Immunology Department,
Animal Health Research Institute, Giza, Egypt.

ROLE OF SELECTIVE ANTIOXIDANTS AND ANTI-INFLAMMATORY DRUG IN PREVENTING UDDER CELL DAMAGE IN VITRO

(With 13 Figures)

By

A.A. RAMADAN; UMIMA M. MANSOUR* and Y.G.M. ABD EL-HAFEIZ **

* Biology of Reproduction Department, Research Institute, Giza, Egypt.
**Virology Research Unit, Animal Reproduction Research Institute, Giza, Egypt.

(Received at 22/5/2007)

دور بعض مضادات الأكسده وعقار مضاد للألتهاب فى منع تلف خلايا الضرع معمليا"

أحمد عبد الفضيل رمضان ، أميمه مبروك منصور ، ياسر جميل عبد الحفيظ

تهدف هذه الدراسه الى استيضاح دور الميلاتونين والسيلينيوم والبيتا كاروتين والهيدروكورتيزون فى حماية خلايا الضرع (فى حالات التهاب الضرع) من التأثيرات الضاره لمشتقات الأوكسجين الحره المفروزه من خلايا النيتروفيل المنشطه معمليا. أضيفت تركيزات مختلفه من الميلاتونين (٠,٥- ٥- ٥٠- ٥٠٠ ميكرومولار) أو البيتا كاروتين (٠,١- ١٠- ١٠٠ ميكرومولار) أو السيلينيوم (٠,٠٠١- ٠,٠١- ٠,١ ميكرومولار) أو الهيدروكورتيزون (٠,١- ١- ١٠ ميكرومولار) الى خلايا الضرع المنماه معمليا من ضرع الجاموس و التى تم اضافة خلايا النيتروفيل المنشطه اليها أيضا (٥×١٠^{٦} خليه لكل عينه). تم تكرار كل تركيز ٣ مرات كما تم عمل عينات تحكم تكونت من خلايا الضرع مضافا اليها خلايا النيتروفيل المنشطه معمليا فقط. بعد تحضين العينات لمدة ٢٤ ساعه تم تدويرها لفصل السائل عن الخلايا، ثم تم قياس تركيزات أنزيم لاكتات ديهيدروجينيز ومالون داي ألديهايد وأكسيد النيتريك. أيضا، تم فحص التغيرات المورفولوجيه لخلايا النيتروفيل المنشطه معمليا بعد صباغتها بصبغه ذات خاصيه فلوريسنتيه (الأكريدين البرتقالى) لفحص تأثير مشتقات الأكسجين الحره عليها. قد وجدت تركيزات عاليه من أنزيم لاكتات ديهيدروجينيز ومالون داي ألديهايد وأكسيد النيتريك فى عينات التحكم التى لم يتم معالجتها بمضادات الأكسده مما يدل على تحطم وتلف خلايا الضرع وخلايا النيتروفيل. أما الخلايا التى تمت معالجتها بتركيزات مختلفه من مضادات الأكسده أوالهيدروكورتيزون فقد كان تركيز أنزيم لاكتات ديهيدروجينيز و مالون داي ألديهايد و أكسيد النيتريك منخفض جدا مما يدل على انخفاض معدل تحطم الخلايا نتجة عمل مضادات الأكسده. وقد أظهرت الصور الملتقطه لخلايا النيتروفيل المنشطه معمليا والتى تم معالجتها بمضادات الأكسده كثره عدد

الخلايا المصبوغه باللون الأخضر بالصوره بعكس صورة الخلايا فى مجموعة التحكم والتى تظهر قلة الخلايا المصبوغه باللون الأخضر. هذه النتائج توضح أن مضادات الأكسده التى استخدمت فى هذه الدراسه وكذا الهيدروكورتيزون، كنوذج لمضادات الالتهاب، تؤثر تأثير مباشر وقوى على مشتقات الأكسجين الحره (مثل سوبر الأكسيد القاعدى- الهيدروجين بير أوكسيد- أكسيد النيتريك) المفروزه من خلايا النيتروفيل المنشطه معمليا وذلك بمعادلتها أو بمحو أثارها الضاره.

SUMMARY

The current study aimed to investigate the role of melatonin, β-carotene, selenium, and hydrocortisone in protecting a primary mammary cell line, as a model of mastitis, from the deleterious effects of reactive oxygen species (ROS) secreted from activated neutrophils in vitro. Different concentrations of melatonin (0.5, 5, 50, and 500 μM), β-carotene (1, 10, and 100 μM), selenium (0.001, 0.01, and 0.1 μM), and hydrocortisone (0.1, 1, and 10 μM) were added separately in triplicates to primary mammary epithelial cell lines prepared from active mammary tissue of female buffalo co-incubated with activated buffalo neutrophils (5 X 10^6/well). Mammary cell damage was evaluated by measuring lactate dehydrogenase (LDH), malondialdehyde (MDA) and nitric oxide (NO) concentrations in culture media as indicative of ROS, and by morphological observations of neutrophils after acridine orange staining. Activated neutrophils damaged the mammary epithelial cell line as demonstrated by an increase in LDH, MDA, and NO concentrations in control mammary cells that did not receive any treatments. Meanwhile, treated mammary epithelial cells with melatonin, β-carotene, selenium, and hydrocortisone secreted, considerably, low concentrations of LDH, MDA, and NO. Also photographs of the neutrophils treated with antioxidants or hydrocortisone did not show much damage as compared with non-treated control cells. Our results suggest that ROS such as superoxides, hydrogen peroxide, hydroxyl radical, and nitric oxide released by activated neutrophils cause damage to mammary epithelial cells indicated by increased concentrations of LDH, MDA, and NO and this deleterious effect could be quenched by the use of antioxidants such as melatonin, β-carotene, and selenium or by the use of anti-inflammatory compound such as hydrocortisone.

Key words: *Udder, antioxidants, anti-inflammatory drugs.*

INTRODUCTION

Despite major advances in herd management and antibiotic therapeutics, bovine mastitis continues to be a major source of economic loss in dairy industry (Crist *et al.*, 1983 and Smith, 1983). During the course of mastitis, the inflammatory reaction induced by the activated immune cells is accompanied by the secretion of many metabolites and molecules belong to reactive oxygen species (ROS). This species plays a central role in killing the invading microorganisms that cause mastitis, unfortunately it also have destructive effect on the udder tissues and consequently reduces milk production (Oliver and Calvinho, 1995 and Rajala-Shultz *et al.*, 1999). Accumulation of neutrophils in the udder tissues is a major sign during mastitis (Paape *et al.*, 2000) and is accompanied by the secretion of ROS such as hydrogen peroxide, superoxide anion, hydroxyl radicals, and nitric oxide (NO). These molecules are destructive to both invading pathogen causing mastitis and udder tissues (Weiss, 1989; Cuzzocrea *et al.*, 1998; Knaapen *et al.*, 1999 and Ledbetter *et al.*, 2001). The destructive cytotoxic effects of these molecules include protein oxidation, lipid peroxidation, DNA damage, and the inhibition of cellular metabolic pathways. Also, stimulated neutrophils generate hypochlorite (OHCl) via the release of the enzyme myeloperoxidase and hydrogen peroxide. Hypochlorite damages proteins by reaction with amino acid side-chains or backbone cleavage (Hawkins and Davies, 1998). Apparently, the process of inflammation during mastitis is devastating to udder tissue as well the invading organisms. Controlling the severity of inflammation and protecting the udder tissue from both pathogens and ROS seems to be a requisite for the future health of the mammary tissues.

