NUTRITIONAL SUPPLEMENTS IN MILK FROM MOTHERS OF PREMATURE INFANTS: THE IMPACT OF IRON, VITAMIN C, TRIVISOL® AND MICROLIPIDS ON LIPID OXIDATION

BY

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for a Degree of

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ABSTRACT

Iron, vitamin C supplements for premature infants may induce lipid oxidation due to free radical formation by Fenton chemistry when added to breast milk (BM) *in vitro* and on cultured intestinal cells. Fox-2, TBARS and conjugated dienes assays measured lipid oxidation. Fatty acid composition was measured by GC. Immunostaining, redox potential and caspase-3 activation assays examined cellular changes.

Lipid peroxides increased from 8.8±0.7μM to 24.7±1.2μM and TBARS increased from 0.36±0.1μM to 0.96±0.1μM with 1.8mM iron in BM. Vitamin C and iron in BM led to decreased peroxides than iron in BM alone. Vitamins A and D exhibited antioxidant activity. Superoxide dismutase and catalase decreased the effects of ROS. Iron exhibited significant oxidative stress in all cellular assays. Oxidative stress due to enteral supplementation may contribute to necrotizing enterocolitis. SOD and CAT used clinically may decrease oxidative. Further research is required to investigate the effects of lipid oxidation *in vivo* with an animal model.

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DEDICATION

This thesis is dedicated to my mother Mrs. Pradnya Shirwadkar who has been a great source of motivation and inspiration. I also dedicate this thesis to the memory of my late grandmother Ms Vaze for her dreams and ambitions.

LIST OF ABBREVIATIONS

8-oxodG	8-hydroxy-2'-deoxyguanosine
AAP	American Academy of Pediatric Society
ATCC	American Type Culture Collection
BHT	butylated hydroxy toluene
BM	breast milk
CAT	catalase
CPS	Canadian Pediatric Society
DMEM	Dulbecco's Modified Eagle Medium
Fe^{+2}	ferrous ion
Fe^{+3}	ferric ion
FT	full term
GC	gas chromatography
GIT	gastrointestinal tract
GPx	glutathione peroxidase
Hb	hemoglobin
HMF	human milk fortifier
H_2O_2	hydrogen peroxide
$HOO^{\cdot}$	hydroperoxyl radical
LBW	low birth weight infants
MDA	malonaldehyde
ML	microlipids
NaCl	sodium chloride
NaOMe	sodium methylate
NEC	necrotizing enterocolitis
NICU	neonatal intensive care unit
NP	nonpasteurised
$O_2^{\cdot}$	superoxide radical
$OH^{\cdot}$	hydroxyl radical
P	pasteurized
PBS	phosphate buffered saline
PT	premature/ preterm
PUFA	polyunsaturated fatty acids
RBC	red blood cells
RDA	recommended dietary allowance
$RO^{\cdot}$	alkoxyl radical
$ROO^{\cdot}$	peroxyl radical
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RDS	respiratory distress syndrome
SEM	standard error of mean
SOD	copper/zinc superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TPN	total parenteral nutrition

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CHAPTER 1 RATIONALE

Premature (PT) infants are babies born before 37 weeks of completed gestation. The survival of PT infants is increasing due to new medical technology and improved understanding of perinatal pathophysiology. The infant mortality rate is decreasing and about 90% of all PT infants survive (Health Canada, 2004). Significant improvements have been made in feeding PT infants but their growth does not match the growth pattern of term infants.

Canadian Pediatric Society (CPS), American Dietetic Association and American Academy of Pediatrics Society (AAP) recommend exclusive breast-feeding of infants for at least four to six months after birth. BM has antimicrobial factors, antibodies, hormones, enzymes and growth factors that are lacking in infant formulas. Thus, breast-feeding may provide more advantages than formulas.

Postpartum, PT infants often face hypoxia as the external environment has lower oxygen concentration than the placenta (Saugstadt, 1990). Consequently, postpartum oxygen therapy to hypoxic PT infants may form reactive oxygen species (ROS) (Saugstadt, 1990). As most systems (e.g. gastrointestinal, respiratory and cardiac and immunological systems) are immature in PT infants, they have decreased resistance to oxidative stress (Saugstadt, 1990; Raghuveer et al., 2002). PT infants also have low levels of iron binding proteins (Frank and Sosenko, 1987; McElroy et al., 1992) and may lead to oxidative stress in the infants' gastrointestinal tract. Bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis and patent ductus arteriosus may have their origin due to ROS (Saugstadt, 1990; Rao et al., 1999).

Necrotizing enterocolitis (NEC) is a disease which predominantly affects PT infants and causes inflammation and necrosis of small intestinal tissue. It has an incidence of about 10- 40 % in PT infants (Stoll et al., 1994). It usually occurs a week to ten days after the initiation of feedings (Fell, 2005). Damage to the intestinal mucosa, oral feeding, and presence of bacteria have been attributed as causative agents in NEC (Horton, 2005).

Iron supplements are beneficial for prevention of iron deficiency anemia in full term infants. Their efficacy in PT infants is uncertain for two months after birth (Raghuveer et al., 2002) the reason being diminutive erythropoisis takes place during this period and physiological anemia occurs even with iron supplementation before 2 months of age (Canadian Pediatric Society, 1991).

PT infants are administered supplemental iron, vitamin C and microlipids in human milk and formulas. Iron forms ROS according to the Haber-Weiss reaction (Wardle et al., 2002). Vitamin C may regenerate ferrous iron from ferric iron, continuing the cycle of ROS production according to the Fenton chemistry (Wardman and Candeias, 1996). Hydroxyl radicals produced by Fenton chemistry or Haber-Weiss reaction are highly reactive and may cause lipid peroxidation and cell injury (Wardle et al., 2002). This could be one of the etiological factors behind NEC.

The measurement of oxidation products in BM will give an estimate of lipid oxidation *in vitro*. Infant intestinal cell lines can be used as a bioassay of ROS. Fox-2 assay to measure peroxides, TBARS measurement and fatty acid analyses will be used to estimate lipid oxidation. The effect of ROS on intestinal mucosa, BM with supplements will be investigated on FHs 74 Int and Caco-2BBe cell lines. We

ultimately plan to enhance oxidative resistance in infant formulas by examining addition of exogenous antioxidants in BM.

CHAPTER 2 LITERATURE REVIEW

2.1 Premature infants

The normal gestation period for a human infant is from 38-42 weeks. PT infants are those babies born before 37 weeks of completed gestation, counting first day from the last day of menstrual cycle of the mother (Samour et al., 2004). They usually weigh less than 2,500g (Cuddy, 2002) while very low birth weight premature infants are those infants weighing less than 1,500g. Previous PT birth, stillbirth, small-for-gestational-age fetus, maternal age of 35 years and older and smoking are the risk factors for PT birth (Surkan et al., 2004). Meeting the nutrient needs of the PT infants to achieve optimal growth is enfeebled by an underdeveloped suckling reflex and immature gastrointestinal tract. Therefore, PT infants are at a higher risk of developing NEC, an inflammatory disease of the intestines, than full term (FT) infants.

2.1.1 Prevalence

PT birth accounts for 7.1% of the births in Canada. The incidence of PT birth has increased 3.2% between 1978 and 1996 and continues to increase. Manitoba has the fourth highest infant mortality rate in Canada according to 'Health Canada census for 1990–1994' birth and death records. PT birth is a major risk factor for perinatal mortality and accounts for 75-85% of all perinatal mortality in Canada (McLaughlin et al., 1999).

2.1.2 Complications of premature birth

Immature body systems

1. Many organs in the body may be physiologically immature in the PT infant compared to the FT infant. The hypothalamus responsible for temperature regulation in the body is not fully developed in PT infants. This may cause hypothermia in PT infants.
2. The respiratory system develops after 28 weeks of gestation. Therefore in neonates born before 28 weeks there may be respiratory problems. Infants with an underdevelopment of the respiratory system are kept on mechanical ventilation and/or oxygen inhalation. This may play a role in oxidative stress in the infant, since 100% oxygen inhaled for a long time causes chest discomfort, pain and pulmonary edema (Weaver and Churchill, 2001).
3. The heart and various blood vessels may not have fully developed in the PT infant. A common example is patent ductus arteriosus where a patent duct connects left pulmonary artery and aorta.
4. Red blood corpuscles and white blood corpuscles may be low in the PT infant leading to anemia and infections.
5. A coordinated suck and swallow reflex is not developed in PT infants until 32-34 weeks (Samour et al., 2004).
6. PT infants are susceptible to infections due to immature immune system. Sedaghatian and Kardouni (1993) carried out a study in which FT and PT infants were immunized with BCG vaccines. PT infants showed decreased development of BCG scar than term infants, portraying lower maturity of the immune system. Table 9 shows some problems faced by PT infants.

Decreased nutrient stores

1. Glycogen, fat, protein, fat-soluble vitamins, calcium, phosphorus, magnesium and trace elements may be low in PT infants (Samour et al., 2004).

Illnesses

1. PT infants often have difficulty in feeding. Many infants are unable to coordinate sucking and swallowing before 35 weeks of gestation. This may lead to poor digestion and gastrointestinal diseases.
2. Respiratory distress syndrome leads to increased risk of aspiration in PT infants.
3. Hyperbilirubinemia in infants may be treated with phototherapy. This may lead to insensible water loss and dehydration in infants.

2.2 Necrotizing enterocolitis

NEC is a gastrointestinal disease affecting gastric and intestinal mucosa in PT infants. It causes inflammation and necrosis of intestinal tissue. It affects mainly PT infants (10 % FT infants) and occurs a week to ten days after the initiation of feedings. The damage due to ROS has been suggested as one of the causative factors of NEC (Rao and Georgieff, 2001).

2.2.1 Prevalence, risk factors, complications and treatment

The incidence of NEC is about 10 % in very low birth weight infants (Uauy et al., 1991) and the death rate is about 25 % (Hack et al., 1991; Berseth and Abrams, 1998). Hypoxia, asphyxia, prematurity, bacteria and formula feeding are some of the risk factors for NEC (Pereira, 1995; Caplan et al., 1994).

Aw (1999) attributes thiol redox imbalance due to oxidative stress as the cause of apoptosis and necrosis of tissue. The mechanism involves modulation of nuclear transcription factor NF-κB by increase in cytokines and leukotrienes. Hsueh et al. (2003) attribute the cause of NEC to bacterial invasion of intestine, leading to inflammation and the production of platelet activating factor-AH, NF- κB and tumor necrosis factor progressing to tissue injury, sepsis, shock and intestinal necrosis.

NEC may be a complication with exchange transfusion (Samour et al., 2004). NEC may lead to fever, apnea and lethargy in the PT infant (Pereira, 1995). Lucas and Cole (1990) did a study on formula-fed PT infants. They found PT infants who received formula and milk had higher risk of NEC than milk-fed only group and concluded formula feeding might be a risk factor for NEC.

The treatment for NEC includes monitoring parenteral nutrition, as oral feeding is not recommended (Stoll et al. 1994 and Fell, 2005) and minimal advancement of feedings. Other preventive measures may include the addition of amino acids to enteral feeds (Diehl-Jones and Askin, 2004).

2.3 Premature infant diet

2.3.1 Breastfeeding

Breastfeeding is the preferable feeding method in infants whether PT or FT. Breast-feeding is a more economical choice of feeding, than formulas for the family and beneficial in providing immunity, nutrition and protection against a number of diseases for the infant (Diehl-Jones and Askin, 2004). Mothers' milk unlike formulas contains natural macronutrients, vitamins and minerals.

PT infants less than 34 weeks have difficulty in sucking and swallowing (Gewolb at al., 2003). In such infants gavage feeding may be done, which consists of inserting a gastric tube via the nose or mouth of the infant into the stomach. Milk may be given with a syringe through the other end of the tube. The expressed BM may be stored in a fridge (freezer compartment) for up to 48 hrs (NICU, St. Boniface General Hospital guidelines, 2002). The frozen milk is usually thawed for 15-30 minutes before given to infants, although there seems to be little consensus regarding the clinical practice after thawing and supplementation. Babies may also be bottle-fed with cow's milk-based commercial formulas like Enfamil formula (Mead Johnson, Evansville, USA) and Similac infant formula (Ross products, Abott Park, USA).

2.3.2 Feeding protocol

American Academy of Pediatrics recommends breastfeeding PT infants (32 weeks gestation onwards) by exclusive breastfeeding every 3-4 hours daily. Infants 34-36 weeks old are breastfed depending on the infants' energy level, and whether the mother is able to breastfeed (NICU, St. Boniface General Hospital guidelines, 2002). The infants' energy level is determined by its consciousness (level of arousal), weight gain, wet diapers, bowel movements, temperature and movement (St. Boniface General Hospital guidelines, 2002).

Infants weighing less than 1,000 g receive minimum enteral feeding, as gastrointestinal reflexes are not well developed. Infants less than 1,200 g are fed approximately < 20 ml BM every 2 hours and those weighing more than 1,200 g are fed approximately 20 ml every 3 hours (Health Sciences Centre guidelines, 2003). Infants more than 37 weeks are breastfed > 20 ml every 4 hours, on demand or based on physicians order (Health Sciences Centre guidelines, 2003). The approximation is based on a generalization as every individual has different feeding needs and methods, depending on the case and response.

2.3.3 Composition of human milk and infant formula

After birth, exclusive breastfeeding is recommended for the first four-six months of life, as it provides all the nutrients, growth factors and immunological components to an infant. Milk is predominantly composed of water, carbohydrates (lactose), fat, protein, minerals and vitamins. Milk is a very complex solution with many components. The nutrient and fatty acid composition of human milk is shown in tables 6 and 7.

Infant formulas are usually made from cow milk or soybean ingredients. Human milk has casein: whey protein ratio of 40:60 whereas cow milk has a ratio of 80:20. β-Lactoglobulin is present in cow milk and is not present in human milk. BM and cows' milk also differ in several amino acids and electrolytes such as potassium. Human milk has a higher cysteine: methionine ratio and some amounts of taurine in comparison to cow's milk, which is deficient in taurine. This may cause a dietary protein allergy in human infants. Human milk also has higher lactose content (Hurley,

2002). Current infant formulas contain about 36 g/L fat, providing 48% of total energy from fat to the infant (Raiten et al., 1998).

2.3.4 Nutritional supplements

Nutritional supplements are extra nutrients that are added to formula or BM to increase the caloric, protein and mineral content of feedings. Some examples of nutritional supplements are

1. Iron supplements (FERINSOL): These are liquids added to infant formula or expressed BM to provide extra iron.
2. Vitamins (POLYVISOL/ TRIVISOL): These are supplements added to formula to provide vitamins A, D and C.
3. Microlipids (ML): Microlipids (50% fat emulsion with 4.5 Kcal/ml) (Novartis, Basel, Switzerland formerly owned by Mead Johnson, Evansville, USA) are lipids that contain mono and polyunsaturated fats like palmitic, stearic, oleic and linoleic fatty acids primarily the latter.
4. Medium-chain triglycerides: These are supplements given to provide extra calories and consist of medium chain fatty acids ranging from C_8-C_{10} carbons in chain length.
5. Human milk fortifier (HMF): This is a supplemental powder added to mothers expressed BM to increase calories (through proteins and carbohydrates) and provide extra calcium and phosphorus.
6. Casec: This is a concentrated protein solution added to formula or expressed BM to provide extra proteins.

The following are two commonly used fortifier powders for PT infants:

1) Enfamil HMF: a Mead Johnson, Evansville, USA product contains fat 1g, linoleic acid 140 mg, alpha- linolenic acid 17 mg, vitamin C 12 mg, iron 1.44 mg, among other substances like vitamin A, D, E, K, calcium, phosphorus, copper, zinc and magnesium per 4 packets.

2) Similac HMF (Ross Products, Abott Park, USA): another nutritional supplement intended for PT infants contains 0.36 g fat, 25 mg vitamin C, 0.4 mg iron, vitamins A, D, E, K and other minerals like calcium, phosphorus, copper, zinc and magnesium per 3.6 g powder.

These supplements contain iron, vitamin C and E. Some like microlipids contain mono and polyunsaturated fatty acids. Fenton chemistry or the Haber-Weiss reaction predict these supplements may contribute to ROS formation. Given the putative link between ROS and neonatal morbidity and mortality, the studies described herein were undertaken.

2.3.5 Milk from mothers of premature and term infants

The PT infant's diet requirements are different from the FT infant. The mineral composition of term and preterm milk also vary. Nitrogen (protein) composition, lactoferrin, lysozymes, immunoglobulins A, G and M of preterm milk are higher than the term milk (Atkinson, 1995). Stage of lactation, changes in maternal hormones and mammary gland morphology may be responsible for the some of the differences in the composition described above (Atkinson, 1995). The concentration of minerals sodium, potassium, calcium and phosphorus; and trace elements zinc, copper and iron in preterm is higher than full term milk (Jensen, 1995).

2.4 REACTIVE OXYGEN SPECIES

When stable molecules lose electrons from the outer orbital shell, they become unstable, forming reactive oxygen species (ROS). ROS react with other molecules to acquire electrons to regain stability, thereby making them unstable. In turn this reaction can cause a chain reaction unless the progress of the reaction is sequestered by antioxidants. Examples of ROS include superoxide anion, hydroxyl radical, ozone, singlet oxygen and hydrogen peroxide (Gille and Joenje, 2002).

2.4.1 Oxidative stress

ROS are produced during normal cellular metabolism. They have physiological roles such as acting as cell signaling molecules (Nindl et al., 2004). ROS cause oxidative stress when free radicals and their products overwhelm cellular antioxidant defense mechanisms. Oxidative stress is defined as a disturbance in pro-oxidant antioxidant balance in favor of the pro-oxidant, leading to potential damage in the body (Sies, 1991). Free radical damage accumulates in the body progressively as a person ages. Oxidative stress may be potential causative agent of cancer, atherosclerosis, degenerative neurological diseases. Oxidative stress is associated with mutagenesis, carcinogenesis and genetic instability (Gille and Joenje, 2002). It is also associated with necrotizing enterocolitis (NEC) and bronchopulmonary dysplasia in infants (Van Zoeren-Grobben et al., 1993). Thus oxidative stress in PT infants may be a possible cause of NEC. We plan to study *in vitro* addition of iron and vitamin C supplements to human milk and their co-incubation with fetal and adult intestinal cells.

2.4.2 ROS and diseases of prematurity

PT infants show lower resistance to oxidative stress than FT infants (Van Zoeren-Grobben et al., 1994) and have lower level of Cu/Zn SOD than FT infants (Saugstad, 1996). Respiratory distress syndrome (RDS), NEC, periventricular leukomalacia, retinopathy of prematurity (ROP) and patent ductus arteriosus are some diseases of prematurity which may be linked to ROS (Saugstad, 1998). NEC primarily occurs in PT infants and there is an inverse correlation between the incidence of NEC and gestational age (Pereira, 1995; Caplan et al., 1994).

RDS is a lung disease that may occur in PT infants due to ROS (Gitto et al., 2004). Mechanical ventilation of the lungs in PT infants has been suggested as the possible cause of RDS by Northway et al. (1967). ROP may be caused by vasoconstriction, ischaemia and hyperoxic exposure of premature retina (Hardy et al., 2000 and Hou et al., 2000). PT infants have less ability than FT infants to cope with the oxidative stress produced by ROS and therefore are more susceptible to these diseases (Saugstad, 1996 and 1998).

2.4.3 Cellular defense mechanisms

Oxidative stress ultimately affects cellular processes. Normally, there exists a balance between ROS and antioxidants in the body. Antioxidants vitamin E, C and enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) protect cells from ROS damage. Lactoferrin found in milk also has antioxidant properties. The normal levels of SOD, GPx and CAT in human milk are 35 U/mL, 0.1U/mL, 15 U/mL, respectively. SOD acts by converting superoxide anion into hydrogen peroxide, which are in turn converted to water and oxygen by

CAT (GPx converts hydrogen peroxide to water) (Frankel, 1998). Some cellular defenses like glutathione, beta-carotene and uric acid also protect against cellular damage (Gille and Joenje, 2002; Constantinescu et al., 2000). When ROS increase out of proportion to antioxidants, cellular damage occurs.

Oxidative stress tips the balance between cellular oxidants and reductants. Redox potential is defined as a measurement of the ease by which a substance gains or loses electrons (USFDA, 2005). Depending on their redox potential, the cells in the body may be induced to enter quiescent, apoptotic or necrotic states (Wang et al., 2000). Alteration in the redox potential has a profound influence on tissue structure and functions like apoptotic cell death. This may also lead to cell death and non-functioning of an organ like the lungs, intestine and/or the central nervous system. These effects are important in the intestinal cells in the PT infant, as these are the main portals of entry of pathogens in the gut.

2.5 LIPID OXIDATION

2.5.1 Dietary fat

The lipid component of human milk consists of fatty acids, triglycerides, monoacylglycerol, cholesterol, phospholipids and cholesterol esters (Jensen, 1995). Triglycerides are the major lipids (> 98 %) in human milk. The total fat content of milk ranges from 31 to 53 g/L or 45 to 58 % of total energy intake (Raiten et al., 1998). Refer Table 7 for the fatty acid composition of human milk. Lipids are a good source of energy and useful for the transportation of the fat-soluble vitamins A, D, E and K. Essential fatty acids linoleic acid, alpha-linolenic acid and arachidonic acid are necessary for body development. Fats are required for eicosanoid production (Leslie, 2004). Eicosanoids play a vital role in regulating blood clotting, inflammation and blood pressure regulation.

2.5.2 Harmful effects of lipid oxidation

Unsaturated fatty acids are susceptible to oxidation due to their double bonds. Fatty acids with a greater number of double bonds oxidize more readily than fatty acids with a lower number of double bonds. Linoleic and arachidonic acids are more susceptible to oxidation than monounsaturated fatty acids (MUFAs). Some enzymes in milk such as xanthine oxidase stimulate oxidation (Frankel, 1998).

Marshall and Roberts (1990) conducted a study on formula, milk and lipid emulsions in rat tissue. They found the survival of the rat depended on diet indicating lipids in the diet had a role to play in oxidation; iron and PUFA's enhanced oxidation while vitamin E reduced it. Granot and colleagues (1999) worked with 2-4 month infants grouped into BM-fed and formula-fed groups. The investigators found higher

TBARS in BM fed group and concluded long chain fatty acids (C_{20-22}) in BM may act as substrates for oxidative damage. This study implies the addition of exogenous long chain PUFA's could increase the lipid oxidation in BM.

Kimura et al. (2004) studied oxidized oil and its acceptability in rats, and found the rats preferred non-oxidized to oxidized oil. Rodriguez et al. (2003) studied the effects of medium-chain triacylglycerols (MCT) on linoleic acid metabolism and concluded MCT decrease lipid oxidation of long chain PUFA's in PT infants. DeRouchey et al. (2004) conducted an experiment on weanling piglets by feeding one group oxidized lipid meal and the other normal soybean meal. They found no change in digestion of oxidized lipids compared to the control and attributed it to decreased uptake of rancid fats by piglets.

Perjesi et al. (2002) fed rancid corn oils rich in omega-6 unsaturated fatty acids to mice. They found an increased expression of c-myc and the p53 genes in mice fed rancid oils, concluding an increase in number of tumor promoting genes occurs with ingestion of rancid corn oil. P53 is a proapoptotic gene; therefore its increase would increase cell death. Tulova et al. (1975) fed rancid lard to pigs and observed lack of appetite due to decrease in intake of oxidized fat, walking changes, dystrophic changes in muscles and heart as well as decrease in these effects with vitamin E supplementation in pigs.

Most of these studies have been done in animals. There are some reports of accidental ingestion of rancid fat and nausea. Detrimental effects on health could easily occur in adults, as well as children, on consumption of rancid fats. Their effects on infants could be worse due to decreased resistance to inflammatory and infective

agents, than in adults. Feeding oxidized lipids to PT infants could thus lead to neonatal diseases like NEC, ROP and RDS. This may affect the health and well being of the infant and thus morbidity and mortality of PT infants.

2.5.3 Factors causing lipid oxidation in human milk

Oxygen present in the atmosphere is a necessary factor leading to oxidative damage in food substances including BM (Friel et al., 2002). Free radicals target the double bonds in fatty acids causing oxidation and lead to oxidation of lipids present in the gut when the lipids are exposed to ROS (Atkinson, 1995; Billeaud et al., 1990; Laudicina and Marnett, 1990).

Temperature plays a pilot role in oxidation of lipids. Lipid oxidation is enhanced at 60°C and higher temperatures. Light exposure also causes oxidation of lipids. Rancidity, defined as disagreeable odor or taste of decomposing oils or fats, is affected by time. Prolonged storage (>4 days) may also oxidize fats in the milk (Van Zoeren-Grobben et al., 1993). Catheters are used in gavage feeing of infants. The material used in the catheter tubes may also affect oxidation (Laborie et al., 1999; Van Zoeren-Grobben et al., 1993).

PUFAs are prone to oxidation because of their double bonds. Conjugated dienes and lipid peroxides are the primary products of lipid oxidation. Conjugated dienes are also formed by the breakdown of long chain (> C20) fatty acids (Leigh-Firbank et al., 2002). TBARS, ketones, aldehyde, alcohol, epoxides and acids are also formed. Alkanes like ethanal, butanal, propanal, hexanal, pentanal and malonaldehyde are the final products of oxidation.

