EFFECT OF DIETARY CHOLESTEROL ON BRAIN CHOLESTEROL IN DEVELOPING RATS

By

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Abstract

Cholesterol is a significant component of brain and is found in both neuronal membranes and myelin. All mammalian milks provide cholesterol during periods of maximal brain growth and myelination. However, infant formulas contain only traces of cholesterol. The purpose of this study is to determine if dietary cholesterol alters rat brain composition during development. Pregnant Long-Evans rats were randomly assigned (12/group) to a semi-synthetic diet with or without cholesterol. On postnatal day 1 (P1), litters were culled to eight and weaned on P17. Litters received the same concentration of cholesterol as their dam from P17 to P32. On P32, pups were sacrificed. The left brain cortices were analyzed for cholesterol and protein concentration. Developing rats exposed to the cholesterol diet had an increase in left brain cortex size (p=0.003), cholesterol (p=0.006) and protein concentration (p=0.0005). Exposure to exogenous cholesterol increased brain cholesterol and protein concentration in developing rats.

Keywords: cholesterol, infant formula, brain
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Chapter 1

Introduction

Cholesterol is an abundant and important constituent of all eukaryotic membranes and plays a pivotal role in membrane synthesis, organization, dynamics, function and sorting (1-4). The adult rat brain is approximately 1% by weight cholesterol (5) and approximately 25% of the total body cholesterol in humans is found in the brain (6). Brain cholesterol is present in oligodendrocytes that wrap axonal regions of the neuron (in a process called myelination) and in plasma membranes of astrocytes and neurons (2, 6).

Myelin is a phospholipid layer that surrounds neuronal axons and is important to nerve conduction. Myelin is composed of approximately 70% lipids and 30% proteins (2, 6). In humans, brain myelination starts in the second trimester of gestation and continues up to at least two years of age (6-8). In rats, myelination begins between 9 to 12 days and reaches a maximum between 15 to 20 days of life (9, 10). During this period there is a significant increase of cholesterol in the brain. During the maximal periods of brain myelination in rats, milk from the dam provides 0.78-2.07 mmol/L (30-80 mg/dL) of cholesterol (10-12). As well, human milk provides significant amounts of cholesterol to human milk-fed infants during this important period of brain cholesterol accumulation (0.26-0.28 mmol/L or 10-11 mg/dL). In contrast to human milk, cow milk based formulas contain less cholesterol.
(0.08-0.13 mmol/L or 3-5 mg/dL) and soy based formulas do not contain cholesterol (13).

The cholesterol content in brain must be derived either from synthesis within the brain or from an exogenous source such as diet or maternal to fetal/newborn transfer (14). Animal studies using radiolabeled cholesterol reported that circulating cholesterol can be incorporated into the brain, suggesting that there is no blood-brain barrier for cholesterol (15-18). However, a study conducted by Morris et al demonstrated that minimal amounts of labeled cholesterol was supplied to 20 day suckling rat pup brains from exogenous sources like mothers milk (19). A study in piglets concluded that animals fed dietary cholesterol during the first 4 to 8 weeks of life had higher cholesterol concentration in cerebrum compared to piglets fed with diets that did not contain cholesterol (14). However, other studies suggest that all cholesterol in the brain is synthesized de novo (10, 20).

In mammalian nervous systems, proteolipid protein and myelin basic protein constitute nearly 80% of total myelin proteins (6). Lipid and protein interactions play a major role in maintaining structure and function of biological membranes (1, 21, 22). By measuring cortex protein and considering the ratio of cholesterol to protein, it is possible to gain some clues about any effects of increasing cholesterol exposure on brain composition.

Since cholesterol content in the brain is crucial for adequate myelination, neuronal growth and synaptogenesis factors that influence the
accumulation of cholesterol in the brain during the early development period of an animal are important and need to be further explored. Ultimately, the answer to the question could have relevance for the newborn as human milk contains higher amounts of cholesterol when compared to commercial formula milk (13). In this study, we asked if feeding dietary cholesterol to the rat during pregnancy and lactation and to pups after early weaning influenced brain cholesterol concentration.

A rat model was chosen for this study as rats have short gestation, lactation and weaning periods to monitor the effect of interventions. It is not feasible to measure brain cholesterol in live human infants.

Statement of purpose

The purpose of this study was to determine if feeding cholesterol (0.5% by weight) to the rat during gestation and lactation and to pups at weaning would increase brain cortical cholesterol concentration per unit brain weight or per unit gram cortex protein concentration.
Research questions

1. Will exposure to dietary cholesterol during gestation, lactation and after weaning increase brain cortical cholesterol in developing rats?

2. Will exposure to dietary cholesterol during gestation, lactation and after weaning alter the brain cortical cholesterol to total protein ratio in developing rats?
Chapter 2

Literature Review

Cholesterol is an abundant and important constituent of all eukaryotic membranes and plays a pivotal role in membrane synthesis, organization, dynamics, function and sorting (1-4). It was discovered by Poulletier de la Salle in 1769 and was named by Michel Chevreul in 1815 (23).

Cholesterol is present in tissues and plasma lipoproteins in the form of free cholesterol or in combination with a long-chain fatty acid as cholesterol ester (24). Cholesterol is a hydrophobic molecule with a planar tetracyclic fused steroid ring and a flexible isooctyl hydrocarbon tail. The molecular formula of cholesterol is $\text{C}_{27}\text{H}_{46}\text{O}$ and molecular mass is 386.65 g/mol. The chemical name of cholesterol is 5-cholesten-3β-ol.

In adult humans, approximately 700 mg of cholesterol is synthesized in the body per day and about 300 mg per day is obtained from the diet. Egg yolk, butter fat, poultry, liver, shrimp and beef are the main sources of dietary cholesterol after weaning. In infants the main source of cholesterol is mother’s milk. About 10-25% of cholesterol synthesis occurs in the liver and 10% occurs in the intestines in humans (24). The remaining cholesterol is synthesized in adrenal glands, reproductive organs, brain and spinal cord.

Cholesterol is synthesized from acetyl Co-A and HMG-Co-A reductase is the rate limiting enzyme in the biosynthetic pathway. Cholesterol
biosynthesis is regulated by dietary cholesterol that reaches the liver via chylomicron remnants. In tissues, cholesterol balance can be maintained between the factors that increase cholesterol (cholesterol synthesis, hydrolysis of cholesteryl ester) and those that decrease cholesterol (synthesis of steroid hormones, synthesis of bile acids). Cholesterol is excreted from liver in bile either in the form of free cholesterol or as bile acids (24).

Total plasma cholesterol of about 200 mg/dL is considered normal. Cholesterol is transported to various tissues via lipoproteins of plasma. Lipoproteins include chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Cholesterol transported via LDL is related to atherogenesis whereas that transported via HDL is related to protection from cardiovascular disease (24).

