The Effect of Dexamethasone on Growth and Arachidonic and Docosahexaenoic Acids in the Piglet Model for Infant Nutrition

By

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A Thesis
Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements of the Degree of

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FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of Master of Science

Dedication

This thesis is dedicated to my mother, Lorraine Petryk, who instilled in me the desire to learn, my father Michael Petryk, who taught me that hard work is the key to success and to my husband Marc, who's love and support has been my inspiration.

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The Effect of Dexamethasone on Growth and Arachidonic and Docosahexaenoic Acids in the Piglet Model for Infant Nutrition.

Introduction: Dexamethasone (DEX) is a corticosteroid used as therapy for premature infants with chronic lung disease. DEX has been shown to reduce growth and decrease essential fatty acid (FA) synthesis in vitro.

Objective: To determine somatic growth, brain, liver, retina, red blood cells and plasma arachidonic acid (AA) and docosahexaenoic acid (DHA) in piglets fed standard formula or sows' milk and treated with or without DEX.

Methods: Twenty 10 day old male piglets were randomised to receive 15 days of DEX or placebo and either sows' milk (S) or formula (F). Measurements were final weight and length and AA and DHA (expressed as % w/w of total phospholipid) in total lipid of brain and phosphatidylethanolamine (PE) and phosphatidylcholine (PC) extracted from liver, retina, plasma, and RBC. Plasma triglyceride and total cholesterol were also examined. All data are mean ± SEM, unless otherwise stated.

Results: DEX treated piglets had reduced weight (DEX 4.3 ± 0.4 < placebo 5.5 ± 0.4 kg, $P<0.05$) and length (DEX 49.2 ± 1.6 < placebo 53.4 ± 1.4 cm, $P<0.05$) regardless of feeding group. Sows' milk attenuated DEX-induced reductions in liver PC DHA and AA and PE DHA. DEX reduced plasma DHA, and a significant correlation was found between liver and plasma FA, PC AA $r^2=0.45$, $P=0.002$ and PC DHA $r^2=0.43$, $P=0.002$. A main treatment affect of DEX was observed in brain AA (telencephalon) (DEX 8.6 ± 0.2 < placebo 9.6 ± 0.3, $P<0.05$). Sows' milk feeding had an overall affect in retina PE AA (S 11.7 ± 1.4 > F 6.15 ± 1.25). In retina PC DHA placebo was significantly greater then DEX, (placebo 3.9 ± 0.4 > DEX 2.6 ± 0.5), and formula feeding was significantly greater then sows' milk feeding (F 3.8 ± 0.3 > S 2.5 ± 0.4). No other significant

differences were observed in Retina FAs. Formula–DEX treated piglets had significantly higher PC DHA in RBC than sow-DEX (F-DEX 2.6 ± 0.4 > S-DEX 0.7 ± 0.4). In the non-fed state, plasma triglyceride (DEX 1.1 ± 0.2 > placebo 0.2 ± 1.8 mmol/L, $P<0.05$) and total cholesterol (DEX 6.2 ± 1.3 > placebo 3.9 ± 0.4 mmol/L, $P<0.05$) were significantly higher in the DEX-treated versus the placebo-treated piglets.

Conclusion: The results indicate a protective affect of sows' milk on DHA status, but not somatic growth. Further research is necessary to determine if the reduced liver and plasma DHA in the formula-DEX piglets and AA in brain reflect greater utilisation or reduced endogenous synthesis and what the consequences to growth and development are in the long term.

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AA	arachidonic acid
ALA	alpha linolenic acid
CV	coefficient of variance
DHA	docosahexaenoic acid
DEX	dexamethasone
FA	fatty acid
LA	linoleic acid
LCPUFA	long chain polyunsaturated fatty acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
RBC	red blood cells
SEM	standard error of the mean
SD	standard deviation

LITERATURE REVIEW

1.0 DIETARY FAT

1.1 The Function of Dietary Fat

Dietary fat is a major nutrient and an important source of body fuel. Fat consists of a mixture of triacylglycerol and their constituent fatty acids (FA), which are crucial to growth, development and cellular processes. Dietary FAs differ considerably in their carbon chain length and number of double bonds between carbon atoms. Various combinations of different kinds of FAs make up different species of triacylglycerol. Typically the FAs are divided into three groups: saturated, monounsaturated and polyunsaturated. It is the polyunsaturated FAs arachidonic (AA) and docosahexaenoic acid (DHA) that are the focus of this thesis because of their importance to growth and development.

1.2 Digestion of Dietary Fats

Digestion of dietary fat starts in the oral cavity with saliva, which contains lingual lipase. The lipase is responsible for the hydrolysis of the triglyceride to release free FA. Hydrolysis continues in the stomach where gastric lipase promotes further lipid digestion. Fat then enters the duodenum, and it is here that it comes into contact with pancreatic lipase and bile salts. Intestinal lipase hydrolyzes the stereospecific number (sn) sn-1 and sn-3 positioned FAs from the glycerol backbone, which are then considered free FAs. The FA left in the sn-2 position remains attached to the glycerol backbone and subsequently is called a monoacylglycerol. Polar lipids of bile: bile acids and phospholipids solubilize FAs in the gut. This promotes the formation of micelles that can then pass into the mucosal cells of the small intestine.

1.3 Absorption of Fat

Once in the intestinal mucosa cell, monoacylglycerols and FA are recombined into triacylglycerol. The triacylglycerols are incorporated into lipoproteins or chylomicrons. Once the fat is in the chylomicron it can then be secreted into the intestinal lymph, where it then passes through the thoracic duct into the systemic circulation. When chylomicrons pass into the peripheral circulation, they come into contact with lipoprotein lipase, the enzyme that is located on the endothelial surface of capillaries. Lipoprotein lipase hydrolyses the triacylglycerols of chylomicrons and free FAs are released. Most of these FAs are re-synthesized into triacylglycerol and are stored by cells. Some FAs that are released into the capillaries of skeletal muscles are taken up and used for energy, as FAs are the major fuel of resting muscles. Still other free FAs bind to albumin and re-enter the systemic circulation. The liver can also utilise free FA, which it can convert into glycerolipids (triacylglycerol and phospholipids).

1.4 Fatty Acids and Cell Structure

There is a limited amount of dietary lipid that occurs as phospholipid. Phospholipids occur almost exclusively in cellular membranes of plants and animals. Phospholipids are different from triglycerides in that they contain a polar head group (hydrophilic) and two nonpolar tails (hydrophobic) that are composed of longer-chain FAs and are therefore, amphipathic, a critical property for their structural role in membranes. There are many different individual phosphoglycerides. Depending on the two FA and the polar head group, the phosphoglycerides can vary in size and charge (and include inositol, choline, serine, ethanolamine, glycerol and phosphatidyglycerol).

Phospholipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the most abundant phosphoglycerides in animals and are a very important part of the structural matrix of all cell and subcellular membranes. The fluidity and other physical properties of membrane phospholipids are largely determined by the chain length and degree of unsaturation of the component FA. The n-3 and n-6 FA composition of the diet will determine the FA composition of the membrane phospholipids in turn influencing several membrane related functions, such as hormone binding and structural functions such as the maintenance of normal activities of membrane-bound enzymes (Innis, Present Knowledge in Nutrition 1996).

2.0 Fatty Acids and Human Growth and Development

2.1 Fatty Acid Synthesis

Fat has been recognised as an essential part of the human diet for nearly 70 years. Evans and Burr demonstrated in 1927 that animals fed fat-free diets would have impaired growth and reproductive failure (Burr & Burr, 1929). In adults and infants, the liver and some cells of the central nervous system, are able to synthesise saturated fatty acids and n-9 monounsaturated FA (oleic acid), but do not have the enzymes necessary to insert the double bonds at the n-6 and n-3 positions which are needed to synthesise the long chain polyunsaturated FA (LCPUFA) linoleic acid (LA) and alpha linolenic acid (ALA) (Innis, 1993). Since humans do not have the capability to synthesise these FA, which are considered essential, the diet must provide them in amounts consistent with need.

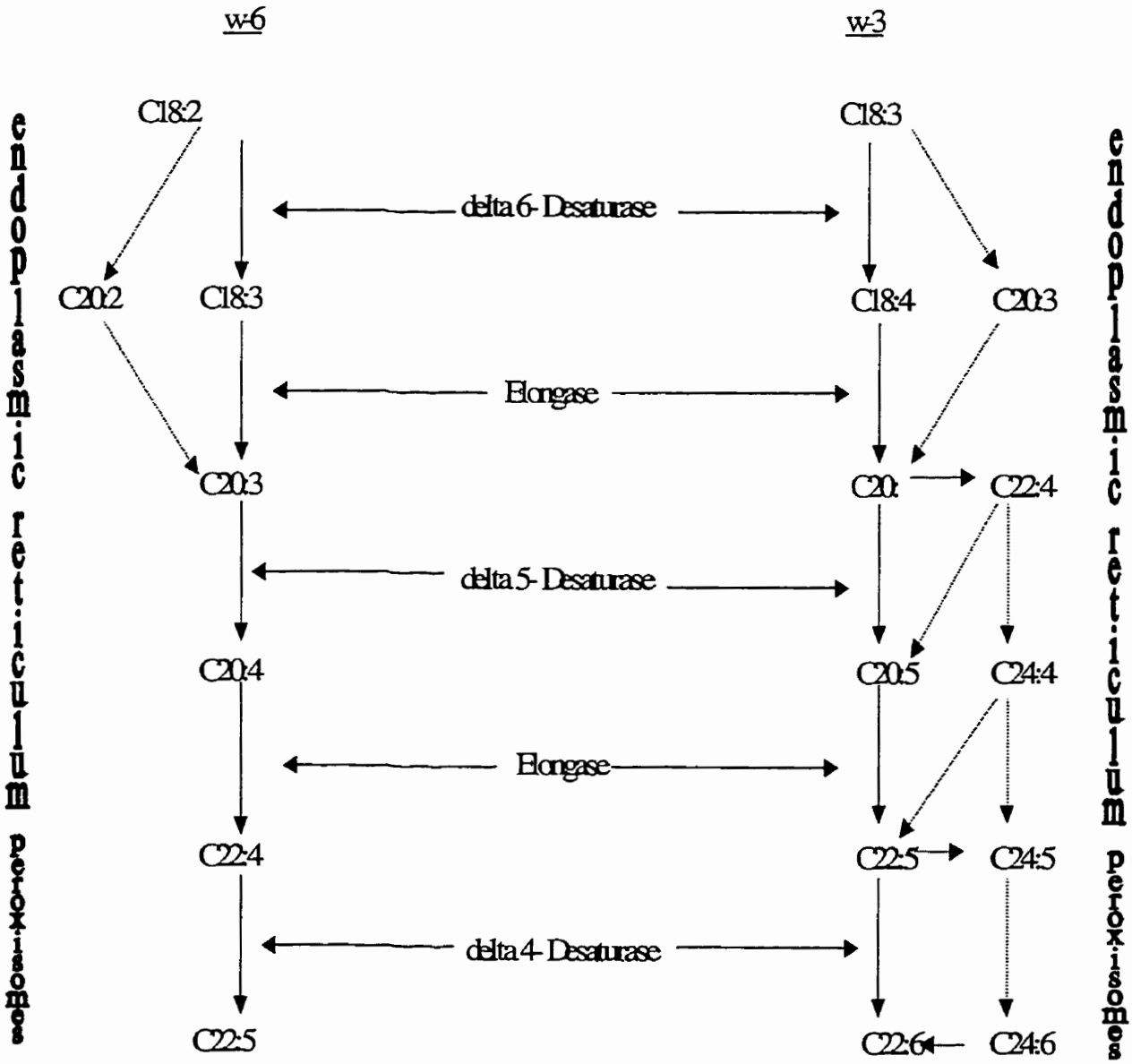
LA and ALA are necessary for energy, growth, cellular metabolism, muscle activity and neural development. Essential FA deficiency is associated with a set of specific findings, and has been related mostly to combined n-3 and n-6 deficits which

include poor growth; skin lesions; decreased skin pigmentation; loss of muscle tone; degenerative changes in kidney, lung and liver; increased metabolic rate; impaired water balance; increased fragility and permeability of cell membranes and increased susceptibility to infection (Uauy, Treem & Hoffman, 1989; Hansen, Wiese, Boelsche, & Haffard, 1963; Caldwell, Johnson, & Othersen, 1972; White, Turner, Turner, & Miller, 1973; Paulsrud, Pensler & Whitten, 1972). If these EFA deficiencies take place just prior to birth, during the most critical time of growth and development, it can be very detrimental to an infants well being.

Another important function of ALA (C18:2n-3) and LA (C18:3n-6) is that they are the precursors to the LCPUFAs DHA and AA respectively. Until recently the accepted pathway for endogenous syntheses of AA (C20:4n-6) and DHA(C22:6n-3) has been a series of alternating desaturation and elongation steps beginning with delta 6 desaturation of ALA and LA and termination with delta 4 desaturation of C22:5n-3 and C22:4n-6, respectively, to C22:6n-3 and C22:5n-6 (figure 2.1.0). An alternative pathway independent of delta 4 desaturation has been demonstrated in isolated rat hepatocytes (Voss, Reinhart, Sankarappa, & Sprecher, 1991; Sauerwald, Hachey, Jensen, Chen, Anderson, & Heird, 1997, Mohammed, Sankarappa, Geiger, & Sprecher, 1995), human skin fibroblasts (Moore, Hurt, Yoder, Sprecher, & Spector, 1995) and, most recently, in preterm infants (Sauerwald, Hachey, Jensen, Chen, Anderson, & Heird ,1997). If the alternative pathway, which includes two delta 6 desaturation steps, is operative in vivo, C18:3n-3 and C18:2n-6 will compete not only with each other but also with C24:5n-3 and C24:4n-6 for delta 6 desaturation. Due to the effects this pathway may have on LCPUFA production and status, it highlights the potential importance of the dietary

intake of ALA and LA, and their amounts in infant formula. These desaturase enzymes, which are membrane-bound, complete their conversion mainly in the endoplasmic reticulum of tissues i.e., the liver, brain, and retina (Innis, 1991; Sprecher, 1992; Moore, Yoder, & Spector, 1990; Morre, Yoder, Murphy, Dulton, Spector., 1991; Wang & Anderson, 1993; Wetzel, Li, & Alverez, 1991). It has also been reported from a number of laboratories that the LCPUFA containing 5 and 6 double bonds such as DHA are not synthesised in the endoplasmic reticulum, but rather in a complex process that occurs in the peroxisomes of the liver (Voss, Reinhart, Sankarappa, & Sprecher, 1991; Sauerwald, Hachey, Jensen, Chen, Anderson, & Heird, 1997). This is of importance because the extra steps occurring in the peroxisomes could limit DHA production, and therefore make intake of DHA of critical importance.

