

ANTIOXIDANT PROPERTIES OF MILK FROM
MOTHERS OF PRE-TERM AND FULL-TERM INFANTS
COMPARED TO INFANT FORMULA

CENTRE FOR NEWFOUNDLAND STUDIES

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MATTHEW D. LANGDON



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TERM AND FULL-TERM INFANTS COMPARED TO INFANT
FORMULA**

By: Matthew D. Langdon

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Department of Biochemistry and Nutrition

Memorial University of Newfoundland

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... ABSTRACT ...

Early feeding of low birth weight (LBW) infants with their own mothers' milk has been shown to decrease the incidence of oxygen radical related disease states. Milk from mothers of pre-term (PT) and full-term (FT) infants may exhibit antioxidant and free radical scavenging ability not provided by humanized formulas. This study investigated the ability of milk from weeks 1, 2 and 12 of lactation (17 PT and 28 FT mothers) to resist oxidative stress by free radical attack as compared to formula. The hypoxanthine/xanthine oxidase (HX/XO) system was utilized to generate the radicals and subsequent oxygen consumption was measured. The samples were also tested for catalase (CAT) activity, and for malondialdehyde (MDA) levels by HPLC following stress. As well, normal (3 mg/L) and high (12 mg/L) iron formulas were tested for their ability to resist oxidative damage with and without the presence of added antioxidant enzymes CAT, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx). There was no difference ($p > 0.05$) between the PT and FT groups with respect to oxygen consumption (nmoles O_2 /min) at any stage of lactation. The means \pm SEM of O_2 consumption reported for the groups respectively following HX/XO stress, were for week one, 7.97 ± 0.56 and 6.68 ± 0.88 ; for week two, 4.62 ± 0.42 and 5.17 ± 0.36 ; and for week twelve, 4.08 ± 0.39 and 4.80 ± 0.49 . There was also no difference in MDA levels or CAT activity between groups. This indicates that PT milk has similar antioxidant capability as FT milk. A correlation ($r^2 = .443$, $p < 0.05$) existed between O_2 consumption and MDA levels, and a negative correlation ($r^2 = -0.30$, $p < 0.01$) between the amount of oxygen consumed, and the activity of CAT for all weeks, demonstrating a possible protective role of this enzyme

in human milk (HM). A difference ($p < 0.001$) was reported in total O_2 consumption (nmoles) over time between the HM (44.75 ± 1.66) and both the normal (98.88 ± 3.11) and high-iron (168.94 ± 9.47) formulas. Addition of CAT, SOD and GSHPx together was shown to increase ($p < 0.01$) the antioxidant capacity of the normal and high-iron formula by decreasing the oxygen consumption to 66.11 ± 6.48 and 104.81 ± 8.65 respectively. Furthermore, the addition of Fe to HM resulted in an increase in O_2 consumption compared to HM controls providing an indication of iron as a free radical generator in infant formula. There was no catalase activity detected in the formula and upon pasteurization of the HM to inactivate the enzymes, antioxidant capability was not compromised.

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... LIST OF ABBREVIATIONS ...

ATP	→	Adenosine Triphosphate
AMP	→	Adenosine Monophosphate
BHT	→	Butylated Hydroxytoluene
BPD	→	Bronchopulmonary Displasia
CAT	→	Catalase
DTPA	→	Diethylenetriaminepentacetic acid
FT	→	Full-term
GI	→	Gastrointestinal
GR	→	Glutathione Reductase
GPx/GSHPx	→	Glutathione Peroxidase
GSH	→	Glutathione
GSSG	→	Glutathione Disulfide
H ₂ O ₂	→	Hydrogen Peroxide
HM	→	Human Milk
HPLC	→	High Pressure Liquid Chromatography
HX	→	Hypoxanthine
IVH	→	Intraventricular Hemorrhage
LBW	→	Low Birth Weight
MDA	→	Malondialdehyde
NEC	→	Necrotizing Enterocolitis

$O_2^{\bullet -}$	→	Superoxide Radical
PT	→	Pre-term
PUFA	→	Polyunsaturated Fatty Acids
RDS	→	Respiratory Distress Syndrome
ROP	→	Retinopathy of Prematurity
ROS	→	Reactive Oxygen Species
SOD	→	Superoxide Dismutase
TBA	→	Thiobarbituric Acid
TPN	→	Total Parenteral Nutrition
VLBW	→	Very Low Birth Weight
XO	→	Xanthine Oxidase
$^{\bullet}OH$	→	Hydroxyl Radical

... CHAPTER 1: LITERATURE REVIEW ...

Recently, oxygen-derived free radicals have become increasingly implicated in a wide variety of human health problems (Halliwell, 1994). Free radical-mediated cell injury occurs both in situations of increased radical exposure or in instances where the protective antioxidant systems are depleted or impaired. Much research is still required to produce a thorough understanding of specific free radical involvement in any particular disease, but current evidence clearly states that reactive oxygen species (ROS) often play a significant role.

All classes of biological molecules are sites of potential ROS attack, especially in the LBW infant, where defense mechanisms are not fully developed and the patient is susceptible to increased oxygen stress. Newborn infants are faced with an abrupt change to a relatively hyperoxic extrauterine environment where oxygen concentrations are five times higher than during intrauterine development (Stone, 1999). Because oxygen radicals can be generated as a consequence of acute and chronic inflammation (Baggiolini & Thelen, 1991), ischemia-reperfusion injury (Oldham & Bowen, 1998.; Sokol & Hoffenberg, 1996), and various other environmental factors it is not surprising that free radicals and antioxidant defense mechanisms have been extensively studied [See figure 1.1].

Very little is known, however, about the antioxidant properties of HM and the possible ROS-antioxidant interactions. Buescher and McIlheran (1988) have demonstrated the antioxidant properties of human colostrum by its ability to deplete hydrogen peroxide and protect epithelial cells from polymorphonuclear leukocyte mediated damage. HM has

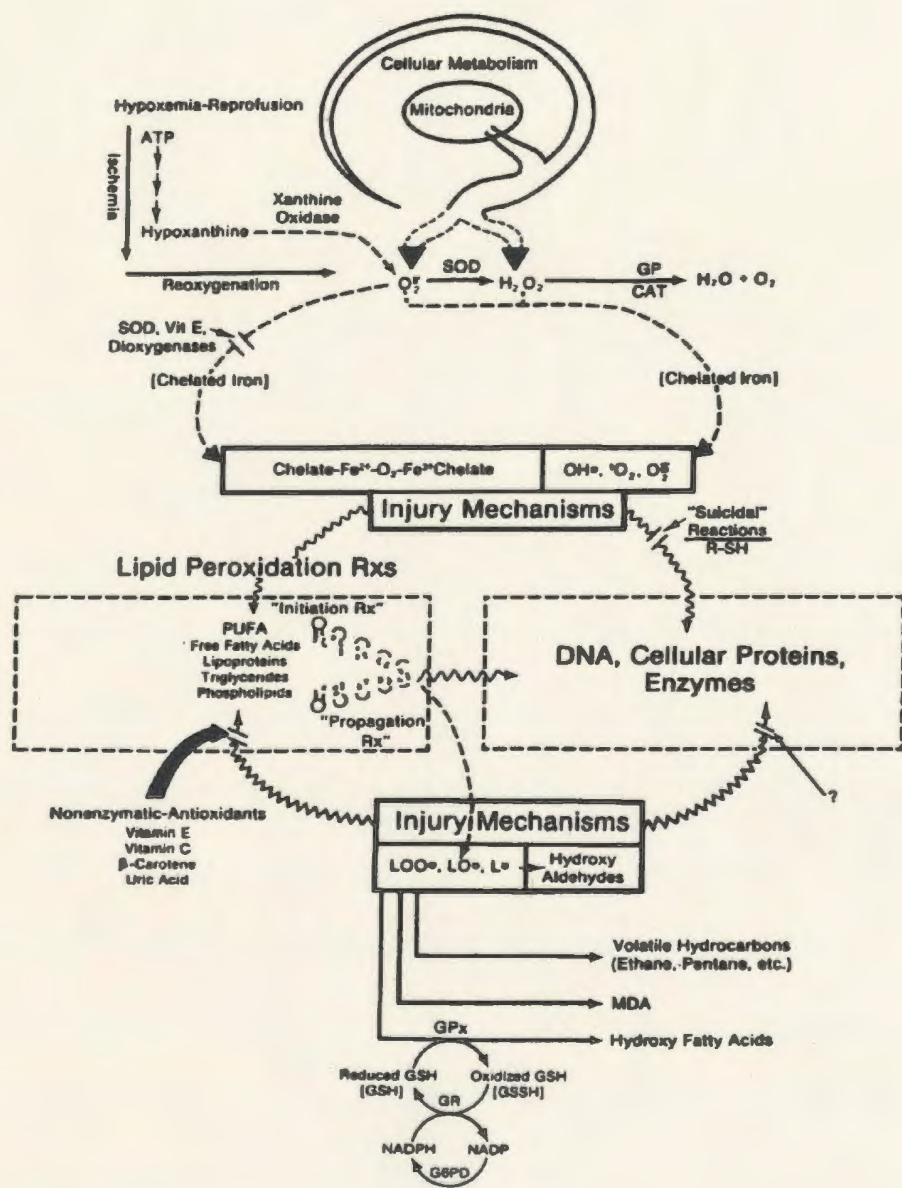


FIGURE 1.1 The source and generation of free radicals and ROS intermediates, and the enzymatic and nonenzymatic protective systems available to the cell. (From Roberts and Frank: Developmental consequences of Oxygen toxicity.)

also been shown to protect against the pathogenesis of necrotizing enterocolitis (NEC) (Marshall & Roberts, 1990). In the latter experiment it was demonstrated that upon pasteurization of the milk, protective effects were lost, leading to the hypothesis that enzymatic defenses that would be denatured by heat are vital to the milk's protective nature. Almass et. al. (1997), found that $^{\bullet}\text{OH}$ production in breast milk in response to oxidative stress was lower compared to humanized formulas thus demonstrating an additional free radical sequestering ability.

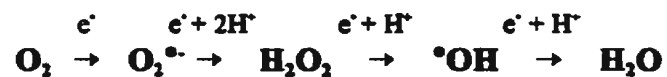
Human milk and infant formulas provide approximately the same level of nonenzymatic antioxidants such as vitamin E and vitamin C. But due to processing procedures such as pasteurization, the cow's milk used for the formula loses some of its functionality, namely enzyme activities, and therefore cannot provide enzymatic defenses to free radical attack. Enzymes such as SOD, GSHPx and CAT present in the mothers milk but, not in humanized formulas, may provide the same physiological protection as in other body tissues. Other, less prominent, enzymes such as myeloperoxidase and lactoperoxidase have also demonstrated antioxidant capability. It has also been shown that premature infants can absorb intact proteins, such as apolactoferrin (Sullivan, 1988) and boost antioxidant capacity. It may be that these predominant antioxidant enzymes in the mother's milk can also cross the neonatal gut intact and increase antioxidant status and prevent disease.

The study of the antioxidant systems in HM in comparison with humanized formulas is important in understanding how these agents may provide protection against disease in the LBW infant which may experience significant oxidative stress in the first few weeks of life.

1.1 Reactive Oxygen Species

A free radical is defined as an atom or molecule that contains one or more unpaired electron(s) in its outer orbital rather than the usual paired electron spins found in most stable molecules (Sokol & Hoffenberg, 1996). An agent becomes a free radical then, by either gaining or losing an additional electron. Thus the free radical generally becomes very reactive as it attempts to re-establish a paired electron system by abstracting electrons from other molecules. ROS, on the other hand, include not only oxygen-centered radicals but also non-radical derivatives of oxygen such as H_2O_2 which may cause damage to body tissues.

In the well condition the major source of ROS produced in the body occurs in mitochondrial and microsomal electron transport chains as O_2 is reduced to superoxide ($\text{O}_2^{\bullet-}$). Subsequent one-electron reductions may then take place to form other ROS as follows:



Each intermediate has the potential to initiate and propagate radical reactions with proteins, DNA and lipids. The most important ROS involved in biological systems are listed in Table 1.1. $\text{O}_2^{\bullet-}$ is the most commonly formed ROS during normal physiological processes. About 1% to 3% of the O_2 we metabolize is converted to $\text{O}_2^{\bullet-}$ (Oldham & Bowen, 1998). As demonstrated by figure 1.2 below, phagocytic cells may release an additional endogenous source of $\text{O}_2^{\bullet-}$ during the inflammatory response (Kilgore & Lucchesi, 1995). Figure 1.3 demonstrates how the antioxidant enzymes work in concert to significantly delay or inhibit the formation of harmful free radicals such as $\text{}^{\bullet}\text{OH}$ and therefore prevent major tissue

damage. During conditions of oxygen stress, if these defense mechanisms are available in adequate quantities in the developing LBW infant, tissue (lipid membranes, protein, DNA) damage will occur leading to the onset of oxygen radical-related diseases.

1.1.1 Oxidative Stress

The term free radical, when referring to oxidative stress may be more specifically described as an oxygen free radical or a reactive oxygen species (ROS). It is here that electron transfer reactions, or redox reactions, occur with oxygen as the central atom (Borg, 1993). These radicals are central to the onset of tissue damage mediated by oxidative stress.

Oxidative stress was defined by Seis (1985) as a disturbance in the pro-oxidant-antioxidant balance in favor of the former leading to potential tissue damage. Under normal conditions the amount of ROS present at any specific time in tissue is extremely small relative to the corresponding concentration of oxygen. For example, the instantaneous concentration of a semi-reduced oxygen molecule in the brain (which consumes about 20% of the body's oxygen) is estimated to be about 10^{-11} M (Floyd, 1993). Therefore, when all systems are functioning normally the well infant has very little trouble dealing with the levels of endogenously produced free radicals. On the other hand, during situations of immature development, such as in the LBW infant, the infant may not be capable of withstanding the elevated levels of endogenous free radical production as well as the added stress applied by

TABLE 1.1
Reactive oxygen species of physiological interest.
 (From Muggli, R. 1993.)

