DIETARY N-3 FATTY ACIDS AND CEREBRAL ISCHEMIA/REPERFUSION

by

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ABSTRACT

Many populations have low intakes of n-3 fatty acids, yet there is substantial evidence that the long chain n-3 fatty acid docosahexaenoic acid (DHA; 22:6n-3), found at high concentrations in the brain, is required for the proper development of the nervous system. However, less is known about requirements of long chain n-3 fatty acids for maintenance and function of the nervous system in later life. Several recent studies have reported that high amounts of long chain n-3 fatty acids reduce the extent of brain damage caused by cerebral ischemia in animals. However, whether or not a dietary deficiency of n-3 fatty acids increases the extent of injury when cerebral ischemia occurs has not been previously reported. The present studies, therefore, sought to determine if a diet deficient in n-3 fatty acids influences the extent of brain injury in the rat following cerebral ischemia. Male rats were fed an n-3 fatty acid adequate (control), an n-3 fatty acid deficient, or a high DHA diet for 5 weeks from weaning. Middle cerebral artery occlusion (MCAO) was induced and infarct volume was measured by 2,3,5,-triphenyltetrazolium chloride staining 24 hours after the procedure. Brain and platelet fatty acids were analyzed by gas liquid chromatography. DHA (22:6n-3) was 21-28% lower in brain phospholipids, and 17% lower in brain total fatty acids in the n-3 fatty acid deficient compared to control group, while 22:6n-3 was 12% higher in total brain fatty acids in the high DHA group than the control group. There was no significant difference in infarct volume (203, 220 and 218 mm^3) among the control, n-3 fatty acid deficient, and high DHA groups, respectively. Platelet fatty acids and platelet aggregation were assessed to determine if these were influenced by the high DHA diet, and could possibly explain the observation of an apparent, but not statistically significant, higher number of rats with hemorrhages in the high DHA diet group. Platelet

lipid arachidonic acid was not lower and platelet aggregation, assessed *ex vivo* using whole blood with a platelet function analyzer, was not longer in rats fed the high DHA compared to control or n-3 fatty acid deficient diets. In summary, dietary n-3 fatty acid deficiency did not increase the extent of brain injury following cerebral ischemia. The possibility that high dietary 22:6n-3 might increase susceptibility to cerebral hemorrhage will require further study.

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LIST OF ABBREVIATIONS

ADP: adenosine diphosphate

ANOVA: analysis of variance

CCA: common carotid artery

DHA: docosahexaenoic acid, 22:6n-3

GLC: gas liquid chromatography

HPLC: high performance liquid chromatography

hr: hour

kg: kilogram

MCA: middle cerebral artery

MCAO: middle cerebral artery occlusion

mg: milligram

min: minute

PC: phosphatidylcholine

PBS: phosphate buffered saline

PE: phosphatidylethanolamine

PFA: platelet function analyzer

PI: phosphatidylinositol

PS: phosphatidylserine

TTC: 2,3,5-triphenyltetrazolium chloride

SPSS: Statistical Package for Social Sciences

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DEDICATION

I dedicate this thesis to Kal. Words cannot convey how much your willingness to stand

behind my pursuits has meant to me over the years. Thanks for always reminding me to stop

and smell the roses, and listen to the music.

CO-AUTHORSHIP STATEMENT

A version of chapter 2 from this thesis will be submitted for publication: Slack, P.J., Wadsworth, L.D., and Innis, S.M. I completed the work described in this paper. Wadsworth, L.D. offered expertise in platelet aggregation studies. Innis, S.M. is the principal investigator and my supervisor, and was involved in all stages. All co-authors edited and reviewed this paper.

CHAPTER 1: INTRODUCTION

1.1Background

High concentrations of the n-3 fatty acid docosahexaenoic acid (22:6n-3) in ethanolamine (PE) and serine (PS) containing glycerol phospholipids is a specific feature of mammalian brain gray matter membranes (1, 2). A substantial amount of research supports the role of 22:6n-3 in the proper development and maintenance of the nervous system, although many details of the roles of 22:6n-3 remain incompletely understood (3-5). Mammalian cells lack the enzymes necessary to form n-3 fatty acids and therefore rely on a dietary source of n-3 fatty acids for the 22:6n-3 needed for brain membranes (6). Several studies have suggested that high intakes of fish oil or 22:6n-3 can reduce the extent of brain damage in animal models of stroke (7-13), which is the 3rd leading cause of death in North America (14, 15). Despite a decrease in the incidence of stroke in the last 50 years, the severity has not changed; 30-50% of those who have suffered a stroke are dependent on others for care (16). Although high intakes of n-3 fatty acids appear to decrease the extent of damage in animal models of stroke, human diets in many nations are low in n-3 fatty acids (17) . The question of whether or not reduced levels of 22:6n-3 in the brain might worsen the extent of damage caused by cerebral ischemia, however, has not been addressed.

1.2 Introduction to Dietary Fatty Acids

The diets of mammals include 3 macronutrient groups: carbohydrates, protein, and fat.

A major component of dietary fat is triglyceride, which is composed of glycerol plus three fatty

acids. This thesis is concerned with dietary fatty acids and in particular the n-3 polyunsaturated

fatty acids.

Fatty acids are hydrocarbon chains with a carboxyl group at one end, known as the delta (Δ)

end, and a methyl group at the opposite end (methyl, n). The basic structure of a fatty acid is

denoted below:

(Methyl end) $CH_3 (CH)_n COOH$ (Carboxyl end)
(n) (Δ)
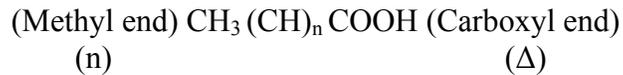
Each carbon atom in a fatty acid chain is either saturated (all four bonds are linked to a separate

carbon, hydrogen, or oxygen), or unsaturated (2 adjacent carbons share two bonds), shown in

Figure 1-1.
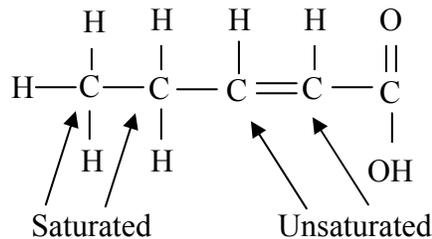
Figure 1-1: Schematic representation of saturated and unsaturated bonds.

Fatty acids not containing any double bonds belong to the saturated family, whereas those with

one carbon-carbon double bond belong to the monounsaturated family, and those with more

than one carbon-carbon double bond belong to the polyunsaturated family **(Figure 1-2)**. Two

important families of polyunsaturated fatty acids are the n-3 and n-6 fatty acids.

n   Δ

CH$_3$ 3   4   6   7   9   10   12   13   15   16   18   COOH   **Saturated (stearic acid)**

CH$_3$ 3   4   6   7   9   10   12   13   15   16   18   COOH   **Monounsaturated (oleic acid)**

CH$_3$ 3   4   6   7   9   10   12   13   15   16   18   COOH   **Polyunsaturated (n-6, linoleic acid)**

CH$_3$ 3   4   6   7   9   10   12   13   15   16   18   COOH   **Polyunsaturated (n-3, alpha-linolenic acid)**
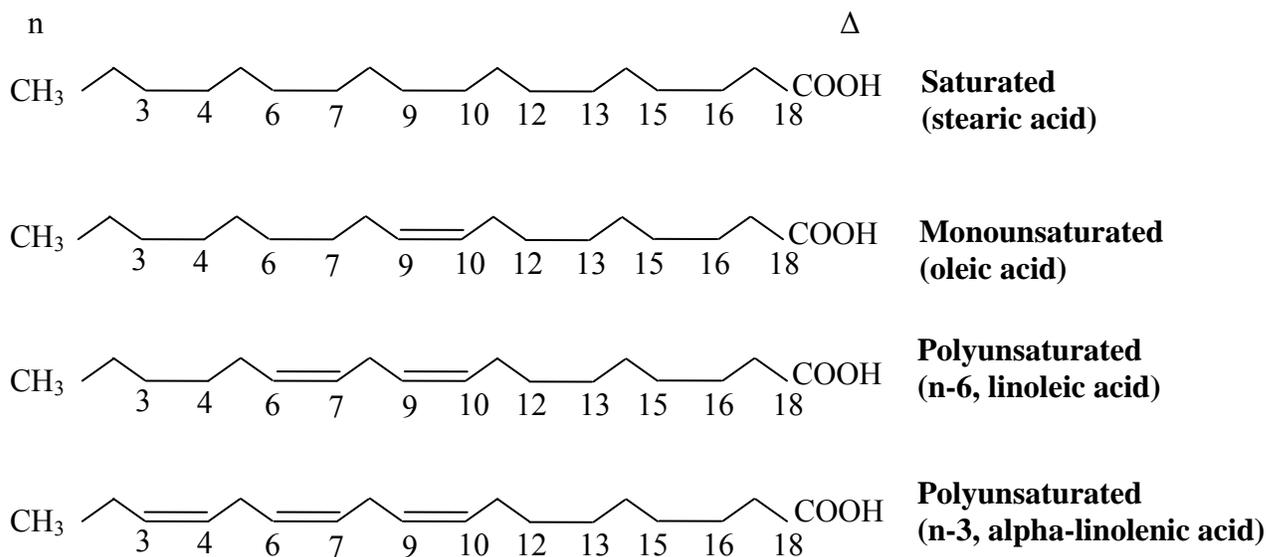
Figure 1-2: Schematic representations to show a saturated (stearic), monounsaturated (oleic), n-6 polyunsaturated (linoleic), and n-3 polyunsaturated (alpha-linolenic) fatty acid.

1.3 Fatty Acid Nomenclature

In common nomenclature, fatty acids are denoted by the length of their carbon chain, followed by a colon, then the number of double bonds, and finally by the position of the 1st double bond from the methyl (n) terminus. For example, the polyunsaturated fatty acid 22:6n-3, shown in **Figure 1-3,** has 22 carbons and 6 double bonds, with the 1st double bond occurring at the 3rd carbon from the methyl (n) terminus (2).

n   Δ

CH$_3$ 1   3   4   6   7   9   10   12   13   15   16   18   19   22   COOH
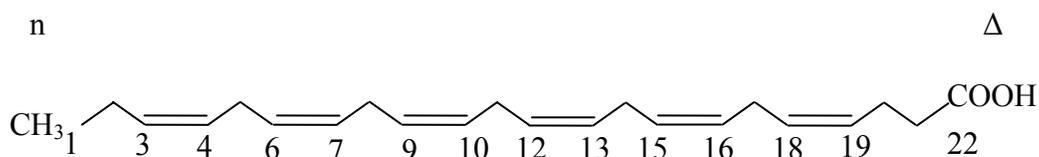
Figure 1-3: Schematic representation of docosahexaenoic acid (22:6n-3) to show the positions of the unsaturated (double) bonds.

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1.4   n-3 and n-6 Fatty Acids and their Metabolism

In mammals, saturated fatty acids and monounsaturated fatty acids of the n-9 and n-7 series can be synthesized *de novo* from acetyl CoA. The n-6 and n-3 polyunsaturated fatty acids, however, cannot be synthesized by mammalian cells; this is due to the absence of the $\Delta 12$ and $\Delta 15$ desaturase enzymes required for insertion of a double bond at the 12th and 15th carbons from the carboxyl terminus, respectively, of 18 carbon chain fatty acids (2, 18). Linoleic acid (18:2n-6) and alpha linolenic acid (18:3n-3) are the precursors to longer chain, more highly unsaturated n-6 and n-3 fatty acids, respectively; these fatty acids, containing 20 or more carbons, are sometimes referred to as long chain polyunsaturated fatty acids (2). Because the n-3 and n-6 polyunsaturated fatty acids are needed, but cannot be synthesized (19-22), they must be obtained from the diet and are termed essential dietary nutrients (23). It is important to note that n-6 fatty acids cannot be converted to n-3 fatty acids, and vice versa (24). This thesis focuses on the n-3 family of polyunsaturated fatty acids. However, because the n-3 and n-6 fatty acids interact in metabolism, and the n-6 fatty acids are also important in many physiological functions, the literature review also includes a discussion of these fatty acids.

The n-3 fatty acid 18:3n-3 and the n-6 fatty acid 18:2n-6 are the essential dietary fatty acids for almost all animals (2). Plants are the major dietary sources of 18:3n-3 and 18:2n-6. Oils such as canola, soybean, and flaxseed, and certain nuts are rich dietary sources of 18:3n-3, while corn, soybean, sunflower, and safflower oils are rich dietary sources of 18:2n-6 for humans. The amounts of 18:3n-3 and 18:2n-6 in common oils and fats are given in **Table 1-1**. Oleic acid (18:1n-9) is a monounsaturated fatty acid commonly found in vegetable oils, and its content is also provided in Table 1-1.

Table 1-1. Levels of oleic acid (18:1n-9), linoleic acid (18:2n-6), and alpha-linolenic acid (18:3n-3) in common dietary oils and fats[1].