Figure 2.1.1. Potential pathways of long chain polyunsaturated fatty acid synthesis. The classical pathway involving delta-6, delta-5 and delta-4 desaturation followed by elongation is depicted by solid arrows. Putative alternative steps consistent with data from Sauerwald are depicted by broken arrows. Adapted from Sauerwald et al., 1997.



2.2 Importance of Arachidonic and Docosahexaenoic Acids in Infant Nutrition

The amount of DHA and AA produced endogenously is of utmost importance to growth and development of infants even though only small amounts are needed for survival. Therefore adequate essential FA precursors are necessary, combined with an adequate ability to synthesize LCPUFA. Recommended dietary intakes of LA are usually around 3-5% of dietary energy (Food and Agriculture Organization/World Health Organization, 1993) even though intakes of about 2.4% of LA support maximum tissue levels of AA in rodents and also prevent the appearance of signs of AA deficiency in human infants and adults (Hansen, Wiese, Boelsche & Haffard., 1963; Adam, Hansen, & Wiese, 1958; Innis, 1991; Bourre, Piciotti, Dumont, Pascal & Durand., 1990). Approximately 0.5-1.0% of dietary energy needs to come from ALA, to achieve maximum tissue levels of DHA and to avoid any apparent deficiency symptoms (Arbuckle, Mackinnon & Innis, 1994; Bourre, Francois, Youyou, Durmont, Piccotti & Pascal, 1989; Innis, 1992; Neuringer, Connor, Van Petten & Barstad, 1984; Neuringer, Connor, Lin, Barstad, & Luck 1986). A deficiency of LA and subsequently AA results in a wide range of disorders, such as decreased growth of the brain and body, and dermatitis (Burr & Burr, 1929; Burr & Burr, 1930; Paoletti &, Galli, 1972).

DHA is scarce in most tissues but has a very high concentration in the brain and retina, the highest concentration being found in rod photoreceptors (Anderson & Risk, 1971) and in neural synapses (Bourre, Durand, Masson, Dummont & Piciotti, 1984). Although DHA is found in some tissues in scarce amounts, there is no question that it is essential for optimal tissue function (Innis, 1991). A decrease in DHA can be due to decreased synthesis, from limited intake, or both. A deficiency of ALA, and therefore

DHA, causes changes in retinal function and vision (Wheeler, Benolken, & Anderson, 1975; Neuringer, Connor, Van Petten, & Barstad, 1984; Lamptey & Walker, 1976) and may also affect learning and behaviour (Lamptey & Walker, 1978).

There is some controversy over whether term infants need supplementation. Healthy term infants fed human milk will acquire approximately 50-60% of energy as fat, of which about 5% is essential FA and 1% is LCPUFA, (table 2.2.0) therefore providing a relatively stable supply of AA and DHA in amounts that meet their requirements (Koletzko, Thiel, & Abiodun, 1992). Infants fed the current formula in North America will receive no AA and DHA through formula (table 2.2.1) however they can acquire DHA and AA while in utero through placental transfer, or after birth through endogenous synthesis. A study by Innis, Akrabawi, Diersen-Schade, Dobson, & Guy (1997) found no difference in growth or preferential looking acuity at 3 months of age among breast-fed term infants and term infants who were fed two different formulas, one with 18% LA and 1.9% ALA and another with 34% LA and 4.7% ALA. On the other hand, a different study using a similar formula that Innis used (Carlson, Carver, & House, 1986), did find lower looking acuity in formula-fed term infants compared to breast fed infants at two months of age. This study did not however, find any differences at four months of age. A few other studies did report a higher increase in looking acuity from two-four months (Jorgensen, Hernell, Lund, Holmer, & Michaelsen, 1994) and another found still a lower visual evoked potential (VEP) at 5 months (Makrides, Simmer, Goggin, & Gibson, 1993). It is not clear if differences in the results among these studies are explained by differences in ALA content, socio-economic or environmental differences in the groups of non-randomised breast-fed and formula-fed infants. In the study by Innis, Akrabawi,

Diersen-Schade, Dobson, & Guy (1997) they also suggested that different methodologies used to assess visual function could potentially explain some of the discrepancies in the findings.

Unlike healthy term infants, some investigators have suggested that the desaturation enzymes in premature infants may not be fully mature (Anderson, Connor, & Corliss, 1990; Carlson, Rhodes, Rao, & Goldgar, 1987). Consequently, infants born premature and denied AA and DHA via placental transfer would have considerable deterioration of these essential FAs unless fed breast milk or formula supplemented with AA and DHA. Formula companies in North America try to develop formulas that mimic the composition and functional outcomes of breast milk by including LA and ALA, but not include DHA and AA at the present time. Current premature formulas in Canada provide 34-41g/L of fat (Enfalac Premature 20 and Enfalac Premature Plus respectively) with the ratio of LA to ALA in formulas ranging anywhere from 16:1 to 4:1, with the majority using a 9:1 ratio (FAO/WHO Expert Committee, 1994, Health Canada 1996, Nutrition Committee, Canadian Paediatric Society 1995). Despite combining vegetable oils, (table 2.2.2) formula feeding has not supported the circulating levels of AA and DHA observed in omnivorous breast-fed infants.

It is important to keep in mind that there are many nutritional differences between breast milk and infant formula, other than the LCPUFAs. Iron is more bio-available in breast milk and nucleotides, as well as other growth and hormonal factors are present that may affect neural function. To control for these confounding variables, studies have to be conducted comparing infant formulas with and without DHA.

Table 2.2.1. Composition of total milk lipids (% of total FA) in breast milk of mothers of term and preterm infants days 5 and 30 with no significant differences noted (Genzel-Boroviczeny, Wahle, Koletzko, 1997).

Fatty Acid	5 days term	5 days preterm	30 days term	30 days preterm
C10:0	0.50	0.46	1.01	1.08
C12:0	3.42	4.17	5.21	6.07
C14:0	5.86	7.65	6.90	7.93
C16:0	24.97	24.79	22.47	22.84
C18:0	7.07	6.34	7.40	7.65
Total Saturated	43.65	44.16	44.30	46.78
C18:1n-9	32.10	33.83	31.50	31.38
Total trans	0.96	1.01	1.13	1.26
C18:2n-6	8.86	9.88	11.33	10.49
C18:3n-6	0.14	0.11	0.18	0.17
C20:2n-6	0.57	0.54	0.30	0.32
C203n-6	0.53	0.50	0.38	0.41
C20:4n-6	**0.72**	**0.74**	**0.45**	**0.48**
C22:4n-6	0.23	0.24	0.08	0.11
Total n-6 LCP	2.15	2.13	1.28	1.31
Total n-6	11.57	12.2	13.06	12.02
C18:3n3	0.65	0.67	0.90	0.73
C20:3n-3	0.09	0.09	0.05	0.00
C20:5n-3	0.04	0.00	0.05	0.00

Fatty Acid	5 days term	5 days preterm	30 days term	30 days preterm
C22:5n-3	0.22	0.22	0.15	0.15
C22:6n-3	**0.46**	**0.43**	**0.23**	**0.24**
Total n-3 LCP	0.80	0.73	0.18	0.42
Total n-3	1.51	1.38	1.52	1.13
Total LCP	3.08	2.85	1.80	1.66
N6/n3 LCP	2.60	2.71	2.58	2.88

Table 2.2.2. Composition of fat in current premature formula from Enfalac Infant Nutritional Product Information (Mead Johnson Nutritionals, 1999).

Fatty Acid %w/w	Enfalac, with iron, for term infants	Enfalac Premature Plus 24	Enfamil Human Milk Fortifier
C6:0	0.1	0.5	2.3
C8:0	1.6	30.0	1.1
C10:0	1.2	12.0	2.0
C12:0	9.3	9.4	3.1
C14:0	4.1	3.6	11.8
C16:0	22.0	5.9	26.4
16:1	0.09	-	1.9
C18:0	4.3	2.4	12.6
C18:1n9	38.0	11.2	28.5
C18:2n6	17.2	22.0	2.0
C18:3n3	1.8	3.1	0.5
C20:0	0.26	-	-
C20:4n6	-	-	0.1

Table 2.2.3. Percent of total Kilocalories from fat in term, preterm and human milk fortifier, of Mead Johnson, Nutritional products, 1999.

	% total kcalories		
Fat composition	Enfalac, with iron, for term infants	Enfalac Premature Plus 24	Enfamil Human Milk Fortifier
Palm olein oil	22	N/A	N/A
High Oleic sunflower oil	7.3	N/A	N/A
Emulsifiers	0.9	N/A	N/A
MCT oil	N/A	16.6	N/A
Soy oil	9.8	18	N/A
Coconut oil	9.8	9	N/A
Total Fat	50	44	3.9*

*From milk protein

2.3 Essential Fatty Acid Status in Premature Infants

The rate of a human infants brain growth is fastest in the last trimester before birth (reviewed in Innis, 1991), and continues to develop after birth until approximately two years of age (Dobbing & Sands, 1970). Since LCPUFA's support growth, neurological development and functioning, it is especially important that infants born premature receive the FAs they need at this critical time in development.

Premature infants have multiple contributing factors that place them at greater risk of growth failure and sub-optimal development compared to term infants such as: deprivation of the vital intrauterine fat accretion that normally takes place during late pregnancy, very low fat stores, and having to rely on immature gut and metabolic function (ESPGAN Committee on Nutrition 1991). Within 3-6 days of being born premature, the premature infant's essential FA concentrations may fall to less than one-fifth of that of the placental-fetal supply. It is speculated that this is because the amount of AA and DHA the infant synthesises does not parallel the amount it would have received while remaining in utero. Premature infants that have the added problem of bronchopulmonary dysphasia also have an elevated metabolic rate caused by the "work of breathing"; inadequate intake or malabsorption of nutrients; and medical treatment such as fluid restriction, catabolic steroids, and diuretics, which all jeopardise nutritional status and growth (Kurzner, Garg, Bautista, Sargen, Bowman & Keens, 1988).

Early nourishment of preterm infants has a major effect on long-term neurological and developmental outcomes (Lucas et al., 1990; Lucas, Morley, Cole, Lister, & Leeson-Payne, 1992; Lucas, Morley, Cole, & Gore, 1994; Lucas, 1994). The undisputed knowledge that FAs are needed for growth and development may have special functional

significance for the premature infants who are fed formulas with low ALA and LA. For example, DHA is needed for the optimal functional maturation of the retina and visual cortex. An infant must be able to manufacture sufficient DHA on its own from the formula it receives, thus formula low in ALA coupled with the infant being premature, may create a situation in which the infant does not have the capability to synthesise enough DHA to meet its needs, and therefore may be dependent on dietary sources of preformed DHA. Recently the ability of the preterm infant to synthesise AA and DHA has been confirmed (Carnielli, et al., 1996), but it remains questionable if endogenous syntheses coupled with minimal tissue stores will result in optimal growth and development, when no dietary source is available (Neuringer, Connor, Van Pette, & Barstad, 1984). The consistent observations of declining LCPUFA after birth poses the question as to whether the small preterm infant is capable of biosynthesising enough AA and DHA to meet the requirements for maximum growth and development. In addition, synthesis of LCPUFA may be limited by illness or catabolic states (Cogo, et al 1997) and further limit growth and development.

2.4 Arachidonic and Docosahexaenoic Acid in Brain and Retinal Development

It is known that a deficiency of both AA and DHA alters the learning capacity in animals (Caldwell & Churchill 1966; Paoletti & Galli 1972; Lamptey & Walker, 1978), however, there is significant controversy in the scientific literature over whether DHA or AA should be supplemented in infant formulas, and if supplemented in what amounts. A study by Lucas, et al. (1992) actually found that preterm neonates fed their own mother's milk, compared to premature infants that were not, had intelligence quotients that were

8.3 points higher at 7.5-8.0 years of age, even after social and educational confounding factors were adjusted for. A study by Crawford (1993) claimed that premature babies fed breast milk were more intelligent than those fed formula, and that breast-fed infants score better on visual and developmental tests than formula-fed infants do. In a recent review by Gibson and Makrides, 1998 (table 2.4.0, #1-5), they found five studies conducted on neurodevelopmental outcomes of preterm infants in randomised clinical trials of LCPUFA supplementation. They concluded from the outcomes of these trials that preterm infants benefit from a supply of dietary LCPUFA. It is important to note however, that the infants in the trials were born less than 32 weeks gestation and only the relatively healthy infants were studied. It would not then be appropriate to state that preterm infants born after 32 weeks gestation are in need of supplementation. A recent study by Bougle, Denise, Vimard, Nouvelot, Penniello & Guillois, 1999, did however find that formula without the addition of LCPUFA allowed for the adequate early maturation of the central nerve system (table 2.4.0, #6).

When researchers compare physiological parameters such as visual acuity between breast and formula-fed term infants, and relate the results to infant FA profiles, often they report better indices of visual function and higher erythrocyte DHA levels in breast-fed infants compared to formula-fed infants (Makrides, Neumann, Byard, Simmer, & Gibson, 1994; Makrides, Simmer, Goggin, & Gibson, 1993; Birch, Birch, Hoffman, Hale, Everett, & Uauy, 1993). An earlier study by Hoffman, Birch, Birch, & Uauy, (1993) found that at 57-weeks follow-up, premature infants fed human milk and marine-oil supplemented formula, and therefore both receiving DHA, had improved visual function relative to infants fed formulas devoid of DHA. The results of Hoffman's work, along

with several similar preliminary studies reported by Carlson (Carlson, Rhodes, Rao, Goldgar, 1987; Carlson, Rhodes, Ferguson, 1986; Carlson, Cooke, Rhodes, Peeples, Werkman, Tolly, 1991) support an essential role for LCPUFA in normal eye and brain development. Therefore the FA requirements of very low birth weight infants are very important to establish in order to maximise the growth, nutritional status, and therefore the health of the infant.