REACTIVE OXYGEN SPECIES

Radical	Name	Typical biological target
$O_2^{\cdot -}$	Superoxide	Enzymes
H_2O_2	Hydrogen peroxide	Unsaturated fatty acids
HO^{\cdot}	Hydroxyl	All biomolecules
R^{\cdot}	R-yl	Oxygen
RO^{\cdot}	R-oxy	Unsaturated fatty acids
ROO^{\cdot}	R-dioxy (R-peroxy)	Unsaturated fatty acids
ROOH	Hydroperoxide	Unsaturated fatty acids
1O_2	Singlet molecular oxygen	H_2O
NO^{\cdot}	Nitroxyl	Several

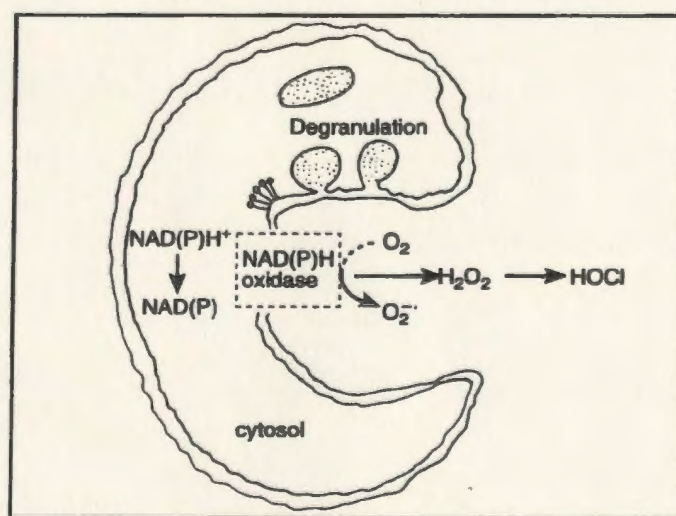


FIGURE 1.2 Production of reactive oxygen species by phagocytic cells. (From Rice-Evans, 1995)

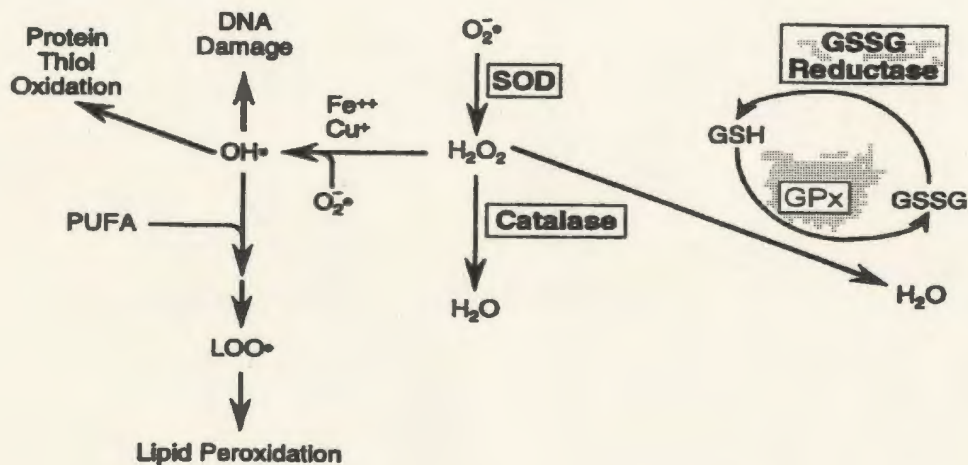


FIGURE 1.3 Antioxidant enzymatic pathways for reduction of $O_2^{\bullet -}$ to H_2O . (Modified from Sokol & Hoffenberg, 1996)

the increased oxygen administration by supplementation due to poor lung development. Lubec et. al. (1997), state that hydroxyl radical attack may be the primary mechanism of injury responsible for the acute and chronic disease states in infants receiving long-term oxygen therapy.

Fortunately oxygen itself does not react with biological tissues because of its stable triplet spin state. Its function physiologically is as a terminal electron acceptor during the formation of ATP and becomes fully reduced to water. It is the semi-reduced states of oxygen that are responsible for the oxidative damage that occurs (Floyd, 1993). Therefore, there needs to be a balance between the amount of ROS that are produced and the level of defense systems available to the tissue in order to prevent the onset of tissue damage. Certain conditions, imposed on the system however, as in the case of LBW infants, may cause a large increase in the amount of ROS produced, thus causing in increased amount of oxidative

TABLE 1.2
Possible factors and mechanisms for oxidative stress in
premature infants. (From: Stone, 1999)

OXIDATIVE STRESS

- **Prematurity**
 - **Cigarette smoking during pregnancy**
 - **Hyperoxia therapy for respiratory distress syndrome**
 - ▶ **superoxide radicals**
 - ▶ **hydroxyl radicals**
 - ▶ **peroxynitrite**
 - **Lack of adequate antioxidant defense systems**
 - ▶ **deficiency of antioxidant enzymes**
 - ▶ **vitamin E deficiency**
-

damage eventually leading to oxygen related disease of the immature neonate. Table 1.2 illustrates some of the possible means by which oxidative stress may be incurred on a premature infant.

1.1.2 Ischemia-Reperfusion Injury

Ischemia refers to a decreased supply of oxygenated blood to a body tissue or organ, and often results in tissue damage and organ dysfunction (Mosby's Medical Dictionary, 4th Ed., 1994). When tissues are deprived of oxygen they are injured, and after a certain period, the damage becomes irreversible. The duration of this period depends on the tissue in question and the extent of the oxygen deprivation (Rice-Evans, 1995). The main therapy for ischemia related injury is reperfusion of the deprived tissue with oxygenated blood.

Unfortunately, even though the reperfusion process is necessary to prevent ischemic death or tissue necrosis, it is also thought to be accompanied by its own component of injury (Omar et. al., 1991). Therefore both processes can be linked together as a single form of injury referred to as ischemia-reperfusion injury.

During ischemia, anaerobic ATP generation is not sufficient enough to supply the normal metabolic requirements of the tissue cells, and thus ATP content of the tissue falls rapidly (Omar et. al., 1991). During the process of ATP metabolism, substrates and metabolic byproducts are formed such that, when reperfusion occurs, production of oxygen radicals will ensue and cause further tissue damage. The HX/XO system (figure 4) is one such mechanism that leads to the production of the $O_2^{\bullet -}$ (Saugstad, 1988) and is commonly found in most tissues, especially the heart, GI tract, kidneys (Omar et. al., 1991), lungs, brain and retina (Saugstad, 1988).

Ischemia-reperfusion injury plays an important role in oxygen related diseases of the LBW infant. Saugstad (1996) suggests reoxygenation injury results due to an explosive production of ROS generated by the HX/XO system, following hypoxia during the perinatal period. Following birth the LBW infant frequently has poor lung development which may temporarily leave the child deprived of oxygen, therefore requiring the necessary high levels of oxygen administration. Coupled with undeveloped defense mechanisms, these processes lead to the pathogenesis of oxygen radical related diseases such as bronchopulmonary displasia (BPD), retinopathy of prematurity (ROP), intraventricular hemorrhage (IVH) (Kelly, 1993) and NEC (Clark et. al., 1988).

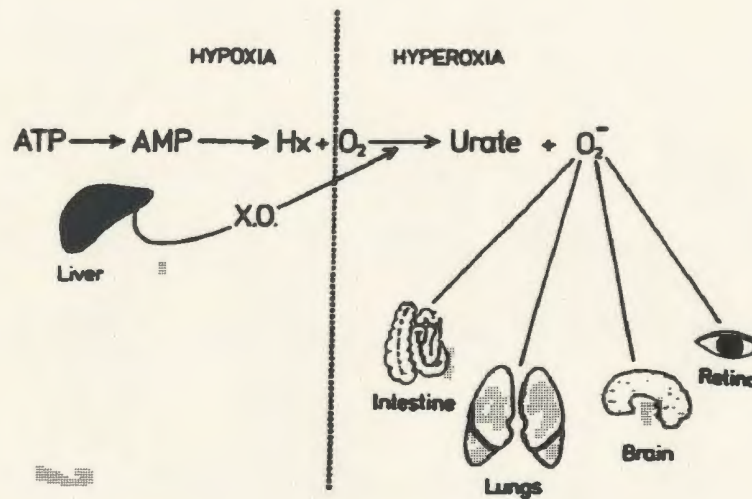


FIGURE 1.4 Proposed mechanism of reperfusion injury.(From Saugstad, 1988)

1.2 Oxygen Related Diseases of the Neonate

The following disorders are some of the most common oxygen radical-related diseases in the neonatal period. LBW infants are very susceptible to the development of these diseases after birth due to increased oxygen stress and underdeveloped defense mechanisms because of poor antioxidant protection. This section will briefly describe the special requirements of the LBW infant and some of the common diseases and their etiologies related to ROS, and to demonstrate how antioxidants play a role in preventing or prohibiting further development of the conditions. If antioxidant enzymes can be shown to play a beneficial role in dealing with such neonatal diseases as BPD, ROP, NEC and IVH, and these same enzymes are found in substantial quantities in mother's milk, there may be yet another added benefit to HM feeding rather than with artificial formula.

1.2.1 Requirements of the LBW Infant

The change from intrauterine life to extrauterine life for the LBW infant places enormous stress upon its respiratory and cardiac systems, along with increased stress in its thermoregulation capabilities (Ballard, 1991). The normal term infant can become stressed too, yet their bodies are more prepared for this change. In the full term infant, catecholamines contribute greatly to many processes that are important to the infant's adaptation at birth. These processes include *"reabsorption of lung fluid, release of surfactant into the alveoli, mobilization of ready usable fuel for nutrition, defense against cold stress, and modulation of cardiac output to ensure preferential flow of blood to vital organs, such as the lungs and brain"* (Ballard, 1991). In a LBW infant, the levels of catecholamines are much lower than those of a full term infant.

Once an infant is born, the primary concern is to establish a patent airway, proper level of oxygen saturation, and a stable respiratory status because the infant lung has not yet fully matured. To avoid hyperoxia, the oxygen blender should be set at 40% oxygen when resuscitation has begun, as compared to 100% as used in the 50's and 60's, and then turned down as quickly as possible as the infant tolerates. It should only be increased to 100% if the infant shows clinical signs of cyanosis of the skin or other respiratory malfunctions (McAteer, 1997).

Once the infant has been stabilized and placed into an isolette, the plan of care is outlined for each individual infant. Even though each plan of care is different, there are general guidelines that should be followed when caring for a LBW baby. Keeping in mind

that increased physiological stress in the infant can cause a decrease in oxygen saturation and lead to ischemia-reperfusion, it is important to minimize the amount of handling the infant undergoes. Minimal handling can be achieved by centering activity such as obtaining vital signs, turning and positioning, and feeding around the infant's sleep-wake cycle. Controlling the noise levels and the temperature of the environment can also maintain adequate stress levels for the infant (Weaver, 1991).

Minimal handling does not imply minimal care. The observation skills of the primary care giver with respect to infant's behaviors and responses to its environment are key indicators in detecting something may be wrong with the infant. Any sudden change in color, tone, feeding patterns, and activity warrants the attention of the physician. Nutrition is also very vital in ensuring recovery from disease maintaining the integrity of the VLBW infant (Kovar et. al., 1984). As a result HM is becoming more recognized as a primary feeding choice for these infants.

1.2.2 Bronchopulmonary Displasia

Also referred to as chronic lung disease, this disorder develops in infants partially due to administration of high concentrations of oxygen to immature lungs (McAteer, 1997). BPD most likely complicates the respiratory distress syndrome found in most LBW newborns. It has been estimated that between 20 and 45% of infants born prior to the 32nd week develop the condition (Kelly, 1993). With classical BPD, destructive inflammatory changes in the lung tissue eventually lead to the destruction of alveolar walls and bronchiolar epithelial linings

(McAteer, 1997). The condition therefore results in an increased need for oxygen administration , thus adding to the problem of excess oxidative stress.

New research poses a view that reactive oxygen species may be largely responsible for the clinical events associated with BPD development. Studies by Varsila et. al. (1995), and later Ogihara et. al, (1996) provide evidence of the involvement of ROS in triggering BPD and oxidation of protein leading to BPD respectively. Furthermore, Rosenfeld et. al. (1984), before any true evidence of ROS involvement in BPD development and the observation of their role in general inflammation, demonstrated that BPD could be prevented upon the administration of SOD.

1.2.3 Retinopathy of Prematurity

Retinopathy of prematurity, or retrolental fibroplasia, refers to the formation of fibrous tissue behind the lens and on the retina of the eye, resulting in blindness This disorder is caused by various factors, including administration of excessive concentrations of oxygen leading to high oxygen levels in retinal vessels premature newborns (Mosby's Medical Dictionary, 1994). Most cases occurred during the time when technology did not permit the clinician to assess appropriate inhaled oxygen concentrations, thus many babies lost their eyesight before the association with oxygen was determined (around the 1960's) (Kelly, 1993). ROP has also been linked with the formation of ROS during ischemia reperfusion of the retinal capillaries as the retinal wall thickens.

The relationship between HM feeding and the development of ROP has been examined. Uauy et. al. (1990) demonstrated a significant improvement in retinal development in infants receiving HM as the primary feeding source, and later, Birch et. al. (1993) confirmed the relationship between breastfeeding, and components of HM, and optimal visual development.

1.2.4 Necrotizing Enterocolitis

Necrotizing enterocolitis is a disease of the neonate characterized by necrosis of the bowel and is a significant cause of death in the LBW infant population. 80% of the infants with NEC are LBW newborns and the incidence increases with decreasing gestational age (MacKendrick, 1993). NEC is the most common serious gastrointestinal disease seen in neonatal intensive care units (Stoil, 1980). Clinical manifestations of the disorder include lethargy, abdominal distension, temperature instability and inability to feed. X-ray indicates tissue damage to the GI tract and bubbles of gas in the intestinal wall (McAteer, 1997).

Oxygen-derived free radicals, particularly $O_2^{\bullet-}$, are important mediators of intestinal tissue injury induced by ischemia-reperfusion (Clark et. al, 1988). Such is the case with NEC. Gastrointestinal hypoxia and later reperfusion of oxygen has resulted in the development of oxygen-derived free radicals leading to intestinal tissue damage in the LBW infant causing the development of NEC (Clark et. al., 1988, Granger et. al. 1996). This reasoning is supported by Babbs (1992) in a review of oxygen radical mediated ulcerative colitis.

There are many separate studies that have suggested that HM feeding provides protection from the development and/or worsening of NEC. Lucas & Cole (1990), found that babies fed breast milk had observed a sharp decline in incidence of NEC and infants who received only formula developed NEC at a rate six to ten times higher than their HM-fed counterparts. A study by Benhamou et. al. (1998) has shown protection by breastfeeding against severe gastric lesions in the neonate, and suggests that breastmilk contains substances which provide protection against inflammation. More research is required to determine the effect of antioxidant enzymes on NEC protection.