Interestingly, many studies demonstrated that most of the antioxidants improved the neutrophils phagocytic functions in vitro and in vivo (Hemingway, 1999; Spears, 2000; Ramadan *et al.*, 2001 and Politis *et al.*, 2004). Melatonin, which is a hormone secreted by the pineal gland during night, recently showed great activity in scavenging hydroxyl radical (Marshall *et al.*, 1996) and peroxynitrite also melatonin prevented DNA damage and tissue injury (Cuzzocrea and Caputi, 1999). Selenium (Se) is an integral part of the antioxidant enzyme glutathione peroxidase (GSH-Px) and plays an important role in the maintenance of the oxidation reduction state of a cell. Selenium supplementation in the bovine has been shown to improve the outcome of acute mastitis caused by coliform bacteria (Maddox *et al.*, 1999). β-carotene is known to

activate neutrophil killing functions in vitro (Ramadan *et al.*, 2001). Moreover, this molecule proved to be effective antioxidant in protecting cells from damage during inflammation (Michal *et al.*, 1994). Hydrocortisone, anti-inflammatory agent, has been recommended in the treatment of some forms of clinical mastitis (Rossoff, 1974 and Mercer and Teske, 1977). However, this drug has been reported to cause significant decreased neutrophil functions in bovine (Paape *et al.*, 1981 and Roth and Kueberle, 1981). Therefore, this study aimed to test the hypothesis that could antioxidants diminishes and quench the destructive effect of the secreted oxidants and, hence, protect the udder tissue. We constructed a model of mastitis in vitro to test this hypothesis and we used some selective antioxidants such as melatonin, β-carotene, and Se, beside hydrocortisone as an anti-inflammatory compound to study their effect on decreasing mammary epithelial cell damage in vitro.

MATERIALS and METHODS

Primary cell culture:

Primary cell culture of active mammary tissue was prepared as standard method described by Mahy and Kangro (1996). A section of mammary tissue, from freshly slaughtered buffalo, was transferred aseptically to the laboratory in Hank's transport medium containing penicillin G-sodium (100 IU/ml), streptomycin (100 µg/ml) and antimycotic amphotricin B (0.25 µg/ml). The tissue was minced into small pieces using sterile scissor in a sterile beaker containing suitable amount of pre-warmed 0.22% trypsin and transferred onto a magnetic stirrer for 2 minutes, the tissue was digested and the supernatant was discarded. Then the tissue was digested several times in a new volume of trypsin for 20 minutes each time till the tissue was exhausted. The suspension was filtered through two layers of sterile gauze in a bottle containing 50 ml of growth minimum essential medium (GMEM; containing 10% fetal bovine serum, 200 mM HEPES buffer, 0.75 mg/ml sodium bicarbonate, 0.29 mg/ml L-glutamine) and antibiotic/antimycotic mixture as mentioned above. The cellular suspension was centrifuged at 1000 *xg* for 20 minutes at 4°C. The supernatant was discarded and the digested cells re-washed two extra times. Finally the cells re-suspended in GMEM. The number of live cells, stained with filtered neutral red stain solution (0.1 gm in 100 ml phosphate buffered saline, PBS) as 5:1 V/V at room temperature was calculated using hemocytometer. The cells then were dispensed in 24-well plates as 15 X 10^4 cells/well and the

plates were incubated overnight at 37°C and 5% CO_2 in humidified incubator till a monolayer cell line was completed.

Preparation of treatments:

Melatonin, β-carotene, Se and hydrocortisone all were used as treatments in this study. Four concentrations of melatonin were prepared (0.5, 5, 50, and 500 µM) first by dissolving in 50% methanol then diluted to the final concentration in assay medium (minimum essential medium; MEM). Three concentrations of β-carotene were prepared (1, 10, and 100 µM) first by dissolving in dimethylsulfoxide (DMSO) then diluted in assay medium (final concentration of DMSO was 0.2%) of the medium. Three concentrations of Se were prepared (0.001, 0.01, and 0.1 µM) and three concentrations of hydrocortisone (0.1, 1, and 10 µM). All of the above mentioned concentrations of the four treatments are the final concentrations distributed in the plates.

Isolation and preparation of neutrophils:

Neutrophils were isolated from whole blood of one female buffalo. Briefly, blood collected in sterilized Vacutainer tubes containing 25 µl of heparin. Blood was layered onto Ficoll solution and centrifuged at 3000 *xg* for 25 minutes at room temperature. Neutrophils settled in the bottom were collected and erythrocytes were removed using cold distilled water and warm 2.7% NaCl. Cells were washed twice, using assay medium without fetal bovine serum, and suspended in the whole medium (GMEM) at concentration of 5×10^6 cells/ml.

In vitro mastitis model assay:

The GMEM in the 24-well plates was discarded and fresh medium added to primary mammary epithelial cell line. Neutrophils then were added to each well (0.5 ml containing 2.5×10^6 neutrophil) and 50 µl opsonized zymosan (0.5mg/ml) also added to activate neutrophils and to initiate inflammation. Various treatments of melatonin, β-carotene, Se, and hydrocortisone were added to assigned wells (four wells for each concentration) to reach the final concentrations indicated above. Controls included mammary epithelial cell culture alone (basal LDH release by the primary tissue), activated neutrophils alone (basal LDH release by phagocytic cell), GMEM (basal LDH release by the medium). Plates then were incubated at 37°C and 5% CO_2. After 24 hours, supernatants were collected from each well, centrifuged, and stored frozen at -70° C until assayed for lactate dehydrogenase (LDH), malondialdehyde (MDA) and NO.

Effect of oxidative metabolites on cell viability:

After removal of the supernatants from the wells, the plates washed gently with PBS then 1 ml of acridine orange solution (8 µg/ml PBS) was added to each well and incubated at room temperature for 10 minutes to allow the stain to penetrate the cells. After incubation, acridine orange was removed from the wells and the cells fixed with 0.5 ml 4% formaldehyde solution. Wells were visualized using fluorescent microscopy and pictures of the live and dead cells were photographed.

Analyses for lactate dehydrogenase and malondialdehyde activity:

The samples collected from the wells were analyzed speactrophotometrically for both lactate dehydrogenase (LDH) (Friedman and Young, 1997) and malondialdehyde (Satoh, 1978) activities according to manufacturer manual using special kits.

Analysis for nitric oxide:

Nitric oxide was assessed according to the assay described by Rajaraman *et al.* (1998). Briefly, 100 µl from each sample was transferred into flat-bottom 96-well ELISA plate and 100 µl of Griess reagent (0.5% sulfanilamide; Sigma Chemical Co.) in 2.5% phosphoric acid (Mereck Co.) and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Chemical Co). The mixture was incubated at 21° C for 10 minutes. Absorbency of the samples and standards was measured at 570 nm using ELISA reader (Dynatech MR7000; Dynatech Laboratories Inc.). Absorbency of test samples was converted to micro molar (µM) of nitrite by comparison with absorbance values of sodium nitrite (Sigma Chemical Co.) standard curve within a linear curve fit.