Oil/Fat	18:1n-9	18:2n-6	18:3n-3
		% of fatty acids	
Flaxseed	18	16	57
Canola	61	21	11
Soybean	23	54	8
Safflower (high oleic)	77	14	1
Safflower[2]	13	78	< 0.1
Sunflower	16	71	1
Olive	75	9	1
Corn	29	57	1
Lard	47	9	1
Butter	28	3	1
Peanut	48	33	< 0.1
Cottonseed	19	54	< 0.1
Palm	39	10	< 0.1
Coconut	7	2	< 0.1

[1] Adapted from (25). Oleic acid (18:1n-9), linoleic acid (18:2n-6), and alpha linolenic acid (18:3n-3) in some common dietary oils and fats. The remainder of the fatty acids in each oil are monounsaturated and/or saturated.
[2] Fatty acid composition is from (23).

Longer chain n-3 fatty acids, such as eicosapentaenoic acid (20:5n-3) and 22:6n-3, and the longer chain n-6 fatty acid arachidonic acid (20:4n-6) are all present in the acyl moieties of cell membrane phospholipids. These fatty acids can be obtained from the diet, but are only found in animal tissue lipids. The richest dietary sources of 20:5n-3 and 22:6n-3 are fatty fish, whereas the predominant sources of 20:4n-6 in most human diets are poultry, eggs and other animal tissue lipids (23) . Arachidonic acid (20:4n-6) is also present in fish, although at lower levels than 20:5n-3 and 22:6n-3 (23). The content of 20:4n-6, 20:5n-3, and 22:6n-3 in some selected foods is shown in **Table 1-2**.

Table 1-2. Arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), and docosahexaenoic acid (22:6n-3) content of selected foods.

Food	20:4n-6	20:5n-3	22:6n-3
		g/100 g food	
Seal blubber	0.40	4.30	5.40
Mackerel (salted)	0.26	1.62	2.97
Salmon, (Atlantic, wild)	0.34	0.41	1.43
Tuna (bluefin)	0.06	0.36	1.14
Herring (Atlantic)	0.08	0.91	1.11
Halibut (Atlantic or Pacific)	0.18	0.09	0.37
Shrimp (canned)	0.10	0.29	0.25
Tuna (canned)	0.03	0.05	0.22
Chicken	0.15	0.01	0.06
Egg	0.15	0.01	0.04
Pork	0.08	<0.00[1]	0.01
Beef	0.07	<0.00	<0.00

Adapted from The Canadian Nutrient File [database on the internet]. Ottawa, ON: Health Canada; c2007 [cited June 24, 2008]. Available from: http://205.193.93.51/cnfonline/newSearch.do?applanguage=en_CA (26).
[1]Reported as 0.

Alternatively, the longer chain polyunsaturated fatty acids can be synthesized from their 18 carbon n-6 and n-3 fatty acid precursors as shown in **Figure 1-4**. Linoleic acid (18:2n-6) can be desaturated and elongated to several 20 and 22 carbon chain n-6 fatty acids, among which 20:4n-6 is particularly important. Alpha linolenic acid (18:3n-3) is desaturated and elongated to longer chain n-3 fatty acids including 20:5n-3 and 22:6n-3 in animals, including humans (Figure 1-4), (2, 18). Linoleic acid (18:2n-6) is metabolized to 20:4n-6, by Δ6 desaturase, elongation, and an additional desaturation by Δ5 desaturase on the endoplasmic reticulum (Figure 1-4). Alpha linolenic acid (18:3n-3) is similarly desaturated and elongated to 20:5n-3, using the same enzymes. For conversion to 22:6n-3, the currently accepted pathway includes two additional elongations from 20:5n-3 to 24:5n-3, followed by desaturation again using Δ6 desaturase to form 24:6n-3. Then, 24:6n-3 is transported to the peroxisome where chain shortening by two carbons (partial beta-oxidation) yields 22:6n-3 (27-29). The same process is believed to lead to the formation of docosapentaenoic acid (22:5n-6) (27, 28). Levels of 22:5n-6 in tissue lipids are usually very low, but increase in the liver, brain, and other organs of animals fed an n-3 fatty acid deficient diet (30-34).

```
18:3n-3                              18:2n-6

   ↓          Δ6-desaturase            ↓

18:4n-3                              18:3n-6

   ↓          Elongase                 ↓

20:4n-3                              20:3n-6

   ↓          Δ5-desaturase            ↓

20:5n-3                              20:4n-6

   ↓          Elongase                 ↓

22:5n-3                              22:4n-6

   ↓          Elongase                 ↓

24 :5n-3                             24 :4n-6

   ↓          Δ6-desaturase            ↓

24:6n-3                              24 :5n-6

   ⇩                                   ⇩

24:6n-3                              24:5n-6

   ↓          Chain Shortening         ↓

22:6n-3                              22:5n-6
```

Endoplasmic Reticulum

Peroxisome
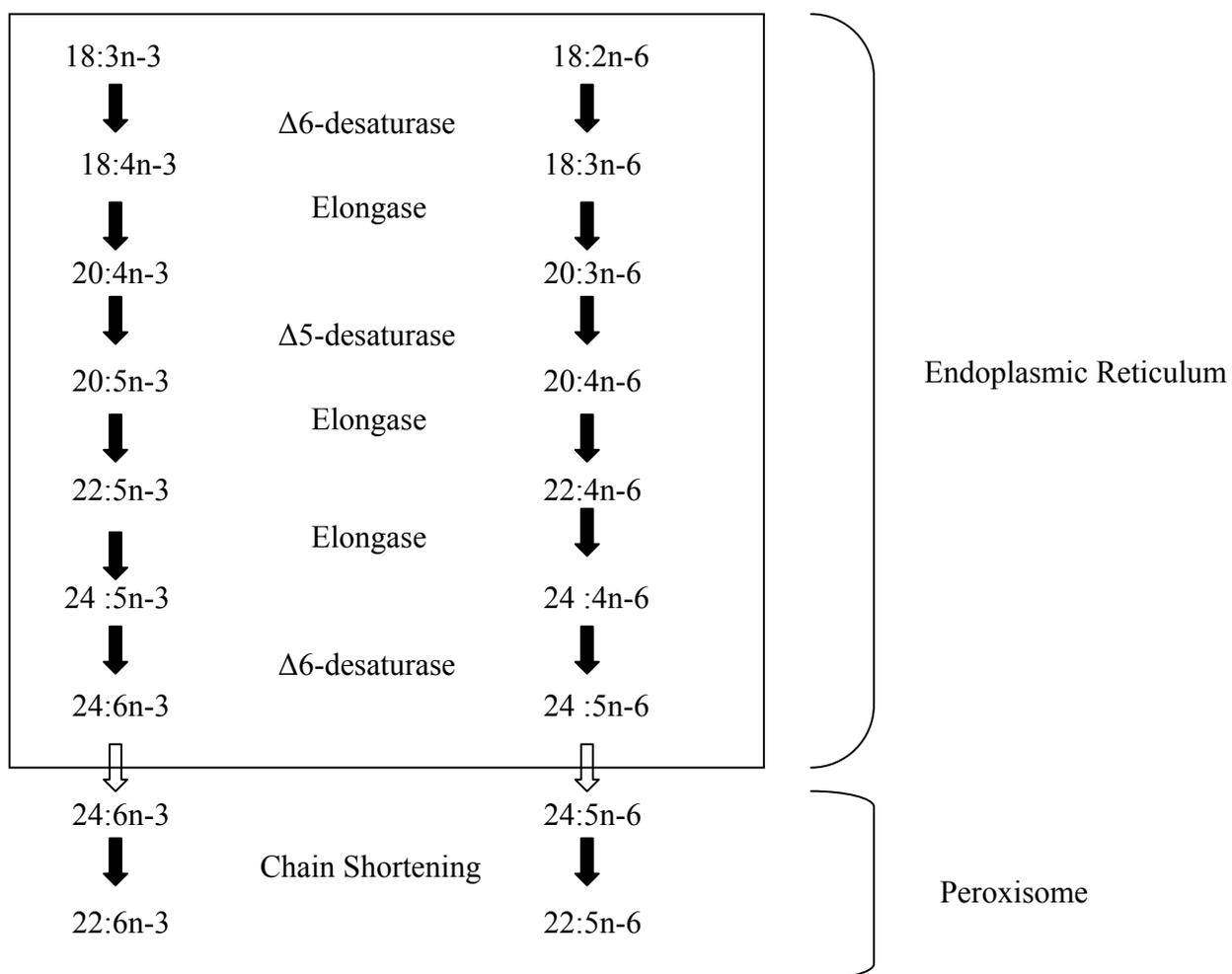
Figure 1-4: Schematic of n-3 and n-6 fatty acid desaturation and elongation in animal cells. Adapted from (18)**.**

The activity of the essential fatty acid desaturation pathway is generally believed to be more active in rodents than in humans (2). This interpretation is based on the higher levels of 22:6n-3 in plasma and liver lipids of rodents fed diets containing soybean or other oils containing 18:3n-3 but not 22:6n-3, when compared to the usual circulating plasma levels of 22:6n-3 in humans. However, some caution is warranted as humans do not usually consume diets containing a single oil source. In humans, about 90% of dietary 18:3n-3 appears to be directly β-oxidized (2), with < 1% of a trace dose of stable isotope labeled 18:3n-3 converted to 22:6n-3 (35-37). Interpretation of the proportion of dietary 18:3n-3 converted to 22:6n-3 is not necessarily an indication that the conversion is limited by the activity of the $\Delta6$ and $\Delta5$ desaturases, or that the conversion is too low. For example, the intake of 18:3n-3 may be greatly in excess of that needed for 22:6n-3 synthesis for incorporation into membranes. Alternatively, inhibition of 18:3n-3 conversion to 20:5n-3 and 22:6n-3 by high dietary intakes of 18:2n-6 could also explain low recovery of stable isotopically labeled 22:6n-3 in plasma following administration of the labeled 18:3n-3 as a precursor.

As introduced above, it is important to note that the relative amounts of n-3 and n-6 fatty acids in the diet have important ramifications for the fatty acid elongation and desaturation pathways. In vitro studies have shown that the $\Delta6$ desaturase enzyme has a preference for 18:3n-3 over 18:2n-6 (38-40) . However, numerous animal studies have shown that high dietary intakes of 18:2n-6 reduce the desaturation of 18:3n-3 and decrease 20:5n-3 and 22:6n-3 in tissue lipids (41, 42). Current mean intakes of 18:2n-6 in Canada and the U.S.A. are about 5-7% dietary energy, while 18:3n-3 represents about 0.5% energy; this results in an 18:2n-6 to 18:3n-3 of 10:1 or higher (42-44). The activities of the $\Delta6$ and $\Delta5$ desaturase enzymes are also influenced by the dietary intake of preformed 20:4n-6, 20:5n-3, and 22:6n-3; this is consistent with the expected product inhibition of the desaturation-elongation pathway. For example, high

intakes of fish (high in 20:5n-3 and 22:6n-3) are associated with lower blood levels of 20:4n-6 and decreased synthesis of eicosanoids formed from 20:4n-6 (2). The median intake of long chain n-3 fatty acids (20:5n-3 plus 22:6n-3) is estimated to be about 200-300 mg/day in the U.S.A. and in Canada (17, 44, 45). It is believed that up until 150 years ago humans consumed a diet with an n-6 to n-3 fatty acid ratio of about 1:1 (46). The high, approximate 10:1 ratio of n-6 compared to n-3 fatty acids, in modern diets, has been suggested to antagonize n-3 fatty acid metabolism, resulting in elevated n-6 fatty acids and a pro-inflammatory environment (46). The low intakes of n-3 fatty acids, but high intakes of n-6 fatty acids that characterize typical western diets have been suggested to be a cause for concern; this is based largely on the opposing biological roles of the metabolites of 20:5n-3 and 20:4n-6, and the important role of 22:6n-3 in the central nervous system (2).

Phospholipids and their fatty acids are continuously turned over as part of cell signaling pathways, leading to release of unesterified 20:4n-6 and 20:5n-3 for further metabolism to eicosanoids (2), and unesterified 22:6n-3 for further metabolism to resolvins and docosanoids (47). Eicosanoids have multiple roles, among which are the initiation, potentiation, and termination of inflammatory pathways, as well as platelet aggregation and vessel wall constriction and permeability (48). In many of these pathways, metabolites derived from 20:4n-6 are pro-inflammatory, while those derived from 20:5n-3 are either weakly-inflammatory or anti-inflammatory; however, the lipoxins derived from 20:4n-6 are important in the termination of inflammatory responses. Eicosapentaenoic acid (20:5n-3) is high in circulating blood cells, but is low in the brain, and is reported to be much lower than 20:4n-6 (49). Recently, a series of metabolites from 22:6n-3, termed resolvins and docosanoids, were identified and shown to have important anti-inflammatory actions in neural cells (47). The release of 20:4n-6, 20:5n-3, and 22:6n-3 from cell membrane phospholipids is important under conditions of injury or disease.

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Under pathological states, the activities of phospholipase enzymes that regulate fatty acid cleavage from phospholipids are elevated (50). In addition, cyclooxygenase and lipoxygenase enzymes, induced in eicosanoid production, are elevated during injury (50). Due to their important roles in the body, and because the n-3 fatty acids are essential dietary nutrients, a substantial amount of research has recently been dedicated to understanding the relationship between the n-3 and n-6 fatty acid intakes, eicosanoids, inflammation and human health.

