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CICLO XXV

RELATIONSHIP BETWEEN AOPP (Advanced Oxidation Protein Products) AND BOVINE NEUTROPHILS "IN VITRO": AOPP PRODUCTION BY NEUTROPHILS AND AOPP EFFECTS ON NEUTROPHILS ROS PRODUCTION AND VIABILITY

Direttore della Scuola: Ch.mo Prof. Gianfranco Gabai

Supervisore: Ch.mo Prof. Gianfranco Gabai

Dottorando: Milena Bordignon

Riassunto

Lo scopo del presente studio è stato quello di valutare le relazioni tra AOPP (prodotti avanzati di ossidazione proteica) e i neutrofili di bovino "in vitro". A questo scopo le AOPP sono state generate "in vitro", ossidando l'albumina sierica bovina con HOCl (acido ipocloroso) mentre i neutrofili di bovino sono stati isolati da sangue intero di bovine da latte. Le AOPP-BSA sono state incubate con i neutrofili di bovino appena isolati in condizioni di assenza di stimolo o stimolati con PMA un forte attivatore del "burst" respiratorio. La produzione di ROS da parte dei neutrofili e la loro vitalità, sono state misurate rispettivamente mediante chemiluminescenza amplificata dal luminolo e dai saggi MTT e lattato deidrogenasi (LDH). I risultati ottenuti hanno mostrato che le AOPP-BSA sono in grado di ridurre significativamente la produzione di ROS da parte dei neutrofili stimolati con PMA e la loro vitalità, misurata con il saggio MTT mentre non è stata rilevata lisi cellulare mediante saggio LDH. Sulla base di questi risultati il presente lavoro si è proposto di studiare se le AOPP sono in grado di scatenare eventi apopotici. A questo scopo le caspasi 3, 8 , 9 e la frammentazione del DNA sono stati utilizzati come marker con l'obiettivo di discriminare tra la via intrinseca e quella estrinseca di apoptosi. I risultati ottenuti hanno mostrato che i neutrofili di bovino non stimolati e incubati con AOPP-BSA per 1 ora e 6 ore, presentano una maggiore ma non significativa produzione di caspasi 8 attiva, se comparati con l'incubazione con BSA. Anche la caspasi 3 mostra un incremento, non significativo in neutrofili non stimolati incubati con AOPP-BSA per 6 ore, rispetto all'incubazione con BSA. Non è stata ottenuta alcuna differenza per quanto riguarda la caspasi 9 e la frammentazione del DNA. Tuttavia, in queste condizioni sperimentali è possibile concludere che la via intrinseca dell'apoptosi non è coinvolta nella riduzione della funzionalità dei neutrofili di bovino o nella loro vitalità ma i neutrofili di bovino incubati con AOPP-BSA sembrano piuttosto essere "accompagnati" verso le fasi precoci della via estrinseca dell'apoptosi.

Inoltre, il seguente studio ha voluto valutare la capacità dei neutrofili di bovino attivati di generare AOPP "in vitro". La BSA è stata incubata con neutrofili di bovino non stimolati e stimolati con PMA per 1-2-3 ore, ed è stata misurata la

formazione di specifici marcatori di ossidazione proteica come le AOPP le ditirosine e i carbonili. La BSA incubata con neutrofili stimolati con PMA, presenta un livello significativamente alto di AOPP e ditirosine rispetto aill'incubazione con neutrofili non stimolati. I carbonili invece sembrano non essere prodotti in queste condizioni, almeno nelle fasi inziali dell'incubazione. In parallelo, la BSA incubata con la stessa concentrazione di HOCl prodotta dai neutrofili stimolati, per 1-2-3 ore, presenta livelli più elevato di AOPP, ditirosine e carbonili. Tuttavia è possibile concludere che i neutrofili di bovino sono in grado di ossidare la BSA e generare modificazioni chimiche e strutturali come AOPP e ditirosine nelle condizioni sperimentate. I carbonili invece sembrano non essere un marcatore specifico di ossidazione proteica mediata dai neutrofili. In aggiunta la diretta esposizione della BSA all'HOCl non è in grado di mimare completamente la complessità degli eventi che portano all'ossidazione della BSA e alla produzione di AOPP da parte dei neutrofili attivati.

Abstract

The aim of the present study was to evaluate the relationship between AOPP (Advanced oxidation protein products) and bovine neutrophils "in vitro". For this.purpose AOPP were produced "in vitro" by oxidizing bovine serum albumin with HOCl (hypochlorous acid) and bovine neutrophils were isolated from whole blood of dairy cattles. AOPP-BSA were incubated with freshly isolated bovine neutrophils, unstimulated and stimulated with PMA a strong activator of the respiratory burst. Neutrophils ROS production and viability were measured by luminol-amplified chemiluminescence and by MTT and lactate dehydrogenase assays (LDH), respectively. Results obtained have shown that AOPP-BSA are able to reduce significantly ROS production of PMA stimulated neutrophil and their viability measured by MTT assay, no cell lysis was detected by LDH assay. On the basis of these results, our work has studied if AOPP are able to trigger apoptotic events. For this purpose, caspase 8-9-3 and DNA laddering were used as markers in order to discriminate between the "intrinsic" and the "extrinsic" pathway of apoptosis. The results obtained showed that un-stimulated bovine neutrophils incubated with AOPP-BSA show a higher but not

significant production of active caspase 8 in comparison with the incubation with BSA. Also caspase 3 display an increase, but not significant, in un-stimulated neutrophils after 6 hours of incubation with AOPP-BSA, respects the incubation with BSA. No differences were obtained for caspase 9 and for DNA laddering. Therefore, in these experimental conditions is possible to conclude that the "intrinsic" pathway of apoptosis was not involved in the reduced functionality of neutrophils or in their reduced viability, but bovine neutrophils incubated with AOPP-BSA seem to be "accompanied" to the early phases of the "extrinsic" pathways of apoptosis.

In addition, the present work wanted to evaluate the capacity of triggered neutrophils to generate AOPP "in vitro". BSA was incubated with un-stimulated and PMA-stimulated bovine neutrophils for 1-2-3 hours and the production of specific markers of protein oxidation such as AOPP, dityrosines and carbonyls was assessed. BSA incubated with stimulated neutrophils presents a significant higher level of AOPP and dityrosines respects the incubation with un-stimulated neutrophils. Carbonyls don't seem to be produced in these condition, at least at the beginning of the incubation. In parallel, BSA incubated with the same concentration of HOCl produced by PMA-stimulated neutrophils, for 1-2-3 hours, presents a higher level of AOPP, dityrosines and carbonyls. Therefore, it's possible to conclude that bovine neutrophils are able to oxidize BSA "in vitro" and generate chemical and structural modification such as AOPP and dityrosines, in the experimental condition used. However, carbonyls seem to be a non-specific indicator of neutrophils-mediated protein oxidation. The direct exposure of BSA to HOCl couldn't fully mimic the complex events leading to BSA oxidation and AOPP production by activated neutrophils.

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CHAPTER 1.

Preface and purpose

An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed "oxidative stress". Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathological conditions (Sies 1997). During acute infections or inflammations, activated neutrophils become the major source of ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radical produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and hypochlorous acid (HOCl) by myeloperoxidase (Iwao et al. 2006). In this setting, the oxidant scavenging potential of plasma components is likely to be overwhelmed as evidenced by a decrease in ascorbic acid, superoxide dismutase and the glutathione system (Witko-Sarsat et al. 1999). The resulting oxidative stress could be an important event in the pathogenesis of atherosclerosis (Steinberg et al. 1989); thrombogenesis processes (Azizova, et al. 2007); diabetes (Krapfenbauer et al. 1998); rheumatoid arthritis (Lunec et al. 1985); neurodegenerative diseases, such as alzheimer's disease (Kato et al. 1998); chronic renal disease, (Witko-Sarsat et al. 1998). Amino acids, peptides, and proteins are vulnerable to attacks by a variety of free radicals and related oxidants (Wolf et al. 1986, Davies 1987). Oxidation of particularly sensitive amino acid residues provokes aggregation, cross-linking, fragmentation, as well as loss of enzymatic or other functional properties (Capeillere-Blandin et al. 2004). In the search for specific markers of protein oxidation, has been recently described a novel family of oxidized protein compounds, which has been designated advanced oxidation protein products (AOPP) (Davies 1987). AOPP contain abundantly chloramines, dityrosines and carbonyl groups, and are formed mainly by chlorinated oxidants resulting from the myeloperoxidase activity of activated neutrophils (Capeillere-Blandin et al. 2004). In humans, the generation of AOPP could also be obtained "in vitro" by exposing serum albumin, the mayor plasma protein target of oxidative stress, to various oxidants, among which HOCl was the most powerful compound (Himmelfarb et al. 2001). Oxidative stress and protein oxidation seem to have a role in the cause and progression of several reproductive events both in humans and in animals. Radical mediated protein oxidation is responsible for reduced fertilization,

oocyte functions, embryo implantation and embryo viability in women (Guerin et al. 2001, Ruder et al. 2008). Reproductive failure carries significant societal and financial consequences. In the last few years, the detection and the explanation of free radical damage has become increasingly important both in Human and in Veterinary Medicine. The modern dairy cow is unique in her experience of repeated lifetime cycles of pregnancy and parturition, followed by lengthy lactation producing high volume of milk. Maximizing reproductive efficiency is the goal of reproductive management programs in dairy. Embryonic mortality represents a major limitation in dairy system, in particular late embryonic mortality that occurs between days 24 and 42 after artificial insemination, accounts for 5-10% of pregnancy failure. Recent studies have observed that there is a correlation between plasma AOPP levels and embryonic mortality in dairy cattle. In particular, the ration of AOPP:albumin, was significantly greater in embryonic mortality than both animals with and without pregnancy, at days 15, 28, 45 and 60 after insemination. These observations suggest that oxidative stress is implicated in late embryonic mortality, and the increase in plasma AOPP in cows with embryonic mortality might also be interpreted as an indication of sub-clinical uterine infection (Celi et al. 2011). Indeed the development of sub-clinical endometritis was observed in 42,8% of cows 4 hours following artificial insemination (Kaufmann et al. 2009). Neutrophils represent a uterine physical defence against bacterial infections. Leucocytes activation related to the inflammatory events can, in turn, increase ROS generation and blood vessel permeability such that serum may leak into the endometrial surface. (Singh et al. 2008). The cause-effect relationship between AOPP production and neutrophils is not fully understood. The capacity of activated neutrophils to oxidize proteins has been demonstrated by "in vitro" oxidation of proteins (serum albumin) and free amino-acids (Methionine – Lysine – Taurine) directly with HOCl (Hawkins et al. 1998, Beal et al. 2009) or with a myeloperoxidase-H_2O_2 system (Heinecke 2002). AOPPs are also clearly able to trigger human neutrophils and monocyte "in vitro" (Witko-Sarsat et al. 2003). The observation that AOPPs are closely related to neutrophil activation

and are involved in pathological events raised the interest in the biological role of AOPP in dairy cattle.

Therefore, in this PhD thesis were analysed:

1. The effects of AOPPs on bovine neutrophils. AOPPs were produced "in vitro" by oxidizing BSA with HOCl, and then it was incubated with either activated and not activated neutrophils. The production of ROS was detected by luminol-amplified chemiluminescence over a 3 hours period, cell viability was measured by LDH and MTT assays and apoptosis was tested determining DNA laddering and caspases 8-9-3 production.

2. The capacity of triggered neutrophils to generate AOPPs from non-oxidized BSA. Stimulated neutrophils were incubated with non-oxidized BSA for 1-2-3 hours. The formation of chloramines and dityrosines was measured spectrophotometrically. Carbonyls generation was detected by Western Blot after a derivatization of the protein with dinitrophenylhydrazine.

CHAPTER 2.

Introduction

2.1 Innate immunity and inflammation

Vertebrates are transiently or constitutively colonized by a variety of microorganisms that can engage in mutualistic or antagonistic interactions with their hosts. The nature of these interactions is still poorly understood, although recent studies have begun to elucidate the host receptors and signaling pathways involved in sensing both commensal and pathogenic microbes. These microbial sensing pathways are used by the immune system to maintain host-microbial homeostasis and to induce antimicrobial defense mechanisms (Iwasaki et al. 2010). The mammalian immune system is comprised of three levels:

1. The anatomic and physiologic barriers.
2. Innate immunity.
3. Adaptive immunity.

Anatomic and physiologic barriers provide the crucial first line of defense against pathogens. These barriers include intact skin, vigorous mucociliary clearance mechanism, low stomach pH and bacteriolytic lysozyme in tears, saliva and other secretions. Innate immunity augments the protection offered by anatomic and physiologic barriers (Janeway et al. 2002). The innate immune system relies on a limited repertoire of receptors, called germline-encoded pattern recognition receptors (PRRs), to detect invading pathogens, but compensates by targeting conserved microbial components that are shared by large group of pathogens (Akira et al. 2006). Speed is a defining characteristic of the innate immune system: within minutes of pathogen exposure, the innate immune system starts generating a protective inflammatory response. Innate immune protection is a task performed by cells of both hematopoietic and non-hematopoietic origin. Hematopoietic cells involved in innate immune response include macrophages, dendritic cells, mast cells, neutrophils, eosinophils, and natural killer cells (NK). In addition to hematopoietic cells, innate immune

responsiveness is a property of the skin and the epithelial cells lining the respiratory, gastrointestinal and genitourinary tracts.

The innate immunity plays a central role in activating the subsequent adaptive immune response. T and B lymphocytes are the main self-defensive weapons of the adaptive immune system, so named because this system is shaped by antigen exposure. In contrast to the limited number of pathogen receptors used by the innate immune system, the adaptive immune system boasts an extremely diverse, randomly generated repertoire of receptors. The benefit of this receptors diversity is that the adaptive immune system can recognize virtually any antigen.

First comes the risk of autoimmune disease. Receptors specific for self-proteins (insulin and myelin) expressed by T and B cells are created by gene rearrangement, and a consequent tolerance mechanism have evolved to eliminate or regulate self-reactive cells.

Second comes the time delay required to generate a protective adaptive immune response after the first exposure to a pathogen: up to 5 days are necessary for clonal expansion of antigen-specific T and B cells (Turvey et al. 2010).

Inflammation is the biologic response of the innate and adaptive immunity. The process of inflammation is initiated by pro-inflammatory agents that include pathogens or their products (e.g. endotoxins/lipopolysaccharides or exotoxins/superantigens), foreign tissue (intravascular or extravascular implants), chemical agents, allergens and ischemic episodes (coronary ischemia and reperfusion injury) (Pahl et al. 1999). The major responses to inflammatory stimuli are mediated by phagocytes (granulocytes, monocytes, macrophages and dendritic cells) and inflammatory T cell subset (Th1 cells and NK T cells). The first cellular subset that responds to pro-inflammatory stimuli is granulocytes (neutrophils, eosinophils and basophils). These cells migrate toward chemotactic stimuli emanating from sites of initial inflammatory insult, and respond by generating toxic oxidants and secreting proteases that contributes significantly to tissue damage. For example, reperfusion injury of coronary vessels after restoration of blood flow is caused by proinflammatory

oxidants generated by neutrophils (Inauen et al. 1989, Lefer et al. 1994). A massive activation of neutrophils in the pulmonary microcirculation, followed by their emigration to the interstitial and alveolar compartments, can lead to Acute Respiratory Distress Syndrome (Fowler et al. 1983, Lu 1997, Matute Bello 2000). Similar to neutrophils, eosinophils and mast cells, play an important role in the inflammatory response underlying allergic syndromes such as asthma and pneumonitis (Martin 1989). These examples mean to underscore the importance of granulocytes and mast cells as cellular effectors of inflammatory response that lead to human disease. Pro-inflammatory agonists are able to trigger an oxidative burst, secretory responses, upregulation of cell adhesion molecules and a network of signal transduction pathways. The ultimate outcome of these signaling networks is to reprogram genes under the control of stress-responsive transcription factors (SRTFs). The endotoxin LPS is one of the most potent proinflammatory agonists for human neutrophils, monocytes and macrophages (Ulevitch et al. 1995). Upon LPS stimulation robust production of the cytokines TNFα, IL-1, IL-6, IL-8, IL-10, IL-12 and IL-18 ensues. The genes encoding these cytokines and other pro-inflammatory products, such as chemochines, inflammatory prostanoids, leukotrienes and oxidants, are controlled by SRTFs. The LPS-induced shift of neutrophils, monocytes, macrophages from a resting to "activated phenotype", is mediated by the activation of the "nuclear factor kappa-light-chain-enhancer of activated B cells" (NF-κB) and other SRTFs including the activator protein 1 (AP1) and the "nuclear factor of activated T-cells" (NFAT) (Cordle 1993, Mackman 1995, Liu et al. 2000, MaciaÂn 2001). Cellular activation by LPS requires the complex of "pattern recognition receptors" (PRRs), which are essential elements of innate immunity (Medzhitov 1997). These receptors encompass the Toll-like-receptors (TLR) family characterized by a leucine-rich extracellular domain and an intracellular toll homology domain (Rock 1998). The LPS binding to TLRs result in the translocation of SRTFs from the cytoplasm into the nucleus. Nuclear import of transcriptionally active NF-κB is required for its binding to cognate sites in the enhancer/promoter regions of some 150 known genes encoding cytokines activators or inhibitors of apoptosis, cell adhesion molecules and pro-

coagulant factors. In human studies of the systemic inflammatory response (sepsis), the persistent activation of NF-κB and its nuclear localization is correlated with an ultimately fatal outcome (Böhrer 1997).

2.2   Neutrophils

As described before, neutrophils with monocytes/macrophages, mast cells, natural killer cells and dendritic cells are very important innate immune cells comprising the first lane of innate immunity. Neutrophils are produced in the bone marrow, migrate to the bloodstream and about 12 hours later move into the tissues (Kumar et al. 2010). Neutrophils constitute about 55 to 60 per cent of the blood leucocytes in most mammals but only about 30 to 35 per cent in ruminants such as cattle and sheep. In these animals, in addition to circulating PMNs there is a pool of mature cells adhering to walls of blood vessels, recruited during infections (Paape et al. 2003).

2.2.1   Structure and morphology

The fine structure of circulating PMNs has been carefully defined. The most prominent characteristic is the multilobated nucleus, which allows PMNs to line up its nuclear lobes in a straight line, giving a more rapid migration between endothelial cells. Macrophages on the other hand have a large horseshoe shaped nucleus that makes migration between endothelial cells difficult. Thus PMNs are the first phagocytic cells to arrive at an infection site. Within the cytoplasm, there are large isles of glycogen for energy supply and numerous bactericidal granules that are used by PMNs for killing bacteria. Electron microscopy shows two types of enzyme – rich granules: the primary and the specific granules. Azurophilic or primary granule is the first granule to appear during the promyeloid stage of granulopoiesis and it contains principally bactericidal enzymes such as myeloperoxidase and lysozyme. The secondary or specific granules appear during myelocyte stage and they contain enzymes such as collagenase and alkaline phosphatase, and integral membrane proteins

such as integrins and NADPH oxidase (Bainton et al. 1971, Bainton 1973). A third "novel" large granule has been identified in cows, goats and sheep. These granules contain the majority of the antimicrobial proteins such as lactoferrin, bactenecins and defensins. These are considered powerful antibiotics peptides due to their affinity to lipopolysaccharide of bacterial walls, which affects the membrane permeability and results in the cell lysis (Farnaud 2003, Wu 1999, White 1995). Mature neutrophil cytoplasm possesses also a small Golgi apparatus and some mitochondria but very few ribosomes or rough endoplasmic reticulum. Thus PMNs cannot synthetize large quantities of protein (Gennaro et al. 1983, Paape et al. 2003).

2.2.2 Functions

Neutrophils primary function is to localize destroy and eliminate a wide variety of microbial pathogen. Bactericidal action of the neutrophils at site of infection involves several cellular processes, namely adherence of cells to the vascular endothelium, chemotactic migration across the vessel wall, microbial recognition and ingestion by the phagosome, metabolic stimulation, degranulation and killing or destruction of pathogens (Kantari et al. 2008).

2.2.2.1 Adhesion and phagocytosis

Bacterial invasion cause the production of many difference chemotactic factors: C5a is generated by activation of the complement system and lipopolysaccharides are released from invading bacteria. Thus neutrophils are attracted to the source of chemotactic stimuli and, because they can crawl but cannot swim, they must attach to a surface before they can respond to chemicals. Neutrophils are triggered to leave the circulation because in the area of bacteria invasion there is a transient increase in adhesiveness of both neutrophils and vascular endothelial cells (Zarbock, Ley 2009). As neutrophils roll toward a chemotactic source, a pseudopod advanced first followed by the main portion of the cells. When the pseudopod meets an ingestible particle, the pseudopod flows over and around it and binding occurs between ligands on the

organism and receptors of the neutrophil. Once the pseudopod completely covers the particle, the particle is enclosed in the phagosome into which cytoplasmic vacuoles are discharged (Nüsse 2011). The process of phagocytosis and bacterial destruction is markedly enhanced when microorganisms are recognized coated with serum proteins known as opsonins, such as complement components (C5a – C3b), antibodies (IgM – IgG) and fibronectin (Semnani et al. 1993). Once opsonins bind to receptors on the PMNs surface, the cell becomes activated and generation of oxidative burst is initiated. The response is followed by increase oxygen consumption and hexose monophosphate activity and is called the "respiratory burst": the plasma membrane and the intracellular granule have now a pivotal role.

2.2.2.2 Respiratory Burst

The plasma membrane is the site of NADPH oxidase, that underline the cell ability to generate a family of reactive oxidizing chemicals, whereas the granules contain microbicidal peptides, proteins, enzymes such as myeloperoxidase (Fig. 2.1). When neutrophil is specifically triggered by pro-inflammatory signals, the oxidase begins to generate and release oxygen metabolites. Almost simultaneously, the granules fuse with the plasma membrane and discharge their contents both into extracellular medium and into the phagocytic vacuole (Weiss 1989). NADPH oxidase is a complex multicomponent enzyme that forms a trans membrane electron transport with cytosolic NADPH as the electron donor and oxygen as the electron acceptor. Activated NADPH oxidase thus converts NADPH (the reduced form of NADP, nicotinamide adenine dinucleotide phosphate) to $NADP^+$ with the release of electrons. The molecule of oxygen accepts two donated electrons resulting in the generation of superoxide anion $\cdot O_2^-$. The $NADP^+$ generated by the NADPH-oxidase accelerates the exose monophosphate shunt, a metabolic pathway that converts sucrose to a pentose and CO_2 and release energy for use by the cell. The $\cdot O_2^-$ interacts spontaneously with water to generate H_2O_2 under the influence of the enzyme superoxide dismutase.
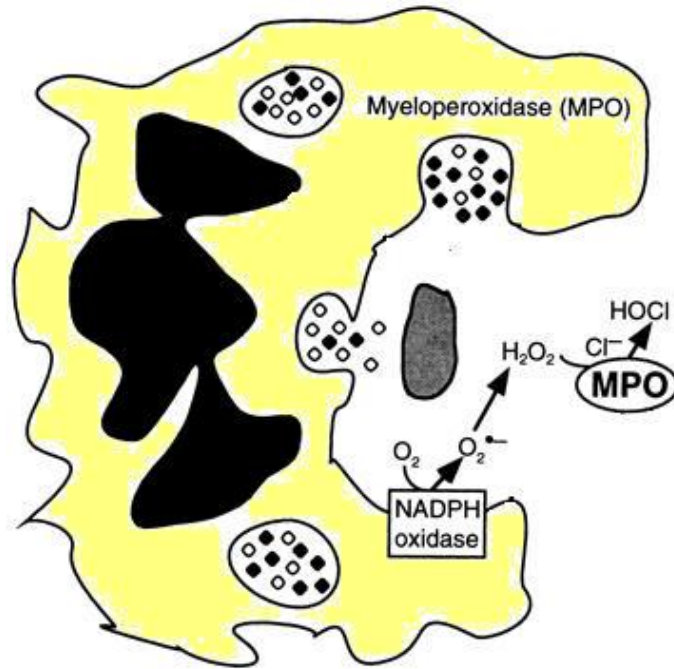
Figure 2.1. The major features of the respiratory burst pathway in neutrophils. Triggered neutrophils generate bactericidal products such as hydrogen peroxide and hypochlorous acid.

Because this reaction occurs so rapidly, superoxide anion does not accumulate. In contrast H_2O_2 is more stable and persists. The hydrogen peroxide may then be converted to bactericidal compounds through the action of myeloperoxidase, the most significant respiratory burst enzyme in neutrophils. Myeloperoxidase is found in large amounts in the primary granules. It catalyzes the reaction between hydrogen peroxide and extracellular or plasmatic Cl^- to produce hypochlorous acid (HOCl). Quantitative analyses demonstrate that 10^6 maximally triggered neutrophils produced approximately 2×10^{-7} mol of HOCl during two hours incubation. Indeed at neutral pH, the 2×10^{-7} mol of HOCl generated by 10^6 neutrophils is enough to destroy as many as 150 million *Escherichia coli* organisms in milliseconds (Test et al. 1986).

Respiratory burst can be monitored by measuring chemiluminescence activity of PMNs. Highly instable oxygen metabolites are in electronically excited states

and excitation is accompanied by emission of light (Allen et al 1976). Chemiluminescence is enhanced by use of the substrates luminol and lucigenin. Luminol seems to largely detect HOCl production, while lucigenin appears more specific for $\cdot O_2^-$ (Myhre et al. 2003).

However, hypochlorous acid is a very powerful oxidant that acts predominantly as bactericidal agent within the phagolysosome but it can also act extracellularly, resulting in very specific oxidative modifications to lipids (Malle et al. 2006), enzymes (Hawkins et al. 2005) and extracellular matrix (Czapski et al. 2002), all of which are components in the inflammatory microenvironment. In addition HOCl and HOCl-exposed biomolecules present a role in the innate and adaptive immunity. Indeed, chlorinated lipids are able to induce dendritic cells maturation (Alderman, Bunyard et al. 2002) while chlorinated glycoproteins are able to scavenger receptors on the dendritic cells, promoting antibody uptake and processing. Therefore HOCl derived from neutrophils may represent a natural innate immune derived adjuvant that can promote induction of adaptive immunity (Fig. 2.2) (Prokopowicz, Arce et al. 2010).
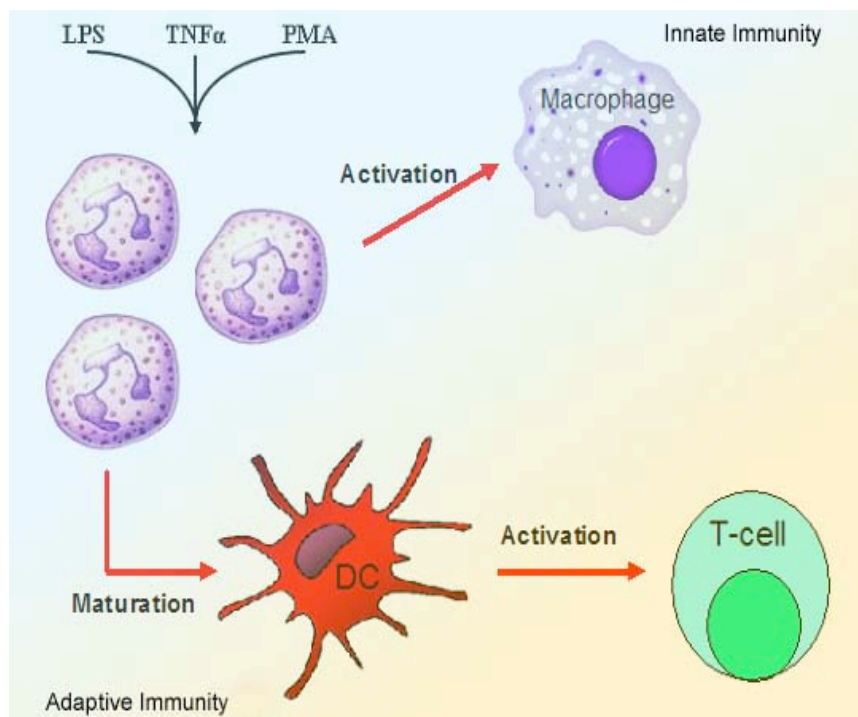
Figure 2.2: Role of activated neutrophils in innate and adaptive immunity.

Neutrophils activation during acute infection of inflammation leads also to synthesis and release of various pro-inflammatory cytokines, such as IFN-γ , IL-8 and TNF-α which act as potent activators of monocytes/macrophages. Once these macrophages are activated, they prolong neutrophils life span at the site of inflammation of infection by releasing IL1β, TNF-α , G-CSF and GM-CSF: the short life span of resting neutrophils (6-12 hours) increases to 24-48 hours (Yamashiro et al. 2001). Triggered neutrophils play also an important role in activation of T-cell immune response by DCs (Megiovanni et al. 2006). Upon activation, neutrophil granules release several inflammatory proteins such as defensins, cathelicidins and lactoferrin, with the potential to induce maturation of immature DCs (Yang et al. 2004). These protein molecules are released as a consequence of first host innate immune response to infectious or inflammatory agents and lead to activation of both innate as well as adaptive immunity (Oppenheim et al. 2005).

2.2.3   Neutrophil programmed cell death: apoptosis

Neutrophils have the shortest life span among leucocytes. After egress from the bone marrow, neutrophils leave the circulation within 6-10 h and migrate into the tissue, were they undergo constitutive (spontaneous) apoptosis in 1-2 day (Maianski et al. 2003).

2.2.3.1   Morphological aspects

Morphological changes of neutrophils undergoing apoptosis are typical for the general apoptotic scenario. Characteristic features are condensation of cytoplasm and intracellular organelles, aggregation and subsequent cleavage of nuclear chromatin, DNA fragmentation and formation of apoptotic bodies (Fig. 2.3).
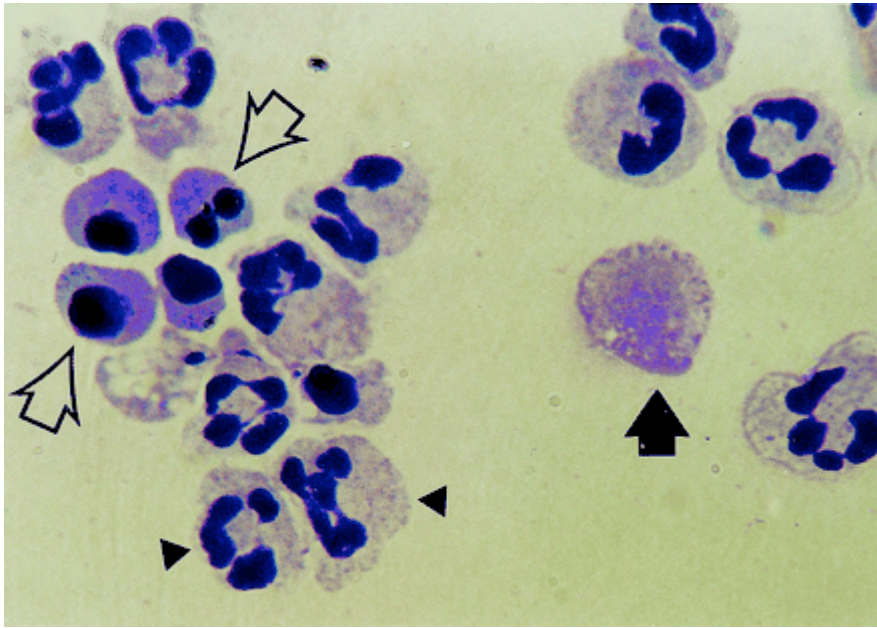
Figure 2.3: Heterogeneity of neutrophils aged in culture (22 h). Light microscopy (×1000) showing non-apoptotic neutrophils, (▲), early apoptotic neutrophils, showing nuclear coalescence and chromatin condensation (⇧), and a late apoptotic neutrophil with nuclear degradation and lack of chromatin (⬆).

