giorni con un terreno di mantenimento.

Il terreno di induzione era costituito da HG- DMEM, supplementato con il 10% FBS, 100 U/ml penicillina, 100 µg/ml streptomina, 0,25 µg/ml amfotericina B, 2 mM L-glutammina, 10 µg/ml insulina, 150 µM indometacina, 1 µM desametasone e 500 µM 3-isobutil-metil-xantina (IBMX). Il terreno di mantenimento era costituito da HG-DMEM, supplementato con il 10% FBS e 10 µg/ml insulina. Le cellule di controllo sono state coltivate per lo stesso periodo di tempo unicamente nel terreno di mantenimento. Dopo 3 settimane, il trattamento differenziativo è stato bloccato e l'adipogenesi è stata analizzata usando la colorazione convenzionale Oil red O (0,1% di Oil red O in 60% di isopropanolo) per visualizzare le gocce lipidiche.

Differenziamento condrogenico

Il differenziamento condrogenico è stato indotto incubando le cellule in monostrato per 2-3 settimane in DMEM low-glucose (LG-DMEM, Euroclone), supplementato con 100 nM desametasone, 50 µg/ml acido-L-ascorbico-2-fosfato, 1 mM sodio piruvato (BDH Chemicals Ltd., Poole, UK), 40 µg/ml prolina, ITS (insulina 5 µg/ml, transferrina 5 µg/ml, selenito di sodio 5 ng/ml) e 5 ng/ml TGF-β₃ (Pe- provet, DBA Italia). Le cellule di controllo sono state coltivate per lo stesso periodo di tempo nel terreno di crescita standard per le cellule amniotiche (HG-DMEM supplementato). Dopo 3 settimane, la presenza di matrice metacromatica è stata dimostrata dalla colorazione con Alcian Blue pH 2,5.

Differenziamento neurogenico

Il differenziamento neurogenico è stato eseguito con una pre-induzione di 24 ore in un terreno costituito da HG-DMEM addizionato con 20% FBS e 1 mM β-

mercaptoetanolo (BME) (Mitchell *et al.*, 2003). Successivamente, l'induzione neurogenica è stata effettuata con un terreno costituito da HG- DMEM supplementato con 2% FBS, 2% DMSO e 200 μ M idrossianisolo butilato (BHA) per 3 giorni. Le cellule di controllo sono state mantenute per lo stesso periodo di tempo nel terreno di coltura standard delle cellule amniotiche. Il differenziamento neurogenico è stato valutato attraverso la morfologia delle cellule e la colorazione di Nissl (0,1% Cresyl violetto) che mostra l'incremento di corpi tigroidi.

Analisi dei marker di espressione attraverso RT-PCR Mediante RT-PCR, è stata indagata sia l'espressione di marker specifici (*CD34*, *CD29*, *CD44*, *CD166*, *CD105*, *MHC I* e *MHC II*) caratterizzanti le AMC's indifferenziate a P1 e P5, sia l'espressione di marker specifici espressi dalle cellule differenziate ottenute dopo specifica induzione.

L'RNA totale è stato estratto a P1 e P5 dalle AMC's indifferenziate usando TRIZOL® Reagent (Invitrogen Carlsbad, CA, USA) seguito da un trattamento con DNasi accordandosi alle specifiche tecniche del produttore. La concentrazione e la purezza di RNA sono state misurate usando lo spettrofotometro NanoDrop (NanoDrop ND1000). Il cDNA è stato sintetizzato dall'RNA totale usando il kit iScript retrotranscription kit (Bio-Rad Laboratories, Hercules, CA, USA). La PCR convenzionale è stata effettuata in un volume finale di 25 μ l con DreamTaq DNA Polymerase (Fermentas GmbH, St. Leon Rot, Germany). I primer oligonucleotidici specifici per l'equino sono stati disegnati tramite il software PerlPrimer v.1.1.17, basandosi sulle sequenze disponibili presso l'NCBI per la specie *Equus caballus* o su sequenze consenso costruite mediante multi-allineamento di geni omologhi di interesse nei mammiferi. Le sequenze oligonucleotidiche sono state disegnate a cavallo tra esoni, con lo scopo di evitare l'amplificazione del DNA genomico.

Per gli esperimenti di differenziamento, l'RNA totale è stato estratto da cellule indifferenziate (cellule di controllo) e da cellule indotte ai differenziamenti. L'analisi della RT-PCR è stata effettuata come precedentemente descritto. Tessuti di equino adulto (osseo, adiposo, cartilagineo e midollo spinale) sono stati impiegati come controllo positivo per analizzare l'espressione dei marker osteogenici (osteocalcina [*BGLAP*] ed osteopontina [*OPN*]), adipogenici (peroxisome proliferator activated receptor- γ [*PPAR- γ*] e adiponectina [*ADIPQ*]), condrogenici (collagene tipo 2 α 1 [*COL2A1*] ed aggrecano [*ACAN*]) e neurogenici (Nestina [*NES*] e Proteina Acida Glio Fibrillare [*GFAP*]).

I primer sono stati utilizzati ad una concentrazione finale pari a 200 nM. Il GAPDH è stato utilizzato come gene di riferimento.

Immunocitochimica

Sono stati valutati gli antigeni Oct-4, TRA 1-60 e SSEA-4, a P3, in quanto marker di pluripotenza tipici delle cellule staminali embrionali (ESCs). Gli anticorpi sono stati scelti sulla base dei risultati ottenuti da Hoynowski *et al.* (2007). Gli anticorpi primari sono stati acquistati dalla ditta Abcam, (Cambridge, UK), mentre quelli secondari sono stati forniti da Invitrogen.

Per l'immunocitochimica le cellule sono state fissate in 3,7% paraformaldeide per 15 minuti e lavate tre volte in PBS. Per valutare la presenza dell'antigene Oct-4, le cellule sono state permeabilizzate in 0,4% Triton-X100 diluito in PBS per 10 minuti a temperatura ambiente. Dopo tre lavaggi con PBS, tutte le cellule sono state bloccate in PBS addizionato con il 2% di albumina sierica bovina (BSA) per 4 ore a 4°C, e sono state, quindi, incubate con gli anticorpi primari per 12 ore a 4°C. Successivamente, sono stati effettuati tre lavaggi e le cellule sono state incubate con gli anticorpi secondari coniugati con il fluoroforo AlexaFluor-488 (Invitrogen, diluizione 1:250) per 1 ora. Infine, per la colorazione nucleare, l'Hoechst 33342 (1 mg/ml) è stato diluito 1:100 in PBS e caricato sui campioni per 15 minuti. Tutte le analisi sono state basate su cellule di controllo incubate con isotipi specifici IgGs per stabilire il segnale di background. Le immagini sono state visualizzate attraverso il microscopio Olympus BX 51.