Table 2.4.1. Neurodevelopmental outcomes of preterm infants in randomised clinical trials of LCPUFA supplementation. Adapted from Gibson and Makrides, 1998.

Reference	Diet	Test	Age	Results
#1 Uauy et al., 1992	HM (supplemented with FO formula) LA:ALA 24:0.5 LA:ALA 21:2.7 LA:ALA 20:1.4 + FO	rod ERG cone ERG SS-VEP acuity acuity cards	36 wks PCA 57 wks PCA 36,57 wks PCA 36,57 wks PCA 57 wks PCA	HM & FO >LA:ALA 24:0.5 no difference with diet no difference with diet HM & FO > LA:ALA 24:0.5 HM& FO > LA:ALA 24:0.5
#2 Carlson et al., 1993	SF, LA:ALA 33:4.8 + FO	acuity cards novelty preference visual attention Bayley's test	48 57 wks PCA 68,79,93 wks PCA 68,79,93 wks PCA 68,79,93 wks PCA 93 wks PCA	FO>SF no difference with diet no difference with diet FO<SF no difference with diet
#3 Carlson et al., 1996	SF, LA:ALA 21, 2-2.4 + TO	acuity cards novelty preference visual attention Bayley's test	2 mths CA 4,6,9,12 mths CA 12 mths CA 12 mths CA 12 mths CA	TO > SF, if no BPD no difference with diet no difference with diet FO < SF FO > SF
#4 Faldella et al., 1996	HM SF, LA:ALA18:0.3 + egg PL	ERC latencies ERG amplitudes flash VEP latency	52 wks PCA 52 wks PCA 52 wks PCA	no difference with diet no difference with diet N4:HM & + egg PL < SF
#5 Damli et al., 1996	SF, LA:ALA not known + DHA, AA	acuity cards		no difference with diet
#6 Bougle et al, 1999	HN, SF, LA:ALA 14:1.3 LA:ALA 18:1.2	Flash VEP BAEP nerve conduction RBC plasma phospholipids	<34 wks PCA	no difference with diet

Table 2.4.1. Abbreviations:

HM human milk

SF standard formula

PL phospholipid

FO fish oil

TO tuna oil

SS-VEP steady state -visual evoked potential

ERG electroretinogram

PCA post conceptional age

CA corrected age

wks weeks

mths months

BAEP brain-stem auditory evoked potentials

(When the abbreviations DHA and AA are used in table 3, no information regarding the source of these fatty acids was provided).

Although it is known LA, ALA, AA and DHA are necessary for the proper growth and development of infants, it is not quite clear in what amounts or ratios they should be added to formulas to have maximum accretion in tissues. A study by Su, Keswick and Brenna (1996) found that when rat pups consumed various levels of LA (with the same ALA levels), retinal, liver and plasma FA composition responded sensitively to the different FA compositions, while the unsaturated FA composition in the brain was nearly independent of dietary composition.

The half-life of PE and PC are estimated to be less than 20 days in the adult and developing rat (Jungalwala, & Dawson, 1971), however, conflicting reports exist concerning the rate of turnover of brain lipids in vivo. It is important to know how fast the turnover rate is occurring in different tissues, so that you can make sure your study is long enough to detect a difference. A study by Clandinin, Jumpsen, and Suh (1994) conducted in rats fed from birth to 6 weeks to assess whether FAs altered brain development, found that diet could alter the phospholipid fatty acid composition in brain membranes within seven days in new-born animals (rats), and within three weeks in weaning animals. They concluded that:

1. "The maximal AA content of PE is attained before the maximum DHA content is attained".
2. "Specific brain regions and cell types vary in the amount and rate of DHA and AA accretion".
3. "Raising the content of n-3 fatty acids in the diet increases preweaning levels of DHA in PE".

4. "Providing both AA and DHA increase the AA but not the DHA content in the PE of the developing brain".

5. "That the brain was sensitive to alterations in dietary lipid intake, even in a nutritionally complete diet".

Rioux, Innis, Dryer & Mackinnon, 1997, reported that the proportions of FA in plasma phospholipids are significantly associated with the proportions of the same FAs in the liver and bile, but not the brain. In vivo studies in mice (Scott & Bazan, 1989) showed that DHA appears in liver 2 hours after injection with C-14 ALA, and later in brain and retina. They suggested that DHA synthesised from ALA by the liver is secreted into the bloodstream in lipoproteins, taken up by brain and retina and incorporated into cell membranes. Since the retina and visual cortex are high in DHA, insufficient amounts of DHA result in the retina having abnormal physiological responses to light (Benolken , Anderson & Wheeler, 1973), and delayed visual-acuity development in animals (Neuringer, Connor, Van Petten & Barstad, 1984). Preterm infants fed diets that improved their n-3 FA status, also improved their visual acuity in early infancy (Benolken, Anderson & Wheeler, 1973).

The ratio of LA to ALA is an important determinant of the amount of DHA and/or AA that must be added to formulas to achieve the same plasma and/or erythrocyte lipid DHA and AA levels observed in breast-fed infants. Manufactures of some infant formulas suggest that the decision to use a LA: ALA ratio of 10:1 is based on the mean LA: ALA ratio of human milk. A study completed by Gibson (Gibson, Makrides, Neumann, Simmer, Mantzioris & James, 1984) looked at a series of factors that might affect the LA: ALA ratio. They found evidence that ALA is metabolised more readily for

energy than LA, which may explain the low levels of ALA in plasma lipid fractions. LA, unlike ALA, readily incorporates into membrane fractions and competes with n-3 LCPUFAs for space in the membrane phospholipid molecule. Finally, the major metabolite of LA, AA, is actively conserved by tissues and its concentration in cells is generally unchanged over a wide range of dietary LA intakes. In contrast, the LCPUFA derivatives of ALA change and depend on a steady supply of n-3 PUFAs. It is likely, therefore, that the LA: ALA ratios of formulas should deviate significantly from the ratios in human milk. For the LCPUFAs levels in breast-fed infants to be duplicated, formulas that contain only vegetable oils should have lower ratios as suggested by Gibson et al. (1994) of LA to ALA of 4:1 or as suggested by Decomps & Rodriguez (1995) who showed plasma phospholipid and red blood cell DHA status to be close to human milk feeding when fed a formula with a ratio of LA:ALA of 6.4:1 and a LA intake of 4.95% of total energy.

3.0 Dexamethasone

3.1 Dexamethasone and the Premature Infant

Neonatal chronic lung disease is a well-recognised complication of preterm birth associated with increased mortality and morbidity, not only in the early weeks after birth, but also continuing after discharge from the neonatal unit (Lindroth, Svenningsen, Ahlstrom & Johnson, 1980; Vohr, Bell & Oh, 1982; Sauve & Singhal, 1985). The lungs of prematurely born infants are particularly sensitive to the injurious effects of oxygen and mechanical ventilation, yet approximately 25% of very low birth weight infants (<1500g) require oxygen supplementation for four weeks or longer to facilitate oxygen transfer and the generation of blood (Yoder, Chua & Tepper, 1991). This unbalanced

oxidant-rich exposure results in significant cell damage and a marked inflammatory response, which seems important in the pathogenesis of neonatal lung injury such as acute respiratory distress syndrome and chronic bronchopulmonary dysplasia. The incidence of chronic bronchopulmonary dysplasia among ventilated infants is estimated to be between 4% and 40% depending on gestation, but undoubtedly the highest incidence, in excess of 70%, occurs in infants weighing 1000 grams or less at birth (Northway, 1990). This is of concern considering the incidence of chronic bronchopulmonary dysplasia may further increase in the coming decade as advances in neonatal intensive care enable clinicians to care for even smaller, more premature, and potentially more critically ill infants. Infants in whom chronic bronchopulmonary dysplasia develops have prolonged hospital stays, and thus the cost of their medical care is also higher. After discharge from the hospital they remain at high risk for recurrent respiratory infection, cardiovascular disease, delayed growth and poor neurodevelopmental outcomes.

Common therapy for chronic bronchopulmonary dysplasia has consisted of fluid restriction, diuretics, bronchodilators, supplemental oxygen and nutritional support. DEX, a synthetic long acting steroid with almost exclusive glucocorticoid property, has been used for the last decade in the management of respiratory distress syndrome. DEX differs from the endogenous cortisol in that it does not possess the salt retaining effects that adrenal cortisol produces, and posses a longer half-life of 36-72 hours, compared to 8-12 hours of endogenous cortisol. Despite the decreased capacity for sodium retention, DEX is more potent then endogenous cortisol and therefore has numerous undesirable side effects NG PC, 1993 as shown in table 3.1.0.

The administration of DEX to infants can vary anywhere from 10 to 42 days. Studies in preterm infants with respiratory distress syndrome, have shown DEX will improve pulmonary mechanics and decrease the period of ventilator dependency but does not affect mortality, the duration of hospitalisation, or the amount of time in which the infant receives supplemental oxygen (Yoder, Chua, & Tepper, 1991; Cummings, D'Eugeno & Gross, 1989; Harkavy, Scanlon, Chowdry & Grylack, 1989; Kazzi, Brans & Poland, 1990; Mammel, Fiterman, Coleman & Boros, 1987; Avery, Fletcher, Kaplan & Brudno, 1985; Mammal, Johnson, Green & Thompson, 1983). Although there has been intensive research, the mechanism(s) by which DEX improves pulmonary function has not been fully explained. Since the pathogenesis of chronic bronchopulmonary dysplasia is complex and multifactorial, it is likely that more than one mechanism is responsible for the acute and rapid improvement of pulmonary function seen with steroids. To date, it is known that DEX acts by reducing the tracheobronchial alveolar inflammatory response and pulmonary edema, thereby facilitating gas exchange, airway patency and improving lung compliance that enables successful extubation (NG PC, 1993).

Table 3.1.1. Side effects of DEX reported in preterm infants and animal models. Adapted from NG PC, 1993.

1. Central nervous system	Gross motor developmental retardation Abnormalities of electroencephalography New ultrasonographically diagnosed echodensites Adrenal suppression
2. Ophthalmological	Retinopathy of prematurity
3. Cardiovascular	Hypertension Myocardial hypertrophy Sustained bradycardis
4. Respiratory	Pneumothorax
5. Gastrointestinal	Perforation Reduced intestinal calcium absorption
6. Renal	Nephrocalcinosis Calciuria
7. Musculoskeletal	Myopathy Protein catabolism
8. Haematological and immunological	Increases total white cell count Neutrophilia Infection
9. Metabolic and endocrine	Retarded weight gain and linear growth Glucose intolerance Proteolysis Delta 5 & 6 liver desaturation enzyme modulation Impaired calcium regulation
10. Psychological	Irritability
11. Bone	Reduced bone mineralization
12. Vitamin	Reduced vitamin D status

3.2 Dexamethasone and Growth

Growth failure in infants with chronic bronchopulmonary dysplasia is a common problem (Carlson & Lombard, 1992). Increased energy expenditure (Kurzner, Garg, Bautista, Sargent, Bowman, & Keens, 1988; Yeh, McClean, Ajayi, & Pildes, 1989), inadequate nutrient intake (NG PC, 1993; Davidson, Schrayer, Wielunsky, Krikler, Lilos, & Reisner, 1993) as well as poor intestinal absorption of nutrients (Mammal, Johnson,

Green, & Thompson, 1983) have all been offered as reasons for growth failure in infants with bronchopulmonary dysplasia. Conversely, it has also been argued that inadequate nutrition plays a role in the development of chronic bronchopulmonary dysplasia (Wilson, McClure, Halliday, McReid, & Dodge, 1991). Very little has been published regarding digestive function in infants with chronic bronchopulmonary dysplasia. Furthermore, there are conflicting data from nutrient balance studies about fat absorption in these infants (Pereira, Baumgart, Bennet, Stallings, Georgieff, Hamosh, & Ellis, 1994; NG PC, 1993). A study by Boehm, Bierbach, Moro, & Minoli 1996, indicated that very low birth weight infants who develop chronic bronchopulmonary dysplasia have limited fat absorption, which might in part explain the inadequate weight gain.

Steroids have also been shown to impair normal growth in children (Rimsza, 1978) and there is evidence that long-term DEX may impair growth in premature infants (Weiler, Paes, Shah & Atkinson 1997; Wilson, Baldwin & Ariagno, 1988; Noble-Jamieson, Regev & Silverman, 1989; Yeh, Torre, Raslogi, Anyebuno & Pides, 1990; Bruntion, Saigal & Atkinson, 1998). Infants treated with glucocorticoids are of great concern because these infants are already in a catabolic state and the developmental outcome of small immature infants exposed to high doses of corticosteroids is known to cause neuromoter dysfunction (Yeh, Lin, Huang, Chen Lin, Lin, Hsieh, Lien, 1998). Therefore, knowing the importance of EFA for infant development, any further compromise to an infant's FA status such as being born premature and treated with glucocorticoids could be affect the premature infant's neuromoter development.

3.3 Effect of Glucocorticoids on Fatty Acids

Glucocorticoids are hormones with many profound roles in human physiology. They exert their effects on almost all-human tissues and organs, and their presence is crucial for the integrity of central nervous system function and for the maintenance of cardiovascular and metabolic homeostasis. Increased endogenous glucocorticoid secretion during times of stress, alters CNS function, prevents the inflammatory-immune response from overreacting and assists with adjustments in energy expenditure. This could also hold true for exogenous glucocorticoids such as DEX.