1.2.5 Intraventricular Hemorrhage

Intraventricular hemorrhage is a condition resulting from bleeding into the ventricles of the brain due to rupture of the thin walled capillaries surrounding them. It remains one of the most important causes of permanent and devastating disability in the sick LBW infant (Kelly, 1993). The rupture of the capillaries is thought to result from increased cerebral blood flow following a state of hypoxia (McAteer, 1997) that occurs during the perinatal process. The ischemia-reperfusion process may lead to the formation of ROS and hence result in damage to the capillaries causing the rupture and bleeding into the ventricles of the brain. Much research is required on the involvement of ROS in the development of IVH, and the possible preventative role of antioxidants.

1.3 Feeding the Premature Infant

Adequate nutrition is essential for the development and well-being of the newborn, especially the premature infant. LBW infants require specialized nutritional support because of a high degree of biochemical immaturity, fast growth rates, and a greater incidence of medical complications (Pereira, 1995). This section briefly reviews the present clinical methods of feeding the LBW infant and the developmental status of the developing infant gut, and evaluates the general benefits of breast-feeding in contrast to artificial formula administration.

1.3.1 Clinical Feeding Practices

The precise nutritional requirements for LBW infants are highly debated and not well established. The Committee on Nutrition of the American Academy of Pediatrics (1985) suggests that the ideal diet for LBW infants is the one that supports growth at intrauterine rates, without imposing stress on the infant's immature metabolic and excretory functions.

Immediately following birth, and during the first few days of life, the goals for nutritional support are the maintenance of fluid status, glucose homeostasis and electrolyte balance (Pereira, 1995). Since LBW newborns are susceptible to many forms of water loss, due to the high surface area to volume ratio, radiant warmers and urine excretion (McAteer, 1997) they require a balanced fluid intake. As well as body fluid requirements, sick LBW infants in particular have decreased body stores of nutrients when compared with their FT counterparts and require nutritional support in the form of parenteral feeding to prevent

weight loss and promote postnatal growth. Pereira (1995) states that even though most LBW infants are maintained on total parenteral nutrition (TPN) for several days after birth, an attempt should be made to begin enteral feeding of a milk as soon as the gastrointestinal tract is functional. It is at this stage that the choice of formula or mother's milk administration can play a role in the prevention and/or development of oxygen related diseases.

It is presently common practice to begin enteral feeding in small amounts until the gut develops. Gastric gavage is the preferred method of enteral feeding to LBW infants who cannot be fed orally because the sucking and swallowing reflex has not yet developed (McAteer, 1997). This method allows the infant to benefit from any properties that the HM or PT formula may afford. If mother's milk is not available, premature infant formulas are the best substitute, and which contain a higher concentration of protein, vitamins and minerals and trace elements than regular infant formula.

6.2 General Benefits of Human Milk Feeding for the LBW Infant

Mother's own milk is the most suitable form of feeding for the LBW infant because the composition of the milk produced is designed to meet the specific needs the premature newborn possesses. Premature milk exhibits increased bioavailability of nutrients, immunologic properties, hormones, enzymes and growth factors (Pereira, 1995) which allow it to provide protection and suitable growth and development for the infant. A study by Narayanan et. al. (1981) demonstrated the value of HM in the prevention of general infections in the high-risk low birth weight infant. They observed that infections were fewer in babies

who received expressed HM compared to those receiving the nursery formula. Gastric emptying is also found to be faster following the feeding of HM than with commercial bovine formula, and gastrointestinal growth and motility is enhanced due to increased postprandial plasma hormone concentrations (Schanler, 1995). An extensive review by Kovar et. al. (1984) also attributes lower infant mortality, decreased disease-specific morbidity, and lower rates of allergic disease to feeding HM to the LBW infant. They also attribute increased psychological and intellectual development of the baby to mother's milk feeding. Meier and Brown (1996), also elude to the health benefits of breastfeeding for mothers and LBW infants, including protection from NEC, protection from infection, greater feed tolerance, reduced risk of later allergy and enhance neurocognitive development.

Can mothers of LBW infants breastfeed? According to a prospective study by Byrne and Hull (1996) who examined the breastfeeding intentions and achievements of mothers of infants born prematurely, over 60% of mothers succeeded in producing milk for their infants, including those of 28 weeks gestation and less. They also stated that many more mothers might do the same with more encouragement and improved facilities in neonatal units. On the other hand, the volume of milk expressed initially is generally small which often leads to slower growth rates than the baby's full-term counterpart, indicating that it is often necessary to supplement the HM administration following the feeding with a nutrient-rich formula fortifier (Schanler, 1995). Whatever the case, the evidence clearly shows that feeding the LBW infant with any quantity of mother's milk provides numerous benefits above and beyond commercial formula feeding.

1.4 Antioxidant Properties of Human Milk

Human milk is presently considered to be the best food source for infants (Byrne & Hull, 1996). It is well known for its anti-inflammatory and anti-allergenic properties, and for its ability to provide protection for the developing infant gut. Recent research has demonstrated increased antioxidant capabilities of HM as compared to formula. Goldman et. al. (1990), state that antioxidants in general are either absent or poorly represented in cow's milk or other artificial feedings, and that attainment of adequate plasma antioxidant levels in the new born is dependant upon HM feeding. Buescher and McIlheran (1988) have reported on the antioxidant properties of human colostrum, demonstrating that HM exhibits antioxidant capabilities by being able to spontaneously reduce cytochrome c and deplete H_2O_2 produced by phagocytic cells and thus prevent damage to epithelial cells. Almass et. al. (1997) demonstrated that $^{\bullet}OH$ production in breast milk, in response to imposed oxidative stress, was lower compared to humanized formulas upon measurement of ROS production in both groups in vitro. They stated that HM appeared to contain "substances" that reduce the formation of $^{\bullet}OH$.

Formula feeding, on the other hand, has been shown to place infants at risk for oxidant stress due to excessive intakes of iron and vitamin C (Marshall & Roberts, 1990), and riboflavin (Friel et. al., 1996) which may lead to excess free radical production. Even though artificial formulas are fortified with ample levels of small molecule antioxidant scavengers such as vitamin E, they are not capable of demonstrating antioxidant enzyme capabilities comparable to untreated HM.

1.4.1 Antioxidant Enzymes

Human milk proteins such as apolactoferrin (Sullivan, 1988) and sulfhydryl oxidase (Isaacs et. al., 1984) have been found intact and active in the premature infant gut and feces. Thus it is possible that other proteins, particularly the antioxidant enzymes, may be stable in the GI tract and absorbed intact and capable of improving the antioxidant status of the LBW infant. The main enzymes of interest in HM are superoxide dismutase, glutathione peroxidase and catalase.

Superoxide Dismutase

Superoxide dismutase is responsible for the catalysis of the reaction responsible for conversion of two molecules of $O_2^{\bullet -}$ to H_2O_2 and the regeneration of O_2 . There are three forms of SOD; copper-zinc, manganese, and iron. Copper-zinc and manganese SOD are found in eucaryotic cells (including human) in the cytosol and mitochondrial matrix respectively, whereas iron-SOD is found in bacteria (Florence, 1992). All three forms catalyze the same reaction with similar efficiency.

The general catalytic reaction for SOD at physiological conditions is demonstrated by the following reaction:



CuZnSOD has been extensively studied for its antioxidant properties and protection against disease, including prevention of motor neuron degeneration (Gurney et. al., 1994) and

ALS in adults (Wiedau-Pazos, 1996) and treatment for BPD in infants (Rosenfeld et. al., 1984). This enzyme seems to be the first line of defense against ROS and can be rapidly induced during periods of oxidative stress (Michiels et. al., 1994).

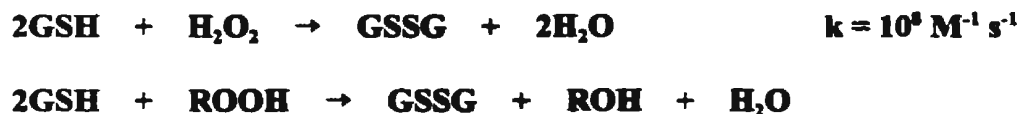
There is presently very little knowledge pertaining to the level and activity of SOD in milk of mothers of both LBW and full term infants. One preliminary communication by Willinger et. al. (1990) examines the difference in SOD activity between the two groups, and an ongoing study by Friel et. al. (1998, Personal communication) demonstrates the first systematic analysis of SOD in longitudinal samples of HM from mothers of PT and FT infants. Both these studies expressed SOD activity in Units/ml and Units/mg protein and found that activity was higher in the FT group (4.8 U/mg protein) than the PT group (3.2 U/mg protein), and the latter showed increasing SOD activity over time. SOD activity in humanized formula is absent due to the pasteurization temperature during processing of the cow's milk.

The high activity of SOD in HM as compared to infant formula suggests a possible functional or protective role of this antioxidant enzyme. For example, such evidence can be seen in the previously mentioned study by Rosenfeld et. al. (1984), where SOD administration was seen to prevent the formation of BPD in infants. Detection of the enzyme involves a slight modification of the method by L'Abbe and Fischer (1986) to account for the interference of myeloperoxidase in the milk, which may also cause reduction of the cytochrome c used in the assay.

Glutathione Peroxidase

With the label, 'peroxidation-inhibiting protein', GSHPx is responsible for protecting cells from further oxidative damage and repair of lipid peroxides formed in the cell membrane. The function of GSHPx is dependant upon levels of GSH, glutathione reductase (GR), NADPH and selenium in the cytosol and mitochondrial matrix of the cell. This system works cooperatively to ensure there is a constant supply of GSH in the medium and hence a reducing atmosphere (Scaduto, 1990). For example, Scaduto (1990) demonstrates the importance of the GSH system in ischemia reperfusion. GSHPx has also been found to reduce the enhanced susceptibility to lipid peroxidation of plasma and LDL in kidney transplant patients (Hussein et. al., 1997).

The main function of GSHPx is to catalyze the oxidation of reduced glutathione to glutathione disulfide (GSSG), and by doing so, reduce any unwanted hydroperoxides or organic peroxides. These two reactions can be written as follows (pH 7, 37° C):



ROOH in the second reaction may be a lipid peroxide formed from previous free radical propagation (Monday & Winterbourne, 1989) or may be a form of xenobiotic compound such as the metabolic by-product of acetaminophen (Mirochnitchenko et. al, 1999).

There is a strong correlation between selenium concentration and GSHPx activity in HM (Hamosh, 1995). The milk enzyme is 90% immunologically identical to the plasma enzyme and acts in the same fashion catalytically. It was observed by Ellis et. al., (1990) that the activity of the enzyme in milk of mothers of LBW infants paralleled changes in PUFA content over the course of lactation, demonstrating a possible function of the GSHPx in milk lipid protection from oxidation, or for oxidative repair. Hamosh states that the enzyme could also maintain the integrity of the milk by neutralizing the damaging action of oxidants that could be produced by the activity of other milk enzymes such as SOD.

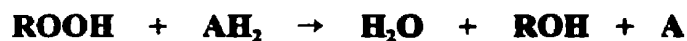
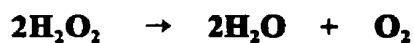
Mannan and Picciano (1987) reported a mean GSHPx activity of 77.1 units/liter for HM in Illinois subjects, and Funk et. al. (1990) reported a similar value of 51.0 units/liter in HM of African women. In an ongoing study by Friel et. al. (1998, Personal Communication) GSHPx levels in milk from mothers of PT and FT infants were compared. This group reported values of 73.0 mU/ml (9.8 mU/mg protein) and 85.8 mU/ml (8.4 mU/mg protein) for the PT and FT groups respectively. There is only one other longitudinal study examining GSHPx activity in milk from mothers of pre term and full term infants. This is the previously mentioned study by Ellis et. al. (1990). There is much work to be done to fully understand the antioxidant capabilities of CAT in HM

Catalase

Like the other antioxidant enzymes, CAT has also been implicated in the prevention and/or treatment of various human health disorders, such cell survival against O₂ stress (Michiels et. al., 1994), ischemia-reperfusion injury and BPD (Grisham et. al., 1990).

CAT activity in cow's milk has been extensively researched in the field of Dairy Science as an indicator of shelf life for cheese products (Hirvi & Griffiths, 1998), but very little is known about the levels of CAT in HM and possible antioxidant properties provided by this enzyme.

CAT is responsible for catalysis of the following reactions:



Heyndrickx (1963) reported CAT activity in HM to be about 10 times greater than unprocessed cow's milk. Research is presently being performed to learn more about the CAT system in mother's milk and the possible beneficial antioxidant properties for the newborn. The enzyme has been found to be absent in infant formulas and has a very low activity in fresh, unprocessed cow's milk, thus supporting Hendrickx' observations in 1963.

1.5 Problem of Investigation

The major enzymes involved in the detoxification of reactive oxygen species and lipid hydroperoxides include CuZn SOD, GSHPx, and CAT. In the absence of adequate endogenous antioxidant defenses, and under added oxygen stress, the propagation of free radical events in LBW infants can lead to the co-oxidation of nucleophilic cellular constituents as well as reaction of secondary lipid oxidation products with other constituents such as protein and DNA (Yuan & Kitts, 1997), thus leading to the onset of various forms of disease.

The purpose of the present study was to determine the antioxidant properties of HM as compared to humanized infant formula, and to make recommendations on both the present neonatal feeding practices and general overall health benefits of breast feeding. The hypotheses and objectives for this study are outlined below:

- ◆ **Hypothesis 1:** Pre-term human milk may exhibit increases antioxidant and free radical scavenging ability compared to full-term human milk, thus supporting it as the feeding method of choice for at-risk low birth weight infants.

Objective 1: To determine the ability of pre-term human milk to resist oxidative stress as compared to full-term milk at the same period of lactation.

- ◆ **Hypothesis 2:** The antioxidant potential of human milk from mothers of pre-term and full -term infants is partially attributed to the presence of the antioxidant enzymes, catalase, superoxide dismutase and glutathione peroxidase.

Objective 2: To determine if the increased antioxidant status of human milk is partly attributed to the presence of the antioxidant enzyme systems comprised of catalase, superoxide dismutase and glutathione peroxidase [see figure 3].

... CHAPTER 2: MATERIALS AND METHODS ...

