Journal of Animal and Veterinary Advances 10 (19): 2575-2581, 2011
ISSN: 1680-5593

Effects of Long Term Polyunsaturated Fatty Acids Supplementation on Membrane Lipid Composition and Growth Characteristics in Rats

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Abstract: This study was conducted to investigate the effects of modifying the n-6:n-3 Fatty Acid Ratio (FAR) of diets using fish oil, soybean oil and butter on growth performance and the fatty acid profile of rat fed with commercial concentrate diet. Fifty individually housed Sprague-Dawley rats (average of 130±2 g of body weight) were fed concentrates at 5% of body weight daily. The treatments consisted of dietary n-6:n-3 FAR of 1.94:1, 5.67:1, 51.82:1 and 61.69:1. After feeding for 20 weeks in individual cages, rats were sacrificed 15 h after feeding and samples of blood were collected. Increasing dietary saturated fatty acid increased the body weight significantly in compared to other treatment groups. Concentrations of n-6:n-3 FAR in plasma increased linearly ($p<0.05$) with increasing dietary n-6:n-3 FAR. Concentrations of C18:3 n-3 decreased ($p<0.001$) in the diet with high level of soybean oil whereas that of C18:2n-6 did not change in all treatment groups ($p>0.05$) in rat plasma with different n-6:n-3 FAR. Proportions of oleic acid in plasma were unchanged by diet. The proportion of Arachidonic Acid (AA) decreased ($p<0.05$) in plasma of rat that fed with low level of n-6:n-3 FAR in compare to the diet with higher level. Proportions of all measured long chain n-3 fatty acids were greater in plasma when diets contained more C22:5 n-3 and 22:6 n-3 from fish oil. By decreasing the dietary n-6:n-3 FAR, the proportions of long chain n-3 fatty acids in plasma increased dramatically; specifically, 22:6 n-3 and 22:5 n-3.

Key words: n-6:n-3 ratio, dietary fatty acid, LC-PUFA, soyeabn oil, rats, plasma

INTRODUCTION

A significant percentage of the daily caloric intake in the animal and human is contributed by lipids (Bialostosky *et al*., 2002). Therefore, altering the lipid content and Fatty Acid (FA) composition of foods can be an effective way to improve the animal and human health even more so for essential fatty acids that must be acquired from dietary sources. The different composition of dietary fatty acid strongly reflects the plasma fatty acid profile in tissue (Rajion *et al*., 2001). Animal cells are not able to synthesized by *de novo* pathway, two independent families of Polyunsaturated Fatty Acid (PUFA) which are Linoleic Acid (LA, 18:2 n-6) and α-Linolenic Acid (ALA, 18:3 n-3) (Simopoulos, 2008). Since, animal cells cannot synthesize these two fatty acids in body, the intake of this two fatty acid from diet is necessary. These Essential Fatty Acids (EFA) are precursor of Long Chain Polyunsaturated Fatty Acid (LC-PUFA) (Bezard *et al*., 1994). These two classes of EFA are non-convertible to each others. They often have important opposing physiological functions. The balance of both EFA is important for good health and normal development (Simopoulos, 2006). In the absence of EFA, growth, learning and visual activity are negatively affected (Bezard *et al*., 1994) and the risk for cardiovascular disease is increased (Williams, 2000). LA can be desaturated and elongated to Arachidonic Acid (AA, 20:4 n-6) in animal tissues (Kinsella *et al*., 1990). AA works as the main substrate for eicosanoid synthesis via the Cyclooxygenase (COX) and lipoxygenase pathways (Smith, 1992). Long chain omega-3 (n-3) PUFA is important to tissues such as the brain and retina and may be important in the maintenance of health by protecting against metabolic diseases (Kris-Etherton *et al*., 2002). Dietary ratios of omega-3 (n-3) to omega-6 (n-6)