Istologia dell'allantoamnios

Opportuni campioni di allantoamnios sono stati fissati in 10% formalina a temperatura ambiente per 24 ore, inclusi in paraffina e tagliati con microtomo (Leica Instruments GmbH) per ottenere sezioni dello spessore di 5-7 μm . I vetrini sono stati colorati con Ematossilina/Eosina col metodo di Mallory.

Crioconservazione

Le cellule sono state congelate a P0 in HG- DMEM con il 50% di FBS ed il 10% di DMSO per 6 mesi a -80°C. Dopo scongelamento alcune cellule sono state usate per lo studio del doubling time, mentre altre cellule sono state espanse fino a P3 per gli studi di immunocitochimica e per valutare l'espressione dei marker specifici di mesenchimalità e di differenziamento multilineare. I risultati sono stati confrontati con quelli delle cellule fresche.

Casi clinici

In questo studio sono stati impiegati 3 cavalli ai quali è stata diagnosticata una lesione spontanea a livello delle strutture teno-legamentose degli arti. Nello specifico sono riportati i seguenti casi: il primo cavallo (caso 1) era affetto da una lesione traumatica acuta del tendine flessore superficiale del dito (SDFI) coinvolgente l'80% della sezione trasversale del tendine (Fig. 10, caso 1 A, B); nel secondo caso (caso 2) è stata individuata una lesione severa di 0,58 cm² nel

medesimo tendine (Fig. 10, caso 2 A, B); il terzo caso era rappresentato da un cavallo con una lesione acuta del legamento accessorio del tendine flessore profondo (AL-DDFT) la cui area danneggiata è stata quantificata in 2,32 cm² (Fig. 10, caso 3 A, B).

Impianto delle MSCs all'interno della struttura teno-legamentosa Previo consenso informato dei proprietari, 1 milione di cellule mesenchimali allogene a P3 diluite in 100 µl di plasma autologo sono state inoculate per via intradermica in alcuni cavalli con anamnesi conosciuta, per valutare eventuali reazioni avverse. L'osservazione è durata 6 mesi. In seguito, nelle lesioni dei tre casi clinici precedentemente descritti, sono state impiantate aliquote di 1 milione di AMC's allogene sempre a P3, diluite in 800 µl di plasma autologo. La procedura prevedeva la tricotomia della regione in cui era presente la lesione, in modo da poter effettuare un'ecografia di controllo prima di procedere all'impianto. I soggetti sono stati sedati con Detomidina (0,01 mg/kg) e successivamente si è proceduto alla preparazione del campo chirurgico previa anestesia tronculare, o cerchiante, prossimale rispetto al sito di lesione impiegando una soluzione di Mepivacaina al 2%. La sonda dell'ecografo è stata inserita all'interno di una guaina sterile o all'interno di un guanto sterile e l'area veniva cosparsa di alcool a 90% per permettere la diffusione delle onde ultrasonore. Le iniezioni sono state eseguite sotto controllo ecografico. I tendini flessori superficiali delle falangi (SDFT) sono stati iniettati con tecnica longitudinale (preferita in questi casi dall'operatore) con un ago 20 gauge e 70 mm, in direzione prossimale-distale. Le lesioni a carico di tutte le altre strutture sono state eseguite con tecnica trasversale usando un ago 21 gauge e 40 mm, inserito lateralmente.

L'arto è stato poi fasciato con cotone garzato sterile e fasce elastiche (Vetrap®). Tale fasciatura è stata mantenuta per 48 h ed i cavalli sono stati confinati in box, prima di consentire 15 minuti di passo a mano per 15 giorni, aumentati a 30 minuti per un medesimo periodo. Non sono stati mai somministrati antibiotici o farmaci anti-infiammatori non steroidei (FANS).

9.3 Results

Raccolta delle membrane amniotiche ed isolamento di AMC's Dalla porzione lavorata di ciascun amnios equino a termine sono state generalmente isolate circa 25x10⁶ di AMC's. La vitalità iniziale è stata pari al 75%. Tutte le cellule isolate sono state seminate e durante la coltura si sono selezionate cellule in base alla loro capacità di aderire alla piastra. Le AMC's hanno mostrato una morfologia fibroblast-like (Fig. 2A). Le colonie cellulari, osservate ai primi stadi di coltura, hanno avuto la capacità di formare cluster (Fig. 2B).

Dopo scongelamento (a P0), la vitalità delle cellule è stata dell'80% per le AMC's che hanno conservato la loro forma fibroblast-like.

Analisi della proliferazione cellulare

Doubling Time

La capacità proliferativa delle AMC's è diminuita a partire dal P8 ($p < 0,05$), ma è stata molto intensa tra P4 e P6. Il valore medio di DT è stato di $1,17 \pm 0,15$ giorni (Fig. 3).

Dopo scongelamento il DT medio osservato è stato di $1,88 \pm 0,51$ giorni.

Analisi delle unità formanti colonie fibroblastoidi Nelle AMC's è stato osservato un incremento statisticamente significativo ($p < 0,05$) della frequenza di CFU-F all'aumentare della densità di semina (Tabella 1). Un esempio di colonia è rappresentato nella Fig. 4.

Differenziamenti in vitro

È stato valutato il potenziale multidifferenziativo delle AMC's (Fig. 5).

Differenziamento osteogenico

Dopo 10 giorni di induzione il differenziamento osteogenico delle AMC's è stato confermato dalla colorazione Von Kossa, che ha evidenziato i depositi di calcio. Le cellule, inoltre, hanno modificato la propria morfologia aumentando anche le proprie dimensioni. Il controllo è risultato, invece, negativo alla colorazione non mostrando matrice mineralizzata. L'analisi dell'espressione dei marker osteogenici *BGLAP* e *OPN* attraverso RT-PCR ha confermato l'induzione osteogenica.