Cogo et al., 1997 also reported that term and preterm infants who are critically ill have metabolic stresses, which elevates the beta-oxidation of LA. The infants were receiving only intravenous glucose and electrolytes at the time of the study, and were infused with albumin-bound [U-^{13}C] palmitic and [U-^{13}C] linoleic acids. A needle biopsy of the s.c. adipose tissue was obtained for FA composition. It was found that the rate of appearance of LA was higher than could be expected from the FA composition of adipose tissue, thus indicating a preferential release of LA instead of palmitic acid during lipoysis (P=0.02). Cogo stated that the different mobilisation of the two FA could be due to the lower "retention" of LA in fat cells. LA as with all the PUFA is a polar FA. The more polar triglycerides, which are the molecules rich in polar FAs, are preferentially located at the interface of cells due to their hydrophilic properties and thus more accessible to hormone sensitive lipase. This could mean that when premature infants are in a negative energy balance, as they often are, the selective mobilisation of certain FA probably would affect adipose tissue FA composition and the qualitative FA supply to tissues and organs such as the brain and retina. Since preterm infants that are formula fed rely solely on their

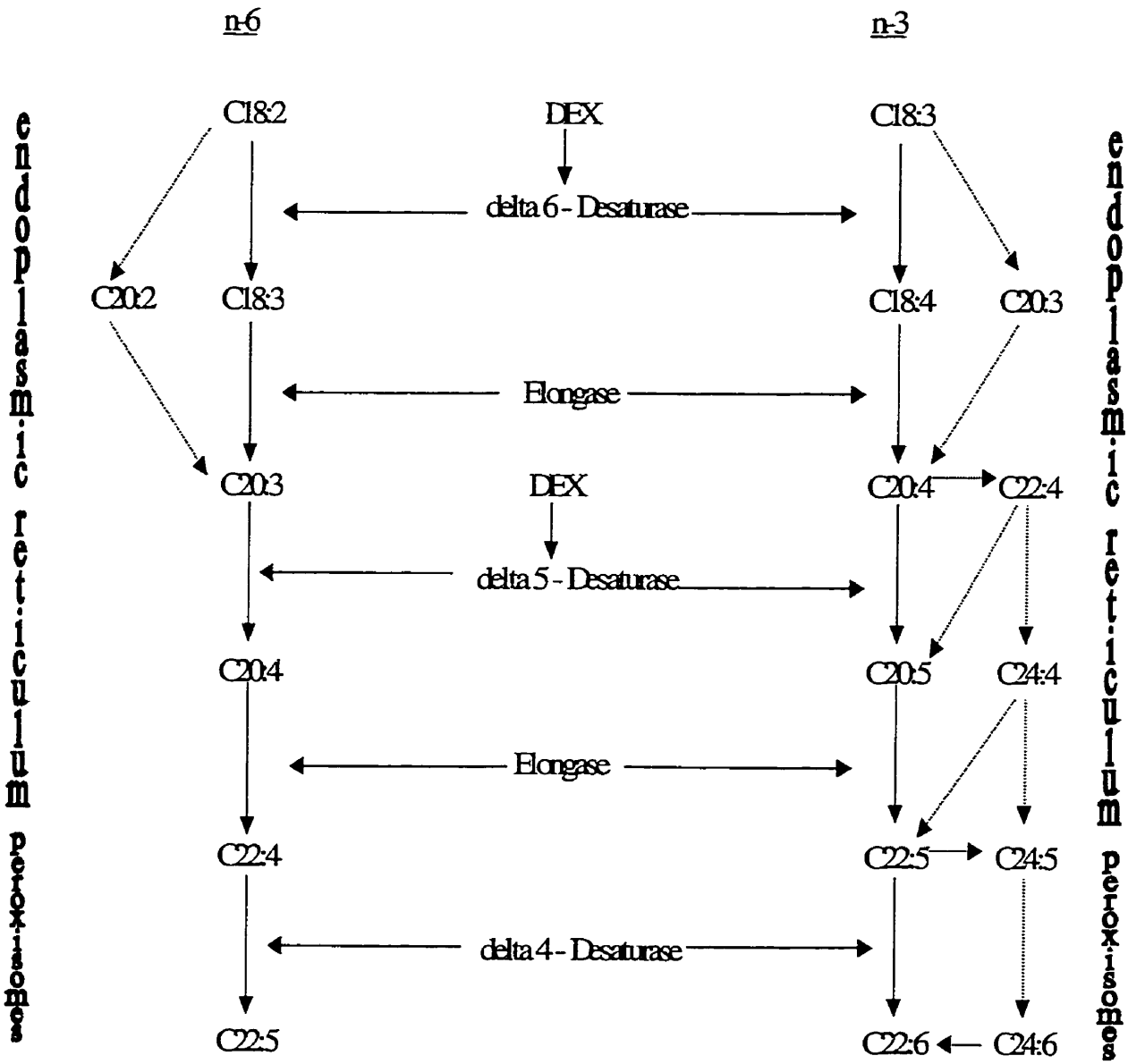
synthesis of AA and DHA from the PUFA LA and ALA respectively, the use of the essential FA for energy rather than for LCPUFA synthesis could very well cause a reduced production of AA and DHA.

As stated previously, PUFA biosynthesis follows an alternating sequence of desaturation and elongation reactions that take place in the microsomal and possibly the peroxisomal fraction of the cell. The control of this biosynthesis is mainly evoked by the regulation of the different microsomal desaturases, which introduce double bonds at the delta 9, delta 6 or delta 5 positions in the FA chains. In vitro, DEX reduces the activity of the liver enzymes, which are responsible for the elongation and desaturation of the essential FAs LA and ALA to AA and DHA respectively (figure 3.3.1). In 1986, Marra, Alaniz, & Brenner demonstrated that when they injected intraperitonely adult female Wistar rats (weighing 160-180 g) with glucocorticoids (DEX 1 mg/rat) it evoked a decrease in delta 6 and delta 5 liver microsomal desaturase activity 12 hours after the injection and blocked AA biosynthesis in the whole cell. This depression in the AA biosynthesis produced by DEX reaches approximately 50% after three hours of treatment. They also showed the addition of DEX to drinking water produces a strong inhibition of desaturase activity studied.

Due to the physiological complexity of the whole animal it is very difficult to illustrate in animals, in vivo, the intimate mechanism of glucocorticoid action. Marra, Alaniz & Brenner, 1986, therefore used isolated hepatic cells to investigate the specific mechanisms responsible for the decrease in desaturase. They found that the addition of DEX to the incubation media of isolated cells leads to a decrease in the biosyntheses of AA in a dose dependent way. They speculate that DEX may inhibit a protein, which is

needed for delta 5-desaturation activity. This is a very import aspect considering LCPUFA are need for proper growth.

Figure 3.3.1. Potential pathways of long chain polyunsaturated fatty acid synthesis with DEX interactions. The classical pathway involving delta 6, delta 5 and delta 4 desaturation followed by elongation is depicted by solid arrow. Putative alternative steps consistent with data from Sauerwald are depicted by broken arrows. Adapted from



3.4 Fatty Acids, Growth and Dexamethasone

Research conducted on DEX-treated piglets, where a high degree of experimental control was possible and disease was not a variable, strongly indicated that steroid-induced reductions in growth had taken place, despite adequate nutrition (Weiler, Wang & Atkinson, 1995). Weiler et al. (1997) also demonstrated disproportionate growth and continued delays in linear growth to six months corrected age in preterm infants previously treated with DEX. Therefore, it is important to understand the effects of DEX on FA metabolism and how it pertains to a premature infant's growth and development.

Brunton, Saigal, & Atkinson (1998) report growth failure commonly seen in preterm infants with chronic bronchopulmonary dysplasia was not due to malabsorption of nutrients, but rather due to an inadequate nutrient intake. Infants were given two different formulas. One with a composition based on the current feeding practice of high energy added to a standard term infant formula, vs a formula with a high protein to energy ratio and a greater concentration of minerals. Although the best formula composition for optimal growth of premature infants before and after term age is unknown (MacLean, 1990), infants fed the high protein to energy formula had improved growth, similar to that of healthy preterm infants (Kashyap, Schulze, Forsyth, Dell, Ramakrishnan, & Heird,1990). There is a possibility that inadequate intake of AA and DHA in premature infants could contribute to growth failure. Achieving a positive energy balance in low birth weigh infants becomes difficult with the need to administer antibiotics and other medications intravenously while faced with restricted fluid intakes (Heird, Jensen, & Gomez, 1992).

To date, no research has investigated the effect of DEX therapy on FAs, namely the production of AA and DHA, and the relationship between formula and breast-fed infants. Further, the implications to growth and development need to be investigated, to assess whether the use of DEX is affecting long-term neurological function and/or growth retardation. To assess the effects of DEX, human infant autopsy samples or an animal model are needed for the study of LCPUFA ratios in internal organs.

4.0 Comparison Between the Piglet Model and Human Infants

Human infant autopsy samples can be used to study the affects of DEX, however samples are difficult to obtain and are often limited by confounding variables of disease, drug regimes and a varying diet. Piglets serve as good experimental models for the investigation of lipid metabolism, as it relates to humans, because of the correlation in physiology and metabolism, brain lipid composition, as well as diet and body composition (Yoder, Chua, & Tepper, 1991; Cummings, D'Eugeno, & Gross, 1989; Harkavy, Scanlon, Chowdry, & Grylack, 1989; Kazzi, Brans, & Poland, 1990). There is also a high degree of control over DEX dosage, time of outcome measurements, nutrient intake and response of tissue FAs to dietary manipulation identified over 16 days (Rioux, Innis, Dyer, & Mackinnon, 1997). An animal model also allows assessment of the relationship between blood and tissue FA composition after DEX treatment, and eliminates the disease variable that is not possible when studying human premature infants who receive DEX therapy. Further, sows' milk contains a similar 18:2n-6, 18:3n-3, and long chain polyunsaturated FA content (Hrboticky, MacKinnon, & Innis, 1990) to human milk (Lammi-Keefe & Jensen, 1984), which is beneficial when studying nursing animals as it enables the researcher to study how these different FA are used in vitro.

Piglets have been shown to be similar morphologically to the human neonate, but with faster postnatal growth (Frank, 1991) and brain development. For example, in humans the brain growth spurt happens in the third trimester and continues into the first 18 months after birth. In comparison piglets take just weeks (Dobbing & Sands, 1979). One might say the two-week postpartum period in piglets is comparable with the nine-week postpartum period in full-term infants. Therefore, piglets appear to be very good models to study the effects of early lipid nutrition and DEX therapy on FA accretion.

The fact that the brain is susceptible to dietary change, depending on the timing of the brain growth spurt, is a significant factor and consequently, premature infants when given DEX are at a different stage in their brain development compared to the healthy term infant. Therefore, if DEX therapy affects the production of DHA and AA in a healthy animal, it could be speculated that reductions in the production of these FA at a time of high demand (such as prematurity), would lead to more severe reductions of DHA and AA accretion.

4.1 Tissues Appropriate to Study Fatty Acid Status

The major tissues responsible for synthesis of AA and DHA are liver, retina and brain (brainstem, cerebellum, diencephalon, and telencephalon), (Clandinin, Wong & Hacker, 1985a, Clandinin, Wong & Hacker, 1985) however tissues such as plasma and erythrocytes are the most attainable markers of FA metabolism in paediatric clinical investigations. Because the FA composition of plasma and erythrocytes are influenced by diet and organ uptake, the degree to which their analysis can reflect FA changes in deeper tissue sites is an important consideration. It has also been postulated that plasma contains FA precursors, which may be used for conversion of DHA in the retina and the brain.

Therefore this thesis also examines the relationship of the changes in plasma and RBC FA to those occurring in the brain, liver and retina.

4.2 Structural Fatty Acids

PE and PC are usually measured in tissues, as they have been found to be sensitive indicators of changes in AA and DHA status. They also can be used to determine whether there is a relationship between status and functional outcome. For example: The concentration of AA in the plasma PC between 2 and 6.5 months past term has been shown to be highly correlated with growth in the first year of life (Carlson, Werkman, Peeples, Cooke & Tolly, 1993, Koletzko & Braun, 1991). The use of RBC lipids as a potential index of EFA status in retinal membranes is based on correlation's between FA profiles in RBC and neural/retinal tissue from rats and non-human primates and in human infants (Makrides, Neumann, Byard & Gibson, 1994; Carlson, Carver & House, 1986; Connor, Lin & Neuringer, 1993). Parameters of post-natal brain growth have been shown to be related to RBC DHA (Woltil, Vanbeusekom, Schaafsma, Muskiet & Okken, 1998) and RBC PE DHA has been shown to correlate with visual acuity in infants at 2 and 4 months of age (Carlson, Werkman, Rodes & Tolly, 1993). The concentration of DHA in retinal PE has been shown to be correlated to the concentration in plasma phospholipids, and the latter a direct reflection of dietary DHA intake (Hrboticky, MacKinnon & Innis, 1991, Foote, Hrboticky, Mackinnon & Innis 1990, Alessandri, Goustard, Guesnet & Durand, 1998). Also ideally, all investigators in a field of study should use the same methods of evaluation, to make the comparison between studies easier.

4.3 Brain Sections

The brain can be divided into various sections depending on the area of interest based on what it is responsible for. Four key areas of the brain include the brainstem, the diencephalon, the telencephalon and the cerebellum. The functions of the different areas of the brain are summarised briefly. The brainstem is where neuronal replication occurs earliest. It is the central core of the brain consisting of mostly nerve fibers. It contains important pathways connecting the spinal cord and cerebellum with the diencephalon and the telencephalon and is where the control of eye movement takes place. The cerebellum, where the majority of neurones replicate after birth, is a coiled structure located under the occipital lobe. It is concerned with motor co-ordination of voluntary movements, equilibrium, and muscle tone. It does not initiate movement, but co-ordinates and smoothes it. It operates entirely below the conscious level. The diencephalon is the part of the brain located between the cerebrum and the telencephalon. Although the diencephalon consists of several structures located around the third ventricle, the main ones are the thalamus and the hypothalamus. The thalamus performs many functions, its main ones being that it is responsible for sensations and emotion, the arousal or alerting mechanism, and plays a part in the mechanism that produces complex reflex movements. The hypothalamus serves as a regulator and co-ordinator of autonomic activities. It helps control and integrates the responses made by visceral effectors all over the body, maintains water balance and some neurones function as endocrine glands. The telencephalon consists of the two cerebral hemispheres, which are the largest part of the brain. A layer of grey substance, called the cerebral cortex covers the cerebral hemispheres. Within each hemisphere are large masses of basal ganglia, and cerebral

fluid. The cerebral cortex is responsible for most sensory, motor and integrative functions (consciousness, memory, use of language, and emotions).