2.2.3.2 Biochemical and regulatory aspects

Importantly, apoptotic changes are accompanied by down regulation of cellular functions, especially receptor-mediated reactions. Neutrophils that have entered the apoptotic process lose the ability of chemokinesis, chemotaxis, oxidative burst and degranulation (White et al. 1993, Dransfield et al. 1995). However, at inflammatory foci, the expression of neutrophils apoptotic program can be regulated by a number of agents such as cytokines, pathogens and environmental stressor (Maggini et al. 2009). In general myelopoietic growth factors G-CSF and GM-CSF, and cytokines such as INF-γ, IL-8 and IL-1β have anti-apoptotic potential (Colotta et al. 1992, Akgul et al. 2001). TNF-α has both pro-apoptotic and anti-apoptotic functions: this controversy could depend on the concentration of the cytokine, on the duration of stimulation and on the initial capacity of the neutrophils before exposition to TNF-α (van den Berg et al. 2001). Also LPS and glucocorticoids induce neutrophils survival by suppressing apoptosis (Liles et al. 1995). Delay in apoptosis coincides with preservation of neutrophils functions and it's not only an effect of extrinsic mediators, but also

an action of the intrinsic neutrophil resources of autocrine/paracrine regulation. Neutrophils secrete pro-survival cytokines such as IL-1β and IL-6 (William et al. 1998, Biffl et al. 1996). Apoptotic neutrophils are eliminated by both professional (macrophages) and non-professional (fibroblasts) phagocytes. The recognition of apoptotic cells is determined by their unusual surface structure, which appears on the outer layer of the plasma membrane during apoptosis. Among these are phosphatidylserine (PS) residues, normally located in the inner leaflet of cellular membrane, which exteriorize upon induction of apoptosis and are recognized by specific receptors on disposing cell. Importantly, phagocytosis of apoptotic neutrophils suppresses the production of inflammatory cytokines promoting inflammation resolution (Fig. 2.4) (Giles et al. 2000).
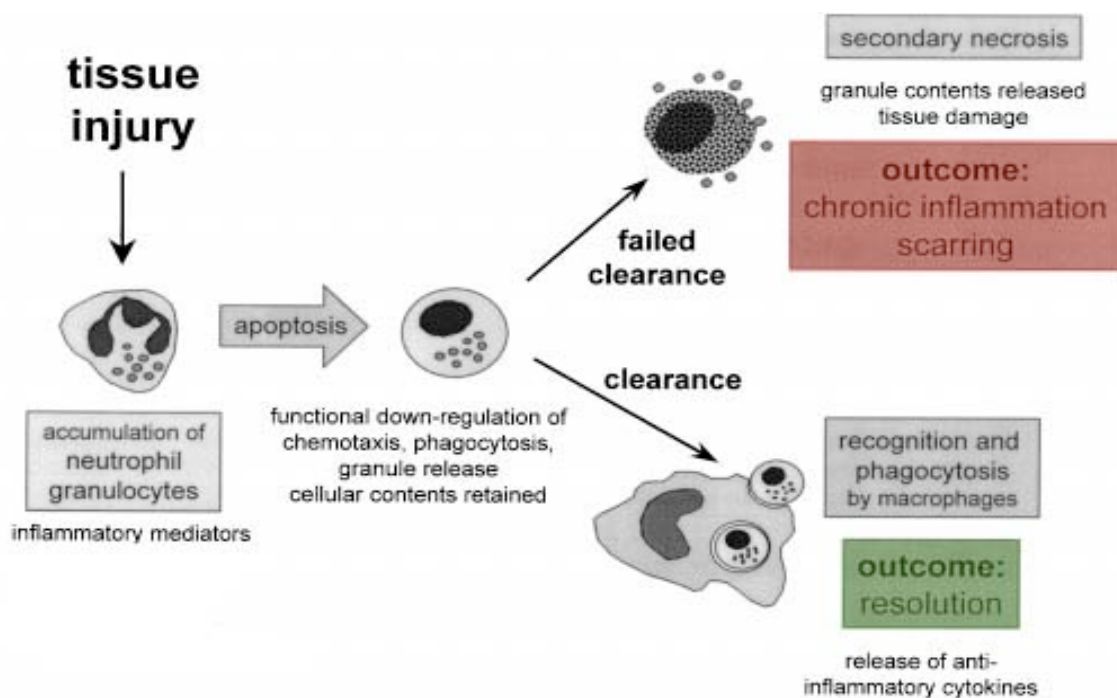
Figure 2.4: Schematic representation illustrating the proposed role of neutrophil apoptosis and phagocytic clearance by macrophages in the process of resolution of inflammation.

The molecular regulation of cell death and apoptosis is very complex and tight. Within each cell, there are two main pathways of apoptosis: an extrinsic or death-receptor mediated pathway and an intrinsic or mitochondrial pathway of cell death. In both pathways a series of molecular and biochemical steps leads

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to the activation of common effector or executioner cysteine proteases, called caspases, resulting in the cleavage of a number of nuclear and cytoplasmic substrates, including those responsible for the maintenance of nuclear integrity, cell cycle progression and DNA repair.

2.2.3.3   Extrinsic pathway

The extrinsic pathway is initiated upon binding of "death receptors" to their ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily and the ligands for these receptors belong to the tumor necrosis factor (TNF) gene family. The most important receptors are CD95 (Fas) and TNFRs. The interaction with specific ligands (FasL and TNF-α) leads to the assembly of the intracellular death-inducing signalling complex (DISC). DISC recruits and activates by autocleavage the upstream initiator pro-caspase 8. Pro-caspase 8 is autolytically cleaved into active caspase 8 and activated caspase 8 serves as an enzyme for downstream effector caspases (caspases 3, 6 and 7). These active effector caspases in turn, cleave a number of cytoplasmic and nuclear substrates, including molecule responsible for DNA and nuclear membrane degradation (Fig. 2.5) (Krammer 2000, Maianski et al. 2003).

2.2.3.4   Intrinsic pathway

A number of stimuli, including chemotherapeutic agents, UV radiation, stress molecule (reactive oxygen species and reactive nitrogen species) appear to mediate apoptosis via the intrinsic pathway. This route of cell death involves mitochondria, which connect Bcl-2 proteins and caspases. Bcl-2 proteins constitute a family of proteins with either pro-or anti-apoptotic properties. The anti-apoptotic members are Bcl-2, Bcl-X_L, Mcl-1 while the pro-apoptotic members are Bid, Bim, Bad, Noxa, Puma, Bax, Bak and Bok. Like in the other cell types, the caspases and the Bcl-2 family proteins occupy a central position in neutrophil apoptosis (Cory et al. 2002). Neutrophils, although having both Bcl-2 and caspase cascade, are an exceptional cell type because they possess only few mitochondria: these organelles seem to have lost their role in the active life

of neutrophils (they mainly use glycolysis for energy supply) but, perhaps, they had preserved a role in cell death (Borregaard et al. 1982, Newmeyer et al. 2003). Mitochondria, upon apoptosis, may release certain proteins into the cytosol that facilitate the activation of the caspase cascade and Bcl-2 homologues are thought to manipulate the integrity of mitochondria. Bax, Bak, Bad, Bid and Bim, indeed, initiate the mitochondrial membrane permeabilization (MMP) by forming a sort or channel in the outer membrane. Bad also interacts with anti-apoptotic Bcl-2 and Bcl-X_L and triggers MMP; Bid is cleaved by caspase 8, traslocates to the outer membrane, causing MMP and the release of cytochrome c. Bind provides a mechanism by which the death receptor pathway is linked to the mitochondrial pathway (Kastan 2005). The release of the cytochrome c triggers the assembly of Apaf-1 (apoptotic protease activating factor) and pro-caspase 9 to form an apoptosome. Pro-caspase 9 is then autolytically cleaved to active caspase 9 which then activates pro-caspase 3 to active caspase resulting in cleavage of its substrates that terminated in cell apoptosis (Fig 2.5) (Adams et al 2002).
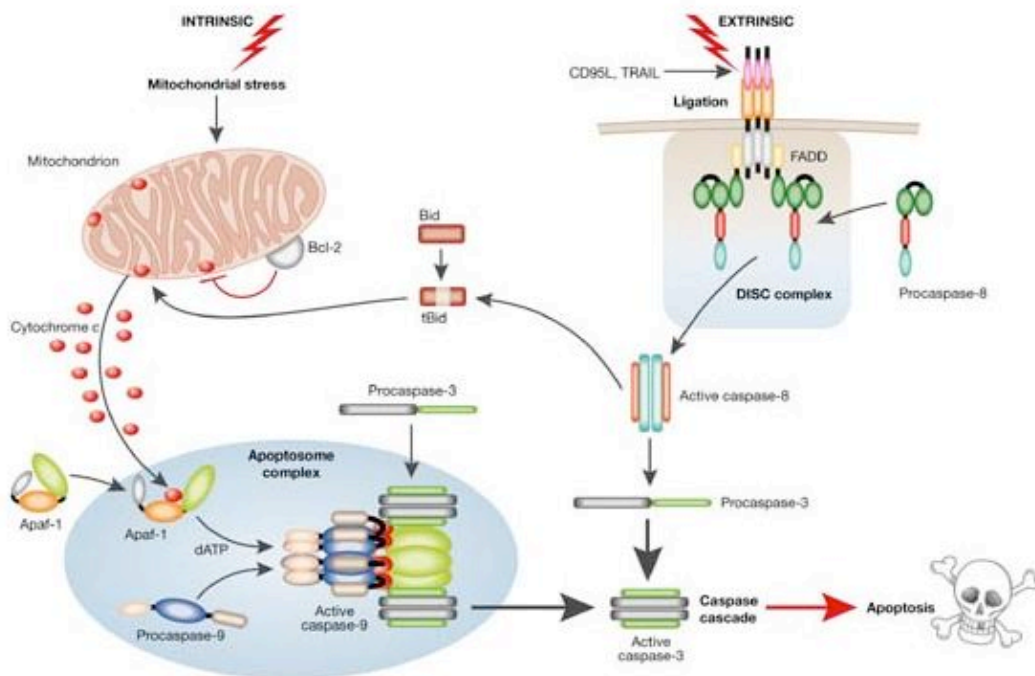
Figure 2.5. Apoptosis: the "extrinsic" and "intrinsic" pathways to caspase activation.

2.3   Free Radicals

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process (reduction and oxidation). These species play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, free radicals and oxidants generate oxidative stress, a deleterious process that can damage cell structures, including lipids, proteins, and DNA (Dröge 2002). Oxidative stress plays a major role in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases (Valko 2007). The animal body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or additives. Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases (Young 2001).

The theory of oxygen-free radicals has been known about fifty years ago by Gershman and Gilbert (cited by: Gerschman et al. 2005). However, only within the last two decades, has there been an explosive discovery of their roles in the development of diseases, and also of the health protective effects of antioxidants.

2.3.1 Characteristics

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the terms collectively describing free radicals and other non-radical reactive derivatives or also called oxidants. Radicals are less stable than non-radical species, although their reactivity is generally stronger.

Normal molecules in the body have two electrons (a paired group) in their outer shell. A molecule with one or more unpaired electron in its outer shell is called a free radical. Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron, by cleavage of a radical to give another radical and, also via redox reactions. Free radicals include hydroxyl (OH), superoxide (O_2), nitric oxide (NO), nitrogen dioxide (NO_2), peroxyl (ROO) and LOO (lipid peroxyl). Moreover, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite (ONOO-), dinitrogen trioxide (N_2O_3), LOOH (lipid peroxide) are not free radicals and are generally called oxidants, although they can easily lead to free radical reactions in living organisms. Biological free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates such as lipids, proteins, DNA (Pacher 2007, Genestra 2007). Details about some of the biologically important reactive species are presented in table 2.6

Reactive species	Symbol	Reactivity / Remarks
Reactive oxygen species :		
Superoxide	$O_2^{\bullet-}$	Generated in mitochondria, in cardiovascular system and others
Hydroxyl radical	$^{\bullet}OH$	Very highly reactive, generated during iron overload and such conditions in our body
Hydrogen peroxide	H_2O_2	Formed in our body by large number of reactions and yields potent species like $^{\bullet}OH$
Peroxyl radical	$ROO^{\bullet}$	Reactive and formed from lipids, proteins, DNA, sugars etc. during oxidative damage
Organic hydroperoxide	$ROOH$	Reacts with transient metal ions to yield reactive species
Singlet oxygen	$^1O_2$	Highly reactive, formed during photosensitization and chemical reactions
Reactive nitrogen species:		
Nitric oxide	$NO^{\bullet}$	Neurotransmitter and blood pressure regulator, can yield potent oxidants during pathological states
Peroxynitrite	$ONOO^-$	Formed from NO. and superoxide, highly reactive
Peroxynitrous acid	$ONOOH$	Protonated form of ONOO-
Nitrogen dioxide	NO_2	Formed during atmospheric pollution

Table 2.6: Reactive oxygen and nitrogen species of biological interest (modified from (Devasagayam, Tilak et al. 2004)

2.3.2 Biological and externals sources

Formation of ROS and RNS can be generated in the cells by two ways: enzymatic and non-enzymatic reactions. Enzymatic reactions generating free radicals include those involved in the respiratory chain, the phagocytosis, the prostaglandin synthesis and the cytochrome P450 system. For example, the super oxide anion radical (O_2) is generated via several cellular oxidase systems such as NADPH oxidase, xanthine oxidase, peroxidases. Once formed, it participates in several reactions yielding various ROS and RNS such as hydrogen peroxide, hydroxyl radical (OH), peroxynitrite (ONOO-), hypochlorous acid (HOCl), etc. H_2O_2 (a non-radical) is produced by the action of several oxidase enzymes, including aminoacid oxidase and xanthine oxidase. The latter catalyses the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid. Hydroxyl radical (OH), the most reactive free radical in vivo, is formed by the

reaction of O_2- with H_2O_2 in the presence of Fe^{2+}or Cu^+(catalyst). This reaction is known as the Fenton reaction. Hypochlorous acid (HOCl) is produced by the neutrophil-derived enzyme, myeloperoxidase, which oxidizes chloride ions in the presence of H_2O_2. Nitric oxide radical (NO·) is formed in biological tissues from the oxidation of L-arginine to citrulline by nitric oxide synthase.

Free radicals can be produced from non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations. The non-enzymatic process can also occur during oxidative phosphorylation (i.e. aerobic respiration) in the mitochondria (Dröge 2002, Valko 2007, Genestra 2007).

ROS and RNS are generated from either endogenous or exogenous sources. Some internal sources of these species are: mitochondria, xanthine oxidase, Fenton reaction, phagocytes (neutrophils, monocytes, macrophages, eosinophils), arachidonate pathways, microsomes, peroxisomes, exercise, inflammation, ischemia, etc.(Willcox, Ash et al. 2004). Many external sources of free radicals and oxidants include: pollutants, cigarette smoke, radiation, medication, etc (Parthasarathy 1999, Valko 2005).

2.3.3   Beneficial activities

At low or moderate concentrations, ROS and RNS are necessary for the maturation process of cellular structures and can act as weapons for the host defense system. Indeed, phagocytic cells release free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against disease. The importance of ROS production by the immune system is clearly exemplified by patients with granulomatous disease. These patients have defective membrane-bound NADPH oxidase system which makes them unable to produce the superoxide anion radical ($O2·$-), thereby resulting in multiple and persistent infection (Valko 2007, Dröge 2002). Other beneficial effects of ROS and RNS involve their physiological roles in the function of a number of cellular signaling systems. Their production by non-phagocytic NADPH oxidase isoforms plays a key role in the regulation of intracellular signaling cascades in various types of non-phagocytic cells including fibroblasts, endothelial cells,

vascular smooth muscle cells, cardiac myocytes, and thyroid tissue. For example, nitric oxide (NO) is an intercellular messenger for modulating blood flow, thrombosis, and neural activity. NO is also important for nonspecific host defense, and for killing intracellular pathogens and tumors. Another beneficial activity of free radicals is the induction of a mitogenic response. In brief, ROS and RNS at low or moderate levels are vital to human health (Pacher 2007, Genestra 2007).

2.3.4   Deleterious activities

When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA). Oxidative stress can arise when cells cannot adequately destroy the excess of free radicals formed. In other words, oxidative stress results from an imbalance between formation and neutralization of ROS and RNS. Endogenous free radicals are generated from immune cell activation, inflammation, mental stress, excessive exercise (sports, hard works), ischemia, infection, cancer, aging. Exogenous ROS and RNS result from air and water pollution (vehicle exhaust, ozone, nitrogen dioxyde, etc), cigarette smoke, alcohol, heavy or transition metals (cadmium, mercury, lead, iron, arsenic, etc), smog, certain drugs (doxorubicin, bleomycin, vinblastine, cyclosporine, tacrolimus, gentamycin, etc), pesticides, industrial solvents, cooking (smoked meat, used oil, fat), radiation (increased exposure to sunlight), etc. After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals. The animal body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally present in situ, or externally supplied through food. If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases as well as the aging process. It has also been involved in the etiology of acute pathologies (trauma, stroke, infection) (Genestra 2007, Dröge 2002, Valko 2004, Valko 2006, Valko 2007, Pacher 2007).

2.3.5   Antioxidant systems

The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated in situ (endogenous antioxidants), or externally supplied through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention.

2.3.5.1   Antioxidant classification

Endogenous compounds in cells can be classified as enzymatic antioxidants and non-enzymatic antioxidants.

The major antioxidant enzymes directly involved in the neutralization of ROS and RNS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx) (Fig. 2.7). SOD, the first line of defense against free radicals, catalyzes the dismutation of superoxide anion radical (O_2-) into hydrogen peroxide (H_2O_2) by reduction: The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein GPx enzyme removes H2O2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH) (Parthasarathy 1999, Frei 1994, Young 2001).

Superoxide dismutase

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 \cdot O_2$$

Catalase

$$H_2O_2 + H_2O_2 \rightarrow H_2O + H_2O + O_2$$

Glutathione Peroxidase

$$H_2O_2 + GSH + GSH \rightarrow GS\text{-}SG + H_2O + H_2O$$

Glutathione Reductase

$$GS\text{-}SG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$
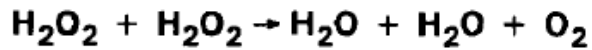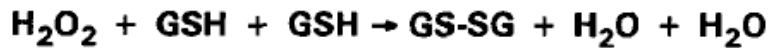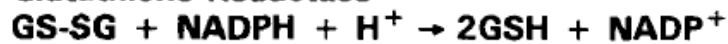
Figure 2.7: Mechanisms of antioxidant enzymes (modified from (Davies 1995)

The non-enzymatic antioxidants are also divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants belonging to endogenous antioxidants are produced by metabolism in the body, such as lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc. While nutrient antioxidants belonging to exogenous antioxidants are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc. (Willcox, Ash et al. 2004)

2.3.5.2 Antioxidant Process.

When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized. Therefore, the antioxidant resources must be constantly restored in the body. Thus, while in one particular system an antioxidant is effective against free radicals: in other systems the same antioxidant could become ineffective. Also, in certain circumstances, an antioxidant may even act as a pro-oxidant e.g. it can generate toxic ROS/RNS (Pham Huy 2001). The antioxidant process can function in one of two ways: chain-breaking or prevention. For the chain-breaking, when a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues

until either the free radical formed is stabilized by a chain-breaking antioxidant (vitamin C, E, carotenoids, etc.), or it simply disintegrates into an inoffensive product. The classic example of such a chain reaction is lipid peroxidation. For the preventive way, an antioxidant enzyme like superoxide dismutase, catalase and glutathione peroxidase can prevent oxidation by reducing the rate of chain initiation, e.g., either by scavenging initiating free radicals or by stabilizing transition metal radicals such as copper and iron (Young 2001, Valko 2005).

2.4 Oxidative stress

Oxidative stress is defined as an increased production of ROS due to an imbalance of oxidant and antioxidant production (Sies 1986). Oxidants are reactive oxygen species (ROS), which have the ability to damage all biomolecules, either directly or indirectly, including proteins, lipids, DNA and carbohydrates. Under normal circumstances, there is a well – managed balance between formation and neutralization of ROS by the cellular antioxidant machinery, so there is minimal modification of biomolecules (Shacter 2000). Oxidative damage to macromolecules due to induced oxidation, cleavage, cross-linking and structural and functional modifications have been implicated in the pathogenesis of several diseases such as atherosclerosis, diabetes, pancreatitis, bowel disease and colitis, pulmonary dysfunction, renal diseases and cancer (Halliwell 1994)

2.4.1 Lipids and Lipid Peroxidation

Membrane lipids present in subcellular organelles are highly susceptible to free radical damage. Lipids when reacted with free radicals can undergo the highly damaging chain reaction of lipid peroxidation (LP) leading to both direct and indirect effects. During LP a large number of toxic byproducts are also formed that can have effects at a site away from the area of generation, behaving as 'second messengers'. The damage caused by LP is highly detrimental to the functioning of the cell. Lipid peroxidation is a free radical mediated process.

Initiation of a peroxidative sequence is due to the attack by any species, which can abstract a hydrogen atom from a methylene group (CH_2), leaving behind an unpaired electron on the carbon atom (•CH). The resultant carbon radical is stabilized by molecular rearrangement to produce a conjugated diene, which then can react with an oxygen molecule to give a lipid peroxyl radical (LOO•). These radicals can further abstract hydrogen atoms from other lipid molecules to form lipid hydroperoxides (LOOH) and at the same time propagate LP further. The process of LP, gives rise to many products of toxicological interest like malondialdehyde (MDA), 4- hydroxynonenal (4-HNE) and various 2-alkenals (Devasagayam 2003).

2.4.2 Carbohydrates

Free radicals such as •OH react with carbohydrates by randomly abstracting a hydrogen atom from one of the carbon atoms, producing a carbon-centered radical. This leads to chain breaks in important molecules like hyaluronic acid. In the synovial fluid surrounding joints, an accumulation and activation of neutrophils during inflammation produces significant amounts of oxyradicals that are also implicated in rheumatoid arthritis (Halliwell 1984).

2.4.3 DNA

Oxidative damage to DNA is a result of interaction of DNA with ROS or RNS. Free radicals such as •OH and H• react with DNA by addition to bases or abstractions of hydrogen atoms from the sugar moiety. The C4-C5 double bond of pyrimidine is particularly sensitive to attack by •OH, generating a spectrum of oxidative pyrimidine damage products, including thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoin and others. Similarly, interaction of •OH with purines will generate 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines and other less characterized purine oxidative products. Several repair pathways repair DNA damage. 8-OHdG has been implicated in

carcinogenesis and is considered a reliable marker for oxidative DNA damage (Cooke, COOKE 2003).

2.4.4 Proteins

Proteins constitute more than 10% of the weight of the blood plasma of living organisms and more than 50 % of the dry weight of cells, and as such can be considered important targets for the effects of oxidants (Griffiths 2000).

Oxidation of proteins by ROS/RNS can generate a range of stable as well as reactive products. Although most oxidized proteins that are functionally inactive are rapidly removed, some can gradually accumulate with time and thereby contribute to the damage associated with ageing as well as various diseases. Lipofuscin, an aggregate of peroxidized lipids and proteins, accumulates in lysosomes of aged cells and brain cells of patients with Alzheimer's disease. Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed. The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (*S*-glutathiolation). In addition, reactive oxygen species may directly alter proteins with the eventual formation of oxidized amino acids, such as *ortho*-tyrosine and methionine sulfoxide (Stadtman 1991). Also carbonyl groups are generated by many different mechanisms and their concentration is a good measure of ROS-mediated protein oxidation. Alternatively, reactive carbonyl compounds formed by the oxidation of carbohydrates and lipids may indirectly lead to advanced glycation or lipoxidation of proteins (Miyata, MIYATA 1999). Increased carbonyl modification of proteins subsequently results in the rise of plasma and tissue contents of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs), whose deleterious biological

effects have been revealed (Dalle Donne et al. 2003, Dalle Donne et al. 2005, Dalle Donne et al. 2006).

2.5 Protein oxidation during inflammatory reactions

During acute infections or inflammations, activated neutrophils become the major source of ROS and the release of myeloperoxidase results in the formation of the most powerful oxidant HOCl (Iwao et al. 2006).

Protein oxidation is defined as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary products of oxidative stress such as oxidized lipids and sugars (Dean et al. 1997). Because there are so many mechanisms for induction of protein oxidation and because all of the amino acyl side chain can become oxidatively modified, there are numerous different types of protein oxidative modification (Table 2.8).

Modification	Amino acids involved
Disulfides	Cys
Methionine sulfoxide	Met
Carbonyls (aldehydes, ketones)	Lys, Arg, Pro, Thr
Dityrosine	Tyr
Chlorotyrosine	Tyr
Chloramine, deamination	Lys, Phe
Lipid peroxidation adducts (MDA, HNE)	Lys, Cys, His
Glycoxidation adducts (Pentosidine, CML)	Lys
Cross-links, aggregates, fragments	Several

Table 2.8: Oxidative modification of proteins.

The two amino acids that are the most prone to oxidative attack are Cys (Lii et al. 1994) and Met (Vogt 1995), both of which contain susceptible sulphur atoms. All oxidizing species can induce modification of Cys and Met. Interaction of

myeloperoxidase –derived HOCl with Lys, Arg, Pro and Thr incurs the formation of carbonyl groups (aldehydes and ketones) on the side chains. Instead, the interaction with Tyr, Phe, Trp and Lys leads to formation of Chlorotyrosine, dityrosine, chloramine and aldehydes (Kettle 1996, Hazen et al. 1998, Simpson et al. 1992). These structural modifications of proteins, induced predominantly by HOCl, were generally termed advanced oxidation protein products (AOPPs) (Capeillere-Blandinn et al. 2004). Indirect oxidative modification of protein amino acyl side chain occurs thought the formation of adducts with products of oxidatively modified lipids and sugars (Fig. 2.9). Lipid peroxidation and glycoxidation induced by oxidants and oxidative stress generate a huge variety of secondary products (reactive carbonyl compound, reactive aldehydes, ketones and alkanes) that react on cellular and tissue proteins to form Advanced lipoperoxidation protein products (ALEs) and Advanced glycation protein products (AGEs) (Kalousova et al. 2005, Negre Salvayre et al. 2008). Products of protein oxidation, compared to products of lipid peroxidation, DNA oxidative base modification or carbohydrates oxidation, offer some advantages as markers of oxidative stress. Proteins have a relatively long half-lives and unique biological functions, so they are stable and there are unique functional consequences resulting from their modifications (Ciolino et al. 1997).
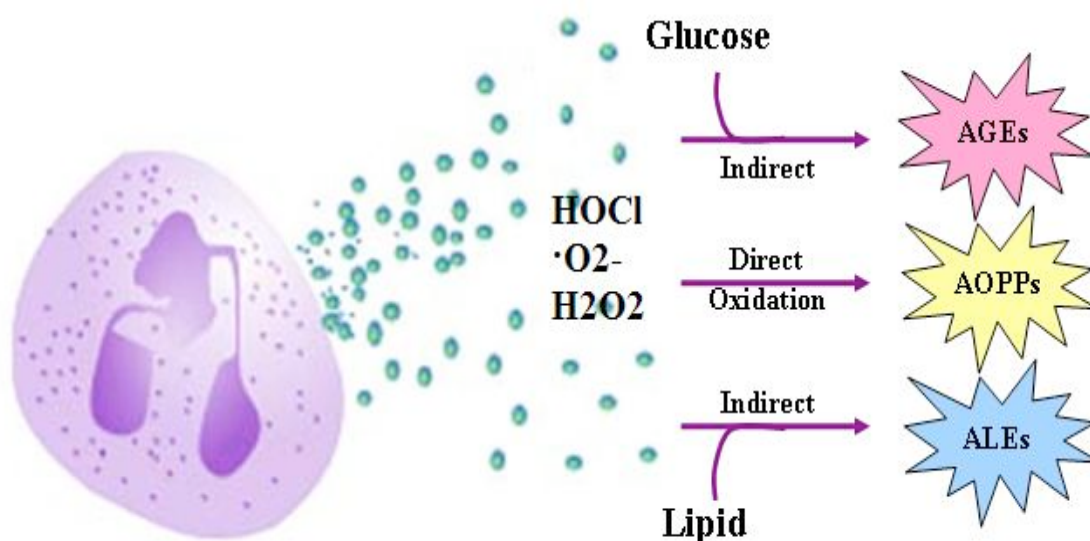
Figure 2.9: Protein oxidation pathways, during neutrophils activation.

2.5.1 Protein oxidation and diseases

Generally the oxidative modification of proteins generates protein which are functionally inactive and rapidly removed, but some oxidized proteins can gradually accumulate with time and be implicated in the ethiology and the development of numerous disorders and "ageing- related" diseases (Mera et al. 2005). These include:

- Atherosclerosis, where oxidation of the apolipoprotein moiety on LDL alters receptor recognition and contributes to foam cell formation (Steinberg et al. 1989) and the oxidation of plasmatic fibrinogen stimulates thrombogenesis processes (Azizova et al. 2007);

- Diabetes, where elevated plasma glucose levels are believed to contributed to protein bound glucose derived oxidizing species (Krapfenbauer et al. 1998);

- Rheumatoid arthritis, where oxidative modification to IgG has been suggested to lead to a self-perpetuating inflammatory lesion within the affected joint (Lunek et al. 1985);

- Neurodegenerative diseases, such as Alzheimer's disease, where altered amyloid precursor protein processing result in a peptide capable

of generating oxidants, and elevated levels of oxidized are reported (Kato et al. 1998);

- Chronic renal disease, where neutrophils were activated during blood passage through dialysis circuits and the consequent production of ROS can damage circulating proteins (Witko-Sarsat et al. 1998).

2.6 Markers of protein oxidation

The oxidative stress modified proteins either indirectly where carbohydrates and lipids, targeted by reactive oxygen species yield, are involved in the formation of AGEs and ALEs, or directly thorough the oxidation of amino acids by reactive oxygen species, that generates advanced oxidation protein products (AOPP) (Miyata et al. 2000).

2.6.1 AGE

2.6.1.1 Chemistry of AGE generation

The AGE formation process, called Maillard reaction, is linked both to oxidative stress and inflammation (Kalousova et al. 2005). It begins, from Schiff bases and the Amadori product, a ketoamine, produced by the reaction of the carbonyl group of a reducing sugar, like glucose, with proteins. During Amadori reorganization, highly reactive intermediates, known as α-dicarbonyls or oxoaldehydes, accumulate. This build-up is termed "carbonyl stress". These carbonyl groups, can react with lysine and arginine functional groups on proteins, leading to the formation of stable AGE compounds, such as N-(carboxymethyl)lysine (CML) and pentosidine (Goldin et al. 2006). These AGE can be formed in inflamed foci both via NADPH oxidase (Anderson et al. 2003) as well as via myeloperoxidase action (Anderson et al. 1999). There are evidences that CML also forms "in vitro" from oxidation of low density lipoproteins (LDL) and during peroxidation of poly-unsatured fatty acid (PUFA) in presence of proteins (Fu et al. 1996).

2.6.1.2 Biological effects of AGE

A common consequence of AGE formation is covalent cross-link formation. Proteins affected by this process are usually stable and long-lived, such as collagen. The chemistry behind cross-linking formation is complex and not fully understood, but is thought to involve lysine residues. Physiological consequences of cross-linking formation include sclerosis of renal glomeruli, thickening of the capillary basement membrane and atherosclerosis development (Monnier et al. 1996). Several different receptors for AGE have been discovered one of which, termed RAGE, initiates the intracellular signalling that disrupts cellular function through the recognition and binding of AGE. RAGE it's a member of the immunoglobulin superfamily receptors and it's expressed on macrophages and microglia. The AGE-RAGE interaction induces oxidative stress and activates nuclear factor NF-κB, which is followed by over expression of genes for cytokines, growth factors and adhesive molecules, increased vascular permeability, a pro-coagulant state and other toxic effects (Fig. 2.10) (Yan et al. 1994). "in vitro" produced AGE-BSA is also able to trigger programmed cell death in (Podesta, Romeo et al. 2000, Yamagishi, Inagaki et al. 2002) mesangial cells and in retinal pericytes of diabetic patients, by inducing pro-apoptotic molecule Bax mRNA and protein in a RAGE-independent way (Podesta et al. 2000, Yamagishi et al. 2002).

2.6.2 ALE

2.6.2.1 Chemistry of ALE generation

Lipid peroxidation induced by oxidants and oxidative stress generates a huge variety of lipid peroxidation products that are precursors of ALE. The oxidation of PUFAs (mainly linoleic acid, arachidonic acid, docohexaenoic acid and eicosapentanoic acid) generates highly reactive aldehydes such as 4-hydroxynonenal (4-HNE), malondialdehydes and acrolein (Esterbauer 1993).

