MATERNAL DIET FAT ALTERS MILK FATTY ACIDS, SUCKLING PUPS' INTESTINAL PHOSPHOLIPID FATTY ACIDS AND INTESTINAL RESPONSIVENESS TO EXPERIMENTAL COLITIS

by

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ABSTRACT

The quantity and quality of dietary lipids is crucial for infant growth and development. The significance of the differences in n-6 and n-3 fatty acids in human milk, and the effect of maternal diet fat on various inflammatory mediators in the gastrointestinal tract of infants have received little attention. The aims of the present study were to determine the effect of maternal dietary fat composition on rat milk, intestinal phospholipid fatty acids and responsiveness to experimental colitis in suckling rat pups.

Female rats were fed isocaloric diets varying only in fat composition throughout gestation and lactation. The oils used were high in n-3 (canola oil, 8% 18:3n-3), n-6 (safflower oil, 72% 18:2n-6), or n-9 (high oleic acid safflower oil, 78% 18:1n-9) fatty acids, n=6 dams/ group. A reference group of rats fed chow (37% 18:2n-6, 4% 18:3n-3, 1%20:5n-3, 2% 22:6n-3) were also studied. Colitis was induced in the pups on postnatal day 15 by intra-rectal dinitrobenzene sulfonic acid (DNBS) administration, with vehicle (50% ethanol) and procedure (0.9% saline) control pups. Jejunal and colonic phospholipids (phosphatidylcholine and phosphatidylethanolamine) and milk fatty acids were determined. The distal colon was assessed for macroscopic damage, histology and myeloperoxidase activity (MPO).

The high n-6 maternal diet increased n-6 fatty acids, whereas the high n-3 diet increased n-3 fatty acids in milk and pup jejunal and colonic phospholipids. Maternal diet, milk and pup intestinal n-6/n-3 fatty acid ratios increased significantly in order: high 18:3n-3 < high 18:1n-9 < high 18:2n-6. DNBS administration in pups in the high 18:2n-6 group led to severe colitis with higher colonic damage scores and MPO activity

than in 18:1n-9 and 18:3n-3 groups. High maternal dietary 18:3n-3 intake was associated with colonic damage scores and MPO activity that were not significantly different from ethanol controls. The composition of rat milk, pups intestinal fatty acids and n-6/n-3 fatty acid ratios in reference group were similar to high 18:3n-3 group, however, DNBS colitis was associated with higher colonic damage scores and MPO activity compared to high 18:3n-3 group. To conclude the maternal dietary fat influences the composition of rat milk fatty acids, intestinal lipids and responsiveness to experimental colitis in nursing offspring. Higher dietary n-3 fatty acids attenuate intestinal responsiveness to colitis. To the best of our knowledge, this is the first report to suggest that the composition of milk fatty acids is associated with the nursing offspring's susceptibility to chemically-induced colitis.

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CHAPTER I

INTRODUCTION

It is well recognized that the composition of dietary essential n-6 and n-3 fatty acids are crucially important determinant of infant growth and development. Studies to date, however, have focused almost exclusively on the developing brain. This is probably largely because of concerns that delays or alterations in brain development can have long term effects beyond the period of dietary deficiency. The intestine, however, is the first organ to be exposed to the diet. Thus it is reasonable to expect that the n-6 and n-3 fatty acid composition of the diet will affect the composition of structural lipids in the intestine and consequently, intestinal function. Indeed, many studies in adult animals have shown that the lipid composition of intestinal cells is readily susceptible to alterations by the dietary fat (Thompson *et al.*, 1987; Clandinin *et al.*, 1994). Limited studies have examined the effect of maternal dietary lipids on the developing intestine in suckling offspring.

The essential n-6 and n-3 fatty acids provide the structural matrix for various cell membranes and are the source of eicosanoid precursors and signal molecules that directly modulate inflammatory responses (Innis, 1991). The content of the n-6 fatty acids specifically 18:2n-6 in human milk has doubled from about 7 to 14 percent since late 1950's (Inull and Ahrens, 1959). The increased intake of n-6 fatty acid rich polyunsaturated vegetable oils by pregnant and nursing women has led to increased intake of n-6 fatty acids in breast fed infants. The amount of 18:2n-6 in infant formulas is also as high or higher than in human milk (Innis, 1992). Higher dietary intakes of 18:2n-6 may lead to excessive production of n-6 fatty acid derived pro-inflammatory

mediators, such as leukotrienes and prostaglandins. This may also trigger a cascade of events resulting in increased production of peptide inflammatory mediators such as interleukins and tumor necrotic factor-α at the site of inflammation.

It is known that the composition of maternal diet fat influences the fatty acid composition of milk (Innis, 1992; Jensen *et al.*, 2000). Despite this, the effect of fat composition of milk diet on the developing intestine has received little attention. Because dietary n-6 fatty acids may be involved in the etiology of inflammatory bowel disease (Belluzzi *et al.*, 2000), some nutrition experts have recommended a decrease in n-6 fatty acid intake. However, the data to support the possibility that high dietary intake of n-6 fatty acids may predispose the neonatal intestine to increased inflammatory responses following chemically-induced colitis is not available. In the present study we sought to establish if the composition of maternal dietary fat during gestation and lactation has functional significance with respect to development of the intestine in the nursing offspring. To the best of our knowledge, this is the first report to suggest that the composition of milk fatty acids is associated with the nursing offspring's susceptibility to chemically-induced colitis.

1. LITERATURE REVIEW

1.1. Fatty acid metabolism

The two independent families of fatty acids, the n-6 and n-3, are known to be essential for normal mammalian cell function. The n-6 and n-3 fatty acids accepted as essential in the human diet are linoleic acid (18:2n-6) and linolenic acid (18:3n-3), respectively (Innis, 1991), because mammalian cells are able to synthesize saturated, n-9 and n-7 series unsaturated fatty acids *de novo* from acyl CoA, but lack the delta 12 and 15 desaturase enzymes necessary for insertion of a double bond at n-6 and n-3 positions, respectively, in a fatty acid carbon chain (Innis, 1991; Sprecher, 1995).

Burr and Burr (1929) were first to recognize the essential nature of n-6 and n-3 fatty acids. The biochemical pathways for essential n-6 and n-3 fatty acids desaturation are only present in chloroplasts. Thus, only higher plants, algae, and some fungi are capable of forming these fatty acids. Hansen *et al.* (1963) firmly established that 18:2n-6 is essential for normal infant nutrition through a clinical and biochemical study of 428 infants fed cow's milk-based formulations that varied in their types of fat. The daily 18:2n-6 intake of the infants ranged from 10mg/kg to 800mg/kg. Infants receiving lower amounts of 18:2n-6 showed clinical signs of 18:2n-6 deficiency such as dryness, skin desquamation and thickening, and growth faltering. Other clinical signs of n-6 fatty acid deficiency include alopecia and histological abnormalities, which are prevented by a dietary intake of 3-4% energy from 18:2n-6 (Innis, 1991; Innis, 2003). The biochemical diagnosis of essential fatty acid deficiency is based on the analysis of quantity or percent of 20:3n-9 relative to 20:4n-6 in plasma or tissue lipids (Holman, 1960). A ratio of

20:3n-9 / 20:4n-6 greater than 0.2 has been considered as essential fatty acid deficiency (Innis, 1992).

Dietary essential 18:2n-6 is metabolised to arachidonic acid (20:4n-6) whereas dietary essential 18:3n-3 is metabolised to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) in the body. Synthesis of 20:4n-6 depends on sequential desaturation with alternate elongation of 18:2n-6 by delta 6 and delta 5 desaturases (Fig I.1). The synthesis of 22:6n-3 from 18:3n-3 involves a similar delta 6 and delta 5 desaturation and elongation to 22:5n-3, then additional elongation and desaturation, by delta 6 desatuarses and β-oxidation (Voss *et al.,* 1988). Due to competition between 18:2n-6, 18:3n-3 and 18:1n-9 for the delta 6 desaturase, the synthesis of 20:4n-6, 22:6n-3 and 22:3n-9, respectively may be altered, however, the preferential substrate affinity of the delta 6 desaturase is well known to be in the order of 18:3n-3>18:2n-6>18:1n-9 (Brenner, 1974; Jefcoat and James, 1984). Thus, the desaturation of 18:1n-9 is competitively inhibited by 18:2n-6 and 18:3n-3 (Brenner, 1974; Mohrhauer and Holman, 1963), which allows for the synthesis of significant amounts of 20:3n-9 and 22:3n-9 only during concurrent deficiency of both n-6 and n-3 fatty acids (Innis, 1991).

1.2. Metabolic roles of essential fatty acids

1.2.1. Fatty acids in cell membranes

Cell membranes are composed of a bilayer of phospholipids in which the fatty acids are oriented inwards to form the hydrophobic core of the membrane interior, and the polar head groups (i.e., choline, ethanolamine, inositol, or serine) are oriented outwards towards the aqueous intercellular and the cytosolic or plasma compartment (Houslay and Stanley, 1982). The membrane can exist as a lipid bilayer due to the amphipathic characteristics of phospholipids. Thus, an individual membrane may possess more than 100 chemically distinct types of lipid molecules (Spector and Yorek, 1985). The predominant phospholipid classes in rat liver plasma membrane are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), (Kinsella, 1990).

The n-6 and n-3 fatty acids are well known to be crucial components of phospholipids, which form the structural matrix of all cell membranes (Kinsella, 1990). Membrane composition, especially the proportions of unsaturated fatty acids in the phospholipids, is regulated to produce an average order of viscosity. Membrane lipids are usually in a fluid state (being 100-fold more viscous than water), which allows for both lateral mobility and rotational diffusion of integral membrane proteins and lipids (Kinsella, 1990). Different lipids have different physical properties and packing behaviors in membranes; some acyl components may affect the specificity and selectivity of enzyme degradation and may be involved in protein-lipid associations. Thus, the lipids of a membrane provide more than an apolar environment that segregates and compartmentalizes different cellular components (Kinsella, 1990). Remodeling of

cellular phospholipids occurs constantly through the exchange with cytostolic and plasma lipids (Finea, 1981).

Phospholipids also serve as a permeability barrier for the cells and cell compartments and the lipid bilayer also forms a structural matrix into which specific proteins are placed, serving as a seal around membrane proteins to prevent non-specific leakage. Lipids also serve to maintain the proteins in their most appropriate functional conformations. The polar position of the phospholipids satisfies the requirements for the electrostatic charge needed for the surface associations of specific cell surface proteins (Clandinin *et al.,* 1991). All of these characteristics are needed and are critical for normal cell function. Therefore, the fatty acid composition of membrane phospholipids, which is partly determined by n-6 and n-3 fatty acids in the diet, can modulate the cell signalling pathways involved in various disease processes such as inflammatory diseases, diseases of liver, kidney etc. (Clandinin *et al.,* 1994). Hence, lipids provide a flexible structure to the membranes where proteins, i.e., enzymes, transporters or receptors for hormones, antigens, or cell growth factors control many metabolic activities. Since dietary fatty acids are exchanged with membrane fatty acids, dietary fat composition is reflected in membrane lipid composition. Thus, dietary fatty acids can influence cell function through effects on membrane properties (Thompson *et al.,* 1989c).

1.2.2. Fatty acids as eicosanoid precursors

Longer chain polyunsaturated fatty acids such as 20:4n-6 and 20:5n-3 are substrates for the synthesis of eicosanoids, which are hydroxylated derivatives of 20-carbon polyunsaturated fatty acids. The two major pathways of eicosanoid synthesis are

cyclooxygenase or lipoxygenase (Lands *et al.,* 1990). Products of the cyclooxygenase pathway are the prostaglandins and thromboxanes. Products of the lipoxygenase pathway are the leukotrienes, hydroxyeicosatrienoic acids, and the lipoxins. Essential 18:2n-6 and its longer chain metabolite 20:4n-6 are the main precursors of the dienoic prostaglandins (PGE_2), thromboxanes (TXA_2), and tetraenoic leukotrienes (LTB_4). On the other hand, 18:3n-3 and 20:5n-3 are precursors of the trienoic prostaglandins (PGE_3) and pentanoic leukotrienes (LTB_5) (Smith, 1978). In addition, n-3 fatty acids directly suppress the activity of cyclooxygenase. Also, the eicosanoids, formed from n-3 fatty acids, are opposite to, or have weaker effects than eicosanoids formed from n-6 fatty acids (Rubin and Laposata, 1992).

Eicosanoids are important and potent mediators of many biochemical processes and play critical roles in coordinating physiological interactions among cells. Eicosanoids formed from n-6 fatty acids can change vascular tone and the concentration of cyclic AMP in tissues. Excessive and / or imbalanced synthesis of eicosanoids can also lead to thrombosis, inflammation, asthma, ulcers, and kidney diseases (Shimizu and Wolfe, 1990). LTB_4 is a chemoattractant and activator of neutrophils and has potent pro-inflammatory activities; PGE_2 can cause pain and vasodilation, and together, they can cause vascular leakage and extravasation of fluid (Powel and Funk, 1987). The net biological effect of the eicosanoids is believed to be determined in large by a balance in tissue levels of 20:4n-6 and 20:5n-3 (Rosenthal, 1987). Hence, altering the polyunsaturated fatty acid profile of biological membranes may modify the inflammatory cell behaviour through membrane effects. The oxygenation of 20:4n-6 may be competitively inhibited by 20:5n-3 in the cyclooxygenase pathways and 20:5n-3

is also a preferred substrate for 5-lipoxygenase, compared to 20:4n-6. Thus, biological membranes rich in n-6 fatty acids may shift the physiologic state to one which is prothrombic and pro-aggregatory, whereas membranes rich in n-3 fatty acids may ameliorate these processes (Endres *et al.*, 1989; Broughton and Wade, 2002).

1.3. Dietary supply of n-6 and n-3 fatty acids

The richest dietary sources of 18:2n-6 and 18:3n-3 include unsaturated plant seeds and polyunsaturated fatty acid-rich vegetable oils (Innis, 1992a). The relative proportions and amounts of 18:2n-6 and 18:3n-3 differ widely among different oils and 18:2n-6 is particularly high in safflower, soybean, sunflower, and corn oils. Soybean and canola oils are high in 18:3n-3, but corn, sunflower, and safflower oils have very low amounts. Canola, a low 22:1n-9 hybrid derived from rapeseed oil, is widely used in cooking and salad oils, table spreads, and in preparing bakery and fast foods. Canola oil is generally considered as a favourable dietary oil because of its relatively high proportion of 18:1n-9 (about 50-60% of the fatty acids) and of 18:3n-3, with modest levels of 18:2n-6 and low levels of saturated fatty acids (Gustafsson *et al.*, 1994).

Vegetable oils and fats do not contain 20-carbon or 22-carbon n-6 or n-3 fatty acids, as the elongation steps leading to 20:4n-6, 20:5n-3, and 22:6n-3 are only found in animal cells (Innis, 1991). As a result, these fatty acids are present in meat, egg yolk, liver, and other organ meats, with the highest concentrations of 20:5n-3 and 22:6n-3 in fatty fish such as herring, halibut, mackerel, and salmon (Achman, 1988). Dairy products contain very low amounts of 18:2n-6 and 18:3n-3, and their longer chain metabolites (20:4n-6 and 22:6n-3, respectively), (Kris-Etherton *et al.*, 2000). The absence of 20:4n-6 and 20:5n-3 in foods from plant origin and the considerable

variability in 18:2n-6 and 18:3n-3 in different vegetable oils results in a wide range of individual intakes of 18:2n-6 and 18:3n-3 fatty acids. Data from Allison *et al.*, (1999) suggested that the intakes of 18:2n-6, 18:3n-3, and 20:5n-3 in the diets of adult population in USA ranged from 12-18 g/d, 1-2 g/d, 0.1g/d, respectively, suggesting 7% and 0.5% energy intake from 18:2n-6 and 18:3n-3 fatty acids, respectively.

Denomme *et al.*, (2005) recently reported the average dietary intakes of 1.3 ± 0.2 g/d for 18:3n-3 and 82 ± 33 mg/d for 22:6n-3 among Canadian women, as determined by direct quantitation of duplicate food collections. Innis and Elias (2003) also estimated a higher intake of 22:6n-3 (160 ± 20 mg/d) and 20:4n-6 (0.12g/d) in pregnant Canadian women in Vancouver, by Food Frequency Analysis compared to average intake of Canadians. Subjects reported consuming 1.5 seafood meals/week. These values correlated with higher plasma 22:6n-3 in these women.

1.4. Fatty acid composition of human milk

Human milk fat is a source of essential n-6 and n-3 fatty acids as well as energy for breast-fed infants. The fat content of mature human milk is about 3.5 to 4.5 g/dl, representing about 40-55% of total milk calories (Jensen, 1989). Milk fat contains about 98% triglycerides and 1.3% phospholipids and small proportions of diglycerides, monoglycerides, cerebrosides, cholesterol esters and free fatty acids. Milk fat is secreted as globules, where triglycerides are surrounded by the phospholipid-rich plasma membrane of the mammary gland epithelial cell (i.e., the milk lipid globule membrane (Neville *et al.*, 1984). Small amounts of cholesterol (0.5%) and proteins are also present in the globule membrane (Jensen, 1989). The milk lipid globule membrane acts as an emulsion stabilizer and represents the membrane of the secretory mammary gland cell.

The globule size ranges from 1 to 10 µM and presents a large surface area (500 cm^2/mL) to lypolytic enzymes (Jensen, 1996).

Milk fatty acids are derived from synthesis in the mammary alveolar cells and by uptake from plasma. Fatty acids, cleaved from plasma triglycerides by the mammary gland-associated lipoprotein lipases, are absorbed by the secretory cells and used with glycerol-3-phosphate for the synthesis of milk fat. Due to the mammary gland-specific enzyme, thioesterase II, fatty acids synthesized in the mammary cells are generally of shorter carbon chain length of 10-carbon to 14-carbon, whereas 16-carbon to 20-carbon fatty acids are taken from the plasma. Medium chain fatty acids are the minor components of milk, usually representing <2% of the total fatty acids (Jensen, 1989; Innis, 1992a).