The dry weight of the human brain is predominantly lipid. Within this 22% of the cerebral cortex and 24% of white matter consists of phospholipid (Uauy, Peirano, Hoffman, Mena, Birch & Birch 1996). Of the total brain phospholipids, AA and DHA alone constitute more than 30%. It was thought that since the brain is composed of mainly fat, it would not then be necessary to look at individual phospholipids to get an accurate picture of FA status. Therefore, for each section of the brain, the total lipid was analysed.

4.4 Limitations

Although the piglet is a very good model to use, it does have its limitations when studying brain development in relation to premature infants. In the human, the brain growth spurt happens in the third trimester and continues into the first 18 month after birth (Altman, 1972). The fact that the brain is susceptible to dietary change depending on the timing of the brain growth spurt may be a significant factor considering that premature infants, when given DEX, are at an earlier stage in their brain development compared to a healthy term piglet. Therefore the term piglets brain FA accretion correlates more to the term infant than it does to the premature infant. However if DEX therapy affects the production of DHA and AA in a healthy animal, it could be speculated that reductions in the production of these FAs at a time of high demand, such as in a premature birth, would lead to more severe reductions of DHA and AA accretion. To help answer these questions it could be beneficial to examine brain FA accretion over longer periods of feeding using an animal model such as a rabbit or a rat, whose brain

growth after birth is closer to that of a premature infant. Both of these animals experience their brain growth spurts just prior to birth, but for the most part after birth (Dobbing & Sands, 1979). In animals such as the rat, growth of the cerebellum occurs almost entirely during the first 15 days after birth (Altman, 1972). Studying the affects of DEX therapy and type of feed on these animals would then provide us a comparable picture as to how a premature infant's cerebellum responds to DEX and FAs at a critical time in the brain's development. Another limitation of using an animal model such as the piglet is that the concentration of DHA and AA are highest in the plasma phospholipids rather than in RBC PE, whereas this distribution pattern has been found to be reversed in newborn infants. In the limited time and scope of this thesis, not all of these avenues could be explored.

5.0 Summary

It has been shown repeatedly that LCPUFA are needed for proper growth and development. However, at this time they are not added to preterm formula in North America. It has been indicated that if intrauterine development is interrupted by premature delivery, the requirements for neural tissue synthesis can be supplied by the liver lipids for only a limited period of time, 9 and 2.3 days for n-6 and n-3 fatty acids respectively (Clandinin, Chappel, Heim, Swyer, & Chance, 1981). Since the essential FA reserves develop in adipose tissue only during the last trimester of fetal growth (Clandinin, Chappel, Heim, Swyer, & Chance, 1981, hepatic and adipose reserves may not meet whole body needs for LCPUFA. Although it is has been shown that preterm infants are capable of synthesising LCPUFA (Carnielli, Wattimena, Luijendijk, Boerlage, Degenhart, Sauer, 1996), it is not known to what extent they are capable of attaining the

accretion rate of LCPUFA attained in premature breast-fed infants, or if they can endogenously synthesis adequate amounts of AA and DHA when subjected to exogenous glucocorticoid therapy and/or faced with critical illness. It is known that DEX therapy has numerous side affects, one being the depression of delta 5 and delta 6-desaturation activity in rodents (Marra, et al 1996; Mandon, et al, 1987). It is also known that preterm infants who are critically ill have metabolic stresses that elevate the beta-oxidation of LA (Cogo et al., 1997). In view of this, it is possible that DEX therapy and critical illness could be detrimental to the endogenous synthesis of LCPUFA in the preterm infant. It therefore is necessary to investigate if DEX therapy causes reductions in essential FA status, and whether feeding a diet containing AA and DHA are beneficial.

In the long term, it is possible that the information concluded in this thesis will facilitate the development of special premature infant formula and/or nutritional supplements to support optimal growth and development, and that this formula will be given not only DEX treated infants, but also to infants who are catabolic due to other illness as well.

6.0 Objectives and Hypothesis

This thesis examines the relationship among a catabolic steroid, FA status and growth.

6.1 Objective:

The objective was to determine somatic growth and brain, liver, retina, RBC and plasma AA and DHA in piglets fed standard formula or sows' milk and treated with or without DEX.

6.2 Hypothesis:

DEX treatment results in low AA and DHA status when no dietary source of AA or DHA is provided.

7.0 Methods

Three-day-old male piglets (n=20) were block randomised within litters to receive either DEX treatment or a placebo drug, combined with either sows' milk or formula feeding using a two by two design. The sample size of five per group was calculated based on a hypothesized difference in brain DHA of 0.25% (w/w of total lipid) with a standard deviation of 0.1% (Hrboticky, MacKinnon & Innis, 1990), power of 80 and alpha of 0.05. All piglets received an intramuscular injection of iron (100mg) at birth. Formula-fed piglets were removed from the sow at three days of age and transported from the Glenlea Research Unit to the University of Manitoba animal housing facility. Formula-fed piglets were housed individually in stainless steel cages with ambient temperature maintained at 29 to 30° C. The standard artificial formula that was used to feed the formula-fed piglets was designed to support a rapid rate of postnatal growth (National Research Council 1998; Weiler, Wang & Atkinson, 1995). To extrapolate the findings to human growth, the goal was to mimic infant formula as closely as possible. Therefore, the formula consumed by the piglets had a ratio of LA:ALA of 4.5:1, (FAO/WHO Expert Committee, 1994; Health Canada 1996; Nutrition Committee, Canadian Paediatric Society,1995) and thought to result in adequate AA and DHA synthesis (Descomps & Rodriguez, 1995). The formula provided dietary fat (37g/L) in similar quantities as measured in formula designed for prematurely born infants (Enfalac Premature 20 with 34-g fat/L and Enfalac Premature Plus with 41-g fat/L Mead Johnson

Nutritionals, Canada). The amount of fat in the formula was lower than in sow's milk (62g/L as measured in milk from the Glenlea Research Unit), but contained enough LA (11.5 to 11.8 g/L) to support growth. (Table 7.0.1). The standard formula contained the following: 55 g/L protein as skim milk powder and whey protein, 101 g/L carbohydrate, 37 g/L fat (70% canola and 30% corn oils) and 2.9 g/L calcium and 2.1 g/L phosphorus from calcium carbonate and minerals in skim milk powder. The formula energy (957 kcal/L) and LA (10.9% of energy) were within recommended limits for healthy growing piglets between 3 and 10 kg as set by the National Research Council, 1998. All formulas were prepared daily by blending double distilled water with the fat to the powder. Formula was not stored for longer than 24 hours at 4°C. Piglets were taught to lap the warmed formula and were fed at 400 ml/kg/d divided in equal volumes at 900, 1300, 1700 and 2100 hours. Piglet morning weight was used to adjust formula and drug dosages given to the piglet daily. The animals were adapted to the formula according to Weiler, et al., 1995. Sow fed piglets housed at the Glenlea facility were allowed to feed ad libitum in litters of less than 10 per sow, to prevent feeding difficulties. Sows-fed piglets were cross-fostered so that the composition of milk was consistent within each group. Aliquots of formula and fore- and hind-milk were taken at baseline, day 5, 10 and 15 for the FA analysis (table 7.0.1). At 10 days of age, DEX (4mg/ml sodium phosphate salt, Hexadrol; Organon Teknika, Toronto) was administered in dosages of 0.5 mg/kg/d for the first five days, followed by 0.3 mg/kg/d for day 6 to day 10, and 0.2 mg/kg/d from day 11 to day 15 based on morning weight. Placebo piglets received an equal volume of normal saline (0.9 %). DEX and placebo were administered by oral gavage in half dosages at 0900 and 1800 hours. All piglets were weighed daily by digital scale with an

animal-weighing program at 0900 hours. To compare FA intake to FA deposition, five ml or less then 10% blood volume, (60-90 ml/kg) was taken from both the DEX treated piglets and the control piglets with hepmized syringes. This was taken at base line and then every five days using the blind stab technique with the piglet restrained. The sample was centrifuged at 2000 x g for 20 minutes at 4^0C to separate plasma and RBC. RBC were washed with equal volume of normal saline (0.9% NaCl) and centrifuged once more prior to final separation. The phospholipid components of cellular membranes are highly vulnerable to oxidative damage because of the susceptibility of their PUFA side chains to peroxidation. Therefore samples were purged with nitrogen, to prevent oxidation and stored at -80^0C.

All procedures were in agreement with the Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993) and reviewed and approved by the "Fort Garry Campus Protocol Management and Review Committee", University of Manitoba.

Table 7.0.1. Proportion of fatty acids >16 carbons in sows' milk and piglet formula[1]

Fatty Acids % w/w	Sows' milk [2]	Formula
16:0	27.3 ± 2.0	5.9 ± 0.2
16:1	7.2 ± 1.6	0.2 ± 0.0
18:0	5.7 ± 0.7	1.8 ± 0.0
18:1	34.5 ± 4.5	48.6 ± 0.2
18:2 n-6	14.4 ± 2.0	32.0 ± 0.1
18:3 n-3	1.8 ± 0.4	6.5 ± 0.0
20:4 n-6	0.9 ± 0.2	--
22:0	0.2 ± 0.3	0.4 ± 0.7
22:1	0.2 ± 0.1	--
22:5 n-3	0.3 ± 0.3	--
22:6 n-3	0.2 ± 0.0	--
Total n-6:n-3 fatty acid ratio	6.7	4.9
20:4 n-6: 22:6 n-3	4.5	--

[1] Data are mean ± SD

[2] Data are averaged from samples of sows' milk taken at baseline, day 5, 10 and 15. sows' milk represents fore- and hind-milk pooled in equal volumes. Formula data is from a single sample measured in triplicate.

7.1 Collection of Tissue

After 15 days of study, sow fed piglets were transported to the University of Manitoba. In all piglets, food was withheld with water ad libitum for 12 hours. Piglets were anaesthetised using sodium pentobarbital (30 mg/kg, Somnotol 65 mg/ml) and length measured from the snout to the base of the tail (to nearest 0.1 cm) using a non-stretchable measuring tape. After deep anaesthesia was achieved, sixty ml of blood was drawn into heparinized syringes via a cardiac puncture. Subsequently, the thoracic cavity of the piglet was opened, and the left ventricle of the heart purfused with 0.9% NaCl through the left ventricle, four times the blood volume (90 ml/kg body weight). The objective was to remove blood from organs of interest; liver, retina, and brain. Whole eyes, brain and liver were excised using standard procedures. Brains were divided into brainstem (excluding cerebellum and diencephalon), cerebellum, diencephalon, and telencephalon. Tissues were removed weighed, flash frozen in liquid nitrogen and stored at -80^0 C until analysis. Blood was centrifuged at 2000 x g for 20 minutes at 4^0 C to separate plasma and RBC's. Plasma was centrifuged again to remove any remaining RBC. RBC were washed with equal volume of normal saline (0.9 % NaCl) and centrifuged once more prior to final separation. Samples were stored with equal volume of saline, purged with nitrogen to prevent oxidation, and stored in glass vials at -80^0 C until analysis. The animal's eyes were removed and frozen whole at -80^0 C. Just prior to FA extraction the anterior segment, lens and vitreous humor were removed and discarded. The retina was detached from the retinal pigment epithelium, immediately emerged in chloroform-methanol (2:1 by vol.), and lipids extracted.

In order to strengthen the stated hypotheses, plasma total cholesterol and triglyceride were measured in triplicate in blood obtained in the 12 hour non-fed state. Plasma triglycerides were measured using a colorimetric assay kit (kit # 210-75A, Diagnostic chemicals Ltd., P.E.I., Canada) and total cholesterol also measured using colorimetric assay kit (kit # 225-26A-1, Diagnostic Chemicals Ltd., P.E.I., Canada).

7.2 Lipid and Fatty Acid Analysis

Total lipids in plasma, liver, brain and retina were extracted according to the method of Folch, Lees & Stanley, 1957. Lipids from RBC were extracted with chloroform and isopropanol by the method of Rose & Oklander, 1965. For the determination of total lipid fatty acid composition, sows' milk, formula and brain tissue, were methylated in 1 ml of methanolic HCL 3N (Supleco Inc, Bellefonte, PA) at 100^0 C for 90 minutes according to Tardi, Mukherjee & Choy, 1992. Total phospholipids specifically PC and PE in plasma, RBC, liver and retina were separated from other lipid classes by two-dimensional thin-layer chromatography. Whatman silica gel 60 A plates, (20cm x 20cm; Fisher Scientific, Nepean, ON, Can), were used and coated with 0.5 M borate. The solvent system used consisted of chloroform/methanol/ammonium hydroxide/water (70:30:2:3, by vol.) followed by chloroform/methanol/water (65:35:5, by vol.). Thin layer chromatography plates were visualised with 2,7-dichloroflouorescein (Superlco Inc, Bellefonte, PA), detected under UV light. Phospholipid classes were compared against appropriate standards and removed under nitrogen for FA analysis .

PE and PC were eluted from the silica gel by rinsing samples with chloroform/methanol/acetic acid/double distilled H_2O (50:39:1:10, by vol.). The lower (organic) layer was then washed twice with 3 ml of methanol/double distilled H_2O (1:1, by

vol.). Samples were stored under N_2 at -20^0 C until ready to methylate (Arvidson, 1968). Methylation of plasma, liver and retina phospholipid fractions was performed using BF_3 methanol reagent. Samples were placed in a water bath at 90^0 C for 10 minutes. 0.5 ml double distilled water, was added, the mixture vortexed and put on ice for 10 minutes. Samples were washed twice with 1.5 ml petroleum ether and pooled. The pooled organic phase was washed twice with 1.5 ml of double distilled water and pooled. The upper phase was dried under nitrogen and reconstituted in 100-200 µL isooctane and stored under N_2 at -20^{0}C until ready to inject into a gas-liquid chromatogram (Tardi, Makherjee & Choy 1992).