2.6.2.2 Biological effects of ALE

ALEs precursors play an active role in signal transduction by altering progressively the structure of tissue and circulating proteins, with consequences on the inflammatory status, cell proliferation and viability (Petersen et al. 2004). One of the best known effects of ALE precursors is their role in the modification of LDL, because they react with Lys residues of apolipoprotein B (apoB), which are required for LDL recognition by its specific apoB/E receptor expressed on most cell types except macrophages. LDL modification alters their affinity for the apoB/E receptor and deviates their metabolism towards scavenger receptor-bearing cells (macrophages and smooth muscle cells), which are progressively transformed in foam cells. The formation of foam cells leads to the formation of chronic inflammation, synthesis of IL-1 and IL-6 cytokines, formation of atherosclerotic plaques and their subsequent rupture and activate thrombogenesis with subsequent development of strokes and infarction (Berliner et al. 1995, Steinbrecher 1999). 4-HNE is able to induce both modification and dysfunction of tyrosine kinase receptors (TKRs) altering cell cycle progression (Liu et al. 1999). It is also able to alter a variety of cell signalling kinases (mitogen-activated protein kinase, protein kinase C β and γ) associated with the activation of transcription factors initiating cellular responses including cell proliferation, inflammatory responses, proteasomal-mediated protein degradation and apoptosis (Leonarduzzi et al. 2004). 4-HNE and acrolein inhibit the activation of NF-κB (Page et al. 1999), while 4-HHE is able to activate NF-κB and induce inflammation (Je, Lee et al. 2004). They also increase the mRNA and protein expression of the pro-apototic adaptors/regulators FasR, FasL, Bax and caspases 1-2-3 and 8, and impair mitochondrial function altering mitochondrial calcium uptake and cytosolic calcium homeostasis, which results in apoptosis (Fig. 2.10) (Kruman et al. 1999).
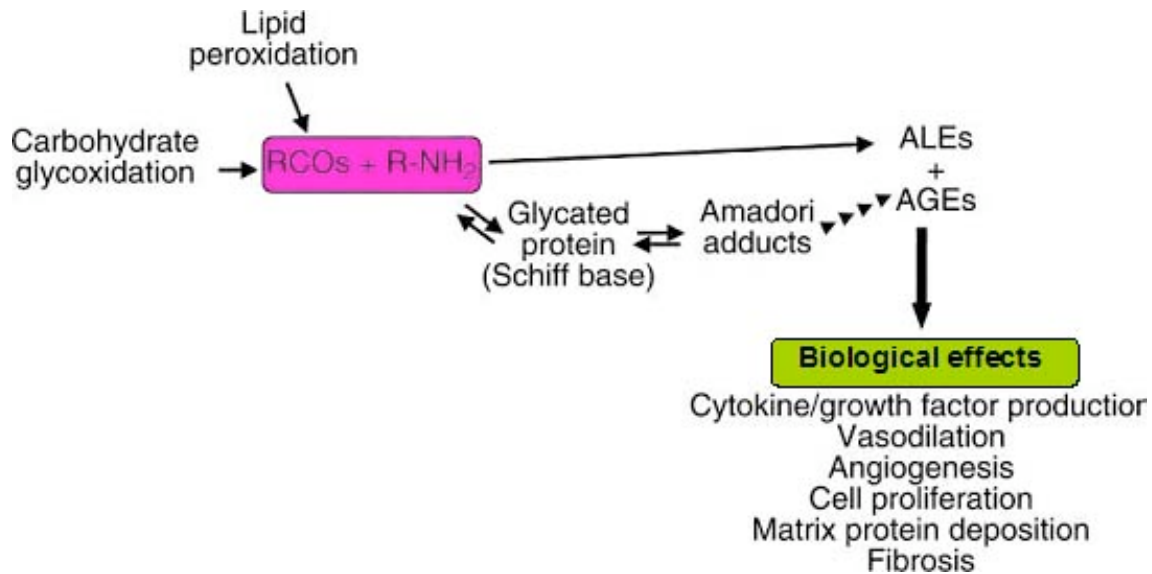
Figure 2.10: Formation and biological effects of advanced glycation end products (AGE) and advanced lipoxidation end products (ALE). R = protein.

2.6.3 AOPP

2.6.3.1 Chemistry of AOPP generation

AOPPs are the dityrosine-containing and cross-linking protein products which were first isolated from plasma of uremic patients (Witko-Sarsat et al. 1996). AOPPs contain abundantly chloramines, dityrosines which allow crosslinking, disulphide bridges and carbonyl groups, and are formed mainly by chlorinated oxidants resulting from myeloperoxidase activity. (Capeillere-Blandin et al. 2004). Activated neutrophils secrete the enzyme myeloperoxidase, which uses H_2O_2 and chloride ions to catalyse the generation of the reactive chlorine species hypochlorous acid. Up to 80% of the H_2O_2 generated by activated neutrophils is used to form $20 - 400$ µM HOCl an hour (King et al. 1997). HOCl can modify proteins in a number of ways, including conversion of cysteine residues into disulphides, conversion of methionine into its sulfoxide, tryptophan oxidation, tyrosine chlorination and dimerization (dityrosines), and lysine and

38

other amino groups (Arg, Pro, Thr) into chloramines (Winterbourn, Kettle , Heinecke 2002). Reaction of chloramines may be of particular biological significance, because these compounds would be expected to diffuse a considerable distance and hence cause damage at remote sites, as a result of their longer lifetimes compared with primary oxidants H_2O_2 and HOCl. Protein carbonyls can form from chloramines via the loss of HCl and hydrolysis of the imine (Hazen et al. 1998). Generation of protein carbonyl derivatives can be elicited by direct oxidative attack in the amino acid side chains or by the modification of side chains with lipid peroxidation products o reducing sugars; thus the carbonyl content of proteins is widely used as a convenient marker of oxidative protein damage during condition of oxidative stress both in vivo and "in vitro" (Stadtman et al. 1998).

Hypochlorous acid-treatment provokes aggregation and fragmentation of a variety of proteins, such as fibronectin, apolipoprotein, ovoalbumin and serum albumin. Intermolecular cross-linking could be attributable to dityrosine formation and the fragmentation could be ascribed to direct reaction of HOCl with the protein backbone (Chapman et al. 2003, Hawkins et al. 2005). HOCl is also able to react both with extracellular matrix (elastin, collagen and proteoglycans) and with intracellular constituents (cytoskeleton proteins, cellular respiration enzymes) generating protein fragmentation and aggregation. As a consequence, vascular matrix components are damaged and cellular adhesion, migration and proliferation compromised, and membrane protein are modified provoking lysis and cell death (Albrich et al. 1986, Pullar et al. 2000, Woods et al. 2003).

The level of antioxidant enzymes in blood plasma is much lower than the intracellular ones, extracellular fluids contain only a small amounts of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase. Therefore ROS released by neutrophils in pathological mechanism, can exert a variety of toxic actions such as tissue damage and oxidative injury. Albumin is the most abundant serum protein with a concentration of about 40 g/l and with a half-life of about 20 days. It possesses a high scavenging activity. The antioxidant activity of BSA might be a direct interaction with ROS (Halliwell

1988). The sulfhydryl (HS-) and the amine (HN-) groups of serum albumin have already been implicated in such antioxidant activity. For instance, Cis-34 in human serum albumin is implicated in the reduction of H_2O_2 and HN- group may react with HOCl to form chloramine (Zgliczyński et al. 1971, Marx et al 1986). Albumin is therefore, highly vulnerable to oxidation and the major representative of circulating advanced oxidized proteins during inflammatory diseases.

AOPP can be formed "in vitro" by exposure of serum albumin to hypochlorous acid (HOCl). In vivo, plasma AOPP are mainly carried by albumin and their concentrations are closely correlated with the levels of dityrosine and carbonyls (Witko-Sarsat et al. 1998).

2.6.3.2 Biological effects of AOPP

Accumulation of AOPP was subsequently found in patients with diabetes (Kalousova et al. 2002), with coronary artery disease (Kaneda et al. 2002) with obesity (Kocak et al. 2007). Recent studies demonstrated that intravenous infusion of AOPP-albumin significantly increases macrophage infiltration in atherosclerotic plaques in hypercholesterolemic rabbits (Liu et al. 2006) and in glomeruli in the remnant kidney model (Li et al. 2007).

HOCl treated albumin and "in vivo" generated AOPP can also trigger oxidative burst in human neutrophils and monocytes "in vitro" (Witko-Sarsat et al. 2003). These data indicate that AOPP are not only the markers of oxidative stress, but a true inflammatory mediator (Witko-Sarsat et al. 1997). Moreover, the intracellular signal transduction processes triggered by AOPP have not been well understood but there are evidences that AOPP could act in two different cellular pathways. "In vitro" and "in vivo" generated AOPP-albumin, as a class of new ligand of RAGE due to the similar characteristics as AGE-modified proteins, are able to damage cells and tissues through a RAGE-mediated signals involving PKC, NADPH oxidase dependent ROS generation, and NF-κB. Induction of pro-inflammatory activities, adhesive molecules and cytokines is even more intensive than that cause by AGE (Guo et al. 2008). At the same time, AOPP-albumin is a high affinity antagonist to the high density lipoprotein (HDL) scavenger receptor class B type I (SR-BI), which acts as the primary

pathway for disposal of HDL-borne cholesterol ester (CE) and triglycerides. The effect is the decrease clearance of HDL, which becomes dysfunctional and pro-inflammatory, and the reduced cholesterol efflux from macrophage that directly contributes to the foam cell formation in the arterial wall. HOCl-modification of HDL and LDL also increases the affinity of AOPP-albumin to SR-BI up to 10 fold. This SR-BI inhibitory activity is not mediated by AGE-like structures (Marsche et al. 2009).

Oxidized proteins have an important role in the induction of cell apoptosis. Studies carried out on human monocytic line, human neuroblastoma cell line and human lung fibroblast have observed that intracellular proteins, synthetized in situ by adding oxidized amino acid such as oxidized phenylalanine and tyrosine in the medium, become proteolytically resistant and accumulate into lysosome compartment. Intra-lysosomal oxidative stress provokes the lysosomal membrane permeabilization (LMP) and the release of lysosomal cathepsins. Cathepsins B and L activate Bax/Bid with consequent creation of pores in the outer mitochondrial membrane resulting in the MMP and release of cytochrome c with activation of the intrinsic way of apoptosis. (Dunlop et al. 2011). In addition, neutrophil-chlorinated taurine, added in the medium of Human B lymphoma cells led to cell death by apoptosis by activating intrinsic pathway, through mitochondrial damaging and caspase 9 activation (Klamt et al. 2005).

2.7 Damage removal and repair systems of oxidized proteins

Damage removal and/or repair systems may be classified as either direct or indirect (Pacifici et al 1991, Davies 1993). Direct repair has so far only been demonstrated for a few classes of oxidized molecules. One important direct repair process is the re-reduction of oxidized sulphydryl groups on proteins. Cysteine residues in proteins are highly susceptible to autoxidation and/or metal-catalyzed oxidation. When two nearby cysteine residues within a protein oxidize they often form a disulphide bond, producing a more rigid protein. Disulphide bonds can also form between two proteins, promoting the formation

of large supramolecular assemblies of inactivated enzymes and proteins; this is called intermolecular cross-linking. Both intramolecular disulphide cross-links and intermolecular disulphide cross-links can be reversed to some extent by disulphide reductases within cells (Davies 1993). Our understanding of such enzymatic reactions is still at an early stage. Another important sulphydryl oxidation process is the oxidation of methionine residues to methionine sulphoxide, typically causing loss of enzyme/protein function. The enzyme methionine sulphoxide reductase can regenerate methionine residues within such oxidized proteins and restore function (Brot et al. 1991)

Indirect repair involves two distinct steps (Davies 1993): first, the damaged molecule (or the damaged part of a molecule) must be recognized and excised, removed, or degraded. Next, a replacement of the entire damaged molecule must be synthesized, or the excised portion of the damaged molecule must be made and inserted. Extensive studies have revealed that oxidized proteins are recognized by proteases and completely degraded (to amino acids); entirely new replacement protein molecules are then synthesized de novo (Davies 1987, Davies 1987, Davies 1987, Marcillat 1988, Giulivi 1993, Grune 2003). It appears that oxidized amino acids within oxidatively modified proteins are eliminated, or used as carbon sources for ATP synthesis. Since an oxidatively modified protein may contain only two or three oxidized amino acids it appears probable that most of the amino acids from an oxidized and degraded protein are re-utilized for protein synthesis. Thus, during oxidative stress, many proteins synthesized as damage replacements are likely to contain a high percentage of recycled amino acids. During periods of particularly high oxidative stress the proteolytic capacity of cells may not be sufficient to cope with the number of oxidized protein molecules being generated. A similar problem may occur in aging, or with certain disease states, when proteolytic capacity may decline below a critical threshold of activity required to cope with normal oxidative stress levels (Pacifici et al.1991). Under such circumstances oxidized proteins may not undergo appropriate proteolytic digestion and may, instead, cross-link with one another or form extensive hydrophobic bonds. Such aggregates of damaged proteins are detrimental to normal cell functions and lead to further problems.

2.8 Oxidative stress in dairy cattle

Under physiological conditions, the body usually has sufficient antioxidant reserves to cope with the production of free radicals, which are produced continuously during metabolism and may increase as a result of pathological and other circumstances (Castillo et al. 2005). However, when free radical generation exceeds the body's antioxidant production capacity, oxidative stress develops.

In dairy cattle, the transition from pregnant, non-lactating state and the non-pregnant, lactating state is especially critical for the animal. This period, termed transition period, begins 2-3 weeks before calving and lasts until 2-3 weeks after calving (Rinaldi et al. 2007). It is characterized by physiological, metabolic and nutritional changes that are often accompanied by the onset of metabolic disorders and the impairment of host immune system.

Parturition and the onset of lactation impose tremendous physiological challenges to the cow's health: the uterus and the mammary gland become very susceptible to infectious diseases, such as metritis, endometritis and mastitis, due to the impairment of host immune function (Fig. 2.11) (Goff et al. 1997).

Neutrophils play an important role in the initial defence of the host against invading microbial pathogens. Impairment of bovine neutrophil recruitment to the site of infection and PMN activity, are influenced by nutritional and endocrine factors.
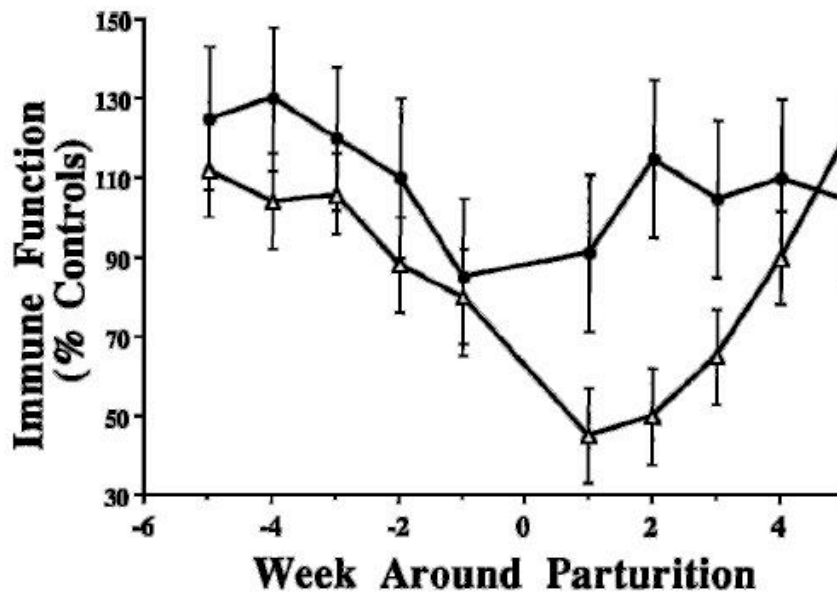
Figure 2.11: Neutrophil function (iodination; △) and lymphocyte function (blastogenesis; ●) are impaired during the weeks immediately before and after parturition. Values are expressed as percentages of control steers (from (Goff, Horst) 1997

During the end of gestation, the parturition and the onset of lactation, the amount of energy that is required for foetus development, maintenance of body tissues and milk production, exceeds the amount of energy that the cow can obtain from dietary sources. This state of negative energy balance could lead to a reduction of dry-matter intake (DMI) and provokes metabolic disorders, such as fatty-liver ketosis, milk fever and hypoglycaemia. Chronic deficiencies of energy are linked to mobilization of lipids, which are released as NEFA (non-esterified fatty acids) from adipose tissue. Elevation of NEFA and suppression of DMI were associated with suppressed PMN myeloperoxidase activity (Fig. 2.12) (Hammon et al. 2006).

44
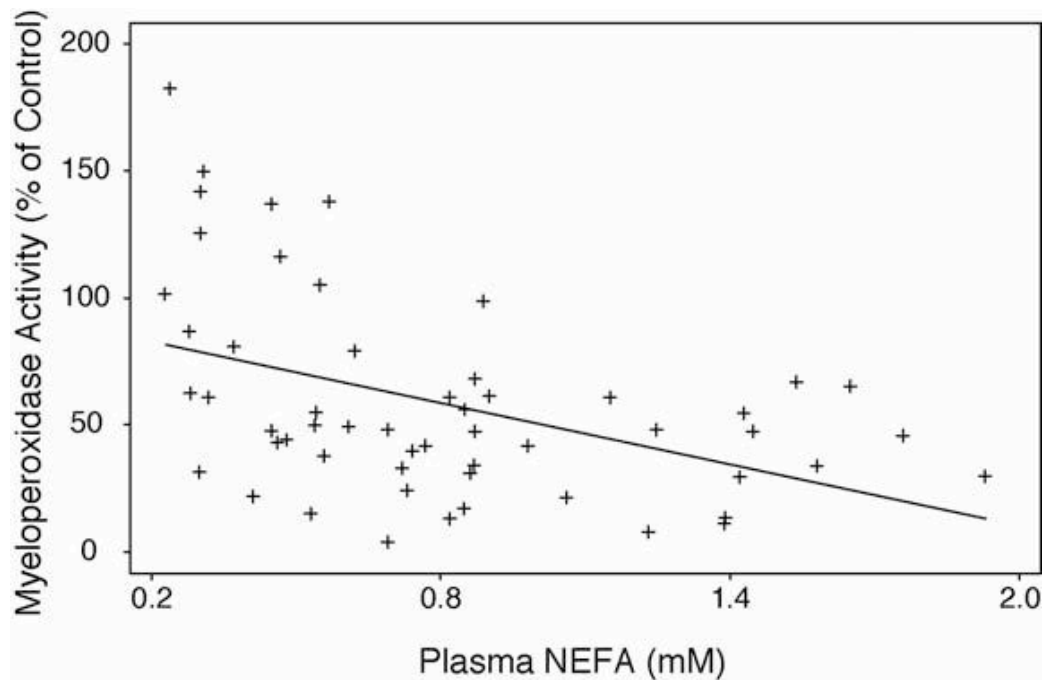
Figure 2.12: Relationship between PMN myeloperoxidase activity and plasma non-esterified fatty acid (NEFA) concentration ($R = 0.44$, $P < 0.001$) (from (Hammon, Evjen et al.) 2006

Moreover, particular endocrine mechanisms regulate gestation and parturition: progesterone is the predominant hormone of pregnancy. Plasmatic levels of progesterone increase steadily until approximately 30 days before parturition and fall precipitously one day prior to calving. Plasma estrogens (17β-Estradiol and estrone) and cortisol, on the contrary, increase rapidly just before calving (Fig. 2.13) (Chew et al. 1977).
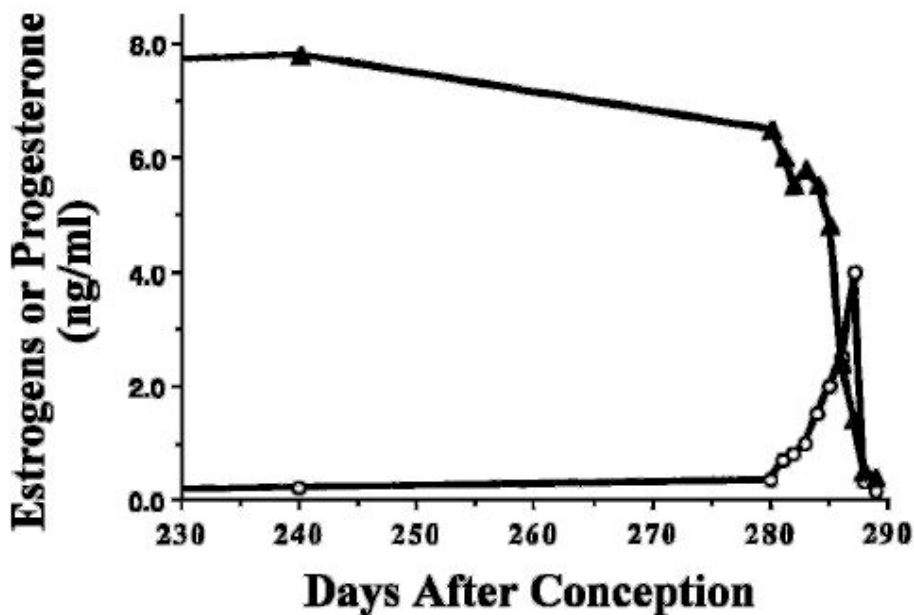
Figure 2.13: Plasma concentrations of estrogens (primarily estrone; O) and progesterone (▲) during late gestation and parturition in the bovine with parturition occurring on d 287 of pregnancy (from (Goff, Horst) 1997

Progesterone, estrogens and glucocorticoids have immunosuppressive effects during the peri-parturient period, in order to prevent rejection of the "foreign" foetus during gestation (Weinberg 1987). Progesterone is able to inhibit lymphocytes activation (Clemens et al. 1979), 17β-estradiol suppresses neutrophil chemotactic ability, influences the diapedesis capacity, affects viability after migration and inhibit their microbicidal activity (Klebanoff 1979, Lamote et al. 2004); Cortisol increases neutrophil survival by suppression of apoptosis but inhibit leukocyte functions and other mechanisms that initiate and amplify inflammation (Cox 1995).

The modern dairy cow is unique in her experience of repeated lifetime cycles of pregnancy and parturition, followed by lengthy lactations producing high volumes of milk. Alteration in host defense mechanisms that occur during peri-partum are associated with changes in hormone profiles and metabolic and physical stress of parturition. These changes may contribute to the high incidence of diseases experienced by the animal (Mallard et al. 1998).

Infection and subsequent inflammation of the reproductive tract and mammary gland compromises animal health, contributes to decrease reproductive efficiency (Coleman et al. 1985) and milk quantity and quality (Seegers et al. 2003), and provokes heavy economic losses in dairy herd. The possibility that oxidative stress during the transition period may be a major underlying cause of inflammatory and immune dysfunction in dairy cattle is supported both in vivo and "in vitro" (Sordillo et al. 2009). In the last few years, the detection of physiological and environmental factors that compromise the cow's immunological defenses, and the protection against them, has become increasingly important in clinical medicine. Several biomarkers can be discovered and monitored in order to assess the oxidative status in dairy cattle during transition period: the most used are metabolic parameters (Glucose, total protein, albumin, urea, creatinine, AST, AP); antioxidant enzymes (SOD, GSH, CAT activity) (Castillo et al. 2003, Sharma et al. 2011); products of lipid mobilization and peroxidation, such as NEFA, triglycerides and MDA; reactive oxygen metabolites (Bernabucci 2002). Reactive oxygen metabolites and oxidative stress generated by increased metabolic demand and/or by neutrophils activation during defence against disease, seem to have a role in the cause and progression of several reproductive events, such as fertilization and embryo development, both in women and in dairy cattle (Guerin et al. 2001). Oxidative stress is also associated with several pathological condition such as retained placenta, udder oedema and mastitis (Miller et al. 1993). The involvement of protein oxidation, in this context has been poorly investigated. Oxidized proteins are often functionally inactive and they leading to accumulation. In addition they may activate the immune reaction and the production of auto-antibodies (Chou et al. 2008). Oxidative damaged proteins could be used as an easy-to use markers of oxidative stress. AOPP, as described previously, are a novel marker of protein oxidation, generated by chlorinated oxidants and able to activate human neutrophil respiratory burst and other pro-inflammatory responses "in vitro". Recent studies have demonstrated that plasma AOPP concentration is significantly greater in dairy cattle with

embryonic mortality respect those with and without pregnancy (Fig. 2.14) (Celi et al. 2011).
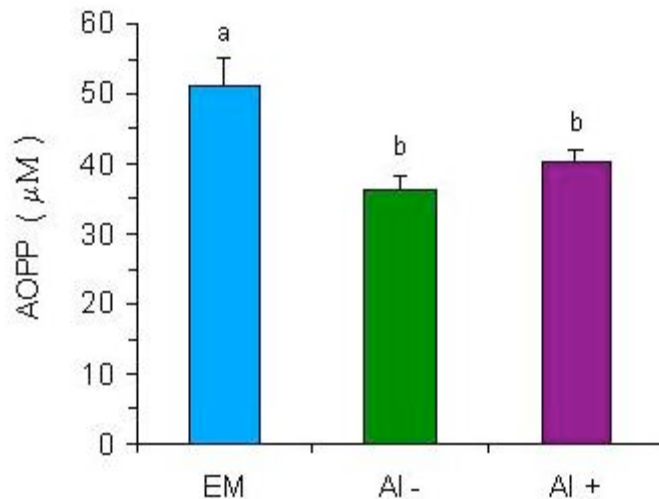
Figure 2.14: Plasma concentration of AOPP in dairy cows following artificial insemination with different outcomes (EM, embryonic mortality; AI-, no pregnancy; AI+, pregnancy). Differentsuperscripts, indicate significantly different means ($P < 0.05$) (from _.

Bovine intrauterine infections contribute significantly to reproductive failure in cattle (Hartigan, Murphy et al. 1972). Intra-uterine infections in cattle have been associated with abortion (Widders, Paisley et al. 1986), early embryonic death (Kaneene, Gibson et al. 1986) and infertility (Studer, Morrow 1978). Understanding uterine defense mechanisms is essential for developing effective treatment and preventative strategies and for optimizing reproductive performance. Polymorphonuclear neutrophils are the predominant phagocytic cell type in the bovine uterus during the follicular phase of the cycle. However, PMNs can reduce fertility through their effects on sperm function and viability (KLUCINSKI, TARGOWSKI et al. 1990) embryo survival and implantation (Waites, Bell 1982). Uterine PMN effusion is known to occur during the follicular phase of the normal bovine estrous cycle. At times other than estrus, the presence of PMNs in the uterine lumen is considered pathological (SKJERVEN 1956). AOPPs levels of clinically healthy cows do not differ during peripartum

because AOPPs are generated during inflammatory processes, where there is a massive recruitment and activation of neutrophils (Fig. 2.15).
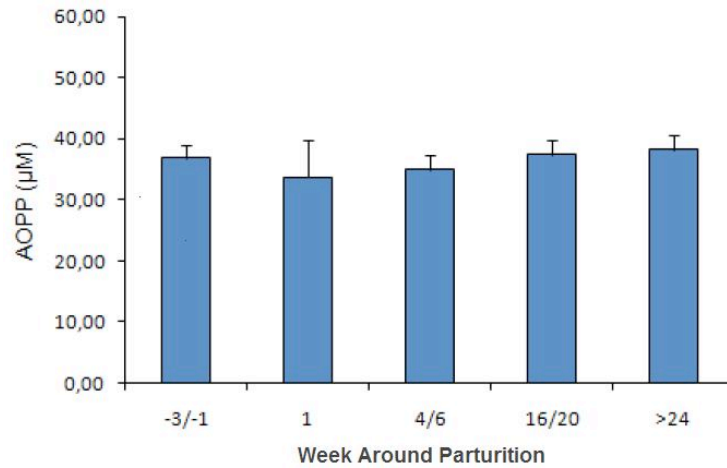
Figure 2.15: Variation of plasma AOPP levels in dairy cattle during different physiological phases.

Indeed studies conducted on cows with acute and chronic inflammations (clinical signs are observed for less than 3 days or for 3 or more days, respectively) shows that plasma AOPP levels are significantly higher in animals with acute inflammation, explaining the pivotal role of neutrophils and innate immune system's cells in the generation of AOPP. Therefore AOPP could be considered a good marker of inflammatory processes (Fig. 2.16) (Da Dalt 2011)
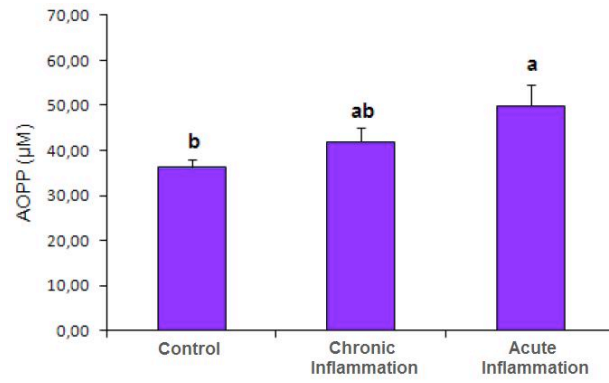
Figure 2.16: Changes in concentrations of AOPP between groups of animals classified on the basis of clinical signs. Different superscripts indicate significantly different averages. (P <0.01).

CHAPTER 3.

"In vitro" production and characterization of AOPP

3.1 Materials and methods

This part of the study aims to produce AOPP "in vitro" in order to evaluate the effects of AOPP on bovine neutrophils activity. Bovine serum albumin (BSA) was used as protein standard because it is the major plasma protein target of oxidative stress and the predominant antioxidant in plasma (Himmelfarb et al 2001, Roche 2008). HOCl was used as oxidant because it is the most powerful compound produced by neutrophils during acute inflammation (Chapman et al. 2003, Stadtman et al. 2003).

3.1.1 Production of standard BSA-AOPP

Commercial NaOCl was used as source of oxidant and the concentration of hypochlorite/hypochlorous acid present, was determined spectrophotometrically using reported extinction coefficient (ε_{290} = 350 M^{-1} cm^{-1}) pH 12. Hypochlorous acid dissociation depends on pH: at pH 10 all chlorine is in the OCl^- form, and the reverse occurs at a 4.5 pH, where all chlorine is in the form of HOCl (Carrel et al. 1966) Therefore at pH 12 the oxidizing solution contains 100% of OCl^- ions and this concentration was measured spectrophotometrically. In this study the term HOCl will be referred to the sum of both species.

BSA (100 mg/ml; Sigma-Aldrich, Milan, Italy) was incubated with increasing concentration of HOCl (0-25-50-75-100 mM) in 50 mM PBS (pH 7.4) for 30 minutes at room temperature (Witko-Sarsat et al. 1998). Then oxidized BSA (1 ml) was separated from the excess of HOCl using a PD-10 Sephadex column (Amersham Pharmacia Biotech, Piscataway, NJ) and eluted with 500 ul of PBS 1X for cell culture (27 mM KCl; 15mM KH_2PO_4; 1,37 M NaCl; 81 mM Na_2HPO_4; pH 7.4) (Hawkins et al. 1998). 30 fractions were collected and the presence of BSA and HOCl presence was monitored spectrophotometrically at 280nm and 290 nm respectively. Also not-oxidized BSA and HOCl alone are loaded into the column and used as control. The aim was to obtain a peak for oxidized BSA and a well separated peak for the unbound HOCl. Fractions representing the peak of BSA were collected and tested for protein content and fractions containing the higher protein concentration were tested for AOPP content, dityrosine levels,

protein integrity and carbonyl groups (Table 3.1). The pH of each fraction was measured with a pH test strip (Sigma-Aldrich, Milan, Italy) in order to check if the neutral range was respected. "in vitro" HOCl-modified BSA was called hereafter as AOPP-BSA.