Data Analyses:

Data were analyzed using General Linear Model (GLM) of Statistical analysis system (SAS) to detect the difference between treated wells and control wells. Data presented as mean ± SE

RESULTS

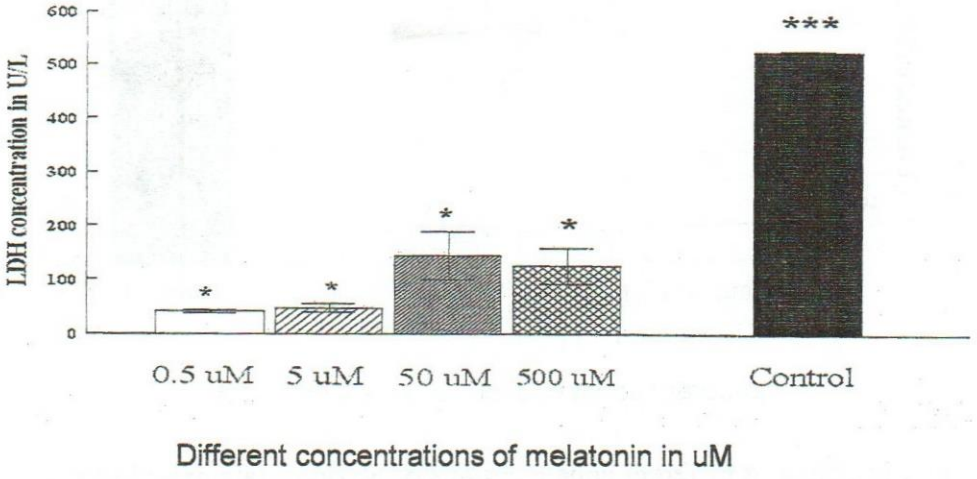

Fig. 1: Effect of different concentrations of melatonin on lactate dehydrogenase production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P < 0.01$).

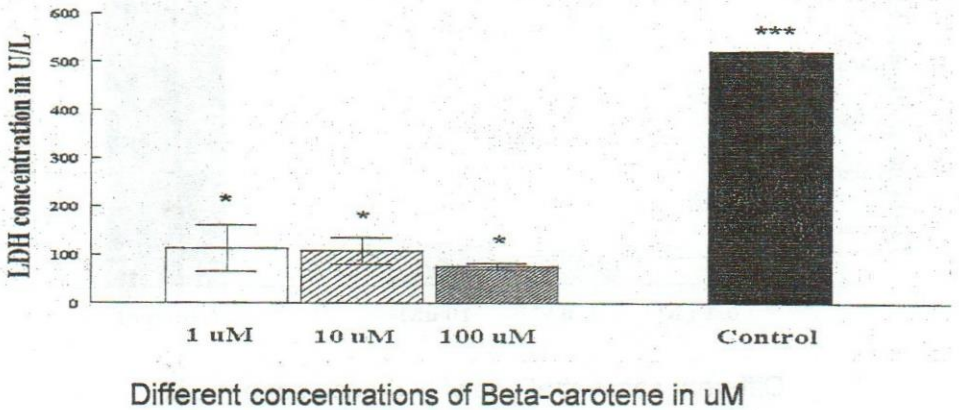

Fig. 2: Effect of different concentrations of β -carotene on lactate dehydrogenase production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P < 0.01$).

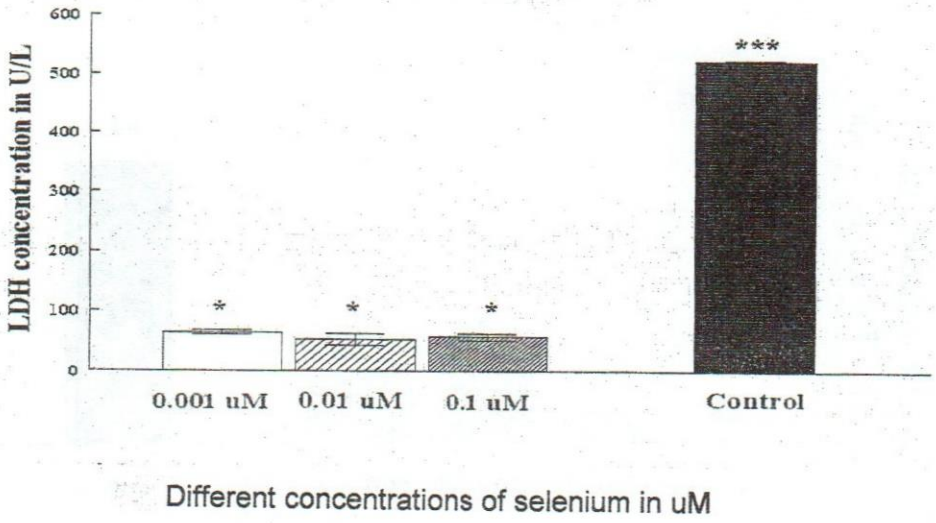

Fig. 3: Effect of different concentrations of Se on lactate dehydrogenase production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P< 0.01$).

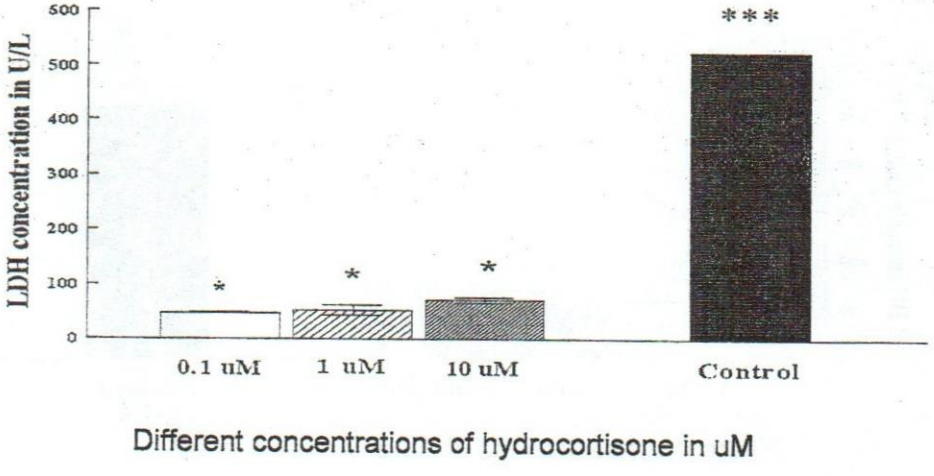

Fig. 4: Effect of different concentrations of hydrocortisone on lactate dehydrogenase production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P< 0.01$).

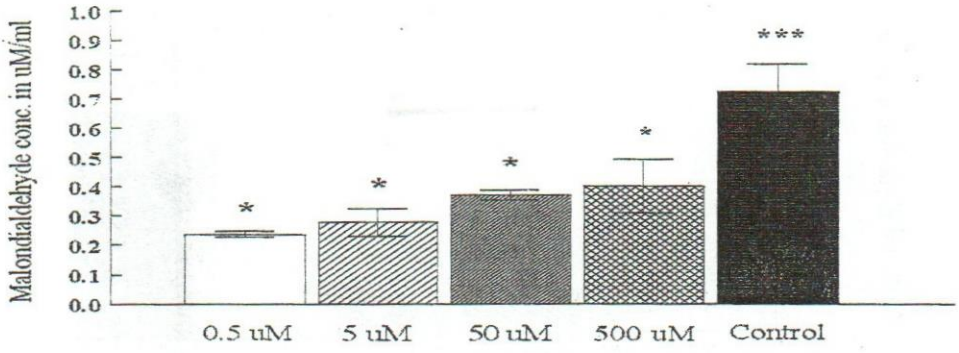

Fig. 5: Effect of different concentrations of melatonin on malondialdehyde production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P < 0.05$).