1.4.1. Supply of n-6 and n-3 fatty acids in milk

The n-6 and n-3 fatty acid concentrations in human milk vary widely, both within and between different populations of women (Innis, 1992; Jensen, 1999) and are influenced by maternal diet (Innis, 1991). Human milk from North American women consuming a mixed diet, contain 30-38% 18:1n-9; 7-18% 18:2n-6; and 1-1.5% 18:3n-3 of total fatty acids (Innis and King, 1999). The desaturation and elongation products of 18:2n-6, including 20:3n-6, 20:4n-6, and 22:4n-6 usually account for about 1% of the total fatty acids, and about 0.5-1.0% are as 18:4n-3, 20:5n-2, 22:5n-3, and 22:6n-3. Major differences are seen in milk fat 18:1n-9, 18:2n-6, 20:5n-3, and 22:6n-3, depending on the type and amount of dietary fat consumed by mothers (Innis, 1992; Innis and King, 1999).

Studies by Inull *et al.* (1959), in the late-1950's reported 7.7% 18:2n-6 in milk from women, following their typical diet, 10.4% 18:2n-6 when 40% of the dietary energy was from lard, and a 4-fold increase to 41.4% 18:2n-6 in milk when the diet had 40% energy from corn oil (52% 18:2n-6), reflecting the composition of dietary fat in mothers' milk. Over the years, the mean amount of 18:2n-6 in milk of women following a Western diet increased to 12-16% (Innis, 1992). The range among individual women, particularly for 18:2n-6, is wide (Innis, 1992; Jensen, 1999; Innis and King, 1999). Levels of 18:2n-6 as high as 28.8% in the breast milk of vegetarian women, and of 31.7% in vegan, compared to 6.9% in omnivorous women, have been reported (Innis and King, 1999; Sanderes and Reddy, 1992).

Analyses of human milk fat from different regions of the world has shown that the intake of 18:2n-6 is also high in some countries that follow non-Western diets (Jensen, 1999). Studies on human milk fat composition from regions of Africa where the total fat intake is low and the diet is largely based on grains, have also found mean concentrations of 11-12% 18:2n-6 in human milk fat (Okolo *et al.*, 2000). About 10-12% 18:2n-6 in the milk fat from Hungarian mothers consuming about 35% energy from fats was reported (Drury *et al.*, 1986). The amount of 18:2n-6 in breast milk samples from French mothers was in a range of 7-31%, and 20:4n-6, 18:3n-3, 20:5n-3, and 22:6n-3 were reported to be 0.4-1.1, 0.06-1.29, <0.03-0.32, and 0.1-1.04%, respectively (Rocquelin *et al.*, 2001). The average amount of 18:2n-6 in human milk was 18.4% in Zhangzi, China (Chulei *et al.*, 1995); 13.3% in Japan (Nakajima, 2000); 15.8% in Peninsula (Auestad *et al.*, 2001); 11.7% in Europe (Koletzko *et al.*, 1992); and 11.5% among African women (Koletzko *et al.*, 1992). Analysis of breast milk samples from

Vancouver mothers showed that the mean percentages of 18:2n-6, 18:3n-3, and 20:4n-6 were 12.1%, 1.4%, and 0.4% of the total milk fatty acids, respectively. The milk concentrations of these fatty acids showed a positive correlation among the above fatty acids and their respective fatty acids in the plasma triglycerides and plasma phospholipids of the breast fed infants (Innis and King, 1999).

Despite wide variations in milk 18:2n-6, levels of 20:4n-6 in human milk are fairly constant (Makrides *et al.*, 1995). Human milk concentrations of 20:4n-6 appear to be much more tightly regulated, and are not reduced by high intakes of 20:5n-3 and 22:6n-3, or in vegetarian or vegan women (Sanders and Reddy, 1992; Nakajima, 2000; Jensen *et al.*, 2000). On the other hand, milk levels of 20:5n-3 can be increased by 10-fold or more, as a result of supplementation with fish oil or by higher dietary intake of fish (Harris *et al.*, 1984; Innis and Kuhnlein, 1988). Many studies have also shown that supplementation of lactating women with fish or fish oil increases the amount of 20:5n-3 and 22:6n-3, while supplementation with single cell oils containing 22:6n-3 increases the 22:6n-3 content of human milk (Helland *et al.*, 2001; Fidler *et al.*, 2000; Harris *et al.*, 1984). The results from studies by Innis (2003), in which lactating women supplemented their diets with flax seed oil to provide 3g 18:3n-3, or salmon oil to provide about 300 mg 22:6n-3 for 14 days (10 women per group) suggest that dietary 22:6n-3 supplementation increases the secretion of 22:6n-3 and 20:5n-3 in human milk, whereas supplementation with 18:3n-3 does not.

Information on fatty acids in the milk from other species is also available. Low levels of 18:2n-6 (2% fatty acids) and negligible amounts of long chain polyunsaturated fatty acids in bovine milk are well known (Jefcoat and James, 1984). When fed to

infants, bovine milk fat results in lower plasma levels of n-6 and n-3 fatty acids (Holman, 1965). The percent of n-6 and n-3 fatty acids in rat milk (Sinclair and Crawford, 1973) are similar or higher than in human milk (Jefcoat and James, 1984) and depends on the fatty acid composition of the diet. As in humans, the fatty acid composition of milk in the rat depends on the maternal diet fat content and composition (Sinclair and Crawford, 1973; Yeh *et al.,* 1990). During lactation, the dietary lipids absorbed by the intestine are readily passed into the milk. An oral load of either long or medium chain triglycerides or the administration of diets with a high concentration of fats to lactating rats inhibits the rate of mammary gland lipogenesis *in vivo* (Grigor and Warren, 1980). Therefore, dietary long chain fatty acids through milk feeding appear to be preferentially channeled into structural lipids.

1.5. Diet fat and intestine development

The intestinal mucosa is a dynamic structure that undergoes biochemical, ultrastructural, and morphological changes throughout life (Thomson and Wild, 1997). The absorptive cells of the small intestine have the capability to adapt their structure and function in response to changes in the intraluminal nutrient content (Henning, 1981). Lipid components of the intestinal membrane are the main determinants of its physicochemical properties (Clandinin *et al.,* 1991). One of the major compositional changes during postnatal development is the increase in lipid-protein ratio in the intestinal microvilli membrane of newborn compared to adults, as shown in rabbits (Pang *et al.,* 1983). Conceivably, alterations in the interactions between lipid-protein and lipid-lipid molecules may be responsible for functional integrity of these membrane barriers (Chu and Walker, 1988). Increases in jejunal and ileal wet weights, during the

first 24-hours after birth, have been reported to occur in response to milk feeding in the nursing pig (Widdowson, 1984) and rat (Berseth *et al.,* 1983). During this period, the weight of the intestinal mucosa increases markedly and this has been attributed to incorporation of milk proteins and lipids in enterocytes (Widdowson *et al.,* 1976).

Several lines of investigation suggest that human milk modulates the functional integrity of the gastrointestinal tract during breast-feeding and long afterwards. The neonatal small and large intestine undergo rapid growth and reorganization as an immediate response to milk (Holt and Buboois, 1991). The interfaces and interactions of milk components with the gastrointestinal tract are complex. Briefly, these interactions involve the action of digestive agents on milk components, the binding of components or their parts with the mucosa, to modulate the growth and development of the gastrointestinal tract, and the absorption of milk components or parts into tissues. Modulation of the microvilli membrane, with respect to changes in the membrane fatty acid composition, begins *in utero* (Chu and Walker, 1988). *In utero,* the fetus is supplied with most of its substrate needs by the placenta. The healthy fetus swallows large amounts of fluid, lung lipids, and oral/nasal secretions, which has an important role in small intestinal development, as well as in the maturation of the small intestine (Pritchard, 1966).

1.5.1. Diet fat and intestinal structural fatty acids

The phospholipid bilayer constitutes the major and basic structural components of the microvillus membrane (Thompson *et al.,* 1989a). It may greatly influence the biophysical and biochemical properties of the microvillus membrane, which is the major component of the intestinal mucosal barrier (Chu and Walker, 1988). In general,

phospholipid alterations may directly affect membrane fluidity (Lands, 1989), activity of membrane-bound enzymes (Sandermann, 1978), the membrane transport system (Green *et al.*, 1980), receptor-binding events, and protein attachment to membranes (Berridge, 1984).

Studies by Chu and Walker (1989) compared newborn and adult rat microvillus intestinal membranes and suggested that the major phospholipid classes in the newborn rat were phosphatidylcholine and phosphatidylethanolamine, which constituted 60-70% of the total phospholipids. With respect to saturated fatty acids, ratios of 16:0 to 18:0 in the newborn, were three-times higher than in adult rats. With respect to unsaturated fatty acids, ratios of 18:1 to 18:2 in the newborn, were twice those of the adult rat. Therefore, differences in phospholipid fatty acid composition between newborn and adult rat intestines may be adaptive response of the intestine to different nutritional conditions, though age-related effects could not be excluded (Thompson *et al.*, 1989b).

Brasitus *et al.* (1985) compared the composition and properties of the brush border and basolateral cell membranes in the small and large intestine of adult rats, given diets rich in unsaturated corn oil (58% 18:2n-6) or saturated butter fat (6% 18:2n-6). After six weeks, the level of 18:2n-6 was increased in the small intestinal microvillus membrane of rats fed the high 18:2n-6, except for the proximal colon basolateral membrane. Rats fed a high 18:2n-6 diet had 33% less 18:1 and 39% more 20:4n-6 in the small intestinal basolateral membranes, but not in small intestinal microvillus membrane, when compared to the saturated fat diet group. The high 18:2n-6 diet increased overall unsaturation of acyl chains and enhanced lipid fluidity of enterocyte

microvillus and basolateral membranes, and the lipid fluidity of colonocyte basolateral membranes.

1.5.2. Diet fat and intestinal uptake of nutrients

Many studies have shown that dietary fat affects intestinal cell membrane properties including the activities of membrane associated enzymes, ion channels, transport properties, signal transduction pathways and intestinal uptake of nutrients. For example, the intestinal uptake of glucose by sodium-dependent glucose transporters (SGLT1) is influenced by dietary fatty acid composition (Thomson *et al.,* 1986). Furthermore, Jarocka-Cyrta *et al.* (1998) suggested that dietary fat selectively altered intestinal glucose uptake in suckling pups. There were no differences in glucose uptake in the jejunum or in the ileum in pups when mothers diet was switched from high 20:4n-6 diet to the low n-6/n-3 diet; in fact, when mothers diet was switched from the 22:6n-3 to the 20:4n-6 diet, this resulted in enhanced ileal, but not jejunal, glucose uptake in pups. Interestingly, when mothers diet was switched during lactation, pups intestinal glucose uptake was not affected. Hence, the intestinal responsiveness to changes in glucose uptake resulting from alterations in the mother's diet during lactation was partly influenced by dietary fatty acid composition during pregnancy (Thomson *et al.,* 1987; Thomson and Wild, 1997).

In later studies, Jarocka-Cyrta *et al.* (1998) also demonstrated that maternal dietary fatty acid consumption during gestation had different effects on the adaptability of jejunal and ileal fructose transport. Although continuous feeding of the high n-6/n-3 diet to rat mothers was associated with higher rates of glucose uptake, the rates of fructose uptake were lower. The differences observed in the adaptation of the intestinal

glucose and fructose transport, in response to the various dietary lipids as a consequence of exposure to different fatty acids during gestation, suggests that transporters (SGLT1 and GLUT5) are influenced differently by various dietary fatty acids. The ontogeny of the intestinal transport of fructose is influenced by the composition of the mother's diet during pregnancy and lactation, and short-term switching of the mother to another diet during lactation did not necessarily correct the low absorption of fructose (Thomson and Wild, 1997).

In later studies, Jarocka-Cytra et al. (1998) further demonstrated that intestinal uptake of long-chain fatty acids in suckling rats is also influenced by variations in dietary lipids in the pregnant dams' diet, but that the pattern of changes was different for various fatty acids and for the jejunum compared with ileum. Further, early exposure to a high n-6/n-3 (14% 16:0; 40% 18:1n-9; 17% 18:2n-6) and a high 20:4n-6 diet (14% 16:0; 40% 18:1n-9; 17% 18:2n-6 plus 1.2% 20:4n-6) through milk feeding, prevented changes in the jejunal uptake of most fatty acids in the suckling pups after the mothers' diet was switched to other diets. In animals whose mother consumed a high 22:6n-3 diet during lactation, the uptake of fatty acids was decreased in the jejunum and ileum. Hence, dietary fatty acids consumed during gestation in rats appeared to have different effects than when consumed during lactation, on the intestinal transport of lipids in suckling pups, and this effect becomes more pronounced after the maternal dietary lipid composition is switched during the suckling period. Taken together, these findings suggest that dietary fat may alter the intestinal uptake of various nutrients.

Despite this, the effect of fat composition of diet on the developing intestine has received little attention. Nevertheless, some information is available on the effects of dietary n-6 and n-3 fatty acids on the composition of various tissues.

1.6. Diet fat and fatty acid composition of different tissues

Numerous studies have shown that the fatty acid composition of triglycerides and phospholipids in several tissues are associated with relationships between the exogenous n-3 and n-6 dietary polyunsaturated fatty acids and the endogenous n-7 and n-9 fatty acid. Lands *et al.* (1990) fed rats diets varying in 18:1n-9 (20-80%), 18:2n-6 (3-58%), and 18:3n-3 (1-15%) and measured fatty acids in liver, plasma, and adipose tissue triglycerides. The tissue concentrations were about 30% as saturated acids, 67% as 16-carbon and 18-carbon unsaturated acids, and only about 2% as 20-carbon and 22-carbon highly unsaturated fatty acids. The tissues maintained a linear relationship between the amount of 18-carbon polyunsaturated fatty acids in the diet and the amount in the tissue triglycerides. The total phospholipids in liver, plasma, and red blood cells were about 45% of the fatty acids as saturated fatty acids and 20-30% as 20-carbon and 22-carbon, highly unsaturated fatty acids, irrespective of the differences in proportions of n-3, n-6, and n-9 fatty acids. In all three tissues, the 20-carbon highly unsaturated fatty acids of the n-3, n-6, and n-9 types were maintained in a competitive hyperbolic relationship. Similar to liver, plasma and adipose tissue triglycerides, Swanson *et al.* (1987) also reported similar proportions of fatty acids in rat kidney. Likewise, the higher amount of 18:2n-6 in diet was associated with increased 20:4n-6 in liver and kidney PE in rats (Croft *et al.*, 1984).

Hence, there is convincing evidence to support the view that dietary n-6, n-9 and n-3 fatty acids influence the fatty acid composition of blood cells, liver, heart, kidney and brain (Putnam *et al.,* 1982; Pita *et al.,*1988; Yeh *et al.,* 1990; Sanders and Reddy, 1992; Bourre *et al.,* 1993; Innis and King, 1999). In addition, the maternal diet has been shown to be important in the development of human and rat fetus. The type of maternal dietary fat consumed during gestation influences the fatty acid composition of lung-phospholipids and fetal growth and development (Clarke *et al.,* 1988). During the postnatal period, the quality and composition of dietary fat supplied to infants has important structural and functional effects on the rapidly growing tissues (Thomson *et al.,* 1989a; Innis, 1991). Therefore, with dietary manipulations, complex interactions and displacements between n-6 and n-3 fatty acids takes place at the tissue level, ultimately affecting the activity of the enzymes responsible for the generation of lipid mediators, such as the eicosanoids (Mostofsky *et al.,* 2000). So, it would be reasonable to expect that with dietary manipulations, intestinal fatty acid composition may be altered. It is also important to understand how these dietary manipulations may influence or affect various disease processes especially diseases of gastrointestinal tract.

1.7. Diet fat and inflammatory bowel disease (IBD)

1.7.1. Overview of IBD

Decades of research have been dedicated to the factors underlying the cellular and molecular responses in IBD. Currently, the term IBD encompasses more than just two diseases (Crohn's and ulcerative colitis), and refers to a group of diseases, triggered and perpetuated by a variety of diverse genetic, environmental, and immunologic factors that share similar clinical manifestations (Kischner, 1989; Oliva-Hemker and

Fiocchi, 2002). In ulcerative colitis, inflammation may be restricted to the mucosa of the colon, or may involve the submucosa of any segment of the intestinal tract, as in Crohn's disease (Fretland et al., 1990).

Reports of both diseases have been made since the latter half of the 19th century. Retrospective studies, however, have shown increases in the incidence of both diseases, starting in the 1930's, with a consistent increase in Europe and the Unites States (Ekbom et al., 1991). The etiology of the disease is complex and multifactorial. Epidemiology studies suggest that the incidence of IBD has increased worldwide with a significant increase in the pediatric population (Armitage et al., 2001; Cosgrove et al., 1996). The incidence of IBD is relatively higher in economically-developed countries of Northern and Western Europe, the United States and Canada. The incidence of IBD has been reported as 1-10/100,000 for Crohn's disease and 5-18/100,000 for ulcerative colitis (Russel and Stockbrugger, 1996). Information on the incidence of IBD in the Canadian population is limited, however, though data from Swedish prospective studies indicate that the incidence of IBD in children increased from 4.6/100,000 during the 1980's to 7.0/100,000 during the late-1990's. The greater contribution of this increase came from ulcerative colitis, while Crohn's disease remained relatively unchanged (Lindberg et al., 2000).