Humans typically consume about 5 mg/kg/day of cholesterol through diet and synthesize about 10mg/kg/day (25). In mice, dietary cholesterol intake from synthetic cereal based animal diet is about 30mg/kg/day and synthesis in the body accounts for about 160 mg/kg/day (25). Total amount of sterol obtained from diet and de novo synthesis in the mouse is approximately 190 mg/kg/day which is about 13 times greater than in a human (15 mg/kg/day) (25). This is due to nearly 40 fold greater hepatic clearance of LDL cholesterol in mice compared to humans requiring high amounts of cholesterol input to maintain steady state cholesterol levels (25).
**Cholesterol composition of the brain**

The central nervous system is composed of gray and white matter. White matter consists of myelin in large quantities, which surrounds axons in the form of myelin sheath (8). Gray matter is made up of neuronal cell bodies and their dendrites (8). Magnetic resonance appearance of white matter is determined by the presence of myelin cholesterol (26). Adult human brain is composed of approximately 80% water, 10-15% lipids and the rest proteins (8).

Cholesterol is a major constituent of the brain (27). Cholesterol comprises approximately 10% of the dry weight of the brain (8) whereas in other organs, cholesterol comprises about 1% of dry weight (8). About 99.5% of cholesterol present in the brain is unesterified (6). The majority of the cholesterol present in the central nervous system (CNS) is present in myelin sheaths and in plasma membranes of astrocytes and neurons. Myelin is composed of 70% lipids and 30% proteins (6, 8). Cholesterol accounts for 27.7% of total lipids in myelin, 27.5% of total lipids in white matter and 22.0% of total lipids in the gray matter (8). Protein accounts for 30.0% of dry weight in myelin, 39% of dry weight in white matter and 55.3% of dry weight in gray matter (8). Other lipids in myelin include sulfatides, ehanolamine phosphatides, sphingomyelin, phosphatidylcholine, phosphatidylserine (8). Myelin contains cholesterol, phospholipids and glycosphingolipids in a ratio of 4:4:2.
Origin and biosynthesis of cholesterol in brain

Cholesterol can be provided to the brain either by endogenous synthesis within the brain or delivery of cholesterol via circulating lipoproteins. There is controversy in the literature regarding the role that circulating cholesterol plays in relation to supplying brain cholesterol (8).

Couerbe first made observations in the nineteenth century that lead him to believe that cholesterol was the principal element of the nervous system (6, 28). Since then, the role of cholesterol in the nervous system gained importance and lead to further investigations in this area.

Initial findings that brain cholesterol was metabolically different from peripheral blood cholesterol were made in 1940s (6, 29). Waelsch et al., showed that administration of $^2$H$_2$O via drinking water to adult male rats did not result in incorporation of the tracer into the unsaponifiable fraction of the brain lipid, i.e. cholesterol (30). A study done on dogs demonstrated that an intravenous infusion of $^2$H-cholesterol for 3 days to a dog did not result in labeled cholesterol in brain and spinal cord (29). Dobbing (1963) injected rats with a $^{14}$C cholesterol tracer from birth to eight weeks of age and found less than 8% of brain sterol came from peripheral blood (17, 20). Edmond et al., administered $^2$H-cholesterol in a milk substitute to rat pups for a period of 10 days (from 5 or 6 days of age to 15 or 16 days of age) did not find any label incorporated in the brain cholesterol (10).
Similar studies were also done on humans. Chobanian et al., administered 4-14C cholesterol by intravenous injection for an average of 226 days to terminally ill patients and did not find any traces of labeled cholesterol in their brains and other nervous tissue after their natural death (31). Morell and Jurevics in their review of literature concluded that all cholesterol accumulating in the brain during development was locally synthesized within the brain and not obtained from circulation (32).

Other studies in rats suggest that dietary cholesterol can increase blood cholesterol and increase brain cholesterol by some mechanism with a conclusion that there is no “blood-brain barrier” for cholesterol (17, 18). A study in piglets by Boleman et al., showed that animals fed cholesterol during the first 4 to 8 weeks of life had higher cholesterol concentration in cerebrum compared to piglets not fed cholesterol (14). There is insufficient evidence in the literature to conclusively determine if exogenous source of cholesterol plays a role in cholesterol accumulation in the brain, especially in the early stages of brain development.

The rate of synthesis and cholesterol content in the brain increases dramatically during brain growth and development (33-36). The rate of cholesterol synthesis then decreases in adulthood (6). The requirement for cholesterol by the nervous system is especially high during the most rapid periods of its myelination (32). In rats, the crucial period for accumulation of cholesterol in brain is the first few weeks after birth during which myelin
formation takes place in the central and peripheral nervous system (32). In humans a crucial period is from the second trimester of gestation to two years of age. The question of how the brain obtains cholesterol is especially important in the early stages of brain development, particularly in conditions that lead to inadequate synthesis of cholesterol. In such circumstances, other means of supplying cholesterol like dietary sources should be further investigated (6-8).

Cholesterol metabolism in the brain

Cholesterol in the central nervous system is metabolized to 24S-hydroxy cholesterol by the enzyme cytochrome P46 which is mainly found in the neurons (37). The metabolite 24S-hydroxy cholesterol can easily cross the blood brain barrier (37). This is the major pathway for removal of cholesterol from the brain and helps to maintain brain cholesterol homeostasis. 24S-hydroxycholesterol is subsequently converted to either bile acids or excreted in a sulfated or glucuronidated form into bile (37). In the central nervous system, cholesterol is mainly distributed in two pools, i.e. cholesterol in the plasma membranes of glial cells and neurons and cholesterol in myelin. About 0.02% of brain cholesterol in the human and about 0.4% in the mouse turns over each day (33). This low turnover of cholesterol in brain is due to efficient recycling and explains its relatively long half life in the brain of 4-6 months (33). This long half life decreases the
requirement of additional new cholesterol and may be especially important during periods of rapid brain growth, synaptogenesis and myelination.

Functions of cholesterol in brain

Cholesterol is an important lipid that regulates the function of neuronal receptors and that may thereby affect neurotransmission related to mood and anxiety disorders (38, 39). Cholesterol also affects membrane thickness and fluidity (40, 41), restricts ion leakage through membranes (42) and is involved in protein sorting (43) and signal transduction (44). Diaz and Monreal demonstrated that myelin lipids without proteins showed poor permeability to H⁺ ions (45). On the other hand, the lipid and cholesterol composition of membranes can affect the activity of membrane proteins. Possible mechanisms by which cholesterol can influence membrane proteins include direct binding to the proteins and activation of the proteins (8). Cholesterol can also interfere with the membrane proteins by altering the physical properties of membranes such as fluidity (8, 46) and by causing phase separations in the plane of membranes, thus stabilizing protein-protein interactions (8, 47).