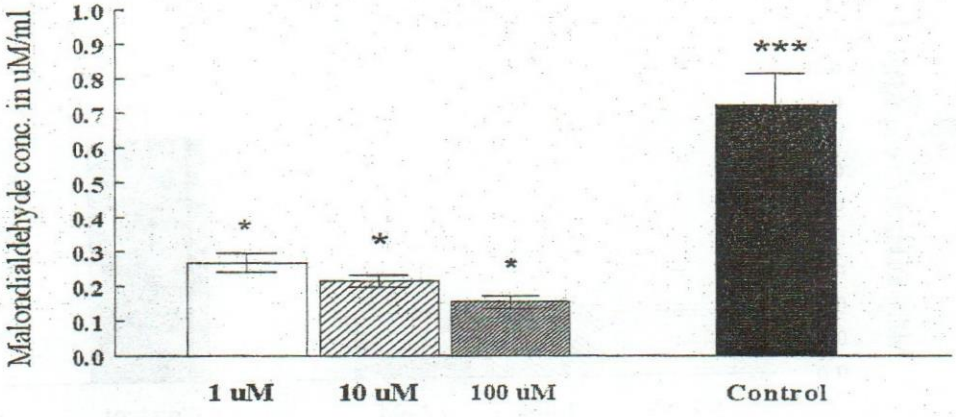

Fig. 6: Effect of different concentrations of β-carotene on malondialdehyde production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P < 0.05$).

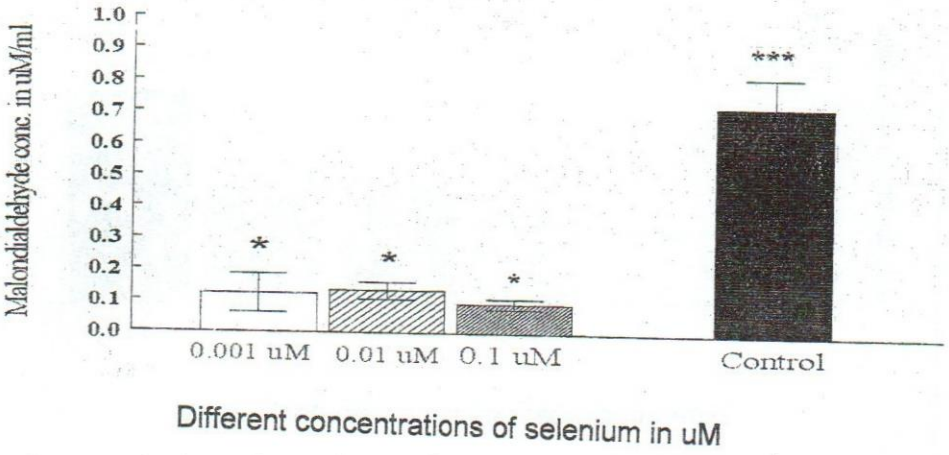

Fig. 7: Effect of different concentrations of Se on malondialdehyde production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P < 0.01$).

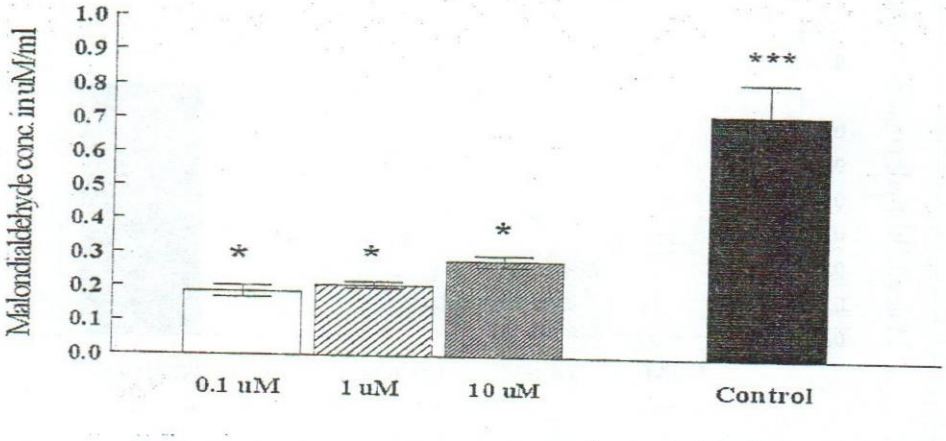

Fig. 8: Effect of different concentrations of hydrocortisone on malondialdehyde production by buffalo mammary epithelial cell line subjected to activated neutrophils in vitro. Stars indicate significant difference from control ($P < 0.05$).

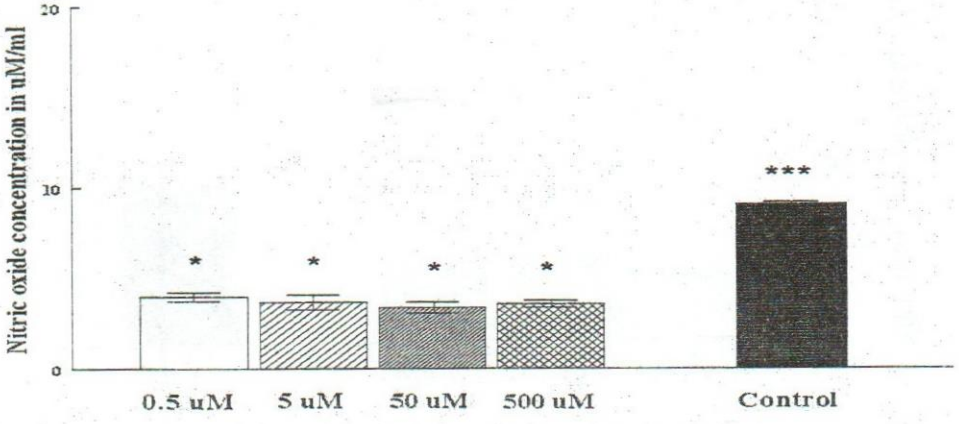

Fig. 9: Effect of different concentrations of melatonin on NO production by activated buffalo neutrophils co-cultured with mammary epithelial cell line in vitro. Stars indicate significant difference from control ($P < 0.05$).

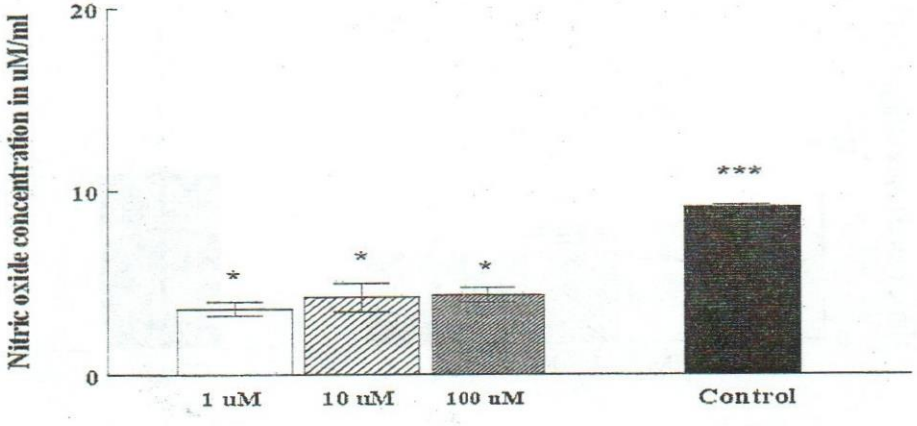

Fig. 10: Effect of different concentrations of β -carotene on NO production by activated buffalo neutrophils co-cultured with mammary epithelial cell line in vitro. Stars indicate significant difference from control ($P < 0.05$).