These diseases are suggested to have a genetic basis, from clinical observation, that included widely different prevalence rates in different ethnic groups, an increased prevalence in first cousins of patients, disease concordance among monozygotic twins, and a high degree of concordance in disease pattern and behaviour among family members (Jewell, 1998). The highest prevalence rates have been reported in Ashkenazi

Jews living in North America. Interestingly, the incidence of ulcerative colitis in Asians living in parts of the United Kingdom is at least as high as that found in the native Caucasian population, reflecting the involvement of both genetic and environmental factors in the disease outcome (Probert CSJ, 1992). In Canada, a number of new cases of IBD have recently been seen in children born to parents who emigrated from countries where IBD is rare (Oliva-Hemker and Fiocchi, 2002).

1.7.2. Alterations in intestinal mucosal defence in IBD

The gastrointestinal mucosa forms the interface between the systemic circulation and the external environment of the gastrointestinal lumen (Powell, 1981). In addition to its digestive and absorptive capacity, this large surface forms both a structural and an immunologic barrier to protect the systemic circulation from the infectious pathogens and toxins. Hence, the structure of the gut, under normal circumstances, maintains a physical barrier and normal bacterial microflora and innate substances (mucus, defensins, lysozyme, and lactoferrin) secreted by the gastrointestinal tract. Its structure is also important in maintaining this physical defense and for containing the bacterial pool (Powell, 1981).

The intestinal mucosal defense consists of two components: extrinsic and intrinsic mechanisms. The intrinsic barrier consists of physical and structural properties of the small intestine. The extrinsic mechanisms consist of mucus, innate humoral factors (lactoferrin, lysozyme, and peroxidase), resident microflora, and the mucosal immune barrier itself (DeWitt and Kudsk, 1999). The gastrointestinal mucosa has a surface area of approximately 300 to 400 m^2 owing to the microvilli that greatly amplify the surface area, which is composed of multiple cell types such as columnar cells, goblet

cells, paneth cells, enteroendocrine cells, and intraepithelial lymphocytes (Bloom and Fawcett, 1994). Goblet cells serve as the mucus-secreting cells of the intestinal epithelium. Mucus covers this cell layer, forming a protective layer and preventing direct contact between the cells and the external environment. Beneath the mucosal cell layer lies the lamina propria, containing numerous immune cells including lymphocytes, macrophages, and dendritic cells that are crucial to the intestinal immune response (DeWitt and Kudsk, 1999). The absorptive epithelial cells of the mucosal layer, the enterocytes, make up the majority of the mucosal epithelium. This continuous single layer of columnar epithelial cells lines both the crypts and the villi (Grumbiner, 1987). The microvillus membrane of the small intestinal epithelial cells are highly specialized to perform functions of nutrient digestion and absorption, and regulate fluid and electrolyte balance by absorptive and secretory mechanisms (Thomson *et al.,* 1989a). Enterocytes are connected to the surrounding cells at the apices, by junctions and desmosomes that are present at all levels of the lateral membranes between adjacent cells. These junctional complexes separate the external from the internal environments, act as a selective barrier, and attach adjacent cells to one another (Boyer and Thiery, 1989). In addition to the cellular elements and antibody-producing cells of the body, a number of substances exist throughout the gastrointestinal tract that have antimicrobial functions. These numerous innate substances are present in many mucosal secretions and produce a wide array of antimicrobial properties to control the bacterial milieu (DeWitt and Kudsk, 1999).

Active episodes of IBD are characterized by the extravasation and infiltration of a large number of neutrophilic polymorphonuclear leukocytes into the lamina propria.

This enhanced inflammatory infiltrate is accompanied by mucosal injury, as in ulcerative colitis, and transmural injury, as in Crohn's disease, that includes disruption of the extra-cellular matrix, epithelial cell injury and ulceration (Riddel, 1988). From the molecular perspective, inflammation is the result of a complex series of cellular interactions between target cells (epithelial cells, smooth muscle cells, and endothelial cells), cells of the immune system (neutrophils, monocytes/macrophages, and lymphocytes), their products (eicosanoids, cytokines, lymphokines, chemokines, and immunoglobulins), and blood components (platelets), (Wallace *et al.*, 1998). Vasodilation, as a result of smooth muscle relaxation, results in increased blood flow, whereas endothelial cell contraction results in increased vascular permeability and edema. These effects are mediated by inflammatory mediators released predominately from activated leukocytes that migrate into target areas (James *et al.*, 2000). Histological analysis of colon mucosa has been shown to be a useful indicator of colonic epithelial damage in experimental animals, which revealed severe alterations in the colonocytes of rats with ulcerative colitis with areas of necrosis and desquamated epithelial cells, as well as loss of intercellular junctions and lobulated nuclei with heterochromatin accumulation in the nuclear envelop (Nieto *et al.*, 2002).

1.7.3. Role of n-6 and n-3 fatty acids in IBD

Epidemiological studies among the Japanese have indicated an association between the high dietary intake of n-6 polyunsaturated fatty acids and their contribution in the development of IBD (Shoda *et al.*, 1996). The suggestion that unsaturated fatty acids modulate immune function arose primarily from animal studies, demonstrating the potent anti-inflammatory effects of n-3 fatty acids (Vilaseca *et al.*, 1990; Yaqoob, 2003).

Some clinical studies have also shown positive effects of n-3 fatty acids in patients with IBD, notably, 20:5n-3 may have anti-inflammatory effects in patients with ulcerative colitis (Belluzi *et al.,* 2000). Previously, dietary fat restrictions, due to steatorrhea associated with ulcerative colitis, were recommended, but the benefits of lipids on the intestinal mucosa are now recognized and promoted (Nieto *et al.,* 2002). In previous studies, Nieto *et al.* (1998) observed that the administration of high amounts of 18:3n-3 to adult rats with ulcerative colitis led to a minimum stenosis score, lowered alkaline phosphatase, and lowered PGE_2 and LTB_4 in colonic mucosa compared to rats fed high 18:1n-9 or high 18:2n-6+18:3n-3 diets. Inui *et al.* (1996) also investigated the efficacy of an 18:3n-3-rich perilla oil, compared with a high 18:2n-6 with 5% energy from fat in adult rats. The levels of 20:3n-6 and 20:4n-6 were significantly higher, while levels of 18:3n-3 and 20:5n-3 were significantly lower in the colonic phospholipids of rats in the high 18:2n-6 group compared to high 18:3n-3 group. Also, no differences were seen in the 18:2n-6 and 22:6n-3 levels in the colonic phospholipids between the groups. In a later study, lower LTB_4 synthesis, and less colonic thickness and damage scores were observed in the high 18:3n-3 group, compared to the high 18:2n-6 group. Likewise, Shimizu *et al.* (2001) fed a perilla oil-enriched diet containing 18:3n-3 (63.2% of total fatty acids) or a soybean oil diet containing 18:3n-3 (5.1% of total fatty acids) to adult Wister rats and further used 4% dextran sulfate sodium as the inflammatory mediator and not surprisingly found lower LTB_4 levels in the colonic mucosa of the rats fed the high n-3 fatty acid diet.

Cell culture studies suggest that short-term dietary exposure to highly purified 20:5n-3 and 22:6n-3 suppresses mitogen-induced mouse T-cell proliferation by

inhibiting interleukin-2 secretion and interleukin-2 receptor mRNA expression, accompanied by reductions in the production of essential lipid second messengers, diacylglycerol and ceramides (Soyland *et al.*, 1993; Arrington *et al.*, 2001). The ability of 22:6n-3 to suppress T-cell interleukin-2 production is consistent with findings in splenic mononuclear cells (Jolly *et al.*, 1997). There is some evidence from human studies suggesting changes in mononuclear cell membrane fatty acid composition are accompanied by changes in LTB_4 generation and endotoxin-stimulated cytokine production (Lee *et al.*, 1985; Endres *et al.*, 1989).

Several human studies have also shown beneficial effects of dietary n-3 fatty acids in IBD. In one study, 96 patients were given either 4.5 g of 20:5n-3/day (treatment group) or olive oil (high 18:1n-9, placebo) for 1 year. A significant lower levels of LTB_4 (<50%) were seen in the patients receiving the fish oil (Hawthorne *et al.*, 1992). Several other clinical trials indicated significant improvement in symptoms and histological appearance in patients with ulcerative colitis who were given 20:5n-3 (in the range of 2.7-5.4 g/day) (Lorenz *et al.*, 1989; Salomon *et al.*, 1990; Stenson *et al.*, 1992). Belluzi *et al.* (2000) administered an enteric-coated fish oil preparation (500 mg) to patients with Crohn's disease for 1 year. The fish oil was effective in reducing LTB_4 and the relapse rate in these patients. A significant reduction in the plasma 18:2n-6 and 20:4n-6 levels was also observed, with increased 20:5n-3 in these patients.

Hence data from experimental and clinical studies suggest that the development of IBD is closely related with fatty acid composition and eicosanoid generation in the intestinal tissue (Belluzi *et al.*, 2000). The colonic mucosa of human patients with ulcerative colitis has an increased capacity to synthesize eicosanoid mediators, such as

LTB$_4$ and PGE$_2$ (Sharon and Stenson, 1984; Raab et al., 1995). PGE$_2$ levels correlates with tissue levels of tumor necrotic factor-α and myeloperoxidase activity (Lauristen et al., 1987). LTB$_4$ alters the absorptive function and cellular immunity of the intestine leading to cell damage (Nieto et al., 1998). High levels of eicosanoids in the colonic mucosa have been suggested to be partly due to an increased synthesis of 20:4n-6 (Inui et al., 1996). One of the proposed mechanisms is that 20:5n-3 competes with 20:4n-6 as a substrate for the cyclooxygenase and lipoxygenase enzymes. LTB$_5$, synthesized from 20:5n-3, differs in its physiologic activity from the various 20:4n-6-derived leukotrienes (Lee et al., 1985, Endres et al., 1993).

Taken together, these findings suggest that diet fat is a major modifiable factor that is known to influence the susceptibility of inflammatory bowel disease. Several studies have begun to examine the effects of dietary fat on the prevalence of IBD. High dietary intakes of n-3 fatty acids have shown therapeutic benefits in experimental and some clinical studies. Conceivably, n-6 fatty acids, which are metabolic precursors of eicosanoids, may be involved in increased incidence of IBD. The increasing incidence of IBD in Western countries with highest prevalence seen in young children (Cosgrove et al., 1996; Phavichitr et al., 2003), warrant further investigation in the role of dietary n-6 and n-3 fatty acids in the development of IBD. It is important to examine the effects of early dietary exposure of n-6 and n-3 fatty acids the intestinal development and further the functional significance of these effects in IBD.

1.8. Summary

Lipids are important for growth and development, and for the long-term health of both healthy children and children with gastrointestinal disorders. During pregnancy and lactation, the maternal diet can affect the growth, development, and function of various tissues including the gastrointestinal tract of the developing infant. Over the years, lipids have been viewed as a largely exchangeable source of energy. Today, however, the quantity and quality of dietary lipids is recognized to be crucial for infant growth and development. The significance of differences in n-6 and n-3 fatty acids in human milk, and the effect of maternal diet fat composition on markers of inflammatory mediators in the gastrointestinal tract of infants have received little attention. Animal models provide an excellent opportunity to explore the advantageous or untoward effects of dietary n-6 and n-3 fatty acids, however, few studies have attempted to provide information using adult animal models. The gastrointestinal tract is the first interface between the diet and body and several lines of investigation suggest that milk feeding modulates the functional integrity of the gastrointestinal tract during breast-feeding and long afterwards. Depending on early dietary experiences, the ability of the intestine to adapt appropriately, in response to various nutritional, physiological, or pathophysiological challenges, may be affected or impaired. Such possible influences make it important to understand in more detail how milk feeding promotes intestinal tissue levels of n-3 and n-6 fatty acids. Presently, the role that diet fat plays in the etiology, pathogenesis, or phenotypic expression of IBD is unclear. High dietary intake of n-3 fatty acids have also shown therapeutic benefit in experimental models of colitis and in several clinical studies including patients with ulcerative colitis and Crohn's disease. The absence of

significant therapeutic efficacy in some clinical trials of n-3 fatty acids in patients with IBD may be explained by differences in study design, patient selection, the formulation, dose and duration of n-3 fatty acids. A plausible link between dietary essential 18:2n-6 and IBD has been suggested, which is the metabolic precursor for synthesis of a range of biologically active compounds such as eicosanoids. As the 18:2n-6 consumption has increased over the last few decades, some nutrition experts have recommended a decrease in n-6 fatty acid intake. However, the data to support the possibility that high dietary intakes of 18:2n-6 may predispose the neonatal intestine to increased inflammatory responses is not available. Therefore, the purpose of the present study was to establish if the composition of maternal dietary fat during gestation and lactation alters the intestinal phospholipid fatty acid composition of the offspring, and if differences have a functional significance with respect to IBD, specifically ulcerative colitis in the nursing offspring.

2. Hypotheses

1) The dietary (milk) fatty acid supply, as determined by maternal dietary 18:2n-6 and 18:3n-3 intake will alter intestinal membrane phospholipid (n-6 and n-3) fatty acids in suckling rat pups.

2) Changes in intestinal phospholipid fatty acids are associated with intestinal inflammatory response to dinitrobenzene sulfonic acid (DNBS) colitis in suckling pups.

3. Objectives

The overall objective of the study was to investigate the effects of a high maternal dietary intake of n-6 fatty acids on the intestinal fatty acids and inflammatory responses in suckling rat pups.

The specific objectives were:

1) To determine the effect of maternal dietary intake of n-6 and n-3 fatty acids on rat milk fatty acid composition.

Specific Aim:

- To study fatty acid composition of rat milk on postnatal day 12.

2) To determine if dietary dependent differences in the composition of rat milk fatty acids alters the composition of intestinal membrane phospholipid fatty acids in suckling rat pups.

Specific Aim:

- To study the fatty acid composition of the major phospholipid classes phosphatidlycholine (PC), and phosphatidylethanolamine (PE) in the jejunum and colon of suckling rat pups.

3) To develop a model of inflammatory bowel disease in suckling rat pups (dose and time studies).

Specific Aim:

- To develop appropriate dose and time frame for the induction of experimental colitis by using DNBS in suckling pups.

4) To determine if the differences in intestinal phospholipid fatty acids are associated with differences in the markers of inflammatory mediators in the colon of suckling rat pups following DNBS colitis.

Specific Aims:

- To determine the macroscopic and histological responses in the colon of the pups in response to DNBS induced colitis.

- To determine the myeloperoxidase activity (a marker of neutrophils infiltration) in the colon of suckling rat pups in response to DNB induced colitis.

Diet

Linoleic acid
18:2n-6

Enzymes

Linolenic acid
18:3n-3

Δ6 desaturase

Gamma-linolenic acid
18:3n-6

elongase

Octadecatetraenoic acid
18:4n-3

Di-homo- gamma-linolenic acid
20:3n-6

Eicosatetraenoic acid
20:4n-3

Δ5 desaturase

Arachidonic acid
20:4n-6

Eicosapentaenoic acid
20:5n-3

elongase

Docosatetraenoic acid
22:4n-6

Docosapentaenoic acid
22:5n-3

Δ 6 desaturase

24:4n-6

24:5n-3

β-oxidation

Δ 4 desaturase

24:5n-6

24:6n-3

Docosapentaenoic acid
22:5n-6

Docosahexaenoic acid
22:6n-3

Figure I.1: Schematic representation of n-6 and n-3 fatty acid metabolism (Innis, 1992).

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CHAPTER II

Maternal diet fat alters milk fatty acids, suckling pups' intestinal phospholipid fatty acids and intestinal responsiveness to experimental colitis.

1. INTRODUCTION

Inflammatory bowel disease (IBD) which includes ulcerative colitis and Crohn's disease is an intestinal inflammatory disorder of unknown etiology that are characterized by frequent remissions and exacerbation of disease (Morris *et al.*, 1989). The incidence of IBD is increasing worldwide and has the highest prevalence observed in economically developed countries and in both younger and older age groups (Armitage *et al.*, 2001; Cosgrove *et al.*, 1996; Phavichitr *et al.*, 2003).

The mechanisms responsible for the increase in IBD, and for the increasing prevalence in Western countries are unknown, however, a multifactorial basis including genetic susceptibility and environmental factors is considered likely to affect it (Holtman and Neurath, 2003). A plausible link between diet and IBD has been suggested, since IBD affects the site of nutrient absorption (Belluzzi *et al.*, 2000). Dietary fat is a major modifiable environmental factor that is known to influence susceptibility to a variety of diseases. Dietary essential n-6 fatty acid, linoleic acid (18:2n-6), which is the metabolic precursor for synthesis of a range of biologically active compounds (eicosanoids) may be involved as 18:2n-6 consumption has increased over the last half-century in many Westernized countries (Broughton and Wade, 2002; Simopoulos, 2002).

Several studies have begun to examine the effects of dietary fat on the prevalence of IBD (Belluzzi *et al.*, 2000; Chuah *et al.*, 1992), and among the Japanese, an increased incidence of Crohn's disease has occurred in association with an increased intake of 18:2n-6 and a relative decreased intake of the n-3 α linolenic acid (18:3n-3), (Shoda *et al*,1996). Consistent with this, the low incidence of IBD among the Inuit has been attributed to their habitual high intake of n-3 fatty acids, particularly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) from marine foods (Belluzzi *et al.*, 2000). High dietary intake of n-3 fatty acids have also shown therapeutic benefit in experimental models of colitis (Andoh *et al.*, 2003; Neito *et al.*, 2002; Yuceyar *et al.*, 1999) and in several clinical studies including patients with ulcerative colitis and Crohn's disease (Aslan and Triadafilopoulos, 1992; Belluzi *et al.*, 1993; Hawthorne *et al.*, 1992). The absence of significant therapeutic efficacy in some clinical trials of n-3 fatty acids in patients with IBD (Loeschke *et al.*, 1996; Lorenz *et al.*, 1989) may be explained by differences in study design, patient selection, the formulation, dose and duration of n-3 fatty acids (Belluzzi *et al.*, 2000).