Oxidized protein	Peak fractions
BSA + HOCl 0 mM	F6-F7
BSA + HOCl 25 mM	F6-F7
BSA + HOCl 50 mM	F6-F7
BSA + HOCl 75 mM	F7-F8
BSA + HOCl 100 mM	F7-F8

Table 3.1: Peak fractions obtained from gel filtration of HOCl oxidized BSA

3.1.2 Measurement of protein concentration

The concentration of BSA in each fraction was determined by Bradford assay following manufacturer's instructions (Bradford reagent; Sigma-Aldrich, Milan, Italy). A calibration curve was constructed, with bovine serum albumin used as a standard (0,25-2 mg/ml). 25 ul of standards or diluted samples (1:10, 1:50, 1:100) were transferred in polystyrene cuvettes (Kartell, 1 cm path length; Milan, Italy) and 750 ul of Bradford reagent was added. Cuvettes were agitated and both standard and samples was incubated for 30 minutes at room temperature. Absorbance was read at 595 nm in a UV-spectrophotometer (Jasco, V-630) and sample values were plotted in the calibration curve. Samples concentration was obtained multiplying the obtained concentration for the opportune dilution factor.

3.1.3 Quantification of AOPP

AOPP concentrations were measured by a spectrophotometric assay first described by Witko-Sarsat (Witko-Sarsat et al. 1996, Witko-Sarsat et al. 2003). Briefly, 200 ul of diluited AOPP-BSA (1:5, 1:20 for each concentration of HOCl)

in 20 mM PBS were placed in a 96-well microplate (Perkin-Elmer Life and Analytical sciences, Shelton, CT, USA) and mixed with 20 ul of acetic acid. In the same plate standard reference wells were prepared, containing 200 ul of chloramine T solution (0-100 umol/L; Fluka, Sigma-Aldrich, Milan, Italy), 10 ul of 1.16 M of potassium iodide (KI; Sigma-Aldrich, Milan, Italy) and 20 ul of acetic acid. The absorbance of the reaction mixtures was immediately read at 340 nm in a microplate reader (Packard Biosciences, CA, USA) against blank containing 200 ul of 20 mM PBS, 10 ul of KI and 20 ul of acetic acid. The chloramine-T absorbance at 340 nm was linear within the range of 0-100 umol/L with a sensitivity of 3.125 umol/L. AOPP concentrations were expressed in umol/L of chloramine-T equivalents/ mg protein.

3.1.4   Measurement of Dityrosine

The formation of dityrosine is an index of oxidant-induced protein cross linking and aggregation. Indeed, AOPP are also defined as dityrosine containing cross-linked protein products (Alderman et al. 2002). Dityrosine content of AOPP-BSA samples, diluted at 1 mg/ml, was calculated spectrophotometrically in polystyrene cuvettes (Kartell, 1 cm path length; Milan, Italy) at 315 nm assuming the absorption coefficient $\varepsilon = 5$ mM^{-1} cm^{-1} at pH 7.5 Results were expressed as umol/ mg protein (Heinecke et al. 2003)

3.1.5   Determination of oxidized protein integrity by SDS-PAGE

Samples of AOPP - BSA, were diluted by the addition of an equal volume of non-reducing sample buffer (Laemmli 2X: 125 mM TRIS-HCl pH 6.8, 4% SDS, 20% glycerol; 0,004% Bromophenol blue; Sigma-Aldrich, Milan, Italy), mixed and boiled at 95°C for 3 minutes. Proteins were then separated using a gradient SDS-PAGE. Two solution of acrylamide-N,N'-methylene-bis- acrylamide (37.5:1; Fluka, Sigma-Aldrich, Milan, Italy) at 4% and 10% were prepared following Leammli protocol (1970) in order to obtain the gradient resolving gel. Stacking

gel was obtained preparing a solution of acrylamide-N,N'-methylene-bis-acrylamide at 3% (Table 3.2).

	Stacking gel 3%	Resolving gel 4%	Resolving gel 10%
H$_2$O	7.790 ml	6.290 ml	4.790 ml
TRIS-HCl	21.250 ml 1M pH 6.8	2.5 ml 1.5M pH 8.8	2.5 ml 1.5M pH 8.8
SDS 10%	0,1 ml	0,1 ml	0,1 ml
Acrylamide	0.750 ml	2.5 ml	2.5 ml
AMPS 10% (ammonium persulphate)	0.1 ml	0.1 ml	0.1 ml
Temed (N, N, N', N'-tetramethylethylenediamine)	0.01 ml	0.01 ml	0.01 ml

Table 3.2: Reagent mixture for gradient SDS-PAGE preparation.

The gradient gel polymerization was performed using a gradient mixer connected with a peristaltic pump (Minipuls 3, Gilson, Middleton,CT, USA). Mixed gradient gels were pumped in a Hoefer SE 245-Dual gel caster (Amersham, Pharmacia, NJ,USA). Samples were loaded at the final concentration of 2 ug/lane and proteins size was detected using a labeled molecular weight markers (11-245 kDa; Genespin, Milan,Italy). The electrophoresis separation was obtained connecting the electrophoresis chamber to the power supply (Amersham Bioscience, NJ, USA) at 30 mA for stacking gel and 60 mA when proteins reached resolving gel. The electrophoresis chamber was filled with running buffer (TRIS-base 0.025 M, Glycine 0.192 M, SDS 0,1%; Sigma-Aldrich, Milan, Italy). Bands were visualized using EZBlue gel staining reagent (Sigma-Aldrich, Milan, Italy) in order to check oxidized protein integrity.

3.1.6 Carbonyl detection by western blot analysis

Carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially of Proline, arginine, lysine, and threonine) where they are oxidized. These moieties are chemically stable, which is useful for both their detection and storage (Berlett et al 1997). Protein carbonyl content is actually the most general indicator and the most commonly used marker of protein oxidation (Chevion et al 2000).

3.1.6.1 Derivatization of sample with Dinitrophenylhydrazine (DNPH)

Highly sensitive assay for detection of proteins involve derivatization of carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable 2,4-dinitrophenylhydrazone product (DNP). The measurement of protein carbonyls following their covalent reaction with DNPH was pioneered by Levine et al. and has become the most widely utilized measure of protein oxidation (Dalle Donne et al. 2003). The availability of commercial antibodies to anti-DNP allows detecting carbonyl groups by immunoblotting analysis. Samples of AOPP-BSA were diluted in 10 mM PBS (0,3 mg/ml) and reacted with a solution containing 12% SDS, and DNPH 20 mM in 10% trifluoroacetic acid (TCA), with the ratio of 1:1:2. In parallel, samples of AOPP-BSA reacted with the same solution without DNPH, and were used as blank controls. Both samples and blanks were incubated for 10 minutes at room temperature. The reaction mixtures were than neutralized with 2M Tris-base containing 30% glycerol and beta-mercaptoethanol. Solutions turned from yellow to orange on neutralization. Samples and relative blanks were mixed and centrifuged at 12.700 g for 1 minute. Supernatants (5 ul/lane) were subjected to SDS-PAGE, electroblotted to a nitrocellulose membrane and immunoassayed for carbonyl content with anti-DNP antibody. Derivatized samples and blanks were loaded in a 4-10% gradient gel as described above (see paragraph 3.1.5).

3.1.6.2 Transfer of proteins

AOPP-BSA, separated by the gradient SDS-PAGE, were transferred into a nitrocellulose membrane (0.45 um; GE healthcare, Uppsala, Sweden) using a trans-blot apparatus (Elettrofor, Rovigo, Italy). The transfer buffer was composed of 25 mM TRIS-Base, 192 mM Glycine, 20% Methanol (Sigma-Aldrich, Milan, Italy), pH 8.3. Transfer parameters were: 250 mA for 2 hours at 4°C. At the end, the membrane was immersed in Red Ponceau solution (0.2% Red Ponceau, 3% trifluoroacetic acid; Sigma-Aldrich, Milan, Italy) for 15 minutes in order to visualize marker and transferred protein bands.

3.1.6.3 Incubation with antibodies

The membrane was firstly washed abundantly with distilled water and incubated overnight at 4°C with the blocking solution (skin milk 3% in 10 mM PBS) in order to occupy non-specific antibody binding sites. Then the membrane was incubated for 1 hour at room temperature with the Rabbit anti-DNP, Primary Antibody (1:35,000; Sigma-Aldrich, Milan, Italy) diluted in T-PBS (10 mM PBS, 0.05% TWEEN 20). The membrane was washed three times with T-PBS solution for 10 minutes and then incubated with the goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:100,000; Bio-Rad Laboratories, Richmond, CA, USA) for 1 hour at room temperature. The membrane was washed three times with T-PBS for 10 minutes.

3.1.6.4 Chemiluminescence reaction

After the last wash, nitrocellulose membrane surface was covered with a solution that contains H_2O_2 and luminol for chemiluminescence reaction (Millipore, Billerica, MA, USA). The solution was discarded after an incubation of 3 minutes and the membrane was exposed to photographic film (GE healthcare, Uppsala, Sweden) for 20 seconds. Bands were visualized in a dark room using developing and fixing solutions (Sigma-Aldrich, Milan, Italy).

3.1.6.5   Densitometric analysis

The photographic film were scanned by ImageScanner (Amersham Biosciences, NJ,USA) and bands were detected and analyzed using the program ImageMaster TotalLab (Amersham Biosciences, NJ,USA). Results were normalized on blanks.

3.1.7 Data analysis

Results were expressed as mean ± SEM. The statistical analysis was carried out by non-parametric ANOVA (Kruskal-Wallis test) followed by bilateral multiple comparisons test.

3.2 Results and discussion

3.2.1 Production of standard BSA-AOPP

BSA (100 mg/ml) was incubated with increasing concentration of HOCl (0-25-50-75-100 mM) in 50 mM PBS (pH 7.4) for 30 minutes at room temperature, and then the excess of free HOCl was removed. In the literature the most used method for the separation of the molar excess of HOCl from "in vitro" oxidized proteins was the dialysis against PBS (Witko-Sarsat et al. 2003). In this study oxidized BSA was filtrated using a Sephadex PD-10 column (Hawkins et al. 1998). Generally, the PD-10 columns were used for desalting, buffer exchange and sample clean up because small molecules are efficiently separated from the high molecular weight substances. This is based on the discrimination of individual sample components by the pores of the packing material: large sample molecules cannot or can only partially penetrate the pores, whereas smaller molecules can access most or all pores, thus, large molecules elute first, smaller molecules elute later. In this study the PD-10 columns were chosen for these purposes: firstly, to separate the molar excess of HOCl, secondly to separate protein dimers from monomers and lastly to obtain a buffer exchange that permit to store the protein in a neutral pH solution of PBS 1X (chapter 4, paragraph 4.1.2) optimal for cell culture. In addition this method permit to monitor the separation of oxidized BSA from free HOCl spectrophotometrically at 280 nm (protein) and 290 nm (HOCl). Oxidized-BSA (66 KDa) was eluted between fraction 5 and 12 (volume fraction between 2,5 and 6 ml) while 100 mM HOCl (52.46 Da) added into the column as control without BSA, was eluted between fraction 14 and 21 (yellow peak; volume fraction between 7,5 and 10.5 ml) as shown in figure 3.4 A. Oxidized-BSA peak width, increases with HOCl concentration and it's due to protein fragmentation: smaller fragments were eluted later from the column. The figure 3.4 B shows peaks of free HOCl separated from oxidized-BSA (fractions 10-24). Unbound HOCl peak high increase with the concentration of oxidant: at low concentrations all free HOCl was consumed during the oxidative processes while at high concentration only

a small part of HOCl remain unbound, if compared to control (yellow peak). The pH of each fraction was measured with a pH test strip in order to check if the neutral range was respected: fractions pH, varied from 6.8 to 7.2.
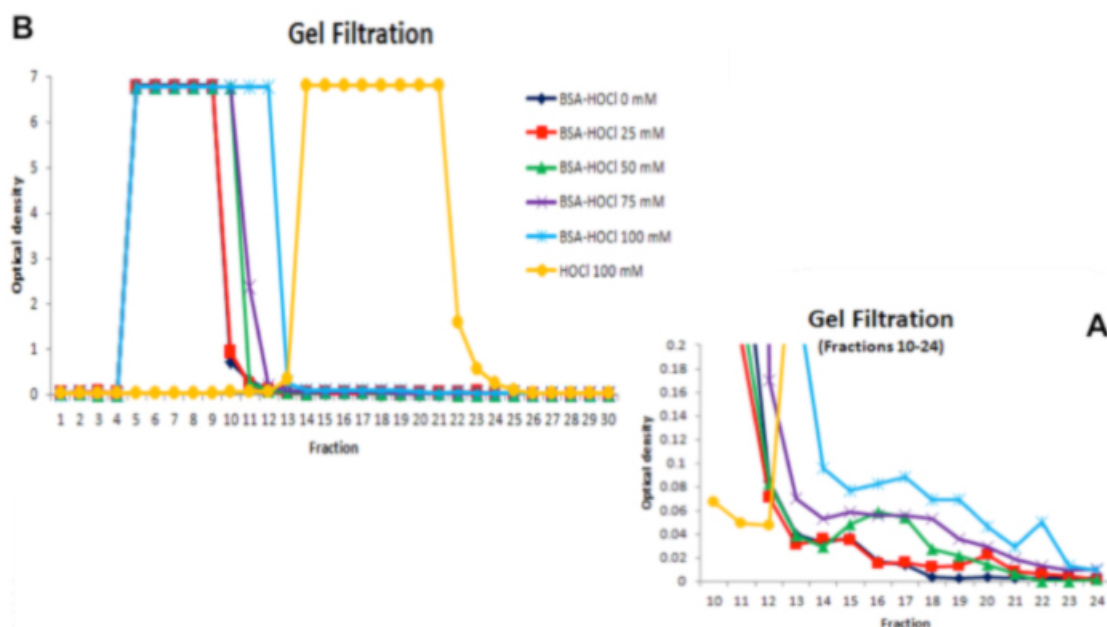
Figure 3.4: Fractions of HOCl-oxidized BSA eluted from PD-10 colums, readed at 280 and 290 nm (A). Graph B represents the detail of free HOCl elution between fractions 10 and 24.

3.2.2 Measurement of protein concentration

The protein content was measured in fractions 5-9 in order to avoid even the slightest presence of unbound HOCl (see figure 3.5 A). The first two fractions containing the highest protein concentrations were used for the analysis of AOPP, dityrosines and carbonyls (see table 3.5 B).
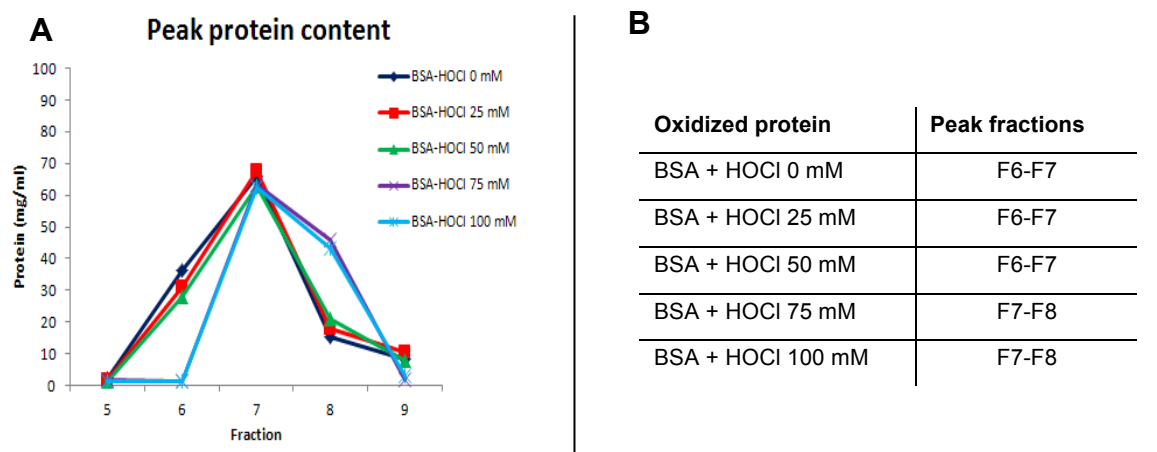
Figure 3.5: Peak fractions obtained from gel filtration of HOCl oxidized BSA

3.2.3 Quantification of AOPP

AOPP levels increase with HOCl concentration in a dose-dependent manner as shown in figure 3.6 B, which represents values of fractions pool. BSA oxidized with 75 mM and 100 mM HOCl presents a concentration of AOPP significantly higher than not oxidized BSA (0 mM HOCl). There were no significant differences between fractions at the same HOCl concentration, as shown in figure 3.6 A.
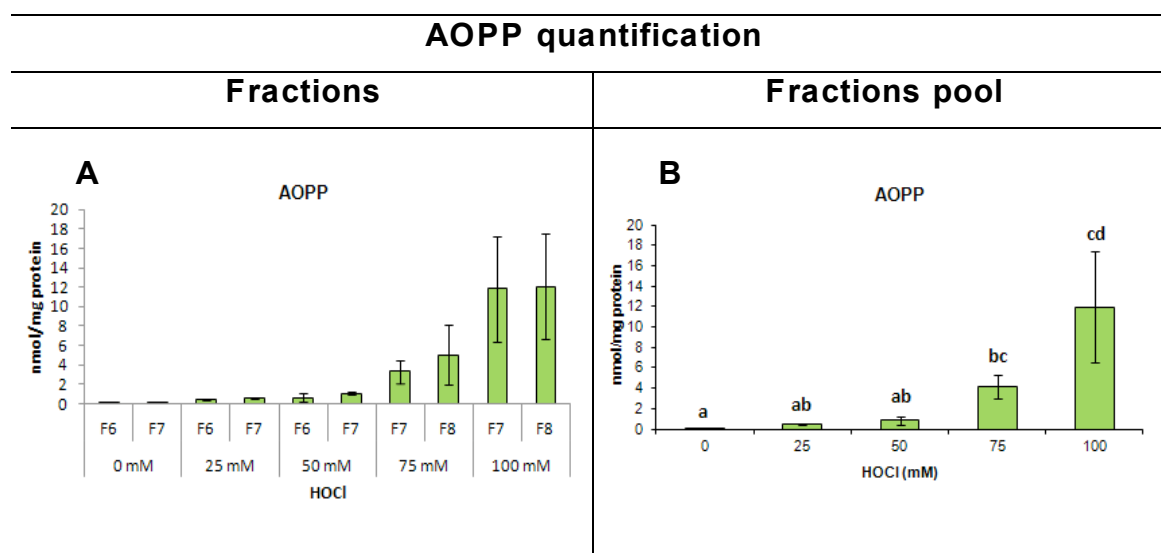
Figure 3.6: Graphs represent AOPP values ± SEM of single fractions (left) and means ± SEM of peak fractions (right), for each HOCl concentration. Statistical significance is indicated by different letters; n=5; p < 0.01.

3.2.4 Measurement of Dityrosine

Dityrosine levels increase proportionally to the HOCl concentration reaching a plateau at 75 mM HOCl. It could be attributable to the saturation reached by dityrosine formation. As shown in figure 3.7 B, BSA oxidized with 75 mM and 100 mM HOCl presents a dityrosine level significantly higher than not oxidized BSA (0 mM HOCl) and BSA oxidized with 25 mM HOCl. In parallel, as shown in figure 3.7 A, dityrosine concentration wasn't significantly different between fractions but, interestingly, dityrosines were noticeably higher in the first fraction respect the second fraction at the same HOCl concentration. The first fraction of the HOCl-modified BSA, subjected to gel filtration, contains protein at a higher molecular weight respect the second fraction, which represent dityrosine cross-linking of proteins.

Dityrosines quantification

Fractions   **Fractions pool**
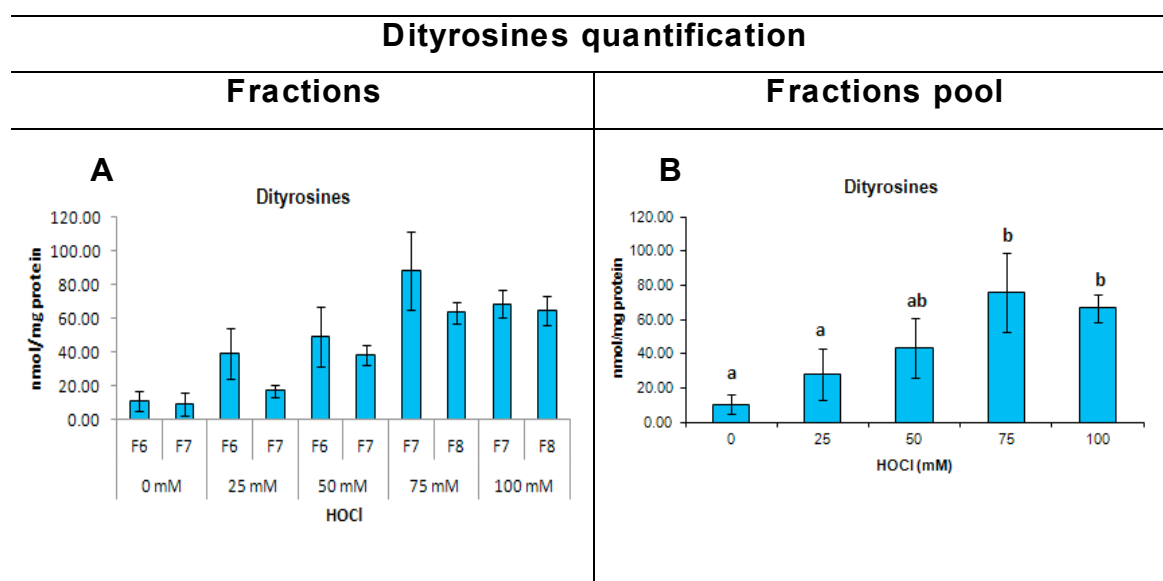
Figure 3.7: Graphs represent Dityrosine values ± SEM of single fractions (left) and means ± SEM of peak fractions (right), for each HOCl concentration. Statistical significance is indicated by different letters; n=5; $p < 0.05$.

3.2.5 Determination of oxidized protein integrity by SDS-PAGE

Samples of AOPP - BSA, were separated using a gradient 4-10% SDS-PAGE. The generation of protein fragments and aggregates were clearly indicated by the presence of the smear at high and low molecular weights, and its intensity increases proportionally to the HOCl concentration as shown in figure 3.8 A and B. Fractions of BSA oxidized with 100 mM HOCl lost completely the native monomeric form. Also fractions of not-oxidized BSA show band of protein fragments and aggregates due to standard impurity but not smears. These results suggest that treatment of BSA with HOCl causes the fragmentation of protein backbone and cross-linking due to oxidation.
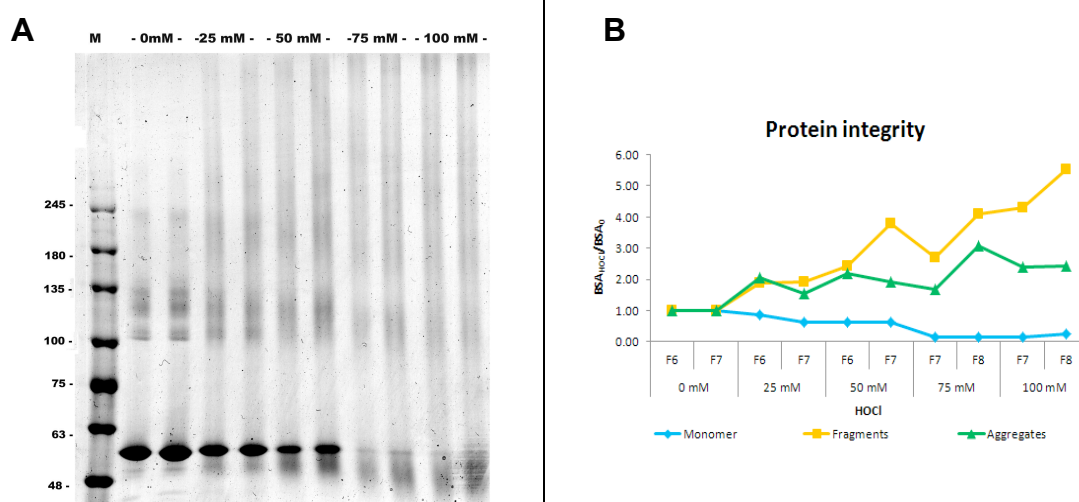
Figure 3.8: Integrity of single fractions of oxidized BSA was examined by 4-10% gradient SDS-PAGE (A). Band intensity of monomers, fragments and aggregates of BSA was measured and plotted in a graph (B). Data are from a single experiment.

3.2.6 Carbonyl detection by western blot analysis

The measurement of protein carbonyls following their covalent reaction with DNPH was pioneered by Levine (Levine et al. 1990). Derivatized samples and blanks were loaded in a 4-10% gradient, electroblotted to a nitrocellulose membrane and immunoassayed for carbonyl content with anti-DNP antibody

(see paragraph 3.1.6) as shown in figure 3.9 A. Carbonyls content, measure as band intensity, increased linearly with HOCl concentration (Figure 3.9 B). At high oxidation levels (75 – 100 mM HOCl) also fragments and aggregates are carbonylated: oxidation generates misfolded proteins that are more susceptible to carbonylation than the native ones.
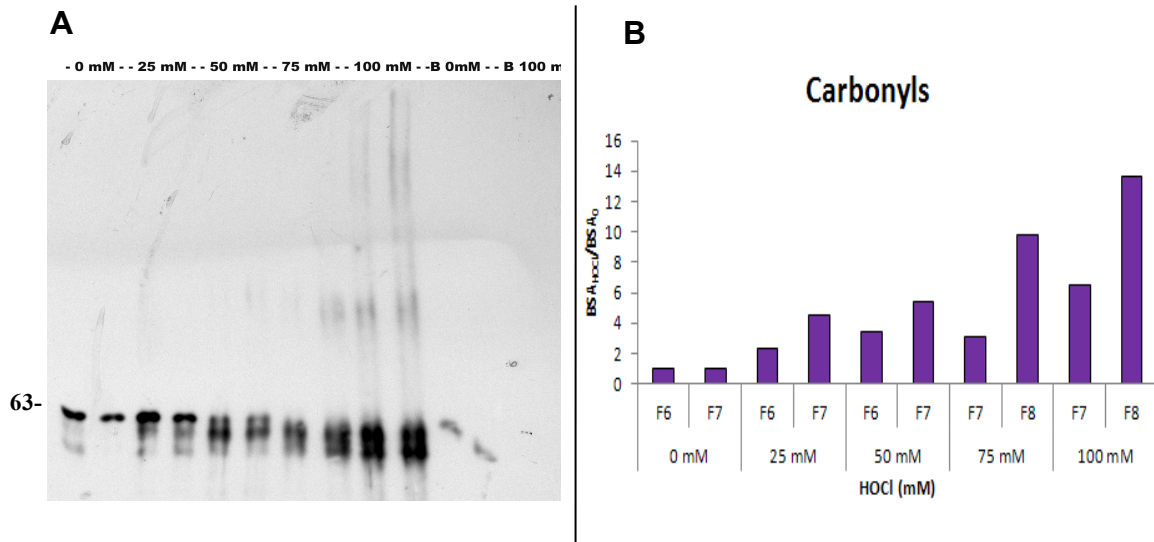
Figure 3.9: Western Blot analysis of single fraction of HOCl-oxidized BSA, for carbonyl detection (A). Bands intensity was measure and plotted in an histogram (B). Data are from a single experiment.

3.3 Analysis of results

Data obtained from the detection of AOPP, dityrosine and carbonyl levels of HOCl-oxidized BSA were plotted and the sample oxidized with the concentration of HOCl that generated a higher level of oxidation in all parameters analyzed and that maintain good protein integrity, was chosen for incubation with neutrophils.

CHAPTER 4.

Set up and validation of the bovine neutrophil isolation protocol and chemiluminescence assay.

4.1 Bovine neutrophil isolation protocol

Assessment of neutrophil functions at laboratory requires a pure population of neutrophils with good viability. Neutrophils are short lived and highly active cells and their isolation requires careful steps to yield a good amount of cells within a shorter period of time. To characterize the specific functions of neutrophils, a high purity, fast and reliable method of separating them from other blood cells is desirable for "in vitro" studies (Maqbool et al. 2011).

In the first part of this study neutrophil isolation protocol was optimized, starting from the conventional Dextran-Ficoll method for human blood in order to harvest a pure population of bovine neutrophils without inducing activation and cellular death. Dextran sedimentation (about 60 min) was removed to obtain a more rapid method (Eggleton et al. 1989). The critical control points of neutrophil isolation protocol that are been checked are: buffering media and temperature.

4.1.1 Animals

Whole blood was collected from jugular vein of clinically healthy dairy cattle of 9-13 months of age; younger cows were avoided because present an immature immune system while older animals encountered a particular hormonal condition, due to puberty, that modulate immune cell functions (Klebanoff 1979). Animals were obtained from the "Lucio Toniolo" experimental farm of the University of Padua.

4.1.2 Reagents

- NaCl 0,9%: 0,9 g of NaCl (Carlo Erba, Milan, Italy) were dissolved in 100 ml distilled water, the pH was adjusted to 7.4 and the solution autoclaved (120°C for 20 minutes).

- Phosphate Buffer Saline (PBS) 1X: A PBS 10 X was prepared dissolving 2g/L KCl (27 mM; Fluka, Sigma-Aldrich, Milan, Italy), 2 g/l KH_2PO_4 (15mM; RP Normapur, Prolabo, Paris, France), 80 g/L NaCl (1,37 M; Carlo Erba, Milan, Italy) and 11,5 g/L Na_2HPO_4 (81 mM; Sigma-Aldrich, Milan, Italy) in 1000 ml of distilled water, the pH was adjusted to 7.4 and

the solution autoclaved (120°C for 20 minutes). For use, the 10X solution was diluted to 1X.

- NaCl 3,6%: 3,6 g of NaCl (Carlo Erba, Milan, Italy) were dissolved in 100 ml distilled water, pH was adjusted to 7.4 and the solution autoclaved (120°C for 20 minutes).

- NH_4Cl – TRIS: a 8,56 g/L NH_4Cl (0,16M; Sigma-Aldrich, Milan, Italy) and a 26,8 g/L TRIS-HCl (0,17M; Sigma-Aldrich, Milan, Italy) stock solutions were prepared. The working solution was obtained mixing 9 parts of the 0,16 M NH_4Cl stock solution and 1 part of the 0,17 M TRIS-HCl stock solution; pH was adjusted to 7.4 and the solution autoclaved (120°C for 20 minutes).

- Ficoll-Paque (premium 1,077 g/L) was purchased from GE healthcare (Uppsala, Sweden).

- HBSS solution without calcium and magnesium was purchased ready to use from Sigma-Aldrich (Milan, Italy).

- Trypan blue solution 0,4% was purchased ready to use from Sigma-Aldrich (Milan, Italy).

- Complete culture medium was obtained by mixing 10 % fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) 25 mM HEPES (Sigma-Aldrich, Milan, Italy), 2 mM L-Glutamine (Gibco Invitrogen; Paisley, Scotland, UK), 100 U/ml Penicillin-Streptomycin (Gibco Invitrogen; Paisley, Scotland, UK), 50 uM beta-mercaptoathanol (Sigma-Aldrich, Milan, Italy), in RPMI 1640 (Gibco Invitrogen; Paisley, Scotland, UK).

- Diff quick staining (Modified Giemsa staining, Sigma-Aldrich, Milan, Italy).

4.1.3 Isolation of bovine neutrophils

In order to maintain the highest neutrophils viability and functionality the period between blood collection and processing time has been reduced to a minimum. In addition, the isolation time was reduced and buffers optimized, because neutrophils were exposed not only to physical manipulation but also to non-physiological condition during the isolation process and this may result in

unwanted effects on basic biological properties of isolated cells (Slama et al. 2006).

Four different protocols were tested as described in the table 4.1.

Protocol	1	2	3	4
Isolation buffer	NaCl 0,9 %	PBS 1X	PBS 1X	PBS 1X
Lysis buffer	H_2O/NaCl 3,6%	NH_4Cl-TRIS HCl	NH_4Cl-TRIS HCl	NH_4Cl-TRIS HCl
Washing buffer	NaCl 0,9 %	PBS 1X	PBS 1X	PBS 1X
Counting solution	NaCl 0,9 %	HBSS	HBSS	HBSS
Isolation temperature	37°	37°	4°	20°

Table 4.1: Description of steps and reagents of bovine neutrophil isolation protocols.