FA methyl esters were separated by gas-liquid chromatography. A Hewlett Packard 5890A gas-liquid chromatogram, with fused silica capillary column 30m-x i.d. 0.25mm coated with polar phase Supelcowax 10 (Supelco Inc., Bellefonte, PA). The gas chromatogram was equipped with autosampler, 3392A integrator and a flame ignition detector. Samples were injected (0.5µl) at an initial temperature of 175° C and increased thereafter at a rate of 3° C/minute to a final temperature of 235° C. Identification of peaks as based on relative retention times compared to the standard Supelco^{tm} 37 component FAME mix (Supelco Inc, Bellefonte, PA).

The FA content was calculated on a percent weight/weight basis (%w/w). The primary outcome measurements were %w/w AA and DHA PE and PC and total brain FA. Plasma and RBC PE and PC were anticipated to reflect changes in liver, brain and retina PE and PC FA. Triplicate test samples of PE liver, retina, plasma and RBC were performed to give an impression of the variability in the measurements of FA and to ensure measurements of samples were accurate (appendices table 9.8.1).

8.0 Statistical Analysis

Values are expressed as mean ± one standard error of the mean (SEM) unless otherwise stated. Statistical significance was accepted for p values < 0.05. Differences in outcome measurements between feeding and treatment groups were detected by two-way ANOVA and post hoc analyses using Student Neuman Keuls all pairwise comparisons test. Differences in weight and rate of weight gain were detected using two-way-ANOVA and post hoc analysis using Student Neuman Keuls all pairwise comparisons test. A two-tailed pearson correlation coefficient was used to assess relationships between tissues and RBC or plasma.

9.0 Results

9.1 Growth

The growth of each piglet was measured daily to assess the affects of DEX vs a placebo. The average piglet weight (DEX 2.5 ± 0.5 vs. placebo 2.3 ± 0.5 kg) was equal between treatment groups at baseline. No differences in weight were observed between sow- and formula-fed piglets throughout the study such that both placebo groups were heavier after 15 days of study (figure 9.1.1). DEX treated piglets had significantly reduced body weight (DEX 4.3 ± 0.4 versus placebo 5.5 ± 0.4 kg $p<0.05$) and were shorter in length (table 9.1.0), as seen in previous studies of DEX treated piglets (Weiler, Wang and Atkinson, 1995). The average cumulative amount of DEX administered over the15 days was 22.5 mg.

In all tables you will note an asterisk indicating one sample is missing from the sow-fed group. A piglet was killed accidentally at the Glenlea facility before the study group was complete and therefore there is missing data for that group.

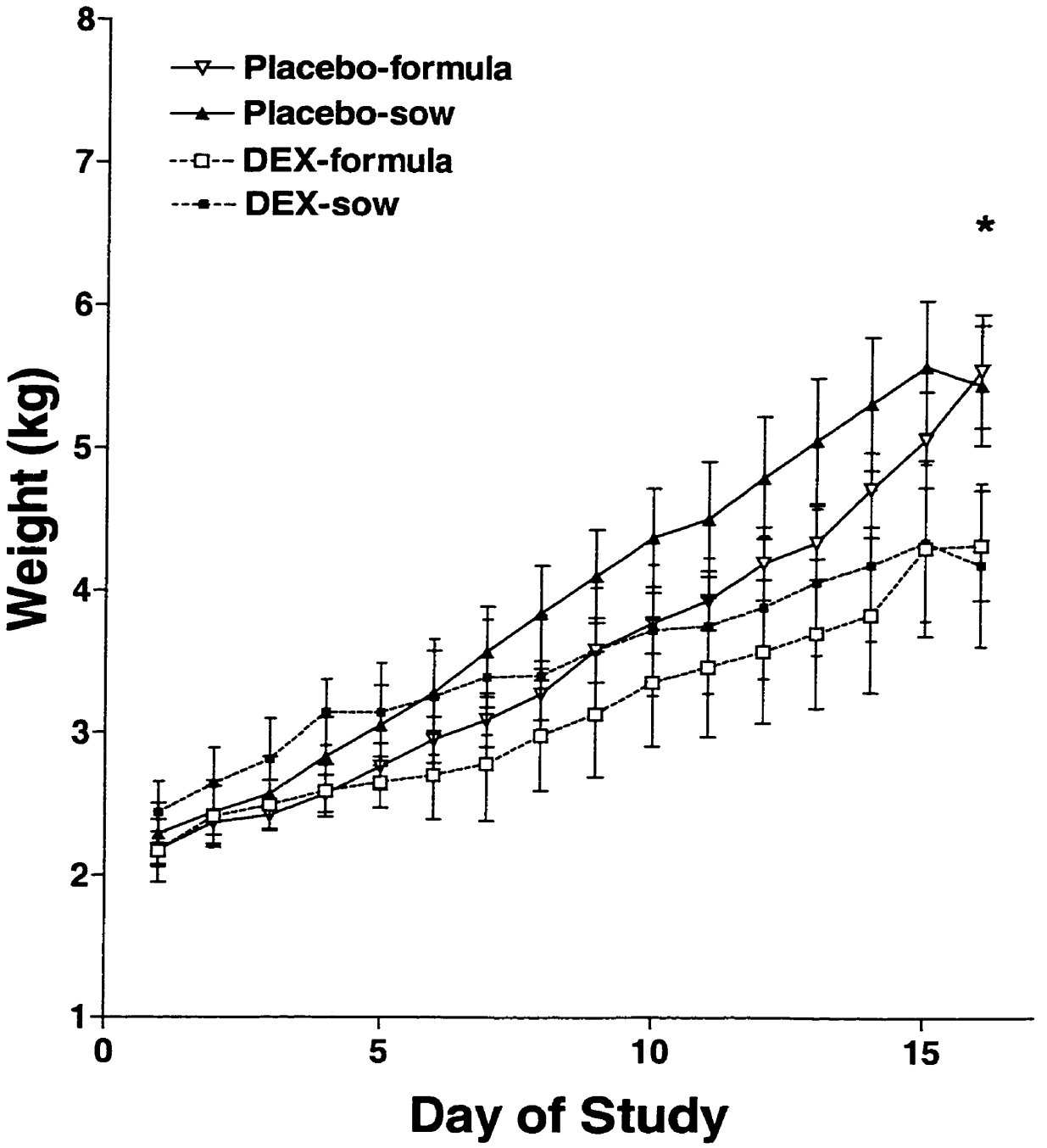


Figure 9.1.1

Weight of sow- and formula-fed piglets treated with and DEX over 15 days. * $p<0.01$ between placebo and DEX groups, n=5 per group.

Table 9.1.1. Piglet weight and length at the end of 15 days of DEX or placebo treatment.

Treatment group	Sow Fed		Formula Fed	
	DEX	*Placebo	DEX	Placebo
Average age at baseline	10	10	10	10
Baseline Wt (kg)	2.5 ± 0.5	2.3 ± 0.5	2.2 ± 0.5	2.2 ± 0.3
Final Wt (kg)	4.2 ± 1.3**	5.4 ± 1.1	4.4 ± 0.8**	5.5 ± 1.0
Weight gain (g/kg/d)	0.04	0.06	0.04	0.05
Final Length (cm)	47.5 ± 2.8**	53.5 ± 2.2	49.2 ± 1.6**	53.4 ± 1.4
Total DEX (g)	24.7	--	20.2	--

Data are mean ± SEM, n=5.
*One measurement missing
** $p < 0.05$

9.2 Blood Lipids

Cholesterol and triglycerides were measured from the blood of each piglet. Results indicated that the piglets treated with DEX in the non-fed state have elevated circulating concentrations of both total cholesterol and triglyceride (table 9.2.1) (DEX 1.1 ± 0.3 vs. placebo 0.2± 0.0 mmol/L, $p<0.05$) and total cholesterol (DEX 6.2 ± 0.1 vs. placebo 0.2 ± 0.6, $p<0.05$).

9.3 Liver

The liver was an organ of interest due to its LCPUFA making capabilities. The liver PC and PE DHA were similar in both sow-fed and formula-fed placebo piglets and significantly greater than the DEX-treated formula-fed group (table 9.3.1). These results indicate that sows' milk can attenuate DEX-induced reductions in liver DHA. A feeding affect was noted in Liver PC AA, in that piglets that suckled milk from the sow had higher levels of PC AA compared to those that were fed formula, however no DEX interactions were noted. No changes were observed from DEX treatment or feed type in PE AA or the precursor FA, ALA or LA (table 9.3.2).

9.4 Brain

In the four areas of the brain were studied, however a main effect of DEX therapy was only seen for AA measured in the telencephalon (Placebo 9.0 ± 0.3 vs DEX 8.0 ± 0.3, $p<0.05$). Type of feed did not make a significant difference (table 9.4.1). No other significant differences were observed in the brain sections studied (table 9.4.1 & 9.4.2. 9.4.3.).

9.5 Retina

Due to the retinas functional dependence on LCPUFA it was important to note any differences in PE and PC, however no differences were seen in retina PC AA or PE DHA (table 9.5.1). A main effect of feeding (sow 11.7 > formula 6.2 ± 1.3) was observed in PE AA ($p<0.05$), however the reverse was found in PC DHA in that formula fed piglets had significantly higher amounts of PC DHA than the piglets that were suckled sows' milk (formula 3.8 ± 0.3 > sow 3.5 ± 0.4). There was an affect of DEX treatment on the retina in that the DEX treated piglets had significantly lower retinal PC DHA than the placebo treated piglets regardless of feed type. No other significant differences were observed in other retinal FA (table 9.5.1., 9.5.2).

Table 9.2.1. Plasma triglyceride and total cholesterol concentration in piglets treated for 15 days with DEX or placebo and fed either sow's milk or formula.

Treatment Group	Sow Fed		Formula Fed	
	DEX	*Placebo	DEX	Placebo
Triglyceride (mmol/L)[1]	0.56 ± 0.18^{a}	0.43 ± 0.20^{a}	1.13 ± 0.18^{b}	0.20 ± 0.18^{a}
Total cholesterol (mmol/L)[1]	10.79 ± 1.3^{a}	8.36 ± 1.30^{a}	6.19 ± 1.30^{a}	3.93 ± 0.30^{b}

Data are mean ± SEM, n=5 per group
1 Treatment effect by two-way ANOVA (feed type and treatment), $p<0.05$
[a, b] Values with different superscripts in rows indicate significant differences by two-way ANOVA (feed group and treatment and post-hoc analysis using Student Newman Keuls test, $p<0.05$)
*One measurement missing

Table 9.3.1. AA and DHA composition of piglet liver PE and PC expressed as % w/w of total phospholipid from sow and formula fed piglets treated with DEX or placebo.

Liver (%w/w)	PC DHA	PE DHA	PC AA[c]	PE AA
Sow-Placebo*	4.4 ± 0.5^{a}	8.6 ± 0.7^{a}	14.0 ± 1.2^{a}	23.8 ± 2.5
Sow-DEX	3.2 ± 0.4^{a}	6.3 ± 0.6^{a}	12.9 ± 1.1^{a}	24.8 ± 2.3
Formula-Placebo	3.7 ± 0.4^{a}	6.9 ± 0.6^{a}	8.9 ± 1.1^{b}	22.5 ± 2.3
Formula-DEX	1.8 ± 0.4^{b}	3.0 ± 0.6^{b}	8.4 ± 1.1^{b}	19.8 ± 2.3

Data are mean ± SEM, n=5
a,b Values with different superscripts in rows indicate significant differences by two-way ANOVA (feed group and treatment and post-hoc analysis using Student Newman Keuls test, $p<0.05$)
c Indicates a main effect of feeding
*One measurement missing

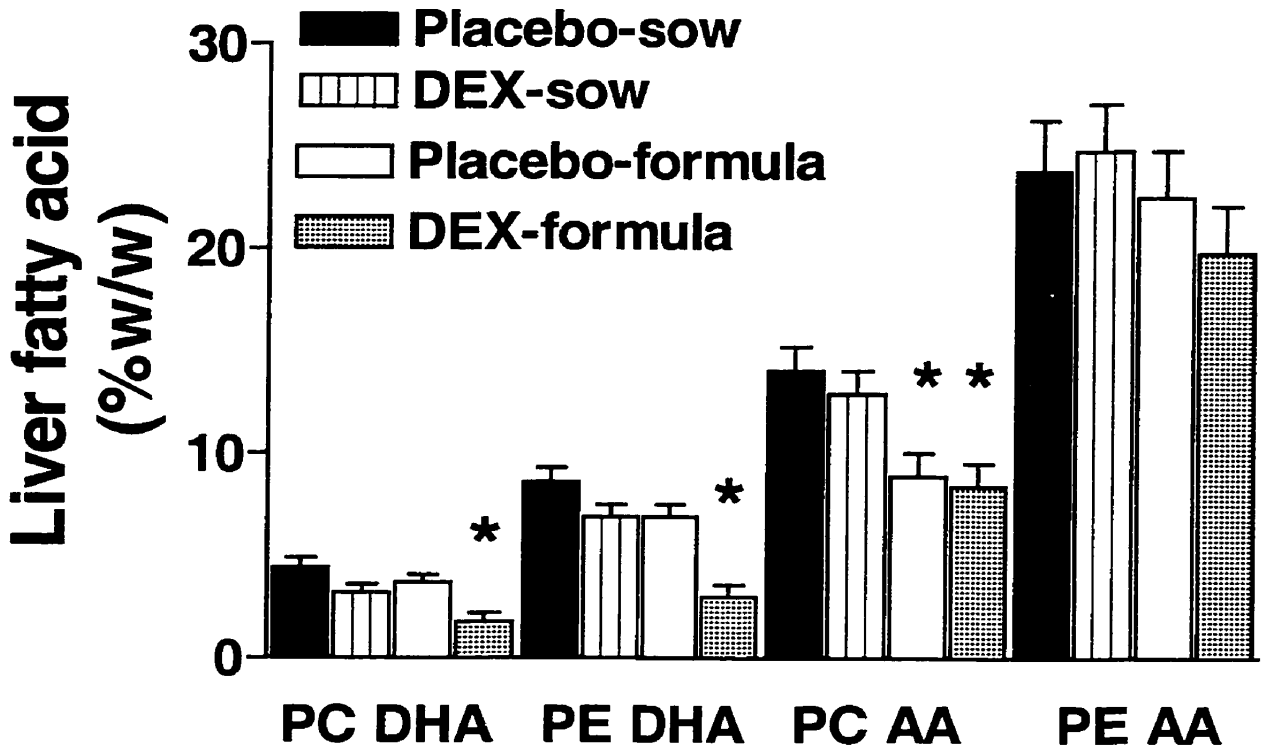


Figure 9.3.1.