2.1 Subject Demographics and Sample Collection

Milk samples for this study were collected from 17 mothers of FT infants and 28 mothers of PT infants once a week beginning on the first day of lactation and continuing weekly for 12 weeks. Each sample was collected by the mother using a manual pump or by hand expression and transported on ice to the laboratory and immediately frozen and stored at -70 degrees Celsius until thawed for analysis. Samples from weeks one, two and 12 were analyzed for the ability to resist stress imposed by $O_2^{\bullet-}$, CAT specific activity, MDA levels after stress and oxidative stress resistance following iron addition.

Low birth weight refers to infants who weigh less than 2,500 grams at birth and are usually born prematurely (< 37 weeks gestation). The average birth weights (mean \pm SEM) of the pre-term and full-term infants were 1810 ± 135 g and 3753 ± 132 g respectively, and the average gestational age was recorded as 32 ± 1 weeks and 39 ± 1 weeks respectively. There was no significant difference between the mothers' ages in the two groups (Table 2.1).

2.2 Experimental Design

The samples in this study were analyzed both longitudinally within groups to determine the change in experimental variables throughout the period of lactation, and between groups as a comparison of means to determine if any differences exist between PT and FT milk (Figure 5). Both groups were subsequently compared with infant formula under the same test conditions in an attempt to understand the increased antioxidant capability

TABLE 2.1
Study subject demographics

	Pre-Term	Full - Term
Number of Subjects	28	17
Mother's age (yrs)	29 +/- 1	31 +/- 1
Gestational age (wks)	32 +/- 1	39 +/- 1
Baby's Birth Weight (g)	1810 +/- 135	3753 +/- 132

Values are mean +/- SEM

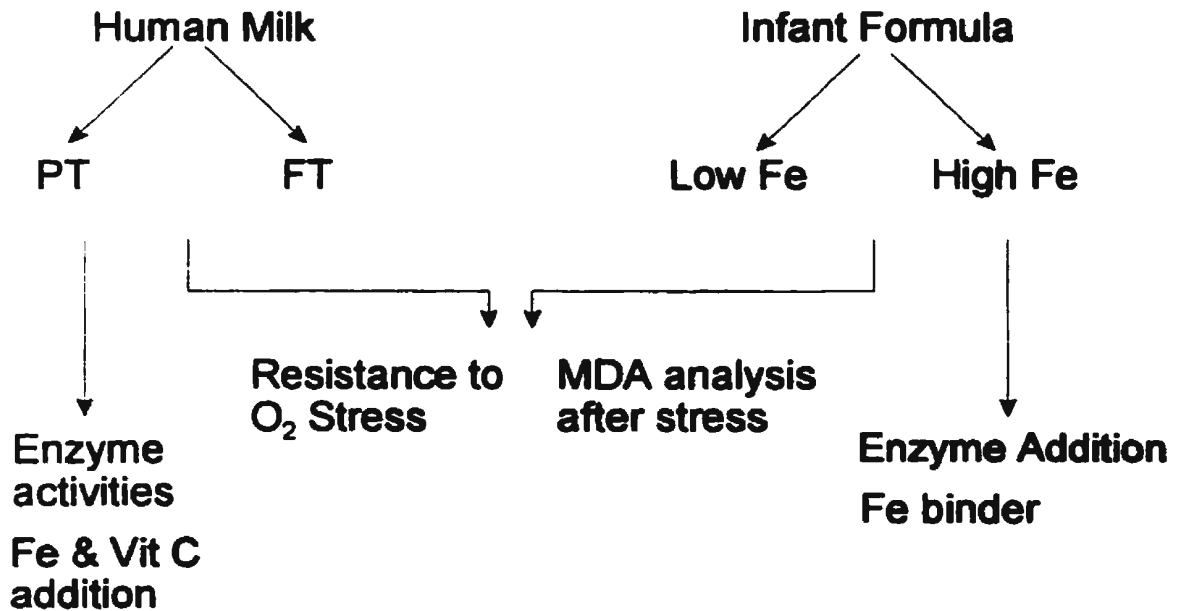


FIGURE 2.1 Flowchart demonstrating experimental design of study.

previously determined in HM. Furthermore, modifications were made to both the HM and formula to determine the action of ROS and antioxidant enzymes under various conditions.

2.3 Methods

2.3.1 Oxygen Stress

2.3.1.1 Antioxidant Capacity Using the Oxygen Monitor

Purpose: To determine the ability of HM to resist oxidative stress by free radical generation via the HX/XO system.

Theory: The HX/XO system is a free radical generating system found in the infant gut [see figure 1.4]. XO catalyzes the reaction of HX and Oxygen to produce $O_2^{\bullet-}$. Therefore the rate of consumption of oxygen measured on the chart recorder corresponds to the ability of the milk to resist the stress incurred.

Method: Resistance of milk samples to oxidative stress was measured polarigraphically as a modification of the method outlined by Hirvi and Griffiths (1998), using a Clark-type oxygen electrode and a YSI 5300 oxygen monitor equipped with a chart recorder. Calibration of the electrode, before use and several times during the period of use, was done by the addition of air-saturated water to the reaction chamber with the magnetic stirrer on and by adjusting the sensitivity control of the polarizing circuit to give a full-scale deflection on the chart recorder. The value for zero oxygen was determined using sodium sulfite, and was equivalent to the electronic zero of the system..

The reaction was carried out at 37° C and the value of 226 nmol oxygen/ml (Handbook of Chem & Phys 40th Ed.; Van Slyke et al, Handbook of Respiration) was used for calculating oxygen consumption at this temperature. The sensitivity control was adjusted to set the baseline to 90 % and chart speed was set to 10 mm/min.

After calibration the water was removed and 1350 µl each of HM sample (or formula) and phosphate buffer (pH 7.4, 100mM) were added directly to the same chamber. The electrode was reinserted and the magnetic stirrer turned on and allowed to equilibrate to 37° C. Once a baseline was established (approx. 3 min), 300 µl of HX/XO solution (50 mM and 25 mU/ml final concentrations respectively; 30:1) was added to the reaction mixture using a syringe through the overflow pore in the plunger. Oxygen consumption was monitored for about 5 min and the slopes were read immediately before and after the HX/XO addition.

Calculation: 1. Determine the nmol of oxygen/min for the before and after slopes.

- multiply the calibrations/min by the nmol/calibration determined during setup (measured to be 2.26 nmol/ml).

- cal/min x 2.26 nmol O₂/calibration = nmol O₂/min.

2. Subtract the before value from the after value to determine the true effect of HX/XO addition.

2.3.1.2 Malondialdehyde by HPLC

MDA is still the most widely used measure of lipid peroxidation despite its limitations, and can be cheaply and easily determined by the thiobarbituric acid (TBA) reaction. The method has been described previously for urine and plasma MDA in pre-term infants by Drury et. al. (1997) and modified here for milk analysis by incorporating a centrifugation step prior to sample injection to remove casein. The introduction of butylated hydroxytoluene (BHT), a common antioxidant, to prevent the formation of MDA by lipid decomposition during the assay, along with chromatographic separation by HPLC has been shown to improve the accuracy of the experiment.

A Beckman 110 pump, Kratos FS970 Fluorometer set at 525 nm, Hewlett Packard 3390A Integrator, Waters C18, 3.9 x 150 mm C18 Symmetry HPLC column and guard column were used. The mobile phase was 60:40 phosphate buffer (pH 6.8, 50 mM) and methanol respectively, set at a flow rate of 0.75 ml/min. The column was washed overnight with 20% methanol and purged on a regular basis with 100% methanol.

To 390 μ l of whole milk sample was added 375 μ l 0.44 M phosphoric acid, 125 μ l 0.6% TBA, 10 μ l BHT (2% in ethanol) and the remaining volume of water for a total volume of 1 ml. Samples were vortexed and incubated at $98^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 60 min and immediately placed on ice until analysis. All samples were filtered using 0.22 μ m pore luer-fitting filters before injection. 100 μ l of sample was injected to rinse and for analysis in each case. 1,1,3,3-Tetraethoxypropane was used to develop a standard curve for each assay and a correlation coefficient of $R^2 = 0.998$ was obtained.

2.3.2 Antioxidant capabilities of human milk and formula

2.3.2.1 Catalase Specific Activity

Purpose: To determine the activity of CAT in HM.

Theory: 1 unit of CAT is defined as the amount of enzyme required to decompose 1 umol of H₂O₂ per min at 25° C.



Method: CAT activity was measured polarigraphically by modification of the method by Hirvi and Griffiths (1998), and calibration of the system was performed as with the method of oxygen stress outlined above. The reaction was carried out at 25° C.

After calibration, 3 ml of tris buffer (pH 7.0; 100mM) and 30 µl of HM sample were added directly to the chamber and the electrode inserted and stirrer turned on. A portion of the same milk sample was set aside for protein estimation. Once a base line was established on the chart recorder (approx. 1 min), 150 µl of 3% (vol/vol) H₂O₂ was added and the oxygen evolution was monitored for about 2 min using the chart recorder set at a chart speed of 0.5 mm/sec. The initial rate of increase in the concentration of oxygen was calculated from the resulting slope.

Calculation: 1. Read initial slope from chart recording (calibrations/min).

2. Multiply by the Oxygen value of each marking determined during calibration - ex: 2.59 nmol oxygen/calibration. This will give the rate of oxygen production (nmol/min).

3. Multiply by 2 for the stoichiometric ratio in the formula above. This will give the rate of hydrogen peroxide decomposition (nmol/min).

4. Divide by 1000 to determine umol/min of hydrogen peroxide which corresponds to units of catalase/ml in the chamber.

5. Multiply by the dilution factor (in this case 100x) to determine the units of catalase/ml in the original sample.

Protein Estimation: Milk protein determination was performed using the Lowry assay and the Folin-Ciocalteu reagent with bovine serum albumin (1 mg/ml) as standard. Spectrophotometric measurements were taken at 750 nm. A slight modification involved sample dilution to reduce particle interference caused by casein during measurement. A commercial infant formula of known protein concentration was used as a control.

2.3.2.2 Pasteurization of Human Milk

Milk samples were pasteurized in a hot water bath for 5 min at 85 °C and tested for the ability to resist oxidative damage before and after heat treatment. This temperature was required to achieve > 95% inhibition of CAT and GSHPx activity (determined spectrophotometrically by modification of method previously described by Paglia & Valentine, 1967). Literature values also suggest that a temperature of 75 °C is sufficient for inactivation of SOD.

2.3.2.3 Enzyme addition to infant formula

The HM antioxidant enzymes CAT, SOD and GSHPx (final concentrations were based upon physiological levels found in HM: 15 U/ml, 35 U/ml, and 0.10 U/ml respectively) were added to both normal iron (Similac Special Care 24, 3.0 mg/L; Similac Advance, 1.5 mg/L, Ross Laboratories, Ohio) and high iron (Similac Special Care 24, 14 mg/L; Similac Advance, 12 mg/L, Ross Laboratories, Ohio) neonatal formula to determine their ability to provide protection against oxidative stress by the HX/XO method previously described. Oxygen consumption was recorded for 5 min prior to the addition of the HX/XO and continued for a total of 20 min. The total oxygen consumed (nmol) was determined before and after the addition of enzymes.

To determine the viability of the antioxidant enzymes in formula, recovery experiments were performed and demonstrated no significant loss in CAT and GSHPx activity. Upon addition to the formula, the enzymes maintained greater than 90% activity.

2.3.2.4 Iron addition to Human milk

Human milk was adjusted to final concentrations of 12 mg/L iron in the form of iron sulfate and 80 mg/L ascorbic acid to comparable levels recorded for common high iron formulas. Samples were then stressed with HX/XO for 20 min and compared against HM controls and HM containing ascorbic acid but not iron. Total oxygen consumption (nmol) was recorded and used to determine the effects of iron and ascorbic acid on the production of ROS and subsequent antioxidant resistance of the HM to added oxidative stress.

2.4 Statistical Analyses

Two-tailed independent samples t-test, using Levene's test for equality of variances, and two-way analysis of variance were performed to determine differences between groups, with the support of the Mann-Whitney U-test. Group descriptives were expressed as mean \pm SEM. Pearson Correlations were used to express relationship between variables. All analyses were performed with $p < 0.05$ taken as significant using the statistical package SPSS (version 9.0).

... CHAPTER 3: RESULTS ...

3.1 Oxygen Stress Resistance (Antioxidant Capacity)

The ability of the milk to resist oxidative stress caused by the formation of the superoxide radical was the main study variable used to determine the relationship between samples from mothers of PT and FT infants, and to investigate whether the PT milk exhibited enhanced antioxidant capabilities. Both groups, however, demonstrated no significant difference at any stage of lactation measured (figure 3.1). The means \pm SEM reported for the pre-term and full-term groups respectively, for the consumption of oxygen (nmoles/ml) during HX/XO stress, were for week one, 7.97 ± 0.56 (n=22) and 6.68 ± 0.88 (n=14); for week two, 4.62 ± 0.42 (n=12) and 5.17 ± 0.36 (n=13); and for week twelve, 4.08 ± 0.39 (n=13) and 4.80 ± 0.49 (n=16).

There was a notable decrease in oxygen consumption (nmoles/ml) during stress after week 1 of lactation as compared with week 2 (7.46 ± 0.49 , n=36; 4.90 ± 0.27 , n=25; $p < 0.001$) when the values for both groups are combined (figure 3.2), but no significant change between weeks 2 and 12.