2.6 IRON

Iron can exist in oxidation states ranging from -2 to +6; the two most common being ferrous (+2) and ferric (+3) states. Iron is involved in hemoglobin-oxygen transport and enzymatic oxidation-reduction reactions. It is also a component of catalase and peroxidase enzymes. Aconitase, an iron enzyme, is involved in Krebs cycle (Disilvestro, 2005). The Recommended Dietary Allowance (RDA) for all age groups of men and postmenopausal women is 10 milligrams of iron per day. The median dietary intake of iron is approximately 16 to 18 mg/day for men and 12 mg/day for women.

Food and Nutrition Board (Institute of Medicine) (2001) states that the normal full term infant has adequate amount of iron in body and a very high hemoglobin concentration at birth; iron can easily move between body iron stores making exogenous iron requirement redundant at birth. After 6-8 weeks from birth, supplemental iron requirements of PT infants (infants with birth weights of 1000 g or more) is 2 to 4 mg/kg per day (AAP, 1999) and 3-4 mg/kg per day according to CPS (1991).

2.6.1 Primary food sources

Green leafy vegetables, lean red meat, beef, liver, poultry, fish, wheat germ, oysters, dried fruit, prune juice and iron-fortified cereals are good sources of iron. Foods high in vitamin C, such as citrus fruits, tomatoes and strawberries increase iron absorption. Therefore iron is co-administered with vitamin C to enhance its absorption. Human milk and cow milk provide approximately 0.35 mg /L and 0.25 mg/L of iron, the former being more bioavailable (Jensen, 1995).

2.6.2 Absorption

There are two pathways for absorption in humans. One involves absorption of heme iron through hemoglobin and myoglobin in meat, and the other involves a gastrointestinal absorption of nonheme iron primarily as salts (Raja et al., 1987). Absorption of nonheme iron is enhanced by ascorbic acid and is a primary reason why vitamin C and iron are co-administered. Most of the iron consumed belongs to the nonheme form. The bioavailable iron is absorbed in a three-step process. It passes into the enterocytes by an energy-dependent, carrier-mediated process (Muir & Hopfer, 1985; Simpson et al., 1986). Later, iron is transported intracellularly and across basolateral membrane into plasma.

2.6.3 Intracellular transport, storage and metabolism

Intracellular iron binds with transferrin, a transport protein, used as a functional component ferritin or in future cellular iron metabolism. All cells have the capacity to store iron. Iron is stored primarily in liver, spleen and bone marrow as ferritin or hemosiderin.

2.6.4 Elimination and excretion

Total body iron is highly conserved. In adults, iron is eliminated from the body in skin, urine and feces approximately 0.2-0.3 mg/day, 0.08 mg/day and 0.6 mg/day respectively; in infants, iron is passed out of the body in skin, urine and feces (Food and nutrition board, 2001). It is about 0.03 mg/kg/day and 6-12 months old babies lose about 0.26 ± 0.03 (SD) mg/day iron. Iron is reabsorbed through the kidneys and is thus conserved in the body.

2.6.5 Iron deficiency and toxicity

Iron deficiency leads to impaired work performance in adults, developmental delay, cognitive impairment and adverse pregnancy outcomes (Willis et al., 1988; Idjradinata & Pollitt, 1993; WHO, 1992). Maternal anaemia is associated with premature delivery, low birth weight and increased perinatal infant mortality (Allen, 1997; Garn et al., 1981; Klebanoff et al., 1991; Lieberman et al., 1988; Murphy et al., 1986; Williams & Wheby, 1992).

Excessive consumption of iron can lead to hemochromatosis and haemosiderosis. Haemosiderosis is a form of excess iron deposition due to excessive iron consumption, liver disease and multiple transfusions (Chen et al., 2004). Hemochromatosis is a more severe form of iron toxicity. Treatment includes phlebotomy or desferoxamine administration.

2.6.6 Indicators for estimating iron requirement

Functional: The important functional indicators of iron requirement are decreased work capacity, delayed psychomotor development in infants, cognitive impairment in infants (Idjradinata & Pollitt, 1993; Lozoff et al., 1987; Oski et al., 1983; Walter et al., 1983).

Biochemical: There are three levels of iron deficiency 1) Depleted iron stores but functional stores are working optimally, 2) Early functional iron deficiency where supply to functional stores is suboptimal, 3) Iron deficiency anemia where a measurable deficit occurs in most functional stores in erythrocytes.

Complete blood count and hematocrit counts are useful in determination of iron status. Table 1 shows some indicators to measure iron status in humans.

Table 1 Laboratory measurements of iron status

Stage of iron deficiency	Indicator	Diagnostic Range
Depleted iron stores	Stainable bone marrow iron Total iron binding capacity Serum ferritin concentration	Absent > 400 µg/dL < 12 µg/L
Early functional iron deficiency	Transferrin saturation Free erythrocyte protoporphyrin Serum transferrin receptor	< 16 % > 70 µg/dL erythrocyte > 8.5 mg/L
Iron deficiency anemia	Hemoglobin concentration Mean cell volume	< 130g/L (male) < 120g/L (female) < 80 fL

Reference: (Food and nutrition board, 2001).

2.6.7 Interactions

Vitamin C enhances nonheme iron absorption. In the presence of ascorbic acid, ferric iron is reduced to ferrous and a soluble iron- vitamin C complex is formed in the stomach (Teucher et al., 2004). Phytate, polyphenols, vegetable proteins (soybean) and calcium inhibit iron absorption (Hurrell, 2004).

2.6.8 Adverse effects

Vomiting and diarrhoea are most common adverse effects of iron overdose. Other systems like the cardiovascular, central nervous system, kidney, liver, and hematological system are also involved with chronic intake. Severity is related to the amount of elemental iron absorbed. Symptoms of acute intoxication can occur with

parenteral iron administration. Hereditary hemochromatosis may occur in iron overload.

2.6.9 Iron requirements

Iron is an integral part of hemoglobin, involved in carrying oxygen to tissues for metabolism. At birth, hemoglobin is the major reserve of iron for PT infants. Iron requirements of the infant vary according to the blood loss associated during birth. Although iron content in milk is low, its bioavailability is high (Jensen, 1995). The concentration of iron decreases as lactation progresses and is highest during the first few days after birth (Jensen, 1995).

After birth, fetal Hb (hemoglobin) concentration starts falling due to rapid growth. Between 4-6 months, active haemopoisis occurs and adult Hb begins to form (Hernell & Lönnerdal, 2002). Therefore, exogenous iron in form of supplements is recommended from 4-6 months by AAP and CPS. Adequate iron is provided by exclusive breast feeding upto 6 months of age; average iron concentration in human milk is 0.35 mg/L; the AI for infants 0-6 months of age is 0.27 mg/day (0.78 L/day x 0.35 mg/L) (Food and nutrition board, 2001).

After 6-8 weeks from birth, supplemental iron requirements of PT infants (infants with birth weights of 1000 g or more) is 2 to 4 mg/kg per day (AAP, 1999). Formula containing iron (12 mg/L) is recommended to prevent iron-deficiency anemia (CPS, 1991). For infants with birth weights of 1000 g or less, supplemental iron at 3 to 4 mg/kg per day after 6 to 8 weeks of birth is recommended (CPS, 1991).

RDA for infants 7-12 months of age is 11 mg/day of iron; RDA for children 1-3 yrs of age is 7 mg/day; for ages 4-8 yrs is 10 mg/day; RDA for boys 9-13 yrs and

14-18 yrs is 8 mg/day and 11 mg/day respectively; RDA for girls 9-13 yrs and 14-18 yrs is 8 mg/day and 15 mg/day respectively (Food and nutrition board, 2001). It is higher in girls to account for menstrual losses.

2.6.10 Iron supplements

Iron supplements are beneficial for the prevention of iron deficiency anemia in full term infants. However, the risk and benefits of iron supplements may be uncertain in PT infants during the first two months of life (Raghuveer et al., 2002). The main reason being that diminutive erythropoisis takes place during this period. Inspite of iron supplementation before 2 months of age, physiological anemia occurs (CPS, 1991). AAP and CPS recommend 2 to 6 mg of elemental iron per kilogram per day after 4-6 months postpartum.

2.6.11 Iron and ROS

Iron can switch between ferrous and ferric states making it a strong oxidant-reductant (Herbert et al., 1994). An excess of iron in the body may lead to cancer and heart diseases due to free radical production (Gutteridge, 1989). Iron added to infant formula or BM produces free radicals (Raghuveer et al, 2002). Ferric iron generates reactive oxygen species according to Fenton chemistry as follows

$$\text{Vitamin C/ E} + Fe^{3+} \rightarrow Fe^{2+}$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + {}^{\cdot}O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$$

Wardle et al. (2002) attribute formation of ROS to excess ferrous iron from blood transfusions by the Haber-Weiss reaction:

$$\cdot O_2 + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

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

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^{-}$$

Thus iron can generate ROS with or without vitamin C or E by the Haber-Weiss reaction. But presence of antioxidants will generate more ferrous iron causing a cyclic reaction. Hydroxyl ions are mostly responsible for the oxidative damage seen due to iron (Herbert et al., 1994). In a study carried out by Friel et al. (2002), vitamin C and iron caused oxidative damage in milk and iron fortified formulas. This may be due to ROS produced according to Fenton chemistry shown above. Lachili et al. (2001) carried out a study with iron fumarate (100 mg/d) and vitamin C (500 mg/d) supplementation on pregnant women. They observed a decrease in plasma levels of vitamin E. There was lipid peroxidation in the plasma, suggesting iron should be used with caution. These studies suggest vitamin C may play a role in peroxide formation in iron containing formulas.

2.6.12 Iron chelaters or binders

Iron binding proteins such as transferrin and lactoferrin act as antioxidants by binding free iron (Gutteridge, 1989). Iron chelators like desferrioxamine and deferiprone are administered in cases of iron toxicity.

2.7 VITAMIN C

2.7.1 Functions and sources

Vitamin C functions as an antioxidant, cofactor of enzymes, and production of collagen, carnitine, and neurotransmitters. Collagen is a protein necessary for the formation of connective tissue in muscles, skin, bones, and cartilage. It aids in the absorption of iron and is needed for the synthesis of bile salts. It protects endothelium by inhibiting LDL cholesterol oxidation, platelet aggregation, and leukocyte adhesion to the endothelium. Citrus fruits, strawberries, cantaloupes, broccoli, spinach, kale, cabbage, and tomatoes are good sources of vitamin C.

2.7.2 Health effects of vitamin C

Vitamin C is important for prevention of atherosclerotic heart disease through its antioxidant action on oxidized LDL cholesterol. It also has a role in cancer, cataract, asthma, COPD and common cold prevention. It also protects cognitive function.

2.7.3 Cellular DNA damage and vitamin C

DNA damage can cause mutation leading to birth defects, genetic diseases, and cancer (Fraga et al., 1991). Fraga et al., (1991) measured oxidized nucleoside 8-hydroxy-2'-deoxyguanosine (8-oxo-7, 8-dihydro-2'-deoxyguanosine; 8-oxodG), one of the major products of oxidative damage to DNA in human sperm. They found dietary vitamin C has a protective role in cellular DNA damage. Subjects consuming higher dietary vitamin C had protective effect in human sperm but not in lymphocyte or urine 8-oxodG (Fraga et al., 1991).

Anderson et al., (1997) did a human study on non-smokers with a wide range of serum cholesterol levels. They observed no effect at either 60 mg/day or 6,000 mg/day supplemental vitamin C on lymphocyte DNA or chromosome damage. The study done by Podmore et al. (1998) showed a decrease in 8-oxoadenine and increase in 8-oxoguanine after 500 mg/day supplemental vitamin C. This study indicated a pro-oxidant role of vitamin C in respect to lymphocyte DNA oxidative damage after 500 mg/day supplemental vitamin C. Cooke et al. (1998) found an increase in serum and urinary 8-oxodG levels and decrease in lymphocyte 8-oxoguanine and 8-oxodG (Food and nutrition board, 2000). This indicated a non-scavenging antioxidant effect of vitamin C.

Increase in oxidized 8-oxoguanine and 8-oxodG would indicate oxidative stress and their decrease in cells would indicate decreased stress to the cells. Increase as well as decrease in lymphocyte DNA was seen in studies with co-supplementation of vitamin E (280 mg/day) and beta-carotene (25 mg/day) (Duthie et al., 1996) and with iron (Rehman et al., 1998), implying mixed results. Summarizing the results, the effects of vitamin C on cell DNA damage are equivocal.

2.7.4 Absorption

Ascorbate is absorbed through a dose dependant Na –dependant active transport. At higher concentration simple diffusion occurs. It is absorbed in the form dehydroascorbic acid and intracellularly converted to ascorbic acid. It exists as ascorbate in plasma.

2.7.5 Pro-oxidant effects of vitamin C

Vitamin C acts as a catalyst in the presence of iron or copper for hydroxyl radical production, by Fenton chemistry (Buettner & Jurkiewicz, 1996). *In vivo* iron is bound to transferrin and ferritin so is not available for catalytic Fenton reaction. Berger et al. (1997) conducted a study on PT infants with high plasma concentrations of ascorbic acid. They found that the combination of high plasma concentrations of vitamin C and redox-active iron did not cause damage to lipids and proteins *in vivo*. Vitamin C acted as antioxidant in their study. *In vitro* vitamin C acts as a pro-oxidant in higher doses and as an antioxidant at lower doses according to Lachili et al. (2001). Thus from the literature studied, vitamin C in presence of iron and PUFAs may form ROS. The effects on lipid oxidation need to be investigated *in vitro* and *in vivo* by examining the effect of iron, vitamin C and ML supplements in BM.

HYPOTHESES:

1. Iron and vitamin C with and without microlipids produces lipid oxidation in human milk *in vitro*.
2. These peroxidation products are harmful to PT infants' intestinal cells causing oxidative damage to the DNA and cell death (apoptosis).
3. Exogenous superoxide dismutase (with catalase) added to milk decreases oxidation when iron with vitamin C and microlipids are added to milk.
4. Pasteurization of milk would negate effect of endogenous enzymes causing more damage to intestinal cells.

OBJECTIVES:

The parameters to test these hypotheses include

1) Measure lipid oxidation products after addition of iron, vitamin C and ML to BM. Measure primary oxidation products conjugated dienoic acid and lipid peroxides by spectrophotometry. Measure secondary oxidation products TBARS by spectrophotometry.
2) Study the effect of lipid peroxidation by ROS on DNA damage and apoptosis in the infants' intestinal cells by indirect immunofluorescence microscopy with a monoclonal antibody to 8-hydroxy-2`-deoxyguanosine (8-OHdG), a nucleotide marker for oxidative stress.
3) Measure the effect of antioxidant enzyme superoxide dismutase (with catalase) and its effect after pasteurization of milk.
4) Quantify lipid peroxidation with addition of human milk fortifier (HMF).

CHAPTER 3: MATERIALS AND METHODS

3.1 Basis of nutritional supplements dosage in human milk

Supplementation of iron in PT infants during the first 2 months of life is doubtful due to low erythropoisis (CPS, 1991). Exogenous iron requirements are minimal as iron is easily obtained from regeneration of RBC's (AAP, 1999). Ziegler et al. (1981) and Lundstrom et al. (1977) suggested 2-3 mg/kg/day iron should be given to low birth weight infants (LBW) from 2 weeks of age or as soon as feeding is tolerated. AAP (1979) advocates 2-3 mg/kg/day elemental iron to be given to LBW infants, as soon as the infants reaches 2000 g weight and/or goes home, in form of ferrous sulfate drops. AAP (1985) recommends 2-4 mg/kg/day elemental iron and up to a maximum of 15 mg/day to LBW infants. Infants receiving erythropoietin treatment could be given 6 mg/kg/day elemental iron according to Shannon et al. (1991) and Ehrenkranz et al. (1994).

In the US iron fortified formulas for full term infants contain iron ranging from 10 mg/L to 12 mg/L (Food and Nutrition Board, 2004). TRIVISOL® with iron (Mead Johnson, Evansville, USA) contains 10 mg/mL iron in addition to the vitamin A 1500 IU/ mL, 400 IU/ mL vitamin D and vitamin C 30 mg/mL. Raghuveer et al. (2002) carried out *in vitro* study in milk from mothers who gave birth to PT infants. On the basis of a telephone survey from NICU in US, they found that most PT infants are given iron supplements once a day. They added 2 to 6 mg/ kg iron for a hypothetical 1.5 kg PT infant. They calculated 16.25 mL/kg milk, as PT infants 3 hourly intake, assuming 130 mL/kg as the daily intake of milk.

We did a pilot study to examine the dose response of iron in BM. A significant increase in peroxides in BM was seen for the iron concentration above 1 mg/kg iron for a hypothetical 1.5 kg infant, among the different concentrations of iron chosen by us. To mimic the practice of PT infant feeding we chose 2 mg/kg/d iron for a hypothetical 1.5 kg infant as the supplemental iron in BM (based on AAP recommendations and the dose of iron administered in the guidelines from NICU, St. Boniface General Hospital). This dose was also used by Raghuveer et al. (2002) (2 to 6 mg/kg/d) and is equivalent to 1.8 mM iron. The relevant calculations are shown in Appendix II and III. We assumed 160 mL/kg as the daily intake of milk of a hypothetical 1.5 kg PT infant. Therefore the 3 hourly BM intake would correspond to 20 mL/kg. Supplements with BM can be administered to PT infants until 4 hours at room temperature (Telang et al., 2004).

Most full term infant formulas contain 2000- 2190 IU/ L vitamin A, 228-438 IU/ L vitamin D and 57-81 mg/ L vitamin C (Food and Nutrition Board, 2004). The American Academy of Pediatrics recommends supplemental 200 IU of vitamin D for the first 2 months of life for a healthy FT. American Academy of Pediatrics (1979) recommends 400 IU supplemental vitamin D in addition to vitamin D in formula in PT infant. TRIVISOL® (Mead Johnson, Evansville, USA) contains 400 IU/ mL vitamin D, in addition to vitamin A 1500 IU/ mL and vitamin C 30 mg/mL. TRIVISOL® with iron (Mead Johnson, Evansville, USA) contains 10 mg/mL iron in addition to the three vitamins mentioned in the previous line. The doses of vitamins A and D were based on American Academy of Pediatrics (1979) and Mead Johnson, Evansville, USA guidelines. Both vitamins A and D were purchased from Sigma

chemicals, Oakville, Canada. We used a dose of TRIVISOL corresponding to 75 IU/mL vitamin A and 20 IU/mL vitamin D in BM for our study.

The dose of HMF (Enfamil Human Milk Fortifier, Mead Johnson, Evansville, USA) was according to the manufacturers' recommendations and guidelines provided by NICU, St. Boniface General Hospital. The guidelines provided by NICU, St. Boniface General Hospital state 1 packet HMF to be added to 25-35 mL BM when infants receives 100 mL /kg/day BM. The dose we used was 1 packet HMF in 25 mL BM. Norris and Hill (1994) recommend microlipids to increase caloric intake in infants. AAP (1979) recommend medium chain triglycerides with long chain PUFAs as a source of energy to PT infants. The dose of microlipids 73.5 µl/mL was based on guidelines for doses in NICU (Zenk et al., 1999).

Catalase (CAT) and superoxide dismutase (SOD) are antioxidant enzymes normally found in human milk. Catalase acts along with superoxide dismutase to decrease hydroxyl radical production in milk (Frankel, 1998). The dose of SOD 100 U/mL was based on the studies by Lopez-Ongil et al. (1998), Rossi et al. (2001), An (2004), Hernanz (2004). The dose of CAT 100 U/mL was based on the studies by Rossi et al. (2001), Khan (2004), An (2004), Buttke and Trope (2003), Kohjitani (2003), Pedrosa and Soares-da-Silva (2002).

3.2 Subject demographics and sample collection

Milk samples for this study were collected from 81 mothers of PT infants (born between 29 and 37 weeks of gestation) during the second week of lactation. Appropriate consent and ethical approval was taken from women who participated in the study. The milk samples were collected by the mother using a breast pump or by

hand expression and transported on ice to the laboratory (Diehl-Jones laboratory) and immediately frozen and stored at – 80°C until thawed for analysis.

3.3 Experimental design

Lipids on oxidation form conjugated dienes and lipid peroxides. They further break down to form aldehydes, ketones and acid. Samples were analyzed for lipid oxidation products (conjugated dienes, lipid peroxides and TBARS) after addition of supplements. For the experiment, physiological doses (the amount of supplemental iron and vitamin C usually given to infants in the NICU as discussed previously) of iron, vitamin C, and microlipids were used. The study was divided into six parts as shown in table 2 on the next page.

Table 2 Parts of the human milk study

Parts	Brief description	Description
I	Dose response	The effect of increasing amounts of iron, TRIVISOL (vitamin C content) in BM to be studied. The guidelines from NICU, St. Boniface General Hospital mention the BM could remain at room temperature until 4 hrs. Therefore, the samples were incubated for 4 hrs. All samples were done in triplicates or more replicates. The samples were analyzed for lipid oxidation products by Fox-2 assay, TBARS assay, Conjugated diene assay and fatty acid analysis by gas chromatography. This would show if oxidation was proportional to the amount of supplements added to milk.
II	Effect of time	Samples were treated with 1.8 mM of iron, 8.52 mM vitamin C and other additives for 1, 2, 3, 4, 8, 12 hrs. This would show if oxidation was proportional to the time after the addition of supplements to milk.
III	Effect of antioxidant enzymes	Samples were treated with 100 U/ml SOD, catalase 100 U/ ml and with 1.8 mM iron. The effects were observed. This would provide a possible solution to decrease oxidation in milk.

IV	Effect of additives after pasteurization of milk	Pasteurization decreases bacterial count and inactivates the enzymes in milk. Enzyme inactivation should increase lipid oxidation in theory. BM samples were pasteurized in a hot water bath for 5 min at 85°C to denature endogenous antioxidant enzymes SOD, GPx, and CAT.
V	Addition of vitamins A and D	Samples were treated with vitamin A (Retinol palmitate) 75 IU/mL and vitamin D (cholecalciferol) 20 IU/mL. This was done to examine the vitamin components of TRIVISOL.
VI	Effect on FHs 74 Int cells and Caco-2BBe adult intestinal cell line	FHs 74 Int is an intestinal cell line obtained from a 3-4 month old infant and CaCo-2BBe is an adult intestinal cell line. Testing the effect of supplemental addition to milk on intestinal cell line would be close to *in vivo* environment in human infants. This will tell us the difference, if any, between *in vitro* lipid oxidation and the cellular changes on the cell culture. There is no study to date looking at FHs 74 Int cells and the effect of iron and vitamin C supplements in milk.

Part I

To test the different objectives different concentrations of iron and vitamin C were used. This would show us the effect of these supplements on lipid oxidation. Milk samples were divided in groups for the first part of the study as follows

Table 3 Concentration of additives

No.	Concentration in BM			Description
	Iron	Vitamin C	Microlipids	
1.	0	0	0	Control BM: No additives
2.	0.9 mM	0	0	BM + Iron
3.	1.2 mM	0	0	BM + Iron
4.	1.8 mM	0	0	BM + Iron
5.	3.6 mM	0	0	BM + Iron
6.	0	4.3 mM	0	BM + Vitamin C or TRIVISOL with 4.3 mM vitamin C
7.	0	5.7 mM	0	BM + Vitamin C or TRIVISOL with 5.7 mM vitamin C
8.	0	8.5 mM	0	BM + Vitamin C or TRIVISOL with 8.5 mM vitamin C
9.	0	17 mM	0	BM + Vitamin C or TRIVISOL with 17 mM vitamin C
10.	0	0	73.5 µl/mL of BM	BM + Microlipids
11.	1.8 mM	8.5 mM	0	BM + Iron+ Vitamin C (or TRIVISOL)
12.	1.8 mM	8.5 mM	73.5 µl/mL of BM	BM + Iron+ Vitamin C (or TRIVISOL) + Microlipids

Part II

Milk samples were treated with additives for 0, 1, 2, 3, 4, 8, 12 hours and the effect on lipid oxidation were observed with Fox-2 assay and TBARS assay. Similarly, for parts III, IV, V results were obtained upon addition of additives.

The samples were analyzed after being subjected to 1) Addition of iron, vitamin C, microlipids, TRIVISOL, superoxide dismutase and catalase and 2) Room temperature and under light. This would mimic the administration practice of adding supplements in NICU and incubating for upto 4 hours at room temperature. The various concentrations are as follows:

Table 4 Concentration of additives in BM

	Concentration				
1.	Iron sulfate mg/ml	1.0	**0.5** *	0.33	0.25
2.	Vitamin C mg/ml	3.0	**1.5** *	1.0	0.75
3.	Microlipids	73.5 µl/ml in BM			

* The concentrations in bold represents the dose of elemental iron and vitamin C advised by AAP (1985) for PT infants after 4-6 months of age. A dose of 1.8 mM iron (equivalent to 2 mg elemental iron/kg/day or iron sulfate 0.5 mg/mL) and 8.52 mM vitamin C (1.5 mg/ml) was chosen as the dose for the study as this is the recommended dose (APS, 1985 and CPS, 1991) as explained in Appendices II and III.