1.5 n-3 Fatty Acid Intakes and Disease

Epidemiological studies, beginning in the 1970's, revealed interesting trends in disease incidence between populations that consumed high amounts of long chain n-3 fatty acids compared to those that consumed low amounts of these fatty acids. The population in Greenland, following a marine diet high in n-3 fatty acids from seal and other marine mammals and fish, were found to have substantially lower than expected rates of myocardial infarction and coronary heart disease (51, 52). However, the incidence of cerebral hemorrhage was substantially higher than expected in the Greenland population (52); estimates on the expected incidence of each type of disease were based on numbers from a predominantly Danish European population.

In regards to coronary health, recent epidemiological studies have reported a lower coronary heart disease rate among men who ate some fish weekly, as well as a lower relative risk of death from coronary heart disease of 0.62, and a relative risk of non-sudden death from myocardial infarction of 0.33 among men who ate at least 35 g of fish per day (about 2 servings/week) when compared to men who ate no fish (53). The Nurses' Health Study reported an inverse relationship between fish intake and between n-3 fatty acids and death from coronary heart disease among women (54) . Similarly, Zhang et al. (55) reported an association across 36

countries between higher fish consumption and lower rates of ischemic heart disease mortality. In clinical intervention studies involving fish or fish oil, the lower mortality from cardiovascular disease appears to be due to lower sudden death (56). Based on epidemiological and clinical studies to indicate that long chain n-3 fatty acids are associated with a decreased risk of cardiovascular disease mortality, particularly sudden death, many expert bodies have recommended 1-2 meals of fish per week as part of a healthy diet (53, 57, 58).

The relationship between dietary n-3 fatty acids and stroke incidence and mortality is more complicated, owing to the presence of two subtypes of stroke; both occur from the cessation of blood flow in the brain, but ischemic stroke results from the occlusion of an artery and hemorrhagic stroke results from the rupture of a blood vessel. Epidemiological studies in this field suggest that long chain n-3 fatty acids have different effects on these 2 subsets of stroke, having a positive association with hemorrhagic stroke and an inverse association with ischemic stroke (52, 59). As introduced above, the early epidemiological study comparing the population of Greenland to a predominantly Danish European population found a higher than expected incidence of cerebral hemorrhage in Greenlanders. Interestingly, other studies have noted that aneurysms and hemorrhages were much more frequent than myocardial infarctions in native Alaskans (60), and a high bleeding tendency in Greenland natives was noted as far back as the 15th century (61). In contrast to the apparent susceptibility to hemorrhage in populations consuming high amounts of n-3 fatty acids, a meta-analysis of cohort studies recently reported that regular fish intake may be inversely associated with ischemic stroke incidence (62). A recent epidemiological study across 38 countries reported an association between lower morbidity and mortality rates from all causes of stroke among countries that had higher intakes of long chain n-3 fatty acids (17); the authors suggested that higher stroke morbidity and

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mortality rates, seen among some countries, may be partially attributable to low intakes of long chain n-3 fatty acids.

1.6 Long Chain n-3 Fatty Acids and Cerebral Ischemia in Animal Studies

The observed beneficial effects of long chain n-3 fatty acids on cardiovascular disease has stimulated interest in the roles these fatty acids have in other aspects of disease. The ability of dietary n-3 fatty acids to modulate tissue fatty acid compositions (2), including their tendency to replace n-6 fatty acids, has brought interest to their possible roles in pathological conditions, where cell membrane destruction leads to a higher than normal level of fatty acids in their free form.

In this regard, several studies have examined the effects of high dietary amounts of fish oil or 22:6n-3 alone on the extent of damage that occurs following cerebral ischemia in animals. A stroke causes an area of damage referred to as an infarct, which is an outcome measured in several of these studies. To date, most animal studies assessing the effects of dietary 20:5n-3 and 22:6n-3 in models of cerebral ischemia have reported evidence to show a reduced infarct volume in animals fed these fatty acids for 3-10 weeks prior to cerebral ischemia (7-11). Black et al. (7) reported that cats fed a diet of cat chow supplemented with an additional 8% of energy as menhaden fish oil for approximately 3 weeks had a significantly smaller average infarct volume after middle cerebral artery occlusion (MCAO) than cats fed standard chow without fish oil.   It must be noted that cats have either no or very low Δ6 desaturase activity and depend on a dietary source of preformed 20:5n-3 and 22:6n-3, as well as 20:4n-6 (63, 64). The species is an obligate carnivore, and also differs from humans in requiring the terminal products of the transulphuration pathway, such as taurine. Relton and colleagues (8) reported a 36% lower infarct volume when a diet containing fish oil (0.9% 20:5n-3 and 0.6% 22:6n-3 by weight of the

diet) was fed to rats for 6 weeks prior to permanent MCAO compared to rats fed a diet with 6% olive oil. Similarly, rats fed a diet containing 14% by weight menhaden fish oil for 6 weeks had significantly smaller infarct volumes when measured after MCAO followed by 24 hrs of reperfusion than in rats fed standard chow (10). Rats fed chow supplemented with 300 mg/kg/day 22:6n-3 for 6 weeks had smaller infarct volumes when compared to rats fed chow only, in a model that involved middle cerebral artery thrombosis, using rose bengal and green light irradiation to induce cerebral ischemia (9). The latter study also administered tissue plasminogen activator 30 minutes after irradiation and found that regardless of whether or not the middle cerebral artery was reopened, infarct volume was smaller in rats treated with 22:6n-3. In other studies, gerbils treated with 200 mg/kg/day of the ethyl-ester of 22:6n-3 by gavage for 10 weeks, in addition to feeding pellets, had a 35% lower infarct volume after bilateral common carotid artery occlusion for 30 minutes, followed by reperfusion, than that observed in animals treated with vehicle alone (11). Beneficial outcomes that have been reported in conjunction with feeding a diet containing high amounts fish oil or following supplementation with ethyl esters of 22:6n-3 that led to a reduction in infarct volume included better gait score and righting reflex (7), and reduced cerebral edema and mortality (11). Marcheselli et al. (65) also reported that infusing 22:6n-3 or 10, 17 *S*-docosatriene (a metabolite of 22:6n-3), immediately following cerebral ischemia, reduced the infarct volume in mice. At the time that the studies described in this thesis were designed and commenced, only one publication contradicted the reduction in infarct volume associated with fish oil or 22:6n-3. This study involved rats fed a diet containing 20% energy as Norwegian cod liver oil for 7 weeks, with the finding that infarct volume, after transient occlusion of both common carotid arteries and the right middle cerebral artery, was higher than in rats fed chow alone (66). However, more recently, Skryzypki et al. (67) reported that rats fed 0.6% 22:6n-3 did not show improvement, and even had tendencies toward larger

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infarct volumes after focal cerebral ischemia. A summary of studies published to date

concerning dietary n-3 fatty acids and infarct volume following cerebral ischemia is provided in

Table 1-3.

Table 1-3. Comparison of published studies assessing dietary n-3 fatty acids and infarct volume.

Reference	Diets	Duration of Feeding	Species	Cerebral Ischemia Model	Infarct Volume	Notes
Black et al. 1979 (7)	Control: cat chow Experimental: cat chow plus 8% energy menhaden fish oil	18-24 day, adult cats, age not specified	Cat	MCAO, permanent	Smaller in experimental group, measured 3 days after	1 animal in the experimental group died of hypotensive shock
Relton et al. 1993 (8)	Control: semisynthetic diet with 6% fat, by weight, from olive oil Experimental: semisynthetic diet with 0.9% 20:5n-3 and 0.6% 22:6n-3 from fish oil, by weight	6 weeks, from weaning	Rat, Sprague Dawley	MCAO, permanent	36% smaller in experimental group, 24 hr after	
Lai et al.1993 (66)	Control: rat chow Experimental: cod liver oil added at 20% energy to rat chow	7 weeks, from weaning	Rat, strain not specified	Focal ischemia (temporary occlusion of MCA and both CCAs for 45 min)	Larger in experimental group, 24 hr after	

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Reference	Diets	Duration of Feeding	Species	Cerebral Ischemia Model	Infarct Volume	Notes
Umemura at al. 1995 (9)	Control: rat chow Experimental: 300 mg/kg/day 22:6n-3 added to rat chow	8 weeks, from 6 weeks of age	Rat, Wistar	Middle cerebral artery thrombosis model (rose green light)	Smaller in experimental group, measured 24 hr after	
Choi-Kwon et al. 2004 (10)	Control: AIN 93G diet Experimental: AIN 93G diet plus 14% menhaden fish oil	6 weeks, from weaning	Rat, Sprague-Dawley	MCAO, 2 hr	Smaller in experimental group, 24 hr after	
Cao et al. 2007 (11)	Control: vehicle Experimental: 200 mg/kg/day ethyl ester 22:6n-3 by gavage	10 weeks, from weaning	Gerbil	Global ischemia, occlusion of both CCAs	35% smaller in experimental group, 24 hr after	

The mechanism by which the long chain n-3 fatty acids, 20:5n-3 and 22:6n-3 or 22:6n-3 alone may reduce infarct volume during cerebral ischemia is not fully understood, but several mechanisms have been suggested. Because high dietary intakes of fish oil are often associated with decreased tissue 20:4n-6 (due to inhibition of 18:2n-6 desaturation and elongation to 20:4n-6), one possible mechanism could involve lower amounts of 20:4n-6 in brain lipids, potentially leading to reduced production of 20:4n-6-derived metabolites that might contribute to tissue damage (68, 69). Eicosapentaenoic acid (20:5n-3) competes with 20:4n-6 for cyclooxygenase and lipoxygenase enzymes for the production of prostaglandin and leukotriene synthesis (70), although 20:5n-3 levels are low in the brain even in animals fed high 20:5n-3 fish oils. The positive results found in animals given ethyl esters of 22:6n-3, or after infusing 22:6n-3 or 10, 17 *S*-docosatriene into the brain, however, suggest that the protective effect on infarct size involves 22:6n-3, rather than 20:5n-3. Although 20:5n-3 levels are low in the brain, some studies have reported that 22:6n-3 is important in regulating brain 20:4n-6 and 20:4n-6-derived eicosanoid production (11, 65, 66).

With respect to the potential benefits of 20:5n-3 plus 22:6n-3 or 22:6n-3 alone, it is important to note that the amounts added to the diet or given in supplements to animals in the studies described above were well above the intakes found in any known human populations including Greenland Inuit. Furthermore, populations with the highest known intakes of long chain n-3 fatty acids that have a lower incidence of some diseases, such as myocardial infarction, also have higher incidences of other conditions, such as cerebral hemorrhage (52). In many, if not most nations, dietary intakes of n-3 fatty acids are low, not high, and this occurs for 18:3n-3, and 20:5n-3 and 22:6n-3; furthermore, the low intakes of n-3 fatty acids often occurs in combination with high intakes of n-6 fatty acids (71). It is known that dietary deficiency of n-3 fatty acids decreases the amount of 22:6n-3 in the brain, with a compensatory increase in n-6

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fatty acids, particularly 22:5n-6, and that this is associated with impaired neurogenesis, altered neurotransmitter metabolism, and altered gene expression during early development (43). The question of whether low dietary n-3 fatty acid intakes lower brain 22:6n-3, which increases the brain n-6:n-3 fatty acid ratio, possibly worsening the extent of brain damage during cerebral ischemia, has not been addressed.

1.7 Primary Research Hypotheses

1)   Feeding a diet deficient in n-3 fatty acids for 5 weeks to young adult rats will reduce the levels of 22:6n-3 in brain phospholipids by at least 20%.

2)   Feeding a diet deficient in n-3 fatty acids will increase brain damage, when assessed as infarct volume, when compared to a diet providing adequate n-3 fatty acids, or containing high amounts of 22:6n-3**.**

1.8 Rationale

One objective was to alter the levels of 22:6n-3 in brain solely by diet to an extent that is considered comparable to the effects of dietary fatty acids on the human brain. Decreasing dietary 22:6n-3 by 20% in rats was considered an acceptable strategy because autopsy studies of brain tissue from infants fed formula containing high 18:2n-6 and low 18:3n-3 with no 22:6n-3 have reported approximately 30% lower brain 22:6n-3 than in the brains of infants fed human milk (72). Previous studies have reported that increasing 22:6n-3 protects the brain from cerebral ischemia damage. Therefore, in addition to an n-3 fatty acid deficient diet, a diet containing supplemental 22:6n-3 was included, therefore, further increasing the range of brain 22:6n-3 levels in which to seek an association between 22:6n-3 and outcome following cerebral ischemia.

1. 9　Thesis Objectives

This thesis had 2 objectives

A) To establish a model of cerebral ischemia/reperfusion in the rat at the Child and Family Research Institute.

B) To address the potential importance of dietary n-3 fatty acid deficiency on brain fatty acids, and determine whether or not dietary induced changes in brain fatty acids alters the extent of damage following stroke using the MCAO model.

1. 10　Subsidiary Objective

Following cerebral ischemia/reperfusion studies, an apparent, but not statistically significant, higher incidence of cerebral hemorrhage was found in rats fed the diet containing supplemental 22:6n-3. Additional exploratory studies were included to address whether or not altered platelet fatty acids and function could contribute to an increased susceptibility to cerebral hemorrhages in rats fed the diet containing supplemental 22:6n-3.