Cholesterol decreases membrane permeability and fluidity by compacting phospholipids and changing the order of fatty acyl chains (35). It is also responsible for determining the functional properties of membrane proteins like neurotransmitter receptors and ion channels (35). Cholesterol is
not evenly distributed in membranes but mostly concentrated in lipid microdomains or rafts (35). Lipid rafts regions of the membrane are believed to be involved in signal transduction, cellular functions involved in neural development, neurotrophic factor signaling, synaptic plasticity and axonal growth (48).

Cholesterol is important for neural signal transduction and ligand binding in neuron transmitters. G-protein coupled receptors (GPCRs) are membrane proteins with seven transmembrane domains and represent one of the largest families of proteins in mammals (1, 49). Cells communicate with the external environment by signal transduction through the GPCRs. These receptors can be activated by various ligands including peptides, amines, lipids, glycoproteins and nucleotides and are important targets for the development of new drugs. A large number of GPCRs are present in the nervous system and are responsible for neuronal function. Lipid protein interactions play a role in the assembly, stability and function of membrane proteins (1, 21, 22). A cell has the capacity to vary its membrane lipid composition in response to stress and stimuli thereby changing the activity of its membrane receptors (1). Since cholesterol is an important membrane lipid, it has an effect on the formation and stability of membrane proteins.

Brain cholesterol plays an important role in maintaining the structure and function of neuronal receptors that are very important for neural signal transduction. Serotonin$_{1A}$ is a neurotransmitter receptor from the family of
GPCRs and plays an important role in generating and modulating cognitive and behavioral functions like sleep, pain, mood, depression and anxiety (1, 39). Serotonergic signaling plays a pivotal role in neurogenesis, dendritogenesis, apoptosis and axonal branching during the developmental stages of the brain (50). Disruption in the serotonergic system has been implicated in the etiology of developmental disorders like infantile autism (39). The role of cholesterol in modulating the ligand binding activity and G protein coupling of the hippocampal serotonin$_{1A}$ receptor was recently demonstrated (51). In this study, cholesterol was depleted from the hippocampal membranes using methyl-$\beta$-cyclodextrin (M$\beta$CD). Depletion of cholesterol was done using various concentrations of M$\beta$CD and it resulted in a concentration-dependent reduction in the binding of the specific agonist 8-hydroxy-2 (di-N-propylamino) tetrailin (8-OH-DPAT) to serotonin$_{1A}$ receptors. Cholesterol depletion also affected the G protein coupling of the receptor. Membranes were subsequently replenished with cholesterol using M$\beta$CD-cholesterol complex and it resulted in recovery of ligand binding activity. This provides evidence that cholesterol is necessary for ligand binding and G protein coupling of serotonin$_{1A}$.

**Cholesterol and brain development**

Cholesterol is required for brain myelination. Formation of myelin sheath around an axon or nerve fiber is called myelination. Myelin provides
electrical insulation to the nerve fiber and is related to the speed of nerve conduction (8). The function of myelin as electrical insulator is most likely due to the presence of lipids, including cholesterol (8). In the mammalian central nervous system, proteolipid protein (PLP) constitutes about 50% of the total myelin proteins (52). PLP is critically important for the development and stabilization of the myelin structure (53). Membrane bound proteins (MBPs) constitute about 30% of the total protein (8). Most important function of MBPs is maintaining the stability and thickness of CNS myelin (8, 54, 55). Mutations in the transmembrane domains of these proteins may cause myelin deficiencies and demyelination.

In rodents, myelination in CNS occurs predominantly during the first four weeks of life (56). In rats, myelination begins between 9 to 12 days and reaches a maximum between 15 and 20 days of life (9, 10). In humans, myelination begins during the second trimester of gestation and extends up to two years of age (6-8) at a rapid rate but continues to occur throughout childhood and adolescence. Myelination in the CNS is preceded by the accumulation of cholesterol esters, desmosterol and 7-dehydrodesmosterol (8, 57, 58). The high levels of these compounds may represent the accumulation of cholesterol for initiation of myelination (8, 59).

Previous studies have shown that cultured neurons from mammalian central nervous system require cholesterol for effective synaptogenesis and neuronal differentiation (60-64). The study by Goritz et al identified dendrite
differentiation as the rate limiting step for astrocyte induced synapse formation in retinal ganglion cells from postnatal rats. Moreover, they showed that glia derived cholesterol directly improves pre synaptic differentiation and continuous synaptogenesis (62). These results provide evidence that cholesterol is essential for growth and effective function of synapses, neuronal differentiation and brain development. Possible mechanism by which cholesterol could promote synapse formation is by serving as building material for synaptic membranes (35), which have been demonstrated to promote synaptogenesis (65). Cholesterol may also be required for synapse stabilization. Neurons form continuous synapses but the connections between these synapses may be lost if adequate cholesterol is not available (35). Adhesion molecules that bind the pre and post synaptic elements are mainly responsible for the stability of synaptic connections. These adhesion molecules are confined to rafts and therefore their function depends on cholesterol concentration and other components of the membrane such as spingolipids (35). Studies have also shown that cholesterol is essential for synaptic transmission and that accurate cholesterol homeostasis is crucial for synaptic plasticity (35, 66)

Volpe et al., studied the role of cholesterol biosynthesis and the biosynthetic enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the developing mammalian brain (67). In this study, cells from the cerebral hemispheres of fetal rats of 15-16 days of gestation were
separated and cultured. Cholesterol biosynthesis was measured by the rate of incorporation of $^{14}$C-acetate into digitonin-precipitable sterols. Two peaks of cholesterol biosynthesis were observed, a first prominent peak after 6 days and a smaller one after 14 days in culture. There was a corresponding increase in HMG-CoA reductase enzyme activity at the same times. The initial prominent peak in both cholesterol biosynthesis and HMG-CoA reductase activity at 6 days coincided with the peaking in DNA synthesis, determined as the rate of incorporation of [3H]thymidine into DNA. The second smaller peak at 14 days coincided with the most rapid rise in the activity of the oligodendroglial enzyme, 2':3'-cyclic nucleotide 3'-phosphohydrolase (CNP). These findings suggest that cholesterol biosynthetic pathway in developing mammalian brain is closely related to periods of rapid cellular proliferation and oligodendroglial differentiation.