Differenziamento adipogenico

Le AMC's hanno mostrato la capacità di differenziare nella linea adipogenica, come dimostrato dal risultato positivo della colorazione con Red Oil O effettuata dopo 3 settimane di coltura nel terreno adipogenico. Le cellule mantenute nel terreno standard non hanno mostrato depositi lipidici e, quindi, la colorazione è risultata negativa. L'analisi dell'espressione dei marker adipogenici *PPAR- γ* e *ADIPQ* attraverso RT-PCR ha confermato l'induzione adipogenica. Le cellule indotte al differenziamento hanno rivelato un'aumentata espressione di *PPAR- γ* e adiponectina rispetto al controllo. Il tessuto adiposo è stato usato come controllo positivo di espressione dei marker adipogenici.

Differenziamento condrogenico

Le AMC's hanno mostrato la capacità di differenziare nella linea condrogenica, come è risultato dalla colorazione con Alcian Blue. Il controllo ha dato esito negativo alla colorazione, come atteso. L'analisi dell'espressione dei marker condrogenici, *COL2A1* e *ACAN* attraverso RT-PCR ha confermato l'avvenuta induzione. Le cellule indotte al differenziamento hanno mostrato un'aumentata espressione di *COL2A1* e *ACAN* rispetto al controllo, che non ha mostrato

l'espressione dei marker. Il tessuto cartilagineo è stato usato come controllo positivo di espressione dei marker condrogenici.

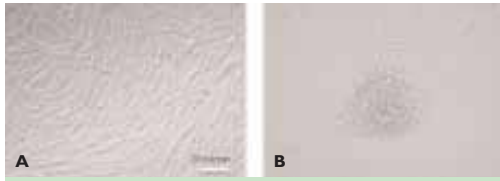


FIGURA 2 - (A) Monostrato di AMCs, 10X e (B) AMCs con un piccolo cluster, 20X. Scale bar 20 microns

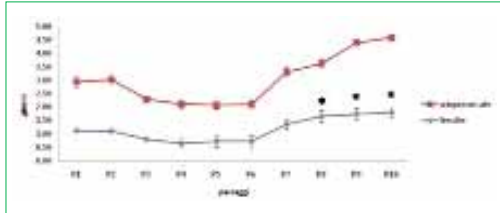


FIGURA 3 - DT a differenti passaggi durante la coltura cellulare delle AMCs. Legenda: asterischi all'interno del grafico indicano differenze statisticamente significative ($P < 0,05$) rispetto a P1 per le cellule fresche.



FIGURA 4 - Colonia cellulare (CFU-F, 20x).

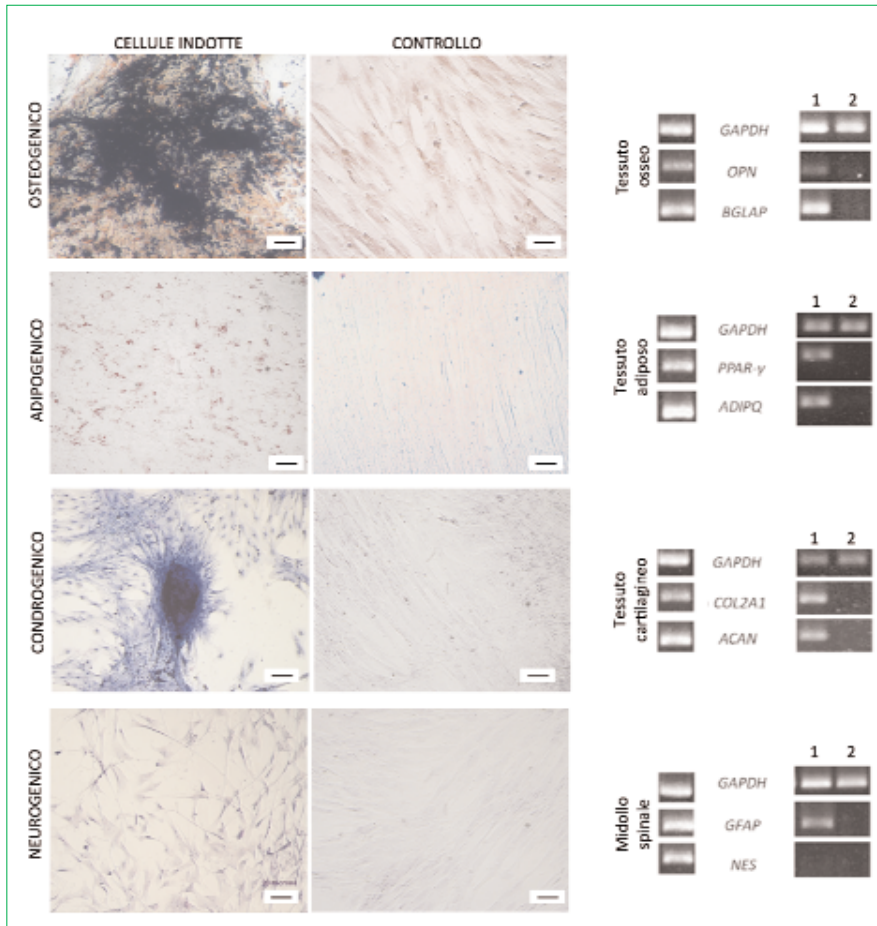


FIGURA 5 - Differenziamenti nelle linee mesodermiche ed ectodermica confermate da colorazioni morfologiche ed espressione genica dei geni specifici delle linee cellulari di induzione.

Differenziamento neurogenico

Dopo 3 giorni di induzione, il differenziamento neurogenico è stato confermato non solo dalla morfologia assunta dalle cellule in piastra, ma anche dalla positività alla colorazione di Nissl. Le AMCs hanno mostrato la tipica morfologia dei neuroni con processi axon-like e dendrite-like e la presenza dei processi primitivi tipici, come quelli osservati nei neuroni. L'espressione del gene *GFAP* ha indicato, però, che in queste condizioni di coltura le cellule amniotiche probabilmente sono state indotte a differenziare in cellule gliali per la mancata espressione della nestina (*NES*).

Il *GAPDH* è stato impiegato come gene riferimento. Le cellule scongelate sono state in grado di differenziarsi verso le stesse linee testate per le cellule fresche isolate (dati non mostrati).