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CHAPTER 2: AN N-3 FATTY ACID DEFICIENT DIET DOES NOT WORSEN INFARCT VOLUME AND A DIET HIGH IN DOCOSAHEXAENOIC ACID DOES NOT IMPROVE INFARCT VOLUME AFTER CEREBRAL ISCHEMIA IN RATS[1]

2.1 Introduction

High concentrations of the n-3 fatty acid 22:6n-3 in phosphoglycerides, particularly PE and PS, is a specific feature of mammalian brain gray matter membranes (1, 2). Mammalian cells lack the enzymes necessary to form n-3 fatty acids and therefore rely on a dietary source of n-3 fatty acids for the 22:6n-3 needed for brain membranes (3). The 22:6n-3 for incorporation into brain phosphoglycerides can be derived from endogenous synthesis by desaturation and elongation of 18:3n-3 provided in the diet, or from a dietary source of preformed 22:6n-3 (3). The n-3 fatty acid 18:3n-3 is abundant in some vegetable oils, such as canola, soybean and flaxseeds, but is very low, <1 g 18:3n-3/100 g fatty acids, in most common vegetable and hydrogenated oils (4). DHA (22:6n-3) is present only in animal tissue lipids, with the richest dietary source being fatty fish, which is also high in 20:5n-3. The n-6 fatty acid 18:2n-6, which is present in high amounts in many vegetable oils, and represents 90% of the polyunsaturated fatty acids in modern western diets (5-7), competes with 18:3n-3 for the desaturase enzymes, and itself is further metabolized to 20:4n-6 (3, 8).

Epidemiological studies have shown an association between higher intakes of 20:5n-3 and 22:6n-3 from fish and other marine foods and decreased mortality from myocardial infarction (9) and all causes of stroke (10). In this regard, several studies have reported that dietary

supplementation with fish oil or 22:6n-3 at doses of 200-300 mg/kg/day 22:6n-3 can reduce the extent of brain damage in animal models of cerebral ischemia (11-17).

However, rather than consuming diets high in n-3 fatty acids, most western diets are high in 18:2n-6 and low in 18:3n-3 with recent estimates indicating that the median intake of 18:2n-6 and 18:3n-3 are about 17 g/day and 1.6 g/day ; this represents 5.5% and 0.6% of energy, respectively (5). In addition, the intakes of preformed 20:5n-3 and 22:6n-3 are also low, with average intakes of 100-200 mg/day 20:5n-3 plus 22:6n-3, equivalent to less than 0.05% of total energy (5). Thus, dietary intakes in humans are in the same range as that given per kg to animals in studies linking 20:5n-3 and 22:6n-3 to protection against brain damage in cerebral ischemia. On the other hand, the important question of whether or not low n-3 fatty acid intakes, sufficient to lower brain 22:6n-3, increases the extent of brain damage during cerebral ischemia has not been addressed. The present studies, therefore, sought to determine if short-term dietary deficiency of n-3 fatty acids decreases brain 22:6n-3 and increases infarct volume after cerebral ischemia/reperfusion, and if supplementation of a diet high in 18:2n-6, deficient in 18:3n-3, but with 22:6n-3 confers protection. To achieve these objectives, rats were fed a diet high in 18:3n-3 (control), deficient in n-3 fatty acids, or the same diet deficient in n-3 fatty acids with supplemental 22:6n-3 for 5 weeks from weaning, then ischemia outcome was addressed after transient MCAO.

2.2 Materials and Methods

2.2.1 Animals and diets

Male Sprague Dawley rats (Charles River Laboratories, Saint-Constant, QC, Canada), 21 days of age, were housed in a humidity and temperature controlled animal facility with a 12 hr:12hr light:dark cycle. The animals were randomly assigned to one of three semi-synthetic diets providing 16.5% of energy from fat, and identical in all nutrients except for fatty acids (18). The diets were provided fresh daily, and were fed ad libitum for approximately 5 weeks. The diets contained per kg, 70 g oil, 200 g vitamin-free casein, 200 g sucrose, 400 g cornstarch, 50 g non-nutritive cellulose, 10 g multi-vitamin mix (Teklad, AIN 93-VX), and 35 g multimineral mix (Teklad, AIN 93G-MX), with an additional 150 μg SeO_2, 1 g choline chloride, and 3 g L-methionine (18). Because the goal was to determine if changes in brain n-3 and n-6 fatty acids influence cerebral ischemia/reperfusion infarct volume, the diets prepared included a control diet with a low 18:2n-6/18:3n-3 ratio to provide as a percent of energy, 3.2% 18:2n-6 and 1.1% 18:3n-3 using canola oil as the fat source; an n-3 fatty acid deficient diet with 12.4% 18:2n-6 and 0.05% 18:3n-3 from safflower oil; and a 22:6n-3 diet prepared by inclusion of 22:6n-3 from single cell triglycerides (Martek Biosciences, Columbia, MD, U.S.A) in the n-3 fatty acid deficient diet, which resulted in a diet providing 0.5% 22:6n-3, 11.5% 18:2n-6, and 0.05% 18:3n-3. Because the total fat content was constant, the three diets also differed in 18:1n-9, with 10.2, 2.4, and 2.4% energy from 18:1n-9 in the control, n-3 fatty acid deficient, and high DHA diets, respectively. All procedures involving animals were approved and carried out in accordance with the animal care committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care Guidelines. A copy of the animal care certificate for this study is located in appendix 1.

2.2.2 Middle cerebral artery occlusion (MCAO) surgical procedure

After feeding the assigned diet for approximately 5 weeks, and at a body weight of 280-340 g, MCAO was performed following the procedures of Longa et al. (19), and Shih et al. (20), with slight modification. Briefly, after an overnight fast, the rats were anesthetized with 5% isoflurane carried by O_2 (1 lb/min), and when adequate anesthesia was reached, the isoflurane flow was decreased to 2.5% and O_2 decreased to 0.5 lb/min. A ventral neck incision was made, and the left external carotid artery ligated and cauterized above the occipital branch using a heat coagulator (Fine Science Tools, North Vancouver, BC, Canada). Branches from the external and internal carotid arteries were cauterized just above the bifurcation, and microvascular aneurysm clips placed on the left common and internal carotid arteries. A small incision was made in the external carotid artery stump and a 3 cm long, 3-0 monosof nylon filament (Assoc. Veterinary Purchasing, Abbotsford, BC, Canada) with a heat blunted end then advanced through the incision into the external carotid artery and advanced through the internal carotid artery to occlude the origin of the middle cerebral artery. A diagram of the occluded artery and surrounding arteries in this procedure can be found in appendix 2.1. Body temperature was maintained between 36.5 and 37.5°C using a feedback regulated temperature controlled heating pad (FHC Inc., Bowdoin, ME, U.S.A.). The incision was then sutured, and the animal returned to room air. After 90 min (occlusion), the rat was re-anesthetized and the filament removed to allow reperfusion. Buprenorphine (0.01 mg/kg) was administered as an analgesic immediately after closing the incision. Sensorimotor testing was done on all animals, immediately before removing the filament to confirm proper placement and occlusion of the artery. Animals who became hypothermic at any time, defined as a temperature < 36°C determined by rectal probe, had a sensorimotor score <10, cerebral hemorrhage evident on brain dissection, or no visible

infarct were not included in analysis of infarct volume, edema, or sensorimotor score. Detailed information on equipment used in this project can be found in appendix 2.2.

2.2.3 Sensorimotor testing

The sensorimotor testing followed published procedures (20-22) and involved 6 tests, scored from 0 to 2, with 0 indicating no deficit, 1 moderate deficit, and 2 a severe deficit in performance, for a maximum potential score of 12. The tests used measured the function of the contralateral (right) side of the body and were: contralateral thorax twisting, forelimb flexion, forelimb bracing against a table edge, resistance to lateral push, table edge sensation, and forelimb placing on a table edge. Sensorimotor testing was repeated twice for each animal, immediately prior to reperfusion and again 24 hr after reperfusion. A score of 10 or higher prior to reperfusion was taken as the index of occlusion, and animals with a score <10 were removed from the study.

2.2.4 Infarct measurement

The rats were euthanized 24 hr after MCAO under isoflurane anesthesia, exsanguinated, and the brains rapidly removed using a rongeur to remove the skull, and sliced into 2 mm coronal sections using a brain matrix (Plastics One, Roanoke, VA). The first, third, fifth, and seventh sections were stained with 2% triphenyl-tetrazolium chloride (TTC) in PBS for 30 min, then fixed in 4% paraformaldehyde for 24 hr (23, 24). Images of the front and back surfaces of each brain section were recorded using a digital camera, and the infarct areas measured using NIH Image J version 1.36b. Infarct volumes in mm^3 were calculated as the sum of the products of the infarct area of the section and the distance between sections. The edema volume was calculated as the difference between the volume of the contralateral hemisphere and the ipsilateral hemisphere.

2.2.5 Brain fatty acids

For analysis of brain phospholipid fatty acids, brains from rats fed the diets not subjected to MCAO, but fed for an equivalent duration, were rapidly dissected into frontal cortex and subcortex (including caudate putamen, corpus callosum, and frontal portion of hippocampus). These analyses provided the ability to establish the extent of alteration in fatty acids in different brain phospholipids caused by feeding the diets in the present studies for 5 weeks from weaning. Previous studies in the field relating diet to ischemia/reperfusion injury have reported brain total lipid fatty acids in animals fed diets with fish oil or supplemented with 22:6n-3 (14, 16, 25). To enable comparison of results from the present study to other published studies, brain total lipid fatty acids were also analyzed. For analyses of total fatty acid, the undamaged hemisphere, collected 24 h after MCAO, was used. All tissue samples were flash frozen in liquid nitrogen, and then stored at -70°C until analysis. For analysis, total lipids were extracted, and the fatty acids directly methylated, or PS, PE, phosphatidylcholine (PC), and phosphatidylinositol (PI) separated, quantified by high performance liquid chromatography (HPLC), and recovered, and their fatty acids then converted to their respective methyl esters, and separated and quantified by gas liquid chromatography (GLC) (18, 26-28). Detailed methods are included in appendix 2.3.

2.2.6 Platelet aggregation and lipid analysis

To address the possibility that diet-induced alterations in platelet fatty acids might contribute to cerebral hemorrhage, analysis of platelet fatty acids and tests of *ex vivo* platelet aggregation were conducted. These tests were done on animals that did not undergo MCAO surgery. Blood was collected by cardiac puncture into tubes containing 3.8% sodium citrate (1.5 v:v) under isoflurane anesthesia, centrifuged 5 min at 4°C and 300 x g, and the platelets recovered by centrifugation at 500 x g for 15 min, after addition of 20% sodium citrate solution.

Platelets were then frozen at -70°C until analysis. For analysis of fatty acids, the platelets were

thawed in cold water, and the fatty acids converted to methyl esters for separation and

quantification by GLC (18, 26, 27, 29, 30). Platelet number was determined on aliquots of

whole blood using an automated blood counter (Toa-Sysmex, Los Alamitos, CA) in the

Division of Hematopathology, B.C. Children's Hospital. Platelet aggregation, *ex vivo*, was

determined using a PFA-100 platelet function analyzer with collagen/ADP test cartridges (Dade

Behring, Mississauga, ON, Canada), following the manufacturer's instructions and as described

previously (29, 30). Detailed methods are included in appendix 2.4-2.6.

2.2.7 Statistical analyses

Data were analyzed using the Statistical Package for Social Sciences (SPSS) for Windows,

version 15.0. Results are given as means ± SEM, unless otherwise stated. Means of the three

groups were compared using one way ANOVA with Fisher's protected least significant

difference for post-hoc analysis. Chi-square was used to determine whether the number of rats

that had cerebral hemorrhages was different among diet groups. Differences with $P < 0.05$ were

considered statistically significant.

2.3 Results

2.3.1 Characteristics of animals

In order to ensure a rigorous approach to the MCAO procedure, male rats were fed their assigned diet until a body weight of 280-340 g was achieved. Body weight at the time of MCAO did not differ among the groups; 313 ± 3.6 g (n=14), 314 ± 4.8 g (n=13), and 319 ± 3.0 g (n=14), ($P > 0.05$) for the control, n-3 fatty acid deficient, and high DHA groups, respectively. The duration of feeding the diets was 35.4 ± 0.9, 34.4 ± 0.8, and 36.1 ± 0.6 days for the control, n-3 fatty acid deficient and high DHA groups, respectively, ($P > 0.05$). A number (4/14, 3/13, and 2/14 in the control, n-3 fatty acid deficient, and high DHA groups, respectively) of rats subjected to MCAO had either no visible infarct or a sensorimotor score <10, this is likely explained by the nylon filament not fully blocking the middle cerebral artery; these animals were excluded from the studies of infarct and edema volume. Cerebral (subarachnoid) hemorrhages were present in 3/14, 3/13, and 7/14 rats in the control, n-3 fatty acid deficient, and high DHA groups, respectively. To address the effects of the diets on the fatty acid composition of the brain phospholipids, without possible influences of the MCAO procedure, a further 6 rats for each diet group were fed for the same duration.