**Clinical significance: Smith-Lemli-Opitz Syndrome**

Defects in cholesterol synthesis cause neurological disorder such as Smith-Lemli-Opitz syndrome (1, 68). This syndrome is present from birth but the symptoms do not become evident until the child grows. In patients with Smith-Lemli-Opitz, most of the cholesterol in brain is replaced with 7-dehydrocholesterol and is not available for myelin synthesis causing mental retardation in affected individuals (69, 70). This disease was first described in 1964 as a severely fatal birth defect syndrome (71). Incidence of the disease
varies from 1 in 10,000 to 1 in 60,000 (72). Carrier frequency is as high as 1 in 30 for Caucasians and lower for Asian and African populations (72). In Caucasians, it is the fourth common recessively inherited disorder after cystic fibrosis, phenylketonuria and hemochromatosis (70).

Clinical symptoms include dysmorphic craniofacial and limb abnormalities like microcephaly, micrognathia, ptosis of eyelids, epicanthal folds, cleft palate, retroverted ears, polydactyly, and foot deformities (8, 70). Disorders of central and peripheral nervous system include mental retardation, abnormal electroencephalograms, small cerebellum, astrocyte proliferation, abnormal muscle tone, loss of nerve cells, absence of cerebellar vermis and irregular gyri. Other organs that are functionally and structurally affected include liver, kidneys, heart, lungs and digestive organs (8, 70).

The biochemical defect underlying the pathogenesis is a defect in 7-dehydroreductase an enzyme that is responsible for the conversion of 7-dehydrodesmosterol to desmosterol and 7-dehydrocholesterol to cholesterol. As a result, both biochemical reactions are blocked and cholesterol is not synthesized. Serum cholesterol level in these patients decreases with the accumulation of 7-dehydrocholesterol. This appears to affect myelin synthesis by delaying the commencement and limiting the degree of myelination (8, 70).

Smith-Lemli-Opitz syndrome is diagnosed by blood sterol test which tests for the presence of high levels of 7-dehydrocholesterol in the blood. This
syndrome can also be identified by an MRI (magnetic resonance imaging) scan which tests for physical deformities.

A study done on Smith-Lemli-Opitz children and adolescents concluded that cholesterol supplementation does not improve their development. According to Macros et al., dietary cholesterol supplementation is the current available method of treatment for Smith-Lemli-Opitz syndrome (72). Simvastatin which inhibits the enzyme (HMG –CoA) reductase is a potential drug for lowering 7-dehydrocholesterol levels in Smith-Lemli-Opitz patients (72).

Human milk and Infant formula

Infants fed with human milk receive considerable dietary cholesterol (73). Human milk contains 10-11 mg/dL of cholesterol (0.26-0.28 mmol/L) (13). In contrast, cow milk based formulas contain less cholesterol (0.08-0.13 mmol/L or 3-5 mg/dL) and soy based formulas do not contain cholesterol (13). Triacylglycerol accounts for 97% to 98% of the total lipid content in milk. Fatty acids constitute about 88% of total milk fat. Total fat content of human milk is between 30 and 50 mg/dL which provides approximately 45% to 55% of total energy. Human milk is also rich in essential fatty acids like linoleic acid (8-17%), and α linolenic acid (0.5-1.0%) along with arachidonic acid (0.5-0.7%) and docosahexanoic acid (0.2-0.5%) (74). Sterol content in human milk varies from 10 to 20 mg/dL, with cholesterol being the major component (75).
Lammi-Keefe et al., collected milk samples from six mothers every four hours from 0600 hr to 2200 hr and found a diurnal pattern in the cholesterol concentration of milk with 8.75 mg/dL at 0600 hour and 11.2 mg/dL at 2200 hour of the day (76).

Boersma et al., (1991) determined cholesterol composition in colostrum, transitional milk and mature milk from mothers in St. Lucia (73). Cholesterol content was found to be 36.0 mg/dL from 0 to 4 days post partum, 19.7 mg/dL from 5 to 9 days and 19.0 mg/dL from 10 to 30 days. This suggests that cholesterol content in human milk decreases progressively over time. Clark and Hundrieser collected twenty five milk samples and analyzed for total cholesterol, total lipid and free cholesterol (77). Mean total cholesterol of the samples was 13.5 +/- 3.1 mg/dL and correlated with total lipid. Mean cholesterol was 10.9 +/- 2.3 mg/dL and correlated with total lipid. During the maximal periods of brain myelination in rats, mother’s milk provides 30-80 mg/dL (0.78-2.07 mmol/L) of cholesterol (10-12).

Finally, studies have shown that breast fed children have higher cognitive development compared to children who are formula fed (78-81). A meta-analysis done on children from infancy to adolescence showed a 3.16 point higher cognitive score in breast fed children compared to formula fed children (82). Angelsen et al., demonstrated that children breast fed for longer duration of time showed improved cognitive development (83). However, the reason for this is unknown.
Chapter 3
Methods

Experimental design

Female virgin Long Evans rats were purchased from Harlan Inc, Indianapolis. Rats were a 70 days (± 1 week) old at the start of the experiment and were bred to proven breeders of the same strain. Light, heat and humidity were kept constant for the duration of the experiment. At the time of initial mating, female rats were assigned randomly to nutritionally complete semi-synthetic diets that differed only in that one contained cholesterol and the other was deficient in cholesterol (see Table 1 for diet details). Two female rats on the same diet were housed with a proven male breeder and left to mate for 5 days, the duration of the estrous cycle in rats. On the sixth day female rats were caged separately. Their weights were monitored as an indication of pregnancy until they gave birth to a litter. That day was designated as postnatal day 0 (P0). Gestation period in rat lasts for 21-22 days. On postnatal day 1 (P1), the litters were culled to eight and the remaining pups were sacrificed.

The eight rat pups were housed with their dam until P17, at which time they were separated from their dam and housed two per cage. The rat pups continued to receive diets with the same cholesterol concentration (0.5% or 0% by weight) as their dam. Two pups from each litter were fed with one of
four levels of sialic acid (see Table 2) from day 17 through day 32 to answer another question. However, the level of dietary sialic acid did not influence brain cholesterol and is not mentioned further in the report. Diet and water were available *ad libitum*. On day 32, the rats were sacrificed and the brains were excised and placed on dry ice until frozen solid. At that time, the sample was stored at -80°C. These samples were later analyzed for cholesterol (Infinity Cholesterol Reagent, Thermo Electron, Louisville, CO) and total protein (BCA Protein Assay kit, Pierce, Rockford, IL) concentration.