Analisi dei marker di espressione attraverso RT-PCR L'analisi RT-PCR ha permesso di evidenziare, a differenti passaggi (a P1 e P5), la presenza degli RNA messaggeri di marker specifici per le MSCs

(*CD29*, *CD44*, *CD166*, *CD105*) e l'assenza del marker ematopoietico (*CD34*) (Fig. 6). Tuttavia, le cellule hanno cominciato ad esprimere quest'ultimo marker a P5 (dati non mostrati). Le AMCs hanno espresso l'antigene di immunoistocompatibilità I (*MHC-I*) sino a P5, mentre non si è manifestata espressione dell'antigene di immunoistocompatibilità II (*MHC-II*) a P1, che si è espresso al passaggio P5. Il *GAPDH* è stato usato come gene di riferimento. Nei campioni congelati è stata osservata una ridotta espressione del *CD105* rispetto alle cellule fresche e l'assenza di espressione del *CD166* (Fig. 6).

Immunocitochimica

È stata indagata l'espressione dei marker di pluripotenza: Oct-4, SSEA-4 e TRA 1-60. Le AMCs hanno rivelato gli antigeni studiati, come mostrato nella Fig. 7.

Oct-4 è espresso nel citoplasma e nel nucleo mentre SSEA-4 e TRA 1-60 sono espressi sulla superficie cellulare come riportato in Fig. 7. L'espressione di TRA 1-60 è risultata molto debole. La presenza combinata degli antigeni Oct-4, SSEA-4 e TRA 1-60 attribuisce alle cellule amniotiche un fenotipo primitivo.

Gli stessi risultati sono stati ottenuti dopo scongelamento (dati non mostrati).

Istologia dell'allantoamnios

L'amnios e l'allantoide delle membrane fetali a termine sono caratterizzati ciascuno da uno strato epiteliale e da uno strato mesenchimale (Fig. 8). L'amnios è un unico tessuto privo di vasi sanguigni, costituito da cellule epiteliali cuboidali nello strato epiteliale (Fig. 9A) e da cellule stromali nello strato mesenchimale (Fig. 9B). Le cellule amniotiche epiteliali, sul versante esterno dell'amnios, sono a diretto contatto con il liquido amniotico.

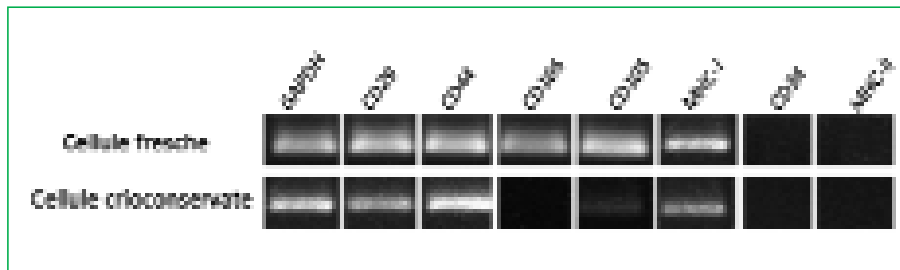


FIGURA 6 - Analisi con RT-PCR per l'espressione di specifici marker di mesenchimalità (*CD105*, *CD29*, *CD166*, *CD44* e *CD34*) e di immunogenicità (*MHC-I* e *MHC-II*) su AMCs prima e dopo crioconservazione. Il GAPDH è stato usato come gene di riferimento.



FIGURA 7 - Presenza degli antigeni associati alla pluripotenza (Oct-4, SSEA-4 e TRA-1-60) nelle cellule mesenchimali amniotiche equine. 20X. Scala: 20 micron.

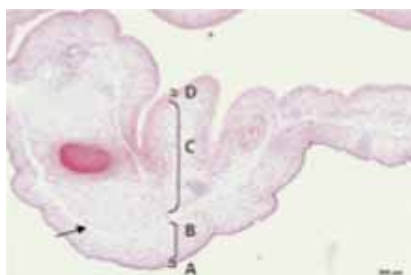


FIGURA 8 - Morfologia delle membrane fetali equine a termine (colorazione con ematossilina-eosina). (A) foglietto amniotico epiteliale; (B) foglietto amniotico mesenchimale; (C) foglietto allantoideo mesenchimale; (D) foglietto allantoideo epiteliale. La regione di contatto tra amnios e allantoide è mostrata dalla freccia. Si può osservare la presenza di un vaso sanguigno nella parte allantoidea della membrana.

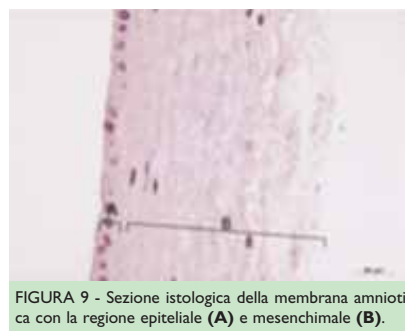


FIGURA 9 - Sezione istologica della membrana amniotica con la regione epiteliale (A) e mesenchimale (B).

Impianto delle MSCs nella struttura teno-legamentosa A seguito delle iniezioni intradermiche, effettuate per valutare eventuali reazioni avverse, non si sono verificate alterazioni, quindi, dopo questa fase si è proceduto agli impianti intralesionali. I soggetti hanno ben tollerato gli impianti intralesionali di cellule mesenchimali allogeniche isolate da amnios equino. Non si sono verificate reazioni

algiche o infiammatorie. Si è verificata, inoltre, già dai primi giorni, una sostanziale riduzione di volume, una diminuita algia alla palpazione del tratto interessato ed una significativa riduzione del grado di zoppia. Ad un primo controllo ecografico a 15 giorni si è evidenziata, in tutti i casi, una precoce riduzione dell'area totale di sezione (T-CSA). Specificatamente, nel caso 1 l'area anecoica rappresentativa della lesione è migliorata marcatamente. L'aspetto ecogenico e l'architettura tissutale nella sezione longitudinale dell'ecografia rivelano un chiaro processo di evoluzione dopo un periodo di 70 giorni (Fig. 10, caso 1 C, D).

Nel secondo caso la lesione anecoica che misurava 0,58 cm² si è ridotta in un'area ipoecoica di 0,18 cm² unitamente ad un aspetto soddisfacente dell'architettura delle fibre in scansione longitudinale dopo i primi 60 giorni (Fig. 10, caso 2 C, D). Nel terzo caso un'acuta e severa lesione di 2,32 cm² del legamento accessorio del tendine flessore profondo delle falangi (AL-DDFT) presentava un aspetto ecografico strutturato ed organizzato a circa 60 giorni (Fig. 10, caso 3 C, D).