3.4 Analysis Methods

3.4.1 Measurement of lipid oxidation products

3.4.1.1 Fox-2 assay measurement of lipid peroxides

Purpose: To find amount of lipid peroxides in treated BM. Peroxides are one of the primary oxidation products of PUFAs.

Theory: PUFA's in the presence of ROS are oxidized as follows

RH + action of pro-oxidants $\rightarrow$R-OOH

where RH is the PUFA and R-OOH is the peroxide product in the reaction.

Lipid peroxides oxidize ferrous to ferric ion selectively in dilute acid and the resultant ferric ions can be determined using ferric sensitive dye, xylenol orange as an indirect measure of hydroperoxide concentration. A colored complex (blue-purple) is formed with the dye xylenol orange and equal molar concentration of ferric ion with an extinction coefficient of $1.5 \times 10^4\ M^{-1}\ cm^{-1}$ at 560 nm wavelength (Wolff, 1994). Undesirable chain oxidation is prevented by addition of antioxidant butylated hydroxy toluene (BHT).

Method: Lipid peroxides were measured spectroscopically by Fox-2 assay based on the method suggested by Wolff (1994) and Nourooz-Zadeh (1999). The plate reader used for the measurements was from Molecular Devices Spectra Max 340, Minnesota, USA. Only deionized distilled water was used in the preparation of all the chemicals.

Fox–2 reagent is comprised of two stock solutions, A and B. Solution A was prepared by dissolving ammonium ferrous sulfate (98.03 mg) in 100 ml of 250 mM H_2SO4. Then xylenol orange (76.06 mg) was added with constant stirring for 10

minutes at room temperature under dark. Solution B was prepared by adding 969.76 mg BHT (Butylated hydroxy toluene) in 900 ml methanol (HPLC-grade). A working Fox –2 reagent was prepared by mixing one volume of solution A with 9 volumes of solution B. The final Fox-2 reagent is composed of xylenol orange (100μM), BHT (4.4mM), sulfuric acid (25 mM), and ammonium ferrous sulfate (250μM). 1.5 mL of milk samples were centrifuged at 13,000 rpm for 5 minutes.

The top lipid layer was separated by placing in a test tube and vortexed for 15 seconds. The lipid layer was added to Fox-2 reagent in the ratio 1:20. The solution was vortexed and kept for 30 minutes under dark. The supernatant 300 μl of the solution was read at 560 nm after centrifugation at 13,000 rpm for 5 minutes. The standards consisted of hydrogen peroxide of concentrations ranging from 0.5 μM to 10 μM. BHT was purchased from Sigma chemicals, Oakville, Canada whereas ammonium ferrous sulfate, sulfuric acid, xylenol orange, methanol and hydrogen peroxide were purchased from Fisher chemicals, Fairlawn, USA.

3.4.1.2 Thiobarbituric acid reactive substances (TBARS) assay:

Purpose: To find amount of TBARS (thiobarbituric acid reactive substances) in treated BM.

Theory: 2- Thiobarbituric acid reacts with secondary oxidation products of lipids in milk to form thiobarbituric acid reactive substances (TBARS). Malonaldehyde (MDA) is a lipid peroxidation product. Sample is heated with thiobarbituric acid at a low pH where one molecule of malonaldehyde reacts with two

molecules of 2-TBA to form a pink colored compound measured spectrophotometrically (Frankel, 1998).

Method: TBARS were measured spectroscopically as a modification of the methods outlined by Raghuveer et al. (2002), Wagner et al. (2000) and Nanua et al. (2000). 1,1,3,3-tetramethoxy propane [Malonaldehyde bis (dimethyl acetal)] standards of concentration 0.1 μM to 1μM were prepared. To 2 mL of milk samples/ standards, 230 μl of 40% trichloroacetic acid (TCA) solution was added. The samples were vortexed and later centrifuged at 13,000 rpm for 5 minutes. This is done to precipitate proteins in samples.

The supernatant (1.6 ml) was placed in glass test tubes and 1.6 ml 2-thiobarbituric acid in 1N HCl was added to the samples. The samples were vortexed and then incubated in 75°C water bath for 30 minutes. The samples were cooled under tap water for 5 minutes. The samples were centrifuged at 2,000 rpm for 5 min (to separate the flocculation) and 300 μl of supernatant was placed in wells of plate.

The absorbance was measured at 532 nm in the micro plate reader purchased from Molecular Devices Spectra Max 340, Minnesota, USA. A standard curve is plotted with the reading of absorbance of the MDA standard. Treated milk samples are plotted against the standard curve. MDA was purchased from Sigma-Aldrich, Oakville, Canada. 2- thiobarbituric acid was purchased from Hallinckrodt Inc., Kentucky, USA and TCA and HCl were purchased from Fisher chemicals, Fairlawn, USA.

3.4.1.3 Conjugated dienoic acid assay:

Purpose: To measure the percentage of conjugated dienes in human milk. A conjugated diene is a molecule in which carbon-carbon double bonds are separated by one single bond. This assay measures diene conjugation of unsaturated linkages present, expressing this value as a percentage of conjugated dienoic acid (Official Methods and Recommended Practices of the AOCS, 1999).

Theory: Oxidation of unsaturated fatty acids results in the formation of conjugated diene structures that absorb light in the wavelength of 230-235 nm and can be quantified spectrometrically.

Method: The chemicals involved in the method were iso-octane and methanol. Iso-octane and methanol were purchased from Fisher chemicals, Fairlawn, USA. Canola oil was purchased from 'CANOLA HARVEST' (Canbra foods, Alberta, Canada). 0.4 ml canola oil was measured in test tubes. Weight of each glass test tube before and after addition of canola oil was taken. 0.8 ml of BM was added to the tubes. Homogenization of the samples was done for 5 min. 3 ml methanol and 5 ml Isooctane was added to each tube. The samples were homogenized for 5 min and centrifuged for 10 minutes at 2500 rpm for 10 min. Upper isooctane layer was separated. 1 mL of this layer was measured at 233 nm wavelength.

Calculation:

$$\text{Conjugated dienes \%} = 0.84\left(\frac{As}{bc} - Ko\right)$$

where –

Ko = absorptivity by acid or ester groups,

= 0.07 for esters

= 0.03 for acids

As = observed absorbency at 233 nm

b = cell length in centimeters (10mm)

c = concentration of sample in grams per liter of the final dilution used for the absorption measurement

3.4.2 Fatty acid analysis

This method is used to measure free fatty acids or lipolysis products. This is done to find the change in fatty acid composition before and after addition of supplements to milk. This would give an indication if the essential fatty acids were undergoing oxidation. Fatty acid analysis of BM is very important as it gives an indication of maternal diet. The gas chromatograph-5890 was from Hewlett Packard, Ramsey, USA.

Purpose: To find fatty acid composition of lipids in BM by gas chromatography

Theory: This involves sealing a measured amount of milk in a vial. The sample is heated to a certain temperature where the products of interest vaporize and approach equilibrium (Frankel, 1998). The vapor phase is injected into gas chromatography column SP-2560 (Supelco capillary column 100m x 0.25μm purchased from Sigma-Aldrich, Oakville, Canada) and the fatty acids are separated.

Method: The frozen milk samples were thawed and homogenized (*to mix lipids in milk solution, otherwise they tend to accumulate at the top of the test tube*). 1 mL milk was taken into a 50 mL test tube. 1 mL C17: 1 standard (1mg/mL in iso-octane) was added (C17: 1 contains methyl 10- heptadecanoate: Nu Chek chemicals,

Elysian, USA). 17 mL 2:1 chloroform: methanol was added. The samples were homogenized for 30 seconds. Addition of 6 mL of 0.7% sodium chloride (NaCl) was done then the tubes were inverted to mix for 30 seconds. The samples were centrifuged at 2000 rpm for 10 minutes. The bottom layer (8mL) was separated and introduced in 20 mL test tubes. Rinsing with chloroform was done between samples. Evaporation of the samples was done under nitrogen for about 20 minutes at 50 °C water bath. Then 1 mL iso-octane was added and the samples were vortexed. Then 6 mL 0.5N sodium methylate (NaOMe) (13.5g NaOMe in 500 mL methanol) (Kept in fridge, which has adapted to room temperature) was added and the tubes were kept in water bath (hot water at 60°C) for 5 minutes, then put in oven for 10 minutes at 50°C.

The samples were vortexed after heating. The samples were cooled and 2 mL iso-octane, 3 mL of 10% acetic acid (10 mL acetic acid + 90 mL deionized distilled water) was added. The samples were mixed (inverted), and centrifuged at 2000 rpm for 10 minutes. The upper layer was separated for GC analysis. 1-1.5 mL of the supernatant obtained was placed in a glass vial and sealed with a stopper. Chloroform, methanol, sodium chloride, sodium methylate and acetic acid were purchased from Fisher chemicals, Fairlawn, USA.

These glass vials were numbered appropriately and placed in the gas chromatography chamber. The head pressure of gas chromatograph was 36 psi. The initial time, temperature and rate were 2 minutes, 70°C and 15 deg/minute respectively for each sample. The final time, temperature and rate were 25 minutes, 155°C and 3 deg/minute respectively for each sample. The GC then analyzed these samples by automatically injecting 1 mL of the sample in the column and plotting the

peaks obtained of different fatty acids with a split of 1:8. The injector and detector temperatures were 250°C and 250°C respectively. A gas chromatograph is obtained, which expresses the quantity, content, and composition of different fatty acids in the sample. Comparing the relative % of different fatty acids, we can quantify the changes seen after addition of iron in BM.

3.4.3 Intestinal cell study

The cell study consisted of incubating FHs 74 Int cells and Caco-2BBe cells with supplements in PT milk. Immunostaining with antibody to 8-hydroxy-2'-deoxyguanosine, intracellular redox potential, and caspase-3 activation were the assays to examine cellular changes. FHs 74 Int cell line was obtained from American Type Culture Collection (ATCC), Manassas, USA ATCC number CCL-241. Caco-2BBe cell line was also obtained from ATCC, Manassas, USA and the ATCC number was CRL-2102.

Dulbecco's Modified Eagle Medium (DMEM) (Gibco chemicals, Paisley, Scotland, UK) with additives was used as the medium for growth of the cells. The additives were insulin 1 mg/mL (total 5 mL), human EGF (Epidermal Growth Factor) 20 μg/mL (total 1 mL), Penicillin/Streptomycin (P 10,000 U/mL, S 10 mg/mL) (total 5 mL), 10 % fetal bovine serum (60 mL) (Sigma chemicals, Oakville, Canada), L-glutamine (10 mL) and 2.5 mM sodium pyruvate (5 mL) (Sigma chemicals, Oakville, Canada) were added to 500 mL DMEM. This was based on the recommendations by Wang et al. (2000). The cell cultures were maintained in humidified atmosphere with 5% CO_2, 95% air at 37°C. The culture medium was changed every 2 days. Only sterile deionized ultra filtered distilled water (Fisher chemicals, Fairlawn, USA) was

used during all steps of cell study. Trypsin, used for splitting of cells, was purchased from Sigma chemicals, Oakville, Canada.

3.4.3.1 Immunostaining

Purpose: To identify nuclear oxidative DNA damage by using the antibody to 8-hydroxy-2'-deoxyguanosine with addition of iron and vitamin C/ TRIVISOL to BM.

Theory: 8-hydroxy-2'-deoxyguanosine is a nucleotide marker for oxidative stress. The antibody to 8-hydroxy-2'-deoxyguanosine (Trevigen, Helgerman, USA) allows the detection and quantification of 8-hydroxy-2'-deoxyguanosine in DNA.

Method: Primary cultures of FHs 74 Int (human small intestine cell line from 3-4 month gestation infant) were prepared. Cell cultures were exposed to milk samples pooled from at least three donors. In brief, using Pasteur pipette in PBS supplemented with 10% fetal bovine serum (Sigma chemicals, Oakville, Canada). The cells were plated into 24- or 6 well Nunc multi-well plates. The medium was changed every other day to ensure nutrition of cells. The cells were observed under microscope to monitor their growth.

The cells were incubated in the treated milk samples for 4 hrs under 5 % CO_2 and 95% air at 37°C. Then they were fixed on coverslips using Poly-L- Lysine (Sigma chemicals, Oakville, Canada). The samples (coverslips coated with cells) were washed twice in 1x PBS, 5 min each. Then the samples were incubated in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.4 mM NaCl and 100 µg/ml RNase for 1 hr at 37°C. EDTA, NaCl and RNase were purchased from Sigma chemicals, Oakville,

Canada. The samples were washed twice with 1x PBS, for 2 min each. The DNA was denatured by soaking in 4N HCl for 7 min. The samples were neutralized by soaking in 50 mM Tris-base for 5 min. Then they were washed twice with 1x PBS, for 2 min each and incubated in 10 mM Tris-HCl, pH 7.5, 10 % fetal bovine serum for 1 hr at 37°C.

The samples were covered with 50 µl of anti-8-oxo-dG monoclonal antibody diluted 1: 300 in 10 mM Tris-HCl, pH 7.5, 10 % fetal bovine serum and incubated overnight in a humid chamber at 4°C. They were washed twice with 1x PBS, for 2 min each the next day. Then they were incubated with Alexa-480 goat anti-mouse (Sigma chemicals, Oakville, Canada) diluted 1:200 in PBS, for 1 hr at 4°C and later 1 hr at 37°C. The samples were then washed thrice with 1x PBS, for 2 min each and mounted on slides in PBS/Glycerol (70:30). They were viewed by indirect immunofluorescence microscopy (Magnification 100X of microscope lens).

3.4.3.2 Intracellular redox potential

Purpose: To study intracellular redox potential by the cell permeant, reactive-oxygen sensitive intracellular probe 2',7'- dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Molecular Probes, Eugene, USA).

Theory: The non-fluorescent dye CM-H2DCFDA undergoes oxidation to an intracellular trapped fluorescent compound.

Method: The method is based on the technique described by Lautraite et al. (2003). The old medium is aspirated from 96 well culture plate (Corning, New York, USA). Then the medium containing 100-µl probe or loading mix [50 µg

CMH2DCFDA in 50 µl DMSO + 950 µl DMEM ± 10 ml with DMEM to reach a concentration of 10 µM] was added to all wells except blank wells (all borders of plate). Then 100 µl DMEM was added to all blank wells. The samples were incubated for 60 min under 5 % CO2; 95% air at 37 °C. All loading mix and medium were then aspirated out from wells.

Then 200 µl of different treatments were added to all the cells except blank and dye (Fe ± Vitamin C/ TRIVISOL, etc.). Then 100 µl DMEM was added to all blank and dye only wells. The readings were taken at 0, 1, 2, 3 and 4 hrs intervals on Fluoroskan Ascent fluorometer (Thermolab Systems, Helsinki, Finland) with 485 nm excitation filter and a 527 nm emission filter.

3.4.3.3 Apoptosis

Purpose: To study apoptosis using caspase-3 activation in human intestinal epithelial (Caco-2BBe) cells.

Theory: The principle involves caspase-3 mediated hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroaniline detected calorimetrically.

Method: A kit was purchased from Sigma, St. Louis, USA. The kit was used according to manufacturers' instructions, with modifications. 96 well culture plate (Corning, New York, USA) were used for growth of Caco-2BBe cells and measurements were taken at 405 nm. In short, the procedure included preparation of cell lysates from apoptotic cells and their assay. The negative control was 2 mM caspase-3 inhibitor Ac-DEVD-CHO (Acetyl-Asp-Glu-Val-al). The positive control was 2 µg/ml caspase-3. A sample of non-induced cells was used as a time control. 1

μg/ml staurosporine was used to induce apoptosis in one well. The specific activity is calculated by the formula OD x dilution factor/10.5 x time x volume where OD is the optical density.

3.5 Statistical analysis

The differences between treatment groups were analyzed using SPSS software (Windows version 12.0.2). Analysis of variance (ANOVA) was performed to determine significant differences between individual means on the control (BM with no additives) and treatment samples (BM with different additives). Probability, $p < 0.05$ was considered as statistically significant. The treatment effects were determined using LSD post hoc test.

CHAPTER 4 RESULTS

4.1 Lipid oxidation

4.1.1 Fox-2 assay

Fox assay is commonly conducted in lipids and oils to find the amount of lipid peroxides. Long et al. (1999) studied milk peroxides and found milk had lower peroxides than other beverages (soft drinks like coke). They concluded milk has more antioxidant potential than other liquids studied. There are no studies with Fox-2 assay analyses on milk from mothers who delivered full term or PT infants till date. After addition of different supplements, the milk samples were incubated at room temperature until 4 hours. The stoppered test tubes were kept on the laboratory desk under room light. Fox-2 assay was performed immediately on most of these samples after the incubation period. The remaining samples were kept at – 80°C until analyses the following day.

4.1.1.1 Pilot study: Dose response curve

A pilot study was conducted to identify the dose of iron which produces significant increase in ROS. A dose response curve was generated by increasing iron doses, after 4 hrs incubation in BM (Figure 1). The different concentrations of iron for this part of study were 0.9 mM, 1.2 mM, 1.8 mM and 3.6 mM in BM. The dose of iron 1.8 mM generated 24.6 ± 1.2 μM peroxides (n=5). There was a significant increase seen in treatments 0.9 mM iron concentration and above, compared to BM (no additives) 8.8 ± 0.7 μM peroxides (n=5). This indicated that a dose of 0.9 mM iron (and over 0.9 mM), significantly increases lipid peroxides in BM after 4 hrs.

A dose response curve was also generated by increasing the dose of TRIVISOL in BM, after 4 hrs incubation as seen in figure 2. The concentrations of vitamin C in TRIVISOL for this part of the study were 4.3 mM, 5.7 mM, 8.52 mM and 17 mM. TRIVISOL with 8.52 mM concentration of vitamin C generated 17.9 ± 1.7 μM peroxides (n=5). There was significant increase seen in treatments 4.3 mM vitamin C concentration and above, compared to BM (no additives) 8.8 ± 0.7 μM peroxides (n=5). This indicated that a dose of 4.3 mM vitamin C and above in TRIVISOL increases lipid peroxides significantly in BM. 1.8 mM iron and TRIVISOL (8.5 mM vitamin C content) dose were chosen as the doses to be used for the study as these were the doses for PT infants recommended by AAP and CPS.

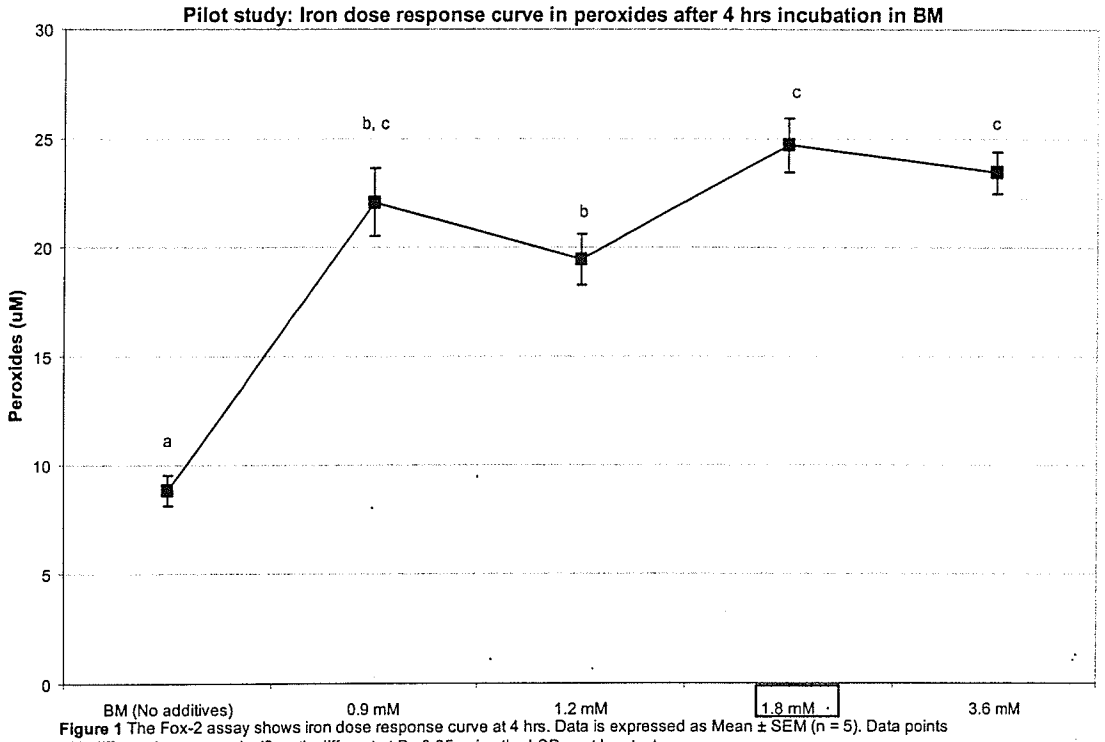


Figure 1 The Fox-2 assay shows iron dose response curve at 4 hrs. Data is expressed as Mean ± SEM (n = 5). Data points with different letters are significantly different at P< 0.05 using the LSD post hoc test.

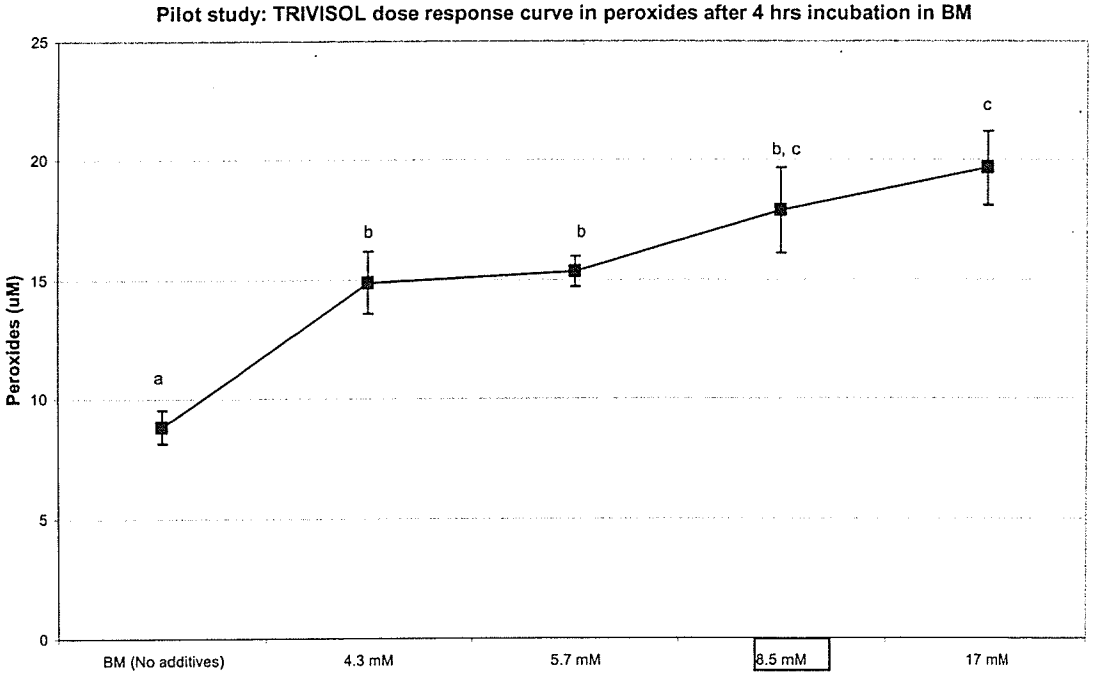


Figure 2 The Fox-2 assay shows TRIVISOL dose response curve at 4 hrs. Data is expressed as Vitamin C concentration in TRIVISOL Mean ± SEM (n =5).

Data points with different letters are significantly different at P< 0.05 using the LSD post hoc test.

The boxed data represents the dose of elemental iron and vitamin C advised by AAP (1985) for PT infants after 4-6 months of age.

4.1.1.2 Addition of supplements at time '0' hrs

The effect of addition of iron, vitamin C, TRIVISOL supplements individually and in combinations was observed at '0' hrs. This was tested by addition of supplements in BM and immediately freezing the samples at – 80°C, until analyses. Figure 3 shows the data obtained. The BM (with no additives) at '0' hrs had 15.8 ± 2.6 μM peroxides (n=3). A statistically significant increase was seen after addition of iron, TRIVISOL (with 8.52 mM vitamin C content) and microlipids together; the peroxide value was 53.3 ± 0.5 μM (n=3) compared BM (no additives) (Figure 4).