2.3.2 Effects of dietary fatty acids on brain fatty acids

The subcortical region represents the core of the infarct in MCAO and is the initial site of cell death where damage is irreversible, whereas the cortex represents a large part of the penumbra, the area of moderate ischemic damage surrounding the area of severe ischemia, which is considered to be potentially salvageable (31). Phospholipid fatty acids have been reported to differ among different regions of the brain (32). Therefore, phospholipid fatty acids were analyzed in both the cortex and subcortex of the rats fed the diet for 5 weeks with the

results shown in **Fig. 2-1**. Rats fed the n-3 fatty acid deficient diet had levels of 22:6n-3 in cortex and subcortex PE, PS, PC and PI that were 21-28% lower than in rats fed the control diet ($P < 0.05$). Conversely, levels of 22:5n-6, in both the cortex and subcortex, were 445-842% higher in rats fed the n-3 fatty acid deficient diet than those fed the control diet, ($P < 0.05$). These results also show significantly higher levels of 20:4n-6 in cortex PE and PI, and subcortex PE and PS in n-3 fatty acid deficient compared to the control diet group ($P < 0.05$). In contrast, the levels of 22:6n-3 were higher in PE and PC of the subcortex, but not cortex, of rats fed the high DHA compared to control diet. Arachidonic acid (20:4n-6) was higher in PI in the cortex and subcortex, and in PC in subcortex, and 22:5n-6 was higher in PC in the cortex and subcortex in rats fed the high DHA diet when compared to those fed the control diet ($P < 0.05$).

The results of brain total lipid fatty acid analysis are shown in **Table 2-1**. When compared to rats fed the control diet, rats fed the n-3 fatty acid deficient diet had lower levels of 22:6n-3, and higher levels of 18:2n-6, 20:4n-6, 22:4n-6 and 22:5n-6 in brain total fatty acids ($P < 0.05$). The brain lipid 20:4n-6/22:6n-3 ratio was 0.71 ± 0.01 and 0.94 ± 0.01 ($P < 0.05$) in the control and n-3 fatty acid deficient group, respectively. The levels of saturated fatty acids were not different, but the levels of 16:1(n-9) and 18:1(n-9) were both lower in brain total fatty acids of rats fed the n-3 fatty acid deficient than in those fed the control diet ($P < 0.05$). Rats fed the high DHA diet had higher levels of 18:3n-3 and 22:6n-3, 18:2n-6, 20:4n-6 and 22:4n-6, but lower levels of 20:5n-3, 16:1n-9 and 18:1n-9 in brain total fatty acids than in rats fed the control diet. The brain lipid 20:4n-6/22:6n-3 ratio, however, was not different between rats fed the control and those fed the high DHA diet 0.71 ± 0.01 and 0.68 ± 0.01 respectively, ($P > 0.05$). Rats fed the high DHA diet, in comparison to those fed the n-3 fatty acid deficient diet, had higher 18:3n-3, 22:6n-3, 18:2n-6, and 20:3n-6, but lower 20:5n-3, 22:4n-6, and 22:5n-6.
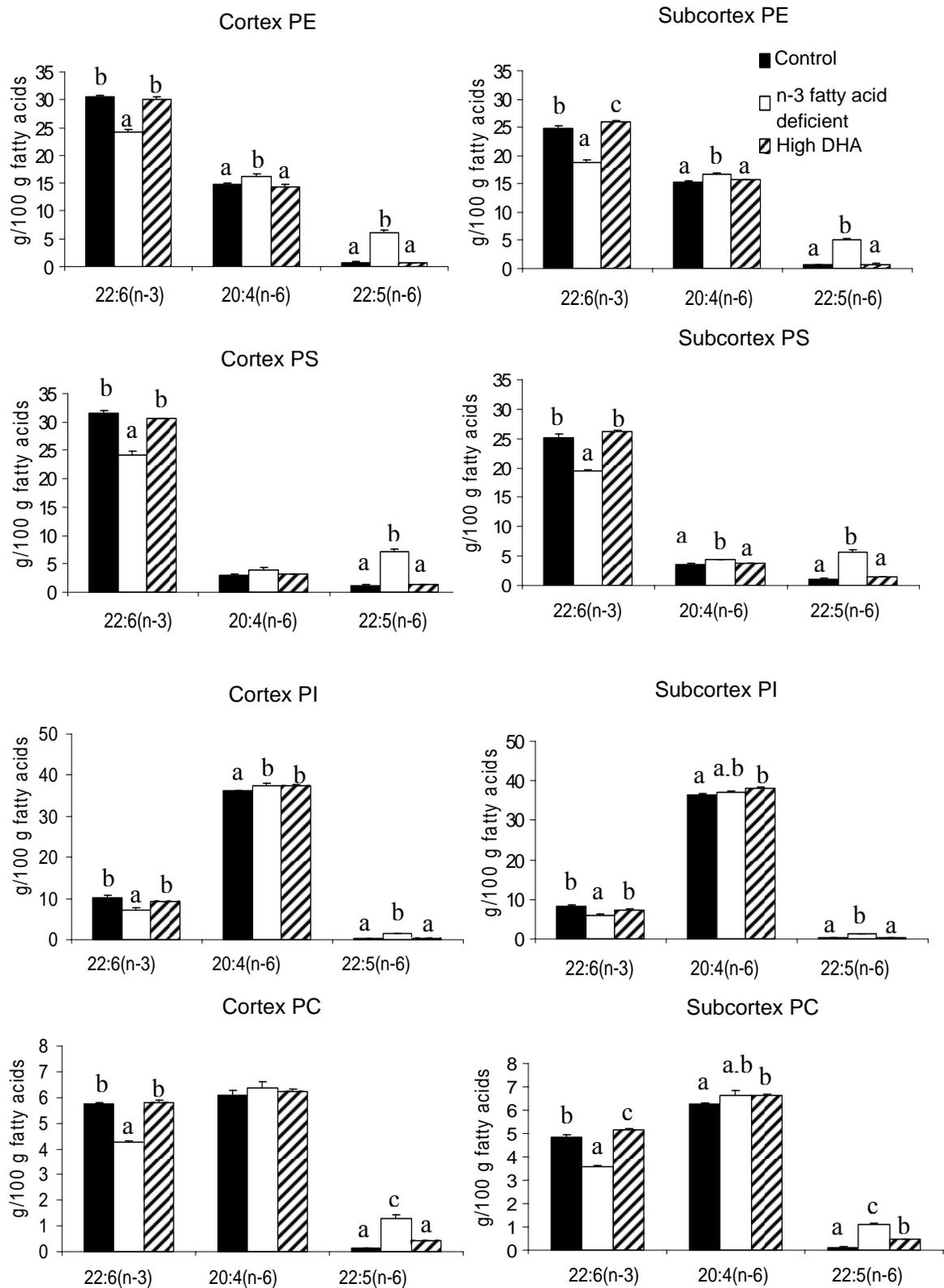
Fig. 2-1 Major fatty acid components in brain cortex and subcortex of rats fed a control, n-3 fatty acid deficient, or high DHA diet for 5 weeks from weaning. Bars are means +SEM, n=5 group. Means with a different subscript are different, $P < 0.05$; a,b,c.

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Table 2-1. Major fatty acid components in brain of rats fed diets varying in n-3 and n-6 fatty acids for 5 weeks[1]

	Control	n-3 Deficient	High DHA
Fatty Acid		*g/100g fatty acid*	
18:3n-3	0.01 ± 0.01^{a}	0.01 ± 0.01^{a}	0.05 ± 0.00^{b}
20:5n-3	0.12 ± 0.02^{a}	0.09 ± 0.02^{a}	0.03 ± 0.00^{b}
22:6n-3	14.4 ± 0.47^{b}	12.0 ± 0.16^{a}	16.1 ± 0.09^{c}
18:2n-6	0.57 ± 0.02^{a}	0.83 ± 0.03^{b}	0.97 ± 0.04^{c}
18:3n-6	0.05 ± 0.00^{b}	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}
20:3n-6	0.51 ± 0.02^{a}	0.48 ± 0.01^{a}	0.58 ± 0.02^{b}
20:4n-6	10.1 ± 0.27^{a}	11.3 ± 0.13^{b}	10.9 ± 0.08^{b}
22:4n-6	3.20 ± 0.08^{a}	4.10 ± 0.07^{c}	3.60 ± 0.04^{b}
22:5n-6	0.52 ± 0.01^{a}	3.40 ± 0.21^{b}	0.59 ± 0.03^{a}
16:0	19.7 ± 0.29	19.6 ± 0.13	19.3 ± 0.14
18:0	21.4 ± 0.30	21.3 ± 0.09	21.1 ± 0.03
16:1(n-9)	0.28 ± 0.01^{b}	0.24 ± 0.00^{a}	0.24 ± 0.00^{a}
18:1(n-9)	19.8 ± 0.44^{b}	17.8 ± 0.15^{a}	17.7 ± 0.14^{a}

[1] Values are means ± SEM, with n = 9, 8, and 8 for the control, n-3 fatty acid deficient, and high DHA diets groups, respectively. Means in the same row with different superscripts are significantly different, $P < 0.05$, by ANOVA.

2.3.3 Sensorimotor scores

Sensorimotor function, when tested immediately prior to or 24 hr after reperfusion, was not different among rats fed the 3 diets, with scores of 10.4 ± 0.29, 10.4 ± 0.20, 10.4 ± 0.24 prior to, and 7.4 ± 1.3, 8.0 ± 0.44, 9.4 ± 0.6 at 24 hr after reperfusion in the control, n-3 fatty acid deficient and high DHA groups, respectively ($P > 0.05$).

2.3.4 Infarct volume and edema volume

The infarct volumes when assessed by TTC staining 24 hr after MCAO were 203 ± 35.5 (n=7), 221 ± 34.8, (n=7), and 218 ± 38.9 mm^3 (n=5) for the control, n-3 fatty acid deficient and high DHA diet groups, respectively, ($P > 0.05$), (**Fig 2-3**). Note that infarct volume was not assessed on rats showing cerebral hemorrhages. Although the mean edema volume appeared higher in the high DHA group, variability was also high in this group of animals, and no statistically significant differences were present among the groups, 43.4 ± 8.1, 50.6 ± 9.8, 67.0 ± 27.3 mm^3 for the control, n-3 fatty acid deficient, and high DHA groups, respectively.
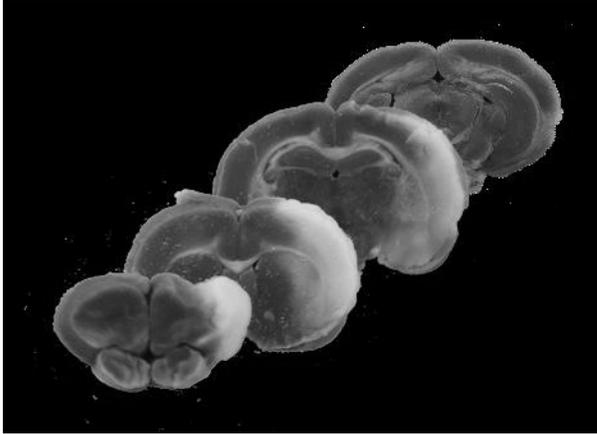
Fig 2-2 Representative TTC stained brain for infarct volume analysis. The brains were sliced into 2 mm coronal sections using a brain matrix, and the 1st, 3rd, 5th, and 7th sections stained with 2% TTC for 30 minutes. Brain slices were photographed, and then infarct areas were measured with NIH image J version 1.36b. Infarct volumes were calculated as the sum of the products of the infarct area of the section and the distance between sections.
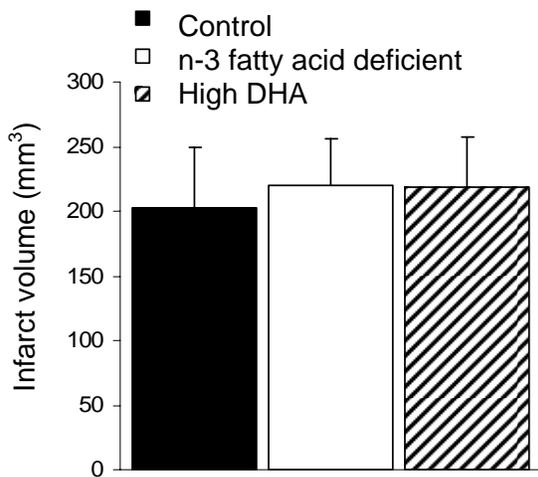
Fig 2-3 Infarct volume (mm^3) in animals, 24 hr after MCAO, which were fed either a control, n=7, n-3 fatty acid deficient, n=7, or high DHA diet, n=5, from weaning to 8 weeks of age.

2.3.5 Incidence of cerebral hemorrhage

A higher number of rats with cerebral hemorrhages was observed in the high DHA diet group compared with the other 2 diet groups. However, using chi-square for analysis of the data, the incidence of cerebral hemorrhage did not differ significantly by diet group, $\chi^2(2, N = 41) = 0.193$. It is important to note some of the diet groups had less than 5 animals with a hemorrhage, indicating that this study methodology was underpowered to detect a significant difference in hemorrhage among the 3 diet groups. There is no statistical significance using chi-square regarding the observation that more rats in the high DHA group having cerebral hemorrhages compared with the other 2 groups. However, an increased incidence of cerebral hemorrhage does occur in populations with high intakes of fish (9), therefore this observation warrants further exploration.