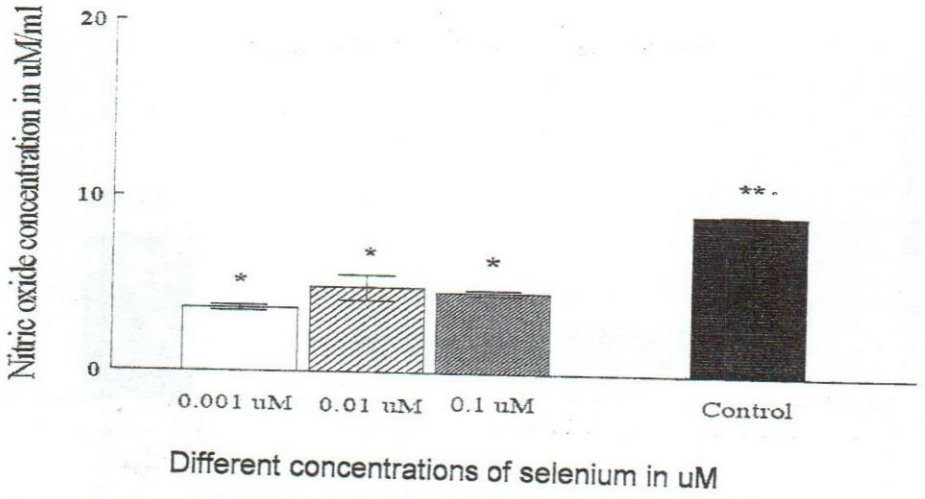

Fig. 11: Effect of different concentrations of Se on NO production by activated buffalo neutrophils co-cultured with mammary epithelial cell line in vitro. Stars indicate significant difference from control ($P < 0.05$).

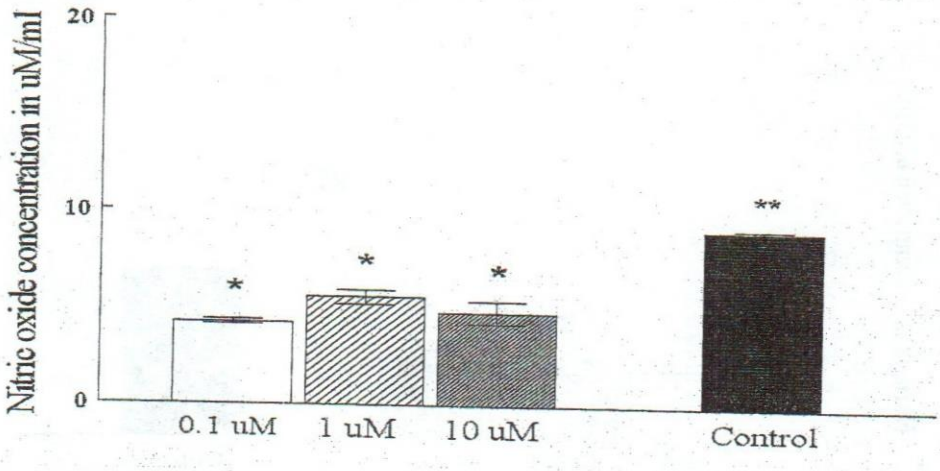

Fig. 12: Effect of different concentrations of hydrocortisone on NO production by activated buffalo neutrophils co-cultured with mammary epithelial cell line in vitro. Stars indicate significant difference from control ($P < 0.05$).

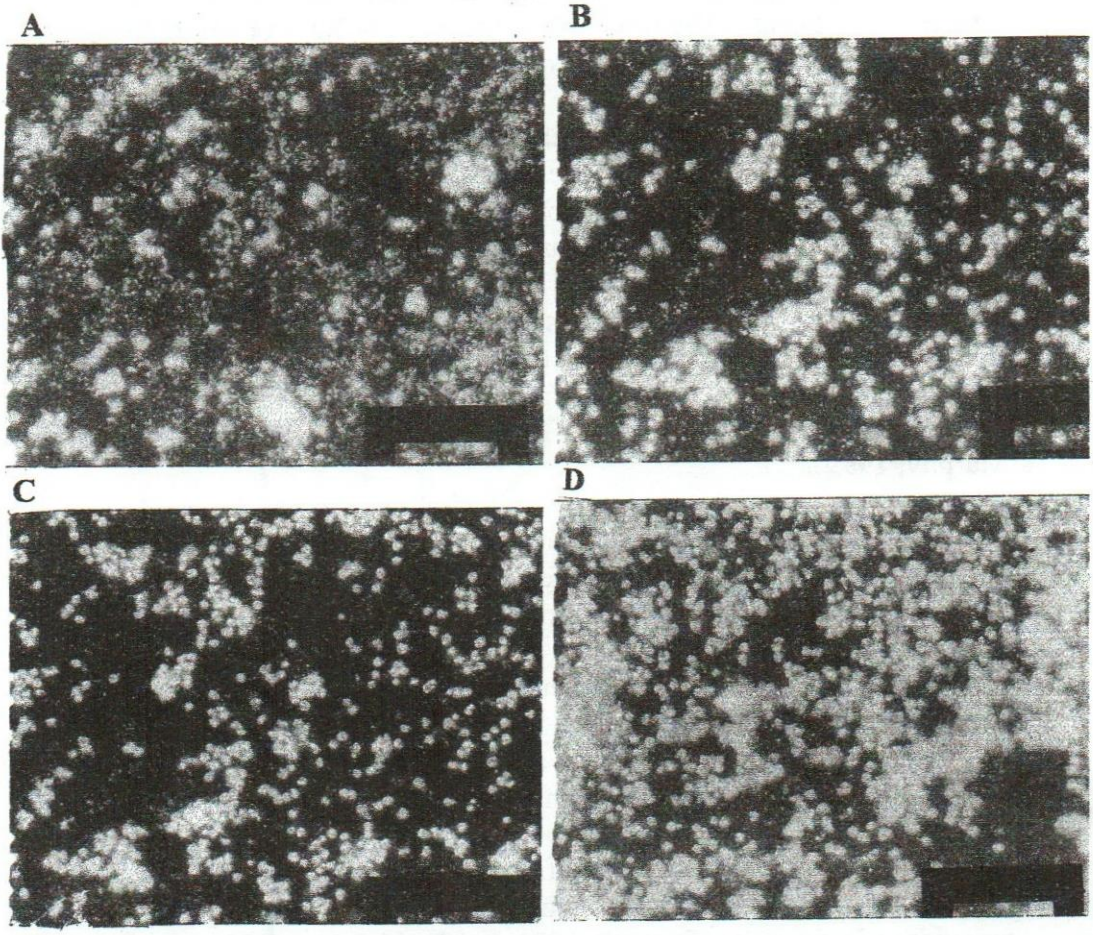

Fig. 13: Acridine orange staining of (A) activated neutrophils without any treatment (control) (B) neutrophils incubated with melatonin (C) neutrophils incubated with β-carotene (D) neutrophils incubated with Se.