Whole blood (25 ml) was collected in EDTA vacuum tubes because EDTA provided the highest number of isolated neutrophils/ml of blood (Freitas et al. 2008). It was transferred in a new sterile 50 ml tube and diluted with an equal volume of isolation buffer. Ficoll –Paque density gradient (1,077 g/L) was used to separate neutrophils from others leucocytes. Ficoll-Paque solution (15 ml) was transferred into a 50 ml sterile tube. Diluted blood (25ml) was slowly added under Ficoll-Paque solution avoiding mixing. This solution was centrifuged at 1500 g for 30 min at 20°C in order to avoid mononuclear cell contamination as described by manufacturer instructions. After the centrifugation, layers containing plasma and platelets, mononuclear cells and the Ficoll-Paque solution, were discarded. The pellet containing granulocytes and erythrocytes (about 5 ml) was maintained and red blood cells (RBC) were lysed by lysis buffer: in protocol 1, RBC were lysed by adding 4 volumes of sterile distilled H_2O for 20 seconds mixing slowly by inversion, and immediately osmolarity was

restored by adding 1 volume of NaCl 3,6% (Nishihira et al. 1985). In protocols 2-3-4, RBC were lysed by adding 10 volumes of NH_4Cl-TRIS/HCl solution for 5 minutes, mixing slowly by inversion (Chou et al. 2009). Subsequently, in all protocols, neutrophil suspension was centrifuged at 500 g for 10 minutes, the supernatant was discarded and lysis was repeated again. Then the supernatant was discarded and cells were centrifuged and re-suspended three times in a row at 500 g for 10 minutes with washing buffer. Neutrophils were re-suspended with the counting solution and counted in a Burker cell counting chamber, at 20x magnification with an inverted confocal fluorescence microscope IX51 (Olympus, Japan). In addition, neutrophil suspension was incubated with trypan blue solution 0.4% for 2 min at room temperature: cells were counted using a Burker chamber, and trypan blue negative cells were considered as viable cells. Neutrophils suspension (10 ul) was smeared on a glass slide and purity was confirmed by Diff Quick staining (Modified Giemsa staining, Sigma-Aldrich, Milan, Italy). All solutions were maintained under sterile conditions at the isolation temperature established by the protocol (see table 4.1).

4.1.4   Results and discussion

Neutrophils isolated with protocol 1 were very few and with a low viability as shown in figure 4.2. This could be attributable to the RBC lysis step: distilled water is strongly hypotonic and could provoke cell swelling and burst in few seconds, not only of RBC but also of neutrophils. In addition, the rapid restore of the osmotic pressure by adding a hypertonic solution (NaCl 3,6%) could contribute to further damage neutrophils membrane.

Protocol 2 permitted to obtain a high number of neutrophils with a low viability. In this case, the isolation procedure and buffers lead to a separation of a high quantity of neutrophils from other leucocytes but the temperature of 37°C provoked a spontaneous activation of cells during isolation (Wu et al. 1999). Neutrophil oxidative metabolism generated an auto-oxidative cell injury which can produce damage on neutrophil cell membrane (Ferrante et al. 1980).

Protocol 3 assessed neutrophils isolation at 4°C, in order to maintain cell in a quiescent state and to avoid spontaneous activations (Salgar et al. 1994). During the isolation procedures, cells maintained in suspension at 4°C formed many clumps, difficult to disperse, which were discarded. As a consequence, isolated neutrophils were very few but with high viability.

Protocol 4 allowed isolating a high number of cells with high viability. This could be due to the entire procedure that seems to be less stressing for cells: isolation temperature (20 °C) seems to be optimal for neutrophils and buffer solutions (PBS 1X and a mild hypotonic solution) seem to have the correct salinity and osmotic pressure that preserve cell membrane integrity.

The morphological analysis (Diff quick staining) of neutrophils isolated with all protocols indicated a purity level of 94% (Fig. 4.2).
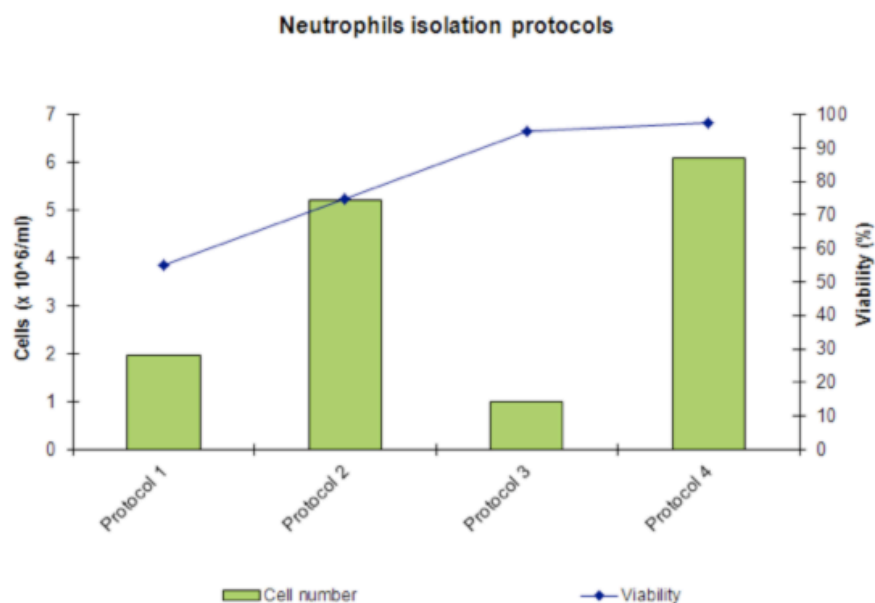
Figure 4.2: Number of isolated neutrophils (from 25 ml of bovine whole blood) and cell viability (Trypan blue staining) of the four isolation protocols.

4.2 Viability assay

Neutrophil viability was also assessed by MTT test. This colorimetric assay measures the reduction of the yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase (Mosmann 1983). The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan. The cells are then lysed and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells and also an index of cell number.

In this study, the aims of the MTT assay were to detect cell viability/number after neutrophils isolation and pre—incubation, in order to choose the best performing protocol, and to understand if chemiluminescence responses were attributable to cell viability or to reaction mixtures and conditions.

4.2.1 Reagents

- HBSS solution without calcium and magnesium, was purchased ready to use from Sigma-Aldrich (Milan, Italy)
- MTT 0,5 mg/ml: 10,5 mg of MTT (Sigma-Aldrich, Milan, Italy) were dissolved in 1 ml of HBSS to obtain the ready to use solution.
- Triton X-HCl solution: 10% TRITON X 100 (Sigma-Aldrich, Milan, Italy) and 0,3 N HCl (Fluka, Sigma-Aldrich, Milan, Italy) were dissolved in distilled water.

4.2.2 Method

Neutrophils isolated with the protocol 1 and 2 (see table 3.1) were centrifuged again at 500 g for 10 minutes, resuspended in complete culture medium, transferred in a sterile 75 cm^2 flask and incubated at 37°C, 5% CO_2 for 24 hours in order to stabilize cells and restore their functions. Then cell suspension was

collected and centrifuged at 500 g for 10 minutes, the supernatant was discarded and the pellet of neutrophils was re-suspended with HBSS at 37°C, counted and plated in triplicates, on 96-well microtiter plates at a final concentration of $1x10^5$/well. Cells were immediately incubated with MTT.

Neutrophils obtained by protocol 3 were too few to test both chemiluminescence and MTT assay.

Neutrophils isolated with protocol 4, at 20 °C without pre-incubation period with complete medium, were plated in triplicates, on 96-well microtiter plates at a final concentration of $1x10^5$/well. Cells were immediately incubated with MTT.

Reaction mixture in the sample wells contained the following reagents at the indicated final concentration (in a final volume of 200 ul): neutrophils 100 ul ($1x10^5$/well), HBSS 100 ul. At the end of the incubation times, MTT (10 ul; 0.5 mg/ml) was added to the samples and the plate was incubated for 4 hours at 37°C in culture hood. Then 50 ul of Triton X-HCl solution was added to the samples in order to lyse cells and to solubilize formazan granules. Plate was covered with aluminum foil and cells were agitated on orbital shaker for 4h. The results were obtained by using Packard Spectracount ELISA plate reader (Packard Biosciences, CA, USA) at the wavelength of 570 nm, with a reference filter of 620 nm (Morgan 1998).

4.2.3 Results and discussion

Data obtained by MTT assay were expressed as optical density (OD) and they could be interpreted both as index of cell viability and as index of cell number. Neutrophils isolated with protocol 4 presented the highest number of viable cells in comparison with the other protocols, as shown in the figure 4.3. The increased mortality of the cells during the 24h culture (protocol 1 and 2) may be the result of intracellular oxidation, which induces the release of proteolytic enzymes from intracellular granules, causing damage to the neutrophils in culture (Urban Chmiel et al 2011). These results demonstrated that pre-incubation of neutrophils isolated with protocol 1 and 2, reduced the number of viable cells and suggested that these protocols weren't optimal to obtain a

higher number of neutrophils with a high viability and functionality, required for the chemiluminescence assay.
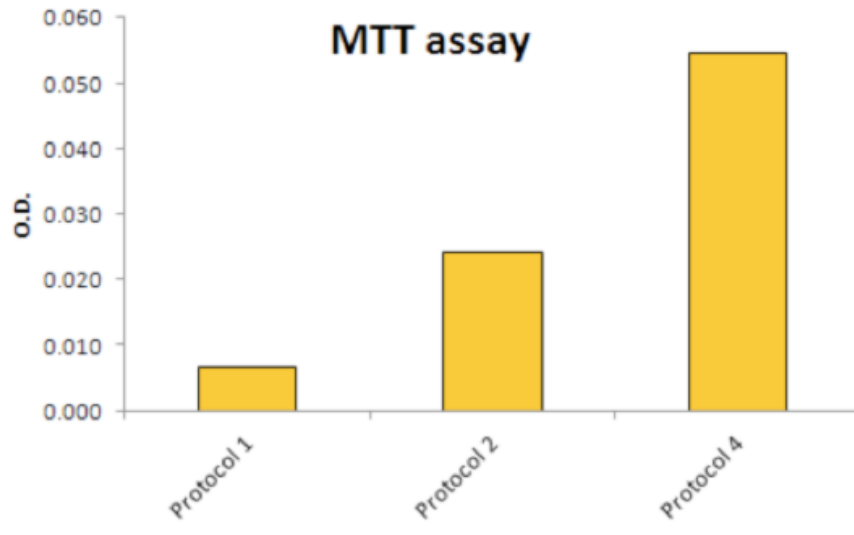
Figure 4.3: This graph highlights the number of viable neutrophils isolated with protocols 1 and 2, immediately after the pre-incubation for 24h at 37°C 5% CO_2, and cell viability of neutrophils immediately after isolation with protocol 4.

4.3 Chemiluminescence assay

The oxidative burst of neutrophils is an important mechanism of defence against pathogens. It is measured by determining individual oxygen metabolites by chemical methods or by measuring chemiluminescence (CL) resulting from a production of reactive oxygen. The generation of CL was originally described by Allen and it was first measured with a liquid scintillation counter (Allen 1972). This method requires a relatively large number of cells and strong neutrophils activation was necessary to generate a measurable signal. Recently, many investigators have employed the compound luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) as a method of amplifying the CL response. Indeed, CL is a natural product of metabolic oxygenation activity (DeChatelet et al. 1982). The light emission is due to the generation of photons from the relaxation of electronically excited substrates molecules after oxidation by reactive oxygen species, i.e. superoxide anion, hydrogen peroxide hydroxyl radical and singlet molecular oxygen. The sensitivity of native CL can be greatly enhanced by the

use of luminol because luminol oxygenation results in a much higher quantum of photons than does oxygenation of native substrates (Lindena et al. 1987). The method becomes extremely sensitive and this make possible to measure the triggering of an oxidative burst in a small number of cells. CL of neutrophils depends on both myeloperoxidase activity and superoxide generation because CL reaction may occur both intracellularly and extracellularly (Albrecht et al 1993). In this part of the study ROS production of isolated bovine neutrophils was measured. PMA, a potent stimulus of the respiratory burst, was used to trigger neutrophils and CL response was amplified by luminol.

4.3.1 Reagents

- HBSS solution without calcium and magnesium, was purchased ready to use from Sigma-Aldrich (H6648)
- PMA (Sigma-Aldrich, Milan, Italy): a stock solution was prepared at the concentration of 0,2 mg/ml in DMSO (Sigma-Aldrich, Milan, Italy).
- Luminol (Sigma-Aldrich, Milan, Italy): a stock solution was prepared at the concentration of 10 mM in DMSO (Sigma-Aldrich, Milan, Italy).

4.3.2 Method

Isolated bovine neutrophils activity was assessed by measuring ROS production. The chemiluminescence assay was set up by determining reagent concentrations and method parameters that permit to obtain a high and stable response. Cell number, PMA and luminol concentrations, incubation times and reading parameters were assessed (Lieberman et al. 1996, Freitas et al. 2009). Neutrophils isolated with protocols 3 and 4, which allowed to obtain a cell viability > 90% (trypan blue exclusion test), were used for chemiluminescence assay. Neutrophils extracted at 4°C and 20°C, without pre-incubation period with complete medium, were plated in triplicates, on a 96-well microtiter plates and their ROS production was tested following chemiluminescence method described in table 4.4.

Method	
Culture medium	HBSS
Cell number	1×10^5/well
PMA (ug/ml)	0-0,0001-0,001-0,01
Luminol (uM)	0-10-50-100
Incubation time (h)	0-2

Table 4.4: Chemiluminescence reaction mixture.

Chemiluminescence reaction mixtures contained reagents at the final concentration indicated in table 4.4 and in a final volume of 210 ul/well: neutrophils 100 ul, PMA 50 ul, HBSS 50 ul, luminol 10 ul. Reaction mixture was added in a black 96-well plate (Perkin-Elmer Instruments, Norwalk, CT), it was subjected to soft agitation and temperature of incubation of 37°C was maintained during the course of the assays. Kinetic readings were started immediately at the end of incubation times analysed. Chemiluminescence signal was monitored every 5 minutes for 15 times in a multilabel reader VICTOR X4 2030 (Perkin-Elmer Instruments, Norwalk, CT). Data were expressed in count per second (CPS) and the result is a chemiluminescence curve. The area under curve (AUC) was also measured in order to detect ROS amount.

4.3.3 Results and discussion

Chemiluminescence profile and AUC obtained from neutrophils isolated with protocol 4 were higher than neutrophils isolated with protocol 3 in both the incubation times tested (Fig. 4.5). These results could be attributable to the isolation temperature. As a matter of fact, a study on equine neutrophils isolated at different temperatures showed that neutrophils processed at 4°C presented a reduced granule release due to microtubule disassociation; in addition cytoplasmic vacuolation has been reported as the earliest sign of degeneration in neutrophils stored at 4°C respect neutrophils processed at room temperature

(Bertram et al. 1982). These effects may explain the low reactivity to PMA of bovine neutrophils isolated at 4°C.

Chemiluminescence response drastically decreased after 2 hours of incubation because the respiratory burst in phisiological condition lasts only 30-60 minutes (Jandl et al. 1978).
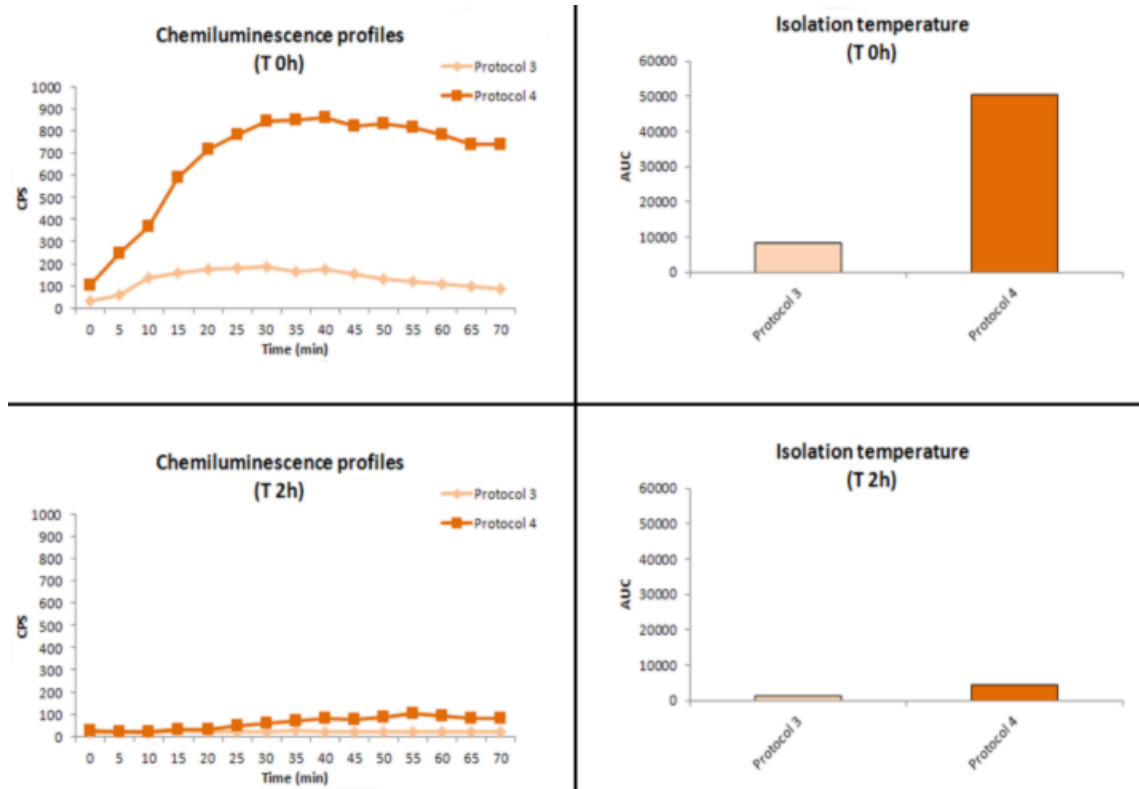
Figure 4.5: Figures represent chemiluminescence profiles and AUC obtained by neutrophils isolated with protocols 3 and 4. Graphs on the right show chemiluminescence profiles neutrophils stimulated with PMA 0,01 ug/ml for 0 and 2 h (Luminol 100 uM). Graphs on the left represent AUC of the respective chemiluminescence profiles.

Results obtained from cell count, trypan blue exclusion test, MTT assay and chemiluminescence suggested that isolation protocol 4 allowed to obtaining a higher number of viable and biologically active bovine neutrophils. Neutrophil isolation protocol 4 was used for the following experiments. The method used preliminarily for chemiluminescence was then modified in order to increase the light signals. For this purpose number of cells and reagent concentrations were increased as described in table 4.6.

Method		
Culture medium	HBSS	HBSS
Cell number	3×10^5/well	5×10^5/well
PMA (ug/ml)	0-0,1-1-10	0-0,1-1-10
Luminol (uM)	0-100-500	0-100-500
Incubation time (h)	0	0

Table 4.6: Validation of chemiluminescence reaction mixture, based on increasing cell number.

Chemiluminescence reaction mixtures contained reagents at the final concentration indicated in table 4.6 and in a final volume of 210 ul/well: neutrophils 100 ul, PMA 50 ul, HBSS 50 ul, luminol 10 ul. Reaction mixture was added, in triplicates, in a black 96-well plate (Perkin-Elmer Instruments, Norwalk, CT), it was subjected to gentle mixing and an incubation temperature of 37°C was maintained during the course of the assays. Kinetic readings started immediately at the end of incubation time. Chemiluminescence signal was monitored every 5 minutes for 15 cycles. Data were expressed in count per second (CPS) and the result is a chemiluminescence curve. The area under curve (AUC) was also measured in order to detect the ROS amount.

Results described in figure 4.7 suggested that the highest chemiluminescence signal was obtained with 3×10^5 PMA-stimulated neutrophils and amplified with 500 uM Luminol. A proportional increase of chemiluminescence with cell number wasn't observed.
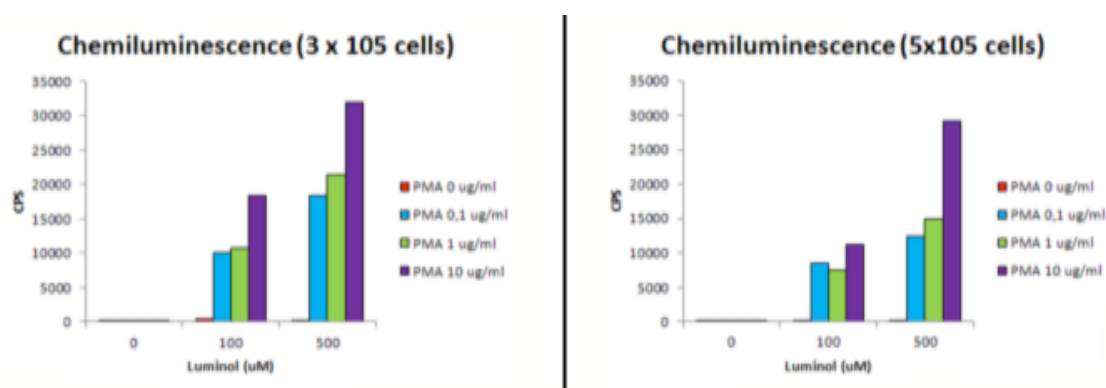
Figure 4.7: Graphs represent the AUC of chemiluminescence profiles of 3×10^5 and 5×10^5 neutrophils stimulated with PMA (0 – 10 ug/ml). Light signal was amplified by Luminol (0-100-500 uM).

CHAPTER 5.

Incubation of AOPP-BSA with bovine neutrophils

5.1 Materials and methods

AOPP formed "in vitro" behaved as potent mediators of human monocytes and neutrophils activation, triggering their respiratory burst. While AOPP, in vivo, are considered a novel mediators of inflammation, notably involved in the monocyte activation state associated with uremia. In this study AOPP, produced "in vitro" by oxidizing BSA with HOCl, were incubated with bovine neutrophils: ROS production and cell viability were assessed.

5.1.1 Bovine neutrophils isolation

Bovine whole blood (25 ml) was collected in EDTA vacuum tubes. It was transferred in a new sterile 50 ml tube and diluted with an equal volume of PBS 1X (2.7 mM KCl, 15mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4; pH 7.4). Ficoll –Paque density gradient (15 ml, 1,077 g/L; GE healthcare, Uppsala, Sweden) was transferred into a 50 ml sterile tube. Diluted blood (25ml) was slowly added under Ficoll-Paque solution avoiding mixing and centrifuged at 1500 g for 30 min at 20°C as described by manufacturer instructions. After the centrifugation, the upper layers were discarded. The pellet containing granulocytes and erythrocytes (about 5 ml) was maintained and red blood cells (RBC) were lysed by adding 10 volumes of NH_4Cl-TRIS/HCl solution (0,16M NH_4Cl, 0,17M TRIS-HCl, ration 9:1, pH 7.4) for 5 minutes, mixing slowly by inversion. Subsequently neutrophil suspension was centrifuged at 500 g for 10 minutes, the supernatant was discarded and lysis was repeated again. Then the supernatant was discarded and cells were washed three times at 500 g for 10 minutes with PBS 1X. Neutrophils were resuspended with the HBSS solution without calcium and magnesium (Sigma, Milan, Italy) and counted in a Burker cell counting chamber, at 20x magnification with an inverted confocal fluorescence microscope IX51 (Olympus, Japan). Neutrophil viability was assessed with trypan blue solution 0,4% (Sigma-Aldrich, Milan, Italy). Neutrophils suspension (10 ul) was smeared on a glass slide and purity was confirmed by Diff Quick staining (Modified Giemsa staining, Sigma-Aldrich,

Milan, Italy). All solutions were maintained under sterile conditions at room temperature (20°C).

5.1.2 Chemiluminescence assay

Neutrophils NADPH oxidase and myeloperoxidase-dependent oxygenation activities were measured by chemiluminescence using a multilabel reader VICTOR X4 2030 (Perkin-Elmer Instruments, Norwalk, CT) as described in paragraph 4.3.2. Luminol was used as chemiluminogenic substrate because it is small and lipophilic, it can diffuse a cross membranes and reacts with both intracellular and extracellular production or release of NADPH oxidase-dependent O_2^- and MPO-dependent H_2O_2 and HOCl (Allen et al. 1976, Albrecht et al. 1993).

Fractions 7 and 8 of BSA oxidized with HOCl 75 mM (called AOPP-BSA) were pulled and used to evaluate the effects of oxidized BSA on bovine neutrophils. Fractions 6 and 7 of BSA oxidized with HOCl 0 mM (called BSA) were pulled and used as control. Two groups of experiments were then set up in order to analyse luminescence activities of neutrophils un-stimulated or stimulated with PMA (Sigma-Aldrich, Milan, Italy) and incubated with AOPP-BSA. The difference between the two experiments was in the adding order of the reagents as described in table 5.1.

Order	Experiment 1	Experiment 2
1	Cells ($3x10^5$/well)	PMA (0 – 0,1-1 ug/ml)
2	PMA (0 – 0,1- 1 ug/ml)	BSA/AOPP-BSA (4 mg/ml)
3	BSA/AOPP-BSA (4 mg/ml)	Luminol (500 uM)
4	Luminol (500 uM)	Cells ($3x10^5$/well)

Table 5.1: Reagents concentrations and addition order used for chemiluminescence experiments.

Chemiluminescence reaction mixtures contained reagents at the final concentration indicated in table 5.1 and in a final volume of 210 ul/well:

neutrophils 100 ul, PMA 50 ul, BSA/AOPP-BSA 50 ul, Luminol 10 ul. Reagents was added, in triplicates, in a black 96-well plate (Perkin-Elmer Instruments, Norwalk, CT), it was subjected to soft agitation and temperature of incubation of 37°C was maintained during the course of the assays. Chemiluminescence signal was monitored every 5 minutes for 30 times (3 total hours, including instrument reading times). Data were expressed in count per second (CPS) and the result is a chemiluminescence curve. The area under curve (AUC) was also measured in order to detect ROS amount.

5.1.3 AOPP-BSA and myeloperoxidase-hydrogen peroxide system

A cell free system was set up in order to evaluate possible interference of AOPP-BSA with components of the neutrophil light generation system. In this study the chemiluminescence of the system $MPO-H_2O_2-Cl^-$ was assessed. The reaction mixture contained reagents at the indicated final concentration and in a final volume of 200 ul/well: BSA/AOPP-BSA (4 mg/ml) 50 ul, Luminol (500 uM) 50 ul, MPO (250 ng/ml) 50 ul, H_2O_2 (50-5000 uM) 50 ul. Reagents were added, in triplicates, in a black 96-well plate (Perkin-Elmer Instruments, Norwalk, CT), it was subjected to soft agitation and temperature of incubation of 37°C was maintained during the course of the assays. Chemiluminescence signal was monitored every minute for 30 times. Data were expressed in count per second (CPS) and the result is a chemiluminescence curve. The area under curve (AUC) was calculated by the Trapezium method.

5.1.4 AOPP and bovine neutrophil viability

The LDH leakage assay and the MTT assay are the most common employed for the detection of cytotoxicity or cell viability. The LDH leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble

tetrazolium salt, which is converted to an insoluble purple formazan that accumulates in healthy cells. The reduction of MTT can also be mediated by NADH or NADPH within the cells and out of mitochondria (Fotakis et al. 2006).

In this study, these assays were used to measure neutrophils viability after incubation with AOPP. 100 ul of freshly isolated bovine neutrophils ($3x10^5$) were plated in triplicates in a 96-well plate, stimulated with 50 ul of PMA (0-0,1-1 ug/ml) and incubated with 50 ul of BSA and AOPP-BSA (4 mg/ml) for 3 hours at 37°C in a humidified incubator.

5.1.4.1 Lactate dehydrogenase assay (LDH)

Cytotoxicity induced by AOPP-BSA was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following incubation of 3 hours, the neutrophils suspension was aspirated and centrifuged at 500 g for 10 min in order to obtain a cell free supernatant. The assay is based on the reduction of pyruvate to lactate in the presence of LDH with parallel reduction of NADH at 25 °C. The formation of NAD^+ from the above reaction results in a decrease in absorbance at 340 nm as described by Mitchell (Mitchell 1980). The absorbance reduction is proportional to the LDH concentration in the medium.

A standard curve was constructed diluting a stock solution of LDH (64,5 UI/ml) in 8 scalar concentrations: 0, 0.06875, 0.1375, 0.275, 0.343, 0.6875, 1.375, 2.75 UI/ml in 0.1 M potassium phosphate buffer, pH 7.4 (KH_2PO_4; Sigma-Aldrich, Milan, Italy). The reaction mixture contained reagents at the indicated final concentration: 1880 ul potassium phosphate buffer (0,1 M, pH 7.4), 70 ul sodium pyruvate (22,7 mM; Sigma-Aldrich, Milan, Italy), 30 ul of NADH (14 mM; Sigma-Aldrich, Milan, Italy), 20 ul of each dilution of LDH added at last. Reagents were transferred in a polystyrene cuvette (Kartell, 1 cm path length; Milan, Italy). Absorbance was read at 340 nm immediately (T0) and after 5 minutes (T5). A standard curve (equation y= ax + b) was obtained plotting in abscissa LDH concentrations (UI/ml) and in ordinate the absorbance variation ΔOD from readings executed (calculated as the difference between the optical density at T5 and the OD at T0).

The culture medium obtained from bovine neutrophils un-stimulated and stimulated with PMA (0.1, 1 ug/ml), incubated with BSA/AOPP-BSA (4 mg/ml) for 3 hours at 37°C, was assessed for the presence of cytosolic LDH. The reaction mixture contained reagents at the indicated final concentration: 1850 ul potassium phosphate buffer (0,1 M, pH 7.4), 70 ul sodium pyruvate (22.7 mM; Sigma-Aldrich, Milan, Italy), 30 ul of NADH (14 mM; Sigma-Aldrich, Milan, Italy), 50 ul of culture medium of each sample added at last. Reagents were added in a polystyrene cuvette (Kartell, 1 cm path length; Milan, Italy). Absorbance was read at 340 nm immediately (T0) and after 5 minutes (T5). The absorbance variation ΔOD from readings executed was calculated and plotted in the standard curve in order to obtain the concentration of LDH (UI/ml) in each sample.

5.1.4.2 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT)

The MTT assay is based on the protocol described for the first time by Mossmann (Mosmann 1983). At the end of the incubation time (3 hours), 10 ul of 0.5 mg/ml of MTT were dispensed in the 96-well plate and neutrophils were incubated for 4 h. Then 50 ul of Triton X-HCl solution (10% TRITON X 100, 0.3 N HCl; Sigma-Aldrich, Milan, Italy) was added to the samples in order to lyse cells and to solubilize formazan granules. Plate was covered with aluminum foil and cells were agitated on orbital shaker for 4h. The plate was read in a Packard Spectracount ELISA plate reader (Packard Biosciences, CA, USA) at the wavelength of 570 nm, with a reference filter of 620 nm. Absorbance values are proportional to the cell viability (Morgan 1998).

5.1.5 Data analysis

Results were expressed as mean ± SEM. The statistical analysis was carried out by non-parametric ANOVA (Kruskal-Wallis test) followed by bilateral multiple comparisons test.

5.2 Results and discussion

5.2.1 Chemiluminescence assay

The effects of AOPP-BSA incubated with un-stimulated and PMA stimulated neutrophils were assessed by measuring ROS production. This assay permitted to measure both intracellular MPO-dependent formation of H_2O_2 and HOCl and extracellular NADPH oxidase-dependent superoxide anion formation (Allen et a1. 1976, Albrecht et al. 1993).