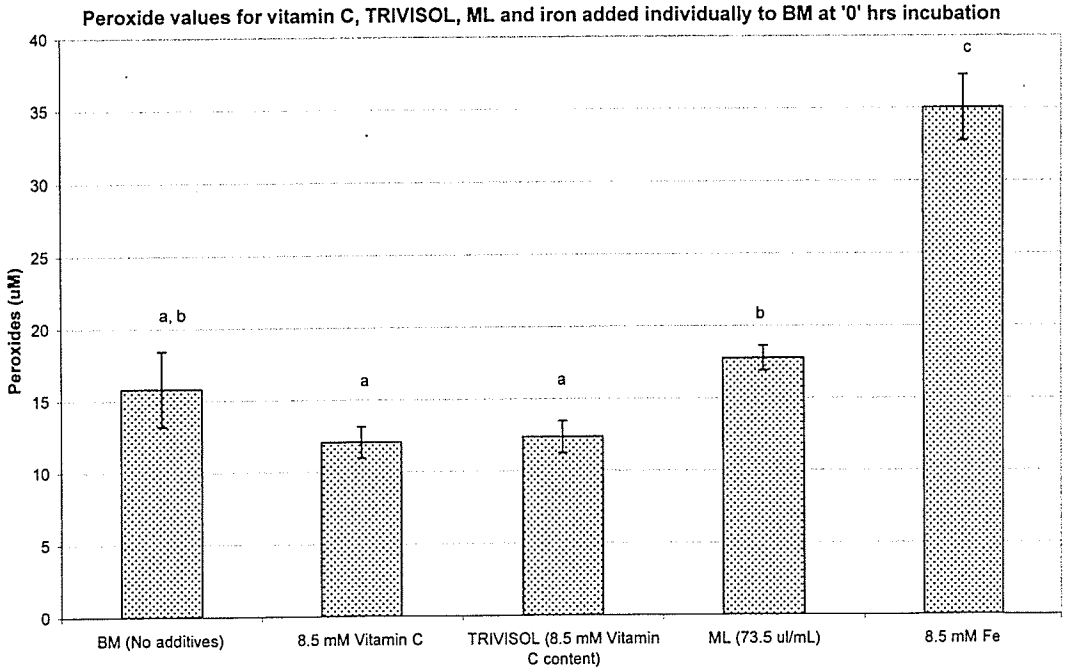


Figure 3 The Fox-2 assay shows the effect of treatment with Vitamin C, TVS, ML and Fe done at '0' hrs incubation. Data is expressed as mean ± SEM (n = 3). Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

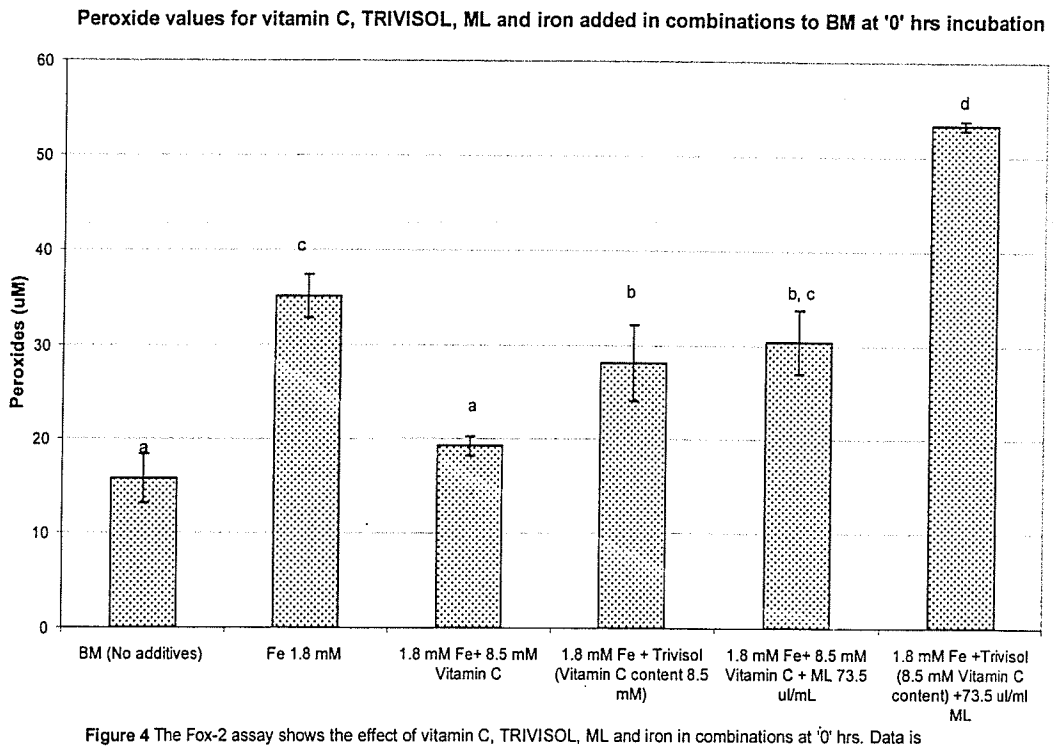


Figure 4 The Fox-2 assay shows the effect of vitamin C, TRIVISOL, ML and iron in combinations at '0' hrs. Data is expressed as mean ± SEM (n = 3)..Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.1.1.3 Effect of supplements after 4 hours incubation with BM

There was a significant increase in lipid peroxides on individual addition of iron and ML to BM (Figure 5). The peroxides mean ± SEM generated in BM alone after 4 hrs incubation under room light were 11.4 ± 1.3 μM; for iron were 31.8 ± 3.6 μM; for iron, vitamin C and ML together in BM were 46.1 ± 3.6 μM (Figure 5). The peroxides were 24.7 ± 1.4 μM on addition of ML and 22.6 ± 3.4 μM after iron and vitamin C were added together in BM as seen in Figure 5.

Iron and TRIVISOL added together in BM led to 19.6 ± 0.3 μM peroxides (n= 5) (Figure 6). BM plus iron, TRIVISOL and ML together led to peroxide value 29.6 ± 0.9 μM. These values were significantly higher than iron in BM which led to 24.6 ± 1.2 μM peroxides (Figure 6).

Peroxide values for vitamin C, ML and iron added individually and in combinations to BM after 4 hrs incubation

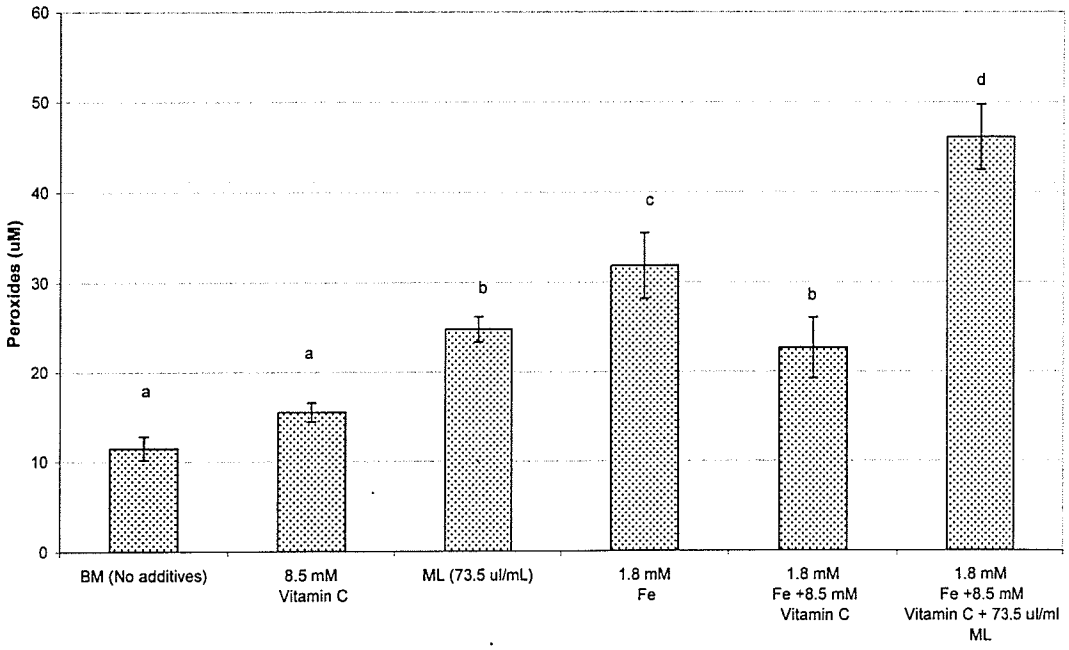


Figure 5 The Fox-2 assay shows the effect of addition of Vit C, ML and iron individually and in combinations to BM at 4 hrs. Data is expressed as Mean ± SEM (n = 3).
Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

Peroxide values for TRIVISOL, ML and iron added individually and in combinations to BM after 4 hrs incubation

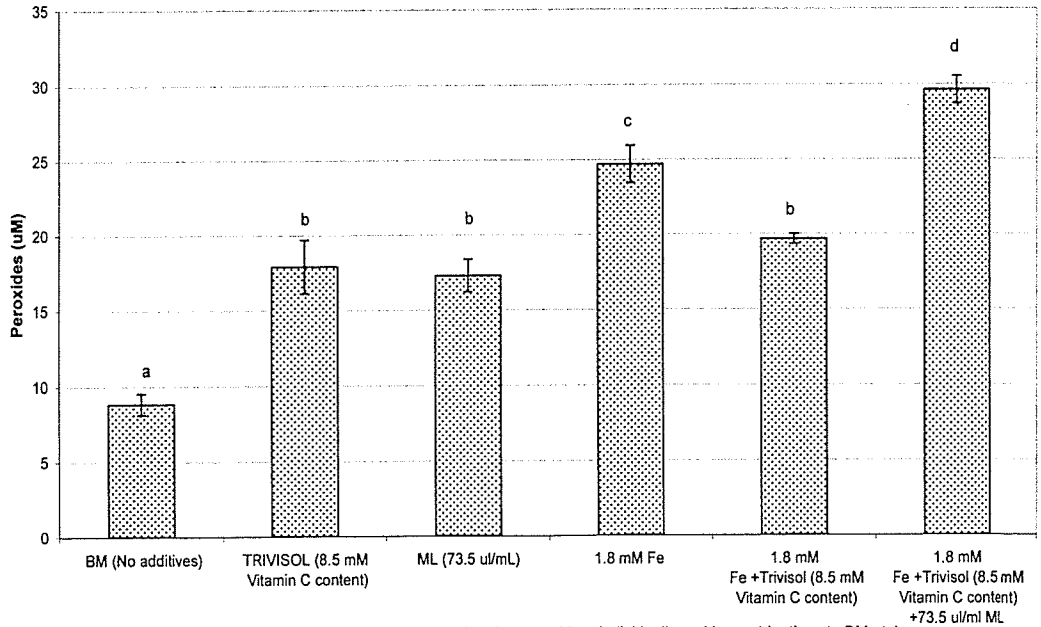


Figure 6 The Fox-2 assay shows the effect of addition of TVS, ML and iron individually and in combinations to BM at 4 hrs.
Data is expressed as mean ± SEM (n = 5). Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.1.1.4 Effect of vitamins A and D

TRIVISOL (Mead Johnson, Evansville, USA) contains vitamin A 1500 IU/mL, vitamin D 400 IU/mL and vitamin C 30 mg/mL. The effects of vitamin C were tested with iron in BM (Figures 3, 4 and 5). Figures 3 and 5 indicate statistically non significant changes on addition of 8.52 mM vitamin C in BM. To observe the effects of the other two vitamins, namely, vitamins A and D, the Fox-2 assay was conducted on BM with iron; and with or without the two vitamins individually. Figure 7 shows a significant decrease 4.1 ± 0.6 μM peroxides after addition of vitamin A (Retinol palmitate) 75 IU/mL in BM compared to the BM alone 7.5 ± 1.0 μM peroxides. A similar decrease was seen with vitamin D (cholecalciferol) 20 IU/mL. Vitamin A and iron in BM also led to a statistically significant decrease 10.3 ± 0.4 μM peroxides compared to iron in BM 13.4 ± 1.0 μM peroxides.

Peroxide values for iron, vitamins A and D added individually and in combinations to BM after 4 hrs incubation

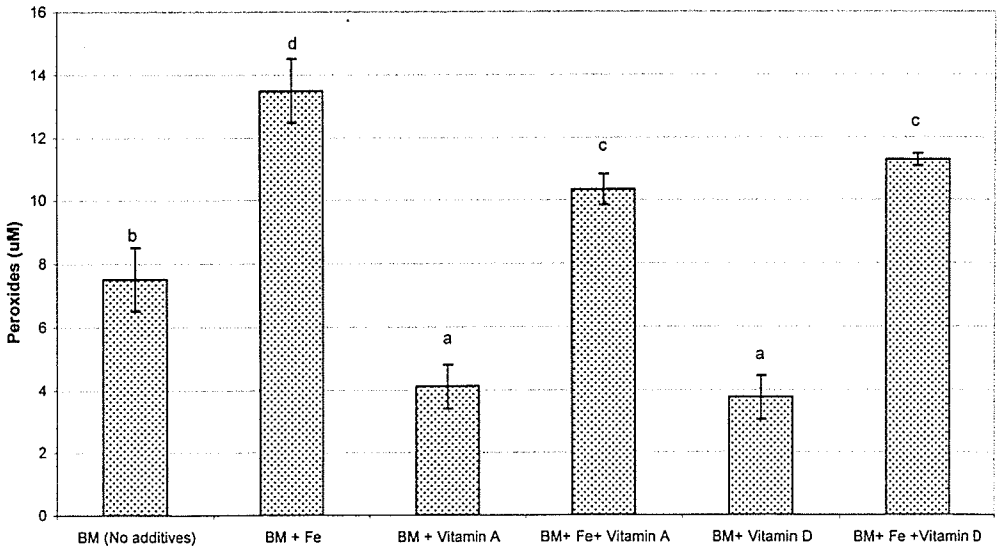


Figure 7 The Fox-2 assay shows the effect of Vitamins A (Retinol palmitate) 75 IU/mL, D (cholecalciferol) 20 IU/mL and 1.8 mM iron after 4 hrs incubation.
Data is expressed as mean ± SEM (n =3). Columns with different letters are significantly different at $P < 0.05$ using the LSD post hoc test.

4.1.1.5 Pilot study with Human Milk Fortifier (HMF)

HMF was added according to the recommended dose by the manufacturers Mead Johnson, Evansville, USA (1 packet per 25-35 mL BM). The dose used in our pilot study was 1 packet per 25 mL BM. This was also the dose recommended by the Health Sciences Centre, Winnipeg, Canada guidelines for PT infants. The doses of iron and TRIVISOL were 0.9 mM iron and TRIVISOL with 4.26 mM vitamin C content. Their concentrations were half the concentrations of the doses of iron and TRIVISOL we used in our focal study.

On addition of HMF in BM the peroxide values were 4.5 ± 0.2 μM; there were no significant changes seen with BM alone 4.9 ± 0.4 μM peroxides. Statistically significant increase was seen on addition of HMF and 0.9 mM iron 14.8 ± 0.4 μM peroxides compared to 0.9 mM iron alone in BM 12.7 ± 0.7 μM peroxides.

No significant changes were seen after addition of 0.9 mM iron, TRIVISOL (with 4.26 mM vitamin C content) and HMF together in BM after 4 hrs incubation under room light 22.4 ± 1.2 μM peroxides with 0.9 mM iron, TRIVISOL (with 4.26 mM vitamin C content) together 22.5 ± 1.1 μM peroxides (figure 8). There was statistically significant increase seen with 0.9 mM iron and HMF together 14.8 ± 0.4 μM peroxides compared with 0.9 mM iron in BM 12.7 ± 0.7 μM peroxides.

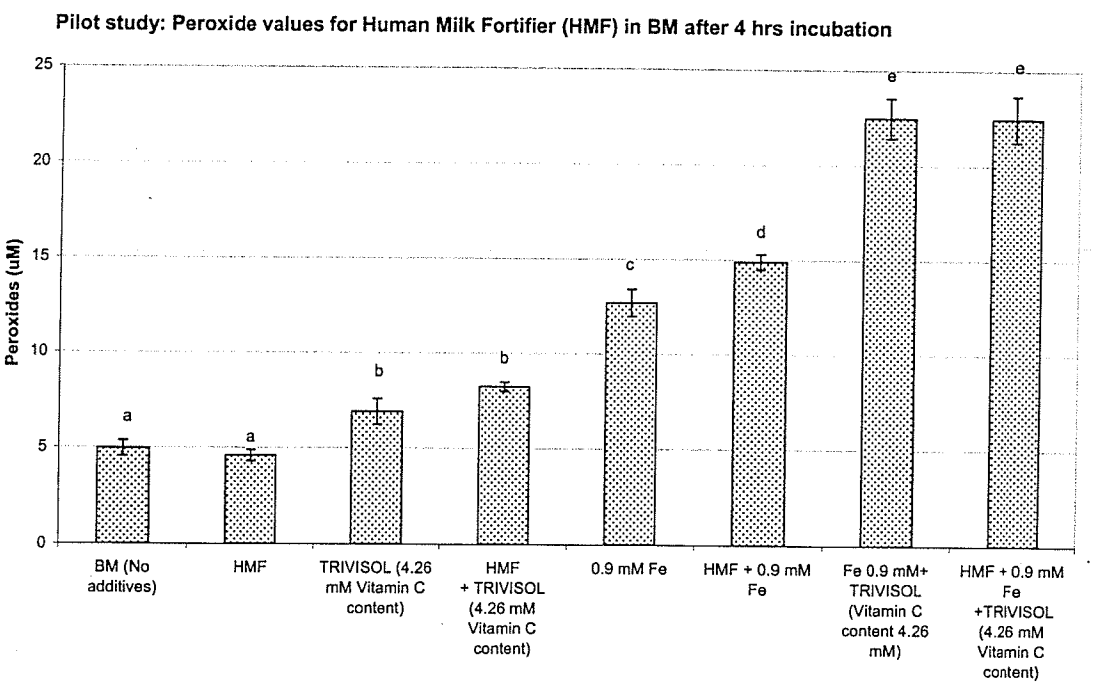


Figure 8 The Fox-2 assay shows the effect of addition of HMF (1 packet in 25 mL BM), TVS and iron to BM at 4 hrs. Data is expressed as Mean ± SEM (n = 10). Columns with different letters are significantly different at $P < 0.05$ using the LSD post hoc test.

4.1.1.6 Pilot study with pasteurization of milk

BM was pasteurized in a hot water bath for 5 min at 85°C to denature the antioxidant enzymes SOD, GPx, and CAT and determine their role in antioxidant protection. Exogenous 100 U/mL SOD was added in pasteurized and nonpasteurized BM with 0.9 mM iron is seen in Figure 9. Statistically significant decrease 0.1 ± 0.04 μM peroxides (n = 3) was seen after addition of exogenous 100 U/mL SOD and 0.9 mM iron from the nonpasteurised BM (with no additives) 16.3 ± 1.9 μM (n =3). A similar decrease was seen in pasteurized BM. However the pasteurized and nonpasteurized BM did not show any significant changes on addition of SOD and iron as seen in figure 9.

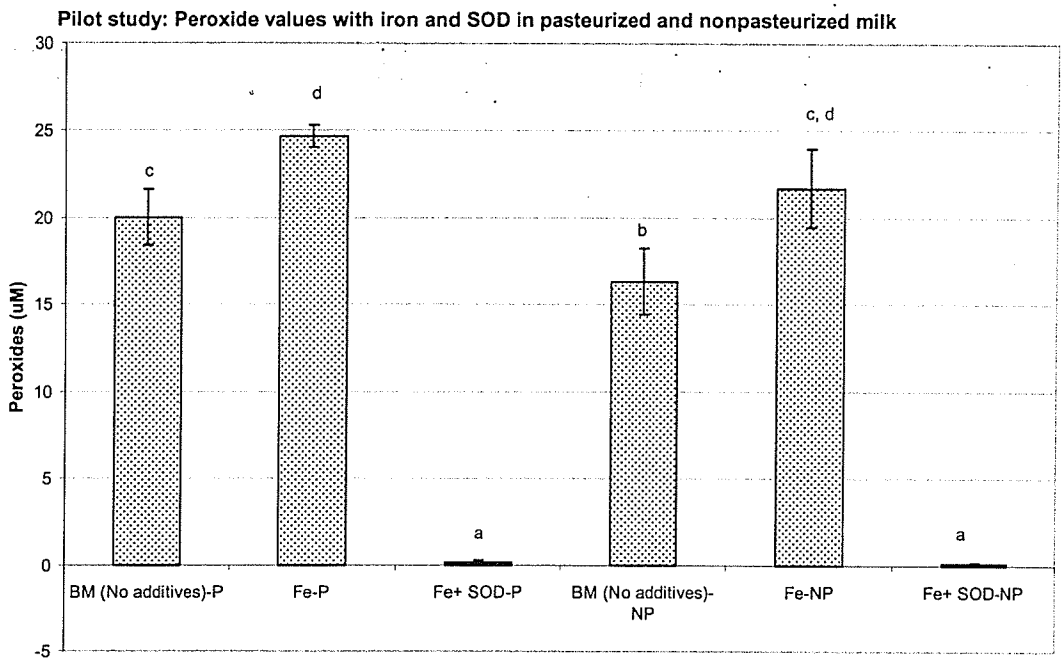


Figure 9 The Fox-2 assay shows the effect of 100 U/mL SOD and 0.9 mM Fe in Pasteurised and Nonpasteurised BM at 4 hrs. P- Pasteurised BM; NP- Nonpasteurised BM
Data is expressed as mean ± SEM (n =3). Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.1.1.7 Effect of SOD and CAT at '0' and '1' hours incubation

The study lacked data to show the effect of supplements after 1 hour's incubation so far. To find the effect of enzymes at '0' (immediately after addition of enzymes) and 1 hour incubation periods Fox-2 assay was performed at these incubation periods. Figures 11 and 12 show the effect of antioxidant enzyme 100 U/mL SOD and 100 U/mL CAT on addition to iron in BM at '0' hours and '1' hours respectively. A statistically significant decrease was seen after addition of 100 U/mL SOD to 1.8 mM iron in BM at both periods.

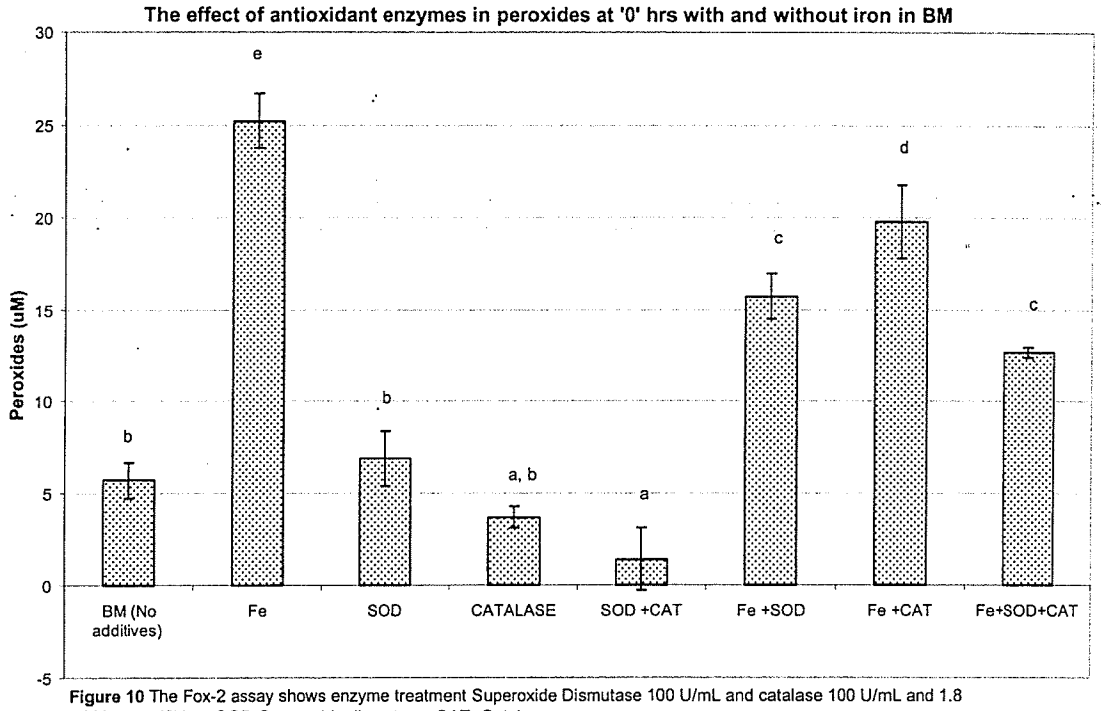


Figure 10 The Fox-2 assay shows enzyme treatment Superoxide Dismutase 100 U/mL and catalase 100 U/mL and 1.8 mM iron at '0' hrs. SOD-Superoxide dismutase; CAT- Catalase
Data is expressed as mean ± SEM (n = 3). Columns with different letters are significantly different at $P < 0.05$ using the LSD post hoc test.