DISCUSSION

The primary role of polymorphonuclear neutrophils (PMNs) is to destroy pathogenic microorganisms after phagocytosis by producing reactive oxygen species (ROS) and toxic molecules. The respiratory burst by neutrophils is characterized by marked changes in oxygen metabolism that result in increased production of superoxide and hydrogen peroxide (Baboir, 1984). Although neutrophil-generated oxygen metabolites are necessary in antimicrobial defense mechanisms,

these free radicals also can damage the neutrophil and surrounding tissues (Weiss and LoBuglio, 1982). Indeed, PMNs produce sufficient amounts of ROS during an oxidative burst to be autotoxic and detrimental to their own functions and to possibly cause DNA damage, protein and lipid oxidation and cell membrane destruction (Walrand *et al.*, 2005). Both malondialdehyde and lactate dehydrogenase enzymes are metabolites of lipid peroxidation and cellular damage (Shiono *et al.*, 2003; Claassen *et al.*, 2005; Chagunda *et al.*, 2006a and b). Elevation of both enzymes in serum or milk is associated with severe cell damage. Nitric oxide which is secreted in large amounts from the activated immune cells such as neutrophils and macrophages, is part of what is known as killing system of the immune cells. It is known to cause cellular damage during respiratory burst (Maeda and Akaike, 1998 and Murphy, 1999).

In current study, mammary epithelial cell line co-cultured with activated neutrophils produced great concentrations of LDH, MDA, and NO in vitro. Indeed, we can refer these elevated concentrations to mammary cell damage by neutrophil respiratory burst and not to bacterial cause since zymosan was used to activate neutrophils in vitro (non-bacterial activator to the neutrophils). The addition of known antioxidants (Se, melatonin, or β-carotene) or anti-inflammatory (hydrocortisone) compound greatly reduced the concentrations of LDH, MDA, and NO in vitro. Selenium in different molar concentrations, greatly reduced LDH secretion by 8-10 folds, MDA by 5-8 folds and NO by 2-3 folds compared with control non-treated mammary epithelial cells. Selenium is an integral component of the enzyme glutathione peroxidase in the cells (Diplock, 1981 and Erskine, 1993). The specific function of glutathione peroxidase is the conversion of hydrogen peroxide to water and lipid hydroperoxides to the corresponding alcohol (Smith *et al.*, 1997). Selenium is expected to play its antioxidant effect on the cytosolic level of the cell (Baker and Cohen, 1983). Many reports indicated the importance of Se in many physiological aspects of the immune and non-immune cells. Smith *et al.* (1985) noticed that heifers supplemented with Se had significantly fewer quarters infected at calving, reduced prevalence of infection throughout lactation, fewer cases of clinical mastitis, infections of shorter duration, and lower milk somatic cell count (SCC) compared with un-supplemented heifers. Boyne and Arthur (1986) reported that neutrophils from Se-deficient mice, rats and cattle were able to ingest pathogens in vitro but were less able to kill them than did neutrophils from Se-sufficient animals. This

defective function has been associated with decreased cytosolic glutathione peroxidase (GPx1) activity in the neutrophils, which allows the free radicals that are produced in the respiratory burst to kill the neutrophils themselves. Also Vitamin E and Se were related to rate of clinical mastitis and bulk tank milk SCC in a survey of Ohio herds that had controlled contagious mastitis (Hogan *et al.*, 1989; Weiss *et al.*, 1990). Grasso *et al.* (1990) showed that PMN from Se-deficient cows had increased accumulation of hydrogen peroxide, decreased viability, and reduced ability for intracellular kill of mastitis-causing pathogens. Smith *et al.* (1997) pointed out that Se plays important role in udder health through quenching ROS from the mammary cells during mastitis. Ramadan *et al.* (2001) reported that Se enhanced both phagocytic and killing activities of neutrophils isolated from peripartal buffaloes in vitro. Cebra *et al.* (2003) suggested that neutrophils from postparturient dairy cows with higher blood concentrations of Se have greater potential to kill microbes. Kommisrud *et al.* (2005) described a positive association between increased blood Se concentration pre partum and decreased incidence of mastitis, ovarian cysts and anoestrus/silent estrus post partum. Gumustekin *et al.* (2004) reported that Se supplementation may accelerate wound healing by preventing induced oxidative stress and lipid peroxidation in injured tissues. It is worth mentioning that NO was not dramatically decreased like MDA or LDH. This may be due to the fact that NO is integral part of the killing system of the neutrophils and its presence is required at this point to eliminate infection.

Melatonin is synthesized and secreted from the pineal gland during the night and is involved in various physiological processes (Borjigin *et al.*, 1999). Melatonin is a hydroxyl radical scavenger and has been shown to protect cells against the damage induced in experimental models of inflammation (Reiter *et al.*, 1995; Marshall *et al.*, 1996 and Cuzzocrea and Caputi, 1999). Melatonin, lately, received much attention from many researchers in many aspects. Lopez-Gonzalez *et al.* (1993) described the presence of receptors of melatonin in neutrophils. Silva *et al.* (2004) proved that neutrophils are one of the main targets for melatonin and that melatonin induces cytokine production by neutrophils. Cos *et al.* (1998) described a direct anti-proliferative effect of melatonin on estrogen-responsive MCF-7 cells (human breast cancer cells) in culture. In our study, melatonin reduced the production of LDH by 3-13 folds, MDA by 2-3 folds, and NO by 2-3.5 folds by buffalo mammary epithelial cell line co-cultured with activated neutrophils compared with control non-treated cells.

Melatonin has been reported to prevent LDH release from MAC-T cells cultured with activated neutrophils and prevented morphological changes of MAC-T cells (Boulanger *et al.*, 2002). Chang and Mun (2004) reported that the level of MDA generated in neutrophils treated with Cyclosporine (which contributes to oxygen free radical metabolism in neutrophils) was increased, suggesting that damage resulted from oxygen free radicals by this compound. This damage was reduced by the addition of melatonin to the culture. Also, Barlas *et al.* (2004) reported that melatonin injected intra peritoneal in rats with acute pancreatitis, reduced the level of MDA secretion in both liver and pancreatic tissue samples. They concluded that melatonin may by its free-radical scavenging and antioxidant activity, which involves an inhibitory effect on tissue neutrophil infiltration, protected pancreatic and hepatic tissues.

β-carotene, in this study, reduced the production of MDA and LDH by 2-5 folds and 4-7 folds respectively by buffalo mammary epithelial cell line co-incubated with activated neutrophils compared with control non-treated cells in vitro. It is obvious that β-carotene acted to prevent or block the damage of both mammary cells and neutrophils. Recently, Walrand *et al.* (2005) indicated that carotenoids have quenching capacities that control both in vivo and in vitro the neutrophils generation of ROS and probably protect these cells against DNA, membrane lipid and protein damages during oxidative burst. β-carotene also been reported to enhance the phagocytic and killing activity of neutrophils in vitro (Ramadan *et al.*, 2001). Moreover, Michal *et al.* (1994) described multiple effects of β-carotene in dairy cows such as enhanced host defense mechanisms by potentiating phagocytic function and decreased the incidence of certain reproductive disorders. Bohne *et al.* (1997) reported that β-carotene significantly and concentration-dependent inhibited the ROS generation by stimulated neutrophils in vitro. Our results also showed a dose-dependent effect of β-carotene on the production of MDA, LDH, and NO in vitro. Jiang *et al.* (1996) described that β-carotene could significantly inhibit the damage of rat neutrophil membrane protein caused by either intracellular or extracellular ROS.

In current study, hydrocortisone significantly reduced mammary cell damage in the form of decreased LDH by 8-11 folds, MDA by 2-4 folds and NO by 1-2 folds compared with control non-treated cells in vitro. Hydrocortisone is used, pharmacologically, to suppress the immune system reaction in certain diseases such as autoimmune diseases and in some allergic conditions. Recently, Kraemer *et al.* (2005)

reported that cortisol significantly reduced plasma MDA concentrations in volunteers after 65 minutes post exercise. Also hydrocortisone inhibited NO production by rat cardiomyocytes and decreased cellular lipid peroxidation (Florio, 2003). Lactate dehydrogenase release from activated immune cells was blocked by treating the cells with corticosteroids (Banks and Michales, 1985).