3.2 Malondialdehyde Levels Following Stress (Lipid Damage)

MDA levels were determined as an estimate of the amount of lipid damage inflicted upon the milk samples during oxidative stress. Both the PT and FT groups provided the same protection against potential free radical damage. The means \pm SEM observed for MDA ($\mu\text{mol/L}$) for the PT and FT groups respectively after addition of HX/XO (figure 3.3)

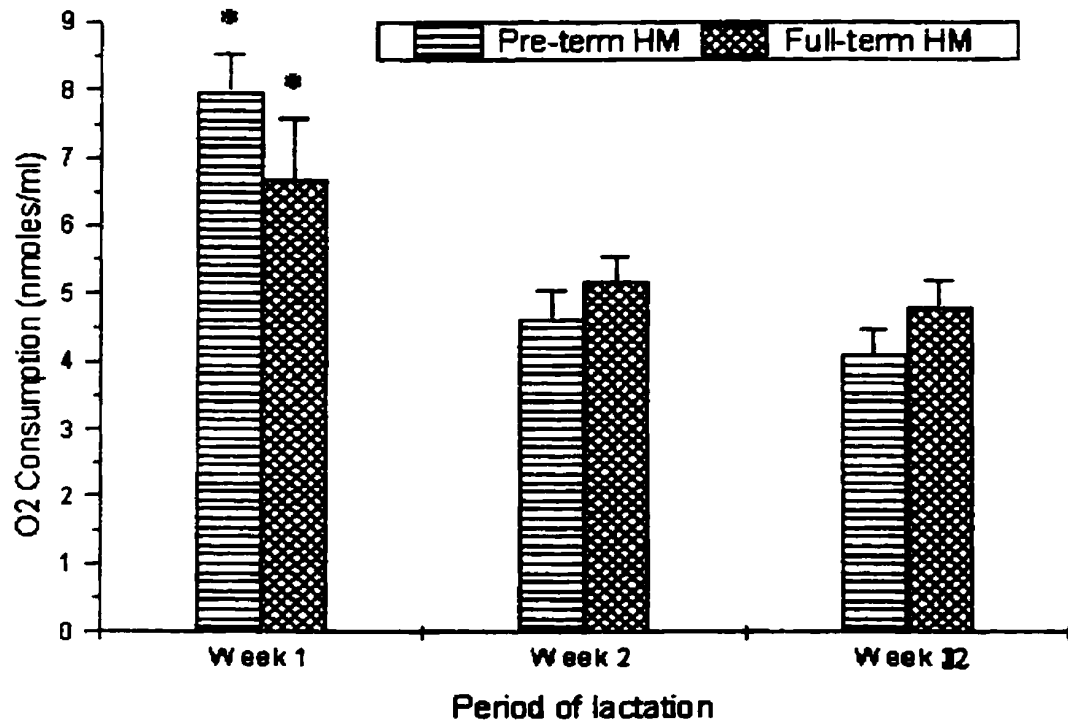


FIGURE. 3.1 Resistance of Human Milk to oxidative stress (Ability to prevent O_2^- radical formation). Mean \pm SEM; * $p < 0.001$.

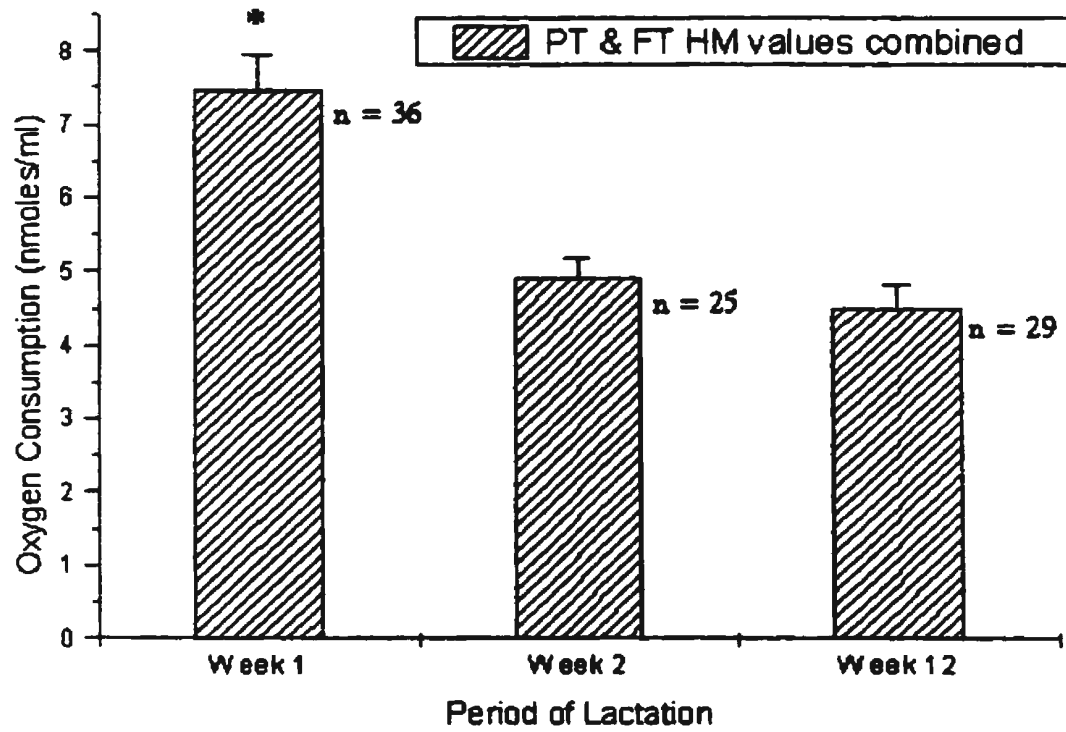


FIGURE 3.2 Ability of Human Milk to resist O_2^- stress at various stages of lactation; Mean \pm SEM, *p < 0.00.

were for week one, 0.58 ± 0.08 , $n=14$ and 0.72 ± 0.11 , $n=8$; for week two, 0.64 ± 0.07 , $n=12$ and 0.58 ± 0.08 , $n=15$; and for week twelve, 0.42 ± 0.09 , $n=11$ and 0.27 ± 0.06 , $n=16$. Thus, there was no significant difference observed between the groups with respect to MDA levels at either week 1, 2 or 12 of lactation.

There was no initial decrease in MDA levels from week 1 to week 2 in combined values (figure 3.4), but a significant decrease was observed from week 2 to week 12 of lactation (0.60 ± 0.05 , $n=27$; 0.32 ± 0.05 , $n=28$, $p < 0.001$).

3.3 Catalase Specific Activity

The means \pm SEM reported for the specific activity (Units/mg protein) of CAT in the PT and FT milk samples respectively for weeks 1 (0.50 ± 0.08 , $n=12$; 0.43 ± 0.05 , $n=8$), 2 (0.72 ± 0.10 , $n=11$; 0.82 ± 0.13 , $n=14$) and 12 (0.97 ± 0.21 , $n=13$; $0.84 \pm .12$, $n=16$) of lactation demonstrated no significant difference between groups (figure 3.5). The combined values (figure 3.6) of each group showed an increase in specific activity (units/mg protein) over time of lactation from week 1 to week 2 (0.47 ± 0.05 , $n=20$; 0.78 ± 0.08 , $n=25$; $p < 0.01$) and week 1 to week 12 (0.47 ± 0.05 , $n=20$; 0.90 ± 0.11 ; $p < 0.005$), but no change between weeks 2 and 12. There was also no difference observed when data was expressed as units/ml of CAT. Commercial infant formula has no CAT activity. Specific activity of CAT was not affected by freezing during storage and thawing prior to sample analysis.

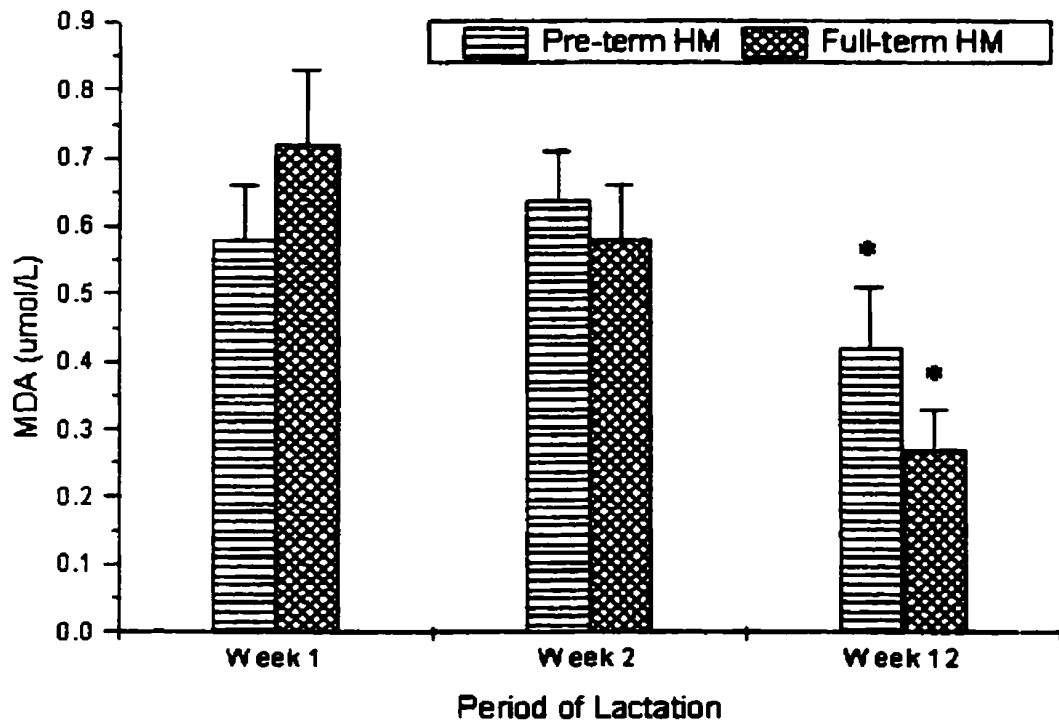


FIGURE 3.3 Malondialdehyde levels in human milk after O_2^- stress by the HX/XO system. Mean \pm SEM; * $p < 0.001$

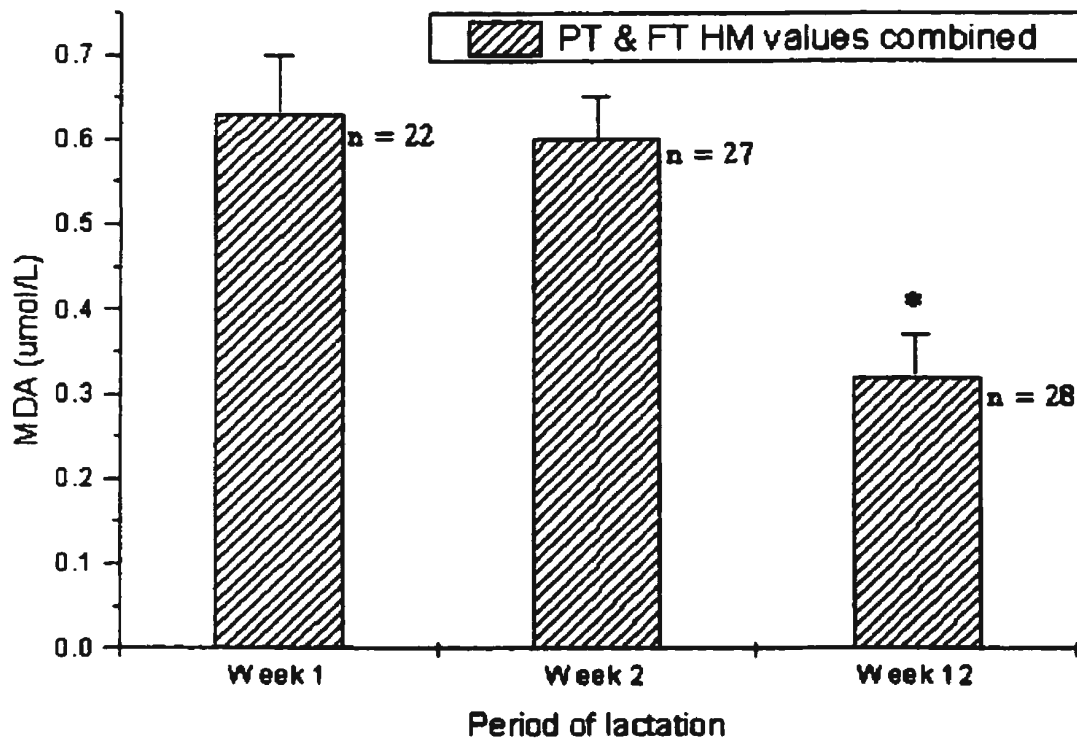


FIGURE 3.4 Malondialdehyde levels in human milk throughout lactation after O_2^- stress by HX/XO; Mean \pm SEM, * $p < 0.05$

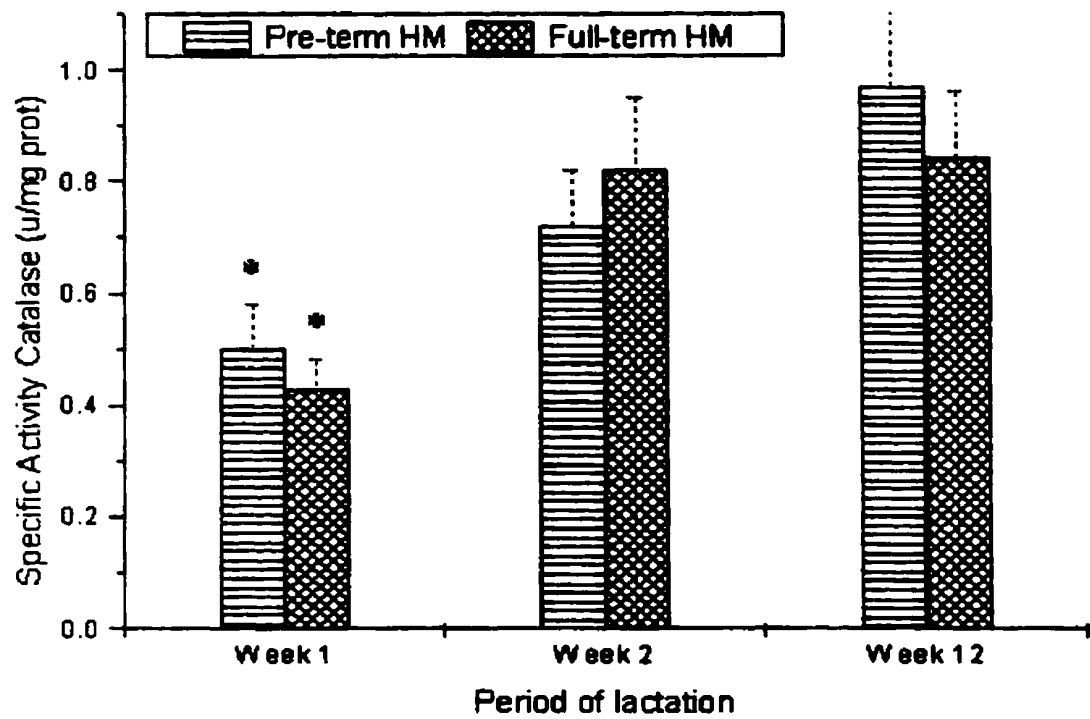


FIGURE 3.5 Specific activity of human milk catalase throughout lactation. Mean +/- SEM; * $p < 0.05$