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Polyunsaturated Fatty Acids (PUFAs) are known to be important to the metabolic syndrome including insulin sensitivity and inflammatory changes. Currently, the consumption of PUFA n-6 had had been emphasized in line with the dietary guidelines promoting the replacement of saturated fats with n-6 rich vegetable oils to lower plasma cholesterol (Kritchevsky, 1998). A typical Western diet typically has a PUFA n-6 to n-3 ratio of 20-30:1, rather far off the recommended limit of 4-5:1 (Krauss *et al*., 2000; Kris-Etherton *et al*., 2002). Many studies have shown that the balance of n-6:n-3 PUFA is very important to prevent many diseases and disorders (Simopoulos, 2008). There is evidence that high intake of PUFA n-6 will lead to development of a range of diseases such as coronary heart disease (Lands, 2005; Hibbeln *et al*., 2006; Okuyama *et al*., 2007; Simopoulos, 2008). Therefore, an increase in the consumption of PUFA n-3, concurrent with a reduction in the consumption of PUFA n-6 is highly recommended. In view that n-3 PUFA and their metabolites are typically anti-inflammatory while that of n-6 PUFA are acknowledged to be pro-inflammatory, the dietary n-3:n-6 ratio should be well balanced (Pischon *et al*., 2003). For balanced production of pro and anti-inflammatory eicosanoids, the ratio of n-6:n-3 PUFA should be between 1:1-6:1 (Gerster, 1998). Therefore, appropriate amounts of dietary n-6 and n-3 PUFA at a ratio of about 1-6 to 1 need to be considered in making dietary recommendations.

This is because PUFA n-6 had an antagonist effect on PUFA n-3. PUFA n-6 can inhibit the formation of Eicosapentaenoic Acid (EPA, 20:5, n-3), Docosahexaenoic Acid (DHA, 22:6, n-3) and other n-3 derived metabolites as well as their incorporation into cell membrane phospholipids (Gerster, 1998). A research finding states that continuous consumption of EPA (20:5, n-3) and DHA (22:6, n-3) will reduce the risk of coronary heart disease (Mozaffarian *et al*., 2005; Mozaffarian and Rimm, 2006).

The correct ratio of n-6:n-3 PUFA obtained is important during embryogenesis and early postnatal stages of development in mammals. The period for AA and DHA accumulation differs depending on the species for rats, it accumulates DHA during the embryonic stage and first 3 postnatal weeks of life (Green and Yavin, 1998). While in humans, it occurs on the last trimester and first 6-10 months after birth (Clandinin *et al*., 1980a, b). The accumulation of DHA correlates with the neurological and visual development (Connor *et al*., 1990; Enslen *et al*., 1991; Carlson and Neuringer, 1999; Moriguchi *et al*., 2000). DHA is also important in normal brain development and function because it is positively correlates with the changes in cognitive and behavioural performance (Innis, 2007; Mccann and Ames, 2005). For EPA, it is found in blood components and it does not accumulate in tissue in great amounts because it is constantly been used in DHA or eicossanoid biosynthesis (Nelson, 2000). This study was designed to determine the long-term effects of dietary supplementation of n-6 and n-3 PUFA in a rat model on growth rate and fatty acid change in the blood plasma.

MATERIALS AND METHODS

Experimental design: This study was conducted using 50 male 12 weeks old Sprague-Dawley rats weighing 275±9.0 g (mean±SE). The 40 rats were randomly selected and assigned into 4 treatment groups (n = 10 group^{-1}). The remaining 10 rats were sacrificed for the baseline blood parameters and tissue samplings after 1 week of adaptation period. This study lasted for 20 weeks inclusive of 1 week of adaptation period. At the end of the feeding trial (20th week), rats were sacrificed and plasma sample were collected. The experiment approved by Institutional Animal Care and Use Committee (IACUC) of Faculty of Veterinary Medicine Universiti Putra Malaysia.