Proportion of docosahexaenoic (DHA) and arachidonic acid (AA) in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fatty acids extracted from liver tissue of piglets fed sows' milk or formula and treated with and without dexamethasone (DEX) for 15 days. *$P<0.05$ lower compared to other feeding or treatment groups, n=5 per group.

Table 9.3.2. LA and ALA composition of piglet liver PE and PC in % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or a placebo.

Liver (%w/w)	PC LA	PC ALA	PE LA	PE ALA
Sow-Placebo*	12.90 ± 0.71	0.47 ± 0.03	5.73 ± 0.56	0.56 ± 0.31
Sow-DEX	14.75 ± 1.03	0.71 ± 0.16	6.62 ± 0.67	0.50 ± 0.13
Formula-Placebo	15.63 ± 1.20	0.83 ± 0.32	11.18 ± 1.51	0.45 ± 0.18
Formula-DEX	19.08 ± 0.75	0.31 ± 0.02	14.72 ± 2.20	0.49 ± 0.10

Data are mean ±SEM, n=5
*One measurement missing

Table 9.4.1. Total piglet brain DHA expressed as % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or a placebo.

Brain DHA (% w/w)	Diencephalon	Telencephalon	Cerebellum	Brain Stem
Sow-Placebo*	1.9 ± 0.4	0.8 ± 0.7	2.2 ± 0.7	3.2 ± 1.1
Sow-DEX	1.6 ± 0.4	1.8 ± 0.6	1.5 ± 0.8	2.1 ± 1.1
Formula-Placebo	1.7 ± 0.4	1.8 ± 0.6	2.6 ± 0.7	2.7 ± 1.0
Formula-DEX	1.7 ± 0.4	0.9 ± 0.6	1.2 ± 0.6	3.2 ± 0.9

Data are mean ± SEM, n=5
*One sample missing

Table 9.4.2. Total piglet brain AA expressed as % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or placebo.

Brain AA (% w/w)	Diencephalon	Telencephalon[d]	Cerebellum	Brain Stem
Sow-Placebo*	7.6 ± 0.4	9.3 ± 0.5[a]	6.9 ± 0.7	5.5 ± 1.7
Sow-DEX	7.8 ± 0.4	8.2 ± 0.4[b]	7.1 ± 0.8	5.6 ± 0.7
Formula-Placebo	8.0 ± 0.4	9.9 ± 0.4[a]	6.3 ± 0.7	5.4 ± 0.6
Formula-DEX	8.3 ± 0.4	9.1 ± 0.4[b]	7.4 ± 0.6	4.9 ± 0.6

Data are mean ± SEM, n=5
a,b Values with different superscripts in rows indicate significant differences by two-way ANOVA (feed group and treatment and post-hoc analysis using Student Newman Keuls test, $P<0.05$)
d Indicates a main effect of treatment group (Placebo>DEX)
*one measurement missing

Table 9.4.3. Composition of piglet telencephalon LA and ALA expressed as % w/w of total phospholipid from sow and formula fed piglets treated with DEX or a placebo.

Telencephalon (%w/w)	LA	ALA
Sow-Placebo*	0.96 ± 0.14	0.34 ± 0.03
Sow-DEX	0.88 ± 0.07	0.31 ± 0.07
Formula-Placebo	1.06 ± 0.37	0.18 ± 0.06
Formula-DEX	1.02 ± 0.05	0.37 ± 0.05

Data are mean ± SEM, n =5
*One sample missing

Table 9.5.1. AA and DHA composition of piglet retina PE & PC expressed as % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or placebo.

Retina (% w/w)	PC DHA[cd]	PE DHA	PC AA	PE AA[c]
Sow-Placebo*	2.4 ± 0.5 [a]	3.5 ± 0.7	7.7 ± 2.0	11.0 ± 2.0 [a]
Sow-DEX*	2.6 ± 0.5 [a]	4.0 ± 0.7	6.2 ± 2.0	12.5 ± 2.0 [a]
Formula-Placebo	5.3 ± 0.5 [b]	3.2 ± 0.7	10.4 ± 1.8	6.9 ± 1.8 [b]
Formula-DEX	2.4 ± 0.5 [a]	2.9 ± 0.7	6.6 ± 1.8	5.4 ± 1.8 [b]

Data are mean ± SEM
a,b Values with different superscripts in rows indicate significant differences by two-way ANOVA (feed group and treatment and post-hoc analysis using Student Newman Keuls test, $P<0.05$)
c Indicates a main effect of feeding
d Indicates a main effect of treatment group
*One measurement missing

Table 9.5.2. LA and ALA composition of piglet retina PE and PC expressed as % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or placebo.

Retina (%w/w)	PC LA	PC ALA	PE LA	PE ALA
Sow-Placebo*	6.22 ± 1.76	0.41 ± 0.11	9.52 ± 0.63	1.42 ± 0.46
Sow-DEX	8.88 ± 0.40	1.16 ± 0.15	5.22 ± 1.57	2.22 ± 0.52
Formula-Placebo	2.57 ± 0.56	1.97 ± 0.50	7.00 ± 1.19	0.81 ± 0.27
Formula-DEX	5.72 ± 1.74	1.70 ± 0.45	8.34 ± 0.52	1.45 ± 0.30

Data are mean ± SEM, n=5
*One sample missing

9.6 Plasma

When the FA were measured in plasma, PC AA, sow fed piglets had significantly higher levels of AA than formula-fed piglets (table 9.6.1). Plasma PC DHA was higher in the sow-fed placebo piglets compared to the formula fed piglets. A significant relationship was observed between liver and plasma in PC AA ($r=0.67$, $p=0.002$) and DHA ($r=0.66$, $p=0.002$), as well a significant difference of $p=0.02$ was found in PE DHA ($r=0.53$), (table 9.6.3). No other significant relationships were found in brain or retinal tissue. No relationships were observed between PE AA in plasma compared to AA or DHA in liver, retina or brain. No significant differences were observed in LA or ALA (table 9.6.2).

9.7 RBC

RBC are often a indication of what is taking place in vitro, however no differences were seen in PE and PC AA or PE DHA in RBC (table 9.7.1). RBC PC DHA was significantly lower for Sow-DEX piglets than formula-DEX piglets. No significant relationships were observed for PE or PC AA or DHA in RBC with liver, brain or retina (table 9.7.3). No significant differences were observed in LA or ALA (table 9.7.2.).

Table 9.6.1. AA and DHA composition of piglet plasma PE and PC expressed as % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or a placebo.

Plasma (% w/w)	PC DHA	PE DHA	PC AA[c]	PE AA
Sow-Placebo*	3.8 ± 0.3^{a}	2.8 ± 0.9	12.9 ± 1.1^{a}	8.4 ± 1.9
Sow-DEX	2.2 ± 0.3^{b}	4.2 ± 0.8	10.8 ± 1.0^{a}	8.5 ± 1.7
Formula-Placebo	2.4 ± 0.3^{b}	2.6 ± 0.8	8.7 ± 1.0^{b}	5.0 ± 1.7
Formula-DEX	1.5 ± 0.3^{b}	2.8 ± 0.8	6.8 ± 1.0^{b}	6.5 ± 1.7

Data are mean ± SEM, n=5
a,b Values with different superscripts in rows indicate significant differences by two-way ANOVA (feed group and treatment and post-hoc analysis using Student Newman Keuls test, $P<0.05$)
c Indicate a main effect of feeding
*One sample missing

Table 9.6.2. LA and ALA composition of piglet plasma PE and PC expressed as % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or a placebo.

Plasma (%w/w)	PC LA	PC ALA	PE LA	PE ALA
Sow-Placebo*	15.40 ± 0.18	0.41 ± 0.11	11.76 ± 2.18	1.32 ± 0.73
Sow-DEX	15.48 ± 0.82	0.45 ± 0.14	10.72 ± 1.28	0.92 ± 0.30
Formula-Placebo	16.00 ± 1.42	0.77 ± 0.22	8.6 ± 0.72	1.67 ± 0.25
Formula-DEX	18.59 ± 3.59	0.61 ± 0.12	10.68 ± 1.93	0.84 ± 0.18

Data are mean ± SEM, n=5
*One measurement missing

Table 9.6.3. Measurement of differences in liver, retina and brain compared to plasma

Tissue	PC DHA		PE DHA		PC AA		PE AA	
	r	p	r	p	r	p	r	p
Liver	0.66	<0.01	0.53	0.02	0.67	<0.01	-0.28	0.24
Retina	0.02	0.94	0.02	0.94	0.42	0.10	0.40	0.09
Brain Stem	0.03	0.90	-0.02	0.93	0.12	0.65	0.00	0.99
Cerebellum	0.40	0.11	-0.4	0.11	-0.42	0.10	-0.19	0.46
Telencephalon	0.02	0.94	-0.2	0.42	-0.1	0.71	0.17	0.54
Diencephalon	0.02	0.94	-0.30	0.28	0.34	0.22	0.09	0.74

n=19
r= Pearson product correlation coefficients

Table 9.7.1. AA and DHA composition of piglet RBC PE and PC expressed as % w/w of total phospholipid from sow and formula fed piglets treated with DEX or placebo.

RBC (% w/w)	PC DHA	PE DHA	PC AA	PE AA
Sow-Placebo*	1.9 ± 0.5^{ab}	1.3 ± 0.4	5.2 ± 0.9	6.8 ± 1.2
Sow-DEX	0.7 ± 0.4^{a}	0.7 ± 0.4	6.0 ± 0.8	4.3 ± 1.1
Formula-Placebo	1.4 ± 0.4^{ab}	0.6 ± 0.3	2.9 ± 0.8	3.2 ± 1.1
Formula-DEX	2.6 ± 0.4^{b}	1.1 ± 0.3	3.5 ± 0.8	6.2 ± 1.1

Data are mean ± SEM, n=5
a,b Values with different superscripts in rows indicate significant differences by two-way ANOVA (feed group and treatment and post-hoc analysis using Student Newman Keuls test, $P<0.05$)
*One measurement missing

Table 9.7.2. LA and ALA composition of piglet RBC PE and PC expressed as % w/w of total phospholipid, from sow and formula fed piglets treated with DEX or placebo.

RBC (%w/w)	PC LA	PC ALA	PE LA	PE ALA
Sow-Placebo*	11.85 ± 1.37	0.97 ± 0.36	13.50 ± 0.22	0.82 ± 0.38
Sow-DEX	14.02 ± 3.18	0.64 ± 0.07	9.03 ± 2.51	2.17 ± 0.80
Formula-Placebo	10.40 ± 0.70	2.10 ± 0.53	9.34 ± 2.67	2.00 ± 0.48
Formula-DEX	9.44 ± 1.83	2.61 ± 0.72	10.74 ± 2.67	2.22 ± 0.35

Data are mean ± SEM, n=5
*One measurement missing

Table 9.7.3. Measurement of differences in liver, retina and brain compared RBC.

Tissue	PC DHA		PE DHA		PC AA		PE AA	
	r	p	r	P	r	P	r	P
Liver	0.3	0.22	0.38	0.10	0.27	0.30	-0.28	0.24
Retina	-0.15	0.54	0.02	0.91	0.42	0.10	0.40	0.09
Brain Stem	0.03	0.90	0.70	0.10	0.12	0.65	0.00	0.99
Cerebellum	0.40	0.11	0.34	0.18	-0.42	0.10	-0.19	0.46
Telencephalon	0.02	0.93	0.16	0.51	-0.10	0.71	0.16	0.53
Diencephalon	0.02	0.94	0.03	0.91	0.34	0.22	0.09	0.73

n=19
r= Pearson product correlation coefficients

Table 9.8.1. Tripilicate data of PE liver, retina, plasma and RBC.

*Tissue Sample	AA PE		DHA PE	
	Mean ± SD	CV%	Mean ± SD	CV%
Liver	23.46 ± 0.03	0.13	8.32 ± 0.96	11.54
Retina	5.07 ± 2.03	40.04	2.03 ± 1.26	62.06
Plasma	13.34 ± 0.46	3.45	4.59 ± 0.13	2.91
RBC	3.17 ± 1.12	35.33	11.83 ± 0.42	3.55

n= 3 per group

CV%= CV=$\frac{SD}{mean}$ x 100

10.0 Discussion

10.1 Growth

This thesis examined the relationships among catabolic steroids, and FA status and growth. Piglet characteristics at baseline were similar between treatment groups in terms of age, weight, and length. By the end of 15 days of study, DEX treated piglets had significantly reduced body weight ($p<0.05$) regardless of feed consumed (sow milk vs formula). This is a new observation as previous studies on DEX have focused solely on formula-fed piglets (Weiler, Wang and Akinson, 1995). It appears that DEX causes a highly significant disturbance of normal growth that cannot be explained by changes in energy input or oxygen requirement (Gibson, Pearse & Wales, 1993). This failure to grow is attributed solely to the DEX, as the piglets were healthy prior to DEX treatment, there were no differences observed in weight or rate of weight gain between sow- and formula-fed piglets fed diets suggested by the daily nutrient intakes and requirements of swine (Nutrient Requirements of Swine, 1998). The failure to grow due to DEX treatment is not as clear in human infants with chronic lung disease, since the growth may be altered by the severity of their lung disease. Whether an increased volume of food could help increase growth in premature infants with brocopulmonary dysphasia is difficult to know, due to the volume restrictions that minimize nutrient intake.