The preventative and therapeutic efficacy of n-3 fatty acids, however is believed to involve both the reduction in cell membrane arachidonic acid (20:4n-6) and partial replacement of 20:4n-6 with 20:5n-3 and 22:6n-3 (Calder, 2003). Numerous studies have shown that changes in membrane phospholipid fatty acids affect cell membrane properties including the activity of membrane associated enzymes, ion channels, signal transduction pathways and synthesis of 20:4n-6 and 20:5n-3 derived eicosanoids (Calder, 2003; Innis, 2003). In addition, convincing evidence has been published to

show that the n-6 and n-3 fatty acid composition of the diet during development influences the composition of membrane phospholipid fatty acids of blood cells, the liver, heart and brain (Bourre *et al.*, 1993; Innis and King, 1999).

It is also known that the composition of maternal dietary fat greatly influences the fatty acid composition of milk (Innis, 1992; Jensen *et al.*, 2000). Maternal diets high in 20:5n-3 and 22:6n-3 are associated with high levels of 20:5n-3 and 22:6n-3 in milk, whereas diets high in n-6 fatty acids are associated with high levels of n-6 fatty acids (Helland *et al.*, 2001; Insull *et al.*, 1959). Furthermore, the composition of fat fed to animals after weaning influences intestinal membrane phospholipid fatty acids, intestinal transport processes and possibly early response genes (Perin *et al.*, 1999; Thiesen *et al.*, 2003; Thompson *et al.*, 1988; Thompson *et al.*, 1989). Despite this, the effect of the fat composition of the milk diet on the developing intestine has received little attention. In the present study we sought to establish if the composition of maternal dietary fat during gestation and lactation has functional significance with respect to development of the intestine in the nursing offspring. To the best of our knowledge, this is the first report to demonstrate that the composition of milk fatty acids influences the nursing offspring's susceptibility to chemically-induced colitis.

2. MATERIAL AND METHODS

Animals and diets

Female Sprague-Dawley rats (175-200 g) obtained from Charles River laboratories (Wilmington, MA) were housed individually in plastic cages in a controlled temperature (23°C) and 12-hour light-dark cycle in the animal unit at British Columbia Research Institute for Child and Family Health, Vancouver. Two weeks prior to mating, rats were randomly divided into semi-synthetic isocaloric diet groups with 20% weight as fat (n=6 litters/diet group). The diets differed in unsaturated fatty acids, oleic acid (18:1n-9), 18:2n-6 and 18:3n-3, but not in total fat or macro or micro nutrients (Innis and *de la* pressa Owens, 2001). Fat was provided as canola oil high in 18:3n-3, safflower oil high in 18:1n-9 or safflower oil high 18:2n-6 (Table II.1). The amounts of 18:2n-6 or 18:3n-3 in the semi-synthetic diets met the minimum dietary requirements of these fatty acids for pregnant and lactating rats (Reeves et al,1993). Reference diet group rats were assigned to receive a standard laboratory chow (Pico Lab Rodent diet 20, #5053; Purina Mills, St Louis) was high in saturated fatty acids (14:0, 16:0, 18:0), moderate 18:1, high 18:2n-6, 20:5n-3 and 22:6n-3, and low 18:3n-3 compared to high 18:3n-3 diet. Reference diet differed in total fat content (5% weight as fat) compared to other diets.

Each litter was randomly reduced to 12 pups/ dam within 24 hours of birth to minimize litter effect. Studies on the nursing pups were conducted on postnatal day 15, at which time no food other than the mother's milk had been consumed. All protocols were approved by the University of British Columbia Committee on Animal Care and Ethics Committee.

Milk collection & quantification of milk fatty acids

Rat milk from all dams in all diet groups was collected on postnatal day 12 by modifying the method of Jensen *et al.* (1996). Rats were given 0.4 ml oxytocin intraperitoneally (10 IE/ml, Sigma Chemical Co., St. Louis, MO, USA) and were sedated with isoflurane in a small induction chamber of 30cm X 20cm X 20cm. The papillae were cleaned with ethanol and the rats were manually milked to yield about 0.5 ml milk. Each session was about 5-7 minutes and the pups remained in their respective cages during the procedure. The milk samples were collected on ice and immediately frozen in nitrogen and stored at -70°C for further analysis purpose.

Fatty acids in rat milk sample (100μl) were determined following direct esterification to prevent loss of medium chain fatty acids using tridecanoic acid (C13:0) and heptadecanoic acid (C17:0), (500μl) as internal standard mix. Samples were mixed with two ml benzene/methanol (1/4, v/v); then added acetyl chloride and heated at 100°C in heating blocks for 60 minutes. Five ml potassium carbonate solution (6%) was added, and centrifuged at 2000g for 10 minutes. Pentane layer was evaporated in nitrogen, leaving methyl fatty acids. Samples were resuspended in hexane and fatty acids were quantified for their respective fatty acid methyl esters using a Varian 3400 GLC equipped with a flame ionization detector, Varian Star data system and 30 m x 0.25 mm ID glass capillary SP 2330 columns (Innis and King, 1999; Lepage and Roy, 1986).

Tissue preparation and lipid analysis

On postnatal day 15, all rat pups were anesthetized using isoflurane and were sacrificed by cervical dislocation. The small intestine from the level of the ligament of

treitz to an area just proximal to the ileocecal valve and the large intestine from the cecum to distal rectum were excised and removed. The jejunum (proximal one third of the small intestinal section measured) and large intestine were removed, flushed with ice-cold phosphate buffered saline (50 nmol/L phosphate, 100 nmol/L Nacl, pH 7.4). For lipid analysis, jejunum and colon samples within each litter were pooled, frozen in nitrogen and stored at -70°C until analyzed. The respective pooled tissues were homogenized in their entirety and total lipids extracted (Christie, 1982; Folch *et al.*, 1957), using Folch extractions with chloroform, methanol, saline (6:3:2.5, v/v) mix. Samples were centrifuged at 2000 rpm for 5 min and lower organic layers were collected and dried under nitrogen. Samples were reconstituted in hexane and further analysed for phospholipid classes separated by high-performance liquid chromatography (HPLC), (Waters 2690 Alliance HPLC, Milford MA) using a quaternary solvent system of hexane-petroleum ether, 97:3 (v/v); methanol-triethylamine-acetic acid, 765:15:13 (v/v/v); acetone-triethylamine-acetic acid, 765:15:13 (v/v/v); isopropanol-acetic acid, 800:40 (v/v) in a linear gradient with a flow rate of 2 ml/min. The sample chamber was kept at 18°C and the column heater at 35°C. The column was Waters YMC-Pack Diol 120NP, 25 cm x 4.6 mm ID, 5 µm particle size and 12 nm pore size. The column eluant was split 10:90 to an ELSD (Evaporative Light Scattering detection), (Alltech, model 2000; Mandel Scientific, Guelph, Canada) and a fraction collector (Gilson FC204, Mandel Scientific). ELSD and quantification of the separated lipid classes was performed with a nitrogen flow rate of 1.8 ml/min, a drift tube temperature of 60°C, and the impactor OFF. Calibration curves to determine the linear range of the analysis were

established using authentic standards for each lipid class, and samples were quantified using the external standard (betuline) method (Innis and Dyer, 2002).

Fatty acids in the recovered phospholipids were methylated using 2 ml hexane plus 1 ml of BF3 standard and samples were heated in heating blocks for 30 min. Samples were cooled at room temperature and added 3 ml saline and 6 ml pentane and centrifuged 2500 g for 10 min, then drawn the top (pentane) layer and dried under nitrogen. Fatty acid methyl esters were resuspended in an appropriate volume of hexane and transfered to a gas chromatograph autosample vial and quantified by gas liquid chromatography (GLC) using a Varian 3400 equipped with a flame ionization detector, Varian Star data system and 30 m x 0.25 mm ID glass capillary SP 2330 columns (Innis et al., 2001; Farquharson et al., 1995). Authentic standards such as PUFA I and PUFA II (Supelco); O7A and O8A (New Ceck); Rapeseed Oil (Supelco); RM5 (New Ceck) and individual fatty acids such as 14:1, 18:4n-6 and 18:3n-6 (Supelco) were quantified on GLC before the samples were quantified. A quality control sample of Cod Liver Oil was also quantified on GLC.

Dinitrobenzene sulfonic acid-induced colitis

On postnatal day 15, pups from each litter were randomly assigned (n=4 pups/litter/group) to receive 2, 4-dinitrobenzene sulfonic acid (DNBS), the vehicle control (50% ethanol), (n=4 pups/group) or a procedure control (0.9% saline), (n=4 pups/litter/group). Pups were sedated with isoflurane in a small induction chamber of 30cm X 20cm X 20cm and DNBS (100 µl of 30 mg/ml 50% ethanol), or an equivalent volume of ethanol or saline was instilled via a polyethylene tube (PE90, 1.27 O.D)

inserted 1 cm proximal to the anus (Morris *et al.*,1989). Pups were kept in the *Trendelenburg* position (head down) for 1 min then placed back in the cages. Pups in each treatment were coded by ear punching. Twelve hours later, the pups were sedated with isoflurane and were sacrificed by cervical dislocation. The inflammatory response was assessed in the distal colon using a macroscopic damage score, a histology score and through assay of myeloperoxidase (MPO) activity.

Macroscopic damage score

Rat pups colonic tissue was excised, cut along the mesenteric border, opened lengthwise and rinsed with phosphate buffered saline. Two observers blinded to the treatment assigned a macroscopic damage score by modifying the criteria used by Galeazzi *et al.* (2000), (Table II.2). Briefly, the macroscopic damage criteria were based on the presence or absence of each focal hyperemia, tissue adhesion and diarrhea in the animals. A mean macroscopic damage score and standard error of the mean was calculated for each group of rat pups with less than 9% scoring variability between observers.

Histological damage score

Rat pups colonic tissue was excised and a colonic segment (0.5 cm) was taken from the proximal margin of the site used for the assessment of myeloperoxidase activity. A corresponding site was obtained from control animals. The segments of colon were then fixed in 10% neutral buffered formalin for 24 hours prior to paraffin sectioning and staining with haematoxylin and eosin. Tissue cross-sections were

examined under a Nikon Eclipse E400 light microscope by 2 independent blinded observers and given a histological damage score using criteria adapted from Galeazzi *et al.* (2000), (Table II.3). Briefly, the histological damage score consisted of loss of mucosal architecture (graded 0-3, for absent, mild to severe), the extent of inflammatory cell infiltrate (graded 0-3, for absent to transmural) and the presence or absence of epithelialitis and crypt abscess formation (0-1) and goblet cell depletion (0-1). In each case a numerical score was assigned. Three tissue sections from each animal (each separated by at least 500µm) were assessed by two observers blinded to the treatment and averaged to obtain a mean histological damage score for each group with less than 5% scoring variability between observers.

Myeloperoxidase (MPO) activity

Rat pups colon was excised and a colonic segment (0.5 cm) was taken from the proximal margin of the maximum macroscopic damage site. A corresponding site was obtained from control animals. MPO activity was assayed (Boughton-Smith *et al.*, 1998) as a marker of inflammation (Smith and Castro, 1978). Briefly, colon tissue samples were placed into a solution of (1ml/50mg tissue) of hexadecyltrimethylamonium bromide, HTAB (0.5%, w/v) in potassium phosphate buffer (50 mM, pH 6.0). Samples were homogenized on ice for 30 sec, centrifuged for 30 min at 10,000 g and at 4°C. Aliquots of the 100 µl of the supernatant was added to 2.9 ml of 0.05% hydrogen peroxide in a o-dianisidine dihydrochloride (Sigma) solution in a cuvette and the absorbance was measured at 460 nm using Spectronic Genesys 5 Spectrometer (Milton

Roy, Rochester, USA). MPO activity was defined as the amount of enzyme able to convert 1 μmol H_2O_2 to H_2O/min at 25°C, and was expressed as U/mg of tissue.

Statistical analysis

The effects of diet and DNBS treatment were analysed by analysis of variance followed by post-hoc comparisons using the Tukey's HSD test to determine significant difference among the means, with the level of statistical significance set at $p<0.05$. Results within each litter were pooled and each litter was considered as n=1, 12 pups/ dam, with n=6 litters per diet group. The effect of DNBS and ethanol treatment were analysed using unpaired student's t-test. All the statistical procedures were performed using Statistical Package for the Social Sciences (SPSS, Version 12, Chicago, IL). Results shown are expressed as mean ± SEM.

3. RESULTS

Rat milk fatty acids

Rats fed the high 18:2n-6 (safflower oil) diet produced milk containing significantly higher 18:2n-6, 22:2n-6, 20:3n-6, 20:4n-6 and 22:5n-6 than rats fed the diets either high 18:1n-9 (high oleic safflower oil) or 18:3n-3 (canola oil) (p<0.05, Table II.4, Fig II.1). Rats fed the diet with high 18:3n-3 produced milk with significantly higher 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3 and a significantly lower 18:2n-6/18:3n-3 and 20:4n-6/20:5n-3 ratio than rats fed the other diets (p<0.05).

The differences in the total n-6/n-3 fatty acid ratio among the dietary oils were clearly reflected in the difference in the milk, with a significant increase in the n-6/n-3 fatty acid ratio among the groups in order: high 18:3n-3 < high 18:1n-9 < high 18:2n-6

(p<0.05). As in the reference diet, rats in this group produced milk high in saturated fatty acids such as 8:0, 10:0, 12:0 and 16:0, and high monounsaturated fatty acids such as 16:1, 18:1n-9 compared to other diet groups. There was no difference in the 18:3n-3, 20:5n-3, 22:6n-3, or ratio of total n-6/n-3 and 20:4n-6/20:5n-3, but the ratio of 18:2n-6/18:3n-3 in reference group rat milk was different compared to rat milk of high 18:3n-3 group. High 18:1n-9 diet which was both low in 18:2n-6 or 18:3n-3, showed no differences in n-6 fatty acids compared to high 18:3n-3 diet and no differences in n-3 fatty acids compared to high 18:2n-6 diet.

There was no difference in the birth weight of pups in all diet groups. On day 3, pups in high 18:3n-3 group weighed 10.2 ± 0.2 g, pups in high 18:2n-6 group weighed 10.1 ± 0.2 g, pups in high 18:1 group weighed 9.9 ± 0.2 g and pups in reference group weighed 10.0 ± 0.2 g. At 15 days of age, pups in the high 18:2n-6 group had a significantly higher body weight (30.4 ± 0.7 g) than pups in the high 18:1n-9 (22.2 ± 0.4 g), high 18:3n-3 group (23.5 ± 0.3 g) or reference pups (26.9 ± 0.9 g), p<0.05. There were no statistical significant changes in body weight among the groups following DNBS, ethanol or saline administration. The analysis by sex at 15 days of age showed that body weights were consistent across male and female offsprings in all diet groups.

Jejunal phospholipid fatty acids

Pups in the high 18-3n:3 group had significantly higher n-3 fatty acids (18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3) in jejunal phosphatidylcholine (PC) and phosphatidylethanolamine (PE) than pups in the 18:2n-6 group (p<0.05, Tables II.5, II.6). Pups in high 18:2n-6 group had significant higher n-6 fatty acids (20:2n-6) in

jejunal PC and both 22:4n-6 and 22:5n-6 in jejunal PC and PE than in high 18:1n-9 or high 18:3n-3 groups (p<0.05). Despite the high 18:2n-6 in milk, the amounts of 20:4n-6 in the jejunal PC and PE were not significantly different among the groups. Of note, the high 18:1n-9 diet, which was low in both 18:2n-6 and 18:3n-3, also resulted in significantly higher 22:6n-3 in the pups' jejunal PC and PE than in pups in the high 18:2n-6 group. The differences in fatty acid intake from milk were reflected in significantly different ratios of 18:2n-6/18:3n-3 and 20:4n-6/20:5n-3 in the pup intestinal lipids, with consistently lower 18:2n-6/18:3n-3 and 20:4n-6/20:5n-3 ratios in the jejunal PC and PE of pups in the high 18:3n-3 groups than in the high 18:1n-9 or high 18:2n-6 groups. Similar to the diet and milk fat, the n-6/n-3 fatty acid ratio in jejunal PC and PE were significantly lower than high 18:3n-3 compared to high 18:1n-9 or high 18:2n-6 (p<0.05).

Interestingly, there was no difference in 22:6n-3 in jejunal PC and PE of pups in both high 18:3n-3 and 18:1n-9 group despite low 22:6n-3 in high 18:1n-9 milk. However, n-6 fatty acids in jejuanl PC and PE of pups in high 18:1n-9 were similar to high 18:3n-3 whereas n-3 fatty acids were similar to high 18:2n-6 group compared to high 18:3n-3 group.

Pups in reference group had higher n-3 fatty acids (20:5n-3, 22:5n-3 and 22:6n-3) in jejunal PC and PE than pups in the 18:2n-6 group. Pups in reference group also had lower total n-6 fatty acids in jejunal PC and PE compared to other diet groups, along with consistently lower ratio of n-6/n-3 and 20:4n-6/20:5n-3 compared to pups in high 18:2n-6 group.