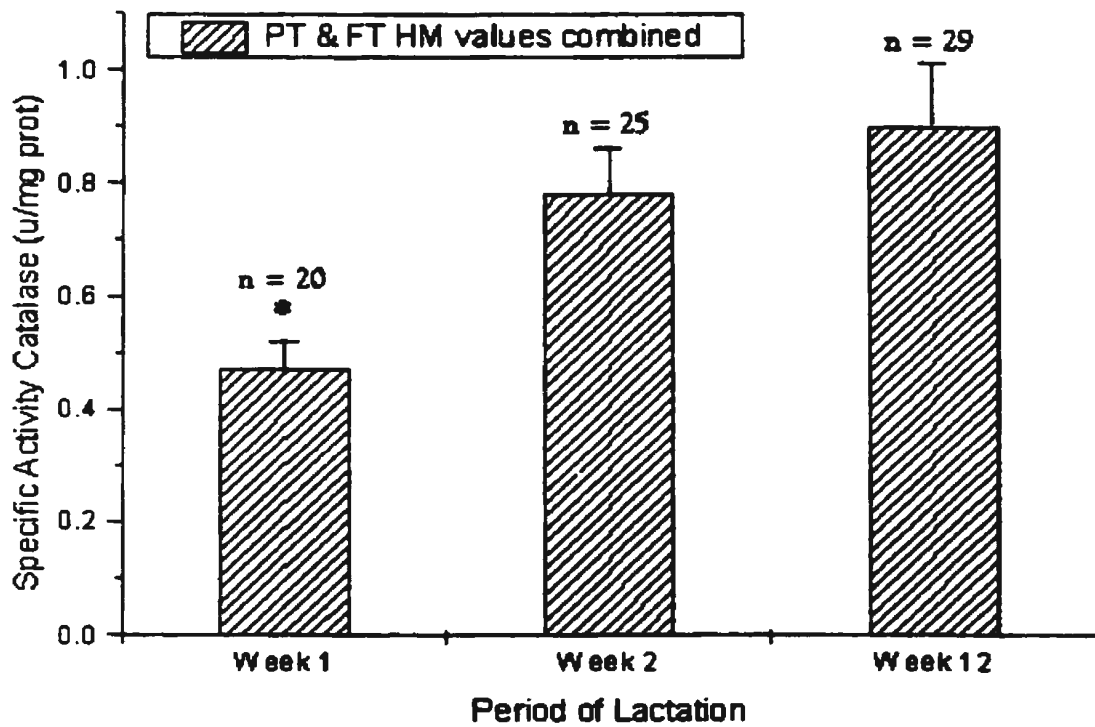


FIGURE 3.6 Total specific activity of catalase throughout lactation; Mean +/- SEM, *p < 0.05

3.4 Human Milk Pasteurization

There was no significant difference observed between milk samples before and after heat treatment with respect to oxygen consumption measured during oxidative stress. Figure 3.7 demonstrates the effectiveness of heat pasteurization on the specific activity of CAT. Heating milk samples at 80°C for 5 minutes is adequate for > 90% inactivity. The same relationship was observed with GSHPx.

3.5 Antioxidant Enzyme Addition to Formula

Normal and high-iron formulas were tested for their ability to resist oxidative damage with and without the presence of the antioxidant enzymes CAT, SOD and GSHPx. A difference ($p < 0.001$; mean \pm SEM) was reported in total O₂ consumption (nmoles) over time between the human milk (44.75 ± 1.66) and both the normal (98.88 ± 3.11) and high-iron (168.94 ± 9.47) formulas after the addition of the free radical generator. Addition of the enzymes was shown to significantly ($p < 0.01$) increase the antioxidant capacity by decreasing the total oxygen consumption (nmoles) of the normal and high-iron formulas to 66.11 ± 6.48 and 104.81 ± 8.65 respectively (figure 3.8).

There was no significant difference observed between the two styles of Similac formulas tested (figure 3.9), hence the values were combined for comparison purposes. Furthermore, it is of interest here that the formulas with high levels of iron exhibited nearly a two-fold increase in oxygen consumption compared to the same formula with low iron levels.

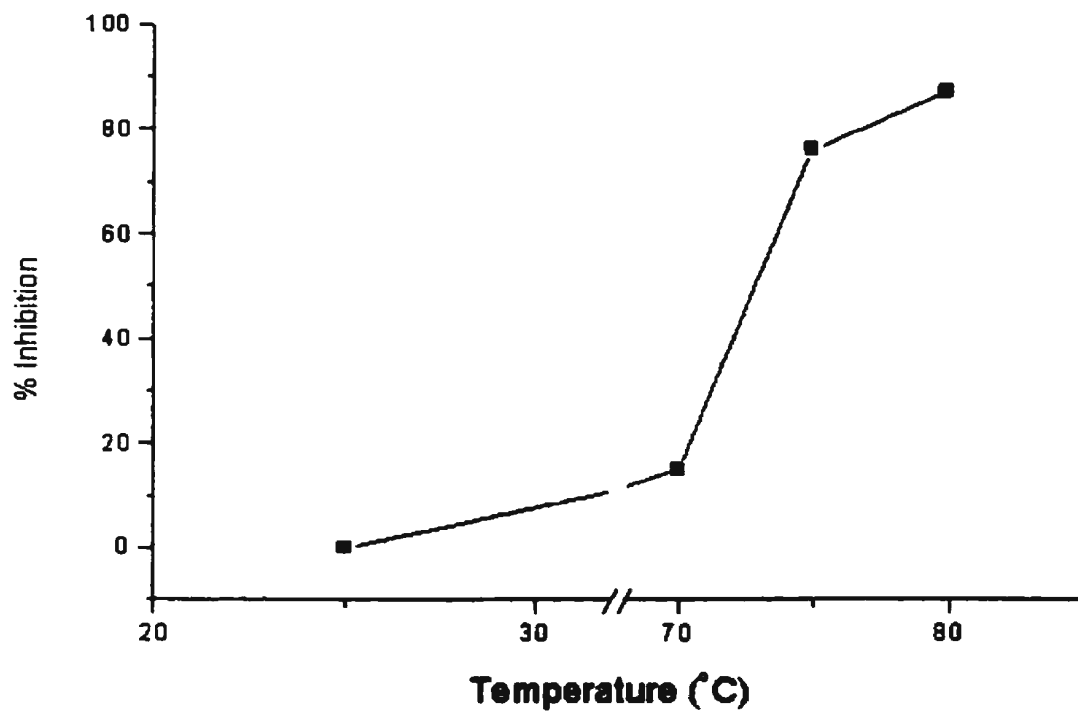


FIGURE 3.7 Pasteurization effect on specific activity of catalase in Human Milk. 5 min incubation time; 1.5 ml sample.

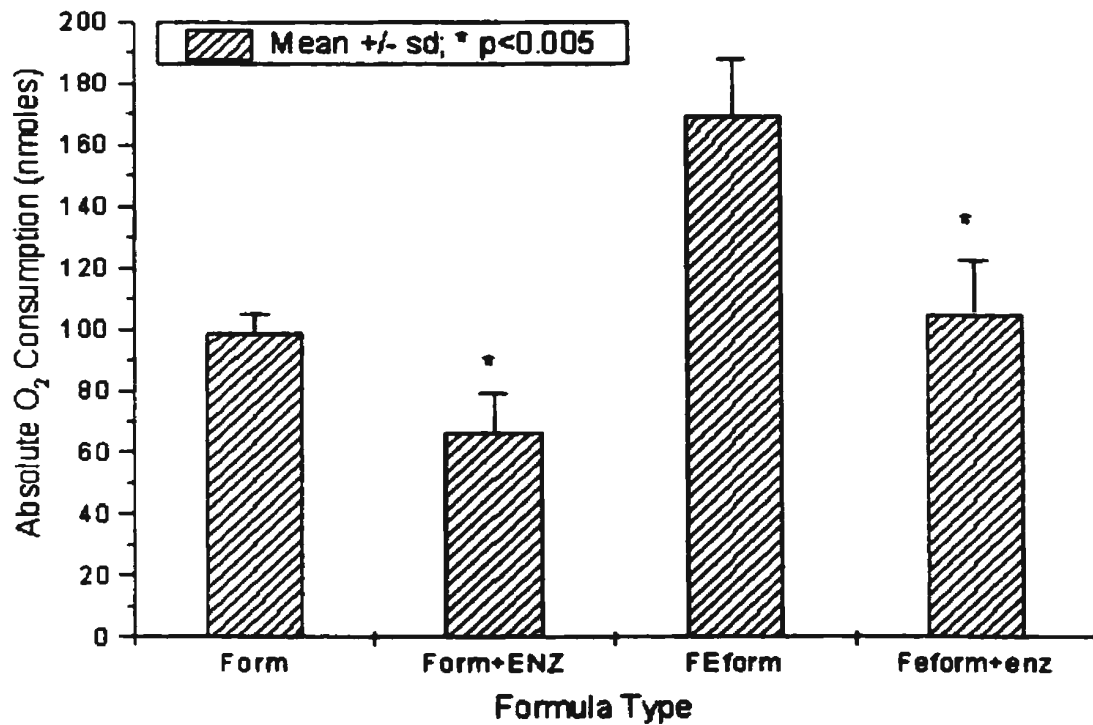


FIGURE 3.8 Indication of free radical formation in Fe and non-Fe infant formula before and after the addition of CAT, SOD & GSHPx

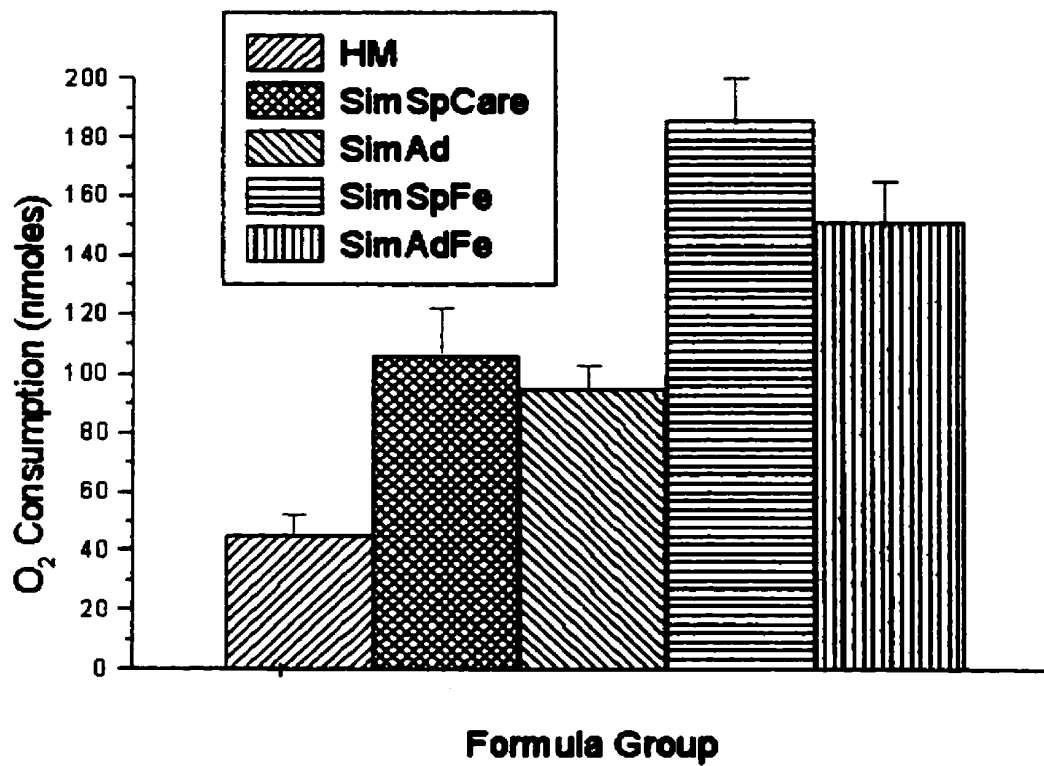


FIGURE 3.9 Comparison of HM and various infant formulas following oxidative stress; Mean +/- SEM.

3.6 Iron Addition to Human Milk

To determine the extent to which high levels of iron in commercial formulas play a role in oxygen consumption as a measure of free radical formation and subsequent tissue damage, equivalent levels of Fe and Vitamin C (ascorbic acid) were added to HM. This addition resulted in a two-fold increase in O₂ consumption (81.53 nmoles) upon addition of HX/XO in the samples containing iron as compared to HM controls (figure 3.10). Samples with only vitamin C added to HM were not significantly different from controls (49.27 nmoles).

3.7 Correlations

A significant ($R^2 = .443$, $p < 0.05$) positive correlation exists between the level of MDA observed in both groups combined at week 2 of lactation and the level of oxygen consumption observed during week 1 (figure 3.11). A negative correlation ($R^2 = -.30$, $p < 0.01$) also exists between the overall level of oxygen consumption observed and the specific activity of CAT for values of weeks 1, 2 and 12 combined (figure 3.12). No correlation was found between the level of MDA and specific activity of CAT at any stage of lactation or with all weeks combined.