Experimental animals and housing: The male Sprague-Dawley rats (n = 50) were obtained from Universiti Putra Malaysia (UPM) Animal Resource Centre and they were rear in the Laboratory Animal House Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The animals were housed individually in a polycarbonate cage (45×30×30 cm) with wood shavings as bedding (changed every week) and stainless steel mesh cover. Fresh diet was provided at 25 g/animal, once daily in individual bowl and clean water was provided *ad libitum*. Rats were exposed to a 12 h light/dark cycle and maintained at a constant temperature between 23±1°C. The room was well ventilated.

Experiment diet preparation: The initial body weight of each rat was recorded and during the whole experiment the body weight and feed intake of each rat was taken every week. After adaptation period the rats were randomly assigned to dietary treatments. The three treatment diets were prepared by supplementation of 10% w/w butter (Auric Pacific Food Processing Sdn., Bhd.) (BCD), supplementation of 6.67% w/w menhaden oil and 3.33% w/w soybean oil and supplementation of 3.33% w/w menhaden oil (Sigma Aldrich, Inc, Missouri USA) (MCD) and 6.67% w/w soybean oil (Unilever Malaysia

Holdings, Malaysia) (SCD) to standard rat chow diet (Table 1), respectively. The final treatment group was the control group which contains the standard rat chow diet without addition of treatment oils. Treatment diets were prepared daily. All the treatment oils were stored under refrigerated condition.

Sample collection: At the end of week 20th of dietary intervention, rats were fasted overnight. Blood sampled from terminal intracardiac were collected using 10 mL plastic syringe with 25 gauge needle with injection of Xylazine and Ketamine anaesthesia. The 5 mL of blood was collected in EDTA coated vacutainer tubes and centrifuged at 1000 G for 10 min to separate the plasma. The plasma was then stored at -80°C for fatty acid profile determination.

Fatty acid extraction for treatment oils and plasma: The total fatty acids were extracted from 0.5 g of feeds and 2 mL of plasma based on the method of Folch *et al.* (1957) with modifications described by Karami *et al.* (2011) using chloroform; methanol 2:1 (v/v) containing butylated hydroxytoluene to prevent oxidation during sample preparation. The extracted fatty acids were transmethylated to their Fatty Acid Methyl Esters (FAME) using 0.66 N potassium hydroxide (KOH) in methanol and 14% methanolic boron trifluoride (BF_3) (Sigma Chemical Co. St. Louis, Missouri, USA) according to methods by AOAC. The FAME were separated by gas chromatography (Agilent 5890N) using a 30 m×0.25 mm ID (0.20 μm film thickness) Supelco SP-2330 capillary column (Supelco, Inc., Bellefonte, PA, USA). About 1 μL of FAME was injected by an auto sampler into the chromatograph, equipped with a Flame Ionization Detector (FID). The split ratio was 1:30 after injection of 1 μL of the FAME. The injector temperature was programmed at 250°C and the detector temperature was 300°C. The column temperature program initiated runs at 100°C for 2 min, warmed to 170°C at 10°C min^{-1}, held for 2 min, warmed to 220°C at 7.5°C min^{-1} and then held for 10 min to facilitate optimal separation. The fatty acid proportions are expressed as percentage of total identified fatty acids.

Table 1: Composition of standard rat chow diet

Nutrient composition	Diet (%)
Crude fat	3.0
Crude protein	20.0
Crude fibre	7.3
Ash	9.2
Nitrogen free extracts	59.4
Calcium	0.9
Phosphorus	0.5

Data analysis: All the data generated in this study were analyzed by the PROC GLM of SAS (SAS Inst. Inc., Cary, NC). Body weight and fatty acid profiles were compared across treatment groups. Significantly different means were then further differentiated using the Least Significant Difference (LSD) comparison procedures. All statistical tests were conducted at 95% confidence level.