Table 1: Composition of control and experimental group diets of dams

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol-Free Diet (0% by weight)</th>
<th>Cholesterol-Containing Diet (0.5% by weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Diet</td>
<td>4560 g</td>
<td>Basal Diet</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>350 g</td>
<td>Soybean oil</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0 g</td>
<td>Cholesterol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 shows the formula for cholesterol free diet (control diet) and cholesterol containing diet (experimental diet) of dams. Soy bean oil was purchased from local grocery store and cholesterol was purchased from Harlan Teklad, Madison, Wisconsin.
Composition of basal diet: (AIN 93G)

<table>
<thead>
<tr>
<th>Formula</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal mix</strong></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
</tr>
<tr>
<td>Corn starch</td>
<td>397.486</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
<tr>
<td><strong>Mineral mix</strong></td>
<td>35</td>
</tr>
<tr>
<td><strong>Vitamin mix</strong></td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
</tr>
<tr>
<td>TBHQ antioxidant</td>
<td>0.014</td>
</tr>
</tbody>
</table>

**Basal mix**: Harlan Teklad TD00235

**Mineral mix**: Harlan Teklad TD94046 (AIN 93G-MX)

**Vitamin mix**: Harlan Teklad TD94047 (AIN 93-VX)

The above table shows the formula for basal diet used by Harlan Teklad, Madison, Wisconsin. The basal diet was mixed with soy bean oil to make cholesterol free diet (control diet) and it was mixed with soy bean oil and cholesterol to make the cholesterol containing diet (experimental diet)
Table 2: Composition of control and experimental group diets with added levels of sialic acid

<table>
<thead>
<tr>
<th>Cholesterol-Free diets</th>
<th>Cholesterol-Containing diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Non-cholesterol diet+sialic acid 0 mg/kg/d</td>
<td>1. Cholesterol diet+sialic acid 0 mg/kg/d</td>
</tr>
<tr>
<td>Basal diet</td>
<td>Basal diet 4650g</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>Soybean oil 350g</td>
</tr>
<tr>
<td>Casein</td>
<td>Cholesterol 25g</td>
</tr>
<tr>
<td>Corn starch</td>
<td>Casein 48g</td>
</tr>
<tr>
<td></td>
<td>Corn starch 8.2g</td>
</tr>
<tr>
<td>2. Non-cholesterol diet+sialic acid 20 mg/kg/d</td>
<td>2. Cholesterol diet+sialic acid 20 mg/kg/d</td>
</tr>
<tr>
<td>Basal diet</td>
<td>Basal diet 4650g</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>Soybean oil 350g</td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>Cholesterol 25g</td>
</tr>
<tr>
<td>Casein</td>
<td>Glycomacropeptide 14.05g</td>
</tr>
<tr>
<td>Corn starch</td>
<td>Casein 36g</td>
</tr>
<tr>
<td></td>
<td>Corn starch 6.15g</td>
</tr>
<tr>
<td>3. Non-cholesterol diet+sialic acid 40 mg/kg/d</td>
<td>3. Cholesterol diet+sialic acid 40 mg/kg/d</td>
</tr>
<tr>
<td>Basal diet</td>
<td>Basal diet 4650g</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>Soybean oil 350g</td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>Cholesterol 25g</td>
</tr>
<tr>
<td>Casein</td>
<td>Glycomacropeptide 28.1g</td>
</tr>
<tr>
<td>Corn starch</td>
<td>Casein 24g</td>
</tr>
<tr>
<td></td>
<td>Corn starch 4.1g</td>
</tr>
<tr>
<td>4. Non-cholesterol diet+sialic acid 80 mg/kg/d</td>
<td>4. Cholesterol diet+sialic acid 80 mg/kg/d</td>
</tr>
<tr>
<td>Basal diet</td>
<td>Basal diet 4650g</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>Soybean oil 350g</td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>Cholesterol 25g</td>
</tr>
<tr>
<td></td>
<td>Glycomacropeptide 56.2g</td>
</tr>
</tbody>
</table>
Table 2 shows the formula for control diet (non cholesterol fed group) and experimental diet (cholesterol fed group) with added levels of sialic acid. The basal diet mixture, cholesterol, casein and corn starch were purchased from Harlan Teklad, Madison, Wisconsin. Glycomacropeptide was obtained from Tatua co-operative Dairy Company Limited, New Zealand.

Ethics

Prior consent was obtained from The Animal Care and Use Committee of Kansas University Medical Center by Susan E. Carlson, PhD before conducting research on rats. Protocol # 2006-1585 was given by Institutional Animal Care and Use Committee for performing our research on rats.

Sample

A total of 86, P32 rat left brain cortices (43 non cholesterol and 43 cholesterol samples) were analyzed for cholesterol. Protein concentrations were determined on 78 samples (39 non cholesterol and 39 cholesterol samples) as one sample from each of the four cholesterol-sialic acid groups was lost.
**Procedure**

**Homogenation**

Animals were decapitated at 32 days of age and the brains removed and placed on dry ice. The samples were stored at -80°C until analysis. The brains were thawed on ice and the left brain cortices removed, weighed and homogenized in an equal weight of lysis buffer (800µl of 20mM Tris HCl, 100µl glycerol, 10µl igepl CA-630 and 10µl protease inhibitor cocktail). Samples were sonicated for one minute at 20% amplitude and stored at -80°C freezer until they were extracted.

**Extraction**

A 50µl aliquot of the homogenated sample was pipetted in to a 15ml clean glass tube (tube 1) with a 100 µl Hamilton syringe followed by the addition of 2.5 ml of chloroform: methanol (2:1). The sample was vortexed for 15 minutes then centrifuged at 1500 x g for 10 minutes. The supernatant was transferred to a clean glass tube (tube 2) and the precipitate re-extracted with 1ml of 2:1 chloroform: methanol by again vortexing for 10 minutes and centrifugation at 1500 x g for 10 minutes. The supernatant from the second extraction was combined with the first extraction. The solvent was vaporized under nitrogen, and the extracted lipids re-dissolved in 75µl of isopropanol. Samples were stored at -80°C freezer until analysis for cholesterol.
Analysis

The cholesterol assay was performed using Infinity Cholesterol Reagent, Thermo Electron, Louisville, CO.

Preparation of standards

A stock cholesterol solution was prepared by dissolving 75 mg of cholesterol in 10 ml of isopropanol. The stock solution was diluted with isopropanol as described below to make a standard curve for the cholesterol assay.

Preparation of diluted cholesterol standards:

<table>
<thead>
<tr>
<th>Serial No</th>
<th>Standards(mg/dl)</th>
<th>Cholesterol stock(ml)</th>
<th>Isopropanol(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>450</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>600</td>
<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>750</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

The standards were stored at -20°C until use.