The effect of Se, β-carotene, and melatonin on activated buffalo neutrophils in vitro has been demonstrated in Figure 13 (B, C and D) compared with non treated cells (A). The overall effect of these antioxidants was protective. The integrity and number of live neutrophils was greater, as indicated by increased fluorescent green stain, while the fluorescent stain decreased in control non-treated cells.

In conclusion, results of this study demonstrated the protective effect of Se, melatonin, and β-carotene, as antioxidants, and hydrocortisone, as anti-inflammatory, on buffalo mammary epithelial cell line in vitro. During mastitis, excessive cell damage due to the inflammatory process may be considered more dangerous than the infection itself since ROS are secreted in great quantities enough to damage mammary cells. Anti-inflammatory agents seems to act locally on the immune cells through specific cell receptor and their duration of activity seems also to be limited, so their use for prolonged time will not affect ROS secretion but on the other hand will suppress the immune cells. Therefore, the use of anti-inflammatory agents to control inflammation during mastitis should be well controlled. On the contrary, we encourage the use of antioxidants during mastitis for long periods because they will not be detrimental or suppressive to the immune cells, but they may enhance the phagocytic activity of neutrophils and protect them from ROS. Both MDA and LDH are known enzymes associated with lipid peroxidation and hence they consider as marker for cellular damage. Nitric oxide, in current study, although it was significantly decreased in response to antioxidants and hydrocortisone treatment, yet the decrease was not dramatically like LDH and MDA. May be it is essential metabolite necessary for controlling infection and antioxidants or hydrocortisone could not suppress it more than that.

REFERENCES

Babior, M. (1984): The respiratory burst of phagocytes. J. Clin. Invest. 73: 599.

Baker, S. and Cohen, H. (1983): Altered oxidative metabolism in Se-deficient rat granulocytes. J. Immunol. 130: 2856.

Banks, K. and Michaels, F. (1985): Stimulation and killing of bovine mononuclear leukocytes by bacterial lipopolysaccharide (endotoxin). Am. J. Vet. Res. 46 (7): 1568-1572.

Barlas, A.; Cevik, H.; Arbak, S.; Bangir, D.; Sener, G.; Yegen, C. and Yegen, B. (2004): Melatonin protects against pancreaticobiliary inflammation and associated remote organ injury in rats: role of neutrophils. J. Pineal Res. 37 (4): 267-275.

Bohne, M.; Struy, H.; Gerber, A. and Gollnick, H. (1997): Protection against UVA damage and effects on neutrophil-derived reactive oxygen species by beta-carotene. Inflamm. Res. 46 (10): 425-426.

Borjigin, J.; Li, X.; and Snyder, S. (1999): The pineal gland and melatonin: molecular and pharmacologic regulation. Annu. Rev. Pharmacol. Toxicol. 39: 53–65.

Boulanger, V.; Zhao, X. and Lacasse, P. (2002): Protective effect of melatonin and catalase in bovine neutrophil-induced model of mammary cell damage. 85 (3): 562-569.

Boyne, R. and Arthur, J. (1986): The response of selenium deficient mice to Cand. albicans infect. J. Nutr. 116: 816–822.

Cebra, C.; Heidel, J.; Crisman, R. and Stang, B. (2003): The relationship between endogenous cortisol, blood micronutrients, and neutrophil function in postparturient Holstein cows. J. Vet. Intern. Med. 17 (6): 902-907.

Chagunda, M.; Friggens, N.; Rasmussen, M.; and Larsen, T. (2006a): A model for detection of individual cow mastitis based on an indicator measured in milk. J. Dairy Sci. 89 (8): 2980-2998.

Chagunda, M.; Larsen, T.; Bjerring, M. and Ingvartsen, K. (2006b): L-lactate dehydrogenase and N-acetyl-beta-D-glucosaminidase activities in bovine milk as indicators of non-specific mastitis. J. Dairy Res. 73 (4): 431-440.

Chang, E. and Mun, K. (2004): Effect of melatonin on the malondialdehyde level of neutrophils in cyclosporine-treated rats. Transpl. Proc. 36(7): 2165-2166.

Claassen, H.; Schunke, M. and Kurz, B. (2005): Estradiol protects cultured articular chondrocytes from oxygen-radical-induced damage. Cell Tissue Res. 319(3): 439-445.

Cos, S.; Fernandez, R.; Guezmes, A. and Sanchez-Barcelo, E. (1998): Influence of melatonin on invasive and metastatic properties of MCF-7 human breast cancer cells. Cancer Res. 58(19): 4383-4390

Crist, W.; Harmon, R. and Newman, L. (1983): How much does mastitis cost? Udder Tropics 6: 6.

Cuzzocrea, S.; Zingarelli, E.; Hake, P.; Salzman, A. and Szabò. (1998): Antiinflammatory effects of mercaptoethylguanidine, a combined inhibitor of nitric oxide synthease and peroxynitrite scavenger, in carrageenan-induced models of inflammation. Free Rad. Biol. Med. 24: 450-459.

Cuzzocrea, S. and Caputi, A. (1999): Protective effect of melatonin on zymosan-induced cellular damage. Biol. Signals Recept. 8: 136–142.

Diplock, A. (1981): The role of vitamin E and selenium in the prevention of oxygen-induced tissue damage. In: Spallholz, J.E.; Martin, J.L. and Ganther, H.E. (Ed.) Selenium in Biology and Medicine. AVI Publishing, Hartford, CT. p 303.

Erskine, R. (1993): Nutrition and mastitis. In: Andersen, K. L. (Ed.) The Veterinary Clinics of North America: Food Animal Practice. W. B. Saunders, Philadelphia, PA. p 551.

Florio, S.; Ciarcia, R.; Crispino, L.; Pagnini, U.; Ruocco, A.; Kumar, C.; D'Andrilli, G. and Russo, F. (2003): Hydrocortisone has a protective effect on Cyclosporin A-induced cardiotoxicity. J. Cell Physiol. 195 (1): 21-26.

Friedman and Youmg (1997): Effects of disease on clinical laboratory tests, 3rd ed. AACC Press.

Grasso, P.; Scholz, R.; Erskine, R. and Eberhart, R. (1990): Phagocytosis, bactericidal activity, and oxidative metabolism of mammary neutrophils from dairy cows fed selenium-adequate and selenium-deficient diets. Am. J. Vet. Res. 51: 269.

Gumustekin, K.; Seven, B.; Karabulut, N.; Aktas, O.; Gursan, N.; Aslan, S.; Keles, M.; Varoglu, E. and Dane, S. (2004): Effects of sleep deprivation, nicotine, and selenium on wound healing in rats. Int. J. Neurosci. 114 (11): 1433-1442.

Hawkins, C. and Davies, M. (1998): Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. Biochem. J. 15: 332 (Pt 3): 617-625.

Hemingway, R. (1999): The influences of dietary selenium and vitamin E intakes on milk somatic cell counts and mastitis in cows. Vet. Res. Commun. 23 (8): 481-499.

Hogan, J.; Smith, K.; Hoblet, K.; Schoenberger, P.; Todhunter, D.; Hueston, W.; Pritchard, D.; Bowman, G.; Heider, L.; Brockett, B. and Conrad, H. (1989): Field survey of mastitis in low somatic cell count herds. J. Dairy Sci. 72: 1547.