Inoltre, un follow up è stato condotto, dopo la ripresa dell'attività agonistica, in tutti i casi fino a 12 mesi dopo il trattamento e non sono state osservate recidive.

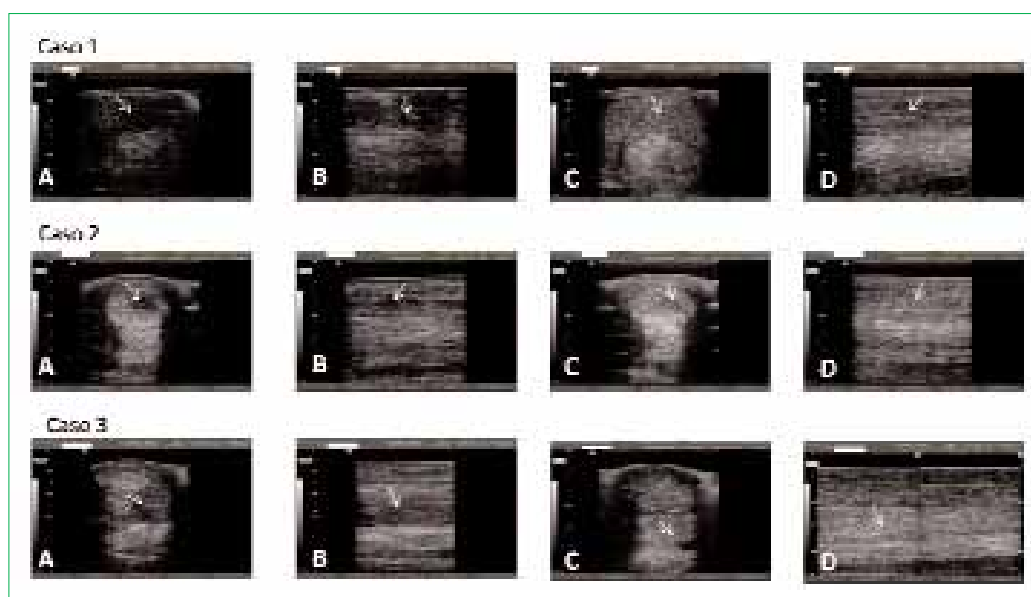


FIGURA 10 - Tre casi di lesioni tendinee prima e dopo trapianto di cellule mesenchimali allogeneiche di derivazione amniotica.
 Caso 1: lesione traumatica acuta del tendine flessore superficiale del dito (SDFT). Le frecce mostrano un'ampia area anecogena nell'ecografia in sezione trasversale (A) e longitudinale (B). Le frecce in C e D mostrano un'omogenea area ecogenica rispettivamente nell'immagine ecografica trasversale e longitudinale nella stessa lesione dopo 50 giorni dall'impianto.
 Caso 2: lesione severa del SDFT. Le frecce indicano un'area anecoica nell'ecografia in sezione trasversale (A) e longitudinale (B), ed una leggera area ipoecoica rispettivamente nella sezione trasversale (C) e longitudinale (D) della stessa lesione dopo 50 giorni dall'impianto.
 Caso 3: Lesione acuta del legamento accessorio del tendine flessore profondo (AL-DDFT). Le frecce mostrano un'area ipoecoica nell'ecografia in sezione trasversale (A) e longitudinale (B) e un'area ecogenica in sezione trasversale (C) e longitudinale (D) rilevabile dopo 50 giorni dall'impianto.



FIGURA 1 - La membrana amniotica è separata meccanicamente dalla sovrastante membrana allantoidea. La freccia indica il punto di sovrapposizione delle membrane.

TABELLA 1
CFU-F per le AMC a P0

Densità cell/cm ²	CFU-F	I CFU-F ogni
100	1,33±0,58	712,50 ^a
250	20,33±2,52	116,80 ^b
500	59,67±4,93	79,61 ^c
1000	74,67±2,52	127,23 ^d

Legenda: lettere diverse all'interno della stessa colonna indicano differenze statisticamente significative per P<0,05.

9.4 Discussion

Terapie basate sul trapianto cellulare sono emerse come un nuovo potenziale approccio negli ultimi anni in svariati settori della medicina rigenerativa. Le MSCs possono essere isolate da molteplici tessuti e possiedono proprietà differenti, in base alla fonte di origine ed al loro grado di differenziamento. Le MSCs ottenute dal BM sono le più studiate e conosciute, come dimostrano i lavori scientifici dell'ultimo decennio (Digirolamo et al., 1999; Gronthos et al., 2003; Vidal et al., 2011). Per tale motivo, esse sono anche le più utilizzate per la terapia rigenerativa tissutale ed, in particolare, in medicina veterinaria, per trattare le patologie dell'apparato muscolo-scheletrico e del sistema ematopoietico.

Le problematiche relative al prelievo ed alle caratteristiche delle cellule mesenchimali isolate da BM hanno portato, però, alla necessità di individuare nuove fonti di cellule staminali.

La recente scoperta in campo umano dell'esistenza di MSCs in annessi fetali, di facile accesso e privi di controversie etico-morali, rende queste cellule interessanti agli occhi dell'intera comunità scientifica e questo vale soprattutto per il fatto che questa popolazione cellulare si colloca in una posizione ontogenetica intermedia tra le ESCs e le cellule staminali adulte (Bajada *et al.*, 2008; Siegel *et al.*, 2007). Infatti, le cellule isolate da queste fonti, in campo umano, sono caratterizzate da proliferazione più rapida, una maggiore espansione *in vitro*, dall'espressione dell'enzima telomerasi (Mitchell *et al.*, 2003) e da un notevole potenziale differenziativo, rispecchiando, quindi, i criteri di staminalità definiti dal Comitato delle cellule staminali.