Two groups of experiments were set up and chemiluminescence profiles are reported in figure 5.2. These experiments differ by the order of addition of reagents, as described in paragraph 5.1.2. Chemiluminescence profiles show differences between experiments: during EXPERIMENT 1, a pre-incubation interval of about 10 minutes occurred between the addition of cells and "Time 0" (beginning to readings). This interval leaded to a lower chemiluminescence signal during the first 40 minutes of incubation. It can be hypothesized that both BSA and AOPP-BSA in the culture medium reacted with ROS making them less available for luminol, which was added as the last reagent in the reaction mixture. Possible interactions between reagents (PMA, proteins and Luminol) were excluded, because EXPERIMENT 2 gave different results. Indeed, chemiluminescence profiles in EXPERIMENT 2, where neutrophils were added 15 seconds before "Time 0", chemiluminescence profiles of controls, BSA and AOPP-BSA show the same starting point and generate two peaks (Fig. 5.2 D, F). In the litherature, chemiluminescence profiles of triggered neutrophils were obtained with continuous readings for approximately 30 minutes. Neutrophils stimulated with fMLP showed an initial peak, found within 2 minutes which is attributable to the extracellular ROS production and a second peak at ca. 10 minutes is attributable to the intracellular ROS production. Otherwise, PMA-stimulated neutrophils present a single peak reached around 20 minutes, indicating that extracellular reactions with luminol are of importance early, whereas intracellularly reactions predominate later on in the response. (Briheim et al. 1983, Dahlgren et al. 1984). The lack of a positive feedback in

the litherature permits only to suppose that the two chemiluminescence peaks obtained in this study over a longer period, by PMA-stimulated bovine neutrophils could be attributable to the extracellular ROS production, where luminol reacts with ROS present in the medium (first peak) and the intracellular ROS production, where luminol enter into the cell and react with reactive oxygen species present into granules (second peak).

EXPERIMENTS 1 and 2 gave the same interesting results: neutrophils stimulated with PMA (0.1 ug/ml and 1 ug/ml) and incubated with AOPP-BSA showed a reduced chemiluminescence signal in comparison with controls and neutrophils incubated with BSA.
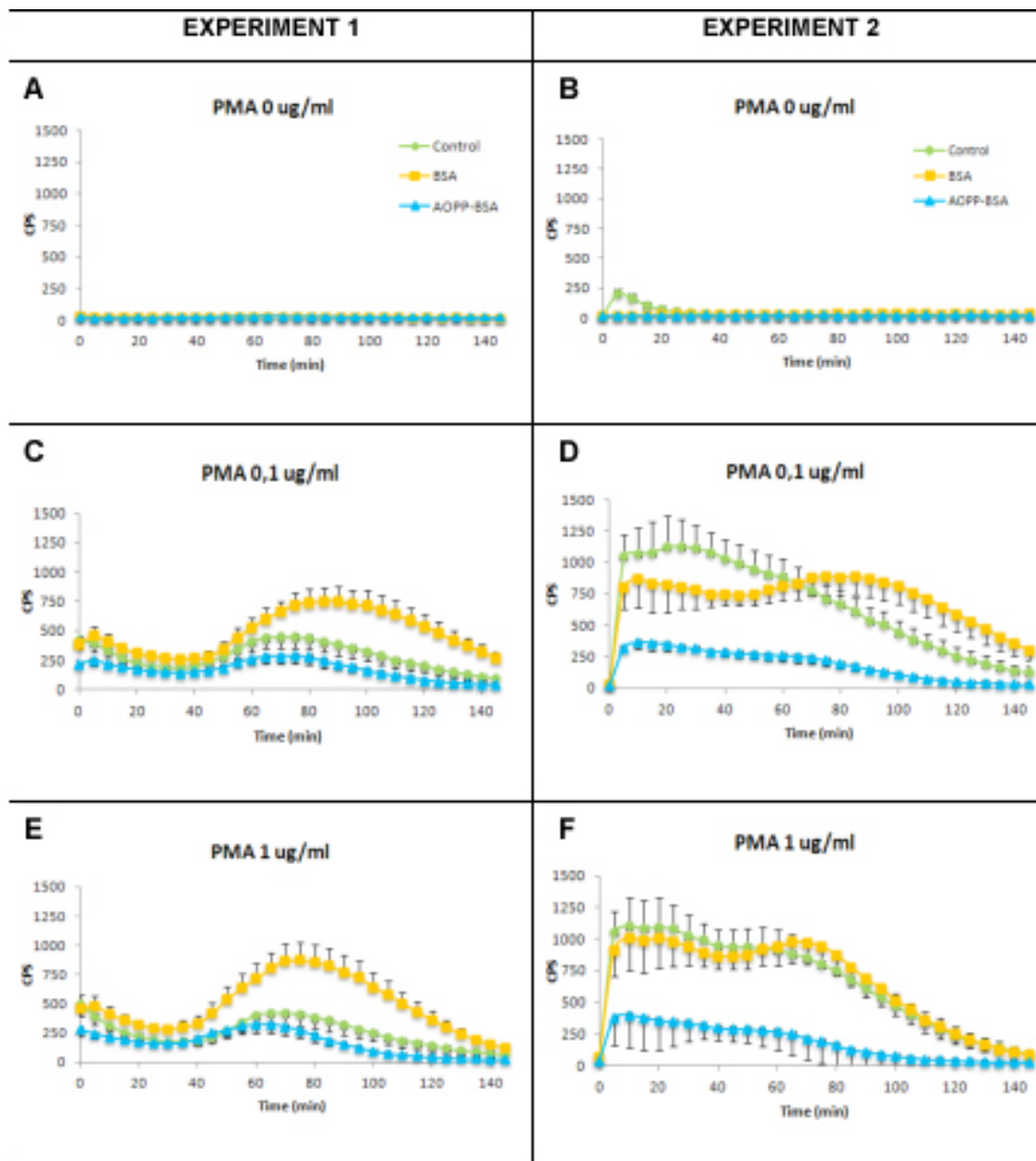
Figure 5.2: Chemiluminescence profiles of freshly isolated bovine neutrophils un-stimulated and stimulated with PMA (0.1, 1 ug/ml), incubated with BSA and AOPP-BSA following protocols of EXPERIMENT 1 (graphs A,C,E) and EXPERIMENT 2 (Graphs B,D,F). Controls represent neutrophils un-stimulated and stimulated with PMA without protein in the medium. Data are expressed as mean ± SEM of 10 experiments, performed in triplicate. a,b, p<0.05.

For each chemiluminescence profile, the area under curve (AUC), the higher luminescence value (CPS max) and the time of maximum CPS achievement (T max) were calculated and represented in figures 5.3 and 5.4. In EXPERIMENT 1, un-stimulated cells incubated with BSA, AOPP-BSA or without protein in the

medium (control) show no differences in each parameter considered (Fig. 5.3 A, D, G). The AUC obtained from PMA-stimulated neutrophils incubated with AOPP-BSA is significantly lower (p< 0.01) respect to PMA-stimulated neutrophils incubated with BSA, no differences was obtained respect the control (figure 5.3 B, C). These results indicate that PMA-stimulated neutrophils incubated with AOPP-BSA produced a significantly lower amount of ROS. CPS values give the same results indicating that the chemiluminescence profile with the higher CPS value has the higher AUC. Also for this parameter, PMA-stimulated neutrophils incubated with AOPP-BSA present a chemiluminescence curve with a significant lower CPS max value (p<0.01) respect PMA-stimulated neutrophils incubated with BSA, no differences was obtained respect the control (figure 5.3 E,F). No differences were obtained for the Tmax value (Fig. 5.3 G, H, I).
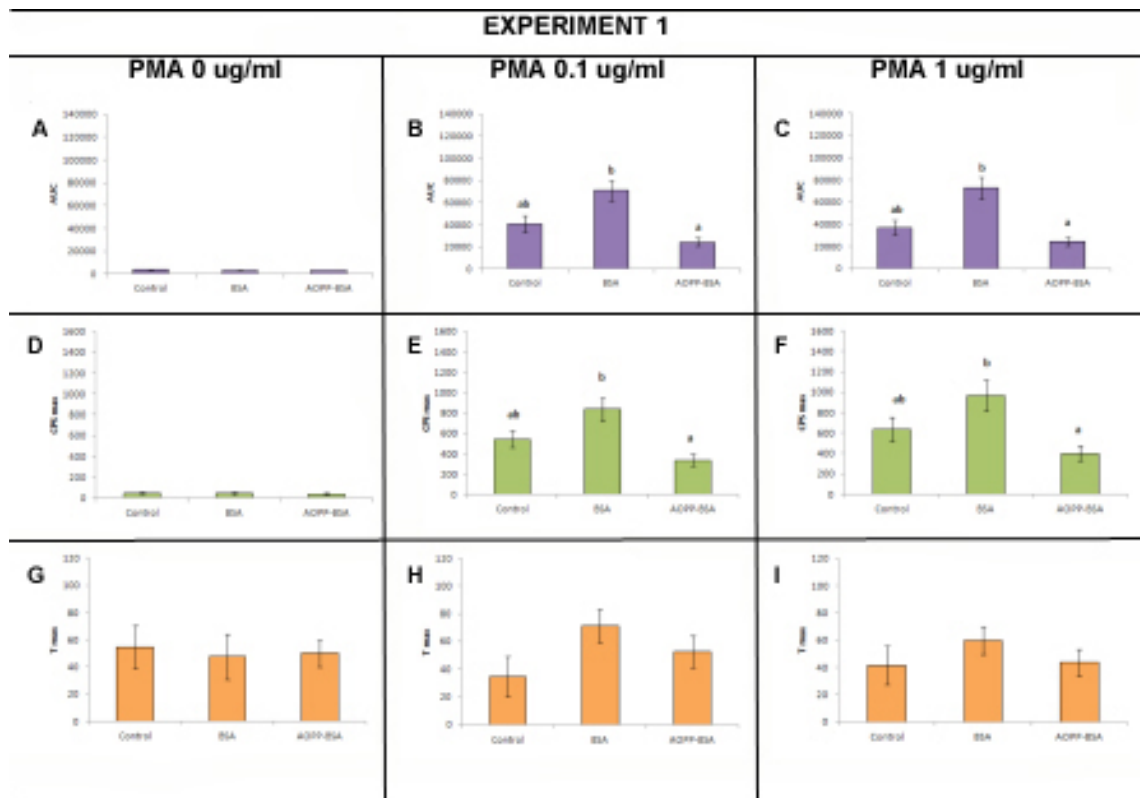
Figure 5.3: Graphs represents the area under curve (A,B,C), CPS max (D,E,F) and Time max (G,H,I) extrapolated from the analysis of chemiluminescence curves obtained from freshly isolated bovine neutrophils un-stimulated and stimulated with PMA (0.1, 1 ug/ml), incubated with BSA and AOPP-BSA following protocols of EXPERIMENT 1. Controls represent

neutrophils un-stimulated and stimulated with PMA without protein in the medium. Data are expressed as mean ± SEM of 10 experiments, performed in triplicate. a,b, $p<0.05$.

EXPERIMENT 2 gave similar results for the three parameters considered. The AUC obtained from un-stimulated and PMA-stimulated neutrophils incubated with AOPP-BSA is significantly lower than both un-stimulated ($p<0.05$) and PMA-stimulated neutrophils ($p<0.01$) incubated with BSA and, the control (figure 5.4 A,B,C). Un-stimulated neutrophils seem to undergo a mild activation due to the contact with the microplate plastic, and this may explain the differences between the AUC value of un-stimulated neutrophils incubated with BSA and the control in comparison with AOPP-BSA (Fig. 5.4 A).

CPS max values of the control are significantly higher ($p<0.001$) than the values of un-stimulated neutrophils incubated with AOPP-BSA due the triggering effects of plastic well surfaces (Fig 5.4 D). PMA-stimulated neutrophils incubated with AOPP-BSA presented a chemiluminescence curve with a significantly lower CPS max value ($p<0.01$) respect PMA-stimulated neutrophils incubated with BSA and the control (Fig. 5.4 E, F).

T max values showed differences only in un-stimulated neutrophils: controls presented the peak at a significantly lower time ($p<0.01$). The highest CPS was observed earlier in respect of un-stimulated neutrophils incubated with BSA and AOPP-BSA (Fig. 5.2 B and 5.3 G).

Figure 5.4: Graphs represents the area under curve (A,B,C), CPS max (D,E,F) and Time max (G,H,I) extrapolated from the analysis of chemiluminescence curves obtained from freshly isolated bovine neutrophils un-stimulated and stimulated with PMA (0.1, 1 ug/ml), incubated with BSA and AOPP-BSA following protocols of E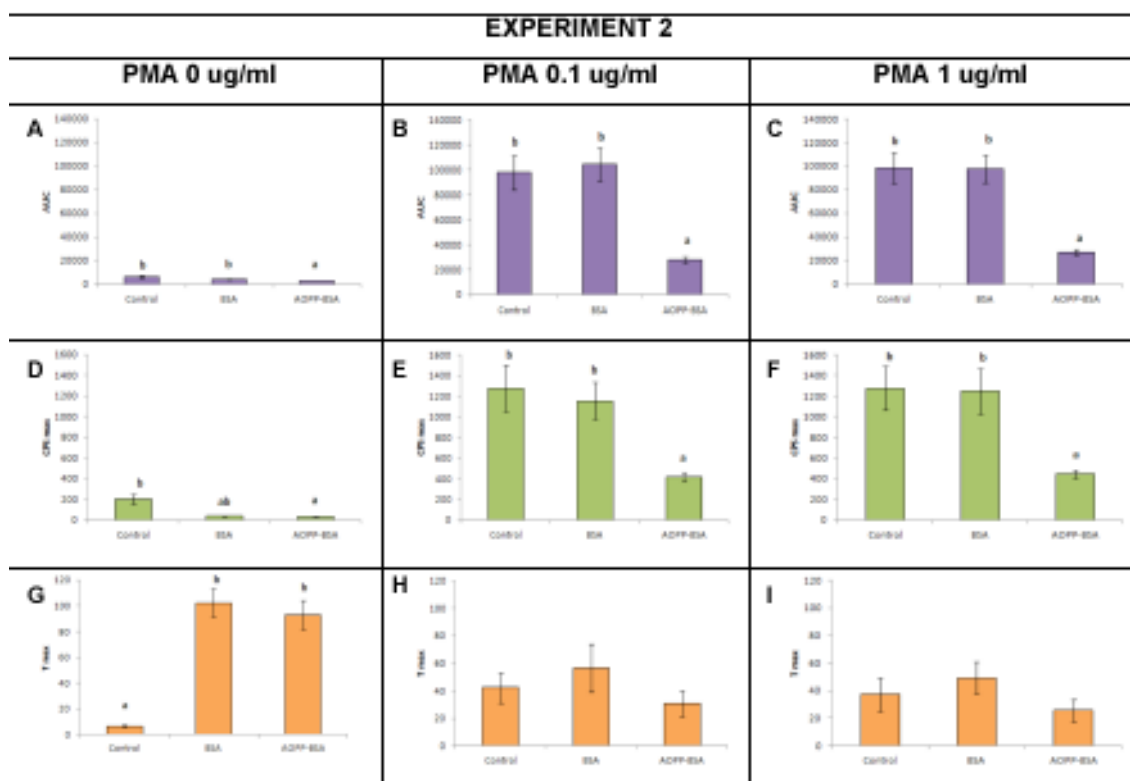XPERIMENT 2. Controls represent neutrophils un-stimulated and stimulated with PMA without protein in the medium. Data are expressed as mean ± SEM of 7 experiments, performed in triplicate. a,b, p<0.05.

In conclusion, the chemiluminescence curves obtained from both un-stimulated and PMA stimulated neutrophils, incubated with BSA, AOPP-BSA and without any protein in the medium (control) have a similar shape, but the AUC showed that the incubation with AOPP-BSA can reduce the ROS production.

5.2.2   AOPP-BSA and myeloperoxidase-hydrogen peroxide system

In order to exclude putative interferences of AOPP-BSA with the mere chemiluminescence reaction, a MPO-H_2O_2-Cl^- system was used as a source of HOCl and the production of light was assessed by luminol amplified chemiluminescence as described in paragraph 4.3.2 Data were expressed as

the area under the curve obtained incubating MPO with increasing concentration of hydrogen peroxide and with BSA or AOPP-BSA or HBSS (control). Results suggested that BSA and AOPP-BSA generate a similar chemiluminescence profile and the relative AUC were not significantly different. In this conditions, MPO produced a very low amount of HOCl that both BSA and AOPP-BSA, which is not completely oxidized, are able to bind (Fig. 5.5).
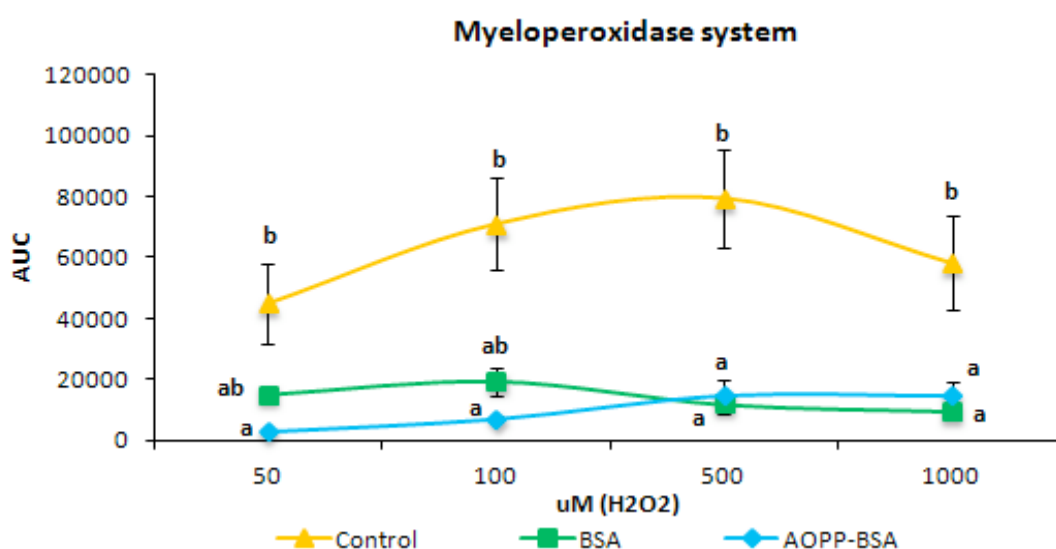
Figure 5.5: The graph represent the area under the curve of chemiluminescence profiles obtained incubating MPO with H_2O_2 and with BSA or AOPP-BSA or HBSS (control). Data are expressed as mean ± SEM of 5 experiments performed in triplicate. a,b, $p<0,05$.

Therefore, BSA and AOPP-BSA behave in the same manner in the MPO-H_2O_2-Cl^- system. It's possible to hypothesize that the differences observed between neutrophils incubated with BSA or AOPP-BSA cannot be attributable to interferences with the light production mechanisms.

5.2.3 AOPP and bovine neutrophil viability

Cell viability was measured in order to investigate the reduced production of ROS of un-stimulated and PMA-stimulated neutrophils incubated with BSA and

AOPP-BSA for 3 hours. For this purpose, the LDH leakage assay and the MTT assay were used.

5.2.3.1 Lactate dehydrogenase assay (LDH)

Cytotoxicity induced by AOPP-BSA was assessed by lactate dehydrogenase (LDH) leakage as describe in paragraph 5.1.4.1. Results obtained haven't revealed significant differences as shown in figure 5.5. Therefore it's possible to hypothesize that the reduction of chemiluminescence produced by PMA-stimulated neutrophils incubated with AOPP-BSA is not attributable to cell lysis.
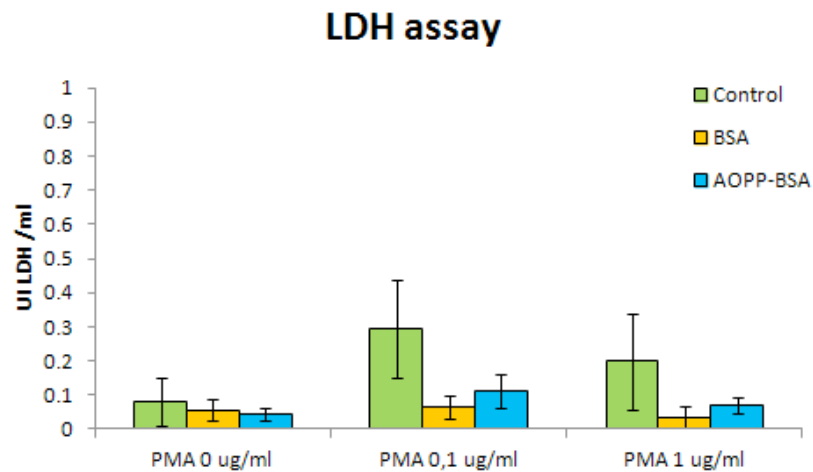
Figure 5.5: Effects of BSA and AOPP-BSA on LDH release in the medium of un-stimulated and PMA-stimulated neutrophils incubated at 37°C, for 3 hours in a humidified incubator. Controls represent neutrophils un-stimulated and stimulated with PMA without any protein in the medium. Data are expressed as mean ± SEM of 7 experiments.

5.2.3.2   3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT)

The results obtained with MTT assay, demonstrated that AOPP-BSA can significantly ($p < 0.05$) reduce the number of viable PMA-stimulated neutrophils (at both concentrations used) in comparison with the incubation with BSA. No significant differences were obtained in un-stimulated neutrophils (Fig. 5.6).
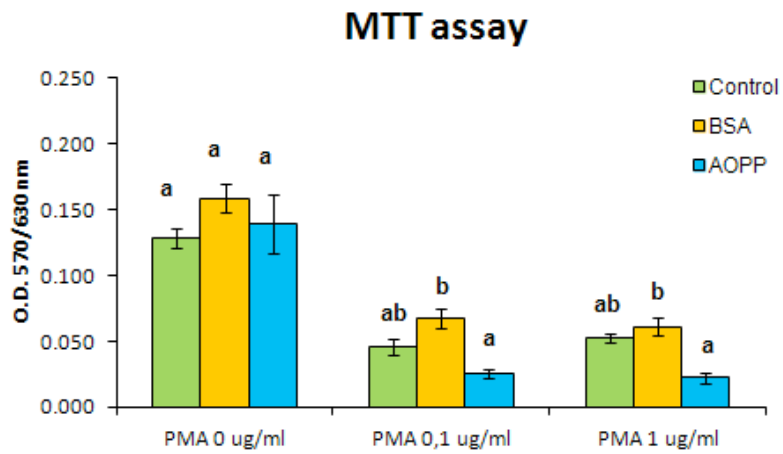
Figure 5.6: Effects of BSA and AOPP-BSA on the MTT assay in un-stimulated and PMA-stimulated neutrophils incubated at 37°C for 3 hours in a humidified incubator. Controls represent neutrophils un-stimulated and stimulated with PMA without any protein in the medium. Data are expressed as mean ± SEM of 6 experiments. a,b, $p<0.05$.

CHAPTER 6.

AOPP induction of bovine neutrophil apoptosis

6.1 Materials and methods

This part of the study was designed to determine the contribution of AOPP-BSA to neutrophils viability and loss of functions, apoptotic events could be involved. In the literature, Zhou et al. (2009) shown that the accumulation of AOPP induces apoptosis in murine podocytes by activation of the p53-Bax–caspase-3 pathway (Zhou et al. 2009). Dunlop (Dunlop et al. 2011) demonstrated that proteins containing oxidized amino acids (among these tyrosine) are able to induce the apoptosis in human monocytes through cytochrome c release and caspase 3 activation; Klamt and Shecter (Klamt et al. 2005) have seen that neutrophil-oxidized taurine chloramine induces apoptosis in human B lymphoma cells provoking mitochondrial loss of permeability and caspase 9 activation.

6.1.1 Experimental conditions

Bovine neutrophils ($2x10^6$) un-stimulated and stimulated with PMA (1 ug/ml) were incubated with BSA/AOPP-BSA (4 mg/ml) for 1-3-6 hours at 37°C. Un-stimulated neutrophils incubated with BSA (4 mg/ml) and analysed at time 0 was used as negative control. Un-stimulated neutrophils incubated with Staurosporine (1 uM, Sigma-Aldrich, Milan, Italy) a pro-apoptotic agent and BSA (4 mg/ml) and analysed at time 6 was used as positive control (Perianayagam et al. 2002, Brets et al. 2004). Reaction mixtures were prepared as described in table 6.1 in a 96-well plate.

	Unstimulated	PMA-stimulated	Positive control	Negative control
Neutrophils	100 ul	100 ul	100 ul	100 ul
BSA/ AOPP-BSA	50 ul	50 ul	50 ul (only BSA)	50 ul (only BSA)
PMA	—	50 ul	—	—
Staurosporine	—	—	50 ul	—
HBSS	50 ul	—	—	50 ul
Time (h)	1-3-6	1-3-6	6	0

Table 6.1: Reagents and experimental conditions of neutrophil apoptosis experiments

6.1.2   Western Blot analysis of Caspases 8,9 and 3

Apoptosis occurs through at least two overlapping pathways referred to as "extrinsic" and "intrinsic". The extrinsic pathway is activated through ligand binding to death receptors (members of the tumor necrosis factor receptor superfamily) at the cell surface and activation of initiator caspase-8. In the intrinsic pathway, mitochondria play a pivotal early role by releasing cell death signals into the cytosol and activating caspase-9 (Fulda et al. 2001). A common feature of the activation of the intrinsic apoptotic pathway is a sudden increase in the mitochondrial permeability transition (MPT), leading to a decrease in membrane potential ($\Delta\psi$m) and causing mitochondrial swelling (Gree et al. 2004. Both pathways converge and activate the executioner caspase-3, which then cleaves intracellular protein substrates and causes cell death (Korsmeyer et al. 2000).

6.1.2.1   Neutrophil protein extraction

Treated neutrophils ($2x10^6$), at the end of incubation times, were pelleted at 500 g for 10 minutes at 4°C. Pellet was resuspended with 100 ul of ice cold protease inhibitor buffer (10 mM Tris-base, 150 mm NaCl, 5 mM EDTA, 10 mm NaF, 10 ug/ml aprotinin, 10 ug/ml leupeptin, 1 mM PMSF, pH 7.4; Sigma-Aldrich, Milan, Italy) (Tsang et al. 2010). Neutrophils were frozen and thawed twice for 5 seconds in liquid nitrogen in order to disrupt cell membranes by thermal shock (Porcelli et al. 2004). Cell Suspension was then centrifuged at 12.000 g for 10 minutes and protein content was determined by Bradford (see paragraph 3.2.2) (Yan et al. 2004). Proteins form cell lysates were diluted with an equal volume of sample buffer 2X (Sigma-Aldrich, Milan, Italy), boiled at 95°C for 3 minutes and separated by 12% SDS-PAGE (10.725 ml H_2O, 6,250 ml TRIS-HCl 1.5 M, pH 8.8, 0.250 ml SDS, 7.5 ml acrylamide, 0.250 ml AMPS 10%, 0.025 ml TEMED; Sigma-Aldrich, Milan, Italy); the upper stacking gel contain 3% of acrylamide (7.790 ml H_2O, 1.250 ml TRIS-HCl 1 M, pH 6.8, 0.100 ml SDS, 0.750 ml acrylamide, 0.100 ml AMPS 10%, 0.010 ml TEMED; Sigma-Aldrich, Milan, Italy). Proteins were subsequently transferred into a nitrocellulose membrane (0.45 um; GE healthcare, Uppsala, Sweden) using a trans-blot apparatus (Amersham Biosciences, NJ, USA) as described in Chapter 3, paragraph 3.2.6.2.

6.1.2.2   Incubation with antibodies

The membrane was firstly washed abundantly with distilled water and incubated for 1h at room temperature with the blocking solution (skin milk 3% in 10 mM PBS) in order to occupy non-specific antibody binding sites. Then the membrane was incubated overnight at 4°C in agitation with the Rabbit anti-caspase 8 specific for cleaved form of ~ 32 KDa (Biovision, Mountain View, CA, USA), rabbit anti-caspase 9 specific for cleaved form of ~ 36 KDa (Biovision, Mountain View, CA, USA) and rabbit anti-caspase 3 specific for cleaved form of ~ 18 KDa (Abcam, Cambridge, UK) Primary Antibodies (1:1,000) diluted in T-PBS (10 mM PBS, 0.5% TWEEN 20). The membrane was washed four times with T-PBS solution for 10 minutes and then incubated with the goat anti-rabbit

horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Richmond, CA, USA) diluted 1:10.000 for caspases 8 and 9 and 1:50.000 for caspase 3 for 1 hour at room temperature as manufacturer's instructions. The membrane was washed four times with T-PBS for 10 minutes and a final wash with PBS 10 mM was done in order to eliminate Tween-20.

6.1.2.3   Chemiluminescence reaction

After the last wash, nitrocellulose membrane surface was covered with a solution containing H_2O_2 and luminol for chemiluminescence reaction (Millipore, Billerica, MA, USA). The solution was discarded after an incubation of 3 minutes and the membrane was exposed to photographic film (GE healtcare, Uppsala, Sweden) for 5 minutes. Bands were visualized in a dark room using developing and fixing solutions (Sigma-Aldrich, Milan, Italy). The photographic film were scanned by ImageScanner (Amersham Biosciences, NJ,USA) and bands were detected and analized using the program ImageMaster TotalLab (Amersham Biosciences, NJ,USA).

6.1.3   DNA Laddering

Neutrophil apoptosis was also determined by characteristic fragmentation of DNA, which was isolated using the Apoptotic DNA Ladder Kit (Roche Diagnostic Systems, Branchburg, NJ, USA) following manufacturer's instructions and the experimental condition described in paragraph 6.1. Briefly, $2x10^6$ neutrophils in 200 ul were collected at the end of every incubation time tested, and were transferred in a tube. Then, 200 ul of Binding/Lysis buffer were added to the samples and the positive control, supplied by the kit. The suspensions were mixed and incubated for 10 minutes at room temperature. After the 10 minutes incubation, 100 ul of isopropanol were added to samples and kit positive control. Then filter tubes were combined to the collection tubes (kit supplied), samples and positive controls were pipetted into the upper reservoir. Tubes were centrifuged at 5500 g for 1 minute, the elute was discarded, 500 ul of Washing buffer were added in the upper reservoir and the tubes were centrifuged at 5500

g for 1 minute. This step was repeated again, and finally tubes were centrifuged for 10 seconds at maximum speed to remove residual washing buffer. Collection tubes were than discarded and filter tubes were inserted in a clean 1.5 ml tube. In order to elute DNA, 200 ul of pre-warmed (70°C) Elution Buffer were added to the filter tube and centrifuged for 1 minute at 5500 g. The DNA solutions obtained were immediately stored at -20°C.

6.1.3.1 DNA quantification

DNA sample (1 ul) was added to 100 ul of distilled water, mixed and transferred in a quartz cuvette (HELLMA GmbH & Co., Müllheim, Germany). The absorbance was read at 260 and 280 nm and the 260/280 ratio was calculated. The amount of DNA was then calculated by multiplying OD_{260} absorbance value by 100 (dilution factor) and by 50, assuming 1 unit od A_{260} = 50.0 ng/uL dsDNA (Haque et al. 2003).

6.1.3.2 Separation of DNA fragments

In cells undergoing apoptosis, a fraction of nuclear DNA is fragmented. When this DNA is analyzed by agarose gel electrophoresis it generates the characteristic "ladder" pattern of discontinuous DNA fragments. This pattern of DNA degradation generally serves as a marker of the apoptotic mode of cell death (Gong et al. 1994).

Agarose-DNA gel 1% was prepared dissolving 1 g of agarose (Sigma-Aldrich, Milan, Italy) in 100 ml of TBE Buffer (45 mM Tris-base, 45 mM boric acid, 0.5 M EDTA, pH 8.0; Sigma-Aldrich, Milan, Italy) into a microwave oven. GelRed nucleic acid gel stain (1ul) was added before gel solidification, as nucleic acid dye in order to replace the highly toxic ethidium bromide (Biotium, Hayward, CA, USA). When agarose was completely dissolved the solution was put into the electrophoresis chamber and the gel comb was placed. After the gel had hardened, 1 ug of purified DNA of each sample and 15 ul of positive control DNA (kit supplied), were mixed with loading buffer (1% SDS, 3,7 mM Bromphenol blue, 3% glycerol; Sigma-Aldrich, Milan, Italy) and loaded in loading pocket. DNA fragments size was detected using a High Ranger Plus

100 bp DNA Ladder (Norgen Biotek Corporation, Thorold, ON, Canada). The gel was covered completely with TBE buffer and the power supply was turned on and the run was set at 75 V for 2 hours. The DNA was finally visualized by placing gel onto a UV transilluminator (Amersham Biosciences, NJ,USA).

6.1.4 Data analysis

Results were expressed as mean ± SEM. The statistical analysis was carried out by a nested ANOVA followed by bilateral multiple comparisons test.