2.3.6 Platelet fatty acids and ex vivo aggregation

The platelet levels of the n-3 fatty acids, 18:3n-3, 20:5n-3 and 22:6n-3 were all lower, and 18:2n-6, 22:4n-6 and 22:5n-6 were higher in rats fed the n-3 fatty acid deficient diet when compared to the control diet ($P < 0.05$), (**Table 2-2**). Rats fed the high DHA diet, on the other hand, had higher levels of 22:6n-3, 18:2n-6 and 20:4n-6, but lower 18:3n-3 and 20:5n-3 in platelet lipids than in the control group ($P < 0.05$). The platelet lipid 20:4n-6/20:5n-3 ratios in rats fed the n-3 fatty acid deficient or high DHA diet were higher than in rats fed the control diet, 151 ± 21.2, 172 ± 53.6, and 25.3 ± 4.4, respectively, ($P < 0.05$). The platelet lipid 20:4n-6/22:6n-3 ratio, on the other hand, was lower in rats fed the high DHA diet, but higher in rats fed the n-3 fatty acid deficient diet than in the control group (5.0 ± 0.67, 272 ± 2.4, 7.9 ± 0.63, respectively, $P < 0.05$). The 20:4n-6/20:5n-3+22:6n-3 ratio was significantly higher in the n-3

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fatty acid deficient than control and high DHA diet groups (24.6 ± 1.9, 5.9 ± 0.6, 4.8 ± 0.7, respectively, $P < 0.05$).

There were no significant differences in platelet number among rats fed the control, n-3 fatty acid deficient, and high DHA diets (data not shown). In an *ex vivo* assay that measures the time required for blood to clot while flowing through a collagen/ADP aperture, the closure times in seconds were 112 ± 11.9 (n=5), 120 ± 10.5 (n=5) and 78.8 ± 5.5 (n=5) for rats fed the control, n-3 fatty acid deficient, and high DHA diets, respectively, with the closure time for the high DHA group shorter, not longer than for the control group ($P < 0.05$).

Table 2-2. Major fatty acid components in platelets of rats fed diets varying in n-3 and n-6 fatty acids for 5 weeks[1].

Fatty Acid	Control	n-3 Deficient	High DHA
		g/100g fatty acid	
18:3n-3	0.41 ± 0.06^b	0.09 ± 0.03^a	0.10 ± 0.05^a
20:5n-3	0.84 ± 0.10^b	0.08 ± 0.03^a	0.19 ± 0.05^a
22:6n-3	2.50 ± 0.15^b	0.81 ± 0.06^a	4.90 ± 0.62^c
18:2n-6	7.10 ± 0.59^a	12.7 ± 2.10^b	14.3 ± 1.60^b
20:4n-6	19.1 ± 0.82^a	$21.5 \pm 1.10^{a,b}$	22.8 ± 0.50^b
22:4n-6	1.80 ± 0.15^a	3.30 ± 0.35^b	2.10 ± 0.53^a
22:5n-6	0.17 ± 0.04^a	1.60 ± 0.27^b	0.29 ± 0.03^a

[1] Values given are means ± SEM, with n=6, 6, and 5 for the control, n-3 fatty acid deficient, and high DHA diet, respectively. Means in the same row with different superscripts are significantly different, $P < 0.05$, by ANOVA.

2.4 Discussion

In the present study, rats were fed diets differing in n-3 and n-6 fatty acids, at the extremes of levels expected in human diets for 5 weeks from weaning, and had altered brain levels of 20:4n-6, 20:5n-3, 22:5n-6 and 22:6n-3. However, using MCAO as a model of cerebral ischemia-induced injury, there was no evidence that the diet-induced differences in brain fatty acids were associated with worsening of the infarct size or extent of edema in animals fed an n-3 fatty acid deficient diet, nor was there evidence of benefit in animals fed the 22:6n-3 supplemented diet.

Whereas the immature brain is generally considered most vulnerable to the effects of unbalanced or inadequate dietary intakes of n-6 and n-3 fatty acids (33, 34), epidemiological studies have drawn attention to a relationship between a decreased intake of fish-derived n-3 fatty acids and increased risk of all causes of stroke (10), as well as aging-related cognitive decline in adults (35-37). The present study demonstrates a decrease by 17% in 22:6n-3 with a substantially higher (550%) 22:5n-6 in brain fatty acids of rats fed an n-3 fatty acid deficient diet compared to control diet for only 5 weeks from weaning. Stable isotope tracer studies have estimated the half-life of 22:6n-3 from adult rat brain phospholipids is 33 days, which increased to 90 days in rats fed an n-3 fatty acid deficient diet for 15 weeks (38). These results show that the more mature brain is plastic to changes in fatty acid composition when a diet high in 18:2n-6 and deficient in n-3 fatty acids is fed. On the other hand, feeding 0.5% energy 22:6n-3 led to an increase of 25% in 22:6n-3, with no increase in 22:5n-6 as found in rats fed the same diet without added 22:6n-3, this being the n-3 fatty acid deficient diet. The results show that rats fed the high DHA diet had lower levels of 22:4n-6 and 22:5n-6 in brain total fatty acids than rats fed the n-3 fatty acid deficient diet. This suggests inhibition of elongation after 20:4n-6 and/or inhibition of Δ6 desaturation at the level of 24:4n-6 to 24:5n-6 by 22:6n-3. The n-3 fatty acid

deficient and high DHA diets provided 12.4 and 11.5% dietary energy from 18:2n-6, respectively, with both diets providing about 0.05% energy from 18:3n-3. The analyses of the brain total fatty acids show higher 18:2n-6, 20:4n-6, and 22:4n-6 in the high DHA group than in rats fed the control diet with 3.2% and 1.1% energy from 18:2n-6 and 18:3n-3. This suggests, however, that feeding a diet high in 18:2n-6 and low in 18:3n-3 results in an increased accumulation of some n-6 fatty acids in the brain, regardless of the addition of 22:6n-3. Epidemiological associations have shown a relationship between habitual diets low in 20:5n-3 and 22:6n-3 from fish, and increased risk of stroke and neurological problems (10, 35-37). The present study provides experimental results to suggest that associated differences in disease could involve differences in tissue n-6 as well as n-3 fatty acids.

This was the first study to assess the effects of n-3 and n-6 fatty acids, over a range of dietary intakes, on the extent of damage after cerebral ischemia. The present study shows that diets ranging in n-3 and n-6 fatty acids, when fed for 5 weeks, results in brain total fatty acid levels of 22:6n-3 that differed between 11 and 25%, and levels of 20:4n-6 that differed between 3 and 10 %. Epidemiological studies indicate that the incidence of morbidity and mortality from stroke is higher among populations consuming diets that are low rather than high in n-3 fatty acids (10); this has, in part, stimulated interest in the possible protective role of n-3 fatty acids in animal models of stroke. At the outset of this study, a number of studies had reported that high dietary long chain n-3 fatty acids reduced the extent of damage caused by cerebral ischemia in animal models; only one study reported larger infarct volumes in rats fed a high fish oil diet. Despite the differences in brain fatty acid levels induced by diets in this study, rats fed these diets did not have statistically significant differences in infarct volumes after cerebral ischemia. Rather, the findings of the current study, and two other publications that have found long chain n-3 fatty acids to have detrimental effects, raise the question of whether or not high levels of n-3

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fatty acids present during cerebral ischemia are safe. Lai et al. (40) reported a 25% larger infarct volume, 24 hr after MCAO, in rats fed chow supplemented with 20% energy from fish oil when compared to rats not given fish oil. In another model, involving intraperitoneal injection of 22:6n-3, 60 min after transient focal cerebral ischemia, there was a dose-dependent increase in cerebral infarct volume in rats (41). However, there are a number of reports that fish oil reduces the extent of damage caused by cerebral ischemia in animals. Relton et al. (12) reported that rats fed a semisynthetic diet with 0.9% 20:5n-3 and 0.6% 22:6n-3/ kg diet from fish oil for 6 weeks had smaller infarct volumes than rats fed a control diet with 6% olive oil/kg diet. Differing from the diets in this study, olive oil is a monounsaturated oil with low amounts of 18:2n-6. In cats, the mean infarct volume was lower 3 days after MCAO in animals fed cat chow with 8% energy fish oil rather than no fish oil (11). Also different from the current study, the activity of $\Delta 6$ desaturase activity is very low in cats (42, 43), and cat chow provides 20:4n-6. Similarly, Choi-Kwon et al. (14) reported smaller infarct volumes 24 hr after 2 hr of MCAO in rats fed a diet with 14% fish oil rather than chow for 6 weeks from weaning. Recently, one laboratory reported that rats given 400mg/kg/day Marincap fish oil for 14 days by gavage had significantly fewer apoptotic neurons in both the hippocampus and prefrontal cortex after cerebral ischemia injury, induced by 45 min of occlusion of both common carotid arteries and hypotension followed by 30 min of reperfusion, compared to controls given saline (44, 45). In addition to dietary supplementation with fish oils, the potential neuroprotective effect of 22:6n-3 has been addressed through administration of ethyl esters of 22:6n-3 by gavage or infusion into the brain. Using an ethyl ester of 22:6n-3 at 300 mg/kg/day in the chow diet for 8 weeks, cerebral lesions in rats receiving 22:6n-3 were lower than in those not receiving it, after 24 hr (13). DHA (22:6n-3), at 200 mg/kg/day given perorally for 3 weeks was also associated with less histological damage in the hippocampus, 8 days after transient forebrain ischemia than in

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rats not given 22:6n-3 (16). Similarly, Cao et al. (15) reported that 200 mg/kg/day ethyl esters

of 22:6n-3 given by gavage for 10 weeks was associated with a 35% lower infarct volume in

gerbils when measured 24 hr after transient global ischemia. Together, the latter studies suggest

that 22:6n-3, rather than, or in addition to 20:5n-3, has neuroprotective effects in decreasing

damage in animal models of cerebral ischemia. Consistent with this, 10,17 *S*-docosatriene, a

metabolite of 22:6n-3, given infused into the brain for 48 hr immediately following cerebral

ischemia was associated with a lower mean infarct volume in mice (46). However, the current

study and that by Lai et al. (40) and Yang et al. (41), outlined above, suggest that 22:6n-3 does

not necessarily have any beneficial effects and can also have detrimental effects on cerebral

ischemia outcome. Understanding the reason for the discrepancy amongst those studies

reporting positive and negative effects of fish oil or ethyl esters of 22:6n-3 in animal models of

cerebral ischemia is complicated by the use of different species, diets, doses and methods of

dietary administration, as well as surgical procedures. Comparison of the reported effects of

fish oil or 22:6n-3 supplementation on brain levels of 22:6n-3, 20:4n-6, or the 20:4n-6/22:6n-3

ratio also offers no clear reason why n-3 fatty acids offer protection in some studies (11-14, 16,

46), adverse effects in others (40, 41), and in the present study had no significant effect on

infarct size or edema volume following cerebral ischemia.

Although it is tempting to speculate that the high DHA diet might have made the rats fed

this diet more susceptible to cerebral hemorrhage in this study, chi-square analysis did not

reveal any statistically significant difference in the incidence of cerebral hemorrhage among the

diet groups. However, this study was neither designed nor powered to test the hypothesis of an

increased susceptibility to hemorrhage among rats fed different diets. Design of an experiment

to properly test this hypothesis may be warranted, due to the epidemiological data to suggest

that populations with high intakes of fish have a higher than expected rate of cerebral hemorrhage (9).

Because some studies have reported that platelet fatty acids and aggregation are altered with high DHA intakes (47-50), experiments were conducted to explore the possibility that platelet fatty acids or aggregation might be altered in the high DHA diet group, which could possibly provide supporting evidence to the observation of more animals with hemorrhage in this diet group. Analyses of platelet fatty acids from rats fed the diets showed the anticipated diet-dependent increase in platelet 22:6n-3 in rats fed the 22:6n-3 supplemented diet, although the 20:4n-6 levels were higher, not lower than in rats fed the control diet. Further, the *ex vivo* platelet aggregation tests found no evidence of decreased platelet aggregation in rats fed the high DHA compared to control or n-3 fatty acid deficient diet.

In summary, although low intakes of n-3 fatty acids have been associated with an increased risk of all causes of stroke (10), in the current study there was no evidence that a diet deficient in n-3 fatty acids increased the infarct volume after cerebral ischemia in the rat, although brain levels of 22:6n-3 were decreased. In addition, feeding a diet supplemented with 22:6n-3 was not associated with a reduction in infarct volume following cerebral ischemia. Further studies are required to address the possible role of dietary fatty acids and particularly long chain n-3 fatty acids in influencing outcome in animal models of cerebral ischemia; this includes the potential influence of the concurrent dietary intake of n-6 fatty acids, presence or absence of 20:5n-3 and 20:4n-6 in the diet, and with hemorrhage as well as infarct volume and potential for later recovery as outcomes.

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CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

3.1General Discussion and Conclusion:

The present studies addresses whether dietary n-3 fatty acid deficiency, or supplementation of an n-3 fatty acid deficient diet with 22:6n-3 alters brain n-3, and n-6 fatty acids, and whether this is associated with changes in infarct volume when measured 24 hrs after MCAO.