To analyze the standards and samples for cholesterol, 5µl aliquots of each standard and sample were pipetted (using a 10 µl Hamilton syringe) into a test tube. To this test tube, 500 µl of cholesterol buffer was added. The tubes containing the reaction mixture were then incubated in a water bath at 37°C for five minutes. The end point of the reaction was read in a
spectrophotometer at 500nm. Cholesterol concentration (mg/dl) was plotted on the x axis and absorbance on the y axis to generate a standard curve, which was linear within the range of standard concentrations chosen. The concentration of the samples was determined from the absorbance using the standard curve and expressed in mg cholesterol/gram wet weight of frontal cortex.

**Principle of colorimetric assay**

The Infinity Cholesterol Reagent Kit contains cholesterol esterase, which will hydrolyze cholesterol esters to cholesterol and free fatty acids. However, brain contains almost no cholesterol esters. Cholesterol is oxidized to cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase. Hydrogen peroxide combines with hydrobenzoic acid and 4-aminoantipyrene to form quinoneimine dye which is responsible for the final pink color. The color intensity is read between 500-550 nm using an absorbance spectrophotometer.

**Protein assay**

The protein assay was performed using a BCA Protein Assay kit, Pierce, Rockford, IL.
Preparation of standards

Standards are prepared in accordance with the instructions and guidelines provided with the BCA protein assay kit. Bovine serum albumin (BSA) is used as a standard. Each 1ml ampoule of the standard contains 2.0 mg/ml in 0.9% saline and 0.05% sodium azide.

Preparation of Diluted albumin standards:

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of diluent(µl)</th>
<th>Volume of BSA</th>
<th>Final concentration of BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300µl of stock</td>
<td>2,000 µg/ml</td>
</tr>
<tr>
<td>B</td>
<td>125</td>
<td>375µl of stock</td>
<td>1,500 µg/ml</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325µl of stock</td>
<td>1,000 µg/ml</td>
</tr>
<tr>
<td>D</td>
<td>175</td>
<td>175µl from vial B</td>
<td>750µg/ml</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325µl from vial C</td>
<td>500µg/ml</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325µl from vial E</td>
<td>250µg/ml</td>
</tr>
<tr>
<td>G</td>
<td>325</td>
<td>325µl from vial F</td>
<td>125µg/ml</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100µl from vial G</td>
<td>25µg/ml</td>
</tr>
<tr>
<td>I</td>
<td>400</td>
<td>0</td>
<td>0µg/ml (Blank)</td>
</tr>
</tbody>
</table>

Working range of the assay is between 125-2,000 µg/ml

Sample dilution

To analyze the total protein concentration in the frontal cortex, 10 µl of the original 1:1 homogenate of the frontal cortex and lysis buffer was combined with 990 µl of deionised water (for a 1:100 dilution) in a 2 ml microcentrifuge tube. The tubes were vortexed thoroughly and the samples
kept on ice until the samples were ready to be loaded into the micro plate wells.

**Preparation of working reagent**

The working reagent for the protein assay was prepared by mixing 50 parts of BCA reagent A with 1 part of reagent B. The working reagent remains stable for several days when stored at room temperature. BCA reagent A contains sodium carbonate, sodium bicarbonate, bicinechinonic acid, and sodium tartrate in 0.1M sodium hydroxide. BCA reagent B contains 4% cupric sulfate.

**Procedure for loading the micro plate**

Triplicate 10µl aliquots of all standards and samples were loaded into a 96 well micro plate. Two hundred micro liters of working reagent was added to each well using a multiple pipettter. The contents of the well were mixed by placing the plate on a micro plate shaker for one minute. The plate was then incubated for 30 minutes at 37°C. The absorbance was read on a micro plate reader at 562nm. A standard curve was plotted with protein concentration (mg/ml) on the x axis and absorbance on y axis. The concentration of the samples was determined from the standard curve in mg/ml and converted to mg/gram of frontal cortex after accounting for the various dilutions.
**Principle of colorimetric analysis**

BCA protein assay kit has bicinchoninic acid for the colorimetric detection of total protein. Total protein present in the sample reduces Cu$^{+2}$ to Cu$^+$ in the presence of alkaline medium. A water soluble complex is formed with the chelation of two molecules of bicinchoninic acid with cuprous ion. The purple colored complex exhibits strong absorbance at 562nm. The macromolecular structure of protein, presence of amino acids like cysteine, cystine, tryptophan and tyrosine, and presence of peptide bonds are said to be responsible for the purple color formation with bicinchoninic acid.

**Data analysis**

Statistical analysis was carried out using Data analysis Tool Pak in Microsoft excel 2007. Results of the cholesterol assay, protein assay, weights of cortices and total body weights of rats were analyzed by one-way ANOVA. A p value< 0.05 was considered as statistically significant.

Power analysis for the study was calculated based on an earlier study by Carlson and House, 1986 that showed an increase in total sialic acid content in the cerebral gangliosides of the brain (84). Based on this information the power for the present study is 99% with a sample size of 12 rats per group.
Chapter 4

Results

We evaluated the effects of sialic acid on brain cholesterol and found none. No significant difference was found for level and level of sialic acid by cholesterol interaction. As a result, statistical analysis was done by one-way ANOVA for cholesterol intake.

The mean body weight of control group (non cholesterol fed) and experimental group (cholesterol fed) rats on day 17 was not significantly different (Table 3). SD is standard deviation, SE is standard error.

Table 3. P17 mean rat weights, control (non cholesterol) versus experimental group (cholesterol)

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Mean weight (g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>39.73</td>
<td>4.65</td>
<td>0.71</td>
<td>43</td>
<td>0.33</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>40.68</td>
<td>4.37</td>
<td>0.66</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>
The mean body weight of control group (non cholesterol fed) and experimental group (cholesterol fed) rats on day 32 was not significantly different (Table 4).

Table 4. P32 mean rat weights, control (non cholesterol) versus experimental group (cholesterol)

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Mean weight(g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>106.13</td>
<td>10.92</td>
<td>1.66</td>
<td>43</td>
<td>0.808</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>105.55</td>
<td>11.24</td>
<td>1.71</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>
The mean cortex weight of control group (non cholesterol fed) and experimental group (cholesterol fed) of P32 rats was not significantly different (Table 5).

Table 5. Mean cortex weights of P32 rat brains, control (non cholesterol) versus experimental (cholesterol) group

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Mean weight (g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>0.580</td>
<td>0.054</td>
<td>0.0083</td>
<td>43</td>
<td>0.33</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.592</td>
<td>0.069</td>
<td>0.0105</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>
The mean right cortex weight of control group (non cholesterol fed) and experimental group (cholesterol fed) of P32 rats was not significantly different (Table 6).

Table 6. P32 mean right cortex weights, control (non cholesterol) versus experimental (cholesterol) group

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Mean weight (g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>0.287</td>
<td>0.044</td>
<td>0.0067</td>
<td>43</td>
<td>0.575</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.281</td>
<td>0.055</td>
<td>0.0084</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>
The mean left cortex weight of experimental group (cholesterol fed) was significantly higher than the control group (non cholesterol fed) of P32 rats (Table 7).