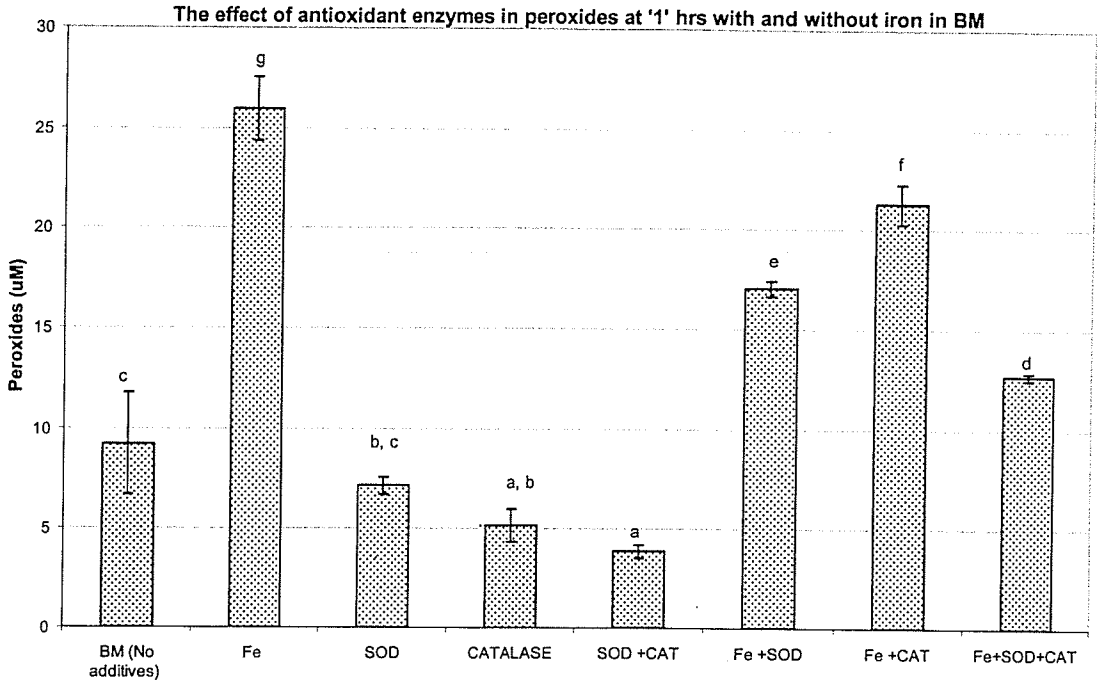


Figure 11 The Fox-2 assay shows enzyme treatment SOD 100 U/mL and CAT 100 U/mL and 1.8 mM iron at '1' hrs.

Data is expressed as mean ± SEM (n = 3). Columns with different letters are significantly different at $P < 0.05$ using the LSD post hoc test.

4.1.1.8 Effect of SOD after 4 hour incubation

The effect of 100 U/ mL SOD with or without 1.8 mM iron after 4 hrs incubation was performed. This was to examine the effect of SOD in BM and the amount of peroxides generated after 4 hours. There was a significant decrease in peroxides with iron and 100 U/ mL SOD together in BM 0.1 ± 0.04 μM compared to iron in BM which led to 31.8 ± 3.6 μM peroxides after 4 hrs incubation under room light (n=3) (Figure 12). Therefore SOD enzyme showed antioxidant activity even in the presence of iron after 4 hours incubation.

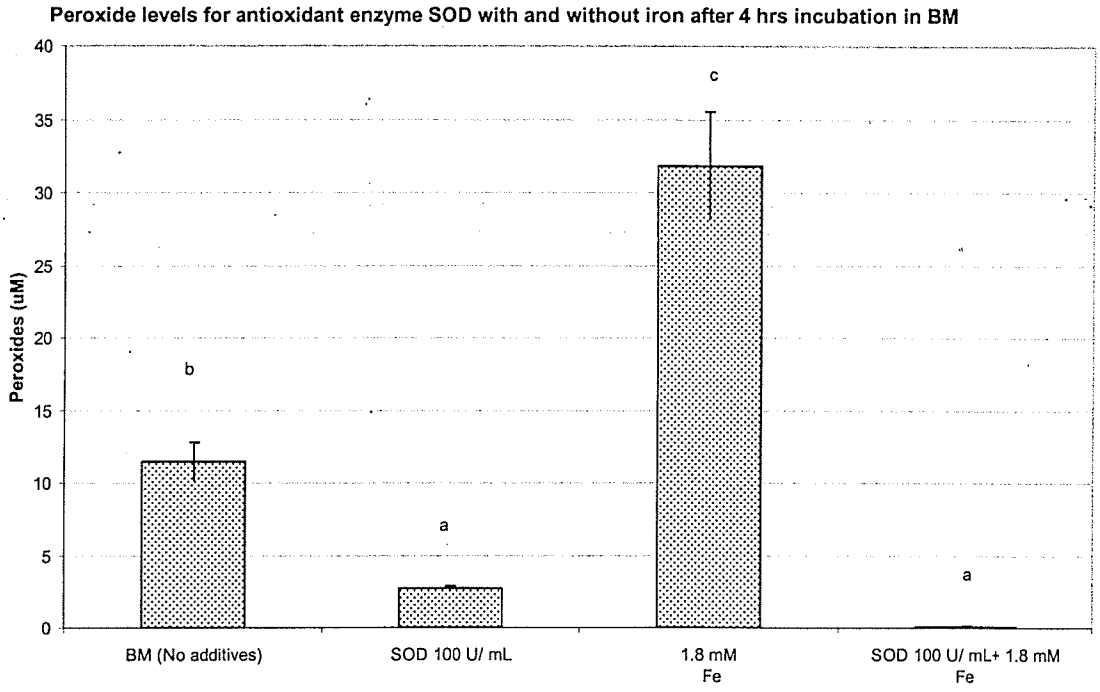


Figure 12 The Fox-2 assay shows the effect of iron and SOD at 4 hrs. Data is expressed as mean ± SEM (n = 3). Columns with different letters are significantly different at $P < 0.05$ using the LSD post hoc test.

4.1.1.9 Effect of Time

The BM samples with additives were incubated for 1, 2 and 3 hours under room light. No significant differences were seen in the treatments with 1.8 mM iron and 8.52 mM vitamin C individually and in combinations for 1, 2 and 3 hours (Figure 13). Statistically significant differences were seen with 1.8 mM iron in BM compared to BM alone at all periods.

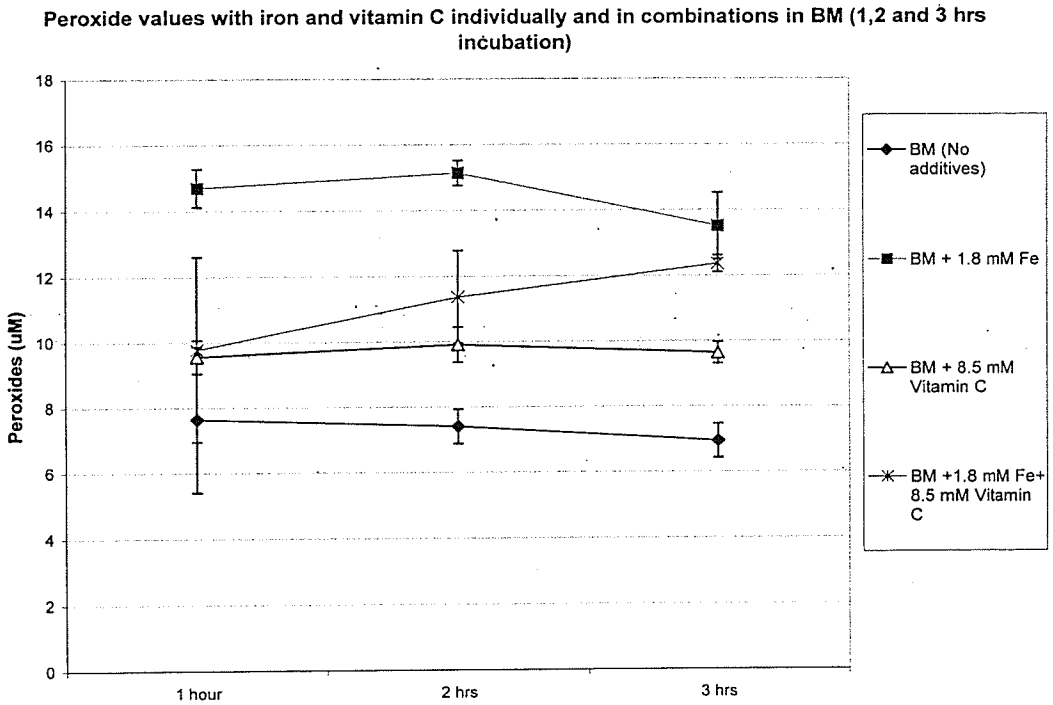


Figure 13 The Fox-2 assay shows 1.8 mM Fe, 8.5 mM Vitamin C treated BM with incubation at 1, 2 and 3 hrs. Data is expressed as mean ± SEM (n = 3).

4.1.1.10 Effect of incubation at 4°C and time

The samples were incubated up to 12 hours at 4°C to test a possible protective effect by keeping the samples at lower temperature than room temperature. No statistically significant differences were seen in the treatments for lipid peroxide values (Figure 14) for time 1, 4, 8 and 12 hours. 1.8 mM iron in BM at all the periods led to significantly higher peroxides than the BM alone. Vitamin C and iron in BM led to significantly lower peroxides at 8 and 12 hours than iron alone in BM.

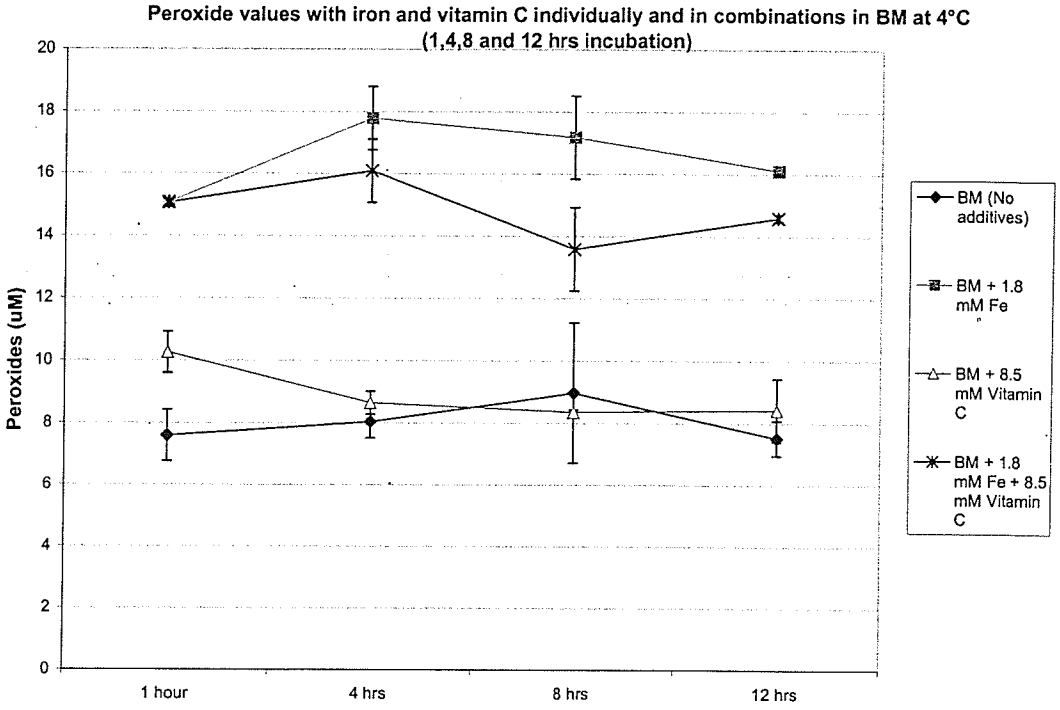


Figure 14 The Fox-2 assay shows 1.8 mM Fe, 8.5 mM Vitamin C treated BM effects at 1, 4, 8 and 12 hrs and incubation at 4 degrees C. Data is expressed as mean ± SEM (n =3).

4.1.1.11 Effect of incubation at 30°C for 2 hrs

The samples were incubated at 30°C for 2 hrs to observe any change in oxidation at that temperature. No statistically significant differences were seen in the treatments at room temperature and 30°C (Figure 15).

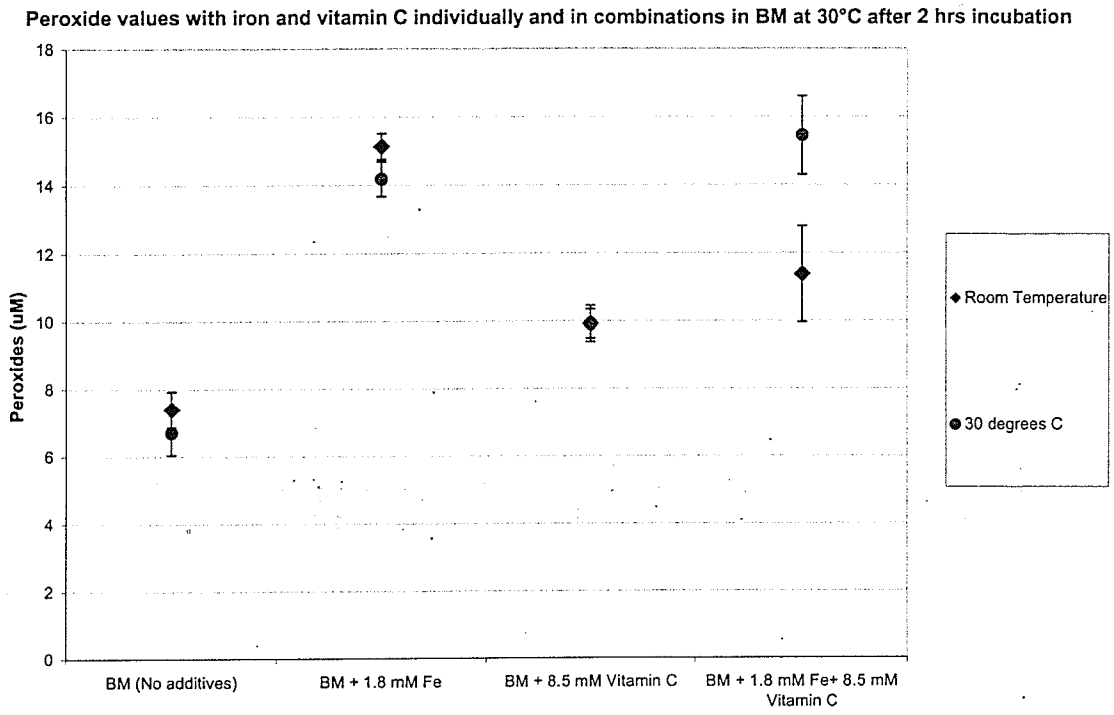


Figure 15 The Fox-2 assay shows the effect of room temperature and 30 deg C on treated BM at 2 hrs. Data is expressed as mean (n = 3).

4.1.2 TBARS

After addition of iron, vitamin C, TRIVISOL and ML supplements, the milk samples were incubated at room temperature until 4 hours. The stoppered test tubes were kept on the laboratory bench under room light. All the samples were kept at – 80°C until analyses.

4.1.2.1 Pilot study: Dose response curve

Iron dose response curve was generated by increasing the dose of iron in BM (Figure 16). The boxed data represents the dose of elemental iron and vitamin C advised by AAP (1985) for PT infants after 4-6 months of age. There were statistically significant increases with 1.2 mM iron concentration and above as seen in Figure 16. BM alone led to 0.3 ± 0.08 TBARS and 1.2 mM iron led to 0.9 ± 0.04 TBARS.

TRIVISOL dose response curve was also generated by increasing the amounts of TRIVISOL in BM as seen in Figure 17. A statistically significant increase was seen at 8.52 mM and 19.8 mM concentrations of vitamin C in TRIVISOL after 4 hrs incubation under room light. BM alone led to 0.3 ± 0.08 TBARS; TRIVISOL (with 8.52 mM vitamin C content) led to 1.25 ± 0.3 TBARS; TRIVISOL (with 17 mM vitamin C content) led to 1.2 ± 0.2 TBARS. A sigmoid curve in TBARS was seen with increasing TRIVISOL concentration added to BM.

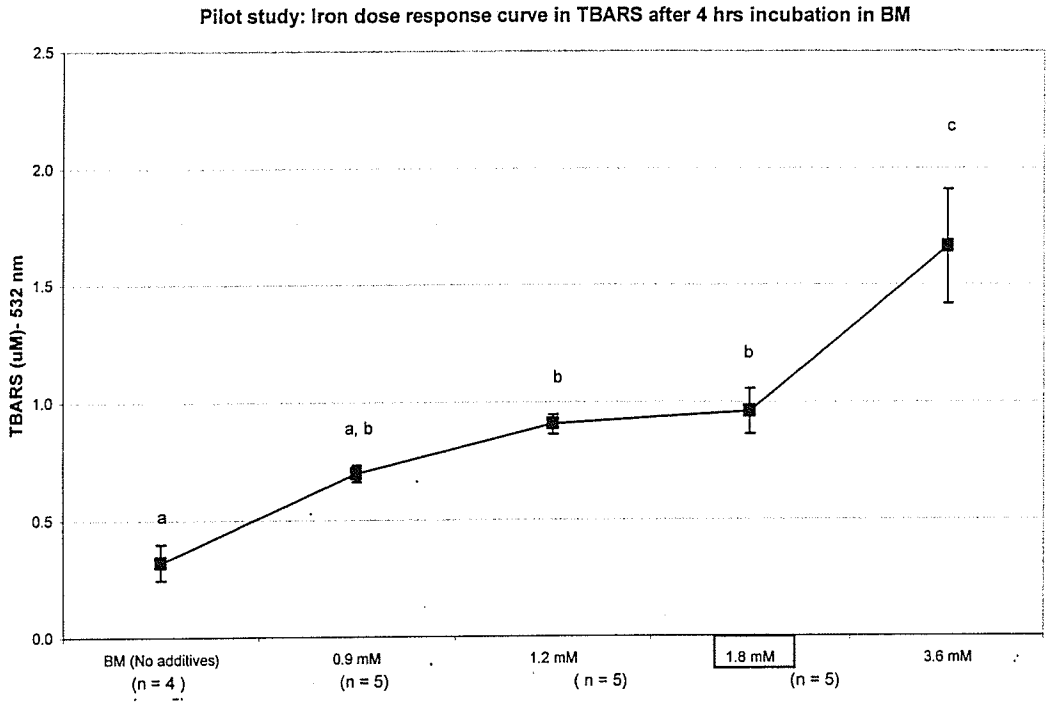


Figure 16 The TBARS assay shows iron dose response curve at 4 hrs. Data is expressed as Mean ± SEM (n ≥ 3). Data points with different letters are significantly different at P< 0.05 using the LSD post hoc test.

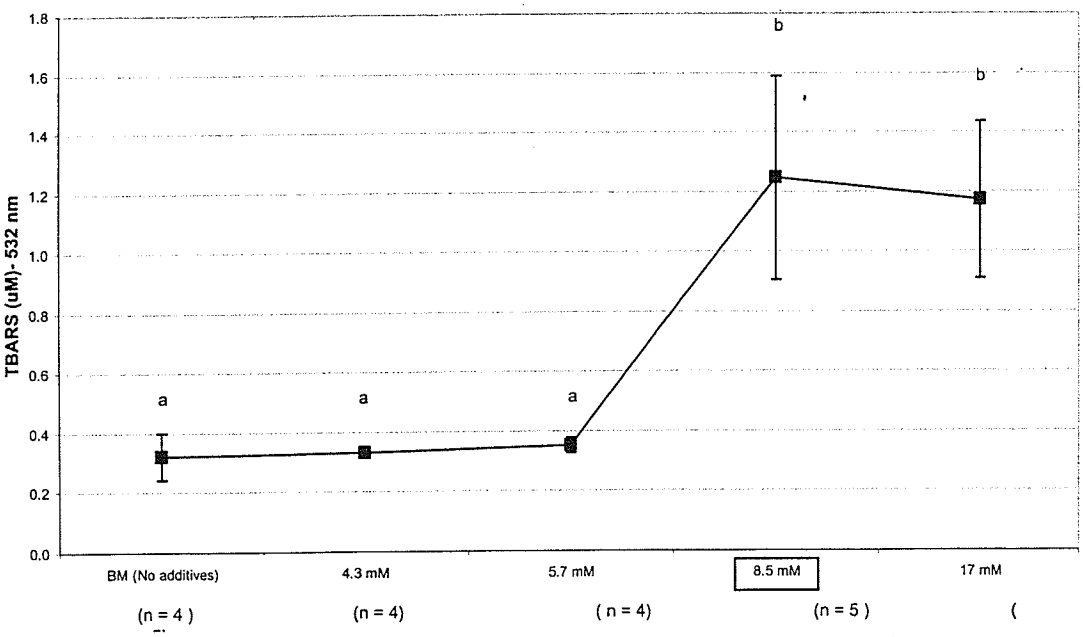


Figure 17 The TBARS assay shows TRIVISOL dose response curve at 4 hrs. Data is expressed as Vitamin C concentration in TRIVISOL Mean ± SEM (n ≥ 3).

Data points with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.1.2.2 Addition of supplements at time '0' hrs

The effect of vitamin C, TRIVISOL, ML and Fe supplements was observed at '0' hrs. This was tested by addition of supplements in BM and immediately freezing the samples at – 80°C, until analyses. The BM alone at '0' hrs did not show statistically significant changes with the addition of vitamin C, TRIVISOL, ML and iron individually to BM (Figure 18).

Statistically significant increases were seen on addition of iron and TRIVISOL together in BM 2.3 ± 0.9 μM TBARS; iron, TRIVISOL and ML together in BM 3.5 ± 0.5 μM TBARS compared to BM alone 0.8 ± 0.4 μM TBARS (Figure 19). Vitamin C with iron in BM did not lead to statistically significant changes compared to BM alone. Therefore TRIVISOL exhibited pro-oxidant activity but not vitamin C in BM.

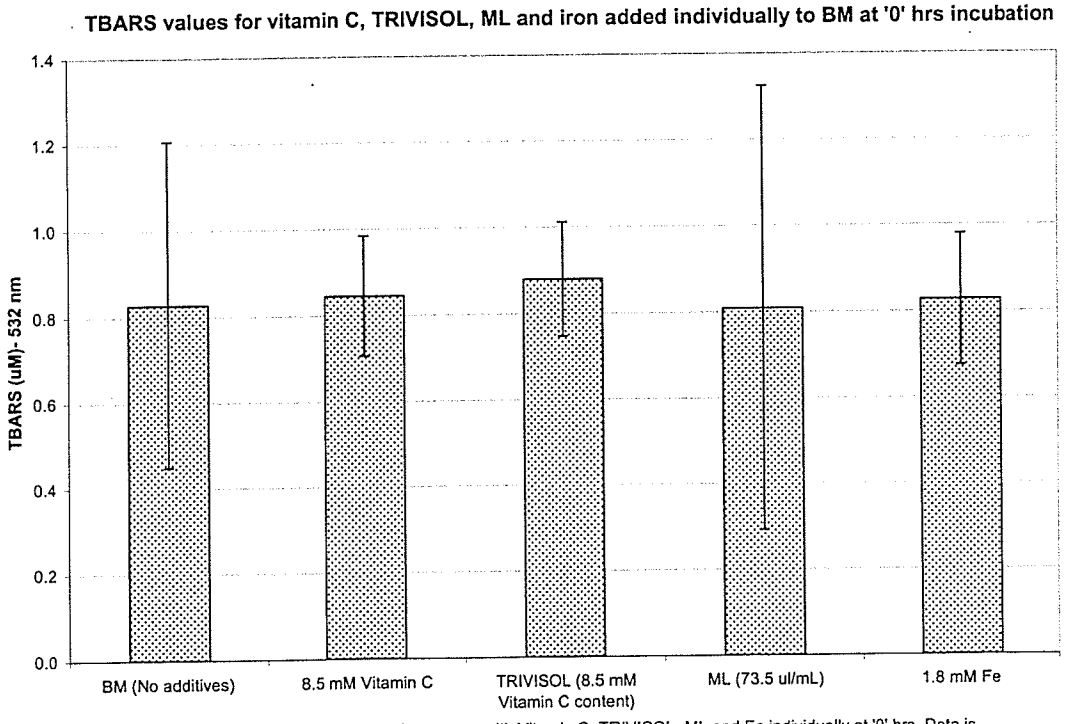


Figure 18 The TBARS assay shows the effect of treatment with Vitamin C, TRIVISOL, ML and Fe individually at '0' hrs. Data is expressed as mean ± SEM (n = 3).

Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

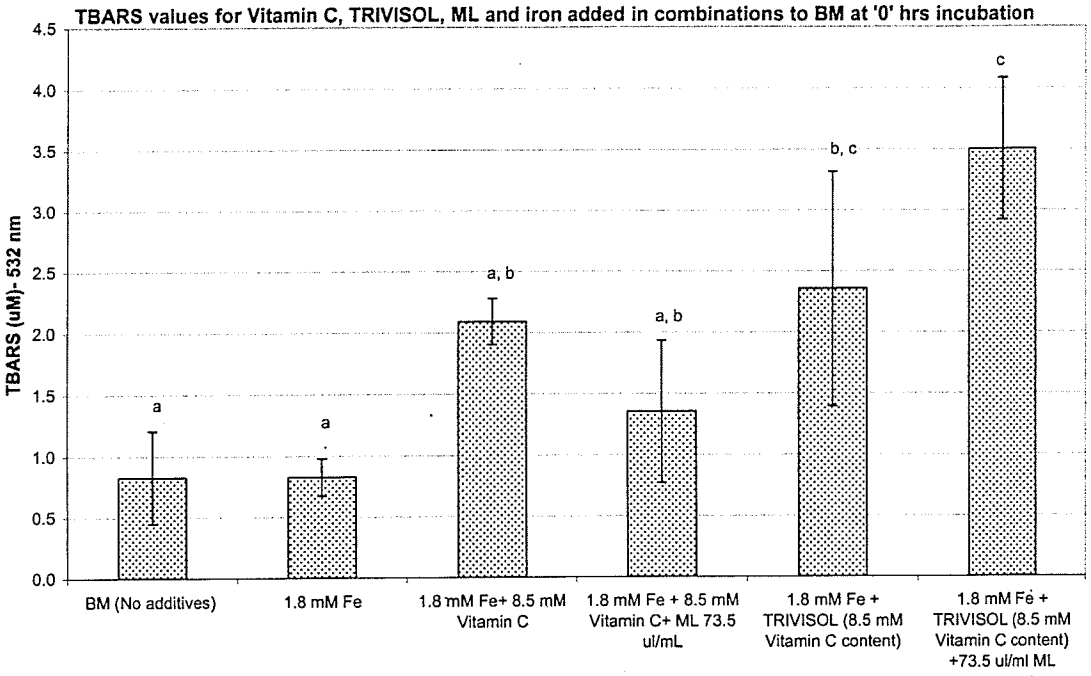


Figure 19 The TBARS assay showing the effect of addition of vitamin C, TVS, ML and iron in combinations to BM at '0' hrs. Data is expressed as mean ± SEM (n = 3).

Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.1.2.3 The effect of supplements after 4 hours incubation

The mean ± SEM TBARS reported for BM alone was 0.38 ± 0.1 μM; for iron in BM was 0.9 ± 0.2 μM; vitamin C in BM was 0.4 ± 0.2 μM as seen in figure 20. BM plus iron and vitamin C led to 4.0 ± 0.8 μM TBARS (Figure 20). This was statistically significant compared to BM alone. Vitamin C exhibited a pro-oxidant activity in this case. The amount of TBARS measured (μM) were lower than peroxides measured by Fox-2 assay. Addition of iron, vitamin C and ML together in BM led to 1.73 ± 0.7 μM TBARS. This was statistically significant compared to BM alone. The TBARS value for TRIVISOL in BM was 1.2 ± 0.3 μM. Statistically significant increase in TBARS 1.4 ± 0.3 μM were seen on addition of ML in BM compared to BM alone 0.3 ± 0.06 μM (Figure 21). BM plus iron and TRIVISOL led

to 2.01 ± 0.10 μM TBARS; iron, TRIVISOL and ML together in BM led to 0.9 ± 0.07 TBARS (Figure 21).

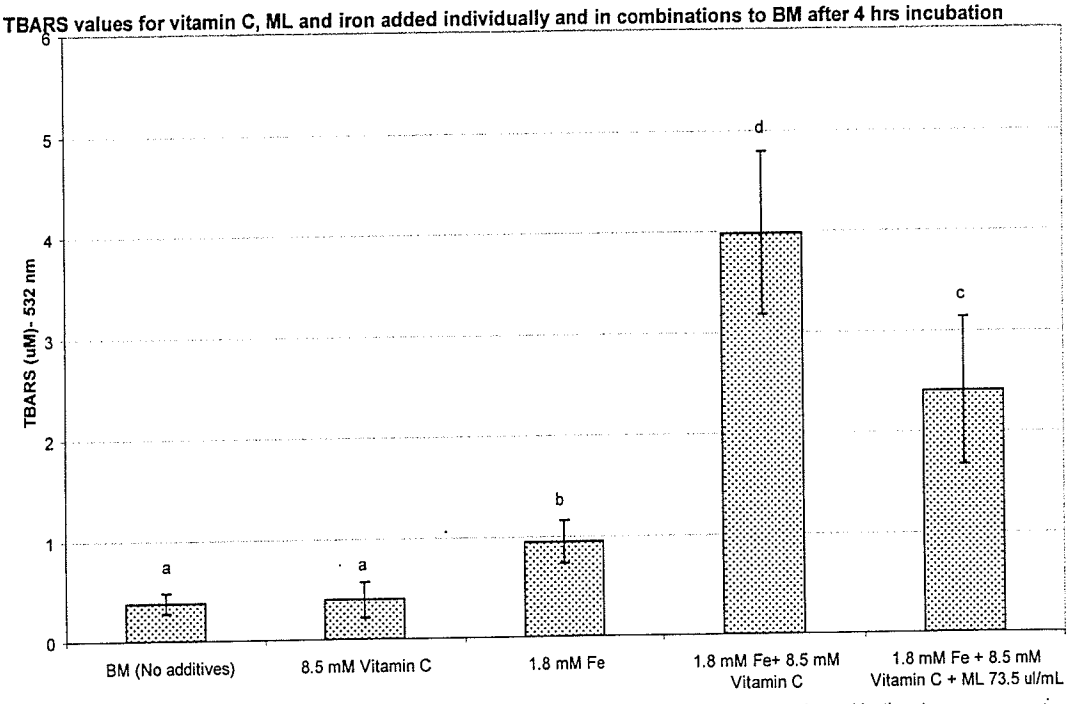


Figure 20 The TBARS assay showing the effect of addition of vitamin C and iron individually and in combinations to BM at 4 hrs. Data is expressed as mean ± SEM (n = 3).

Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

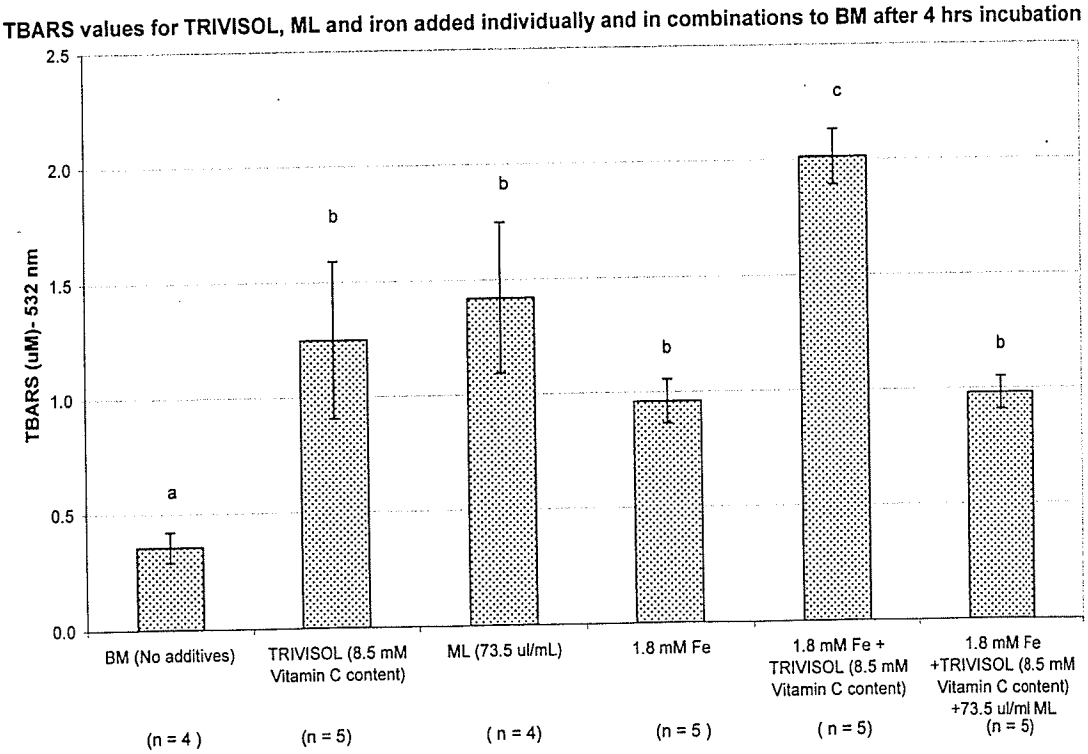


Figure 21 The TBARS assay showing the effect of addition of TVS, ML and iron individually and in combinations to BM at 4 hrs. Data is expressed as mean ± SEM (n = 3).

Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.1.2.4 Effect of vitamins A and D

Figure 22 shows the effect on TBARS after addition of vitamin A (Retinol palmitate) 75 IU/mL and vitamin D (cholecalciferol) 20 IU/mL. Statistically significant decreases were seen after addition of vitamins A and D to BM 0.19 ± 0.002 μM and 0.13 ± 0.002 μM respectively from BM alone 0.36 ± 0.007 μM (Figure 22).

TBARS values for iron, vitamins A and D added individually and in combinations to BM after 4 hrs incubation

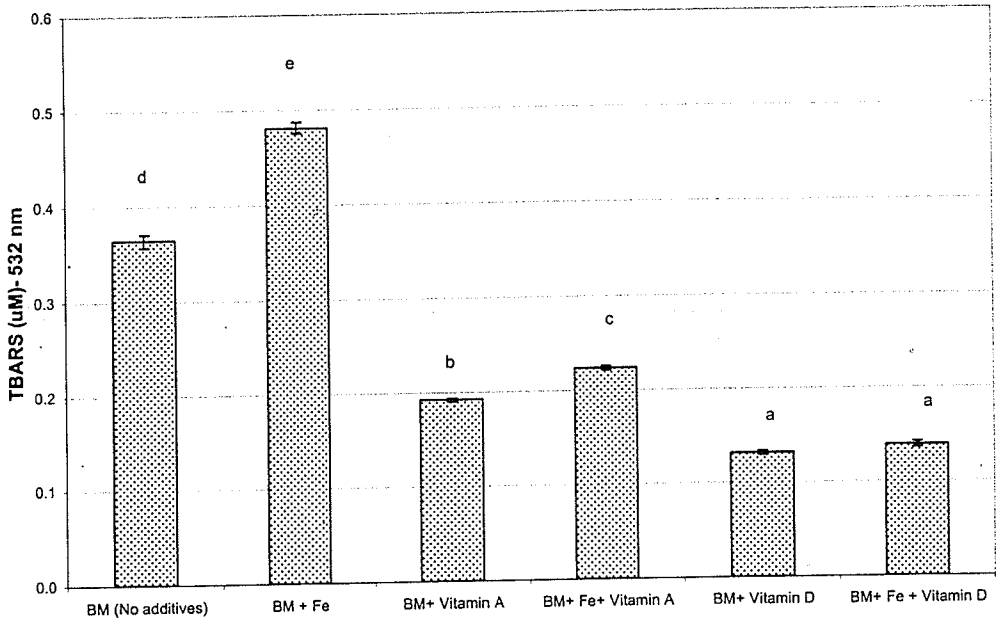


Figure 22 The TBARS assay shows the effect of Vitamins A (Retinol palmitate) 75 IU/mL, D (cholecalciferol) 20 IU/mL and 1.8 mM iron at 4 hrs incubation.

Data is expressed as mean ± SEM (n = 3). Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.1.2.5 Effect of SOD at time '0'

There were no significant changes in TBARS upon addition of 1.8 mM iron and 100 U/ mL SOD to BM as seen in figure 23.

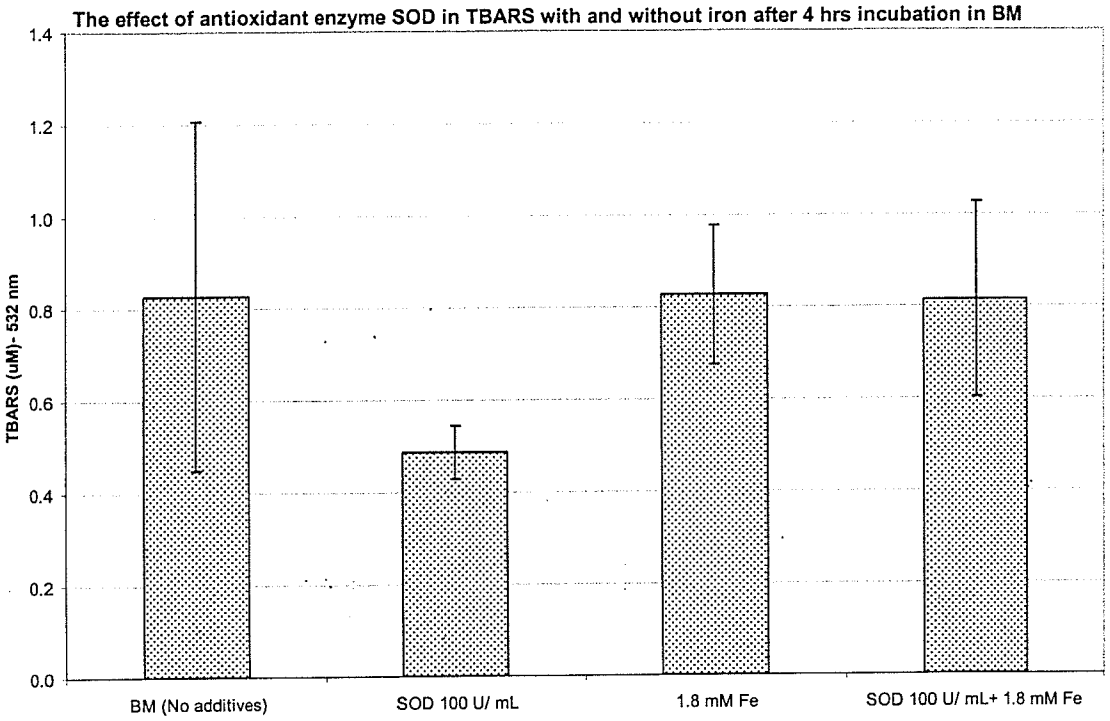


Figure 23 The TBARS assay shows the effect of iron and SOD at '0' hrs. Data is expressed as mean ± SEM (n = 3). Columns with different letters are significantly different at $P < 0.05$ using the LSD post hoc test.

4.1.3 Conjugated dienes

4.1.3.1 Dose response curve

There were no statistically significant changes seen in conjugated diene % after increasing the dose of iron as seen in figure 24. The reason may be that most of the fatty acids had undergone oxidation and were in peroxide or further oxidation stages. The diene stage of the oxidation had passed for the oxidized fatty acids.

There was significant decrease seen after increasing the TRIVISOL concentration (above 8.52 mM vitamin C content in TRIVISOL) as seen in Figure 25. The amounts of diene % for BM alone were 1.5 ± 0.04 and for TRIVISOL were 1.3 ± 0.07.

4.1.3.2 Effect of supplements at 4 hrs incubation

Conjugated dienes % value for BM alone after 4 hrs incubation under room light was 1.5 ± 0.04, for iron was 1.4 ± 0.04, TRIVISOL was 1.3 ± 0.07 as seen in figure 26. Conjugated dienes % with TRIVISOL was statistically significant compared to BM alone. On addition of iron and TRIVISOL together in BM the diene % value was 1.3 ± 0.03 (n=5), and after addition of iron, TRIVISOL and ML together in BM it was 1.38 ± 0.02. These results were statistically significant compared to BM alone.

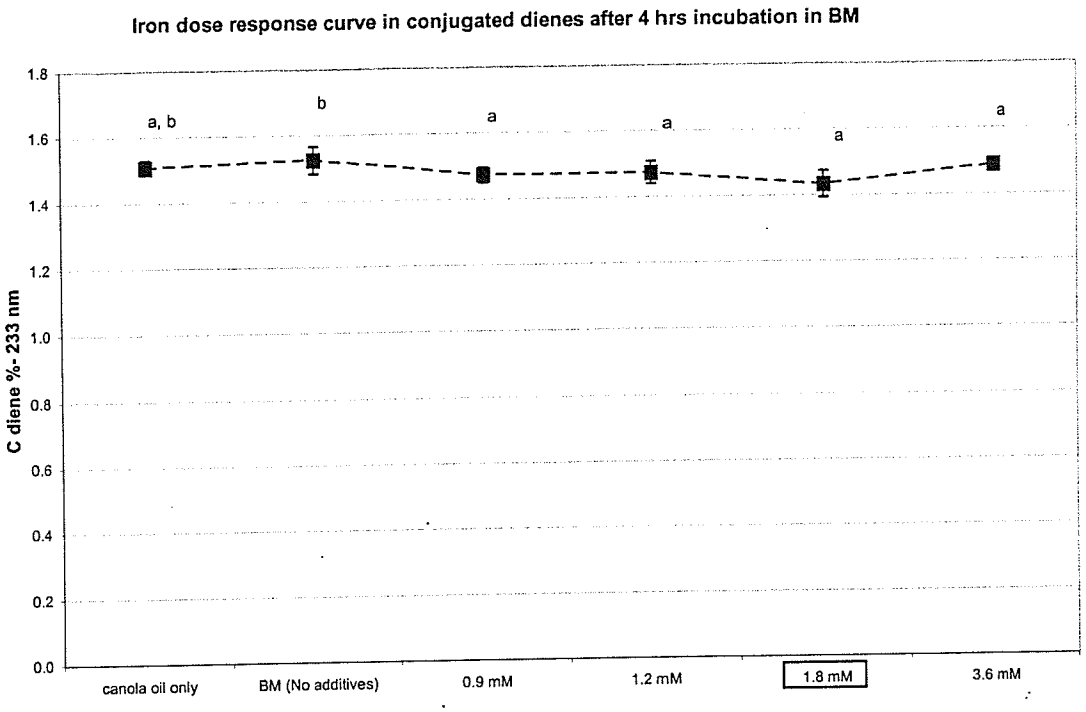


Figure 24 The Conjugated dienes assay shows iron dose response curve at 4 hrs. Data is expressed as Mean ± SEM (n = 5). Data points with different letters are significantly different at P< 0.05 using the LSD post hoc test.

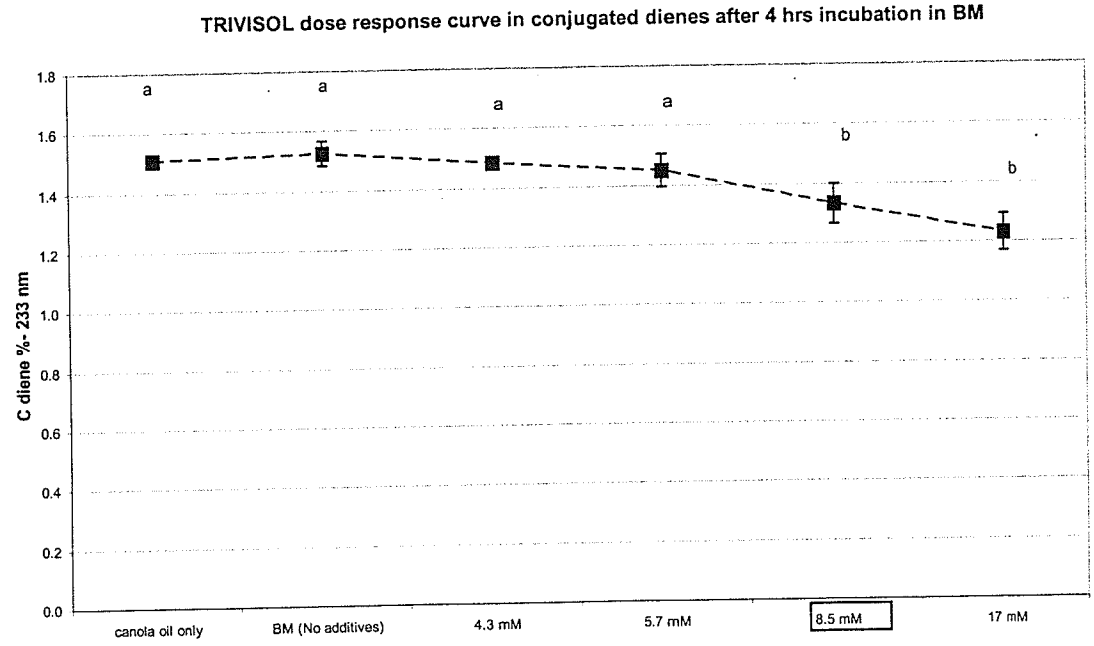


Figure 25 The conjugated dienes assay shows TRIVISOL dose response curve at 4 hrs. Data is expressed as Vitamin C concentration in TRIVISOL Mean ± SEM (n = 5).

Data points with different letters are significantly different from control BM at P< 0.05 using the LSD post hoc test.

The boxed data in the previous two figures 24 and 25 represents the dose of elemental iron and vitamin C advised by AAP (1985) for PT infants after 4-6 months of age.

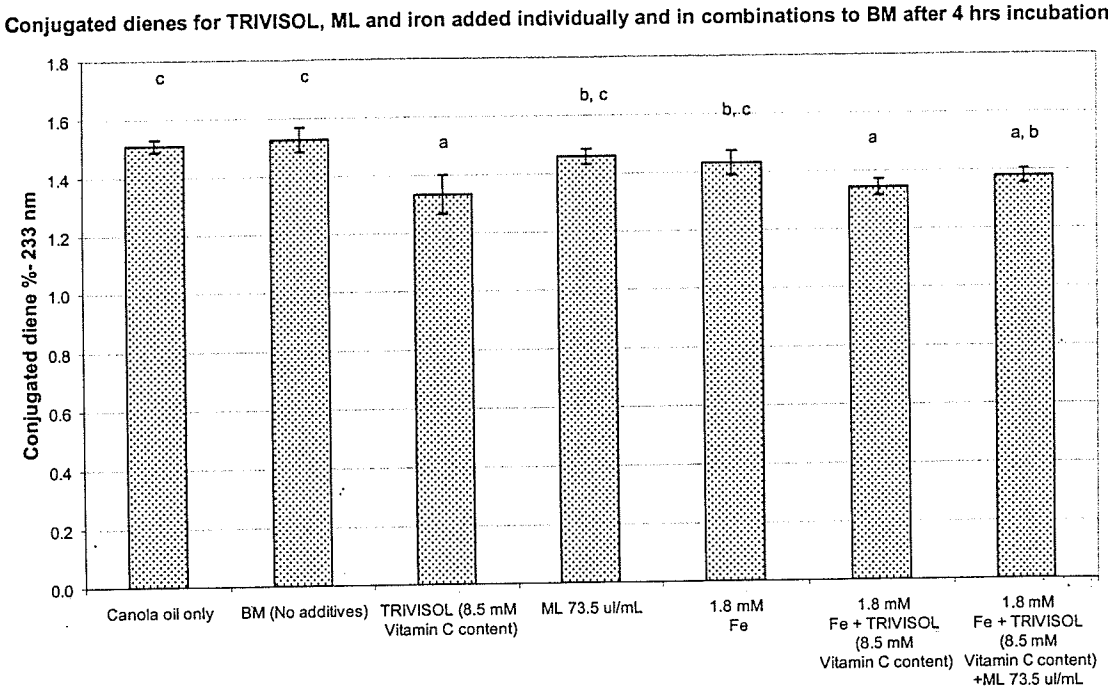


Figure 26 The conjugated dienes assay shows the effect of addition of TRIVISOL, ML and iron to BM at 4 hrs. Data is expressed as mean ± SEM (n = 5). Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.2 Fatty acid analysis

Fatty acid analysis was done by GC on milk samples by increment in dose of iron. There was not enough milk available for other analyses on supplements in BM, therefore only fatty acid analyses on iron supplementation was conducted. Table 5 (next page) shows the data obtained from GC for different fatty acids. Essential fatty acid (EFA) index is calculated based on the total of relative percentage of fatty acids in the formula:

[18:2 (n-6 cis and trans) x 2] + [18:3 (n-3) x 3] + [20:4 (n-6) x 4]

MUFA index is calculated based on the total of relative percentage of fatty acids in the formula:

(14:1n-9) + (16:1n-7) + [18:1(n-9, n-7 and trans n-9, n-7)] + (20:1n-9) + (22:1n-9)

PUFA index is calculated based on the total of relative percentage of fatty acids in the formula:

[18:2 (n-6 cis and trans) x 2] + [20:2(n-6) x 2] + [18:3 (n-6) x 3] + [18:3 (n-3) x 3] + [20:3 (n-6) x 3] + [20:4 (n-6) x 4] + [22:4 (n-6) x 4] + [22:5 (n-3) x 5] + [22:6 (n-3) x 6]

Iron supplementation in BM led to decrease in EFA, MUFA and PUFA indices compared to the BM control. There was a statistically significant decrease in Essential fatty acid and PUFA indices compared to the BM control on addition of iron to BM. This indicated ROS were produced after addition of iron to BM and ROS decreased the number of unsaturated bonds in the fatty acids in milk.