3.2 Influence of Diet on Brain Fatty Acid Composition

The first hypothesis, that an n-3 fatty acid deficient diet when fed to rats for 5 weeks from weaning would lower the level of 22:6n-3 in brain phospholipids by at least 20% when compared to rats fed an n-3 fatty acid adequate (control) diet was proven. In previous studies using similar diets, dams fed an n-3 fatty acid deficient diet throughout gestation produced offspring that, at embryonic day 19, had 55-65% lower 22:6n-3 in brain phospholipids (1). When fed from weaning, n-3 fatty acid deprivation for 15 weeks resulted in 27-43% lower levels of 22:6n-3 in brain phospholipids in rats (2). Very little information is available to relate dietary n-3 fatty acid restriction to brain fatty acids in humans. However, fatty acid analysis of autopsy samples from infants fed formula with high 18:2n-6 and low 18:3n-3 and no 22:6n-3 or 20:5n-3 reported levels of 22:6n-3 that were approximately 30% lower than in the brains of infants who were breastfed before death (3, 4). Current human diets, particularly those in many Western countries are believed to be low in all n-3 fatty acids, and to contain an abundance of n-6 fatty acids (5). The replacement of 0.5% energy from safflower oil with 22:6n-3 from single cell triglycerides resulted in significantly higher levels of 22:6n-3 in brain phospholipids of rats fed the high DHA compared to the n-3 fatty acid deficient diet. The presence of 22:6n-3 in the diet also prevented the accumulation of high levels 22:5n-6 that were present in brain phospholipids of rats fed the n-3 fatty acid deficient diet, and which are a characteristic feature

of n-3 fatty acid deficiency. Several recent studies have linked increased risk of aging-related cognitive decline and incidence of dementia to low dietary intakes of n-3 fatty acids (6-8), raising the question of whether or not low dietary intakes of n-3 fatty acids alter brain fatty acids, with consequences for neurological function after infancy.

3.3 Influence of Dietary n-3 Fatty Acids on Cerebral Ischemia/Reperfusion Outcome

The second hypothesis, that rats fed an n-3 fatty acid deficient diet would have a larger infarct volume 24 hrs after MCAO when compared to rats fed the control diet or diet high in 22:6n-3 was rejected. The edema volume was also not significantly different among the groups, and no differences in the sensorimotor deficits were detected 24 hrs after the MCAO procedure.

The absence of a beneficial effect of the high DHA diet on infarct volume, compared to the control diet is consistent with some other studies that have found 22:6n-3, either added separately or through fish oil, does not benefit stroke outcome. Lai et al. (9) reported larger infarct volumes after temporary focal ischemia in rats fed a diet containing 20% of energy as fish oil for 7 weeks from weaning when compared to rats fed chow. Similarly, Skrzypski et al. (10) found a tendency toward larger infarct volumes in rats fed a diet containing 0.6% energy as 22:6n-3 when focal cerebral ischemia was induced. In another study, Yang et al. (11) reported that intraperitoneal injection of 22:6n-3 given 60 min after transient focal ischemia was associated with a dose-dependent increase in cerebral infarct volume in rats. However, other reports described in Chapter 2 have reported smaller infarct volumes and beneficial outcomes in animals given dietary fish oil or 22:6n-3 (12-18).

Comparison of currently published studies, to address potential reasons for the discrepancy, revealed no clear explanation when considering factors such as the species of animal used, the

model of cerebral ischemia, the duration of feeding, or the amount of fish oil or 22:6n-3 given.

The findings in the present study, together with those from other laboratories show no benefit or

larger infarct volumes after cerebral ischemia in animals given 22:6n-3 or fish oil. This

emphasizes the need for further research to consider the possibility of adverse effects,

particularly using models of diets and supplementation paradigms relevant for human

populations.

Recent studies have suggested that a limiting amount of n-3 fatty acids may be detrimental

to the brain's ability to create new synapses during pathological states, such as stroke and

Alzheimer's disease (19, 20). Whether or not the decrease of 20% in brain membrane

phospholipid 22:6n-3 in rats fed the n-3 fatty acid deficient diet might have any relevance to

later recovery following cerebral ischemia was not addressed in this research, but may be

important. DHA (22:6n-3) can be further metabolized to 10-17 *S*-docosatriene, which is

produced during ischemia/reperfusion injury (21). When infused into the brain, 10-17 *S*-

docosatriene may reduce the extent of injury through inhibition of inflammatory and apoptotic

processes (21). Whether the brain, following injury, can produce 10-17 *S*-docosatriene

endogenously and in amounts sufficient to afford neuroprotection is not known (21). However,

the brain pool of 22:6n-3 is very large, while available evidence indicates 10-17 *S*-docosatriene

production is quantitatively very low (21). Whether dietary deficiency of n-3 fatty acids,

leading to decreased brain 22:6n-3, can alter 10-17 *S*-docosatriene production remains to be

elucidated.

3.4 Cerebral Hemorrhages in Relation to Dietary n-3 Fatty Acid Intakes

The model of MCAO used in the current study is known to sometimes result in cerebral (subarachnoid) hemorrhage. The advancement of a nylon suture through the internal carotid artery to block the middle cerebral artery can result in damage to these arteries which might cause them to rupture. In reviewing published studies using this procedure, many have not reported an incidence of cerebral hemorrhage, however, Shimamura et al. (22) and Yang et al. (23) reported incidences of 16.7% and 20%, respectively. In the current study, the incidences of cerebral hemorrhage were 23.1% and 21.4 in the n-3 fatty acid deficient and control groups, respectively, and 50% in the high DHA diet group. Notably, the assessment of hemorrhage was not an intended outcome of the current study. There was an apparent, but not statistically significant increase using the chi-square, in the incidence of cerebral hemorrhage in the high DHA diet group compared to the other 2 groups. Possible explanations for this observation include the possibility that the DHA feeding increased susceptibility to hemorrhage or that there was an incidental difference in technique during surgery.

There are several risk factors and causes for cerebral hemorrhage in human populations. Causes of cerebral hemorrhage include chronic hypertension, vascular malformations, anticoagulant or antiplatelet therapy, and loss of vascular integrity, among others (24). Platelet fatty acids and aggregation were assessed to determine whether or not rats in the high DHA diet group had characteristics, such as an increased 20:5n-3+22:6n-3/20:4n-6 ratio in platelet fatty acids or decreased platelet aggregation which could increase their susceptibility to bleeding. None of these characteristics were found in rats fed the high DHA diet. Although not measured in this study, it seems unlikely that elevated blood pressure could be involved because there is no evidence that 22:6n-3 increases blood pressure; rather 22:6n-3 is associated with lower blood

pressure in both humans and rats (25, 26). Very little information is available to address

whether or not 22:6n-3 influences vascular integrity. One study reported that treatment of

neutrophils with 22:6n-3 in vitro, increased endothelial detachment (27), and this was suggested

to reflect potential increased susceptibility of endothelial cells to injury. At this time, there is a

paucity of information on the effects of high DHA intakes on vascular tissues.

Alternatively, rather than a cause and affect association between dietary DHA and

cerebral hemorrhage, it is possible that the apparent increased incidence of hemorrhage in the

high DHA group occurred by chance. As is clear from published data, other laboratories

performing this model of MCAO report that about 16-20% of animals experience cerebral

hemorrhage (22), (23). Although it seems unlikely that the apparent higher number of

hemorrhages in the high DHA group occurred by chance observation, the low number of

animals does make a chance observation possible. Two approaches are possible for further

exploring the question of whether or not supplementation with high amounts of 22:6n-3

increases the risk of cerebral hemorrhage; first, the present study could be repeated with

sufficient power to detect a two-fold increase in the incidence of hemorrhage, based on the

present findings. Alternatively, it may be more appropriate, if an increased incidence of

cerebral hemorrhage is of interest, to design a study using an appropriate animal model to enable

this to be rigorously tested and mechanisms explored. A possible example to assess vessel

fragility is a variation is the Rumple-Leede test used in rats (28).

To summarize, further studies will be required to determine whether or not rats fed high

22:6n-3 diets are more susceptible to cerebral hemorrhage than rats fed control diets without

22:6n-3. Consideration of this possibility is important because of the epidemiological reports of

higher incidences of cerebral hemorrhage in populations with high intakes of n-3 fatty acids

from fish and marine animals than among populations with habitual low intakes of the same

foods (29), and the current trends to use high dose fish oil supplementation for primary and secondary prevention of cardiovascular disease (30), as well as widespread addition of 22:6n-3 and fish oils to many foods.

3.5 Conclusion

In conclusion, although lower stroke mortality rates have been reported in populations who consume higher amounts of long chain n-3 fatty acids, the present study found no evidence that a diet deficient in n-3 fatty acids leads to a greater extent of brain damage, or that a diet high in 22:6n-3 leads to a smaller amount of damage after cerebral ischemia/reperfusion in the rat, when measured 24 hrs after the injury. However, the possibility that important differences in recovery may emerge with a longer-term study have not been addressed. The potential higher rate of cerebral hemorrhage in rats fed a high DHA diet requires further research to confirm this observation, elucidate possible mechanisms, and better understand the relevance to long chain n-3 fatty acid supplementation in human populations, particularly because increased bleeding among individuals consuming high amounts of fish is acknowledged as a concern.

3.6 Strengths and Limitations

The current study addressed the effects of diets differing in n-3 fatty acids on infarct volume following ischemic stroke. A limitation in this field of research is the variability in the approaches to provide n-3 fatty acids to animals, including addition to the diet, gavage, infusion into the brain, and intraperitoneal injection. Although addition to the diet may seem simplest, fatty acids also provide energy, thus addition of fat to a complete diet increases the energy content per kg and thus decreases the protein, vitamin, and mineral content per kg. Many studies have compared diets that have different amounts of fat; a common approach was adding fish oil to a stock diet and comparing it to a control diet not containing fish oil. A major strength of the current study was that a rigid dietary design was used in which the energy densities, protein, and vitamin and mineral content were constant, and only the composition of fatty acids was altered. Three diets were included to enable comparison of the effects of n-3 fatty acid deficiency, n-3 fatty acid adequacy, and high amounts of 22:6n-3 on ischemic stroke outcome. An unavoidable limitation is that in order to keep total fat constant, as the amount of one fatty acid is changed, another must also change. Thus, the levels of 18:1n-9 and 18:2n-6 also differed between the 3 diets, since fat was supplied by canola oil in the control group and safflower oil in the n-3 fatty acid deficient and high DHA groups. It would have been possible to limit the difference between the levels of n-6 fatty acids in the control and n-3 fatty acid deficient diets by using high oleic acid safflower oil (77% 18:1n-9, 14% 18:2n-6) rather than high linoleic acid safflower oil (13% 18:1n-9, 78% 18:2n-6). However, since no difference in the size of infarct volume was found at 24 hrs after the procedure, the difference in 18:1n-9 and 18:2n-6 between the control and n-3 fatty acid deficient diets would not likely be an important modifier of the outcome.

The difference in fatty acid composition of the diets fed, and the 5 week feeding duration was successful in altering the levels of 22:6n-3 in brain phospholipids by 20% between the control and n-3 fatty acid deficient diet groups. This relatively large difference in brain phospholipid 22:6n-3, induced solely by feeding diets of different fatty acid composition, adds strength to a conclusion that within the context of usual human diets, diet-dependent differences in brain fatty acids are not likely to influence the extent of injury in the early period following stroke. However, some caution is warranted. First, the analyses included total brain lipid fatty acids and the phospholipid fatty acid composition of the four major species, PC, PE, PI, and PS. The analyses, however, did not include specific cells such as neurons, astrocytes, or capillary endothelial cells. It is possible that the effects of the diet differed across cell types and after 5 weeks, insufficient incorporation or depletion of 22:6n-3 from cell types that might influence the extent of the infarct volume size was not achieved.

Several different animal models of ischemic stroke have been described in the literature. The animal model of cerebral ischemia used in the present research is considered to be among the most relevant to human ischemic stroke (31), and is similar to models used in several other studies assessing the impact of n-3 fatty acids on cerebral ischemia (9, 12, 13, 16). The use of an accepted model of ischemic stroke that has also been used by others investigating the effect of n-3 fatty acids on stroke outcome was a strength of the present study, and it also enabled comparison between the results of the current study and published studies. Regardless of the consistency among studies in terms of the animal model used, both positive and negative effects of n-3 fatty acids on cerebral ischemia outcome have been reported when similar protocols have been used (9, 10, 12, 13, 16).

A limitation of the present study was that outcome was assessed 24 hrs after MCAO, but recovery at later time points was not part of the objective. TTC staining was performed at 24

hrs. This is considered optimal since the size of the infarct does not change significantly after this point, for at least 14 days after MCAO (32). However, recovery at later time points, including improvements in behavioural outcomes may or may not necessarily correlate with the extent of damage measured soon after occlusion. Assessment of longer-term recovery including behavioural testing was considered, but was not included because of the large number of animals that would be required for the procedure, and the absence of any adverse effect of n-3 fatty acid deficiency which was the major focus of this research.

In the present study, the number of animals assigned to each dietary group was relatively large. However, although the careful exclusion of animals that did not have an ischemic stroke was a strength, it also resulted in a smaller number of animals that could be assessed for infarct volume. This raises the possibility that a low sample size might have led to the inability to detect important effects of the diets in modifying infarct size. However, this seems unlikely because the results showed no evidence of any trend towards altered infarct volume. Furthermore, rats expected to have negligible infarct volumes due to insufficient occlusion of the middle cerebral artery were identified by a sensorimotor score of less than 10. If n-3 fatty acids protected the brain by reducing the infarct volume size, then more animals in the control and 22:6n-3 groups might have had a sensorimotor score less than 10 than among those in the n-3 fatty acid deficient group; this was not the case. Rats that had a cerebral hemorrhage were identified and also excluded from analysis. Inclusion of measures of the infarct volume from these groups could have influenced the results, but the interpretation of such results is questionable. A further strength of this study is that the model used and experimental approach enabled the observation that cerebral hemorrhage may be increased in rats fed a diet high in 22:6n-3. Although the mechanism is currently unknown, human populations habitually

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consuming diets high in long chain n-3 fatty acids do have an increased incidence of cerebral hemorrhage (29).