6.2 Results and discussion

Un-stimulated and PMA stimulated neutrophils were incubated for 1, 3 or 6 hours with either BSA or AOPP-BSA (see tab 6.1) in order to analyzed the presence of specific markers of apoptosis. Caspases 8, 9 and 3 and DNA laddering were tested: the purpose was to to investigate whether AOPP-BSA could affect cell viability and, if positive, discriminate between the intrinsic and the extrinsic pathways.

6.2.1 Western Blot analysis of Caspases 8, 9 and 3

Data obtained from the densitometric analysis of cleaved caspase 8 bands (active form), reveals that un-stimulated neutrophils incubated with AOPP-BSA for 1, 3 hours, showed a higher but not significant activation of caspase 8 in comparison with the incubation with BSA (Fig. 6.2 B, D). Instead, after 6 hours of incubation there was a tendentially significant ($p < 0.1$) activation of caspase 8 in un-stimulated neutrophils incubated with AOPP-BSA in comparison with the incubation with BSA (Fig. 6.2 F). In addition, un-stimulated neutrophils incubated with BSA for 1 hour showed a significantly lower ($p < 0.05$) presence of cleaved caspase 8 in comparison with PMA-stimulated neutrophils incubated with BSA (Fig. 6.2 B). No differences were obtained between PMA-stimulated neutrophils incubated with BSA and AOPP-BSA. Indeed, there are evidences in the litherature that ROS produced by PMA-stimulated neutrophils are able to induce apoptosis, thus the possible pro-apoptotic avents triggered by AOPP-BSA could be masked by the PMA activity (Lundqvist et al. 1999).
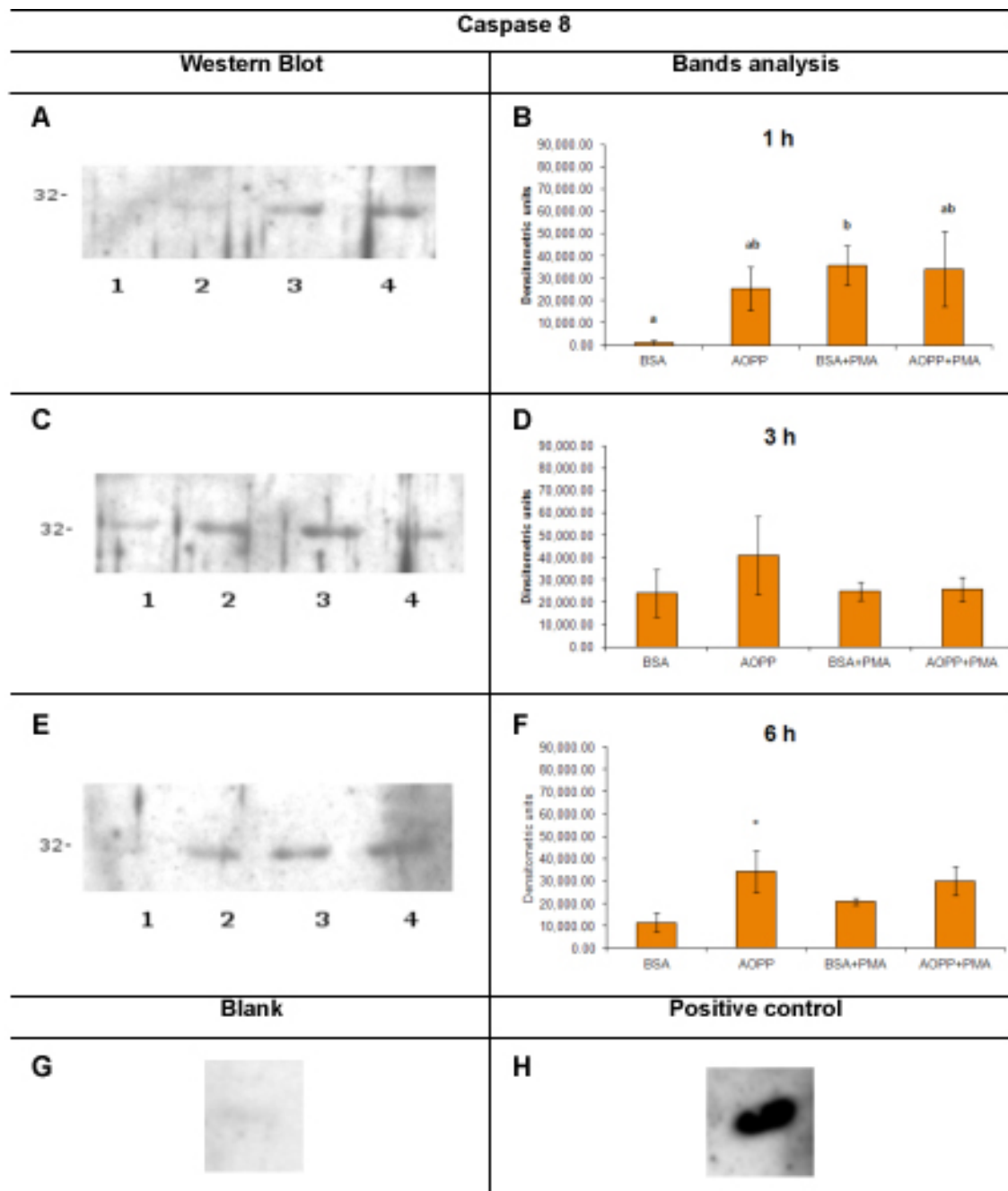
Figure 6.2: Western blot analysis of cleaved caspase 8 (~ 32 KDa) in cytosolic fraction of un-stimulated and PMA-stimulated bovine neutrophils incubated either with BSA or AOPP-BSA for 1h (A), 3h (C) and 6h (E). Lane 1: un-stimulated neutrophils incubated with BSA; Lane 2: un-stimulated neutrophils incubated with AOPP-BSA; Lane 3: PMA-stimulated neutrophils incubated with BSA; Lane 4: PMA-stimulated neutrophils incubated with AOPP-BSA. Blank (G) was obtained by incubating un-stimulated neutrophils with BSA immediately after cell isolation. Positive control (H) was obtained incubating un-stimulated with Staurosporine (1uM) for 6 hours. The western blot images represent one of the 4 experiments. Densitometric analysis of bands obtained by un-stimulated and PMA-stimulated bovine neutrophils incubated with BSA and

AOPP-BSA for 1h (B), 3h (D) and 6h (F). Data are expressed as mean ± SEM of 4 experiments. a,b, p<0,05. *, p< 0.1.

Western blot analysis of the cleaved caspase 9 showed no significant differences between the experimental theses at all incubation time-intervals considered (Fig. 6.3). This observation led to the hypothesis that the intrinsic apoptotic pathway is not involved in the decreased functionality of neutrophils under our experimental conditions.
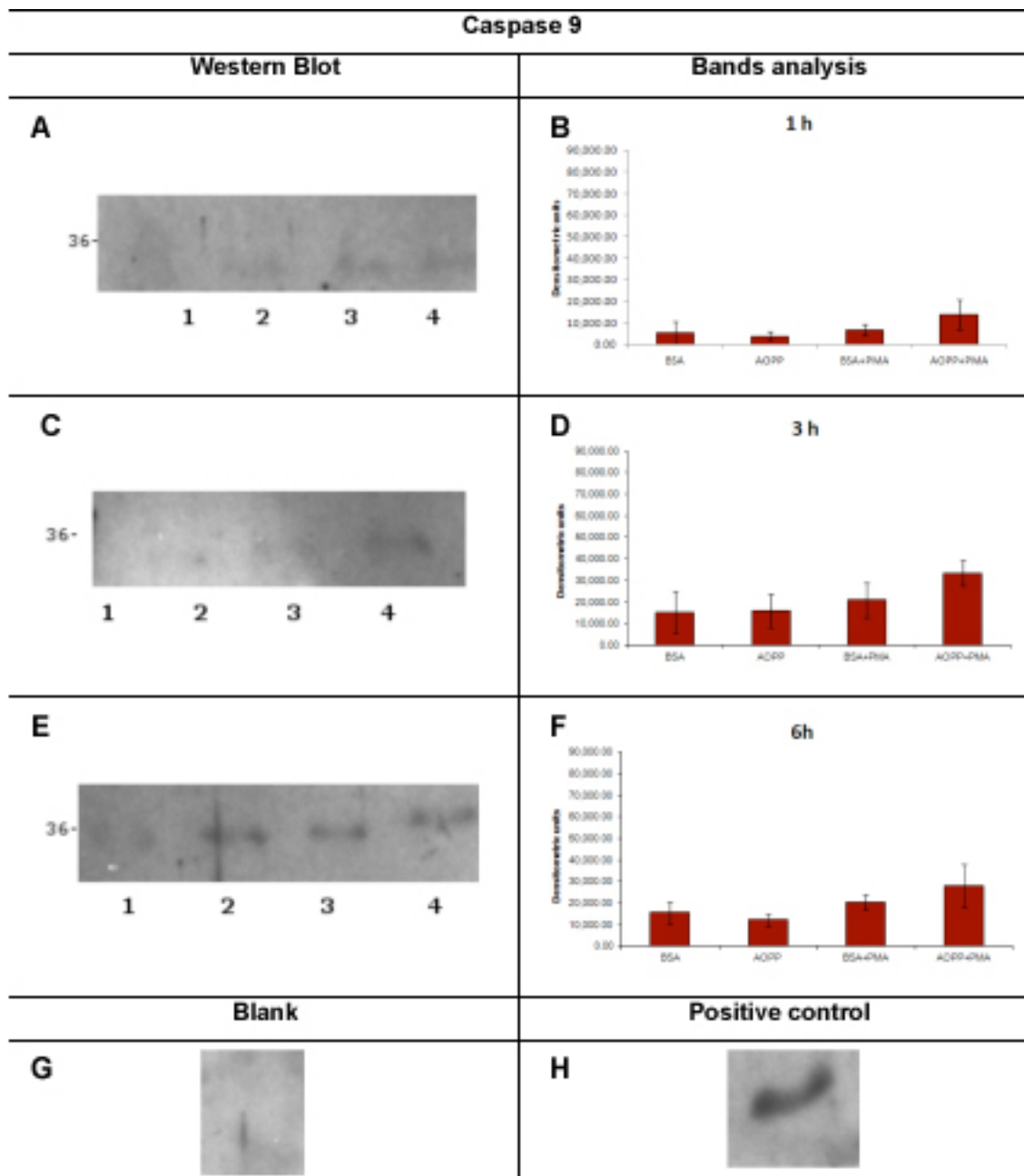
Figure 6.3: Western blot analysis of cleaved caspase 9 (~ 36 KDa) in citosolic fraction of un-stimulated and PMA-stimulated bovine neutrophils incubated with BSA and AOPP-BSA for 1h (A), 3h (C) and 6h (E). Lane 1: un-stimulated neutrophils incubated with BSA; Lane 2: un-stimulated neutrophils incubated with AOPP-BSA; Lane 3: PMA-stimulated neutrophils incubated with BSA; Lane 4: PMA-stimulated neutrophils incubated with AOPP-BSA. Blank (G) was obtained by incubating un-stimulated neutrophils with BSA immediately after cell isolation. Positive control (H) was obtained incubating un-stimulated with Staurosporine (1uM) for 6 hours. The western blot images represent one of the 4 experiments. Densitometric analysis of bands obtained by un-stimulated and PMA-stimulated bovine neutrophils incubated with BSA and AOPP-BSA for 1h (B), 3h (D) and 6h (F). Data are expressed as mean ± SEM of 4 experiment.

Also the western blot analysis of caspase 3 showed no significant differences between the experimental thesis after 1 and 3 hours of incubation (Fig. 6.4 B, D). However, an increase of activated caspase 3, although not significant, could be observed after 6 hours of incubation in un-stimulated neutrophils incubated in AOPP-BSA (Fig. 6.4 E).
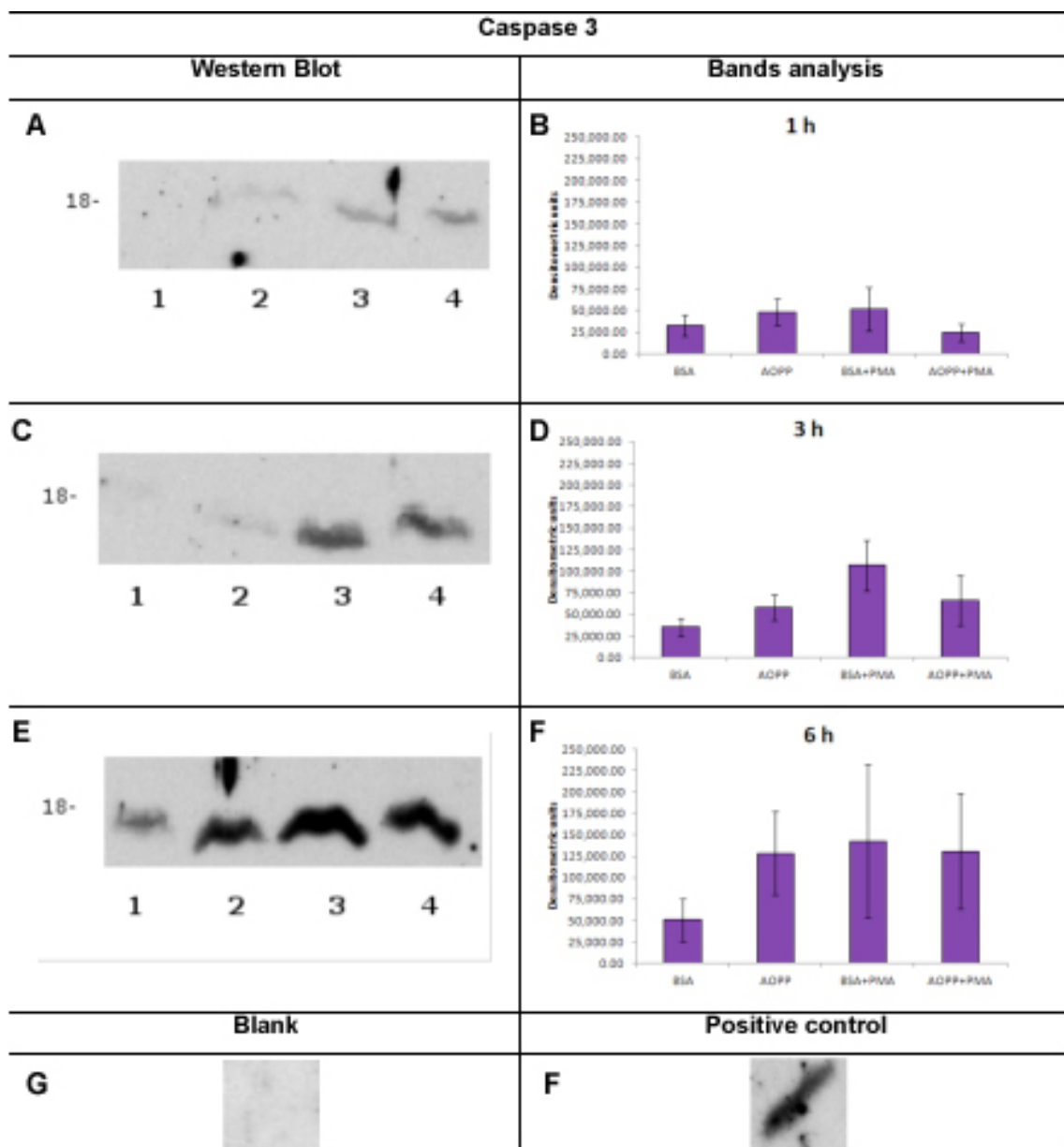
Figure 6.4: Western blot analysis of cleaved caspase 3 (~ 18 KDa) in citosolic fraction of un-stimulated and PMA-stimulated bovine neutrophils incubated with BSA and AOPP-BSA for 1h (A), 3h (C) and 6h (E). Lane 1: un-stimulated neutrophils incubated with BSA; Lane 2: un-

stimulated neutrophils incubated with AOPP-BSA; Lane 3: PMA-stimulated neutrophils incubated with BSA; Lane 4: PMA-stimulated neutrophils incubated with AOPP-BSA. Blank (G) was obtained by incubating un-stimulated neutrophils with BSA immediately after cell isolation. Positive control (H) was obtained incubating un-stimulated with Staurosporine (1uM) for 6 hours. The western blot images represent one of the 4 experiments.. Densitometric analysis of bands obtained by un-stimulated and PMA-stimulated bovine neutrophils incubated with BSA and AOPP-BSA for 1h (B), 3h (D) and 6h (F). Data are expressed as mean ± SEM of 4 experiment.

6.2.2   DNA Laddering

The occurrence of late neutrophil apoptosis was also investigated by the characteristic fragmentation of DNA (Fig 6.5 B, D, F). The densitometric analysis of the bands obtained from agarose gel showed no statistically significant differences between the experimental thesis, none of which presented DNA fragmentation (Fig. 6.5 A, C, E).
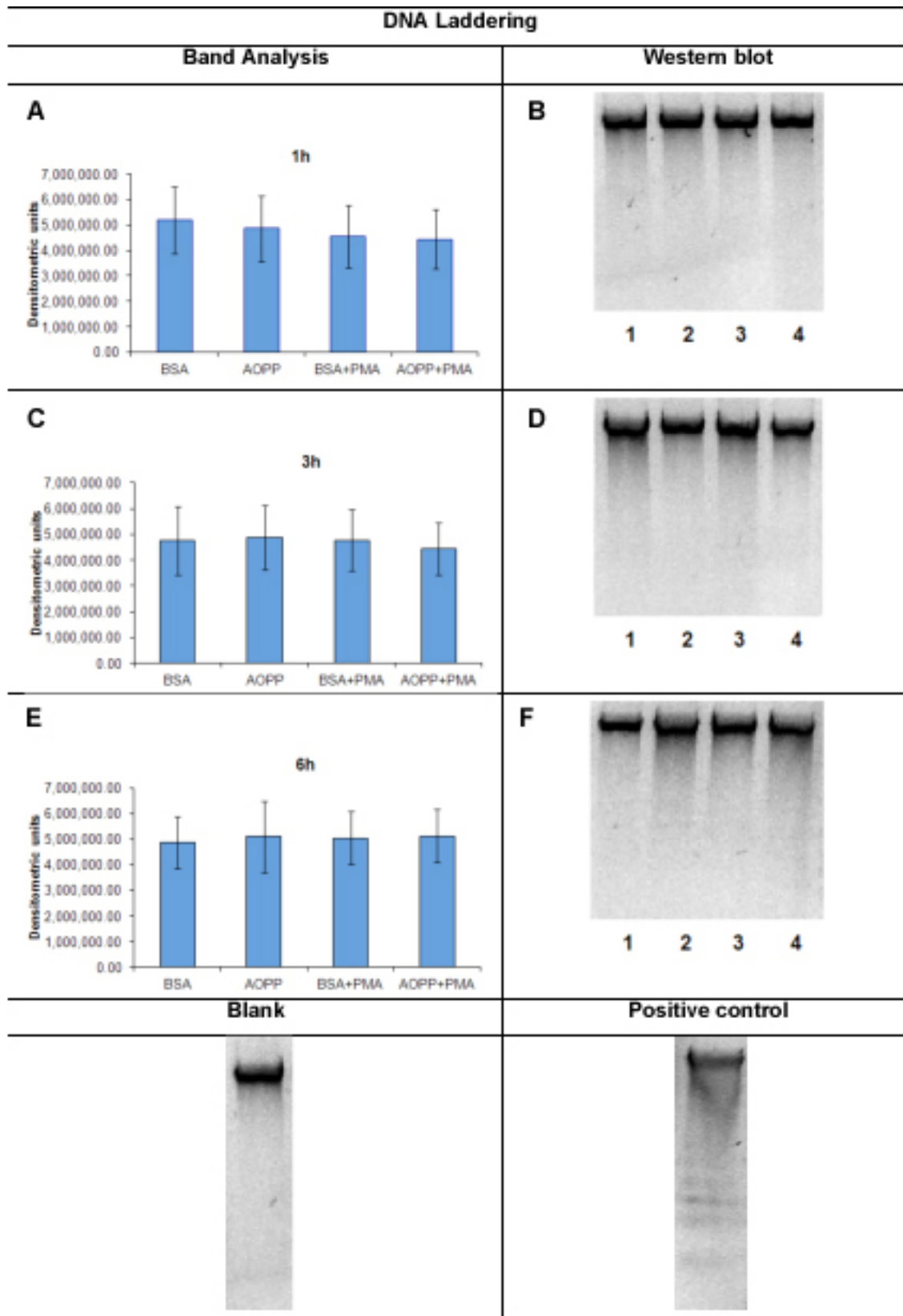
Figure 6.5: Agarose gel electrophoresis of DNA extracted from un-stimulated and PMA-stimulated bovine neutrophils incubated with BSA and AOPP-BSA for 1h (A), 3h (C) and 6h (E).

Lane 1: un-stimulated neutrophils incubated with BSA; Lane 2: un-stimulated neutrophils incubated with AOPP-BSA; Lane 3: PMA-stimulated neutrophils incubated with BSA; Lane 4: PMA-stimulated neutrophils incubated with AOPP-BSA. Blank (G) as obtained by incubating un-stimulated neutrophils with BSA for 0 hours. Positive control (H) was obtained incubating un-stimulated with Staurosporine (1uM) for 6 hours. Images represent an example of 4 experiments. Densitometric analysis of bands obtained by un-stimulated and PMA-stimulated bovine neutrophils incubated with BSA and AOPP-BSA for 1h (B), 3h (D) and 6h (F). Data are expressed as mean ± SEM of 5 experiments.

Taken together, these results support the hypothesis that AOPP-BSA may have a role of mild pro-apoptotic agent, which may lead to an earlier activation of the extrinsic apoptosis pathway.

CHAPTER 7.

Incubation of BSA with PMA-stimulated bovine neutrophils.

7.1 Materials and methods

In this part of the study the formation of AOPP, dityrosines and carbonyls was measured incubating BSA directly with activated neutrophils.

Freshly isolated bovine neutrophils (7.5 x10^6), un-stimulated and PMA-stimulated (1 ug/ml) were incubated with BSA (0,5 mg/ml) for 1-2-3 hours at 37°C in a humidified incubator. Reaction mixture was composed of 1 ml of neutrophils suspension, 250 ul of BSA and 250 ul of PMA and it was transferred in triplicates in a 6-well plate (Iwaki, Tokyo, Japan). Parallel a 6-well plate was prepared by adding neutrophils (7.5 x10^6) un-stimulated and PMA-stimulated (1 ug/ml) without BSA, in order to measure HOCl production. At the end of incubation times neutrophils suspensions were collected and centrifuged at 500 g for 10 minutes. Neutrophils pellet was discarded and supernatants obtained were used for the following analysis.

7.1.1 Bradford assay

25 ul of the supernatant of each sample were used to detect protein content using Bradford assay following manufacturer's instructions (Bradford reagent; Sigma-Aldrich, Milan, Italy) as described in chapter 3, paragraph 3.1.2.

7.1.2 Neutrophil HOCl quantification

HOCl concentration, produced by un-stimulated and PMA-stimulated neutrophils, without BSA in culture medium was detected on supernatants spectrophotometrically at 290 nm, using reported extinction coefficient (ε_{290} = 350 $M^{-1}cm^{-1}$).

7.1.3 Incubation of BSA directly with HOCl

In order to make a comparison between cell-system and cell-free system, BSA (0,5 mg/ml) was incubated with HOCl at the same concentration produced by

PMA-stimulated neutrophils (200 uM), for the same incubation times (1-2-3 hours). At the end, oxidative reaction was stopped by adding methionine (10 mM)

7.1.4 AOPP quantification assay

AOPP production was measured using the spectrophotometric method described by Witko-Sarsat (see paragraph 3.2.3) with little modifications (Witko-Sarsat et al. 1996, Witko-Sarsat et al. 2003). The microplate method was adapted to polystyrene cuvettes (Kartell, 1 cm path length; Milan, Italy) increasing proportionally reagents volume starting from a higher quantity of sample. 750 ul of both supernatants and HOCl oxidized BSA were transferred in a polystyrene cuvette and mixed with 75 ul of acetic acid. Calibration curve was constructed by preparing standard reference cuvettes containing 750 ul of chloramine T solution (0-100 umol/L; Fluka, Sigma-Aldrich, Milan, Italy), 37.5 ul of 1.16 M of potassium iodide (KI; Sigma-Aldrich, Milan, Italy) and 75 ul of acetic acid. The absorbance of the reaction mixtures was immediately read at 340 nm in a spectrophotometer against blank containing 750 ul of 20 mM PBS, 37.5 ul of KI and 75 ul of acetic acid, The chloramine-T absorbance at 340 nm was linear within the range of 0-100 umol/L with a sensitivity of 3.125 umol/L. AOPP concentration was expressed in umol/L of chloramine-T equivalents/ mg protein.

7.1.5 Dityrosine quantification assay

Dityrosine content of both neutrophil culture medium and HOCl oxidized BSA were measured spectrophotometrically in polystyrene cuvettes (Kartell, 1 cm path length; Milan, Italy) at 315 nm assuming the absorption coefficient $\varepsilon = 5$ mM^{-1} cm^{-1} at pH 7.5 Results were expressed as umol/ mg protein as described in paragraph 3.2.4 (Heinecke et al. 1993).

7.1.6 Carbonyl detection by western blot analysis

Samples both neutrophil culture medium and HOCl oxidized BSA were diluted in 10 mM PBS (0,3 mg/ml) and derivatized with a solution of DNPH. In parallel, samples reacted with a solution without DNPH, and were used as blank controls. Samples and relative blanks were mixed and centrifuged at 12.700 g for 1 minute. Supernatants (5 ul/lane) were subjected to 4-10% gradient SDS-PAGE, electroblotted to a nitrocellulose membrane and immunoassayed for carbonyl content with anti-DNP antibody as described in paragraphs 3.2.6.2 – 3.2.6.6.

7.2 Data analysis

Results were expressed as mean ± SEM. The statistical analysis was carried out by parametric ANOVA followed by Duncan's test. Data that were not normally distributed were transformed by using a square root transformation

7.3 Results and discussion

The pivotal role of neutrophils in the production of AOPP is widely claimed in the literature but the demonstration "in vitro" is only indirect. AOPP can be produced "in vitro" by oxidizing proteins (principally serum albumin) with HOCl (Iwao, Anraku et al. 2006) or using a MPO-H_2O_2-Cl^- system as source of HOCl (Heinecke et al. 1993). Weiss (Weiss 1982) demonstrated that activated neutrophil are able to generate chloramines by oxidizing "in vitro" amino acids such as taurine.

In this part of the study, the formation of AOPP, dityrosines and carbonyls was measured by incubating BSA with activated neutrophils (cell system) and results were compared incubating BSA with a solution of HOCl at the same concentration produced by PMA-stimulated neutrophils (cell-free system).

7.3.1 Quantification of HOCl production by un-stimulated and PMA-stimulated neutrophils

Un-stimulated (0 ug/ml PMA) and PMA-stimulated neutrophils (1 ug/ml PMA) were incubated at 37°C in a humidified incubator for 1, 2 and 3 hours. Then, cells were pelletted and the supernatant was used to measure HOCl production ($A_{290}= 350$ M^{-1} cm^{-1}). PMA-stimulated neutrophils produced a significantly higher concentration of HOCl respect un-stimulated neutrophils in all incubation time tested. In addition, no differences were obtained comparing HOCl levels of un-stimulated neutrophils incubated for 1,2 and 3 hours. As expected, HOCl concentration generated by PMA-stimulated neutrophils increase significantly in a time-dependent manner (Fig. 7.1 A) and results expressed as the area under the HOCl concentration-time curve show that PMA-stimulated neutrophil were able to generate a significantly higher amount of HOCl in comparison with un-stimulated neutrophils (Fig 7.1 B).

A HOCl concentration (200 uM) comparable to that produced by PMA-stimulated neutrophils was used to oxidized BSA in a cell-free system.
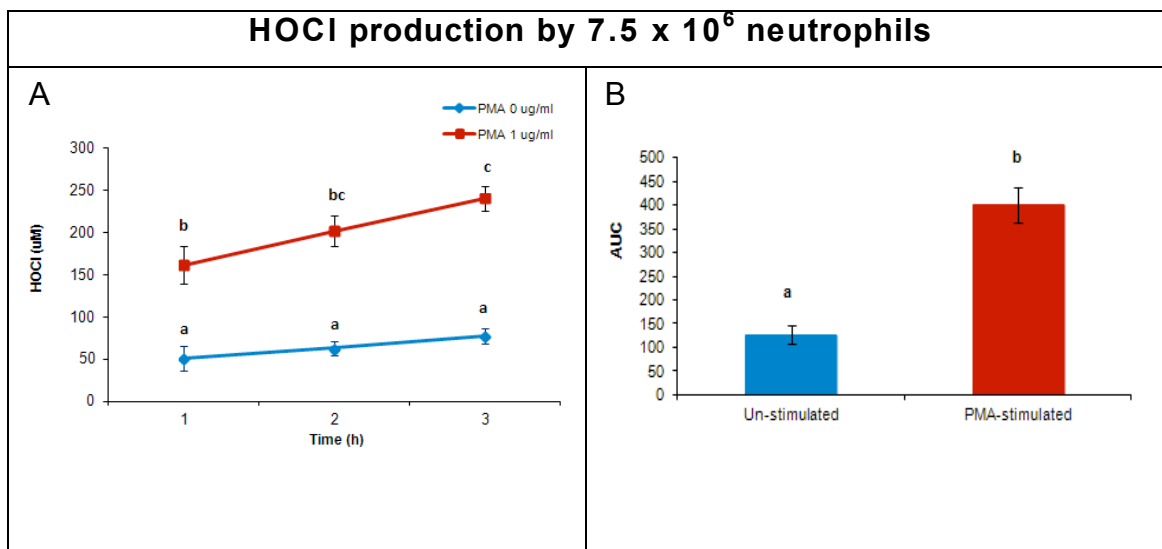
Figure 7.1: HOCl production by neutrophils un-stimulated and stimulated with PMA (1 ug/ml), and incubated with BSA (0.5 mg/ml) for 1, 2 and 3 hours at 37°C in a humidified incubator (A).

The area under curve was calculated from the HOCl concentration – time curve (B). Data are expressed as mean ± SEM of 7 experiments. a,b,c p<0,05.

7.3.2 AOPP quantification

PMA-stimulated neutrophils incubated with BSA for 3 hours are able to produce a significantly (p<0.05) higher concentration of AOPP than un-stimulated neutrophils. No differences were observed after 1 and 2 hours of incubations. AOPP levels produced by PMA-stimulated neutrophils didn't differ between the different incubation time-intervals. Un-stimulated neutrophil were able to generate a small quantity of HOCl that produces a significantly higher AOPP concentration after an incubation time of 1 and 2 hours in comparison with the 3 hour incubation, where the mild un-stimulated neutrophils activation declined (Fig. 7.1 A).

In parallel, BSA incubated with 200 uM HOCl showed levels of AOPP significantly higher than BSA incubated with HBSS alone in all the time intervals investigated. No differences were observed between the three incubation times analyzing AOPP formation in absence of HOCl, while a significantly higher level of AOPP was measured after 1 hour in presence of 200 uM of HOCl. These results suggested that AOPP can be generated rapidly, within an hour of incubation, and then their concentration decline significantly (Fig. 7.2 B).
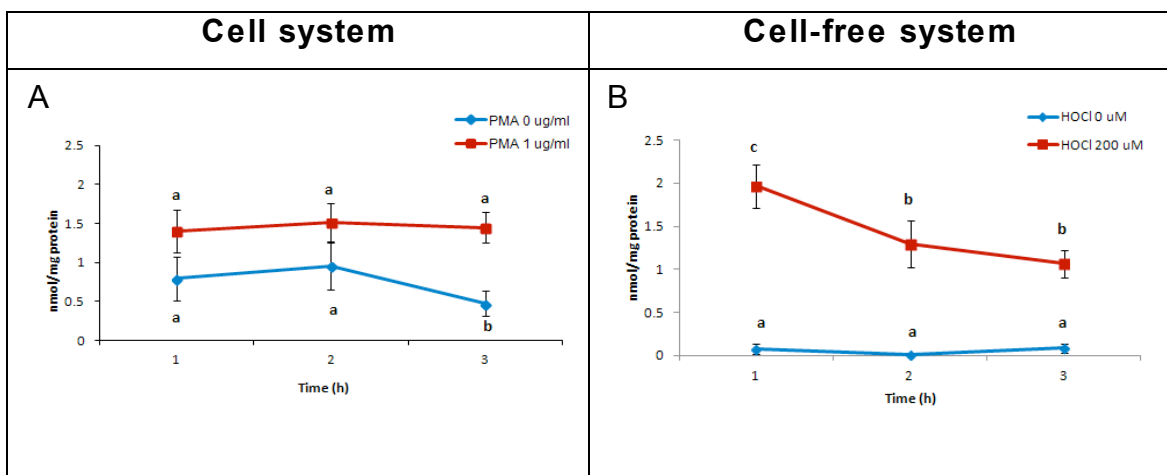
Figure 7.2: AOPP concentration in the medium containing BSA following incubation with un-stimulated or PMA-stimulated (1 ug/ml PMA) neutrophils (7.5 x 10^6) for 1, 2 and 3 hours at 37°C

in a humidified incubator (A), and BSA exposed to HBSS alone and 200 uM HOCl for 1, 2 and 3 hours at 37°C (B). Data are expressed as mean ± SEM of 7 experiments for the cell-system and 5 experiments for the cell-free system. a,b,c, $p < 0.05$.