Jiang, J.; Yu, Z.; Qiu, Q. and Wu, Y. (1996): Protective effects of beta-carotene liposome against rat neutrophil membrane damage caused by intra- or extra-cellular reactive oxygen species. Zhongguo, Yi Xue, Ke Xue, and Yuan Xue Bao. 18 (5): 387-391.

Knaapen, A.; Seiler, F.; Schilderman, A.; Nehls, P.; Bruch, J.; Schins, R. and Borm, P. (1999): Neutrophils cause oxidative DNA damage in alveolar epihelial cells. Free Rad. Biol. Med. 27: 234–240.

Kommisrud, E.; Osteras, O. and Vatn, T. (2005): Blood selenium associated with health and fertility in Norwegian dairy herds. Acta Vet. Scand. 46 (4): 229-240.

Kraemer, W.; French, D.; Spiering, B.; Volek, J.; Sharman, M.; Ratamess, N.; Judelson, D.; Silvestre, R.; Watson, G.; Gomez, A. and Maresh, C. (2005): Cortisol supplementation reduces serum cortisol responses to physical stress. Metabolism. 54 (5): 657-668.

Ledbetter, T.; Paape, M. and Douglass, L. (2001): Cytotoxic effects of peroxynitrite, polymorphonuclear neutrophils, free-radical scavengers, inhibitors of myeloperoxidase, and inhibitors of nitric oxide synthase on bovine mammary secretory epithelial cells. Am. J. Vet. Res. 62: 286–293.

Lopez-Gonzalez, M.; Calvo, J.; Segura, J. and Guerrero, J. (1993): Characterization of melatonin binding sites in human peripheral blood PMN, Biotechnol. Ther. 4: 253–262.

Maddox, J.; Aherne, K.; Reddy, C. and Sordillo, L. (1999): Increased neutrophil adherence and adhesion molecule mRNA expression in endothelial cells during selenium deficiency. J. Leukoc Biol. 65 (5): 658-664.

Maeda, H. and Akaike, T. (1998): Nitric oxide and oxygen radicals in infection, inflammation and cancer. Biochem. 63: 854–865.

Mahy, B. and Kangaro, H. (1996): Virology Methods Manual. Academic Press Limited, London, NW.

Marshall, K.; Reiter, R.; Poeggeler, B.; Aruoma, O. and Halliwell, B. (1996): Evaluation of the antioxidant activity of melatonin in vitro. Free Rad. Biol. Med. 21: 307–315.

Mercer, H. and Teske, R. (1977): Special considerations for the development of drugs for acute clinical mastitis. J. Am. Vet. Med. Assoc. 170: 1190-1193.

Michal, J.; Heirman, L.; Wong, T.; Chew, B.; Frigg, M. and Volker, L. (1994): Modulatory effects of dietary beta-carotene on blood and mammary leukocyte function in periparturient dairy cows. J. Dairy Sci. 77 (5): 1408-1421.

Murphy, M. (1999): Nitric oxide and cell death. Biochem. Biophys. Acta 1411: 401–414.

Oliver, S. and Calvinho, 0. (1995): Influence of inflammation on mammary gland metabolism and milk composition. J. Anim. Sci. 73: 18-33.

Paape, M.; Shafer-Weaver, K.; Capuco, K.; Van Oostveldt, K. and Burvenich, C. (2000): Immune surveillance of mammary tissue by phagocytic cells. Adv. Exp. Med. Biol. 480: 259-277.

Paape, M.; Gwazdauskas, F. and Guidry, A. (1981): Concentrations of corticosteroids, leukocytes, and immunoglobulins in blood and milk after administration of ACTH to lactating dairy cattle: effects on phagocytosis and Staphylococcus aureus by polymorphonuclear leukocytes. Am. J. Vet. Res. 42: 2081-2087.

Politis, I.; Bizelis, I.; Tsiaras, A. and Baldi, A. (2004): Effect of vitamin E supplementation on neutrophil function, milk composition and plasmin activity in dairy cows in a commercial herd. J. Dairy Res. 71 (3): 273-278.

Rajala-Schultz, P.; Gröhn, T.; Mc Culloch, C. and Guard, C. (1999): Effects of clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 82: 1213–1220.

Rajaraman, V.; Nonnecke, B.; Franklin, S.; Hammell, D. and Horst, R. (1998): Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. J. Dairy Sci., 81: 3278-3285.

Ramadan, A.; Ghoniem, A.; Hassan, H. and Youssef, A. (2001): Effects of β-carotene, selenium and vitamin A. on in vitro polymorphonuclear leukocytic activity in peripheral blood of buffalo (Bubalus bubalus). Theriogenology 55: 693-704.

Reiter, R.; Melchiorri, D.; Sewerynek, E.; Poeggeler, B.; Barlow-Walden, L.; Chuang, J.; Ortiz, G. and Acuna-Castro-Vieio, D. (1995): A review of the evidence supporting melatonin's role as an antioxidant. J. Pineal Res. 18: 1–11.

Rossoff, I. (1974): Corticotropin. In Hand book of Veterinary Drugs. New York: Springer Publishing Co. Inc. 139.

Roth, J. and Kaeberle, M. (1981): Effects of in vivo dexamethasone administration on in vitro bovine polymorphonuclear leukocyte function. Infect. Immun. 33: 434-441.

SAS (1993): User's Guide: Statistics, Version 6.08. SAS Inst., Inc., Carry, NC.

Satoh, K. (1978): Clinical Chemical Acta. 90: 37.

Shiono, H.; Yagi, Y.; Chikayama, Y.; Miyazaki, S. and Nakamura, I. (2003): The influence of oxidative bursts of phagocytes on red blood cell oxidation in anemic cattle infected with Theileria sergenti. Free Radic. Res. 37 (11): 1181-1189.

Silva, S.; Rodrigues, M.; Ximenes, V.; Bueno-da-Silva, A.; Amarante-Mendes, G. and Campa, A. (2004): Neutrophils as a specific target for melatonin and kynuramines: effects on cytokine release. J. Neuroimmunol. 156 (1-2): 146-152.

Smith, K. (1983): Mastitis control: a discussion. J. Dairy Sci. 66: 1790.

Smith, K.; Conrad, H.; Amiet, B. and Todhunter, D. (1985): Incidence of environmental mastitis as influenced by vitamin E and selenium. Kiel. Milchwirtsch. Forschungsber. 37: 482.

Smith, K.; Hogan, J. and Weiss, W. (1997): Dietary Vitamin E and Selenium Affect Mastitis and Milk Quality. J. Anim. Sci. 75: 1659–1665.

Spears, J. (2000): Micronutrients and immune function in cattle. Proc. Nutr. Soc. 59 (4): 587-594.

Walrand, S.; Farges, M.; Dehaese, O.; Cardinault, N.; Minet-Quinard, R.; Grolier, P.; Bouteloup-Demange, C.; Ribalta, J.; Winklhofer-Roob, B.; Rock, E. and Vasson, M. (2005): In vivo and in vitro evidences that carotenoids could modulate the neutrophil respiratory burst during dietary manipulation. Eur. J. Nutr. 44 (2): 114-120.

Weiss, S. (1989): Tissue destruction by neutrophils. N. Engi. J. Med. 320: 365-376.

Weiss, W.; Hogan, J.; Smith, K. and Hoblet, K. (1990): Relationships among selenium, vitamin E, and mammary gland health in commercial dairy herds. J. Dairy Sci. 73: 381.

Weiss, S. and LoBuglio, A. (1982): Phagocytic-generated oxygen metabolites and cellular injury. Lab. Invest. 47: 5.