The current study successfully tested the effect of whether or not differences in 22:6n-3 in brain lipids, achieved by feeding an n-3 fatty acid deficient diet compared to an adequate or enriched diet, influences infarct volume in rats subjected to cerebral ischemia/reperfusion. Although there are many aspects of damage and recovery to consider in cerebral ischemia, the results show that using infarct volume as the outcome measure, there is no worsening of infarct volume when brain 22:6n-3 is altered by 20% in brain phospholipids by dietary means.

3.7 Future Directions

1   Assess histological damage beyond 24 hrs to determine whether the diets varying in n-3 fatty acids impact the extent of damage or recovery at later time points.

2   Assess behavioural outcomes, post cerebral ischemia, using tests of sensorimotor function such as the horizontal ladder, or staircase to determine whether the diets affect such outcomes.

3   Explore the possibility of an increased incidence of cerebral hemorrhage with high DHA feeding, using either sufficient power with the current model, or using an animal model which would assess vessel fragility.

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APPENDICES

A ppendix 1: UBC Ethics Board Certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0450

Investigator or Course Director: Sheila M. Innis

Department: Paediatrics

Animals: Rats Sprague Dawley 106

Start Date: September 26, 2006   **Approval Date:**   November 9, 2006

Funding Sources:

Funding Agency: Bristol-Myers Squibb, US (Princeton)

Funding Title: Unrestricted Nutrition Research Grant

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

Appendix 2: Supplemental Methods

Appendix 2.1 Diagram of MCAO
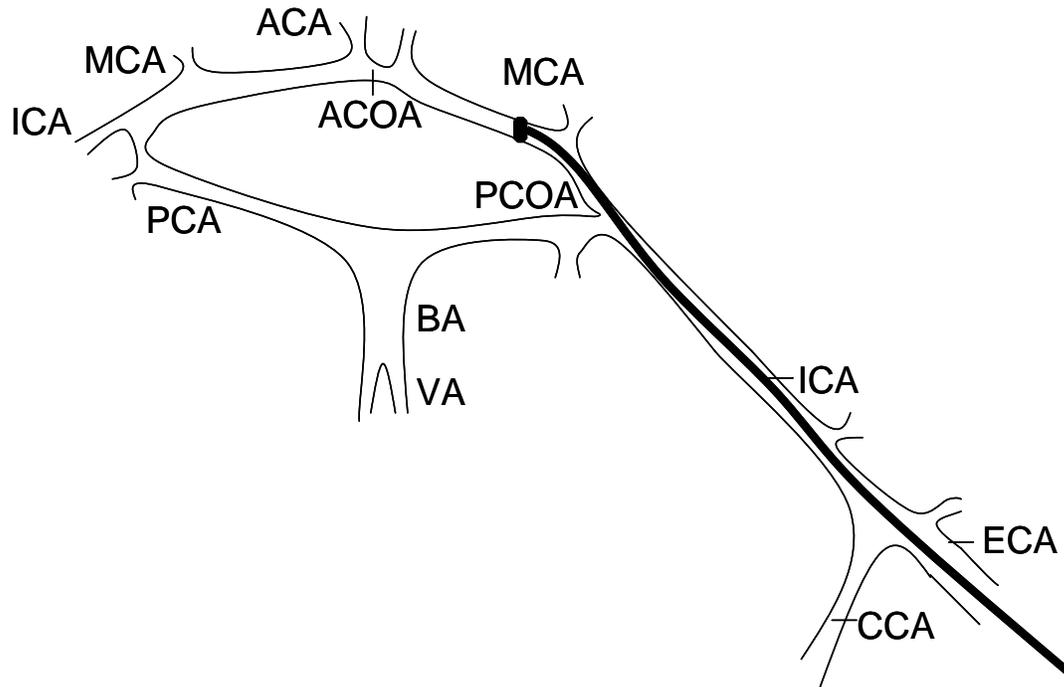
Diagram of filament placement during MCAO in the rat. ACA, anterior cerebral artery; ACOA, anterior communicating artery; BA, basilar artery; CCA, common carotid artery; ECA, external carotid artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; PCOA, posterior communicating artery; VA, vertebral artery. Adapted from (1).

Appendix 2.2 Equipment

Centrifugation was performed using a Sorvall Biofuge Stratus centrifuge with swinging basket rotor (Mandel Scientific, Guelph, ON, Canada). Fatty acid methyl esters were separated and quantified by GLC with a 6850 gas chromatograph from Agilent Technologies (Mississauga, ON, Canada), equipped with flame ionization detection and an IBC computer system with GC chemostation software (Agilent Technologies). Glass capillary columns (30.3 m x 250 um) were from Supelco Canada Ltd. (Oakville, ON). Individual phospholipids (PE, PS, PC, PI) were separated using HPLC with a Waters 2695 Alliance HPLC, (Milford MA), equipped with an auto-sampler and column heater. The column was a Waters YMC-Pack Diol 120NP, 25 cm x 4.6 mm id, 5 μm particle size and 12 nm pore size. The column eluant was split using an Alltech (model 2000) evaporative light scattering detector (ELSD) from Mandel Scientific and a Gilson FC204 fraction collector (Mandel Scientific). Surgery was performed using a Nikon SMZ645 dissecting microscope (Mississauga, ON, Canada), and a Fiber-Lite MI-150 illuminator (Lawrence, MA, U.S.A.). Sterilization of surgical instruments was by autoclave or using a Steri 350 bead sterilizer (Inotech, Rockville, MD, U.S.A.).

Appendix 2.3 Analysis of Brain Fatty Acids

Lipid Extraction

Total lipids were extracted according to the Folch method (2), with minor modifications. Brain tissue (100-150 mg) was added to 17 volumes of saline, and sonicated with a sonic dismembrator, model 500 (Fisher Scientific). Fatty acids were extracted with solvent proportions of chloroform:methanol:saline, 6:3:2.25 (v/v/v) . The samples were centrifuged and the resulting bottom organic layer was filtered into a test tube, and the aqueous layer transferred to a new tube and 6 ml chloroform added. The aqueous layer and chloroform were centrifuged,

and the resulting aqueous layer discarded, and the 2nd organic layer filtered into the test tube with the original organic layer. Samples were dried under nitrogen gas and for phospholipid separation by HPLC, and reconstituted in methanol:chloroform:hexane:acetone, 3:2:0.5:0.5 (v/v/v/v). For fatty acids analyzed in their total fraction, (17:0, heptadecanoic acid) was added prior to methylation as an internal standard. Betulin (50 ul of 1 mg/ml) was added as an internal standard prior to quantification by HPLC for phospholipid fatty acid analysis.

Separation of phospholipid classes by high performance liquid chromatography (HPLC)

Individual phospholipids (PE, PS, PC, PI) were separated using a HPLC. The sample chamber was kept at 18°C and the column heater at 35°C. A quaternary solvent system of hexane-petroleum ether, 97:3 (v,v); methanol-triethylamine-acetic acid, 765:15:13 (v/v/v); acetone-triethylamine-acetic acid, 765:15:13 (v/v/v);isopropanol-acetic acid, 800:40 (v/v) in a linear gradient with a flow rate of 2 ml/min was used. The column eluant was split 10:90 to an ELSD and a fraction collector. ELSD detection and quantitation of the separated lipid classes was performed with a nitrogen flow rate of 1.8 ml/min, a drift tube temperature of 60°C, and the impactor OFF. Calibration curves to determine the linear range of the analysis were established using authentic standards for each lipid class, and samples were quantified using the external standard method.

Methylation of samples

Methylation of fatty acids is carried out to hydrolyze complex lipids to free fatty acids and convert the carboxylic groups to methyl esters in preparation for quantification of individual fatty acids by GLC. This procedure neutralizes functional groups on fatty acids that would otherwise react with the GLC columns and creates compounds that are more volatile and more

easily analyzed by GLC. Phospholipid fractions were methylated with 1 ml of 14 % boron

trifluoride in methanol, and total fatty acids were methylated with 0.7 ml 14 % boron trifluoride

in methanol (Pierce Biotechnology, Rockford, IL, U.S.A.) at 110°C for 30 minutes. Methyl

esters were recovered by two extractions with 3 ml saline and 6 ml pentane, and 3 ml saline,

respectively. The pooled pentane layers were dried under nitrogen gas and resuspended in

hexane for GLC analysis.

Analysis of fatty acids by gas-liquid chromatography

Phospholipid fatty acids and total fatty acids were separated and analyzed as their

respective methyl esters by GLC. Separation was carried out with 30.3 m x 250 μm ID**,** 0.20

μm nominal, fused silica polar SP2380 columns (Supelco, Belefronte, PA, U.S.A.). Helium was

used as the carrier gas, at a flow rate of 25 ml/min, inlet pressure of 18.6 pounds per square

inch, and inlet splitter set at 10 to 1. Samples were injected at 80°C. The temperature program

was as follows: samples remained at 80°C for 2 minutes, then increased to 165°C at 5°C/min,

were held for 14 min, then increased to 180°C at 1.5°C/min, were held for 2.5 min, then

increased to 240°C at 20°C/min and held for 20 min. The temperatures of the injectors and

detectors were 240°C and 260°C, respectively. Retention times of fatty acid methyl esters were

compared with those of authentic standards for identification. Fatty acids identified were 18:3,

20:5, 22:5, 22:6 from the n-3 series; 18:2, 18:3, 20:3, 20:4, 22:4, 22:5 from the n-6 series; 16:1,

18:1 from the n-7 series; 14:1, 16:1, 18:1, 20:1, 22:1, 24:1 from the n-9 series; 22:1 from n-11

series; and 8:0, 10:0, 12:0, 14:0, 16:0, 18:0, 20:0, 22:0, 24:0 of the saturated fatty acids. For

computation of fatty acid peak areas, GC Chemstation software (Agilent Technologies) was

used.

Appendix 2.4: Platelet Fatty Acid Analysis

Methylation of platelet total fatty acids

      Platelet samples were thawed in cold water. A 0.1 ml aliquot of platelets and 10 ul of

C17:0 internal standard was methylated with 2 ml benzene:methanol, 1:4 (v/v) following the

method of Lepage and Roy (1), with slight modifications. Samples were vortexed, and 200 ul of

acetyl chloride added before capping tubes and heating at 100°C for 60 minutes. During the

incubation, tubes were vortexed every 15 minutes. Samples were then cooled at 4°C to prevent

loss of medium chain fatty acid methyl esters, which are very volatile and subject to

evaporation. Five ml of 6 % (weight/v) potassium carbonate solution was then added to the

samples for neutralization, and the samples were briefly shaken. Two extractions with 6 ml

pentane were carried out to extract methyl esters. The pooled pentane layers were dried under

nitrogen gas and then resuspended in 200 μl hexane for analysis by GLC.

Analysis of fatty acids by gas-liquid chromatography

      Fatty acids were separated and analyzed as their respective methyl esters by gas liquid

chromatography, as described above for brain phospholipid and total fatty acid samples.

Appendix 2.5 Platelet Aggregation Studies

Original method of blood collection for complete blood counts and platelet function tests

Rats were anesthetized with isoflurane. Samples for CBC were taken by cardiac puncture (500 ul), using a syringe rinsed with EDTA, and put into an EDTA collection tube (supplied by hospital). Blood samples were taken to BC Children's hospital (Vancouver, BC, Canada) for analysis. For platelet aggregation studies, blood was collected by cardiac puncture, using a 3 ml syringe, with 1" 20 G needle, both rinsed with 3.2% sodium citrate. Blood was put immediately into 2 ml 3.2% sodium citrate vaccutainer tubes. An aliquot of 800 ul of blood was added to each test cartridge, and tested in the PFA-100. The results from this initial attempt resulted in closure times of greater than 300 seconds (maximum time) with both the collagen/EPI and collagen/ADP cartridges for all rats tested.

Revised method of blood collection for complete blood counts and platelet function tests

Rats were anesthetized with isoflurane anesthesia. For complete blood counts, a 500 ul blood sample was taken by cardiac puncture, with 20 G needle and 1 ml syringe, rinsed with EDTA. The sample was collected into an EDTA tube and taken to hematology at BC Children's Hospital (Vancouver, BC, Canada) for CBC analysis. For platelet aggregation studies, the method of Dunleavy et al. (3) was followed. Rats were injected intraperitoneally (IP) with heparin (1000 IU/kg). Blood was collected by cardiac puncture, using a 3 ml syringe, with a 1" 20 G needle, both rinsed with heparin (5000 IU/100 ml), and was immediately put into a 3ml heparin/lithium vacutainer tube. Platelet aggregation was assessed with 800 ul of blood, using a platelet function analyzer (PFA-100, Dade sciences), and collagen/ADP and collagen/EPI cartridges.

Appendix 2 Bibliography

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