Colonic phospholipid fatty acids

Pups in the high 18:2n-6 group had significantly higher 18:2n-6 in their colon PC and PE than pups in the high 18:1n-9 or high 18:3n-3 groups (p<0.05, Tables II.7, II.8). Similar to findings in jejunum, pups in high 18:2n-6 group had significantly higher 20:2n-6 in colon PC and 22:5n-6 in colon PC and PE than other diet groups, whereas pups in high 18:3n-3 group had significant lower 22:4n-6 than other diet groups (p<0.05). Similar to the jejunum, the amounts of 20:4n-6 in the colon PC and PE were not significantly different among the groups. However, pups in the high 18-3n:3 group had significantly higher n-3 fatty acids (18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3) in colonic PC and PE than pups in the 18:2n-6 group (p<0.05). The high 18:1n-9 diet group, which was low in both 18:2n-6 and 18:3n-3, also resulted in significantly higher 22:6n-3 in the pups' colonic PC and PE than in pups in the high 18:2n-6 group. Similar to the jejunum, the differences in fatty acid intake from milk were reflected in significantly different ratios of 18:2n-6/18:3n-3 and 20:4n-6/20:5n-3 in the pup intestinal lipids, with consistently lower 18:2n-6/18:3n-3 and 20:4n-6/20:5n-3 ratios in the colon PC and PE of pups in the high 18:3n-3 groups than in the high 18:1n-9 or high 18:2n-6 groups. Similar to the diet and milk fat, the n-6/n-3 fatty acid ratio in colon PC and PE were significantly lower in high 18:3n-3 compared to high 18:1n-9 or high 18:2n-6 (p<0.05).

Consistent with findings in the jejunal PC and PE, pups in reference group had higher n-3 fatty acids (20:5n-3, 22:5n-3 and 22:6n-3) in colonic PC and PE than pups in the 18:2n-6 group. Pups in reference group also had lower total n-6 fatty acids in colonic PC and PE compared to other diet groups, along with consistently lower ratio of n-6/n-3 and 20:4n-6/20:5n-3 compared to pups in high 18:2n-6 group.

Macroscopic damage of pups colon

Intra-rectal administration of DNBS resulted in macroscopic damage associated with diarrhea, shortening and thickening of the colon and mucosal ulceration, however intra-rectal administration of saline in rat pups did not result in macroscopic damage scores as seen in pups with DNBS colitis (Fig II.2). Pups in all the diet groups who were given ethanol had significantly higher macroscopic damage scores compared with those given saline ($p < 0.05$).

At 12 hours post intra-rectal administration of DNBS, macroscopic damage was most marked in pups in the high 18:2n-6 group ($p < 0.05$, Table II.9). Pups in the high 18:2n-6 and high 18:1n-9 group had a 1.6 and 1.76 fold higher damage score, respectively, in response to DNBS than the respective ethanol control ($p < 0.05$). In contrast, DNBS treatment in pups in the high 18:3n-3 group had a macroscopic damage score that was not significantly different from their ethanol control group, but was significantly lower than pups in other diet groups ($p < 0.05$). Pups in reference diet group had higher macroscopic damage score in response to DNBS and ethanol than in pups in the high 18:3n-3 group, however there was no difference in the macroscopic damage score in ethanol treated pups in reference group compared to ethanol treated pups in other diet groups.

Histological damage of pups colon

Similar to findings of macroscopic damage scores, the histological damage scores in pups were also higher than respective saline-treated controls. Pups in all the diet groups who were given ethanol had significantly higher histological damage scores compared with those given saline ($p < 0.05$).

Pups in the high 18:2n-6 group had a significantly higher histological damage score following DNBS administration than pups in the high 18:1n-9 or high 18:3n-3 group (p<0.05, Fig II.3). Focal epithelial ulceration becoming confluent with superficial fibrinoid necrosis, goblet cell depletion, submucosal haemorrhage and transmural inflammation with acute and chronic inflammatory cells were found in pups in the high 18:2n-6 group. In contrast, pups in the high 18:1n-9 and high 18:3n-3 diet groups treated with DNBS had histological damage scores that were not significantly different from their respective ethanol controls, whereas pups in high 18:2n-6 group had 2.0 fold higher histological damage score in response to DNBS colitis than the respective ethanol controls (p<0.05). Pups in the high 18:2n-6 group treated with DNBS had focal epithelial ulceration, submucosal haemorrhage and a milder infiltrate of acute and chronic inflammatory cells than that observed in the high 18:1n-9 diet group that was limited to the mucosa and submucosa, whereas pups in the high 18:3n-3 diet group given DNBS had an intact epithelium and a slight increase in mononuclear cells in the lamina propria with some extension into the submucosa, with no neutrophils evident. Pups in reference diet group had higher histological damage score in response to DNBS than in pups in the high 18:3n-3 or high 18:1n-9 diet (Table II.10). There were no differences in histological damage scores in pups treated with ethanol in all diet groups.

Myeloperoxidase activity

In keeping with previous observations, MPO activity in ethanol control pups was significantly different from that observed in saline control pups (p<0.05). Consistent with the macroscopic damage and histological changes, intra-rectal DNBS was associated with significantly higher MPO activity in pups in the high 18:2n-6 group than

in pups in the high 18:1n-9 or high 18:3n-3 group ($p<0.05$). MPO activity was also significantly higher in the ethanol-treated pups in the high 18:2n-6 group compared with the high 18:1n-9 and high 18:3n-3 groups ($p<0.05$, Table II.11). Pups in the high 18:1n-9 or high 18:3n-3 diet groups treated with intra-rectal DNBS, in contrast, had MPO activity that was not significantly different from their respective ethanol controls. MPO activity in pups in the high 18:1n-9 group given intra-rectal DNBS or ethanol was not significantly different from the activity in pups in the high 18:3n-3 group given the same treatment. Pups in reference group had higher MPO activity associated with intra-rectal administration of DNBS than pups in high 18:3n-3 or high 18:1n-9 groups, however there were no differences in MPO activity in ethanol-treated pups in reference group compared to other groups.

4. DISCUSSION

This study used a tightly controlled experimental design with the composition of dietary fat as the only variable and to our knowledge provides the first demonstration that maternal dietary fat influences the offspring's responsiveness to experimental colitis. Rats were fed semi-synthetic diets that differed in unsaturated, but not saturated fatty acids, with 40% energy from canola oil (8% 18:3n-3), high oleic safflower oil (78% 18:1n-9) or high linoleic safflower oil (72% 18:2n-6). Canola oil and high 18:1n-9 safflower oil were both high in 18:1n-9 and relatively low in 18:2n-6, but differed considerably in 18:3n-3 and in the 18:2n-6/18:3n-3 ratio (3, 65, respectively). The high 18:2n-6 safflower oil, on the other hand, was high in 18:2n-6 and low in 18:3n-3 and 18:1n-9 and consequently had a considerably higher 18:2n-6/18:3n-3 ratio (360) than the

other two dietary oils. The reference diet was high in saturates, moderate 18:1, high 18:2n-6, 20:5n-3 and 22:6n-3, and low 18:3n-3 compared to high 18:3n-3 diet group, consequently had a considerably higher 18:2n-6/18:3n-3 ratio (12) than canola oil diet group, but lower ratio than other two diets. The reference diet not only differed in total fat content in diet (10% energy from fat), but was also different in macro and micro nutrients such as wheat germ, corn, ground oats, whey, soybean meal, fish meal, dried yeast, alfalfa, beet pulp etc.

The major contribution of this study suggests that maternal rat diet fat composition influenced the fatty acid composition of rat milk. Consistent with clinical studies in lactating women (Innis, 1992; Insull *et al.*, 1959), the maternal rat diet high in 18:2n-6 resulted in secretion of large amounts of 18:2n-6 along with n-6 fatty acids in milk, while the diet high in 18:3n-3, on the other hand resulted in higher milk levels of n-3 fatty acids and consequently lower ratios of 18:2n-6/18:3n-3 and 20:4n-6/20:5n-3.

In the reference group, higher dietary 18:3n-3 was reflected in the maternal milk along with low 18:2n-6 resulting in higher ratio of 18:2n-6/18:3n-3 (16) compared to high 18:3n-3 rat milk (4). Hence, this study clearly shows that type of maternal diet fat is reflected in rat milk. Although the amount of 18:2n-6 in reference diet was moderately high and the amount of 18:3n-3 was low compared to high 18:3n-3, yet the same was not reflected in the rat milk. Rather reference milk 18:2n-6 and 18:3n-3 were not different from the rat milk of high 18:3n-3 diet, resulting in no difference in ratio of total n-6/n-3 fatty acids or 20:4n-6/20:5n-3 compared to high 18:3n-3 group.

The ability of dietary fat to alter intestine membrane lipid composition and function demonstrates the importance of diet in early development (Sardesai, 1992).

Morphological and metabolic changes occur in the small intestine of mammalian neonates during the suckling period, leading to the development of a mature mucosa (Bouhours and Bouhours, 1981). This study paradigm allowed to establish that the maternal diet and consequently the milk diet n-6 and n-3 fatty acids altered the developing jejunal and colonic phospholipid fatty acids. This study also allowed to establish any differences in phospholipid fatty acid composition of developing jejunum or colon. The high intake of n-6 fatty acids from milk increased 22:4n-6 and 22:5n-6 in the pup jejunal and colonic PE, even though 22:4n-6 and 22:5n-6 were not found in significant amounts in the rat milk. This is reasonably explained by increased desaturation and elongation of n-6 fatty acids in the pups in response to the low n-3 fatty acid supply in the high 18:2n-6 safflower oil group (Innis, 2003). However, the high intake of n-6 fatty acids from milk was not associated with a significant increase in 20:4n-6 in the pup jejunal and colonic PC and PE. As expected, high intake of n-3 fatty acids from milk resulted in increase in n-3 fatty acids in the pup jejunal and colonic PC and PE.

Some differences were found in the responses of the colon and jejunum. For example, the maternal diet high in 18:2n-6 resulted in significantly higher 18:2n-6 in colon but not in jejunum PC and PE fatty acids. Possibly this is explained by the higher levels of 18:2n-6 in the colon when compared to the jejunum. Clearly evident from this study is that differences in the dietary n-6/n-3 fatty acid balance were maintained and transferred through the maternal milk to both the small and large intestinal phospholipids of the developing offspring.

In reference group, consistent with maternal diet effect on rat milk, the effect of milk feeding on pups intestinal fatty acids was clearly established. Similar to the findings in the reference milk, the differences in levels of 18:2n-6 and 18:3n-3 in the reference diet were not reflected in jejunal PC and PE. The jejunal and colonic phospholipid fatty acid composition of pups in reference group was similar to pups in high 18:3n-3 group except for high saturated fatty acids, monounsaturated fatty acids, high 22:5n-6 in jejunal PE, high 18:2n-6 and high 20:5n-3 in colonic PE.

In keeping with previous observations (Clearly *et al.*, 1999; Massiera *et al.*, 2003), the higher weight gain observed in pups suckling from mothers consuming the high 18:2n-6 safflower oil diet was likely due to n-6 fatty acid promotion of adipose tissue development during gestation and lactation. No evidence of under-nutrition was observed in all the diet groups. Pups in all diet groups had normal healthy appearing coats and no difference in intestinal wall thickness or histology were observed.

Another major contribution of this study is the demonstration that maternal dietary fat also modulated the intestinal inflammatory response to chemically-induced colitis in offspring not exposed to any diet other than the mother's milk. The DNBS colitis model was chosen because of the rapidity of onset of colitis following intra-rectal administration. In addition, the pups were examined 12 hours post instillation of DNBS so as to eliminate the potential confounding effects of reduced suckling associated with colitis and to prevent maternal killing of sick pups.

Pups in the high 18:2n-6 diet group demonstrated an exaggerated inflammatory response associated with severe macroscopic and histological damage and elevated MPO activity. In contrast, the diet high in 18:3n-3 and with low n-6/n-3 fatty acid ratio

prevented colonic infiltration of neutrophils and abrogated the inflammatory response, such that macroscopic and histological damage scores and MPO activity were not different from ethanol treated control animals. The maternal diet high in 18:1n-9, on the other hand, resulted in macroscopic damage and a histological appearance between that found in the high 18:2n-6 and high 18:3n-3 groups.

Interpretation of these findings suggests an association between the dietary n-6/n-3 fatty acid ratio and consequently the n-6/n-3 fatty acid ratio in colonic phospholipids and the inflammatory response to the colonic insult. However, the absence of differences in the colonic PC and PE 20:4n-6 between pups in the high 18:2n-6 and 18:3n-3 groups suggests that the enhanced susceptibility to chemically-induced colitis may involve the significant increase in colonic 18:2n-6 or reduced levels of long chain n-3 fatty acids, and consequently the high n-6/n-3 fatty acid balance. These results are consistent with other experimental studies who have shown decreased macroscopic and microscopic damage scores, and less myeloperoxidase activity to trinitrobenzene sulphonic acid (TNBS) colitis, when adult rats were fed fish oil (high 18:3n-3) with 10% energy from fats (Nieto et al., 2002); or perilla oil (12% 18:2n-6; 50.2% 18:3n-3) as compared to soy oil (55.6% 18:2n-6; 7.3% 18:3n-3), (Inui et al., 1996). Interestingly, pups in reference group also showed increase in macroscopic, histological damage scores and MPO activity following the insult, compared to pups in high 18:3n-3 group. This is unclear why reference maternal diet and consequently milk feeding failed to attenuate the DNBS colitis in suckling pups.

Experimental and clinical studies have shown that dietary n-3 fatty acids decrease membrane lipid 20:4n-6 with partial replacement with n-3 fatty acids, which

result in decreased synthesis of 20:4n-6 derived eicosanoids and increased synthesis of eicosanoids derived from 20:5n-3 (Calder, 2003; Harbige, 2003; James *et al.*, 2000; Seidman et al., 2002). However, in this study, higher 20:4n-6 was not found in colonic PC or PE. Nevertheless, the colonic 20:5n-3 was significantly higher in pups in the high 18:3n-3 group and in the reference group, while 22:6n-3 was significantly higher in pups in the high 18:3n-3, 18:1n-9 and reference groups than pups in the 18:2n-6 group. Of relevance, 22:6n-3 has recently been shown to be the precursor for 17s-resolvins that have anti-inflammatory properties (Serhan *et al.*, 2002). Possibly, the higher colonic n-3 fatty acids, 20:5n-3 and 22:6n-3 abrogated the intestinal inflammatory response, whereas reduced n-3 fatty acids were associated with a potentiation of the inflammatory response in the semi-synthetic diet groups.

Although the present study did not address the mechanisms involved in the inflammatory response or the potential different mechanisms involved in ethanol mediated epithelial injury and in DNBS-induced colitis, it appears that n-3 fatty acids had little effect on the response to the toxic effects of ethanol, but attenuated the inflammatory response associated with the immune mediated consequences of DNBS. In mature animal models of TNBS colitis, the therapeutic effects of n-3 fatty acids have been attributed at least in part to suppression of mucosal leukotriene-B_4 and prostaglandin E_2 production (Nieto *et al.*, 1998; Neito *et al.*, 2002).

The effects in this study may reflect direct actions of n-3 fatty acids (Harbige, 2003; Nieto *et al.*, 1998; Neito *et al.*, 2002). Consistent with this, the lack of neutrophil infiltration into the colon of pups in the 18:3n-3 fed group suggests that the higher milk and colonic n-3 fatty acids, including 20:5n-3 and 22:6n-3 abrogated the inflammatory

response, possibly by attenuating chemotaxis through reduced endothelial cell adherence and directed migration (Lee *et al.*, 1985). However, others have suggested that n-3 fatty acids may be efficacious in IBD by serving as a free radical scavenger, thereby protecting the mucosa from oxidative cell damage (Barbosa *et al.*, 2003; Fisher and Levine, 1991). The reasons for and the potential biological significance of 20:4n-6 in the pup jejunal and colonic phospholipids in response to high dietary 18:2n-6 are unclear. Therefore, the hypotheses that possible mechanisms of suppression of 20:4n-6 derived eicosanoids in colonic responsiveness to inflammatory stimuli is not carried off. A plausible explanation of this might be that it is not the 20:4n-6 derived eicosanoids alone or the n-3 fatty acids alone which attenuate the inflammatory response, rather a balance between 18:2n-6 or 18:3n-3 might play a key role in inflammatory processes. However, the reference group findings can not be dismissed and suggest that some other mechanisms exist which counteract with the protective action of n-3 fatty acids in experimental colitis and warrant further investigation. It is also important to note that the high 18:3n-3 milk had higher 18:3n-3 and consequently higher 18:3n-3 in pups colonic PC compared to reference milk or colonic PC of reference pups. It is quite possible that high dietary 18:3n-3 through milk feeding might have attenuated the intestinal inflammatory response in pups in high 18:3n-3 group.

In conclusion, the present study has demonstrated that maternal dietary fat influenced the fatty acid composition of rat milk and consequently the intestinal phospholipid fatty acids of the offspring and intestinal responsiveness to an inflammatory stimulus. These data support the integral role of maternal diet on the early developmental pattern of the gut, however further studies are required to determine the

durability of these effects. These data may form basis for the development of dietary interventions aimed at reducing the risk of intestinal inflammatory diseases such as inflammatory bowel disease or gastroenteritis.

Table II.1: Fatty acid composition of diets

Fatty acid	Diet groups			
	High 18:3n-3	High 18:1n-9	High 18:2n-6	Reference
14:0	0	0	0	2
16:0	5	6	7	23
18:0	2	2	3	7
18:1	65	78	18	24
18:2n-6	20	14	72	37
18:3n-3	8	0.2	0.2	4
20:5n-3	0	0	0	1
22:6n-3	0	0	0	2
18:2n-6/18:3n-3	3	65	360	12

The diets contained high 18:3n-3 from canola oil, high 18:1n-9 from high oleic safflower oil or high 18:2n-6 from safflower oil. Individual fatty acids are expressed as g/100g. The semi-synthetic diets contained the following ingredients per kg of the complete diet: 200 g oil, 200 g vitamin free casein (U.S. Biochemical, Cleveland, OH), 160 g sucrose, 329 g cornstarch, 50 g non-nutritive cellulose (Teklad Test Diets, Madison, WI); 10 g vitamin mix (AOAC # 40055, Teklad Test Diets, Madison, WI), providing 20,000IU retinyl palmitate, 2000 IU cholecalciferol, 100 IU α-tocopherol acetate, 5 mg menadoine, 5 mg thiamine-HCL, 8 mg riboflavin, 40 mg pyridoxine-HCL, 40 mg niacin, 40 mg pantothenic acid, 2000 mg choline, 100mg myoinositol, 100 mg para-aminobenzoic acid, 0.4 mg biotin, 2 mg folic acid, and 30 mg cyanocobalamine; 50 g mineral mix (Bernhart-Tomarelli salt mix, General Biochemicals, Chagrin falls, OH) providing 1.1 g calcium carbonate, 36.8 g calcium phosphate, 0.1 g citric acid, 23 mg cupric citrate, 0.5 H_2O, 1.3 g magnesium oxide, 418 mg manganese citrate, 0.5 mg potassium iodide, 3.4 g potassium sulfate, 1.5 g sodium chloride, 1.1 g sodium phosphate, 66.5 mg zinc citrate $2H_2O$ with additional 279.38 mg Mn^{2+}, 0.084mg Se^{2+}, 1.0 g choline chloride and 3.0 g L-methionine (Innis and de la Presa Owens, 2001). Reference diet was Rodent Laboratory Chow (Pico Lab Rodent Diet 20, #5053) obtained from Purina Mills, St Louis.