È con questi obiettivi che è stato affrontato questo studio, nel quale, per la prima volta nella specie equina, la membrana amniotica a termine, che solitamente è scartata ed è di facile raccolta, è stata da noi considerata come una fonte alternativa di cellule con proprietà staminali in campo medico-veterinario. In medicina veterinaria, la membrana amniotica è usata in terapia clinica per la ricostruzione della superficie oculare come membrana *in toto*, ma a parte quest'uso non sono mai state ottenute cellule amniotiche dall'amnios equino per possibile uso terapeutico. In seguito a digestione enzimatica del tessuto amniotico, sono state isolate cellule amniotiche equine di natura mesenchimale che sono state espanse in coltura per 10 passaggi, sono state caratterizzate, differenziate e, dopo inoculo nei siti di lesione, valutate per la loro potenzialità rigenerativa. Essendo questa una prima indagine conoscitiva su quest'invoglio fetale, è stato effettuato uno studio dettagliato relativo alla morfologia di questa membrana ed alla caratterizzazione delle sue cellule.

Dallo studio istologico è emerso che la membrana amniotica equina è composta da uno strato epiteliale e da uno stroma avascolare nel quale è presente una rete di cellule mesenchimali fibroblastoidi. Nelle nostre condizioni, dopo digestione, la vitalità iniziale delle cellule isolate è stata più alta del 75%, che è considerata un

ottimo valore della qualità a lungo termine di queste cellule sia in vista dell'efficienza di semina sia per la crescita cellulare. Durante le fasi di coltura, le cellule che sono rimaste adese alle piastre sono state espanse fino al passaggio P10 ed i nostri risultati hanno dimostrato la presenza di un gruppo di cellule capaci di aderire tenacemente al substrato colturale e di proliferare intensamente *in vitro*.

Le cellule amniotiche equine, in generale, hanno dimostrato un'alta capacità proliferativa fino al 6°- 8° passaggio. Dopo questo periodo, la proliferazione è diminuita, anche se il DT si è attestato, comunque, a valori non superiori alle 48 ore. Comunque, una robusta proliferazione fino al passaggio P6 è stata riportata anche da altri autori per le cellule amniotiche umane (Soncini *et al.*, 2007; Miki *et al.*, 2010).

Le AMC's mantenute in coltura in alta densità hanno sviluppato strutture sferoidali senza mostrare inibizione da contatto. Queste strutture sferoidali si sono sviluppate tridimensionalmente al di sopra del monostrato e sono state descritte nella coltura delle ESCs (Miki e Strom, 2006) ed individuate anche da Carlin *et al.*, (2006) nella coltura delle cellule della matrice del cordone di suino. Miki *et al.* (2005) hanno suggerito che il monostrato di cellule amniotiche, adese alla piastra di coltura, potrebbe giocare il ruolo di monostrato cellulare di sostegno autologo servendo come substrato per l'adesione del cluster o per fornire fattori che potrebbero indurre o mantenere indifferenziate le cellule amniotiche che costituiscono le strutture sferoidali.

Le cellule amniotiche *in vitro* hanno mostrato anche l'abilità di formare cloni, popolazioni omogenee di cellule generate da un unico capostipite. Questa caratteristica, definita *clonogenicità*, è un attributo essenziale delle cellule staminali le quali, contrariamente a quanto avviene con cellule differenziate, sono in grado di ripristinare in tempi brevi una popolazione di cellule uguali tra loro anche quando seminate a diluizioni estremamente elevate. La frequenza di formazione delle CFU-F è aumentata con l'incrementare della densità di semina indicando l'esistenza di qualche segnale paracrino, fra le cellule amniotiche, che avrebbe potenziato la formazione delle CFU-F nella coltura primaria a P0 (Sarugaser *et al.*, 2005).

Le cellule amniotiche equine hanno rivelato il caratteristico modello di espressione degli antigeni delle MSCs espanse in coltura. L'analisi è stata eseguita mediante RT-PCR basata su un pannello di primer oligonucleotidici specificatamente disegnati sulla sequenza genica di questa specie. Il pannello di marker realizzato rappresenta un utile strumento nella ricerca delle cellule staminali equine, poiché molti anticorpi, marker di staminalità descritti nelle altre specie, mostrano poca o nulla reattività nella specie equina, rendendo così difficoltoso il loro utilizzo in questo campo. Queste cellule hanno manifestato positività ai più comuni marker che definiscono le MSCs: *CD29*, *CD44*, *CD105*, *CD166* e *MHC-I* ma non *MHC-II*. La natura non ematopoietica delle cellule staminali è stata suggerita dalla mancata espressione del marker *CD34* che ha iniziato ad esprimersi a P5. Quest'ultimo dato

potrebbe essere un'evidenza che le cellule amniotiche hanno un potenziale angiogenico così come è stato riportato in medicina umana da Alviano *et al.* (2007). A P5 anche il marker *MHC-II* ha iniziato ad essere espresso mentre il marker *CD105* non era più espresso. La scarsa stabilità dei marker di superficie potrebbe indicare fenomeni epigenetici associati con la coltura cellulare che potrebbero influenzare le presunte cellule staminali amniotiche. Ulteriori caratterizzazioni di queste cellule saranno necessarie per comprendere meglio questi cambiamenti.

È importante sottolineare come l'assenza di espressione del marker *MHC-II*, almeno fino al quinto passaggio, renda queste cellule ipo-immunogene e, quindi, possibilmente utilizzabili nei passaggi precoci per terapie cellulari di tipo allogenico. In aggiunta ai caratteristici marker di superficie, le cellule amniotiche hanno mostrato immunopositività a Oct-4, TRA-1-60 e SSEA-4. Questi marker sono tipici delle ESCs, ma rilevati anche in altre popolazioni cellulari derivanti da annessi fetali quali il cordone ombelicale (Carlin *et al.*, 2006; Hoynowsky *et al.*, 2007), la membrana amniotica ed il liquido amniotico umani (Miki *et al.*, 2005; Portmann-Lanz *et al.*, 2006; De Coppi *et al.*, 2007; Ilancheran *et al.*, 2007; Kim *et al.*, 2007; Wolbank *et al.*, 2007). È stato ipotizzato che la presenza di popolazioni cellulari con tali caratteristiche possa essere messa in relazione ad uno stato di "primitività" del tessuto stesso e ad uno stadio cellulare privo di differenziamento, ma caratterizzato da pluripotenza (Miki *et al.*, 2005; Portmann-Lanz *et al.*, 2006; De Coppi *et al.*, 2007; Kim *et al.*, 2007; Ilancheran *et al.*, 2007; Wolbank *et al.*, 2007). Le nostre positività potrebbero ricalcare queste ipotesi. Lo studio del differenziamento *in vitro* suggerisce che queste cellule hanno fenotipo pluripotente, come supportato dalle differenziazioni nelle linee mesodermica ed ectodermica. In particolare, da questo lavoro emerge la capacità di queste cellule amniotiche di differenziarsi nelle linee osteogenica, adipogenica e condrogenica, come riportato da In't Anker *et al.* (2004) per l'amnios umano, ma anche in cellule della linea gliale come mostrato da Miki *et al.* (2005) sempre in campo umano, dove il 95% delle cellule stromali amniotiche ha espresso immunolocalizzazione di *GFAP*. Osservando i dati relativi al potenziale proliferativo e differenziativo delle cellule amniotiche mesenchimali, si può pensare che questa membrana possa offrire vantaggi come una straordinaria fonte di presunte cellule staminali disponibili per i futuri sforzi nell'ambito della terapia cellulare. È per queste caratteristiche che ci è sembrato di notevole interesse un primo studio di queste cellule *in vivo*, per capirne gli eventuali meccanismi riparativi. Gli effetti benefici di queste cellule in questo test iniziale sottolineano il loro potenziale applicativo nella medicina rigenerativa veterinaria.