Table 7. P32 mean left cortex weights, control (non cholesterol) versus experimental (cholesterol) group

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Mean weight(g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>0.292</td>
<td>0.032</td>
<td>0.005</td>
<td>43</td>
<td>0.0039**</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.311</td>
<td>0.026</td>
<td>0.004</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

** p value highly significant
The mean cholesterol concentration (mg/g) of left brain cortex in experimental group (cholesterol fed) was significantly higher than the control group (non cholesterol fed) of P32 rats (Table 8).

Table 8. Mean cholesterol concentration of P32 rat brain left cortices, control group (non cholesterol) versus experimental group (cholesterol)

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Cholesterol conc. (mg/g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>11.71</td>
<td>1.336</td>
<td>0.203</td>
<td>43</td>
<td>0.006**</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>12.606</td>
<td>1.62</td>
<td>0.247</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

** p value highly significant
Figure 1: P32 Rat Brain Left Cortex Cholesterol Concentration

Figure 1 displays the cholesterol concentration of control (non cholesterol) and experimental (cholesterol) group rats analyzed on P32. Mean cholesterol concentration of control group rats is 11.71 mg/g and experimental group rats is 12.606 mg/g respectively. The two standard error bars shown in the figure do not overlap indicating that the two means are statistically significant. *P<0.01 indicates that it is highly significant.
The mean total protein concentration (mg/g) of left brain cortex in experimental group (cholesterol fed) was significantly higher than the control group (non cholesterol fed) of P32 rats (Table 9).

Table 9. Mean total protein concentration in P32 rat brain left cortices, control group (non cholesterol) versus experimental group (cholesterol)

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Protein conc.(mg/g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>50.2</td>
<td>4.82</td>
<td>0.772</td>
<td>39</td>
<td>0.0005**</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>53.7</td>
<td>3.68</td>
<td>0.589</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

** p value highly significant
Figure 2: P32 Rat Brain Left Cortex Total Protein Concentration

Figure 2 displays the total protein concentration of control (non-cholesterol) and experimental (cholesterol) group rats analyzed on P32. Mean total protein concentration of control group rats is 50.2 mg/g and experimental group rats are 53.7 mg/g respectively. The two standard error bars shown in the figure do not overlap indicating that the two means are statistically significant. P<0.01 indicates that it is highly significant.
The cholesterol to total protein ratio in left brain cortex in control group (non cholesterol fed) and experimental group (cholesterol fed) of P32 rats was not significantly different (Table 10).

Table 10. Cholesterol to total protein ratio for P32 rat brain left cortices, control group (non cholesterol) versus experimental group (cholesterol)

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Protein conc.(mg/g)</th>
<th>SD</th>
<th>SE</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Cholesterol</td>
<td>0.235</td>
<td>0.033</td>
<td>0.005</td>
<td>39</td>
<td>0.75</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.237</td>
<td>0.025</td>
<td>0.004</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 5

Discussion

The purpose of this study was to determine if feeding cholesterol (0.5% by weight) to the rat during gestation and lactation and to pups at weaning will increase brain cortical composition of cholesterol and influence the cholesterol to total protein ratio in the brain. The results showed that dietary cholesterol significantly increased brain cortical cholesterol and protein concentration in developing rats when compared to rats fed with no cholesterol diet. The ratio of cholesterol to total protein remained unchanged. Surprisingly, the left cortex weight was significantly higher in the cholesterol fed rats.

Previous studies showed that radioactive dietary cholesterol administered to rat pups for a 10 day period from 5 or 6 days to 15 or 16 days of age via milk substitutes did not result in incorporation of labeled cholesterol in the brain (10). However, a study conducted by Morris et al. demonstrated that minimal amounts of labeled cholesterol was supplied to 20 day suckling rat pup brains from exogenous sources like mothers milk (19). Haque and Mozaffor (1992) demonstrated a 10% increase of brain myelin cholesterol in 20 day old suckling mice pups whose dams were fed with ten times the weight of cholesterol used in our study or 5% dietary cholesterol compared to controls (85). This study implies that supplemental cholesterol fed to the
dams increased cholesterol concentration in dams’ milk thereby increasing the amount of dietary cholesterol available to the pups. A study by Schoknecht et al., demonstrated that dietary supplementation of cholesterol (200 mg/100 g diet for 35 days) increased cerebral brain cholesterol in pigs (86). A study in piglets showed that animals fed with dietary cholesterol during the first 4 to 8 weeks of life had higher cholesterol concentration in cerebrum compared to piglets fed with diets that did not contain cholesterol (14). Ours is the first study in rats to show that feeding dietary cholesterol to rats at gestation, lactation and weaning increased brain cortical cholesterol concentration significantly.

Brain can synthesize cholesterol de novo (10, 20). Morell and Jurevics postulated that cholesterol accumulating in the brain during development is locally synthesized within the brain and found no evidence of a contribution from the circulation (32). However, there were also a few studies that reported that dietary cholesterol can increase blood cholesterol and that blood cholesterol can be incorporated into the brain, suggesting that a tight blood brain barrier may not exist for cholesterol (17, 18). Our study has demonstrated that dietary cholesterol can increase brain cortical cholesterol. This suggests that exogenous source of cholesterol can contribute to accumulated cholesterol in the brain during early developmental stages by some mechanism.
A secondary research question was how dietary cholesterol might influence the ratio of cholesterol to total protein in left brain cortex of developing rats. In mammalian nervous systems, proteolipid protein and myelin basic protein constitute nearly 80% of total myelin proteins (6). Lipid-protein interactions play a major role in maintaining structure and function of biological membranes, regulation and dysfunction of brain cholesterol (1). It is also important to see how dietary changes influence total protein concentration and thus cholesterol to total protein ratio in the brain. Our study showed that dietary cholesterol supplementation to the rat during gestation, lactation and to pups at weaning increased the left cortex brain composition of total protein with no change in the cholesterol to total protein ratio indicating that there was a proportionate increase in cholesterol and total protein. This may mean that an increase in brain cortical cholesterol caused an adequate and proportionate increase in the composition of membrane proteins. Such maintenance of physiological cholesterol to protein ratio is important as lipid function is closely interrelated to protein in myelin and cell membranes.

Although there was no significant difference in overall brain weight between the two groups, left brain cortex weight was significantly higher in the cholesterol fed group when compared to the non-cholesterol group with no difference seen in right brain cortex weights. While an increase in cortical weight can be explained by the proportionate increase in cholesterol and total protein content, it is unclear why the weight increase is limited to the left
cortex. A study on piglets fed with cholesterol rich diet showed a significant increase in cerebrum weight when compared to the group fed with non-cholesterol diet (14). However, separate measurements of left and right cortices were not done in that study. Further investigations may be needed to elucidate the above findings from our study.