Table II.2: Criteria for assessment of macroscopic colonic damage scores in DNBS colitis.

Score	Criteria
0	No Damage
1,2 or 3	Focal hyperemia; no ulcers (Mild → Severe)
Plus	
0	No adhesion
1	Minor adhesion (colon easy to separate from other tissues)
2	Major adhesion
Plus	
0	No diarrhoea
1	Diarrhoea
Total	

Modified from: Galeazzi et al (2000).

Table II.3: Criteria for assessment of colonic histological damage scores in DNBS colitis.

Criteria	Score	
Loss of mucosal architecture	0, 1, 2 or 3	(Absent, Mild →Severe)
Cellular infiltration	0, 1, 2 or 3	(Absent, Mucosal, Sub-mucosal → Transmural)
Muscle thickness	0, 1, 2 or 3	(Absent, Mild →Extensive)
Epithelialitis and Crypt abscess formation	0 or 1	(Absent or Present)
Goblet cell depletion	0 or 1	(Absent or Present)
	Total=	

Modified from: Galeazzi et al (2000).

Table II.4: Fatty acid composition of rat milk

Fatty acid	Diet groups			
	High 18:3n-3	High 18:1n-9	High 18:2n-6	Reference
8:0	0.40±0.24	0.40±0.13	0.53±0.34	1.92±0.47
10:0	3.02±1.04	3.14±0.53	3.3±0.93	8.63±0.98
12:0	3.39±0.62^b	3.54±0.69^b	4.60±0.63^a	8.49±0.97
14:0	7.18±3.58	3.71±0.80	5.18±0.43	9.09±1.17
16:0	12.6±0.58	12.3±0.77	13.3±0.33	22.9±0.87
18:0	3.38±0.13^b	2.73±0.18^a	3.23±0.14^{ab}	5.22±0.28
16:1n-9	0.38±0.01^b	0.37±0.04^b	0.14±0.00^a	0.29±0.04
18:1n-9	46.2±1.90^b	55.2±4.44^b	13.8±0.37^a	19.53±1.58
20:3n-9	0.18±0.03^b	0.23±0.02^b	0.04±0.01^a	0.10±0.02
16:1n-7	0.68±0.06^b	0.44±0.13^{ab}	0.27±0.01^a	1.76±0.16
18:1n-7	2.31±0.13	4.70±2.56	0.62±0.05	2.38±0.58
18:2n-6	11.4±0.67^a	9.43±0.48^a	47.8±0.89^b	12.2±1.06
18:3n-6	0.28±0.03^a	0.28±0.03^a	0.71±0.05^b	0.36±0.05
20:2n-6	0.32±0.01^b	0.21±0.02^b	0.92±0.05^a	0.45±0.08
20:3n-6	0.30±0.01^b	0.27±0.05^b	0.72±0.08^a	0.43±0.05
20:4n-6	1.30±0.07^a	0.98±0.09^a	2.59±0.14^b	1.25±0.21
22:4n-6	0.17±0.04	0.18±0.02	0.15±0.09	0.28±0.10
22:5n-6	0.08±0.01^a	0.10±0.00^a	0.35±0.06^b	0.03±0.01
18:3n-3	2.71±0.35^b	0.12±0.01^a	0.20±0.00^a	0.79±0.06
20:5n-3	0.37±0.05^b	0.01±0.00^a	0.02±0.00^a	0.45±0.04
22:5n-3	0.35±0.02^b	0.03±0.01^a	0.03±0.00^a	0.52±0.12
22:6n-3	0.73±0.06^b	0.25±0.10^a	0.07±0.00^a	1.42±0.51
22:0	0.07±0.02^{ab}	0.10±0.00^b	0.01±0.01^a	0.05±0.03
24:0	0.15±0.03^a	0.17±0.03^a	0.62±0.06^b	0.17±0.10
24:1	0.02±0.00^b	0.00±0.00^a	0.00±0.00^a	0.05±0.01
Total SFA	30.5±2.63	26.3±2.14	31.0±1.34	44.6±7.83
Total MUFA	51.1±2.17^b	61.5±2.08^c	15.2±0.38^a	24.9±1.72
Total PUFA	18.2±0.99^b	12.1±0.42^a	53.7±1.02^c	22.9±3.20
Total n-6	13.9±0.73^a	11.4±0.46^a	53.3±1.03^b	8.01±2.77
Total n-3	4.18±0.35^b	0.42±0.12^a	0.34±0.01^a	3.18±0.66
LA/LNA	4.44±0.40^a	80.7±8.96^b	231±12.3^c	15.6±1.18
Total n-6/n-3	3.40±0.23^a	35.7±8.33^b	145±4.55^c	3.62±1.41
ARA/EPA	3.88±0.60^a	76.6±11.7^b	110±14.7^c	2.70±0.20

Dams were fed canola oil (high 18:3n-3), high oleic acid safflower oil (high 18:1n-9), safflower oil (high 18:2n-6) or reference chow, n=6 dams/ group. Individual fatty acids are expressed as g/100g. Values with a different superscript a, b, c, are significantly different, $p<0.05$. Total SFA, sum of saturated fatty acids; Total MUFA, sum of monounsaturated fatty acids; Total PUFA, sum of polyunsaturated fatty acids; Total n-6, sum of n-6 fatty acids, Total n-3, sum of n-3 fatty acids; LA, 18:2n-6; LNA, 18:3n-3; ARA, 20:4n-6, EPA, 20:5n-3; Total n-6/n-3, sum of n-6/n-3 fatty acids.

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Table II.5: Jejunal phosphatidylcholine fatty acids in suckling pups

Fatty acid	Diet groups			Reference
	High 18:3n-3	High 18:1n-9	High 18:2n-6	
12:0	0.36±0.20	0.05±0.00	0.04±0.00	0.17±0.10
14:0	0.58±0.01	0.68±0.09	0.57±0.16	0.97±0.25
16:0	26.6±0.63	28.3±1.46	29.9±0.92	37.1±2.42
18:0	13.2±0.07	13.4±0.80	15.1±1.04	15.1±1.16
16:1	0.93±0.00	0.64±0.05	5.05±4.51	1.82±0.37
18:1	14.9±0.57[b]	15.0±0.39[b]	9.34±1.82[a]	10.6±2.27
20:3n-9	0.10±0.00[ab]	0.17±0.04[b]	0.02±0.00[a]	0.07±0.02
18:2n-6	15.1±1.71	12.2±2.51	16.9±0.63	14.1±1.46
18:3n-6	0.08±0.02	0.15±0.04	0.13±0.01	0.18±0.02
20:2n-6	0.47±0.04[a]	0.43±0.03[a]	1.60±0.15[b]	0.34±0.11
20:3n-6	1.12±0.05	1.34±0.17	1.26±0.11	1.45±0.12
20:4n-6	19.6±1.10	21.9±2.11	21.9±1.21	14.1±1.93
22:4n-6	0.54±0.05[a]	0.87±0.17[a]	1.20±0.08[b]	0.25±0.09
22:5n-6	0.12±0.03[a]	0.13±0.02[a]	1.01±0.05[b]	0.06±0.01
18:3n-3	0.52±0.18[b]	0.02±0.00[a]	0.02±0.00[a]	0.23±0.07
20:5n-3	0.31±0.07[b]	0.02±0.00[a]	0.01±0.00[a]	0.44±0.04
22:5n-3	0.37±0.03[b]	0.16±0.03[a]	0.09±0.01[a]	0.40±0.07
22:6n-3	2.76±0.28[b]	2.29±0.31[b]	0.78±0.09[a]	2.41±0.31
22:0	0.19±0.02[b]	0.23±0.00[b]	0.01±0.00[a]	0.07±0.02
22:1	0.41±0.01[b]	0.20±0.02[a]	0.23±0.03[a]	0.37±0.08
24:0	0.10±0.01[b]	0.58±0.07[a]	0.06±0.02[b]	0.47±0.15
24:1	0.10±0.01[a]	0.02±0.00[b]	0.02±0.00[b]	0.04±0.00
Total SFA	41.2±0.50[a]	43.6±0.83[ab]	46.2±1.51[b]	54.1±2.17
Total MUFA	17.4±0.62	16.4±0.36	15.0±6.29	13.1±2.60
Total PUFA	41.2±1.12	39.8±1.08	38.7±5.10	32.7±3.94
Total n-6	37.1±1.22[a]	37.1±1.06[a]	44.1±1.29[b]	30.6±2.06
Total n-3	5.10±0.12[c]	3.85±0.19[b]	2.18±0.17[a]	4.94±0.44
LA/LNA	43.4±10.3[a]	622±114[b]	977±437[b]	221±122
Total n-6/n-3	7.32±0.42[a]	9.7±0.47[a]	20.8±1.61[b]	6.35±0.41
ARA/EPA	75.2±13.2[a]	848±97[b]	>999±200[b]	35.8±9.62

Dams were fed canola oil (high 18:3n-3), high oleic acid safflower oil (high 18:1n-9), safflower oil (high 18:2n-6) or reference chow, n=6 dams/ group. Individual fatty acids are expressed as g/100g. Values with a different superscript *a, b, c* are significantly different, $p<0.05$. Total SFA, sum of saturated fatty acids; Total MUFA, sum of monounsaturated fatty acids; Total PUFA, sum of polyunsaturated fatty acids; Total n-6, sum of n-6 fatty acids, Total n-3, sum of n-3 fatty acids; LA, 18:2n-6; LNA, 18:3n-3; ARA, 20:4n-6, EPA, 20:5n-3; Total n-6/n-3, sum of n-6/n-3 fatty acids.

Table II.6: Jejunal phosphatidylethanolamine fatty acids in suckling pups

| Fatty acid | Diet groups | | | Reference |
	High 18:3n-3	High 18:1n-9	High 18:2n-6	
12:0	0.12±0.03	0.04±0.01	0.04±0.01	0.18±0.03
14:0	0.35±0.06^b	0.23±0.05^{ab}	0.08±0.01^a	0.21±0.07
16:0	9.49±1.24^b	7.40±0.82^{ab}	5.88±0.27^a	8.60±0.48
18:0	21.4±1.14	24.3±1.44	25.3±1.23	23.2±1.48
14:1	0.08±0.01^b	0.02±0.00^a	0.01±0.00^a	0.03±0.00
16:1	0.59±0.07^b	0.25±0.09^a	0.31±0.08^{ab}	0.80±0.16
18:1	9.68±0.48^b	7.82±0.78^{ab}	6.36±0.14^a	6.18±0.77
20:3n-9	0.19±0.02	0.17±0.04	0.05±0.04	0.17±0.09
18:2n-6	6.64±1.13	7.10±0.93	7.74±0.68	5.91±0.59
18:3n-6	0.08±0.02^{ab}	0.40±0.16^b	0.03±0.01^a	0.12±0.03
20:2n-6	0.56±0.06^b	0.19±0.02^a	0.66±0.08^b	0.16±0.01
20:3n-6	1.37±0.32	0.94±0.13	1.06±0.12	1.08±0.12
20:4n-6	30.7±1.56	31.8±1.63	35.2±1.36	29.7±2.29
22:4n-6	2.80±0.33^a	3.87±0.43^a	5.82±0.43^b	1.34±0.63
22:5n-6	0.37±0.17^a	0.33±0.15^a	5.60±0.14^b	0.04±0.04
18:3n-3	0.88±0.31^b	0.06±0.02^a	0.06±0.01^a	0.41±0.15
20:5n-3	1.02±0.30^b	0.03±0.01^a	0.01±0.00^a	0.81±0.11
22:5n-3	1.49±0.04^c	0.62±0.03^b	0.30±0.07^a	2.53±0.39
22:6n-3	10.5±1.27^b	10.2±1.39^b	3.88±0.55^a	14.4±1.41
22:0	0.26±0.03^b	0.36±0.03^b	0.02±0.01^a	0.12±0.07
24:0	0.45±0.06^a	2.28±0.44^b	0.07±0.03^a	2.91±0.95
24:1	0.03±0.01	0.10±0.06	0.03±0.01	0.04±0.01
Total SFA	32.5±1.65	35.1±2.03	32.0±0.93	35.6±1.95
Total MUFA	10.7±0.48^a	8.99±0.66^{ab}	7.40±0.16^b	7.62±0.90
Total PUFA	56.7±2.09	55.8±2.14	60.5±0.84	56.7±2.78
Total n-6	42.5±1.37^a	44.7±0.76^a	56.1±0.48^b	38.4±2.08
Total n-3	15.3±1.27^b	11.9±1.37^b	5.33±0.55^a	19.3±1.28
LA/LNA	14.9±5.21^a	210±90^b	211±92^b	44.9±20.6
Total n-6/n-3	2.85±0.23^a	3.97±0.49^a	11.1±1.46^b	2.02±0.14
ARA/EPA	47.6±15.8^a	>999±200^b	>900±100^b	40.9±7.44

Dams were fed canola oil (high 18:3n-3), high oleic acid safflower oil (high 18:1n-9), safflower oil (high 18:2n-6) or reference chow, n=6 dams/ group. Individual fatty acids are expressed as g/100g. Values with a different superscript *a, b, c* are significantly different, $p<0.05$. Total SFA, sum of saturated fatty acids; Total MUFA, sum of monounsaturated fatty acids; Total PUFA, sum of polyunsaturated fatty acids; Total n-6, sum of n-6 fatty acids, Total n-3, sum of n-3 fatty acids; LA, 18:2n-6; LNA, 18:3n-3; ARA, 20:4n-6, EPA, 20:5n-3; Total n-6/n-3, sum of n-6/n-3 fatty acids.

Table II.7: Colonic phosphatidylcholine fatty acids in suckling pups

Fatty acid	Diet groups			
	High 18:3n-3	High 18:1n-9	High 18:2n-6	Reference
12:0	0.01±0.00	0.06±0.01	0.08±0.02	0.47±0.11
14:0	1.10±0.05^a	1.44±0.28^{ab}	2.18±0.23^b	2.35±0.38
16:0	32.1±1.33	30.7±1.03	34.1±1.30	30.3±6.21
18:0	5.68±2.31	10.5±0.30	12.9±1.26	11.0±0.31
16:1	1.83±0.13^c	1.30±0.07^b	0.67±0.01^a	1.77±0.02
18:1	31.0±2.40^c	24.8±0.24^b	10.1±0.24^a	19.6±0.70
20:3n-9	0.15±0.01^{ab}	0.25±0.04^b	0.07±0.02^a	0.11±0.06
18:2n-6	9.63±0.74^a	7.92±0.63^a	18.5±1.31^b	11.1±2.19
18:3n-6	0.10±0.01	0.06±0.02	0.08±0.02	0.10±0.00
20:2n-6	0.40±0.13^a	0.83±0.05^a	2.77±0.68^b	0.30±0.12
20:3n-6	1.58±0.02	1.79±0.02	1.74±0.44	1.76±0.02
20:4n-6	10.1±0.38^a	14.3±1.15^b	12.3±0.82^{ab}	10.0±0.70
22:4n-6	0.96±0.06^a	1.68±0.23^b	1.40±0.11^{ab}	0.82±0.09
22:5n-6	0.03±0.02^a	0.13±0.02^a	0.74±0.07^b	0.00±0.00
18:3n-3	0.20±0.07^b	0.02±0.01^a	0.04±0.02^a	0.04±0.02
20:5n-3	0.36±0.04^b	0.06±0.02^a	0.07±0.02^a	0.46±0.15
22:5n-3	0.60±0.05^b	0.20±0.04^a	0.12±0.02^a	0.47±0.02
22:6n-3	2.02±0.46^b	1.42±0.14^{ab}	0.41±0.15^a	1.75±0.10
22:0	0.13±0.02	0.21±0.00	0.19±0.09	0.16±0.05
24:0	0.12±0.02^a	0.61±0.07^b	0.02±0.02^a	0.17±0.02
24:1	0.07±0.04	0.01±0.00	0.01±0.00	0.00±0.00
Total SFA	39.4±1.14^a	43.9±1.24^{ab}	50.0±2.39^b	50.8±2.07
Total MUFA	34.3±1.79^c	27.3±0.87^b	11.6±0.34^a	22.1±0.81
Total PUFA	26.2±0.77^a	28.7±1.16^a	38.3±2.62^b	27.0±1.25
Total n-6	22.8±0.43^a	26.7±0.95^a	37.5±2.47^b	24.1±1.14
Total n-3	4.78±0.60^b	3.51±0.17^{ab}	2.40±0.48^a	4.50±0.06
LA/LNA	58.8±9.07^a	442±114^b	790±330^b	378±104
Total n-6/n-3	5.01±0.45^b	7.63±0.11^b	19.0±5.00^a	5.36±0.18
ARA/EPA	29.4±3.87^a	290±81^b	275±88^b	24.0±4.29

Dams were fed canola oil (high 18:3n-3), high oleic acid safflower oil (high 18:1n-9), safflower oil (high 18:2n-6) or reference chow, n=6 dams/ group. Individual fatty acids are expressed as g/100g. Values with a different superscript a, b, c are significantly different, $p<0.05$. Total SFA, sum of saturated fatty acids; Total MUFA, sum of monounsaturated fatty acids; Total PUFA, sum of polyunsaturated fatty acids; Total n-6, sum of n-6 fatty acids, Total n-3, sum of n-3 fatty acids; LA, 18:2n-6; LNA, 18:3n-3; ARA, 20:4n-6, EPA, 20:5n-3; Total n-6/n-3, sum of n-6/n-3 fatty acids.