7.3.3 Dityrosine quantification

Dityrosines were produced by both systems in a significant amount (Fig. 7.3 A,B). PMA-stimulated neutrophils incubated with BSA for 1, 2 and 3 hours are able to generate a significantly higher amount of dityrosines ($p < 0.05$) than un-stimulated neutrophils (Fig. 7.3 A). Also BSA oxidized with 200 uM HOCl showed a significantly higher level of dityrosines ($p < 0.001$) in each incubation time in comparison with BSA exposed to HBSS alone, as shown in figure 7.3 B. The cell-system and cell-free system did not show any significant differences in the dityrosines concentration between each incubation time both in the oxidant conditions (1 ug/ml PMA or 200 uM HOCl) and no-oxidant conditions (0 ug/ml PMA or 0 uM HOCl).
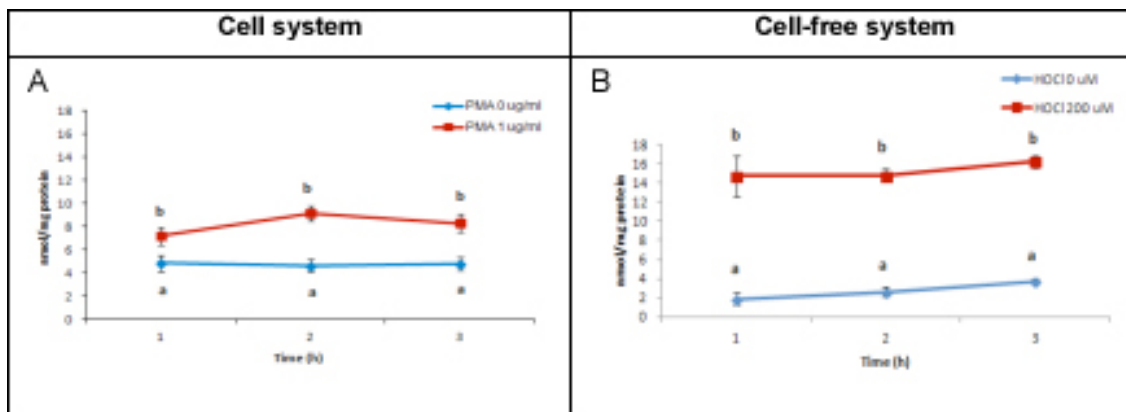
Figure 7.3 Dityrosine concentration of BSA incubated with un-stimulated and PMA-stimulated (1 ug/ml PMA) neutrophils (7.5 x 10^{6}) for 1, 2 and 3 hours at 37°C in a humidified incubator (A), and BSA exposed to HBSS alone and 200 uM HOCl for 1, 2 and 3 hours at 37°C (B). Data are expressed as mean ± SEM of 7 experiments for cell-system and 5 experiments for cell-free system. a,b,c, $p < 0.05$.

7.3.4 Carbonyl detection by western blot analysis

Results obtained from western blot analysis did not show any significant production of carbonyl groups by PMA-stimulated neutrophils in comparison with un-stimulated neutrophils, although a mild production of carbonyls was appreciable in PMA-stimulated neutrophils after 3-hour incubation (Fig. 7.4 A). Conversely, in the cell-free system the carbonyl amount was significantly higher (p<0.01) in BSA incubated with 200 uM HOCl for 1, 2 and 3 hours in comparison with BSA incubated with HBSS alone. No differences were observed between the three incubation time-intervals in BSA exposed to 200 uM HOCl, while a significant increase (p<0.05) of carbonyls production was observed BSA exposed to HBSS for 2 hours in comparison with the 1 hour incubation (Fig. 7.4 B).
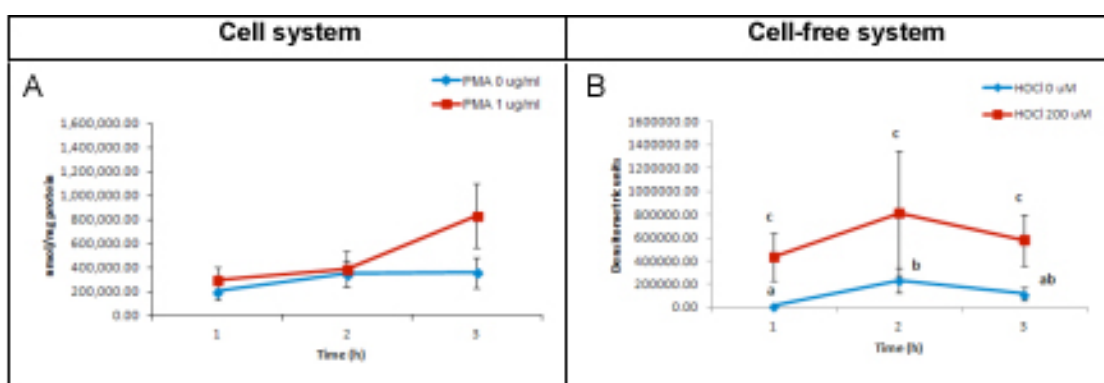
Figure 7.4: Carbonyls concentration of BSA incubated with un-stimulated and PMA-stimulated (1 ug/ml PMA) neutrophils (7.5 x 10^6) for 1, 2 and 3 hours at 37°C in a humidified incubator (A), and BSA exposed to HBSS alone and 200 uM HOCl for 1, 2 and 3 hours at 37°C (B). Data are expressed as mean ± SEM of 7 experiments for cell-system and 5 experiments for cell-free system. a,b,c, p<0.05.

7.3.5 Comparisons between cell-system and cell-free system

The area under curve was calculated from the results obtained by analyzing AOPP, dityrosine and carbonyl levels in the cell system and in the cell-free system at different incubation times. The AUC is an indicator of the whole amount of each marker of protein oxidation produced in the different experimental conditions.

In general, BSA incubated with PMA-stimulated neutrophils or with 200 uM HOCl presented significantly higher AOPP ($p<0.05$ and $p<0.001$ respectively) and dityrosines ($p<0.001$) concentrations (figure 7.5 A, B). Carbonyls, instead, were significantly higher ($p<0.01$) in BSA exposed 200 uM HOCl respects the incubation with HBSS alone on the contrary, PMA-stimulated neutrophils seem to be unable to stimulate a significant BSA carbonylation (figure 7.5 C). It is noteworthy to observe that BSA exposure to HOCl generated a significantly greater amount of dityrosines ($p<0.001$) and carbonyls ($p<0.05$) than PMA-stimulated neutrophils. In addition, un-stimulated neutrophils generated a significantly higher amount of AOPP ($p<0.001$) and dityrosines ($p<0.05$) in comparison to BSA incubated with HBSS alone, indeed Pithon Curi (Curi 1998) have reported that rat neutrophils, in the absence of PMA, produced a small amount of ROS. Physiologically, neutrophils generate ROS during the respiratory burst by membrane bound NADPH-oxidase and by myeloperoxidase in intracellular granules. The balance between oxidant production and antioxidant defense may have an important role in the prevention of oxidative damage. Superoxide dismutase, catalase and GSH-related enzymes are active during respiratory burst and phagocytosis and are able to scavenge efficiently H_2O_2 and O_2^-. In condition of oxidative stress ROS production exceed antioxidant mechanisms and protein oxidation occur (Aruoma 1987, Pietarinen et al. 2000). This could explain the lower oxidation level in BSA incubated with PMA-stimulated neutrophils obnserved in the present study, where the production of ROS could be partially scavenged by the cell antioxidants enzymes.

Taken together, these results suggested that bovine neutrophils can oxidise BSA and generate chemical and structural modification such as AOPP and dityrosines. Conversely, carbonyls seem to be a poor indicator of neutrophils-mediated protein oxidation. The direct exposition to HOCl, commonly used in the literature, couldn't fully mimic the complex events leading to BSA oxidation and AOPP production by activated neutrophils.
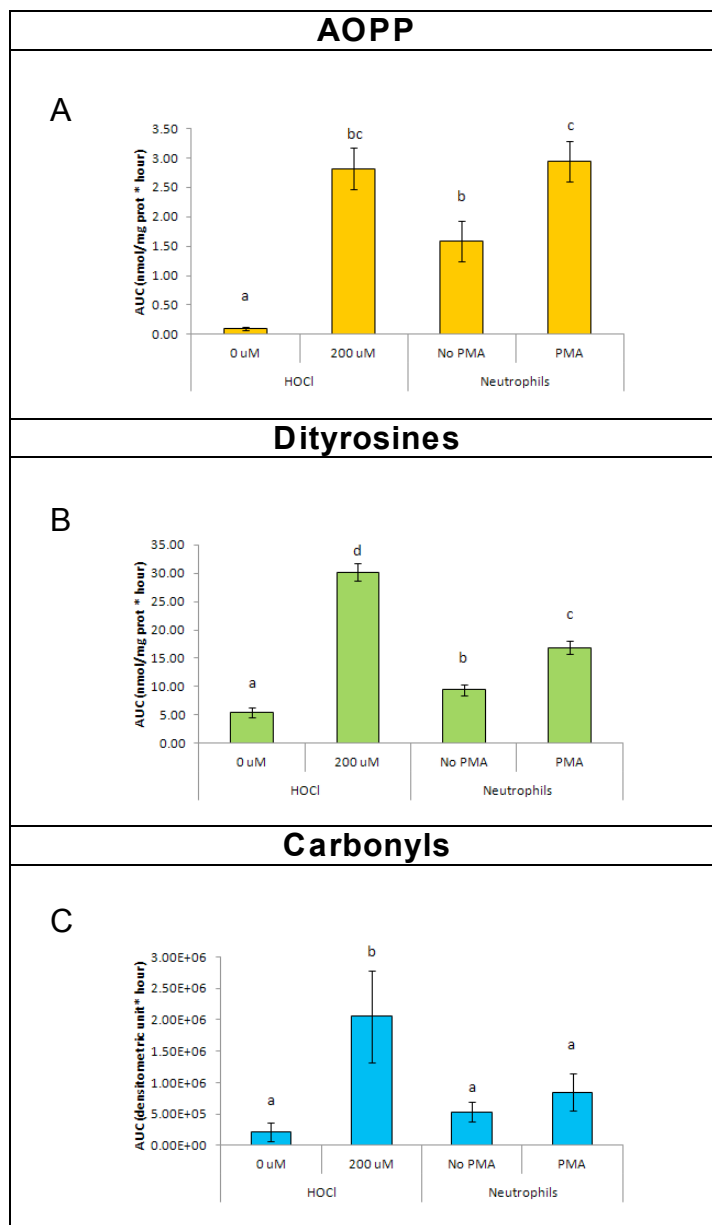
Figure 7.5: Area under curve of AOPP (A), dityrosine (B) and carbonyl concentration calculated over a 3 hours period with trapezoidal method. Results obtained with the cell-systems are

compared with those obtained with the cell-free system. Data are expressed as mean ± SEM of 7 experiments for cell-system and 5 experiments for cell-free system. a,b,c,d p<0.05.

CHAPTER 8.

General discussion and conclusions

Advanced oxidation protein products (AOPP) are novel markers of protein oxidation that were first described and characterised in plasma of uremic patients (Witko-Sarsat et al. 1996). AOPP are mainly generated by chlorinated oxidants produced by the myeloperoxidase (MPO) of activated neutrophils. (Witko-Sarsat et al. 1999, Capeillere-Blandin et al. 2004). They are considered also mediators of pro-inflammatory responses. Interestingly, AOPP are able to trigger monocyte and neutrophils respiratory burst in humans (Witko-Sarsat et al. 1998, Witko-Sarsat et al. 2003) Recent studies have shown that AOPP concentration increases in dairy cows when they are fed maize silage (Celi et al. 2010) and in growing dairy calves (Celi et al. 2010). In addition, mean plasma AOPP concentrations and the ratio of AOPP:albumin were significantly higher in cows undergoing late embryonic losses (Celi et al. 2011). These results raised our interest in the biological role of AOPP in dairy cattle and in the role of neutrophils on oxidative stress and AOPP production, in order to make a comparison with those observed in human, considering species-specific differences.

The first aim of the present study was to evaluate the effects of AOPP on bovine neutrophils "in vitro": for this purpose and on the basis of results obtained, neutrophils ROS production, cell viability and apoptotic markers were assessed. Standard AOPP were produced "in vitro" using hypochlorous acid as oxidant because it is the major strong oxidant generated by neutrophils and serum albumin as protein standard due to its structural and functional characteristics. Stimulated neutrophils generate superoxide and its dismutation product, hydrogen peroxide, and release the heme enzyme myeloperoxidase. Myeloperoxidase has the unique property of converting chloride to hypochlorous acid (HOCl). Under most circumstances, HOCl is likely to be the major strong oxidant produced by neutrophils. Myeloperoxidase is also present in monocytes and there is evidence that it is expressed by macrophages and microglia in vivo. Thus, specific reaction products of HOCl should provide biomarkers of the oxidant activity of neutrophils and other myeloperoxidase-containing cells. HOCl is a highly reactive species that participates in both oxidation and chlorination reactions and it is freely diffusible from the enzyme MPO. It has

many biological targets for oxidation. Thiols and thioethers are particularly reactive and other compounds, including ascorbate, urate, pyridine nucleotides, and tryptophan, are oxidized by HOCl, although not as rapidly. The main biological chlorination reactions are with amine groups to give chloramines; with tyrosyl residues to give ring chlorinated products; with unsaturated lipids and cholesterol to give chlorohydrins; and ring chlorination of cytosine resides in nucleic acids (Fig. 8.1) (Albrich 1981, Winterbourn et al. 2000).
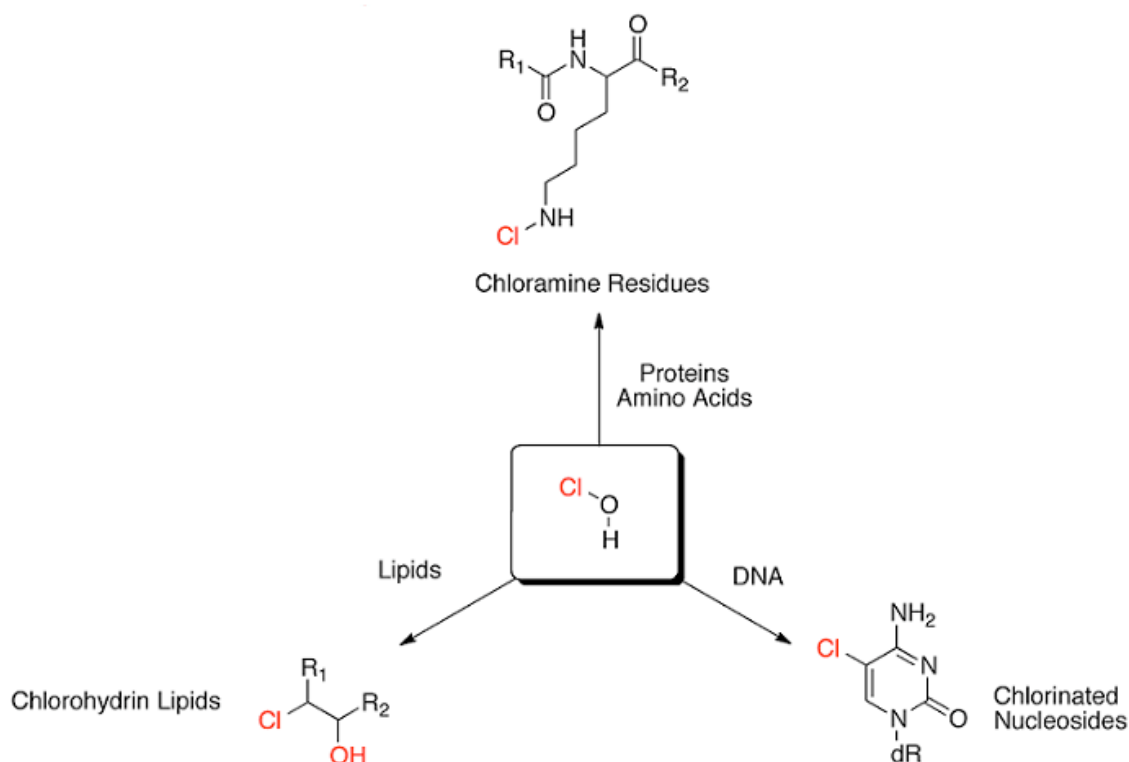
Figure 8.1. Chlorination and oxidation that occur to proteins, lipids and DNA upon exposition to HOCl.

Serum albumin is probably one of the most studied models of globular proteins. It is synthesized by the liver in mammals and has a half-life in the circulatory system of ~19 days. Its concentration varies from 35 up to 55 mg/mL in the blood plasma. It corresponds to the most abundant protein in blood plasma, accounting for ~60% of the total number of globular protein. The sequence of human serum albumin and bovine serum albumin exhibit striking homology. Human and bovine serum albumin is constituted by 585 amino acids residues,

including 35 cysteines (17 disulfide bridges), which confer a relatively strong stability to the protein (Barbosa et al. 2010). They are known to have at least two distinct ligand binding sites, namely Sudlow's Site I and Site II. Amino acid residues of lysine, tryptophan, tyrosine and arginine are related to the ligand binding properties. Serum albumin function is associated with the maintenance of blood osmolarity and with the binding and transport of several small molecules such as fatty acids, dyes, metals, amino acids and pharmaceutical compounds (Peters 1997). More recently, antioxidant activity has been recognized as a significant function of serum albumin in the plasma were only a limited amount of antioxidant enzymes are available (ROCHE 2008).

Plasma proteins are targets of oxidative stress and represent the first line defence against ROS (DalleDonne et al. 2005). The antioxidant activities of serum albumin might be the direct interaction with ROS, which may cause reversible and/or irreversible modification. Chlorination of Met147, Met353, Met572, Trp238 by myeloperoxidase lead to not detectable or negligible changes in serum albumin in vivo. The reaction of ROS with free thiol of Cys34 could lead to the formation of sulfenic (SOH) or sulfinic acid (SO_2H), two reversible intermediates that may shift back to the original protein form or react with glutathione. The stable oxidation of Cys34 to SO_3H-albumin is an irreversible modification that alters structural and functional characteristic of serum albumin, which becomes more susceptible to trypsin digestion and degradation (Kawakami 2006). The oxidation of Trp214 and Tyr410, located near the hydrophobic part of the binding pockets of serum albumin, alter protein structure and pharmacokinetic characteristics: these amino acids are important for holding together the two halves of the heart-shaped albumin and it is affected in the oxidative stress of albumin. In addition the chemical modification of Tyr and Trp slightly increased the hepatic uptake in parallel with the reduction in plasma concentration (Ma et al. 2006).

In general, certain amino acids are particularly susceptible to modification by the MPO-H_2O_2-Cl^- system. Tyr, Phe undergo ring chlorination and form long-lived chloramines. Lys are converted to semi-stable chloramines and subsequently undergo hydrolysis to aldehydes and ammonia, inducing the formation of

carbonyls. In addition protein carbonyl derivatives may be produced by the direct oxidation of Arg and Pro. Myeloperoxidase is able to convert tyrosine in the long-lived tyrosil radical and when two tyrosil radicals productively interact the major product is o,o – dityrosines leading to protein cross-linking (Dalle Donne et al. 2001, Heinecke 2002).

The oxidation of amino acids residues leads also to protein fragmentation, aggregation and cross-linking that could accumulate both in the intra- and extracellular compartment. The presence of elevated levels of oxidized protein products was reported in patients with renal disease, atherosclerosis and diabete mellitus and it is correlated with an inflammatory condition (Witko-Sarsat et al. 1996, Liu et al. 2006, Kalousova et al. 2002).

Treatment of BSA with increasing concentration of HOCl induces protein fragmentation and cross-linking. Fragmentation is ascribed to direct reaction of excess of oxidant with the backbone, while aggregation could be attributable to dityrosine formation. Studies on aminoacid analysis of oxidized serum albumin indicates that oxidation of the protein induces significant modification of tyrosine and basic aminoacids such as lysine and arginine (Iwao et al. 2006). In vivo, protein fragments and aggregates, generated during severe and extensive oxidative stress, becomes progressively resistant to proteolytic digestion and are detrimental to normal cell functions (Davies 2001).

Serum albumin incubated with increasing concentration of HOCl presents concentration of AOPP, dityrosines and carbonyls that increase in a dose dependent manner. A close correlation was found between AOPP and carbonyls and dityrosines levels. In addition, AOPP closely correlates also with pentosidine, a marker of protein glycation (AGE). AGE are modifications of proteins that become non-enzymatically glycated and oxidized after contact with aldose sugars. Both AOPP-human serum albumin (HSA) and AGE-HSA triggered monocyte respiratory burst. Of note, while AOPP-HSA did not contain AGE-pentosidine, a significant concentration of AOPP were found in AGE-HSA preparations. These data suggest that AGE-proteins contain some structural motifs that results from an oxidative process and support the hypothesis that AGE and AOPP were involved in a similar receptor-mediated process due to a

possible structural resemblance (Witko-Sarsat et al. 1998, Guo et al. 2008). Indeed, both AOPP and AGE are able to trigger ROS production through protein kinase C (PKC)-dependent activation of NADPH oxidase via a RAGE mediate signaling pathways in vascular endothelial cells and mesangial cell (Wong 2002, Guo et al. 2008, Wei 2009).

RAGE is the best characterized receptors for AGE. It is present in many cells in human, among them endothelial cells (Schmidt 1994), mesengial cells (Skolnik 1991), T lymphocytes (Imani 1993), monocytes, macrophages (Kirstein 1992) and neutrophils (Collison 2002). AOPP-HSA retain also the capacity to trigger human neutrophil respiratory burst (Witko-Sarsat et al. 2003, Mera, Anraku et al. 2005). Bernheim (Bernheim 2001), similarly to Witko-Sarsat studies, have demonstrated that AGE-BSA were able to increase significantly the superoxide production of resting human neutrophils, but they are also able to decrease significantly superoxide production of PMA-stimulated neutrophils. A possible explanation is that AGE-BSA induce an intracellular disfunction at or distal to PKC, which eventually causes an inhibitory action on NADPH oxidase activity. Sehr (Sehr 1998) have reported that glycosylation of intracellular activators of NADPH oxidase complex inhibits protein interaction, resulting in a reduced respiratory burst. It's necessary to consider that in the study of Barnheim, human neutrophils were incubated with an heterlogous protein such as bovine serum albumin oxidized with glucose.

In the present study AOPP-BSA produced "in vitro", significantly reduce ROS production by PMA-stimulated bovine neutrophils and according with the observations of Sehr et al. this may support the hypothesis that oxidative reactions can contribute to the NADPH-oxidase or MPO inactivation. Therefore, on the basis of our results AOPP-BSA didn't affect un-stimulated neutrophils. A possible explanation may reside in species differences between human and bovine. Bovine neutrophils undergo chemotaxis to certain agents and are able to phagocytise bacteria, produce a respiratory burst and kill bacteria, but their oxidative reactions are of a smaller scale than their human counterparts. Bovine neutrophils don't respond to chemotactic signal derived from bacteria (e.g. undiluited filtrates of E.Coli) or molecules which are similar to synthetic peptides

that are potent for human neutrophils (fMLP). Instead, bovine neutrophils infiltration is stimulated from serum-derived chemortaxins, generated during the complement cascade. In addition, functional chemotaxins for bovine neutrophils might be those derived from the arachidonic acid cascade (e.g. leukotriene B). Bovine neutrophils lack receptors for formyl-peptides (fMLP) (Gray et al. 1982). Additional explanations of the reduced respiratory burst of bovine neutrophils could be imputable to different cellular functions: different isozymes of protein kinase C could be involve in ROS production by PMA-stimulated bovine neutrophils respect to human neutrophils (Coussens 1986). Different proteins phosphorilated during activation pathways could result in the inability of PMA to stimulate bovine neutrophils degranulation (measured in elastase release) and to increase cytoplasmic calcium, which are two characteristic of PMA-stimulated human neutrophils (Brown et al. 1991). Gennaro et al. have observed that oxygen-independent machanisms of bacterial killing may be more important in bovine neutrophils than in human neutrophils (Gennaro et al. 1978). The higher proportion of oxygen released as superoxide by human neutrophils may be explained in terms of the relative initial rates of phagocytosis or in terms of longer lag times before the initiation of superoxide generation by the bovine neutrophils. Superoxide may be scavenged by bovine neutrophils but not the human cells. The mechanisms of host defence are not completely understood in domestic animals (Young et al.1986).

The formation and the accumulation of AOPP and AGE are well documented in many pathological conditions (Miyata 1993, Schmidt 1994, Kaneda 2002, Kalousova et al. 2002, Liu 2006, Li et al. 2007, Krzystek-Korpacka et al. 2008).

The effects observed on cell viability after treatments with AOPP or AGE depend on different parameters such as the cell line, experimental conditions and the assay used in the study. Thus, it is difficult to compare results of independent studies. MTT and LDH assay are the most used methods to investigate the cellular response after AOPP or AGE exposure. Zhong et al. demonstrated that AOPP-rat serum albumin (RSA), incubated with rat calvarial osteoplast-like cells for 24 hours, are able to reduce the number of living cells, showing a decrease in the MTT OD values. This result indicated that AOPP can

induce impairment in osteoblastic proliferation probably mediated by ROS-dependent NF-kB activation (Zhong 2009). In addition the incubation of AGE-HSA for 48 hours, with human brain cells such as embryonic microglia and astroglia seems to provoke significant cytotoxic effects detected by the LDH assay, whereas no inhibitory effects were detected on cell functionality by MTT. Interestingly, the incubation of AGE-HSA with murine microglia presents a clear cytotoxic (LDH assay) and viability decreasing (MTT assay) effect. Differences in viability results obtained in human cell lines compared with murine cell line might result from specie specific differences in enzyme activities or even in signaling cascades (Bigl 2007). A study on murine macrophage cell line incubated with AGE-FBS has observed an increase in LDH release that confirmed membrane perturbation and a loss of membrane integrity after 3 hours of incubation. The MTT test was less markedly affected: the mitochondrial apparatus displayed a mild but significant damage only after 6 hours of incubation (Bassi 2002). Therefore, is it possible to conclude that AOPP and AGE retain the capacity to affects cell functionality and membrane integrity but this depends principally on cell line and experimental conditions. In the present study, bovine neutrophils incubated with AOPP-BSA for 3 hours showed a reduced functionality, assessed by MTT assay but they seem to maintain membrane integrity (LDH assay) respect the incubation with BSA. A possible explanation is that the incubation time tested didn't allow to show marked cytotoxic effects by LDH assay probably because the mithocndrial dehydrogenase activities were affected before than the plasma membrane damage. On the basis of these results, our work has studied if AOPP are able to trigger apoptotic events. For this purpose, caspase 8-9-3 and DNA laddering were used as markers in order to discriminate between the "intrinsic" and the "extrinsic" pathway of apoptosis. The results obtained showed that un-stimulated bovine neutrophils incubated with AOPP-BSA show a higher but not significant production of active caspase 8 in comparison with the incubation with BSA. Also caspase 3 display an increase, but not significant, in un-stimulated neutrophils after 6 hours of incubation with AOPP-BSA, respects the incubation with BSA. No differences were obtained for caspase 9 and for DNA laddering.

The lack of significant differences might be partially attributed to the higher variability of western blot method used for caspases detection. Therefore, in these experimental conditions, the "intrinsic" pathway of apoptosis was not involved in the reduced functionality of neutrophils or in their reduced viability, but bovine neutrophils incubated with AOPP-BSA seem to be "accompanied" to the early phases of the "extrinsic" pathways of apoptosis and probably an increase of the incubation time might have provided clearer results.

In the literature, AOPP and more widely AGE, are linked to apoptosis and the reduction of cell lifespan. Zhou et al. have shown that AOPP are able to induce apoptosis in murine podocytes by the activation of the p53 – Bax – caspase 3 pathway and this is mainly mediated by PKC-NADPH oxidase dependent O_2^- generation (Zhou et al. 2009); In addition, AGE, are able to induce apoptosis in different type of cells such as bovine retinal pericytes (Denis 2002) and human vascular endothelial cells (Yamagishi et al. 2002), umbilical endothelial cells (Min 1999), osteoblasts (Alikhani 2007), fibroblasts (Alikhani 2005), podocyes (Chuang 2007). Generally, the cell lines described above were incubated with oxidized proteins for more than 24 hours. In these studies, AGE binding to their specific receptor (RAGE) has been shown to induce ROS formation and to stimulate NADPH oxidase trough PKC activation. Oxidative stress induction leading to apoptosis through caspase-3, which was activated through both cytoplasmic (caspase-8 dependent) and mitochondrial (caspase-9) pathways.

The second pourpose of the present work was to evaluate the capacity of triggered neutrophils to generate AOPP "in vitro". BSA incubated with PMA-stimulated neutrophils for 1-2-3 hours presents a significant higher level of AOPP and dityrosines respects unstimulated neutrophils. Carbonyls don't seem to be produced in these conditions, at least at the beginning of the incubation. In parallel, BSA incubated with the same concentration of HOCl produced by PMA-stimulated neutrophils, for 1-2-3 hours, presents a higher level of AOPP, dityrosines and carbonyls. Therefore, it's possible to conclude that bovine neutrophils are able to oxidize BSA "in vitro" and generate chemical and structural modification such as AOPP and dityrosines, in the experimental condition used. However, carbonyls seem to be a non specific indicator of

neutrophils-mediated protein oxidation. The direct exposure of BSA to HOCl couldn't fully mimic the complex events leading to BSA oxidation and AOPP production by activated neutrophils. The commonly used method decribed in the litterature to generate AOPP in vitro is based on the oxidation of proteins with HOCl that is the major oxidant produced by triggered neutrophils. (Witko-Sarsat et al. 1998, Dalle Donne et al. 2001, Iwao et al. 2006). In addition methods using MPO-H_2O_2-Cl$^-$ system as source of HOCl (Heinecke et al. 1993) were tested in order to mimic the in vivo production of oxidants during acute inflammation. Weiss et al and Tsan et al., have assumed the oxidation of proteins and the formation of chloramines by incubating in vitro activated neutrophil with free aminoacids such as taurine (Weiss 1982), alanine and methionine (Tsan et al 1981). To the best of our knowledge, this is the first study showing that neutrophils are directly involved in the generation of AOPP in vitro.

Therefore, on the basis of the results obtained, it's possible to conclude that:

1. Triggered bovine neutrophils are able to generate AOPP in vitro and that AOPP retain the capacity to inhibit ROS production of stimulated neutrophils.
2. AOPP can affect neutrophils functionality and viability (MTT assay) and activate the early phases of the apoptotic processes.

A challenge for the future could be to analyze the molecular mechanisms involved in the interaction of AOPP with bovine neutrophil machinery. As described before, both AOPP and AGE are able to activate human neutrophils by activating the PKC-NADPH oxidase pathway, which induces ROS production and apoptosis. These events seem to be triggered by the specific binding of AOPP and AGE with RAGE. A study on the presence of RAGE also in bovine neutrophils could be interesting. In addition, other molecules notoriously able to trigger neutrophils such as LPS and TNFa could be tested, in order to compare results with those obtained with PMA.

CHAPTER 9.

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