Attraverso studi di trapianto cellulare *in vivo*, abbiamo osservato che il trapianto allologo di cellule mesenchimali derivanti da amnios è ben tollerato dal cavallo e che tutti i dati clinici (riduzione dello spessore nelle dimensioni del tendine; sensibilità alla palpazione, misure delle aree delle sezioni trasversali nell'ecografia) forniscono completa evidenza nel supportare l'azione benefica delle cellule

iniettate. L'evoluzione ecografica riportata per l'architettura di tendini e legamenti è simile a quella che è stata precedentemente riportata dopo applicazione di altre colture cellulari multipotenti mesenchimali ed autologhe (Richardson *et al.*, 2007). La possibilità di inoculo terapeutico immediato nelle lesioni di cellule che sono prontamente disponibili prima che ogni cambiamento ultrastrutturale avvenga, e l'effetto plastico dimostrato da queste cellule, rappresentano i maggiori aspetti di interesse di questo nuovo approccio biotecnologico nel campo delle tendinopatie nei cavalli. Ovviamente, questo studio non è stato un trial pre-clinico, ma un'analisi di scoperte preliminari ottenute con le cellule di derivazione amniotica nella specie equina. Inoltre, un punto debole di questo studio è la mancanza di un gruppo di controllo e di una valutazione istologica, quindi, ulteriori studi applicati ad un maggior numero di animali saranno necessari per confermare questi risultati. Infine, abbiamo dimostrato che le cellule amniotiche equine possono essere congelate, conservate e recuperate senza la perdita della loro integrità funzionale in termini di morfologia, presenza di specifici marker e potenziale differenziativo, sebbene la capacità proliferativa sia stata più bassa di quella osservata nelle cellule fresche. Da questo punto di vista i protocolli di crioconservazione dovrebbero essere migliorati al fine di permettere la creazione di un servizio di banca cellulare.

9.6 Conclusions

Questo studio è il primo a documentare le caratteristiche di presunte cellule staminali isolate dalla membrana amniotica equina ed il loro uso *in vivo*. Questi risultati ci conducono a sviluppare protocolli d'isolamento che possano essere utili in futuro per ottimizzare la raccolta e l'espansione di queste cellule ai fini del loro utilizzo e ad ipotizzare la costituzione di riserve crio-preserved utilizzabili in medicina rigenerativa.

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CHAPTER 10

Appendix II

Reconstruction of calf oocytes by germinal vesicle transfer in mature bovine oocytes: preliminary results

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10. Reconstruction of calf oocytes by germinal vesicle transfer in mature bovine oocytes: preliminary results

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10.1 Introduction

The employment of pre-puberal animals as oocyte donors gives the potential to shorten the generation interval and raise the genetic improvement rate by means of embryo transfer reproductive technology. Several studies in this field have shown that prepuberal oocytes can resume and complete the meiotic process after in vitro maturation (IVM) but lack competence to develop into normal embryos due to incomplete or retarded cytoplasmic maturation (Salamone et al. 2001). The ooplasm, indeed, plays a key role in the development of early bovine embryos as is evident in the biochemical discrepancies between the ooplasm of calf and adult oocytes: the altered synthesis and the different phosphorylation status of calf oocyte protein kinases can be considered as an example of the cytoplasmic dysfunction of these young oocytes (Taneja et al. 2000). Among the several options developed to allow the recovery of non-competent oocytes, the cytoplasmic transfer from in vitro and/or in vivo matured oocytes to a non-competent oocyte, has been proposed. This integration can be performed either by transferring small volumes of cytoplasm or by electro-fusion of two demi-oocytes, one bearing the carioplasm and the other the ooplasm, but, based on these techniques, no more than 50% of competent cytoplasm can be integrated. A further approach can be the replacement of the whole cytoplasm by means of germinal vesicle (GV) transfer: this procedure allows the GV of a non-competent oocyte to mature and terminate the first meiotic cycle within an ooplasm of a competent oocyte. In this work we perform the reconstruction of calf oocyte through its germinal vesicle transfer into the ooplasm of an adult bovine oocyte matured in vitro. The reconstructed oocyte is then subjected to the further steps of in vitro fertilization (IVF) and in vitro embryo culture (IVC).

10.2 Materials and methods

Retrival of GV stage oocytes and in vitro matured oocytes. This procedure is based on the use of two distinct oocyte populations: adult bovine oocytes collected after slaughtering and assigned to IVM for 24 hours and calf oocytes collected at the GV stage. Four week old donor calves underwent a four day ovarian stimulation protocol by gonadotropin administration and oocytes were retrieved by laparotomy under general anesthesia. Oocyte collections from calves were performed on the day when adult oocytes ended the IVM step. The mean collection rate (ratio between the number of aspirated follicles and the number of oocytes collected) was 87%. For the micromanipulation processing, both oocyte populations were decumulated by ialuronidase treatment (300 IU/ml) before germinal transfer could be performed.