Table 5 Fatty acid composition of BM samples with iron supplementation at 4 hrs incubation at room temperature under room light *

	Control BM (No additives)	Fe 0.9 mM	Fe 1.2 mM	Fe 1.8 mM	Fe 3.6 mM
Monounsaturated fatty acids					
14:1*n-9*	0.13± 0.003[a]	0.14 ±0.002[a]	0.15±0.003[b]	0.14 ±0.005[a]	0.14± 0.004[a]
16:1*n-7*	2.3 ± 0.03[a]	1.8 ±0.01[b]	1.9 ±0.04[b]	1.8 ±0.02[b]	1.9 ±0.03[b]
18:1**	39.9 ± 0.2[a]	36.8±0.2[b]	36.7 ±0.4[b]	36.8 ±0.4[b]	36.9 ±0.4[b]
20:1*n-9*	0.7 ± 0.01[a]	0.7 ±0.01[a]	0.6 ±0.01[b]	0.7 ±0.02[a]	0.6 ±0.01[b]
22:1*n-9*	0.1 ± 0.01	0.1 ±0.003	0.13 ±0.003	0.14 ±0.004	0.13 ±0.002
Polyunsaturated fatty acids					
20:2*n-6*	0.4 ±0.01	0.4 ±0.01	0.4 ±0.01	0.4 ±0.01	0.4 ±0.003
18:3*n-6*	0.09±0.004[a]	0.06±0.009[b]	0.06±0.003[b]	0.06 ±0.01[b]	0.07±0.004[b]
18:3*n-3*	1.05 ±0.02[a]	0.9 ±0.01[b]	0.9 ±0.02[b]	0.9 ±0.02[b]	0.9 ±0.01[b]
18:2***	11.3 ±0.1[a]	9.4±0.1[b]	9.6±0.1[b]	9.4±0.1[b]	9.5±0.1[b]
20:3*n-6*	0.06 ± 0.003	0.06 ± 0.003	0.06 ± 0.002	0.06 ± 0.001	0.07 ± 0.005
20:4*n-6*	0.33±0.005[a]	0.25±0.004[b]	0.25±0.006[b]	0.25±0.008[b]	0.25±0.007[b]
22:4*n-6*	0.12±0.003[a]	0.09±0.002[b]	0.08±0.003[b]	0.09±0.001[b]	0.09±0.003[b]
22:5*n-3*	0.12±0.002[a]	0.08±0.002[b]	0.08±0.004[b]	0.08±0.003[b]	0.09±0.005[b]
22:6*n-3*	0.21 ±0.03	0.2 ±0.02	0.2 ±0.005	0.2 ±0.01	0.21 ±0.002
EFA index	26.98	23.50	22.93	22.50	22.62
MUFA index	43.11	36.99	39.5	39.6	39.7
PUFA index	30.54	27.25	26.12	25.72	25.86
Other fatty acids					
20:3*n-3*	0.38 ±0.01[a]	0.35 ±0.01[b]	0.34±0.003[b]	0.35 ±0.01[b]	0.34 ±0.01[b]

* Values are mean ± SEM with n = 5 for each supplement. The analyses are done comparing the iron treatment to the control. Values that have different letters are statistically different, at $p < 0.05$ using LSD post hoc test.

** 18:1 (includes cis *n-9, n-7* and trans *n-9* and *n-7*)

*** 18:2 (includes cis and trans *n-6)*

4.3 FHs 74 Int immunostaining

Immunostaining for 8-hydroxy-2`-deoxyguanosine was conducted on FHs 74 Int infant cell line. The BM with supplements was incubated with the cells for 4 hrs at 37°C with 5% CO_2 and 95% air. The cell nucleus appears unstained by the dye (antibody to 8-hydroxy-2'-deoxyguanosine) in the absence of oxidative stress. There is non-specific background fluorescence due to subcellular organelles such as lysosomes and mitochondria.

The cell nuclei appeared unstained by the negative control (absence of secondary antibody Alexa- 480) and the BM alone (figure 27). Qualitative analyses of the data could not be performed at this stage due to time constraints on the research. However, qualitative analysis of the data is underway in Dr. W. Diehl-Jones lab. Fluorescent microscopy of the data was performed in Dr Huebners laboratory, department of Zoology, University of Manitoba. The other treatments iron in BM; iron with TRIVISOL together in BM showed marked staining of cell nuclei (figure 28). The cells incubated with positive control (hydrogen peroxide 1 μM) also showed marked fluorescent green staining of nuclei. All samples were done in 4 replicates. Vitamin C in BM and TRIVISOL in BM did not show nuclear staining compared to

the BM and negative controls (figure 27). The white arrows in figures 27 and 28 point towards the nucleus of the intestinal cells.

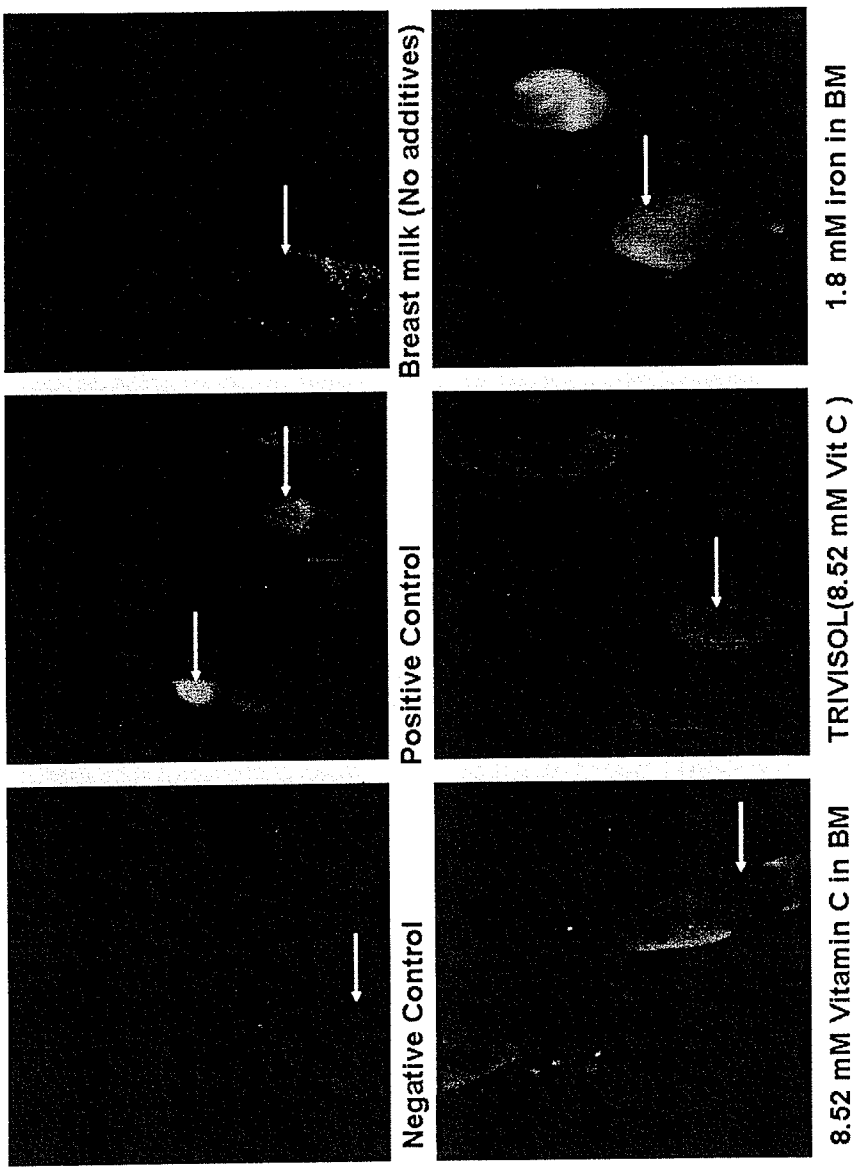


Figure 27 Immunostaining with Fe and vitamin C, TRIVISOL in BM. Magnification 100X of microscope lens. The figure shows negative control (no addition of secondary antibody), BM alone with unstained nucleus and positive control (hydrogen peroxide 1 μM) with fluorescent green staining of nucleus.).

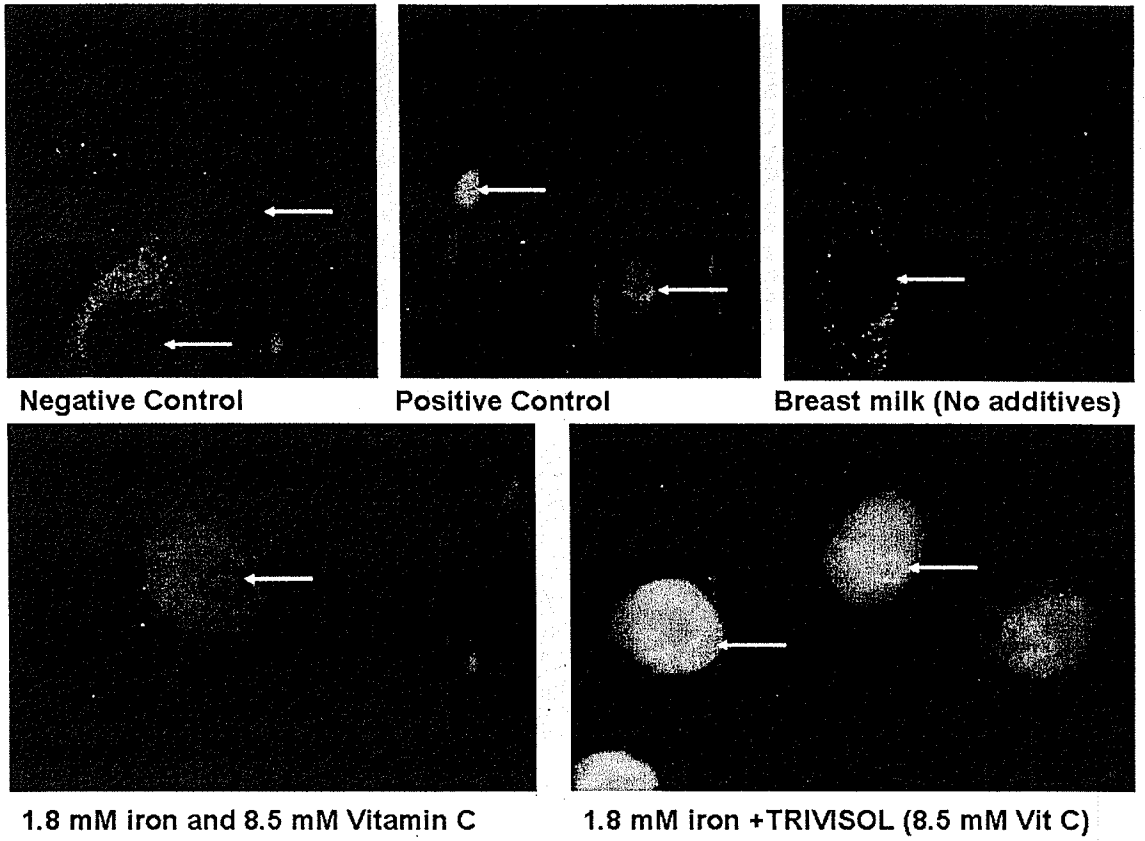


Figure 28 Immunostaining with iron and vitamin C, TRIVISOL in combinations in BM. Magnification 100X of microscope lens. The figure shows iron with vitamin C in BM and iron and TRIVISOL in BM, negative control (no addition of secondary antibody), BM alone with unstained nucleus and positive control (hydrogen peroxide 1 μM)

4.4 Intracellular redox potential with FHs 74 Int cell line

The BM alone led to a fluorecent intensity of 12.6 ± 0.5. There was a statistically significant increase seen with iron fluorecent intensity of 18.2 ± 1.1 compared to BM alone (Figure 29).

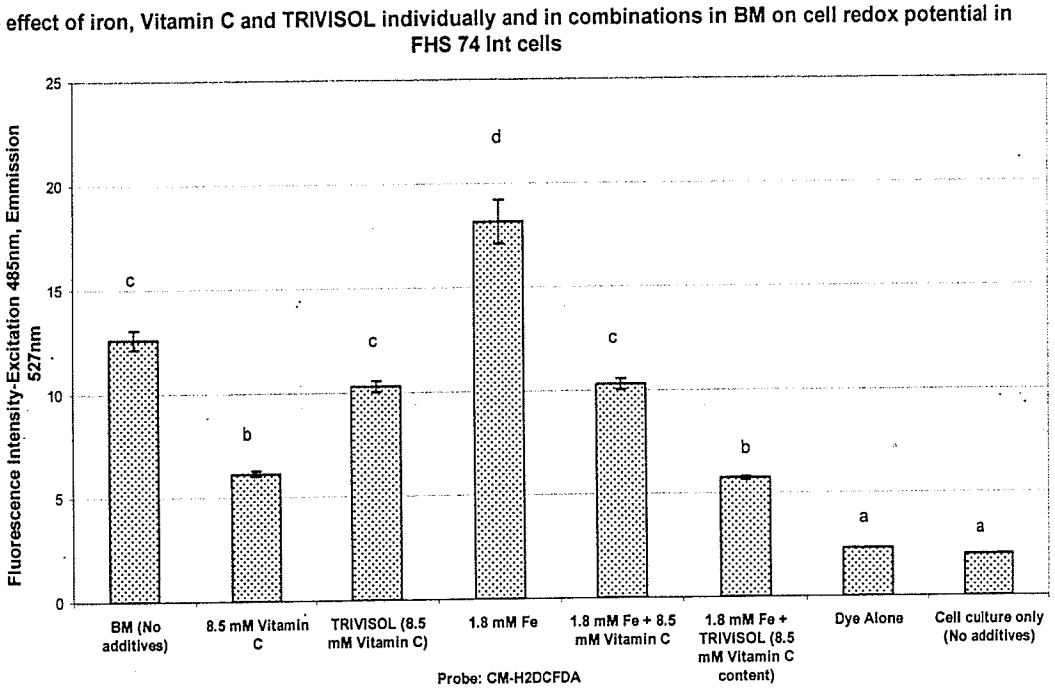


Figure 29 Effect of iron, Vitamin C and TRIVISOL individually and in combinations in BM on cell redox potential in FHS 74 Int cells (n= 12) mean ± SEM. Columns with different letters are significantly different at P< 0.05 using the LSD post hoc test.

4.5 Apoptosis

Statistically significant increase in the specific activity was seen after addition of iron and ML to BM. The BM alone led to specific activity of 0.08 ± 0.009 nM pNA/minute/mg. Iron and TRIVISOL added individually to BM led to a specific activity of 0.43 ± 0.02 and 0.22 ± 0.004 nM pNA/minute/mg respectively. Vitamin C with or without iron did not show significant changes with BM sample figure 30). TRIVISOL and iron in BM led to a specific activity of 0.1 ± 0.004 nM pNA/minute/mg. Vitamin C and TRIVISOL both acted as antioxidants in the presence of iron in BM in this assay.

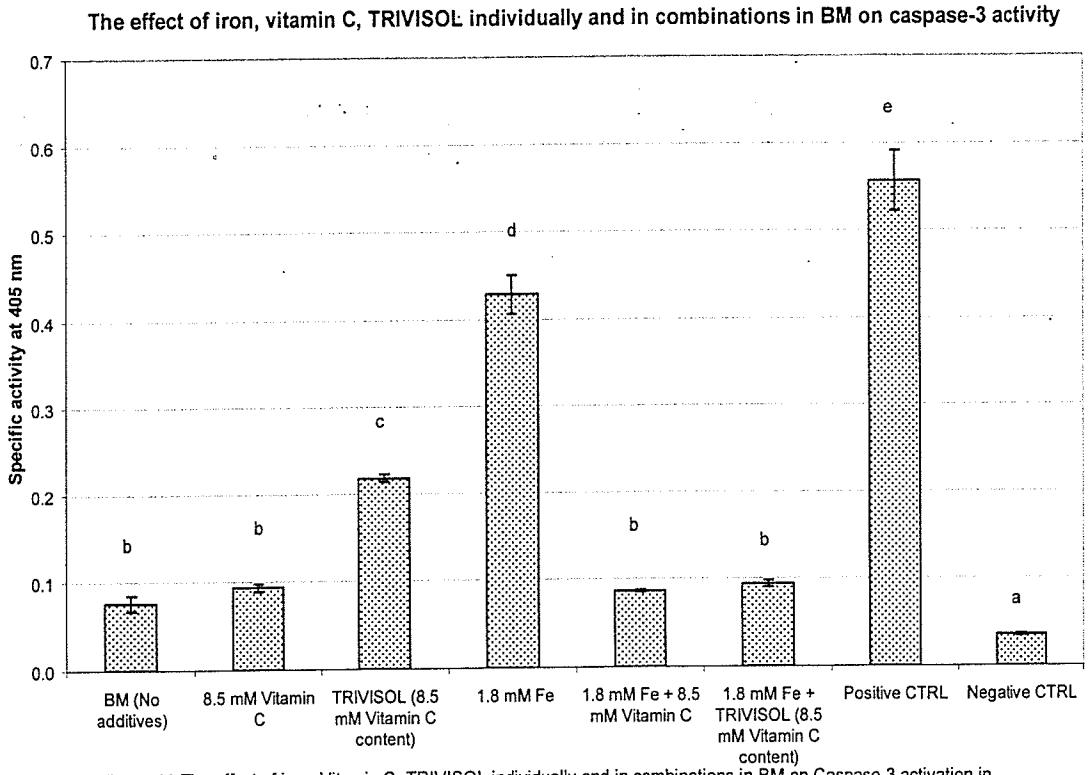


Figure 30 The effect of iron, Vitamin C, TRIVISOL individually and in combinations in BM on Caspase-3 activation in FHS 74 Int cells; mean± SEM (n≥ 3)

CHAPTER 5 DISCUSSION

5.1 Study Review

There is a lack of information upon which to base feeding guidelines for PT infants in neonatal units concerning the storage, shelf life of breast milk with and without supplementation. The shelf life is the duration for which the BM can be stored without being unsuitable for consumption. The present study was designed with the primary goal of examining the storage, shelf life and supplementation of BM from mothers of PT infants and potential oxidative stress generated by the addition of iron and vitamin C supplements. The secondary goal was to find ways to extend the shelf life of BM given with supplements. Finally, the tertiary goal was to study the effect of supplements in BM on oxidative stress in human fetal FHs 74 Int cell line and adult intestinal cells Caco-2BBe, in order to mimic the effect of the infant gut.

The addition of iron in BM increases ROS (Qian and Buettner, 1999). According to Friel et al. (2002), BM from mothers of PT and FT infants had equal resistance to oxidative stress. A larger amount of ascorbate being spared in BM than is spared in formulas may be the reason BM has higher ability to resist oxidative stress (Friel et al., 2002). PT infants may be susceptible to NEC due to their decreased ability to resist oxidative stress (Raghuveer et al., 2002). Iron is a known oxidant-reductant in ROS reactions.

According to Fenton chemistry, iron and vitamin C in the presence of oxygen, with a substrate like lipids in milk, can form ROS. Vitamin C can convert ferritin, a weak catalyst of lipid oxidation, to release iron, which is a well-known *in vitro* catalyst of lipid oxidation (Wills, 1966). Vitamin C at higher concentrations (about >

500 mg/day in adult humans) acts as an antioxidant, but at lower concentrations enhances the catalytic effect of iron and copper (acts as a pro-oxidant) (Wills, 1965 and 1969; Childs et al., 2001). Vitamin C has been reported to release iron from ferritin and hemosiderin in humans (Charlton et al., 1973). Therefore vitamin C could enhance pro-oxidant activity of iron *in vivo* (Sies, 1985).

The ROS formed from iron and vitamin C (Fenton chemistry) can oxidize the lipids in BM. It is recommended that PT infants receive 2-4 mg/kg/day to prevent iron deficiency anemia (AAP, 1985; Siimes and Jarvenpaa, 1982). CPS (1991) recommends 3-4 mg/kg/day iron for PT infants after 4 months of age. There are a number of supplements in the neonatal nursery containing iron (e.g. FERINSOL, Mead Johnson, Evansville, USA) and vitamin C (e.g. TRIVISOL, Mead Johnson, Evansville, USA). Ferrous sulfate and vitamin C added simultaneously to BM increase oxidative stress (Friel et al., 2002). Therefore, supplementation of iron with vitamin C in BM may generate ROS. ROS may cause injury to the small intestine (inflammation or necrosis). NEC is related to damage due to ROS (Rao and Georgieff, 2002). ROS damage through intestinal route could be the one of the etiological causes of NEC.

5.2 Summary of study design

The purpose of our study was to examine different feeding preparations that occur in neonatal units to assess possible oxidative stress that might occur in the milk given to PT infants. There is a possibility that iron and vitamin C supplements with microlipids may be mixed together in BM in some neonatal units. BM samples were collected from 81 mothers who delivered PT infants. Three or more milk samples

(from different mothers) were pooled together to decrease error due to individual variability in fatty acids. These homogenized milk samples were used to study the effect of addition of iron and vitamin C supplements on oxidative stress. Aliquots ($n \geq 3$) were taken from the pooled milk samples. Sample number differed due to availability of BM during the course of the study.

5.3 Iron as a free radical generator in human milk

The appropriate age for starting iron supplements in PT infants has been an issue of debate. Iron is required in the body for synthesis of RBCs and as a cofactor for enzymes. According to the AAP (1985) and CPS (1991) iron supplements in PT infants should be given after 4-6 months. Before that age supplementation appears to show no benefit due to the low rate of erythropoisis and physiological breakdown of RBCs which provide iron to the body (CPS, 1991). Individual requirements have to be taken into consideration (like trauma or bleeding after birth, hereditary enzyme deficiency, the blood indices and anemia) during birth.

ROS have been implicated in neonatal diseases like intraventricular hemorrhage, ROP, bronchopulmonary dysplasia and NEC. Hypoxia and reperfusion is suggested as one of the mechanisms of neonatal diseases by which free radicals act (Saugstad, 1988; Sullivan, 1988). NEC in particular is associated with enteral feedings, prematurity, bacterial infection and oxidative stress. Oral feeding is also done in full term infants but they are not susceptible to NEC as PT infants (Rao and Georgieff, 2002). PT infants have an increased susceptibility to develop neonatal diseases than do FT infants due to their lower immunity (Van Zoeren-Grobben et al., 1994).

The generation of ROS due to iron and vitamin C supplementation together in BM was hypothesized as the cause of *in vitro* lipid oxidation and oxidative stress to intestinal cells in the present study. Iron has the capacity to generate ROS in human milk (Rao and Georgieff, 2002; Raghuveer et al., 2002). Iron supplements can generate ROS by iron- oxygen chemistry, Haber-Weiss reaction and Fenton chemistry (in the presence of vitamin C or E) (Qian and Buettner, 1999). Higher amounts of iron and vitamin C in infant formulas may generate ROS (Herbert et al., 19994; Qian and Buettner, 1999).

Higher amounts of iron supplements in the diet may reduce plasma ceruloplasmin (Lonnerdal et al., 2001) and plasma SOD (Barclay et al., 1991) leading to oxidative stress. The amount of elemental iron used by Barclay et al. (1991) was 13.8 mg, 7 mg, or no elemental iron per day (given as the form iron edentate). These doses are much higher than the dose of elemental iron used in our study 2 mg/kg/day for a 1.5 kg PT infant. Iron was given as ferrous sulfate in our study. The amount of iron used by Lonnerdal et al. (2001) was 12 mg/L fed to infant rhesus monkeys was much lower than the amount of iron used in our study.

Our study showed iron supplements have the potential of causing ROS *in vitro* when added to BM. This was also seen in the experiments with infant and adult cell lines. Vitamin C added to iron containing BM decreased lipid oxidation. Iron in the absence of exogenous vitamin C or TRIVISOL led to an increase in lipid oxidation compared to the BM alone. Thus ROS production may occur via Haber-Weiss reaction or 'Iron- Oxygen chemistry' rather than Fenton chemistry as exogenous vitamin C is absent. Raghuveer et al. (2002) found supplemental addition

of iron in BM led to higher oxidation products than unsupplemented BM. They found antioxidant effects with exogenous addition of lactoferrin to BM. The researchers used human lactoferrin. As bovine lactoferrin has been reported to cause allergic reactions therefore its clinical use to decrease oxidative stress in BM is doubtful (Natale et al., 2004). The antioxidant enzymes SOD and CAT, used in the present study to combat ROS production, were highly effective in decreasing lipid oxidation. SOD converts superoxide radical to hydrogen peroxide which is converted to innocuous water and oxygen by CAT. Therefore this system of enzymes SOD and CAT is very effective in combating ROS.

5.4 Analysis of study components

The study comprised of *in vitro* analysis of iron, vitamin C, TRIVISOL and ML supplements individually and in combinations in BM for lipid oxidation. Iron and vitamin C along with vitamins A, D, E, K, linoleic acid, linolenic acid, calcium, phosphorus, copper, zinc and magnesium are the components of HMF. Therefore, the effect of its addition on lipid oxidation in BM was studied. SOD, CAT and GPX are endogenous antioxidant enzymes in BM. SOD can convert superoxide radical into hydrogen peroxide, which in turn is converted by CAT and GPX into water and oxygen. SOD with CAT, was used in our study to counteract the effects of ROS produced by iron. The effects of ROS were also studied on FHS 74 Int and Caco-2BBe cell lines for presence of oxidative stress.

5.4.1 Lipid oxidation in human milk

In the present study we found that addition of iron to BM increased lipid oxidation. Iron increased lipid peroxides immediately after addition of iron to BM.