The restriction of food intake is not a problem with healthy piglets. While daily feeding volume was unavailable in the sow-fed piglets, it is unlikely that intake was different between placebo and DEX groups since let down of sows' milk results in consistent amounts of milk per piglet at each feeding (Spinka, Illmann, Algers & Stetkova, 1997). No differences were observed in formula intake between formula fed

groups (400 ml/kg/day). Therefore the DEX piglets likely consumed equal total feed volumes (ml) or higher volume corrected to weight (ml/kg) in comparison to the placebo piglets. Thus additional intake per weight does not attenuate the DEX-induced delay in growth. Since no differences in weight gain were observed between feeding types, this indicates that sows' milk is not protective against DEX-induced growth delay. Figure 9.0.0 shows the slightly greater weight in the sows' versus formula fed placebo piglets. This was because the weight of the sow-fed piglets at 9:00 am included the feedings that had already taken place during the early morning. There was no difference in weight obtained on day 16 after 12 hours of withholding all food.

By day 16, length was significantly reduced by DEX treatment regardless of feeding group. It has already been shown that steroid use has a direct affect on the linear growth of infants, however it is not yet known if this growth delay affects neurological development. It is also not known if it is the lack of AA or DHA in tissues that is partly to blame for the growth delays. If it is the lack of very AA or DHA, increasing the amount of AA and DHA in sow milk through supplementation might be helpful, as the maximal accretion of DHA in the retina is not attained through natural milk feeding, but through LCPUFA-supplemented formula (Alessandri, et al., 1998).

It is known that unfortified breast milk fed to premature infants may not reflect the "gold standard", since analysis of the composition of human milk from mothers giving birth to preterm infants (Clandinin, et al., 1981) indicates that mothers' milk provides levels of AA and DHA approximating the predicted requirement at day 16 of life at oral intake levels of approximately 120 kcal/kg of body weight (Clandinin, et al.,

1981), as there is no significant difference between term and preterm mothers milk after 30 days (table 2.2.1).

10.2 Liver

This study tested the hypothesis that the exogenous glucocorticoid DEX results in sub-optimal amounts of AA and DHA in tissue, unless a dietary source is provided. After only 15 days of DEX treatment, significantly lower amounts of PE and PC DHA were found in the liver ($p<0.05$), and the provision of AA and DHA through sow's milk attenuated the DEX induced reductions. Liver ALA (and LA) was similar among groups suggesting that the liver was not able to synthesise adequate DHA to support the requirements of growth and development during DEX treatment. The formula and sow-fed piglets treated with placebo had equal ALA and DHA status regardless of the phospholipid investigated so the reduced DHA in formula–fed piglets may be due to DEX rather than diet- unless the amount of fat in the diet was too low. The sow fed piglets received more fat in their diet than the formula fed piglets (sow 52 vs formula 37g/L). Therefore it is possible that under a stress such as in the administration of DEX, piglets need higher amounts of fat to support normal growth and development.

There are multiple explanations of why DEX piglets that were not sow-fed have decreased levels of the DHA in the liver tissue. Firstly, exogenous DEX injections (de Gomez Dumm, Alaniz, & Brenner, 1979) and the administration of DEX in drinking water (Mandon, Irma, de Gomez Dumm, de Alaniz, Marra, & Brenner, 1987) produce significant reductions in the liver delta 5 and or delta 6 enzymes that desaturate the LA and ALA to AA and DHA respectively. This decrease of enzymes then decreases the amount of AA and DHA produced from the FA precursors LA and ALA respectively.

Secondly, the final steps in the synthesis of DHA are recently reported to occur in peroxisomes in liver (Voss, Reinhart, Sankarappa, & Sprecher, 1991; Sauerwald, Hachey, Jensen, Chen, Anderson, & Heird, 1997). Since delta 6 desaturase is used twice in the production of DHA, the limited enzymatic activity may reduce DHA to a greater degree than AA. Thirdly the catabolic stress DEX induces on piglets may result in increased energy requirements. Stress can cause increased lypolysis of FA's such as LA to be preferentially released from adipose tissue and used as an energy source rather than for AA synthesis (Cogo, et al., 1997). If these energy requirements are not met, 18:2-n6 and 18:3n-3 provided in human milk and formulas will be oxidized to meet the energy cost of essential metabolic and physiologic processes. Under these circumstances, it should be expected that levels of AA and DHA would decline in formula fed piglets that are not provided extra AA and DHA found in sow milk. All of these factors can account for the increased requirements that sow fed piglets and infants not fed mothers milk could endure while on DEX therapy, and cause the ill premature infant to be at risk for AA and DHA deficiencies, and therefore at risk of decreased growth and development.

10.3 Brain & Retina

The liver plays a major role in supplying AA and DHA to the central nervous system during early postnatal development (Uauy, Peirano, Hoffman, Mena, Birch & Birch, 1996; Sauerwald et al., 1997). Animal studies indicate that dietary intakes that produce physiologic change in membrane structure and function in the liver, also result in differences in membrane composition and transitions in the function of other tissues such as specific membranes in brain (Foot, Cruz & Clandinin, 1997; Hargreaves & Clandinin, 1989; Clandinin, Field, Hargreaves, Morson & Zsigmond, 1985; Connor & Neuringer,

1988; Jumpsen, Lien, Goh & Clandinin, 1997) and retina (Suh, Wierzbicki & Clandinin, 1994). This was not found to be true in the brain, as DEX treatment caused the lowering of telencephalon PE AA, yet no differences were found in PE AA in the liver. It may be that DEX treatment has a impact on AA synthesis in the brain. It is known that FA synthesis is affected by DEX in the liver so it is possible that when the brain synthesises its own supply of AA (Scott and Bazan, 1989), DEX may be reducing brain AA synthesis as well.

In explanation for the similar proportions of DHA in the brain of placebo- and DEX-treaded piglet, Morris, Simmer, Van barneveld, & Gibson, 1999 suggests that growing brain tissue and the associated increase in myelination mask changes in AA and DHA accretion because of altered proportions relative to other FA. Since the placebo piglets were rapidly growing, myelination would be expected to proceed normally. The DEX-treated piglets grew slower suggesting myelination may have also been slower and responsible for the similar amounts of AA and DHA in most areas of the brain. Other animal studies (Alessandri, et al., 1996) have found as well, that the low amounts of DHA in sow's milk did not have any effect on DHA accretion in cerebral or retinal phospholipids, (the use of DEX was not a factor in either study). It has also been shown that brain astrocytes (Morre, Yoder, Murphy, Dutton & Spector 1991), have the ability to produce DHA, and could therefore help protect against the lower amounts being produced in the liver.

Since the timing of brain growth spurt differs across species, differences due to nutrient intake may be seen in premature infants or other animals who are treated with DEX and who's brain growth spurt correlates more with a preterm infant, than a term

infant. These results presented in this thesis coincided with previous reports that the CNS- especially brain avidly retain DHA even after lengthy periods of dietary deprivation of essential FAs (Fliesler & Anderson, 1983; Tinoco, 1982), however as Clandinin, Jumpsen, & Suh found in 1994, specific regions of the brain can vary in the amount and rate of DHA and AA accretion and this could help to explain the decrease in telencephalon AA.

In the retina, PE AA indicated a main effect of feeding (sow > formula). A study by Gibson et al, 1984 stated that ALA is metabolised more readily for energy than LA and the LA unlike ALA readily incorporates into membrane fractions and competes with n-3 for space in the membrane phospholipid. It could concluded from this information that AA would be seen in larger amounts when provided, such as from sows' milk.

This study showed that short-term DEX (15 days) decrease the amount of PC DHA seen in the retina, however sow feeding did not attenuate the lowering of FA that DEX induced as it did in the liver. Another significant difference noted was in formula fed piglets, in that larger amount of PC DHA were seen compared to piglets that were suckling. These main affects could have been due to the large difference seen in formula-placebo fed piglets compared to all other groups. It is generally assumed that the liver is the major source of DHA for the retina, however the retinal pigment epithelium has the ability to produce DHA (Wang and Anderson, 1993). The amount of ALA and LA in formula was relatively high based on the corn/canola blend used, and endogenous DHA synthesis could have been increased in both the liver and the retina. If this is true, no differences or even protection may be seen with formula feeding, and therefore justify the increase in PC DHA seen in formula fed piglets. Another reason maybe that in

comparison to human infants, the piglet has a higher rate of DHA synthesis than human infants do based on stable isotope tracer studies. This is a limitation of using the piglet model that has to be considered when attempting to extrapolate data to the human infant.

Other limitations of the study could have been that the sample size was decreased by one piglet death, human error, and low power. Perhaps specific phospholipid FAs should have been measured in the brain and the study could have been too short.

10.4 Plasma and RBC

Studies have found that levels of AA are lower in the plasma phospholipids (predominantly PC) of infants fed formula rather than human milk (Innis, 1992). In this thesis it was also found that plasma PC AA levels were higher in sow-fed rather than formula-fed, however no differences were seen in RBC PC or PE AA. Lower levels of AA are not usually seen in human infant RBC PE, even though AA is present in substantially higher amounts in the RBC PE phospholipid than in the plasma fraction. This was seen in an infant study by Hargreaves and Clandinin (1989) where it was found that reductions in AA and DHA become evident in plasma phospholipids before RBC phospholipids (predominantly PC), in infants fed formula rather than human milk.

Several studies have described the plasma and RBC FA of term and preterm infants fed formulas or human milk, with particular emphasis usually given to DHA (Innis, 1991; Innis, Foot, MacKinnon, & King, 1990; Koletzko, Decsi, Demmelmain, 1996; Putnam, Carlson, DeVoe, Barness, 1982; Uauy, Birch, Birch, Tyson, & Hoffman, 1990). This study showed a significant correlation ($p< 0.05$) between plasma and liver levels specifically for PC DHA and PC AA. Since the FA composition of plasma can be influenced by metabolism (Leyton, Drury, & Crawford; Anderson & Connor, 1988)

dietary intake, as well as preferential tissue uptake (Sinclair, 1975), the correlation to liver status supports previous work on plasma phospholipids showing that they are reliable indicators of liver FA status (Rioux et al., 1997). Future studies have to be undertaken to determine if in piglets treated with DEX, compared to those that are not treated with DEX, have lower liver levels of PC FAs because of an increased demand for DHA and AA by CNS tissue, or due to the livers decreased ability to elongate and desaturate linolenic and linoleic to their metabolic precursors.

Although plasma correlated with liver, there was no correlation of plasma or RBC to the retina and brain. It is unusual not to find correlations in plasma, as both Innis (Hrboticky, MacKinnon & Innis, 1991; Foote, Hrboticky, MacKinnon & Innis, 1990) and Alessandri (Alessandri, Goustard, Guesnet & Durand, 1998) found that changes in retinal PE correlated to plasma phospholipids. This lack of correlation again could have been due to the limited sample size in the retina or a diverse group.

The retina showed significantly lower levels of PC DHA in sow-fed piglets, and in the DEX treated group PC DHA in RBC as also reduced with sows, however no significant correlations were found between retina and RBC PE and PC. RBC is continually modified by deacylation and reacylation with plasma FAs, and by exchange with plasma phospholipid, and therefore it is not as likely to see correlations between RBC and brain or retina. The limitation of using an animal model such as the piglet could also have affected the results, as the concentration of DHA and AA are highest in the plasma phospholipids rather than in RBC PE in the piglet, whereas this distribution pattern has been found to be inverted in newborn infants (Alessandri et al, 1998).

10.5 Fat Metabolism

It was indicated that DEX treatment, at therapeutic dosages, alters fat metabolism in that plasma triglyceride and total cholesterol were higher in the DEX-treated vs the placebo-treated piglets. The significant main effect of DEX to elevate both triglyceride and total cholesterol suggests clearance of FA from circulation is impared or there is an increase in mobilization when combined with no affect on liver ALA or LA, which supports the conclusion that reduced endogenous synthesis was responsible for the reduction in proportion of DHA in phospholipds from liver and plasma. In order to explain these results, tracer studies could be conducted using the same animal protocol but with addition of a radioactive stable isotopes of LA and ALA.

11.0 Summary

Since this study shows that DEX therapy affects the proportion of FAs in the tissues of healthy new-born piglets, it would be likely that DEX therapy would also affect human infants. However, because premature infants do not have the reserves of AA and DHA new-born infants have, nor do they have the ability to produce as much DHA to ALA as healthy piglets, it could be postulated that the low levels of AA and DHA found in the liver, and plasma would eventually occur in the retina and throughout the brain. This also applies to RBC turnover.

When conducting studies on the very low birth weight infant, it is not possible to control the infants' state of well being. It is important to note that this study was done on healthy piglets to eliminate confounding variables, such as poor nutrient intake, that could cause decreased n-6 and n-3 reserves and ultimately reduce AA and DHA accretion. The confounding variables premature infants encounter include: lung disease;

on going difficulties in maintaining adequate energy intakes; possible inappropriate composition of available intravenous lipid emulsion; as well as speculated immaturity of AA and DHA biosynthetic pathways. Premature infants with bronchopulmonary dysplasia may have also received antenatal DEX.

In summary, this study demonstrated that liver AA and DHA in neonatal piglets are altered by DEX treatment over 15 days. This study also showed that the feeding of sows' milk to piglets (which contains AA and DHA) vs formula (which does not contain AA and DHA), results in sow fed piglets having higher proportion of AA and DHA in the liver.

12.0 Future Directions

Further research needs to be done to see if chronic administration of DEX results in more severe consequences such as decreased brain DHA. Although this thesis did not note any differences in brain DHA, differences may have been seen with a larger sample size. The wide range of variation observed in this research limited the power. For example, in brain DHA increasing the sample size to n=50 would compensate for the variation in the samples, and a difference may then have been noted between sow placebo and sow DEX. However, instead of increasing sample size considerably, the use of triplicate samples can also eliminate outliers, creating a lower standard error, and therefore a better reliability of results.

Further research is necessary to confirm if the reduced liver DHA and brain AA in the DEX treated piglets that were formula-fed reflects reduced endogenous synthesis. Tracer studies would be beneficial to understand endogenous FA synthesis. Given that DEX cause lower levels of DHA to be produced in the liver and AA in brain in healthy

term piglets, it could be speculated that more severe reductions would be observed in preterm infants. Investigations in premature infants where DEX is used as therapy for chronic lung disease are necessary to determine if altered AA and DHA status are related to neurodevelopmental delay observed late in infancy.

13.0 References

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