Table II.8: Colonic phosphatidylethanolamine fatty acids in suckling pups

Fatty acid	Diet groups			
	High 18:3n-3	High 18:1n-9	High 18:2n-6	Reference
12:0	0.05±0.01	0.29±0.16	0.11±0.03	0.27±0.14
14:0	0.38±0.04	0.43±0.07	0.23±0.05	0.82±0.16
16:0	5.86±0.86	5.66±0.38	5.89±0.24	9.92±2.06
18:0	14.1±0.89^a	15.7±0.08^a	19.5±0.87^b	15.2±1.44
16:1	0.52±0.06^b	0.29±0.01^{ab}	0.29±0.07^a	0.81±0.11
18:1	21.4±3.10^b	14.0±0.30^{ab}	10.0±0.11^a	12.4±1.1
20:3n-9	0.32±0.01	0.39±0.10	0.80±0.45	0.25±0.16
18:2n-6	4.40±0.44^a	4.43±0.42^a	9.22±0.27^b	8.80±2.10
18:3n-6	0.96±0.93	1.74±0.58	0.16±0.06	0.04±0.02
20:2n-6	0.32±0.05	0.33±0.03	0.78±0.34	0.44±0.15
20:3n-6	1.65±0.11	1.77±0.12	2.13±0.16	2.09±0.14
20:4n-6	32.3±1.71	34.8±1.34	33.0±0.24	29.3±4.21
22:4n-6	4.46±0.52^a	7.47±0.42^b	8.07±0.06^b	3.75±0.68
22:5n-6	0.06±0.01^a	2.74±0.52^b	4.72±0.07^c	0.04±0.03
18:3n-3	0.69±0.46	0.09±0.00	0.55±0.21	0.24±0.03
20:5n-3	1.16±0.16^b	0.01±0.00^a	0.05±0.03^a	1.89±0.26
22:5n-3	2.30±0.29^b	0.70±0.09^a	0.39±0.09^a	1.99±0.26
22:6n-3	6.99±0.84^b	5.99±0.15^b	2.06±0.81^a	9.25±1.89
24:0	0.31±0.10	1.19±1.04	0.10±0.09	0.68±0.16
24:1	0.01±0.00	0.03±0.01	0.00±0.00	0.02±0.00
Total SFA	21.5±0.83^a	24.0±1.56^{ab}	26.4±1.10^b	27.5±3.61
Total MUFA	22.5±3.66^b	15.4±0.45^{ab}	11.3±0.15^a	13.8±1.27
Total PUFA	55.9±3.13	60.5±1.71	62.2±1.18	58.5±4.88
Total n-6	44.1±1.98^a	53.3±1.38^b	58.1±0.17^b	44.5±3.33
Total n-3	12.8±1.50^b	8.58±0.19^a	5.20±0.80^a	15.4±2.25
LA/LNA	21.2±9.91	49.4±8.39	33.4±12.9	39.1±12.4
Total n-6/n-3	3.65±0.44^a	6.20±0.06^a	12.4±2.12^b	3.20±0.55
ARA/EPA	30.0±4.01^a	999±110^b	754±300^b	16.0±5.67

Dams were fed canola oil (high 18:3n-3), high oleic acid safflower oil (high 18:1n-9), safflower oil (high 18:2n-6) or reference chow, n=6 dams/ group. Individual fatty acids are expressed as g/100g. Values with a different superscript a, b, c are significantly different, $p<0.05$. Total SFA, sum of saturated fatty acids; Total MUFA, sum of monounsaturated fatty acids; Total PUFA, sum of polyunsaturated fatty acids; Total n-6, sum of n-6 fatty acids, Total n-3, sum of n-3 fatty acids; LA, 18:2n-6; LNA, 18:3n-3; ARA, 20:4n-6, EPA, 20:5n-3; Total n-6/n-3, sum of n-6/n-3 fatty acids.

Table II.9: Macroscopic damage score in colon of rat pups

Treatment	Diet groups			Reference
	High 18:3n-3	High 18:1n-9	High 18:2n-6	
DNB	1.6 ± 0.2^a	$3.0\pm0.3^{b*}$	$3.9\pm0.3^{c*}$	3.0 ± 0.3
Ethanol	1.5 ± 0.2^a	1.7 ± 0.2^{ab}	2.4 ± 0.2^b	2.2 ± 0.3
Saline	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Macroscopic damage scores in the colon on postnatal day 15 in pups in the high 18:3n-3, high 18:2n-6, high 18:1n-9 or reference groups 12 hours after intra-rectal dinitrobenzene sulfonic acid (DNBS), 50% ethanol or 0.9% saline. Values are means $\pm$ SEM: different superscripts *a, b, c* (significantly different, $p<0.005$) compared with pups in diet groups; *$p<0.05$ DNBS compared to pups that received 50% ethanol.

Table II.10: Histological damage score in colon of rat pups

Treatment	Diet groups			Reference
	High 18:3n-3	**High 18:1n-9**	**High 18:2n-6**	
DNB	2.3 ± 0.4^a	3.4 ± 0.7^a	$7.3\pm0.3^{b*}$	$6.0\pm0.5^*$
Ethanol	2.8 ± 0.5	2.5 ± 0.6	3.6 ± 0.6	2.7 ± 0.6
Saline	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Histological damage scores in the colon on postnatal day 15 in pups in the high 18:3n-3, high 18:2n-6, high 18:1n-9 or reference groups 12 hours after intra-rectal dinitrobenzene sulfonic acid (DNBS), 50% ethanol or 0.9% saline. Values are means $\pm$ SEM: different superscripts a, b (significantly different, $p<0.005$) compared with pups in diet groups; $*p<0.05$ DNBS compared to pups that received 50% ethanol.
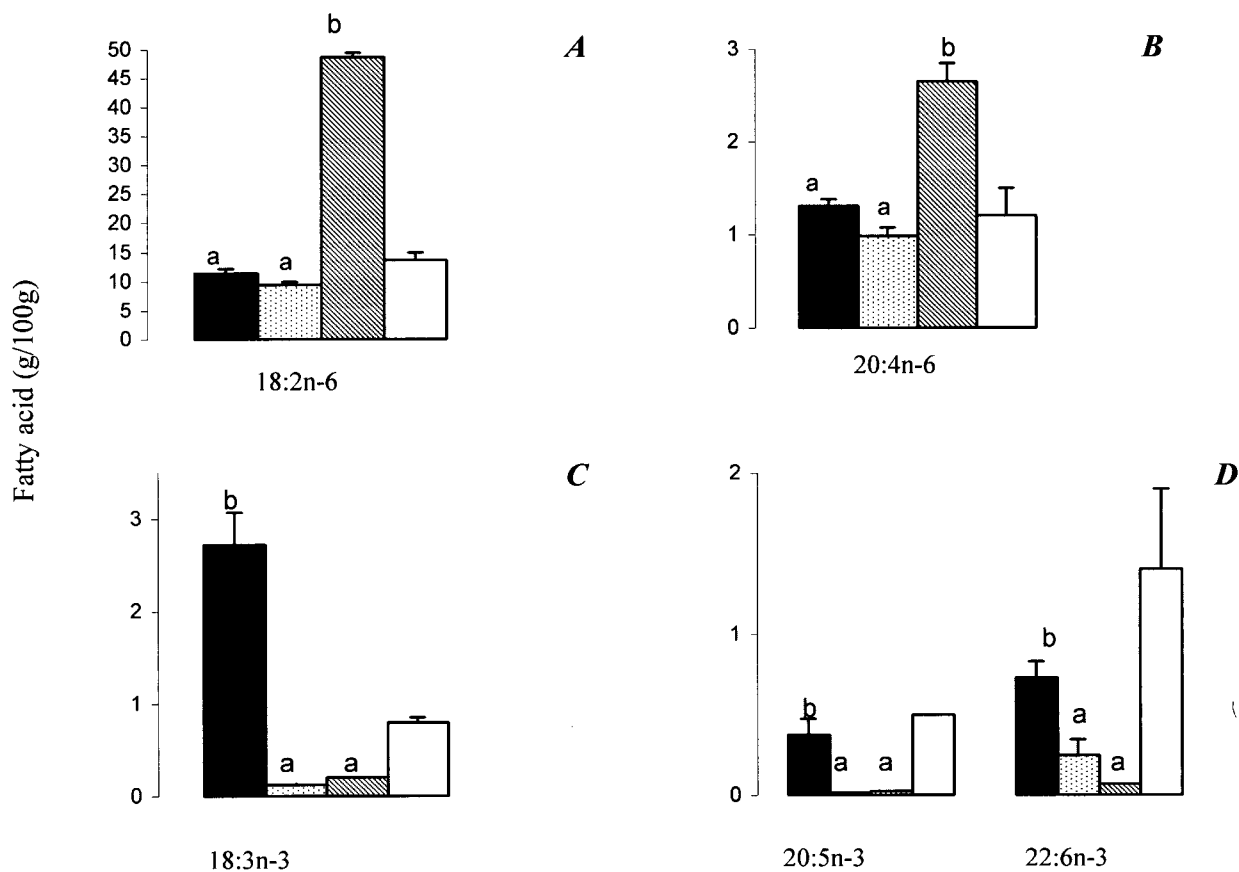
Table II.11: Myeloperoxidase activity in colon of rat pups

Treatment	Diet groups			Reference
	High 18:3n-3	High 18:1n-9	High 18:2n-6	
DNB	4.1 ± 0.7^a	4.2 ± 0.3^a	$23.5\pm6.1^{a*}$	15.0 ± 2.6
Ethanol	2.8 ± 0.6^a	2.9 ± 0.5^a	11.2 ± 2.0^b	2.9 ± 0.3
Saline	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0

Myeloperoxidase activity in the colon on postnatal day 15 in pups in the high 18:3n-3, high 18:2n-6, high 18:1n-9 or reference groups 12 hours after intra-rectal dinitrobenzene sulfonic acid (DNBS), 50% ethanol or 0.9% saline. Values are means $\pm$ SEM: different superscripts *a, b* (significantly different, $p<0.005$) compared with pups in diet groups; *$p<0.05$ DNBS compared to pups that received 50% ethanol.
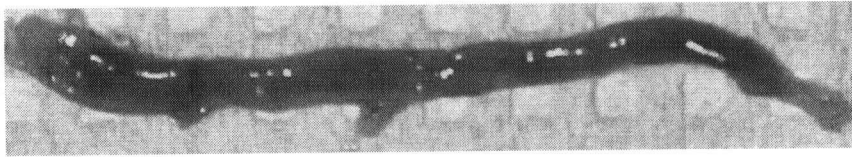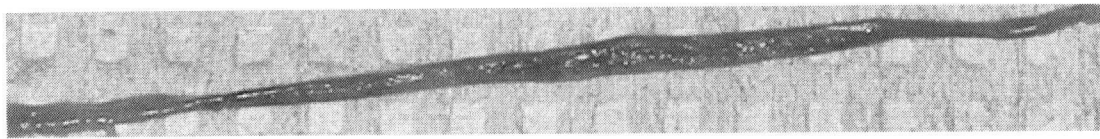
Figure II.1: Fatty acid composition of rat milk for the group fed high 18:3n-3 (■), high 18:1n-9 (▨), high 18:2n-6 (▨) or reference chow (□) for *A*, 18:2n-6 content in milk, *B* 20:4n-6 in milk, *C* 18:3n-3 in milk, *D* 20:5n-3, 22:6n-3 in maternal milk. Values are mean ± SEM: $p<0.05$, significantly different at different superscripts *a*, *b*.
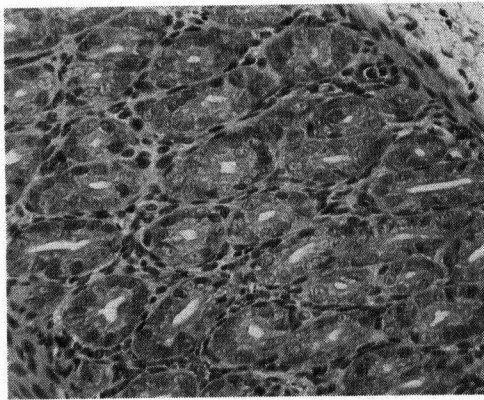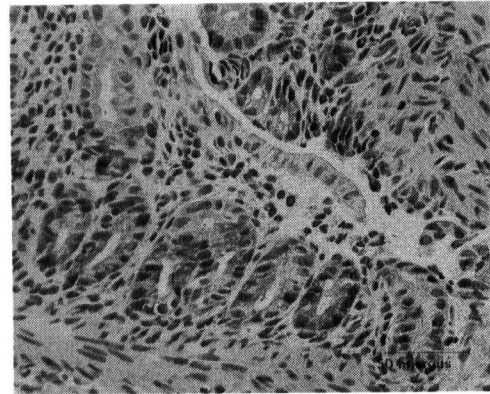
A. High 18:2n-6 plus intra-rectal DNBS

B. High 18:2n-6 plus intra-rectal saline

Figure II.2: A. Macroscopic appearance of the colon from a pup suckled by a rat fed high 18:2n-6 diet (postnatal day 15), at 12 hours post intra-rectal administration of dinitrobenzene sulfonic acid. Note the macroscopic damage with shortening and thickening of the colon.
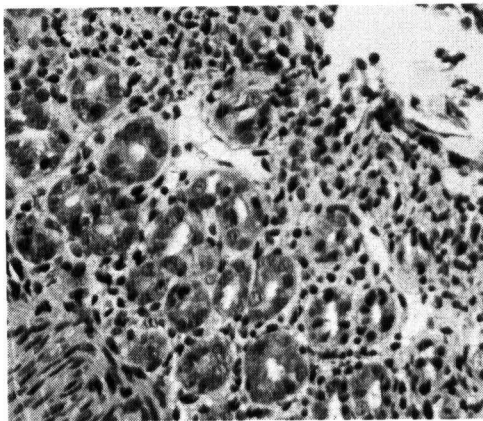
B. Macroscopic appearance of the colon from a pup suckled by a rat fed high 18:2n-6 (postnatal day 15), at 12 hours post intra-rectal administration of saline. Note the normal appearing colon compared to rat pup colon in A.
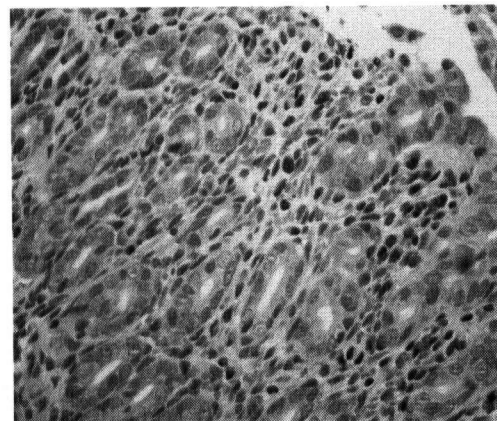
High 18:3n-3   High 18:1n-9

High 18:2n-6   Reference

Figure II.3: Histological appearance of colonic tissue sections (haematoxylin and eosin staining) from pups 12 hours post intra-rectal administration of dinitrobenzene sulfonic acid in pups in the high 18:3n-3, high 18:1n-9, 18:2n-6 groups or reference chow. The bar represents 50 μm. Note the loss of architecture, epithelial ulceration, goblet cell depletion, and inflammation with acute and chronic inflammatory cells in a representative pup in the high 18:2n-6 group. A milder degree of epithelial injury with focal ulceration, and a milder infiltrate of acute and chronic inflammatory cells were present in pups in the order: high 18:1n-9<reference group. Pups in the high 18:3n-3 group showed an intact epithelium and only a slight increase in mononuclear cells.

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CHAPTER III

GENERAL DISCUSSION AND CONCLUSIONS

1. Rat milk fatty acids

One of the main objectives of the study was to determine the effects of maternal diet fat on fatty acid composition of rat milk. Rats were fed semi-synthetic diets that differed in unsaturated fatty acid oleic acid (18:1n-9), linoleic acid (18:2n-6) or linolenic acid (18:3n-3), but not in total fat, macro, or micronutrients. Reference group rats were fed non-purified standard laboratory chow diet which differed in total fat, macro, and micro nutrients, compared to semi-synthetic diets. This study has clearly shown a direct relationship between maternal dietary fatty acid intake and rat milk fatty acids. High maternal dietary intakes of 18:2n-6, 18:3n-3, or 18:1n-9 resulted in higher milk levels of these fatty acids along with a concomitant increase in the respective longer chain fatty acids. Also, the differences in the ratio of n-6/n-3 fatty acids among the diets were clearly reflected in the rat milk. The present findings are consistent with clinical studies (Innis, 1992) and animal studies (Korotkova *et al.*, 2002) that have shown a direct relationship between maternal diet fat and milk fatty acids, with a consequent effect on plasma fatty acid composition. This study shows that the fatty acid composition of maternal diet alters the fatty acid composition of rat milk.

The results from the reference chow milk also suggested an association between maternal diet fat and milk fatty acids, even though total fat was lower in reference diet compared to semi-synthetic diets. Interestingly, the reference diet was also high in 18:2n-6, compared to the high 18:3n-3 diet, however, 18:2n-6 did not increase in

reference milk. This could be explained by a competition between the dietary supply of n-3 fatty acids, such as 18:3n-3, 20:5n-3, and 22:6n-3, and n-6 fatty acids resulting in higher n-3 fatty acids in rat milk. Interestingly, differences in the ratio of 18:2n-6/18:3n-3 among the diets were clearly reflected in rat milk in the order of: high 18:3n-3 diet < reference diet < high 18:1n-9 diet < high 18:2n-6 diet.