An increase in left cortex weight and brain cholesterol and total protein content may theoretically be explained by a relatively higher weight gain in cholesterol fed rats. We measured the weights of all cholesterol and non-cholesterol fed rats at P17 and P32 and there was no significant difference in weight between the two groups. Therefore, results from our study cannot be explained by differences in dietary uptake or weight gain in the two groups. Previous studies also did not show increase in body weights of rats fed with cholesterol (10).

In neonates, cell membrane synthesis, myelin formation and synaptogenesis are the most important functions of cholesterol in the central nervous system. Studies in preterm infants have demonstrated that breast milk fed infants may have better developmental outcomes and higher intelligent quotients than infants fed with formula milk (87, 88). Human milk has higher amounts of cholesterol than formula milk (13) and since we have demonstrated that dietary cholesterol can increase brain cortical cholesterol, there is a possibility that exogenous cholesterol might be making a significant impact on the neurological development of the brain.
Haque and Mozaffar conducted a study that showed the effect of dietary cholesterol on myelination of mouse brain (85). In their study, 20 day suckling pups whose dams were fed with 5% cholesterol showed a 10% higher myelin cholesterol compared to the control group. This data suggests that dietary cholesterol fed to the dams during gestation altered brain myelination of weaning mice. Since cholesterol plays a very important role in signal transduction and ligand binding activities of myelin proteins, insufficient amounts of cholesterol can interrupt these activities including myelin formation. Inadequate synthesis of myelin may in turn result in disruption of cognitive function, loss of memory and speech impairment. Since maximum myelin formation takes place during early developmental stages, it becomes important to provide infants with sufficient cholesterol to supplement de novo synthesis.

Increase in brain cholesterol and protein content may affect the cognitive function, nervous function and behavior of rats. These effects were not studied in our current study. Therefore, further investigations are needed to see if increase in brain cholesterol has any affect on the physiology of nervous system and behavior.

In our study, exogenous cholesterol increased cortical cholesterol and total protein concentration as discussed earlier. A proportionate increase in cholesterol and total protein in the left cortex along with the increase in left brain cortex weight suggests that there was an increase in overall
neurogenesis or synaptogenesis with dietary cholesterol. Another possibility given that final 1:4 ratio of cholesterol to protein is that the increase occurred in white matter. Previous studies have shown that cultured CNS neurons from mammalian nervous system require cholesterol for effective synaptogenesis and neuronal differentiation and addition of cholesterol to cultured CNS neurons increased the number of synapses formed (60-64). CNS myelination and synaptogenesis require cholesterol, which in turn are rate limiting factors for brain maturation. These studies provide evidence that cholesterol is required for normal brain development. Our results suggest that dietary intake of cholesterol during initial periods of brain development increase cholesterol content in the brain, which has implications for neuronal development, myelination, synapse formation and infant feeding.

**Implications**

Since the mean brain cholesterol concentration of the cholesterol fed rat group is higher than the control group, it provides evidence that an external source of cholesterol from the time of conception to 32 days of age in the rat can increase brain cholesterol accumulation. This can lead to studies focusing on the behavior of rats fed with cholesterol or not to determine if cholesterol fed rats demonstrate some different behaviors compared to rats not fed with cholesterol. Such evidence would also suggest that human infants fed human milk might also have higher brain cholesterol than infants
fed formulas (which are low in cholesterol) and could lead to studies of cholesterol supplementation and behavior in human infants

**Limitations**

Animal studies may not accurately reflect human metabolism. For example, mouse studies have shown that total amount of sterol obtained from diet and *de novo* synthesis is approximately 190 mg/kg/day, which is about 13 times greater than that in humans (15 mg/kg/day) (25). Metabolic pathways, membrane composition, developmental stages and maturation at birth may not be exactly similar in rats and humans (89). However, an animal experimental study is the first step towards an investigation like this as it involves procedures that are not feasible in humans.

Analysis of cholesterol and total protein content in the right cortex was not measured in this study because that portion of the cortex was used for analysis of brain gangliosides. Analysis of cholesterol content was attempted on rat milk consumed by pups at P1. However, it could not be accurately measured due to interference of stomach contents, acid and mucus.

Although brain cholesterol was increased by dietary cholesterol, we could not determine when dietary cholesterol exposure produced the effect as cholesterol exposure was increased through gestation, nursing and after weaning.
Future studies

Since the results of this rat study showed that feeding 0.5% cholesterol during gestation, lactation and to pups at weaning increased cholesterol and total protein concentrations in left brain cortices, studies focusing on the behavior of rats fed with and without cholesterol should be carried out to see if the two groups demonstrate different behaviors.

Our study has shown an increase in left brain cortex weight in the cholesterol fed rat group. Studies are needed to evaluate the disparity in weight gain between the right and left cortices. Also, studies evaluating the effect of increasing amounts of cholesterol in rat diets can be done.

Finally, infants fed human milk might accumulate higher brain cholesterol than infants fed formulas (which are low in cholesterol) and could lead to studies of cholesterol supplementation and behavior in human infants.
Chapter 6

Summary

Cholesterol is a significant component of brain. In humans about 25% of total body cholesterol is present in brain. Cholesterol content in the brain is crucial for adequate myelination, synaptogenesis and neuronal function. All mammalian milks, including human milk, provide cholesterol during periods of maximal brain myelination. However, US infant formulas contain only traces of cholesterol. This study examined the effect of dietary cholesterol on brain cholesterol and total brain protein in developing rats. Pregnant Long-Evans rats were randomly assigned to a nutritionally complete semi-synthetic diet (AIN-93G) with or without cholesterol (0.5% by weight or none). Litters were culled to eight on postnatal day one and weaned on day 17. Two pups from each litter received the same concentration of cholesterol as their dam at one of four levels of sialic acid (0, 20, 40, 80 mg/kg/day) from P17 to P32. On P32, rat pups were sacrificed. The left brain cortices were extracted and analyzed for cholesterol and total protein. Developing rats exposed to 0.5% cholesterol diet during gestation, lactation and post weaning had a significantly larger left cortex and the cholesterol and protein concentration of the left cortex (mg/g) were also increased. Higher exposure to exogenous cholesterol during development appears to influence cortex size and the concentration of cholesterol and total protein per gram of brain. Further
studies need to be done to evaluate the effect of dietary cholesterol and increased brain cholesterol on rat behavior and nervous function. Ultimately, it needs to be seen if supplementing infant formulas with cholesterol has a positive effect on brain development in infants.
References


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