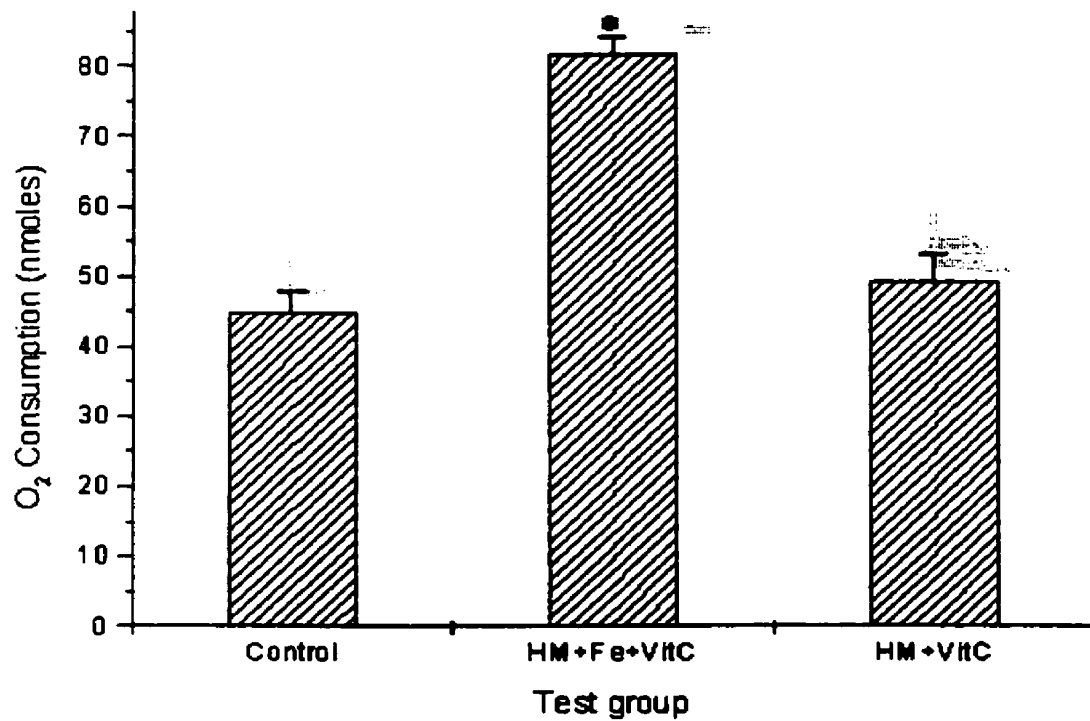


FIGURE 3.10 Effect of addition of formula-equivalent levels of Fe and Vit. C to Human milk on O₂ consumption (Fe=12 mg/L; Vit C=80 mg/L); Mean +/- SEM

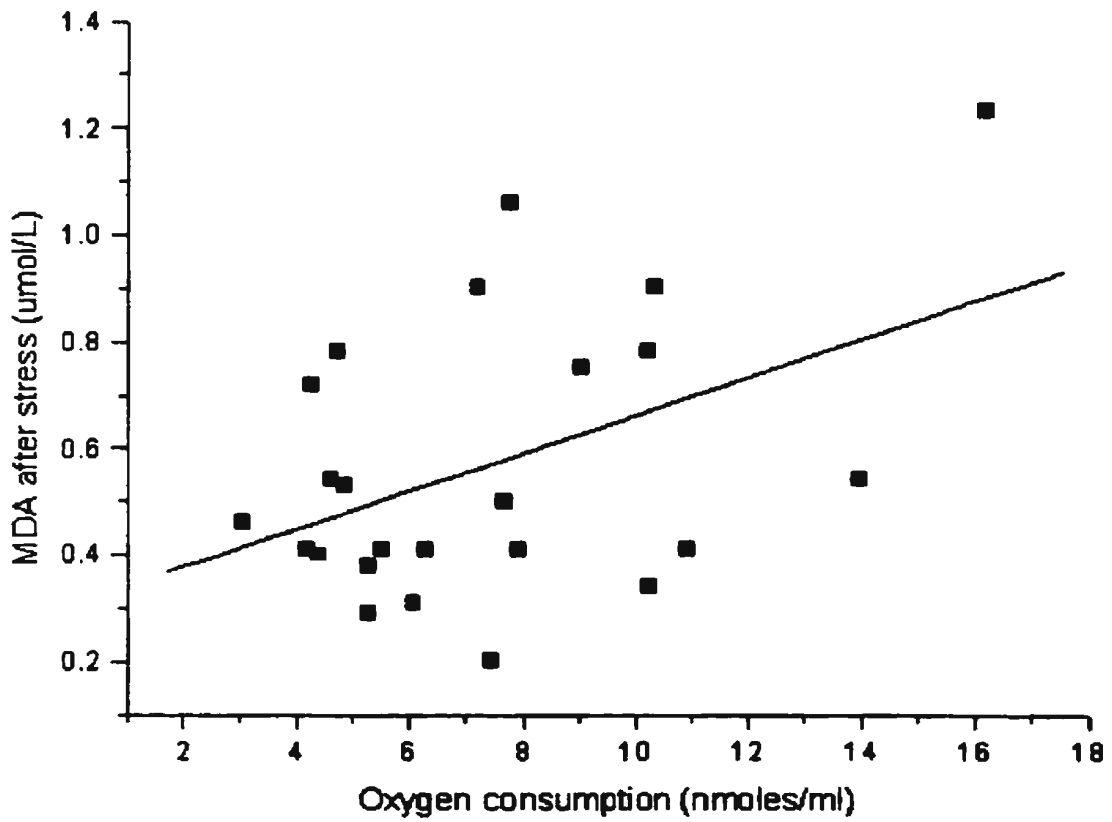


FIGURE 3.11 Correlation of MDA (wk2) following stress with the level of O₂ uptake observed in corresponding wk 1 samples. $R^2 = 0.449$; $p < 0.001$

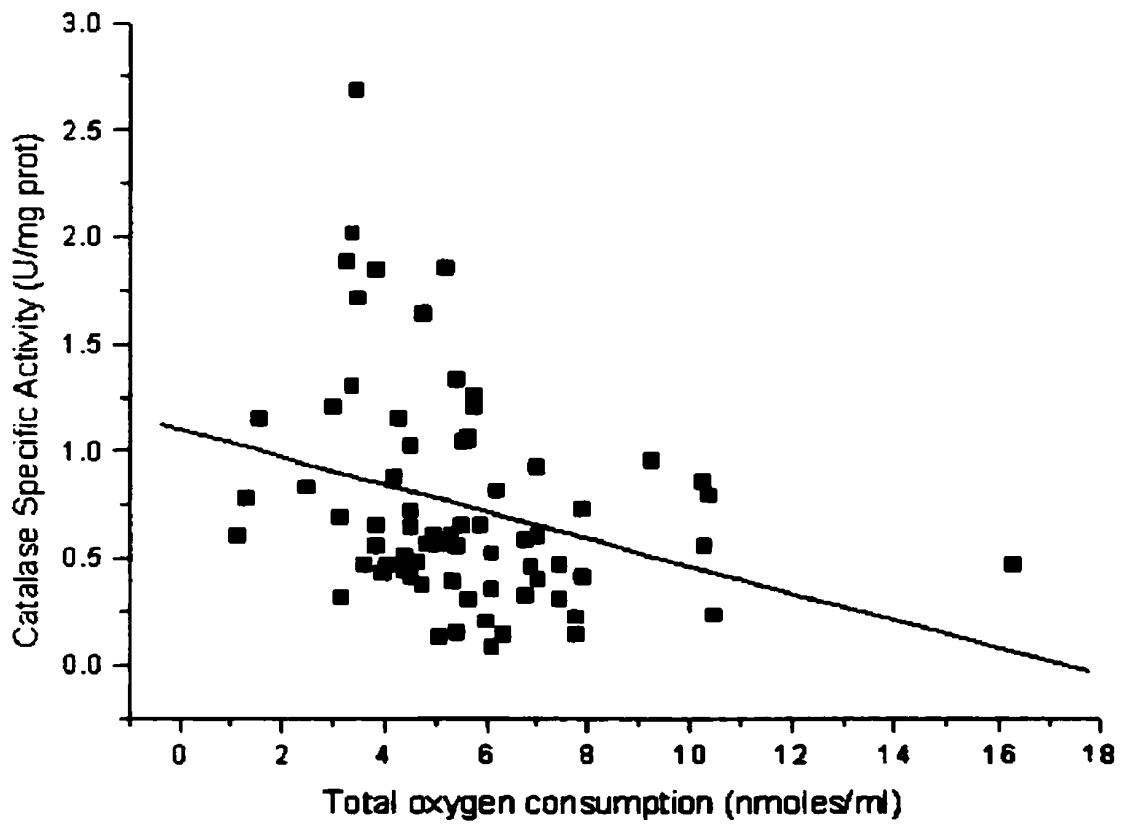


FIGURE 3.12 Correlation of Catalase activity in HM with oxygen uptake over all stages of lactation. $R^2 = -.30$; $p < 0.001$

... CHAPTER 4: DISCUSSION ...

4.1 Study Review

Feeding of newborns with mother's milk provides many benefits leading to optimal growth and development. For the compromised LBW infant the same is true. Human milk has been demonstrated to have increased nutrient bioavailability, enhanced immunological properties, active enzymes and specific growth factors (Pereira, 1995), all of which are essential for the health and maturation of these neonates. Furthermore, common neonatal infections have been shown to be lower in infants receiving milk from mothers of LBW infants than in infants receiving the prescribed nursery formula (Narayanan, 1981).

Breast milk has also been recently shown to exhibit antioxidant properties not seen in cow's milk and/or commercial formula (Goldman, 1990). Such antioxidant characteristics have been implicated in the sequestering of harmful ROS such as H_2O_2 (Buescher and McIlheran, 1988) and $^{\circ}OH$ (Almass, 1997), and therefore aid in the prevention of and recovery from oxygen radical-related diseases (Meier & Brown, 1996; Marshall & Roberts, 1990; Rosenfeld, 1984). It is necessary, then, upon examination of the specific needs of the LBW infant, to study the antioxidant properties of HM compared to the commonly prescribed infant formulas, and to make some recommendations for future clinical practice and formula development based upon these findings.

The present study was designed with two objectives in mind. First, to determine if there were any observable differences in the antioxidant properties between milk samples from mothers of PT and FT infants, and second, to make an attempt at characterizing the specific

components of HM that enable it to exhibit enhanced antioxidant potential compared to that of infant formula. What inherent properties of PT HM provide antioxidant protection against free radical-mediated diseases? Is it possible for these protective agents to be incorporated into commercial infant formulas and implemented under circumstances where mothers are unable provide milk for their infants? The results of the present study are useful in helping to determine the answers to these important questions and are discussed in detail in the following sections.

“It is widely thought that an imbalance between antioxidant defenses and free radical activity may be in part responsible for some of the complications of neonatal intensive care” (Drury, 1998). Since the premature infant is not simply a small term infant but is also developmentally delayed and has decreased enzymatic antioxidant defense systems, it is essential to try and boost these systems with the diet. This study has demonstrated yet another benefit of HM feeding practices, and has characterized some of the key players involved in the fight to maintain a healthy antioxidant balance.

Human milk samples from weeks one, two and 12 of lactation were collected from both study groups for analysis. The first two weeks of lactation were chosen because it is during this time that oxidative damage is most severe, and any antioxidant protection that may be provided is truly beneficial. Week twelve was chosen as mature milk samples, to examine how HM antioxidant properties vary throughout the duration of lactation. Values of n often differed within a group from week to week due to subject dropout and/or inconsistency in self collection of samples.

4.2 Analysis of Study Components

4.2.1 Catalase specific activity in human milk

Heyndrickx (1963) was the first and only to report CAT activity in HM. The study gave an approximation of the activity of the enzyme to be about 10 times greater than the level determined in cow's milk. It appears that all study of the CAT enzyme has been reserved for those in the field of dairy science as an examination of its ability to maintain cheese stability and shelf-life.

The present study is the first to report longitudinal values for the specific activity of CAT in milk from mothers of both PT and FT infants. Both groups demonstrated the same activity at weeks one, two and 12 of lactation, illustrating that milk from mothers of LBW infants are not compromised with respect to this antioxidant defense system. The activity of the enzyme was also seen to increase with the length of lactation to a near two-fold difference between week one and week 12 samples. This was opposite to that which was expected, since it would seem appropriate for the mother to have an increased expression of the gene for the CAT enzyme for the period in which the infant would be at most oxidative risk, and could benefit from extra antioxidant protection.

The uncovering of the presence of CAT in both PT and FT human milk is promising. It's role may be extremely important in the prevention of the harmful $^{\bullet}\text{OH}$, and therefore the prevention of extensive lipid and protein oxidation of the milk constituents themselves; and thus may be partially responsible for protecting the infant gut on site, and at other tissue sites

throughout the body - provided the enzyme can pass through the leaky gut and into the blood stream.

4.2.2 Antioxidant capacity of pre-term human milk

The literature clearly demonstrates that HM from mothers of both PT and FT infants exhibits far greater benefits than that of infant formula (Pereira, 1995; Narayanan et. al., 1981). This evidence leads to support the hypothesis that there are compounds or systems exclusive to HM that provide it with outstanding antioxidant potential. Furthermore, it is also commonly held that FT HM composition adjusts itself slightly depending upon the specific needs of the newborn. Is the same true for PT HM? If this were the case then milk from mothers of LBW infants would exhibit a different antioxidant composition than milk from those of healthy FT infants and would vary depending on the severity of the case.

Pre-term HM samples in this study, however, demonstrated the same antioxidant characteristics by our tests as did the samples from the FT group. There was the same amount of lipid damage, and CAT specific activity at all stages of lactation, clearly illustrating that the PT milk samples do not exhibit enhanced antioxidant properties over and above those seen in FT milk samples. However, although the PT samples did not contain more antioxidant capacity, they were just as effective in resisting oxidative stress and preventing the formation of MDA as were those in the FT group, and much more effective than the infant formula. Therefore, despite the fact that increased antioxidant potential was not observed in the PT HM group, the results may support the premise commonly found in literature that

early HM feeding may lead to a decrease in the incidence of oxygen radical-related diseases in neonates.

In an attempt to explain why the expected outcome was not observed with respect to the antioxidant potential of milk from mothers of LBW infants, it may be safe to suppose that the mother may not be physically able to provide enhanced antioxidant protection for her infant who, until recently, would not be assumed to have survived such an early birth. Advances in technology and clinical interventions now allow infants to be born even as early as 24 weeks gestation and survive (Stone, 1999). The mother's body is simply may not be prepared to deal with such an underdeveloped child's development, and can only provide the maximum antioxidant status that is observed in mothers of term babies.

4.2.3 Protective effects of antioxidant enzymes

Human milk and infant formulas do not differ a great deal in their amounts of nonenzymatic antioxidants such as vitamin E and vitamin C (Appendix A). These components are commonly added to infant formulas by manufacturers as essential components to the infant diet. Infant formulas, however do not contain active antioxidant enzymes. During the processing procedures of formula preparation, the cow's milk used to form the protein base of the formula is pasteurized to ensure the product is free from bacteria. As a result enzyme activity is lost and therefore cannot provide enzymatic defenses to free radical attack. As well, non-human milk sources in their natural state do not contain

the same levels of antioxidant enzymes found in HM (Heyndrickx, 1963; Shahan et. al., 1980).

It was expected, then that the antioxidant enzymes CAT, SOD and GSHPx present in the mothers milk would play a role in providing the HM with its antioxidant capability *in vitro*, and that these antioxidant enzymes in the protection of mother's milk would be capable of crossing the neonatal gut intact and increase antioxidant status and prevent neonatal disease. This result would also have provided evidence that HM antioxidant enzymes may also play a role in effectively sequestering free radicals directly at the site of inflammation in the infant gastrointestinal tract. However, upon pasteurization of the HM, its antioxidant properties were not compromised upon oxidative stress. The fact that there was no change observed in the protective properties of HM following enzyme inactivation indicates that either the enzymes are not involved in the sequestering of free radicals in HM at all, or that HM exhibits alternate, and equally-effective, means of dealing with excess oxidative stress and does not depend solely upon the known antioxidant enzyme defense systems.