2. Jejunal and colonic phospholipid fatty acids

Dietary manipulations can affect the lipid composition of biological membranes, and the gastrointestinal tract is no exception to this effect. The intestinal mucosa is a dynamic structure that undergoes biochemical, ultrastructural, and morphological changes during postnatal development. Absorptive cells of the small intestine have the capability to adapt their structure and function in response to changes in the intraluminal content of nutrients, which can modulate the associated function. This study established that the maternal diet and consequently, the milk diet n-6 and n-3 fatty acids, have marked effects on the developing jejunum and colonic phospholipid fatty acids. The study also evaluated any differences in jejunal or colonic phosphatidylcholine and phosphatidylethanolamine fatty acids in pups, in response to milk diet fatty acids.

High intake of n-6 fatty acids resulted in increased n-6 fatty acids, whereas, high intake of n-3 fatty acids resulted in increased n-3 fatty acids, in both jejunal and colonic phospholipid fatty acids. Nevertheless, some differences were found in the responses of the colon and jejunum. For example, a maternal diet high in 18:2n-6 resulted in significantly higher 18:2n-6 amounts in colon but not in jejunum PC and PE fatty acids. This may be explained by higher levels of 18:2n-6 in the colon, compared to the jejunum. A high intake of n-6 fatty acids from milk was not associated with high 20:4n-

6 in pup jejunum or colon PC and PE, whereas a high intake of n-3 fatty acids was associated with high 20:5n-3 and 22:6n-3 in jejunum or colon PC and PE. Also, marked increases were found in 22:4n-6 and 22:5n-6 in the pup jejunal and colonic PE in high 18:2n-6 group, even though the amounts of 22:4n-6 and 22:5n-6 were not found to be significant in the rat milk. This could be due to the increased desaturation and elongation of n-6 fatty acids in pups, in response to the low n-3 fatty acid supply in the high 18:2n-6 group. Importantly, the amount of 22:6n-3 was also high in jejunal and colonic PC and PE in pups in the high 18:1n-9 group, though the amount of 22:6n-3 in the milk was significantly lower in high 18:1n-9 group than in high 18:3n-3 group.

Clearly, the differences in n-6/n-3 fatty acids and 20:4n-6/20:5n-3 were maintained in jejunal and colonic PC and PE, with respect to the differences in rat milk. Experimental studies have also shown that dietary n-3 fatty acids increased n-3 fatty acids in plasma (Amusquivar *et al.*, 2000), liver, red cells and kidney (Swanson *et al.*, 1987; Lands *et al.*, 1990; Huang *et al.*, 1992; Suarez *et al.*, 1996) and maintained the n-3/n-6 ratios in phospholipid fatty acids, with respect to n-3/n-6 fatty acid ratio in the diet.

3. Intestinal responsiveness to DNBS colitis in pups

The intestinal epithelium is covered by a protective mucus gel composed predominantly of glycoproteins (synthesized and secreted by goblets cells) and structural fats. The gastrointestinal tract is the first interface between the diet and the body. Therefore, maintenance of structural integrity plays a key role in preventing intestinal inflammation. This study suggests that dietary fat, through milk feeding, modulates the intestinal phospholipid fatty acid composition. Depending on early dietary experiences, the ability of the intestine to adapt in response to various pathophysiological challenges

may be impaired. Using macroscopic, histological techniques, and enzyme assay, this study shows an association between milk diet n-6 and n-3 fatty acids and intestinal responsiveness to chemically-induced colitis in offspring fed milk diet.

3.1. Macroscopic and histological damage in pups

The major contribution of this study is to demonstrate that a high maternal dietary intake of 18:2n-6 modulated the inflammatory response associated with severe macroscopic and histological damage. In contrast, a diet high in 18:3n-3 or 18:1n-9 with a low n-6/n-3 ratio showed a lessened colonic infiltration of neutrophils and an abrogated inflammatory response, with histological damage scores that were lower than those seen in the high 18:2n-6 diet group. The inflammatory response was no different from ethanol controls in both high 18:3n-3 or 18:1n-9 diets. A high dietary intake of 18:1n-9 resulted in macroscopic damage scores that were between those found in the high 18:2n-6 and high 18:3n-3 diets.

Pups in high 18:2n-6 group following DNBS colitis showed severe alterations in the colonocytes, with areas of necrosis, desquamated epithelial cells as well as a loss of goblet cells, in comparison to the pups in other groups. DNBS-induced colitis also affected contiguous segments of the colon and the rectum with active lesions showing signs of edema, the lack of a normal looking vascular pattern and bleeding in rat pups in the high 18:2n-6 group. Histologically, lesions demonstrated a mucosal infiltrate with crypt abscesses, edema and mucus depletion from goblet cells. Other symptoms, such as the presence of mucus and watery stools, were also noted at the time of sample collection.

Consistent with macroscopic damage, high histological damage scores were also observed in the high 18:2n-6 group, whereas the lowest scores were seen in the high 18:3n-3 group. Several studies (Inui *et al.*, 1996; Teitelbaum and Walker, 2001) have suggested a link between inflammatory bowel disease and intestinal fatty acid composition. Bernstein *et al.* (1996) also suggested that high dietary n-6 fatty acids may alter mucin maturation in experimental colitis. Protective effects of high 18:1n-9 in inflammation have also been suggested possibly due to suppression of inflammatory mediators such as cytokines and interleukins (Grimble and Tappia, 1998).

Interestingly, pups in the reference group also showed marked increases in macroscopic and histological damage scores following DNBS insult, compared to pups in the high 18:3n-3 group. However, the colonic fatty acid composition of pups in both reference and high 18:3n-3 groups was similar. Importantly, the reference diet was a non-purified diet and was not controlled with respect to macro or micro nutrients. The differences seen in colonic response to DNBS insult in pups in reference diet suggest the importance of other factors in the reference diet that could have exaggerated the inflammatory response. Few studies (Appleyard and Wallace, 1995; Wallace *et al.*, 1998) have examined chow fed adult rats following trinitrobenzene sulfonic acid colitis and shown a marked increase in transmural inflammatory cell infiltrate of both acute and chronic inflammatory cells in the distal colon, whereas the inflammatory infiltrate was less pronounced in ethanol controls.

3.2. Myeloperoxidase activity in pups

A strong evidence indicates the participation of myeloperoxidase in the oxidative damage in ulcerative colitis. Myeloperoxidase reacts with free radicals to produce *N*-

chloramines that are stable oxidants (Segal *et al.*, 1980). Myeloperoxidase, one of the main enzymes in neutrophils, can be used as a marker for their presence in intestinal mucosa, to indicate the severity of the inflammatory process (Nieto *et al.*, 1998).

In the present study, the results of myeloperoxidase activity were no different from those of macroscopic or histological damage. Diets high in 18:3n-3 or 18:1n-9, with a low ratio of n-6/n-3, resulted in low myeloperoxidase activity, compared to other diets. Interestingly, the reference group pups and the high 18:2n-6 pups showed higher myeloperoxidase activity, compared to the other groups, along with significant differences from their respective ethanol controls. Consistent with other experimental studies using mature animals, n-3 fatty acids decreased myeloperoxidase activity (Nieto *et al.*, 1998). Later studies (Nieto *et al.*, 2002) showed a higher degree of epithelial repair than that observed in high 18:1n-9 or chow-fed groups following one week post trinitrobenzene sulfonic acid colitis. In these studies, the chow-fed rats had severe colonic damage with fused apical crypts and excessive mucus in the lumen. In addition, chow-fed rats had more positive PAS (periodic acid-Schiff stain) areas (a marker of lipid peroxidation) than did other groups. Chow-fed rats had higher myeloperoxidase activity (9.7±1.3 U/mg), compared to the ethanol controls (2.3±0.8 U/mg), or the saline controls (0.1±0.0 U/mg), following TNBS insult.

The present findings also suggest associations among the milk n-6/n-3 fatty acids, n-6 and n-3 fatty acids in colonic phospholipids of pups, and consequently the effects of these changes in colonic insult. Enhanced inflammatory response in colon of pups in high 18:2n-6 group may be due to high 18:2n-6 along with low n-3 fatty acids, consequently a high n-6/n-3 fatty acid balance.

Experimental and clinical studies have shown that n-3 fatty acids decrease tissue 20:4n-6, with partial replacement of 20:5n-3 which results in decreased synthesis of 20:4n-6-derived eicosanoids and increased synthesis of 20:5n-3-derived eicosanoids (Empey *et al.*,1991). However, others have suggested that n-3 fatty acids may be efficacious in IBD by serving as free radical scavengers and protect the colonic mucosa from oxidative cell damage. The increased numbers of monocytes and macrophages, cells that produce oxidative bursts, expose the colonic mucosa to oxygen-derived free radicals (Bartnik and Shorter, 1980). Oxygen-derived free radicals, such as the superoxide anion and the hydroxyl radical, are cytotoxic and promote lipid peroxidation and membrane damage by cross-linking proteins, lipids, and nucleic acids (Itoh and Guth, 1985). Since these radicals mediate tissue damage and have been recently shown to be responsible for the development of mucosal injury of the gastrointestinal tract in the rat, (Sam, 1990; Salim, 1990) they may play a role in the mechanism of ulcerative colitis. Some studies have shown a significant reduction in oxidative stress in patients given n-3 fatty acids in combination with drug therapy (Fisher and Levine, 1991; Barbosa *et a.l*, 2003)

Hence, the differences seen in n-3 fatty acids between the high 18:2n-6 and the high 18:3n-3 groups suggest the protective role of n-3 fatty acids in the experimental colitis. Current research also suggests that 20:5n-3 is a precursor of resolvin E1 (Arita *et al.*, 2005) and 22:6n-3 is a precursor of 17s resolvins, which have anti-inflammatory properties (Serhan *et al.*, 2002). As emphasized in many studies, diets rich in n-6 fatty acids enhance interleukin production and the tissue responsiveness to cytokines, whereas diets rich in n-3 fatty acids have an opposite effect (Nieto *et al.*, 1998; Grimble and

Tappia, 1998; Lee *et al.*, 1985). Although this study did not address the mechanisms involved in the inflammatory response in DNBS colitis or ethanol-induced colonic damage, n-3 fatty acids appeared to attenuate the inflammatory response. Likewise, monounsaturated fatty acids also known to diminish the tissue responsiveness to cytokines and interleukin production in inflammatory process (Grimble and Tappia, 1998). Consistent with this, the absence of differences in histological damage and myeloperoxidase activity in high 18:1n-9 or high 18:3n-3 groups suggest beneficial effects of monounsaturated fatty acids in DNBS colitis, however, this study did not address the mechanisms behind the protective effects of monounsaturated fatty acids.

4. OVERALL CONCLUSIONS

In the rat, the lipid composition of the diet was found to influence the milk fatty acid composition. In general, rats fed a high n-6 fatty acid diet produced milk that contained significantly higher amounts of n-6 fatty acids, whereas rats fed a high n-3 fatty acid diet produced milk with significantly higher n-3 fatty acids. The differences in the total n-6/n-3 fatty acid ratio in the dietary oils were clearly reflected in differences in the milk, with a significant increase in the n-6/n-3 fatty acid ratios among the groups, according to the following order: high 18:3n-3 < high 18:1n-9 < high 18:2n-6.

The maternal diet, through milk feeding, influenced jejunal and colonic fatty acids in the sucking rat pups. Colonic, rather than jejunal fatty acids, appeared to be influenced to a greater degree in response to dietary 18:2n-6 fatty acid. Longer chain n-6 fatty acids (22:4n-6 and 22:5n-6), however, increased both jejunal and colonic fatty acids in pups of mother rats receiving a high n-6 fatty acid diet, compared to those receiving n-3 or n-9-rich diets.

Thus, the maternal diet fat influenced the composition of intestinal fatty acids, through milk feeding, in rat pups. In regards to the intestinal inflammatory responses to chemically-induced colitis, maternal n-3 or n-9 fatty acids attenuated the intestinal inflammatory response, whereas, n-6 fatty acids exacerbated the response. Nevertheless, the differences in the inflammatory responses of pups nursed by dams fed chow, compared to pups fed the semi-synthetic diets, suggest that other factors besides colonic fatty acids, may be playing a significant role. The effects of the reference diet on chemically-induced colitis, in the reference pups, may not be directly related to the fatty

acids. Other factors, possibly of the immune system, could also be playing a role to give rise to the differential responses to chemical insult in pups.

5. FUTURE DIRECTIONS

Due to the financial and time constraints of this single student research project, not all variables related to the present study could be covered adequately. During the study, some gaps in knowledge became apparent and further investigations could be useful to explore these areas. In particular, future studies could:

- Systematically explore the therapeutic and dose-dependent effects of maternal dietary 18:2n-6 or 18:3n-3 on intestinal fatty acid composition of newborn and adult experimental animals in normal development and following intestinal inflammatory insult. The efficacy of early, diet-related differences could also be explored to determine whether or not the effects persist into adulthood. Also, experiments could be better designed if isolated fatty acids, particularly, 20:4n-6, 20:5n-3, and 22:6n-3 in semi-synthetic diets could be added to maintain a specific level of n-6 or n-3 fatty acids.

- Be designed to refine the methods of lipid extraction, especially for the fractions to provide information on fatty acid composition of other phospholipid classes such as phosphatidylserine and phosphatidylinositol. Although an attempt was made to measure the other phospholipids such as phosphatidylserine and phosphatidylinositol in the pup jejunum and colon, these phospholipid classes were extracted in smaller fractions, and the inconsistent findings were not used in this study.

- Specifically examine the gestational contribution of the maternal diet. While the effects of the maternal diet and, consequently, the milk were examined on sucking pups, the study did not explore the gestational effects of the maternal diet.

- Focus on exploring various pathophysiological mechanisms that may be acting alone or in combination with dietary modifications such as understanding the role of varying maternal dietary fat on milk peptide mediators such as cytokines, interleukins or lipid mediators such as prostaglandins or leukotrienes and further finding the effect of milk feeding on these markers in inflammatory bowel disease.

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APPENDIX

APPENDIX I.

Dinitrobenzene sulfonic acid dose response and time course studies

The third objective of the study was to develop a model of inflammatory bowel disease in the suckling rat pups. Best to our knowledge, this was the first experiment to develop the neonatal rat model of ulcerative colitis. In many experimental rat models of colitis, a higher dose of 60 mg 2,4-dinitrobenzene sulphonic acid (DNBS) / ml of 50% ethanol has been used. We wanted to test a dose in neonatal rat model which was no high enough to kill the pups. Therefore, we tested several doses of DNBS (20, 30, 40, 60 mg DNBS / ml of 50% ethanol) in rat pups and followed the similar experimental design mentioned earlier. Specifically, on post natal day 15, pups nursed by chow fed rat mothers, were randomly assigned (n=6 pups/dose) to receive DNBS, the vehicle control (50% ethanol) or a procedure control (0.9% saline). Twelve hours later, pups were sacrificed using cervical dislocation and myeloperoxidase enzyme activity was measured. The results (Fig AI.1) indicated the myeloperoxidase activity increased with increasing doses of DNBS. We selected 30 mg DNBS dose to preclude pups being killed by a higher dose and the diet effect, if any, not eluded.
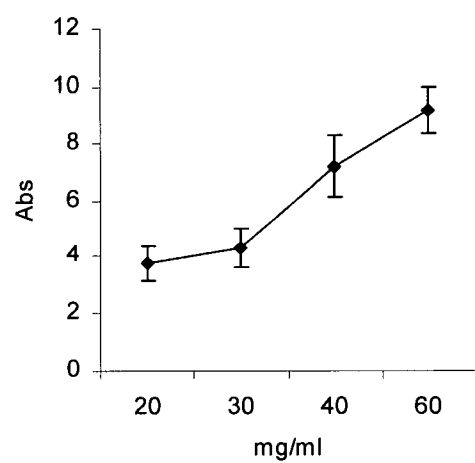
Figure AI.1: Myeloperoxidase activity in the rat pups using different DNBS doses (n=6 pups/dose). Abs: Absorbance.

Time course studies

The 30mg / ml DNBS dose was selected based on myeloperoxidase activity. To establish an appropriate time course following DNBS colitis, pups (n=6 pups/time) nursed on chow fed mothers were sacrificed at different time points (6hr, 12hr, 24hr, 48hr, 72hr). Results of myeloperoxidase activity (Fig AI.2) indicated highest myeloperoxidase activity 12 hours post DNBS treatment. After 12 hours of treatment, the enzyme activity decreased. We selected 12 hours time frame to ensure maximum enzyme activity and the diet effect, if any, not eluded.
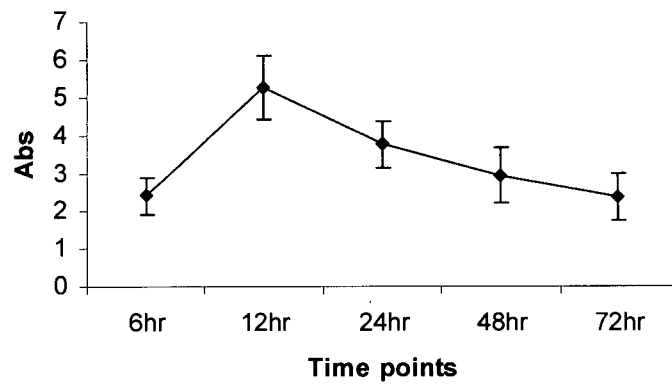
Figure AI.2: Myeloperoxidase activity in the rat pups at different time points of DNBS

treatment (n=6 pups/time point). Abs: Absorbance; hr: hours.