Lipid oxidation was also seen after 4 hours incubation. TBARS showed an increase after addition of iron to BM after 4 hours incubation. Iron increased peroxides but not TBARS at time '0' hours. TBARS are secondary oxidation products therefore they were expected to show an increase after few hours of incubation. Raghuveer et al. (2002) found significant increase in TBARS with 2 mg/kg /day iron dose. The values they obtained are similar to the TBARS values we obtained. Conjugated dienes were not detectable by spectrophotometry in human milk alone. To increase detection we added canola oil to BM samples. The pilot study with iron showed no significant changes even with 3.6 mM iron. Canola oil may have masked the conjugated dienes in BM, however iron plus BM and canola oil did not show an increase than BM plus canola oil.

Vitamin C alone in BM did not increase lipid oxidation products. This was expected as vitamin C is an antioxidant. TRIVISOL alone in BM showed higher levels of oxidation products (peroxides and TBARS). TRIVISOL therefore acted independent of vitamin C, contrary to the hypothesis. Experiments with vitamins A and D alone in BM led to lower levels of oxidation products than did BM alone. Therefore TRIVISOL, acted independent of all three vitamins A, D and C. β-carotene, the precursor of vitamin A, is a known antioxidant. Vitamin A also exhibits antioxidant activity according to Zhou and Zhang (2005) and Zaidi et al. (2005). Vitamin D aids the antioxidant activity of other antioxidant substances (Wang et al., 2005) and may also exhibit antioxidant activity (Noyan et al., 2005). Other ingredients in TRIVISOL that may have contributed to oxidative stress are glycerin, polysorbate 80, sodium hydroxide and artificial flavors.

The vitamins A, C, D or E do not exhibit similarities in their chemical structures. Therefore, vitamin D may exhibit antioxidant activity by donation of one or more electrons to an electron deficient compound. This is a hypothesis which needs to be investigated further.

Vitamin C and iron in BM led to significantly lower peroxides (but not TBARS) compared to iron alone in BM. Though TBARS did not show the same trend as peroxides, the amount of TBARS (in μM) was much lower than the amount of peroxides (in μM). Since TBARS are secondary oxidation products, vitamin C may not be able to quench ROS as effectively as it could in the primary products. Champagne et al. (1990) also noted lower ascorbate free radicals with ascorbic acid solution containing iron and copper than infant formula containing iron and copper. Friel et al. (2002) noted a similar increase with iron and vitamin C in oxygen consumption than BM alone. The amount of iron used by Friel et al. (2002) was 12 mg/L which is lower than the amount of iron used in our study. We are not directly able to compare results with Friel et al. (2002) as they did not conduct a study with iron alone in BM and due to the different methods employed. Vitamin C therefore exhibited antioxidant activity and not pro-oxidant activity with iron in BM contrary to expectations. Raghuveer et al. (2002) observed an increase in TBARS after addition of iron to BM; however, we are not able to compare the results as they did not add vitamin C to BM containing iron. Rosenthal et al. (1993) did not observe an increase in TBARS after addition of iron and vitamin C to milk. The amount of vitamin C (1 g/L) and iron lactate (50 mg/L) were lower than the amount of vitamin C and iron used in our study.

Addition of ML to BM containing iron and vitamin C led to an increase in peroxides compared to BM containing iron and vitamin C but no ML. Microlipids contain PUFAs. Therefore ML acted as substrates for ROS produced by iron and vitamin C. TRIVISOL alone in BM showed an increase in peroxides and TBARS compared to BM alone after 4 hours incubation. TRIVISOL with iron in BM showed a decrease in peroxides and TBARS compared to iron alone in BM. This could be due to the protective effect of antioxidant vitamins A and C in TRIVISOL. Vitamin D in TRIVISOL may have boosted the effect of antioxidant vitamins.

When analyses were done on the samples after 1, 2 and 3 hours incubation no significant increases were seen in peroxides after 1 hour in each treatment group. This indicates that the lipid oxidation was immediate and further incubation did not lead to its progression. A similar effect was observed at 4°C where treatment samples were incubated until 12 hours. In this case storage at 4°C (cooler temperature than room temperature) wasn't protective from lipid oxidation. Even incubation at 30°C showed a similar lipid oxidation to room temperature after 2 hours incubation. Therefore the lipid oxidation appears to be immediate and remains stable over time.

Turoli et al. (2004) did not observe significant changes in TBARS in their full term milk samples at room temperature or at - 20°C. They attribute it to degradation during analysis, because of manipulation and light exposure at room temperature and lipoprotein lipase activity at - 20°C. They did not add iron and vitamin supplements to their samples. They were examining the storage properties of BM. Miranda et al. (2004) studied the effect of storage in full term BM. They found higher MDA in

refrigerated BM than control. This study also examined the storage of BM and not the effect of iron and vitamin supplements.

5.4.2 Effect of HMF

No significant changes were seen with HMF (Enfamil Human Milk Fortifier, Mead Johnson, Evansville, USA) in BM in our study. No statistically significant differences were seen with HMF added to TRIVISOL. HMF and iron in BM led to a statistically significant increase over iron in BM alone. There are no other reported studies examining lipid oxidation in BM with addition of HMF. HMF contains fat 1g, linoleic acid 140 mg, alpha- linolenic acid 17 mg, vitamin C 12 mg, iron 1.44 mg, among other substances like vitamin A, D, E, K, calcium, phosphorus, copper, zinc and magnesium per 4 packets. The presence of a large number of antioxidants in HMF could have prevented lipid oxidation in BM when HMF is added.

5.4.3 Protective effect of antioxidant enzymes

Exogenous SOD and CAT enzymes added to BM led to significant reduction in peroxides, even in the presence of iron. This indicates that exogenous enzymes are protective and addition of enzymes may be considered in supplements to reduce lipid oxidation. Pasteurized BM did not exhibit significant changes in lipid peroxides compared to nonpasteurized BM. The antioxidant properties of milk were not compromised after inactivation of endogenous SOD, CAT and GPx by pasteurization. This indicates the antioxidant effect of BM persists after inactivation of endogenous enzymes. Friel et al. (2002) also reported non significant changes in oxygen consumption between pasteurized and non pasteurized BM and attributed this antioxidant protective effect to unknown substances in BM. Some non-enzymatic

antioxidants like lactoferrin, vitamins C, E and carotenoids (Lindmark-Mansson and Akesson, 2000) not affected by pasteurization could have contributed to this protective effect.

5.4.4 Fatty acid analyses

Analyses for fatty acids revealed a significant decrease in fatty acids when the dose of iron was increased. This was observed for most mono and polyunsaturated fatty acids. This indicates iron produced ROS, which attacked double bonds. Increase in ROS decreased the number of double bonds in fatty acids.

MUFA index showed an appreciable decrease when the dose of iron was increased. The EFA and PUFA index showed a significant decrease in PUFAs when iron dose increased. Therefore iron was responsible (by Haber-Weiss reaction as vitamin C was not added) in production of ROS. The fatty acid composition of milk fatty acids in our study is similar to the fatty acid composition in BM obtained by Mitoulas et al. (2003). Their study was analysis of fatty acids in FT BM over 1 year of lactation. These results imply that milk fatty acid composition is not quite varied between PT and FT milk.

5.4.5 Oxidative stress with FHs 74 Int and Caco-2BBe cell lines

The antibody to 8-hydroxy-2`-deguanosine attaches itself to 8-hydroxy-2`-deguanosine (8-hydroxy-2`-deguanosine is oxidized form of DNA nucleotide deoxyguanosine) (Kasai et al., 1986). We observed considerable staining with the antibody to 8-hydroxy-2`-deguanosine with the FHs 74 Int cell nuclei with iron alone in BM, and iron and TRIVISOL together in BM. Such staining of the nucleus was not observed with BM alone, vitamin C alone in BM, TRIVISOL in BM or iron

and vitamin C together in BM. Therefore vitamin C with iron in BM does not appear to increase ROS damage to DNA as seen in this study. This supports our *in vitro* findings with increase in lipid oxidation and fatty acid analyses with iron in BM, increase with iron and TRIVISOL in BM and a decrease in oxidation with iron and vitamin C in BM. This also shows similar effects between *in vitro* lipid oxidation and the effect in cell line. This is very important in validation of cell lines as bioindicators of ROS. Quantification of the data was not possible during this stage due to time constraints on the research. Quantification of the data is underway in Dr. W. Diehl-Jones laboratory.

Ichiba et al. (1992) observed growth promoting activity on FHs 74 Int cells with BM but not with formulas. They therefore conclude BM stimulates intestinal cell growth in PT infants and progresses the maturation of the intestinal tract. Hirai et al. (2002) reported a study with FHs 74 Int cells, amniotic fluid and human milk, examining the trophic effect of growth factors. There are no other reports of studies conducted with iron, vitamin C and ML addition to human milk. Our study is the first to report these findings with iron, vitamin C and ML supplements in BM using FHs 74 Int cell line.

Redox potential of cells study, with the molecular probe CM-H2DCFDA, showed a significant increase with iron alone in BM treatment. Other treatments with vitamin C or TRIVISOL with or without iron did not show a significant change from BM alone. This indicates iron was primarily responsible for ROS production in this assay. Vitamin C in BM and in TRIVISOL (also vitamins A and D) in BM aided in decreasing redox potential in FHs 74 Int cells. Iron in BM showed an increase in

redox potential. This supports our *in vitro* findings with increase in lipid oxidation and fatty acid analyses with iron in BM. There are no reported studies on redox potential with FHs 74 Int cells. Our study is the first to report findings of supplemental addition in FHs 74 Int cells and its effects on redox potential.

Iron alone in BM and TRIVISOL added individually to BM, increased the caspase-3 activity significantly in Caco- 2BBe cells. Apoptosis occurs normally in the gut but its overactivity can damage intestinal mucosa (Diehl-Jones and Askin, 2004). Caspase-3 activity is an indicator of apoptosis (Dorman et al., 2004). Therefore iron in BM caused apoptosis, as well as, the TRIVISOL in BM treatment. Vitamin C in BM showed non significant changes compared with BM alone. Thus iron in all the three cell assays showed an increase in ROS activity with iron treatment. This supports our *in vitro* lipid oxidation and fatty acid analyses study. Vitamin C did not show such activity. ROS are implicated in cell apoptosis and tissue damage (Rao and Georgieff, 2001). Our results are consistent with this concept. Thus the conditions used in these experiments can lead to cellular damage and apoptosis with iron supplementation.

5.5 Clinical implications

The data suggests that under conditions used in these experiments, iron and microlipid supplements can generate ROS in BM. ROS may cause cellular damage in infant intestinal cells. This *in vitro* damage may be one of the causes of NEC *in vivo*. Antioxidant enzymes SOD, CAT and vitamins A and C decrease *in vitro* lipid oxidation. Exogenous addition of antioxidant enzymes in milk and formulas may be considered in NICUs and in infant formulas. This addition may help decrease the

production of ROS in BM fed to PT infants. This would decrease the oxidative load given to PT infants. The increase in peroxides and TBARS by iron alone in BM could be due to Haber-Weiss reaction as no other supplements (especially vitamin C) were added. ROS may have formed in iron containing BM leading to oxidation by exposure to light and oxygen. More research is required to explore the antioxidant activity of vitamin D.

Summary

The doses of iron, vitamin C and microlipids typically used in NICU recommended by AAP (1985) and CPS (1991) were added to human BM samples. These samples were incubated until 4 hours under room light and room temperature. The samples were also incubated with FHs 74 Int cells for 4 hours, to study DNA damage associated with oxidative stress and with Caco-2BBe cells for 4 hours to study apoptosis. We observed significant lipid oxidation and oxidative stress both *in vitro* and with the intestinal cells with iron and microlipid supplements. Fatty acid analyses revealed significant decrease in polyunsaturated fatty acid indices with the iron treatment in BM. Vitamin C exhibited antioxidant activity in the doses used in this study. SOD and CAT decreased the damage due to ROS. Vitamins A and D also exhibited antioxidant activity. Appropriate caution is required during administering iron supplements as they may lead to oxidative stress in PT infants. This stress may be the cause of NEC and may be avoided by the use of antioxidants.

Strengths and Limitations

Strengths: Although adding iron and TRIVISOL together results in ROS production *in vitro,* vitamin C exhibited an antioxidant role in this study. Therefore TRIVISOL acted independent of vitamin C, contrary to expectations. Microlipids increased oxidative stress. Cell culture studies did not reveal significant oxidative damage with vitamin C. Iron and microlipid supplements increased lipid oxidation in milk. Oral ingestion of these supplements by PT infants may be a possible cause of NEC. This is the first study to investigate oxidative stress in the human fetal cell line FHs 74 Int. The antioxidant effect observed with vitamins A and D in this study can

be investigated further. This would provide new light in the properties of these vitamins.

Limitations: The BM samples should have been collected at one given time for the whole of the experiment and not over a period of time, to avoid possible source of errors due to variation in the composition of milk. Many factors affect the total lipid content. The lipid content increases during a feed, increases with age, post partum, stage of lactation, diurnal rhythm, between breasts, gestational age at birth: PT vs. term and maternal diet (Jensen, 1995). It usually decreases with infections, medication, mothers menstrual cycle or pregnancy, decreases with parity, season changes, age, individual changes (adiposity increases) (Jensen, 1995). Fore, middle-stream or hind milk also have different fatty acid composition (Turoli et al., 2004). The resulting error due to some of these factors may have decreased in our study by pooling 3 or more samples of breast milk, between subjects. However, many of the above factors have not been taken into account during the analysis of data.

Directions for Future research

The following are possible parameters to investigate generation of ROS in the gastrointestinal tract of PT infants:

1. Urinary markers of DNA damage: Urinary production of 8-hydroxy-2'-deoxyguanosine could be used as a marker for oxidative stress with addition of supplements to BM. Urine is an excretory product of PT infants. Therefore its measurement would give us an indication of *in vivo* oxidative stress.

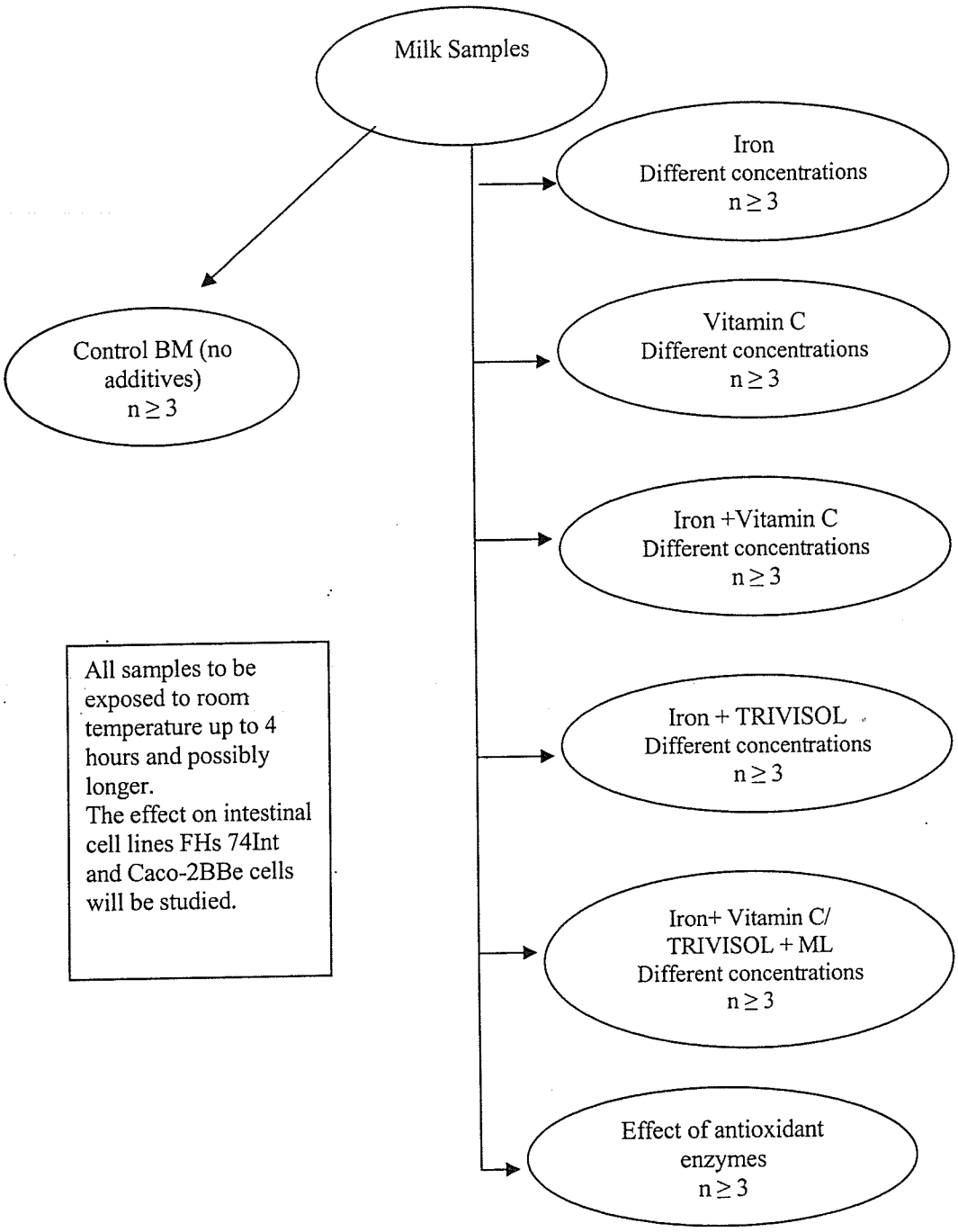
2. *In vitro* studies: Supplement higher doses (> physiological doses) of vitamin C and observe pro/antioxidant effects.
3. Vitamin E: Marshall and Roberts (1990) suggested protective role of vitamin E against lipid oxidation. A study with iron supplements and ability of vitamin E to resist oxidative stress in BM would be useful.
4. Copper: Observe oxidative damage if any on addition of copper. High serum ascorbate can increase reactive ferrous ions by inhibiting the ferroxidase activity of caeruloplasmin (Powers, 1995). Therefore the effect of copper on iron metabolism would provide more information on its bioavailability.
5. Zinc: Zinc has the capacity to decrease oxidative stress due to iron (Zago et al., 2000). Its effect on iron supplementation in BM would be useful.
6. Heat: Effect of heating milk samples upto 100°C and investigate increase/decrease in oxidation.
7. A digestion model: A continuous flow intraluminal digestive phase model can be used to test the effect of ROS.
8. Use of pre and probiotics: Test decrease in incidence of NEC with probiotics (Reber and Nankervis, 2004).
9. Lactoferrin: The effect of lactofferin added to BM containing iron supplements on intestinal cell line.
10. Animal model: A piglet or rat model could be used to conduct an *in vivo* study with iron supplemental addition to milk and gastrointestinal tract.

In addition, the respiratory system could be examined for presence of oxidative stress. The respiratory route is a possible source of oxidative stress, along with gastrointestinal tract, as PT infants are administered hyperbaric oxygen, a source of ROS.

CHAPTER 6 CONCLUSION

Iron and microlipids supplements, necessary for optimal growth of neonatal infant, increase oxidative stress *in vitro.* The lipids in human milk are oxidized due to supplemental iron. Polyunsaturated and monounsaturated fatty acid indices show a decrease due to supplemental iron in BM. Exogenous addition of antioxidant enzymes SOD and CAT decreases the oxidative stress due to ROS. Vitamin C acted as an antioxidant at the doses used in our study. Vitamins A and D also exhibit antioxidant properties.

Iron supplements cause oxidative stress in adult and infant intestinal cell line. This damage due to ROS on intestinal cells, may contribute to *in vivo* oxidative damage to PT infants gastrointestinal tract, leading to inflammatory diseases such as NEC. Further research is required to investigate the *in vivo* effects of lipid oxidation on intestinal cells. This can be conducted by an animal model rat, piglet or monkey.



Appendix I Experimental design of the current study.

Appendix II The basis of dose of iron

Assumptions:

1.Premature infant is 1.5 kg

2.The infants daily enteral intake is 160 mL/kg divided evenly in eight feedings, the volume of individual feeds given at 3-h intervals in a day is 20 mL/kg

3.**We chose 2 mg of elemental iron per kg per day** to be given to the infant as it is the dose recommended by American Academy of Pediatric Society 2-4 mg/kg/day iron for premature infants (AAP, 1985)..

Calculations:

A) Molecular weight $FeSO_4.7H_2O$ 278

Atomic weight Fe 56

The dose of elemental iron is 2 mg/kg/day for a 1.5 kg infant given once a day in a single feed.

The volume of individual feeds is 20 mL/kg given at 3-h intervals in a day to a 1.5 kg infant.

Therefore the **elemental iron** given in mg/mL is

$$\frac{2 \text{ mg/kg/day}}{20 \text{ mL/kg}} = \textbf{0.1 mg/mL breast milk per day}$$

This is equivalent to **0.5 mg/mL per day $FeSO_4.7H_2O$**

B) Molarity of Iron

Molecular weight $FeSO_4.7H_2O$ 278

Atomic weight Fe 56

The amount of $FeSO_4.7H_2O$ is 0.5 mg/mL breast milk

This is equivalent to elemental iron 0.1 mg/mL breast milk

= 100 mg/L

= 0.1 g/L

$$= \frac{0.1 \text{ g. moles}}{56 \text{ g. L}}$$

= 0.0018 moles/ L

Molarity = 0.0018 x 1,000 mM/ L

Molarity = 1.8 mM/L.

Appendix III The basis of dose of TRIVISOL

Assumptions:

1. Premature infant is 1.5 kg
2. The infants daily enteral intake is 160 mL/kg divided evenly in eight feedings, the volume of individual feeds given at 3-h intervals in a day is 20 mL/kg
3. **We chose 1 mL of TVS in one feed per day** administered to the infant, as it is the dose recommended by Mead Johnson; Evansville, USA. The Food and nutrition board (2000) recommends 40 mg/day vitamin C to infants 0-6 months of age. American Academy of Pediatric Society (1979) recommends 35 mg/day vitamin C.

TRIVISOL contains per 1mL:

Vitamin A 1500 IU, Vitamin C 30 mg and Vitamin D 400 IU

Therefore 1 mL is dissolved in one feed of 20 mL. Therefore for vitamin C the amount is 30 mg/20 mL breast milk.

This is equivalent to 1.5 mg/mL vitamin C.

B) Molarity of Ascorbic acid

Molecular weight 176

Molarity of 1.5 mg/mL

$= 1.5$ g/ L

$= \frac{1.5}{176}$ g/ L. moles

$= \frac{1.5}{176} \times 1{,}000$ millimoles/L

= 8.52 mM/L.

Table 6 Human milk composition (Nutrients per liter)

	Constituent per liter	Human milk
	Energy, Cal	700
	Volume, ml	1000
	Protein, g	10.3
	Fat, g	44.0
	Carbohydrate, g	69.0
Vitamins	Vitamin A, IU	3300(+ betacarotene)
	Vitamin D, IU	4- 97
	Vitamin E, IU	20
	Vitamin B1, µg	1000
	Vitamin B2, µg	400
	Vitamin B6, µg	200
	Vitamin B12, µg	0.30
	Niacin, µg	1800
	Folic acid, µg	13.0
	Pantothenic acid, µg	2400
	Biotin, µg	10
	Vitamin C, mg	50
Minerals	Calcium, mg	330
	Phosphorus, mg	140
	Magnesium, mg	30
	Iron, mg	0.50
	Zinc, mg	2.8
	Copper, mg	0.50
	Sodium, mg	170
	Potassium, mg	500
	Chloride, mg	420

References (Jensen, 1995 and Geigy Scientific tables, 1981)

Table 7 Fatty acid composition of PT and FT human milk 20 days postpartum

(% of total fatty acids)

	PT milk	FT milk
C10:0	1.05	1.15
C12:0	5.39	7.17
C14:0	7.03	8.97
C16:0	23.18	22.58
C18:0	7.74	7.85
Total Saturated FA	46.28	48.09
C18:1n-9	31.97	29.81
Total trans FA	1.49	1.13
C18:2n-6	10.47	10.17
C18:3n-6	0.18	0.16
C20:2n-6	0.31	0.32
C20:3n-6	0.38	0.41
C20:4n-6	0.50	0.51
C22:4n-6	0.10	0.11
Total n-6 PUFA	1.35	1.35
Total n-6	12.09	11.64
C18:3n-3	0.78	0.78
C20:3n-3	0.05	0.05
C20:5n-3	0.04	0.05
C22:5n-3	0.15	0.15
C22:6n-3	0.27	0.24
Total n-3 PUFA	0.53	0.42
Total n-3	1.43	1.13
Total PUFA	1.90	1.66
n6/n3 PUFA	2.56	2.88

Reference (Genzel-Boroviczényy et al., 1997)

Table 8 Potential problems of PT infants

Undernutrition	Asphyxia
Anemia	Respiratory distress syndrome
Retinopathy of prematurity	Unconditioned suck and swallow
Poor temperature control	Hyperbilirubinemia
Apnea	Hypocalce,mia
Glucose intolerance	Periventricular leukomalacia
NEC	Infection
Intraventricular hemorrhage	Fat malabsorption
Decreased gastric motility	Limited renal function
Osteopenia	Hypotension
Patent ductus arteriosus	Bronchopulmonary dysplasia

Source: Samour et al. (2004).

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