GV transfer and calf oocyte reconstruction. GV stage calf oocytes and adult bovine oocytes at metaphase II stage were treated with cytochalasin and Hoechst 33342 (5 μ g/ml), in order to make the plasma membrane more suitable to be micromanipulated and to locate the nucleus. The GV of each calf oocyte was transferred into the perivitelline space of an in vitro matured and enucleated adult bovine oocyte (Fig. 1). The fusion between the carioplasts and the ooplasm was performed in an electrofusion chamber in a suitable medium (0.3 M Mannitol, 0.1 mM CaCl₂ and 0.05 mM MgSO₄ in H₂O) with a double pulse (2KV/cm DC for 30 μ s at 0.1 sec intervals).

Maturation, fertilization and embryo culture. After fusing, the reconstructed oocytes at the GV stage were allocated to IVM for 18–20 hours with the aim of completing the first meiotic division (Fig. 2). Afterwards, upon attainment of the metaphase II stage, the reconstructed oocytes were assigned to IVF and IVC (Fig. 3).

Fig. 1 Calf oocyte following GV transfer. Original magnification 40×

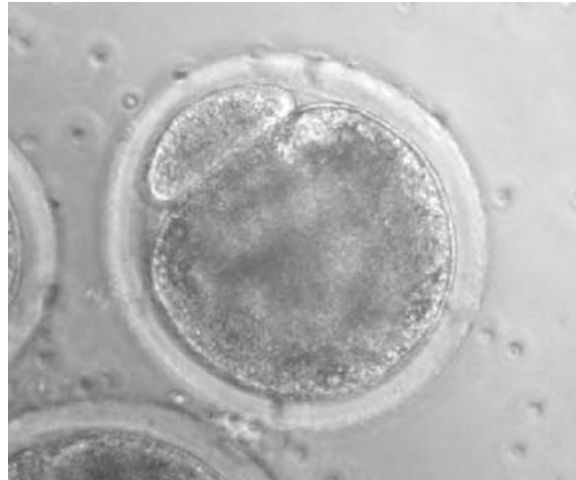
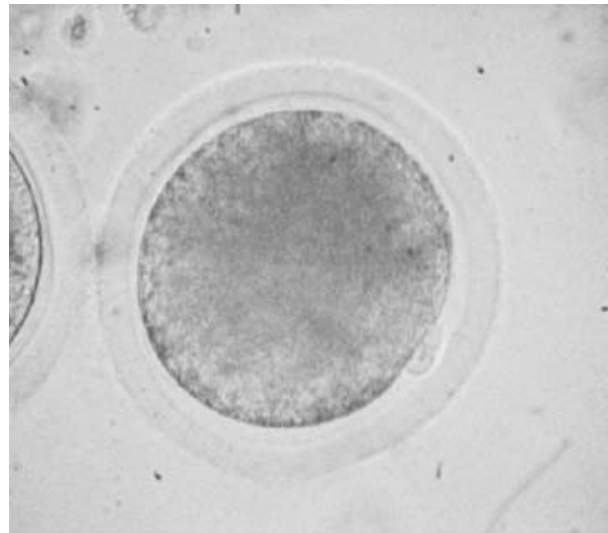


Fig. 2 Reconstructed calf oocyte showing a polar body after attainment of first meiotic division. Original magnification 40×



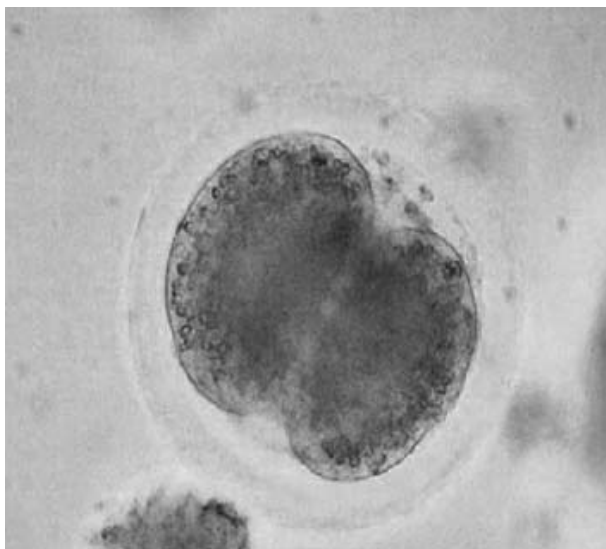
Parthenogenesis and IVM assessment of prepuberal oocytes. To set up the electrofusing technique, oocytes were reconstructed, as described above, starting from ooplasm and GVs collected from adult bovine oocytes which underwent all the IVM/IVF/IVC steps in order to study some of the micromanipulation parameters such as the partenogenetic activation and the polyspermic rate after IVF. A further batch of prepuberal oocytes, assigned to IVM just after collection, served as the control group.

10.3 Results

In the present study the percentage of oocytes reconstructed after GV transfer and electrofusion was 76.34%. The maturation rate of the reconstructed prepuberal oocytes was 56%, the same as that observed in the case of the reconstructed adult

oocytes, but significantly different ($p \leq 0.05$) from the maturation rate of the control adult oocytes (80%) and of the control prepuberal oocytes (37%). The cleavage rate observed for both reconstructed oocyte populations was 40% and lower than that reported, on average, for adult bovine oocytes (60%). Neither parthenogenetic or polyspermic events were detected.

Fig. 3 Two cell embryo from in vitro fertilization of reconstructed calf oocyte. Original magnification 40×



10.4 Discussion

Several, albeit differing data concerning the maturation and cleavage rates of calf oocytes, relatively to the donor age and to the experimental conditions adopted, are present in the scientific literature. In this preliminary work we demonstrated that our nuclear transfer technique is effective, that calf oocytes that have undergone GV transfer mature in vitro and can be fertilized, even if the maturation and the cleavage rates are still lower than those of adult bovine oocytes. A reason for this could be the lack of the selection of the calf oocytes used in the juvenile IVM/IVF/IVC program as almost all of the oocytes collected, excluding the damaged ones, were used regardless of their quality, due to the scarcity and value of the oocytes retrievable from the calves. Another reason could be the absence of contact with the cumulus cells, necessary elements either for the maturation or the competence of embryonic development, that the oocytes are removed from by the micromanipulation step.

Our data are of interest for investigating the relationship between the ooplasm and carioplasm following electrofusion in view of the production of embryos from calves by standard in vitro procedures and could represent a way to enhance, in vitro, the competence toward embryo development of juvenile calf oocytes.

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10.7 References

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