The latter premise, that HM may have an alternate method of dealing with free radicals, and that the activity of the antioxidant enzymes cannot be totally ignored takes precedence over the first upon examination of the results observed following the addition of the same antioxidant enzymes to infant formula. The antioxidant enzymes CAT, SOD and GSHPX, when added together to infant formula were shown to provide increased protection against oxidative stress and lipid damage caused by free radical attack. This indicates that the proteins are active and are likely working with other systems in the HM and functioning as

antioxidants, however to a lesser degree than expected. Research pertaining to these findings has not been available up until this point, therefore it is necessary to reconsider the participation of antioxidant enzymes in HM. Moreover, the addition of these protective agents to clinically administered infant formulas may help reduce the harmful effects of excess oxidative stress and inflammatory induced ROS, resulting in reduced symptoms, faster recovery and normal development.

The most common cause-effect relationship between an antioxidant enzyme and common oxygen radical-related disease occurs with SOD. SOD has been recently found to be highly active in HM (Willinger et. al., 1990; Friel, 1998, personal communication) and subcutaneous administration of the enzyme to LBW infants has been shown to reduce the onset of BPD (Rosenfeld et. al., 1984) and motor neuron degeneration (Gurney et. al., 1994). GSHPx in HM has also demonstrated functional and catalytic similarities with the plasma enzyme (Hamosh, 1995), and has been observed to protect milk lipids from oxidation (Ellis et. al., 1990). In the present study CAT has been found to be present and active in both PT and FT milk and therefore may also provide protection by decomposing the ROS, H₂O₂, and preventing the formation of the more reactive [•]OH in the neonatal gut and in other tissues, including the retinas, lungs, and brain. Each of these enzymes, then, if added to neonatal formula may provide the same protective effects observed in HM.

If a mother is physically incapable of providing milk for her LBW infant or chooses not to breastfeed, an alternative source of nourishment has to be considered. Administering a neonatal formula that contains added 'active' antioxidant ingredients may prove to be

beneficial in minimizing the degree of oxidative damage that would otherwise be no less than severe.

4.2.4 Iron as a free radical generator in formula

The action of iron in the body is a paradox. In ample quantities and under proper storage while attached to protein it is an essential element to cell function and metabolism, but when found in free form it is toxic and damaging to tissues (Herbert et. al., 1994). Herbert's group states that because of its ability to switch back and forth between ferrous and ferric states, iron can be both a strong biological oxidant and a good reductant. Most of the $\cdot\text{OH}$ generated during cell metabolism comes from the iron-dependant reduction of H_2O_2 (Halliwell & Gutteridge, 1986). Therefore, if iron levels are high, there may be an increased risk of tissue damage and onset of oxygen radical-related diseases of prematurity.

When ferrous iron reduces H_2O_2 to produce $\cdot\text{OH}$ it becomes ferric iron. In the presence of vitamin C, the iron gets converted back to its ferrous state and is again available to begin another cycle of $\cdot\text{OH}$ formation if it is not sequestered by the iron storage or transfer proteins. Vitamin C and iron are found in high quantities in most neonatal infant formulas, and potentially at levels which allow for a source of free iron that cannot be sequestered and is capable of initiating free radical reactions which have been shown to lead to tissue damage and neonatal disease.

In the present study, when iron was added to HM at equivalent levels commonly found in neonatal infant formula, there was a much greater increase of oxygen consumption

observed indicating that the iron was playing a role in propagating free radical reactions. As well, preliminary data was obtained demonstrating a decreased production of free radicals in infant formula upon the addition of the iron binder diethylenetriaminepentacetic acid (DTPA), but there was suspicion that the DTPA was simply producing a more reducing environment in the reaction mixture, interfering with the HX/XO reaction and therefore preventing any oxidation from occurring, rather than simply binding the free iron. It was observed that as the amount of DTPA was increased, there was no oxygen consumption upon the addition of HX/XO indicating an inhibition in the production of free radicals. More research is required here.

Comparing the response of the low and high-iron infant formula to oxidative stress, it is apparent that the high level of iron is responsible for increased free radical formation. The high iron formulas (12 mg/L) demonstrated a two-fold increase in the amount of oxygen consumed compared to the same formulas containing low amounts of iron (1.5-3.0 mg/L). Again this illustrates that such extreme levels of iron may indeed be involved in the production of excess free radicals in the LBW infant gut following ingestion of high-iron formula. This may aggravate symptoms of chronic inflammation, or even initiate the process of disease development in the infant gut or throughout the body if the excess iron is not adequately dealt with.

4.3 Clinical Implications

The findings observed in the present study provide interesting insights into present clinical practices regarding the neonate without inflicting unnecessary oxidative damage. First of all, there is a common relationship between the amount of oxygen administered to a LBW infant following birth and the degree of disease onset as measured by MDA levels in plasma (Friel, 1998). This coincides with the results of the MDA analysis in the present study, where there was an increase of lipid damage observed in samples that were also noted to have increased oxygen consumption, and therefore less ability to resist oxidative stress. The amount of oxygen provided is a difficult decision, since it is necessary to deal with respiratory distress syndrome commonly found in the underdeveloped lungs of LBW infants. As illustrated by Friel, if excess oxygen is administered the result is increased tissue damage due to the formation of free radicals, and from the present data, is most likely in the presence of excess iron.

Secondly, HM has been shown to provide much better antioxidant protection than that of commonly administered neonatal formulas (Kovar et. al., 1984; Meier and Brown 1996). Although there appears to be non-enzymatic antioxidant systems at work in human milk when enzymes are inactive, the inverse correlation found between the amount of CAT observed and the corresponding levels of oxygen consumption and MDA following stress throughout the duration of lactation, is indicative of the protective ability of HM against free radical damage. This supports the neonatal recommendations to involve the incorporation of HM feeding practices for LBW infants where possible and it is practical to do so.

4.4 Future Research Recommendations

The following list is a set of recommendations for possible future research related to the field of infant feeding and antioxidant properties of HM. The suggestions given are based upon the findings of the present study, and would help provide further evidence for the claims made as well as provide new and valuable insight into the specific antioxidant systems now known to be present in mother's milk. Such information would prove to be valuable in clinical settings when trying to determine the most effective and practical form of intervention for LBW infants.

1. **Sephadex column fractionation of HM to separate the proteins by molecular weight. This will help determine if the enhanced antioxidant properties observed are attributed in part to small molecule antioxidants not yet identified. Since antioxidant capacity is not lost with pasteurization, the enzymes are clearly not the only agents involved.**
2. **An examination of HM antioxidant enzyme stability in the infant gut. Active proteins such as apolactoferrin have been found in infant feces. This indicates that specific whole protein molecules may remain intact in the infant digestive tract, and may pass unaltered through the 'leaky gut'. Active antioxidant enzymes from mother's milk may behave in the same fashion and be capable of providing the LBW infant with added protection against excess oxidative stress.**

3. **Formula-fed LBW infants have higher incidence of oxygen-related disease states than those fed HM. Therefore fortification of common pre-term infant formula with antioxidant enzymes may prove effective in decreasing symptoms related to oxidative stress. It is necessary to perform a clinical trial involving enzyme fortification and subsequently observe the degree of onset of symptoms related to common oxygen radical-related diseases.**

4. **A re-evaluation of the presence of high levels of Fe (12 mg/ml) in infant formulas intended for low birth weight infants. In the present study iron has been implicated as a free radical generator, which potentially leads to a perpetuation of common neonatal disease.**

... CHAPTER 5: CONCLUSIONS ...

The literature provides a powerful argument for the role of reactive oxygen species in many of the diseases related to low birth weight infants. The underdevelopment and lack of adequate enzymatic defenses in these newborns, along with the clinical requirements to use high oxygen levels to treat respiratory distress are important contributors to this oxidative stress. The extent of such oxidative damage is very often related to poor clinical outcome and physical development in many of these babies. Advances in present clinical feeding practices and the successful development of antioxidant formulations may provide a means of effectively dealing with oxygen-related diseases in neonates and ensuring adequate growth and development. The following points outline the major findings of the present study and demonstrate hope that the results will assist in the achievement of these goals.

- ◆ Milk from mothers of pre-term infants does not differ from that of milk from mothers of full-term infants in providing protection against oxidative damage. In the present study, samples from both the PT and FT milk groups at all stages of lactation analyzed, demonstrated the same response to oxidative stress, the same level of MDA (as a measure of lipid oxidation) following stress, and no difference in the specific activity of the antioxidant enzyme catalase.

- ◆ Antioxidant enzyme systems may not be the only mechanisms of providing human milk with its added antioxidant capabilities. Although the addition of catalase,

superoxide dismutase and glutathione peroxidase to infant formula was able to provide it with added antioxidant potential, pasteurization of the human milk, as a means of enzyme inactivation, was unable to diminish the antioxidant capabilities of the human milk. This indicates that human milk may contain other non-enzymatic free radical-sequestering mechanisms not yet known.

- ◆ **Speculation: Iron in infant formula increases free radical formation and potentially increases risk of oxygen radical-related disease in LBW infants. In the present study, the high-iron formulas were observed to consume a much greater amount of oxygen upon addition of a free radical generator than did the same formulas with low iron. Furthermore, addition of iron to human milk also resulted in a two-fold increase in oxygen uptake upon oxidative stress.**

- ◆ **Addition of antioxidant enzymes naturally found in mothers milk to infant formula may help protect the milk from free radical formation. Formula composition for low birth weight infants is constantly being adjusted to provide the maximum benefit for the compromised neonate. The results of the present study indicate a possible protective role of the addition of catalase, superoxide dismutase and glutathione peroxidase to neonatal infant formulas, which may aid in oxygen radical-related disease prevention and/or treatment.**

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... APPENDIX A: MILK COMPOSITIONS ...
(Nutrients per liter)

	Human Milk*	Similac® Special Care® with Iron 24 (Low Iron)**	Similac® Advance® with Iron (Low Iron)**
Energy, Cal	700	806	680
Volume, ml	1000	1000	1000
Protein, g	10.3	21.85	14.0
Source	-	Nonfat milk and whey protein concentrates	Nonfat milk and whey protein concentrates
Fat, g	44.0	43.79	37.0
Source	-	Medium chain triglyceride, soy & coconut oils	Medium chain triglyceride, soy & coconut oils
Linoleic Acid, mg	-	5645	5500
Carbohydrate, g	69.0	85.5	71.0
Source	-	Corn syrup solids & lactose	Lactose
Vitamins			
Vitamin A, IU	3300(+ betacarotene)	10081	2030
Vitamin D, IU	4 - 97	1210	406
Vitamin E, IU	20	32.3	20
Vitamin K, mcg	-	97	54
Thiamine, mcg	1000	2016	680
Riboflavin, mcg	400	5000	1000
Vitamin B ₆ , mcg	200	2016	400
Vitamin B ₁₂ , mcg	.30	4.44	1.7
Niacin, mcg	1800	40323	7100
Folic Acid, mcg	13.0	298	100
Pant. Acid, mcg	2400	15323	3040

	Human Milk*	Similac® Special Care® with Iron 24 (Low Iron)**	Similac® Advance® with Iron (Low Iron)**
Biotin, , mcg	10	298.4	30
Vitamin C, mg	50	298	61
Choline, mg	-	81	110
Inositol, mg	-	44	32
Minerals			
Calcium, mg	330	1452	527
Phosphorus, mg	140	806	280
Magnesium, mg	30	96.8	40
Iron, mg	.50	14.52 (2.98)	12.0 (1.5)
Zinc, mg	2.8	12.1	5.1
Manganese, mcg	-	97	34
Copper, mcg	.50	2016	610
Iodine, mcg	-	48	41
Selenium, mcg	-	14.5	15.5
Sodium, mg	170	347	160
Potassium, mg	500	1040	710
Chloride, mg	420	653	439

* From Geigy Scientific tables, 1981

* From Abbott Industries, Ross Infant Formula product directory.

... APPENDIX B: SUBJECT CONSENT FORM ...

**FACULTY OF MEDICINE
MEMORIAL UNIVERSITY OF NEWFOUNDLAND
ST. JOHN'S, NEWFOUNDLAND A1B 3V6**

CONSENT TO PARTICIPATE IN BIO-MEDICAL RESEARCH

TITLE: ANTIOXIDANT STATUS IN PREMATURE INFANTS

INVESTIGATOR(S): James K. Friel, Wayne L. Andrews, Brian S. Simmons, Khalid Aziz

You have been asked to participate in a research study. Participation in this study is entirely voluntary. You may decide not to participate or may withdraw from the study at any time without affecting your normal treatment.

Confidentiality of information concerning participants will be maintained by the investigator. The investigator will be available during the study at all times should you have any problems or questions about the study.

1. Purpose of study:

The purpose of this study is to determine if human milk has better antioxidant protective properties for infants than does formula feeding. Because the premature infant is underdeveloped in terms of its defences, it may be that mothers milk has extra benefits for these infants early in life. We would like to collect milk samples from mothers of both premature and full-term infants and assess whether this is true.

2. Description of procedures and tests:

Once you have decided to breastfeed, we would like to collect 10 ml of milk from one feeding a week for 12 weeks. We would ask that for one feeding you use the breast pump in hospital or that we will provide to remove all your milk from one breast. At that time you can give us the milk we need and give the remainder to the infant.

We also wish to get some information from you and ask your permission to obtain that information from hospital records.

3. Duration of subjects participation:

We ask you to be enrolled during the first week of your infants life. We ask that you provide milk until 12 weeks after the birth of your infant.

4. Foreseeable risks, discomforts, or inconveniences:

The only inconvenience will be milk collection.

5. Benefits which the subject may receive:

There will be no direct benefits to you or your infant.

6. Alternative procedures or treatment for those not entering the study:

If you choose not to enter the study your infant will receive the normal care that they would receive at any time.

7. Liability Disclaimer Statement:

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to participate as a subject. In no way does this waive your legal rights nor release the investigators or involved institutions from their legal and professional responsibilities.

I, _____, the undersigned, agree to my participation or to the participation of _____ (my child, ward, relative) in the research study described.

Any questions have been answered and I understand what is involved in the study. I realise that participation is voluntary and that there is no guarantee that I will benefit from my involvement. I acknowledge that a copy of this form has been offered to me.

(Signature of Participant) (Date)

To be signed by investigator:

To the best of my ability I have fully explained to the subject the nature of this research study. I have invited questions and provided answers. I believe that the subject fully understands the implications and voluntary nature of the study.

(Signature of Investigator) (Date)

Phone Number: _____