RESULTS AND DISCUSSION

Table 2 shown the fatty acid composition of treatment oils. Butter had significantly the ($p<0.05$) highest total Saturated Fatty Acid (SFA) when compared to other treatment groups. The total Unsaturated Fatty Acid (UFA) was significantly high ($p<0.05$) in soybean oil in compared to other groups. In menhaden oil, the total LC-PUFA n-3 was the highest in compare to other groups. The fatty acid composition of treatment diets was shown in Table 3. The total PUFA n-6 in SCD and CD group was significantly ($p<0.05$) higher than other groups while PUFA n-3 was significant ($p<0.05$) highest for MCD group. The total SFA was high in BCD group.

Table 4 shows the fatty acid composition of treatment plasma at the end of feeding trial. Generally, the plasma SFA of the BCD group was significantly ($p<0.05$) higher than MCD group but it was not significantly different ($p>0.05$) with other groups. The total UFA were almost 2 times more than total SFA in plasma. However, the levels of individual UFA clearly reflected the type of supplemented fatty acid (Table 4). The SCD group had significantly ($p<0.05$) highest amount of UFA in compare to other groups. The MCD group also showed significantly ($p<0.05$) low amount of AA production when compared to other groups.

Table 2: Fatty acid composition of treatment oils (mean±SE; n = 10)

Fatty acid composition	Butter	Menhaden oil	Soybean oil
Palmitic acid (16:0)	34.54	24.89	11.71
Stearic acid (18:0)	9.96	5.11	3.80
Oleic acid (18:1)	22.60	9.91	24.62
Linoleic acid (18:2 n-6)	0.61	0.96	47.11
Alpha-linolenic acid (18:3 n-3)	0.14	0.27	5.60
Arachidonic acid (20:4 n-6)	ND	0.20	ND
Ecosapentanoic acid (20:5 n-3)	0.10	22.11	ND
Docosahexanoic acid (22:6 n-3)	ND	19.51	ND
Total SFA	73.52	42.20	16.97
Total UFA	26.48	57.80	83.03
Total MUFA	25.18	10.64	30.33
Total PUFA n-3	0.24	46.00	5.60
Total PUFA n-6	1.06	1.15	47.11
n-6:n-3 ratio	4.24	0.03	8.41
U:S ratio	0.36	1.37	4.92
P:S ratio	0.02	1.12	3.14

ND: Not Detected; Total SFA: Sum of 16:0 and 18:0; Total UFA: Sum of 18:1 n-9, 24:1, 18:2 n-6, 20:4 n-6, 18:3 n-3, 20:5 n-3, 22:6 n-3; Total MUFA: Sum of 18:1 n-9; Total PUFA n-3: Sum of 18:3 n-3, 20:5 n-3 and 22:6 n-3: Total PUFA n-6: Sum of 18:2 n-6, 20:4 n-6; n-6: n-3: Total PUF An-6 (Sum of 18:2 n-6, 20:4 n-6): Total PUFA n-3 (Sum of 18:3 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3)

Table 3: Fatty acid composition of treatment diets. (mean±SE; n = 10)

Fatty acid composition	BCD	MCD	SCD	CD
Palmitic acid (16:0)	32.85±2.54	20.82±1.84	18.11±2.43	21.89±1.11
Stearic acid (18:0)	11.55±0.96	8.11±1.21	6.29±0.41	8.33±1.51
Oleic acid (18:1)	10.38±7.50	23.44±1.37	23.08±1.12	24.44±4.31
Linoleic acid (18:2 n-6)	19.30±1.48	26.09±2.99	36.86±2.57	37.82±4.24
Alpha-linolenic acid (18:3 n-3)	0.38±0.01	0.57±0.09	4.55±0.22	0.63±0.06
Ecosapentanoic acid (20:5 n-3)	ND	6.25±0.74	1.00±0.16	ND
Docosahexanoic acid (22:6 n-3)	ND	5.43±0.63	0.58±0.09	ND
Total SFA	66.55±5.62	32.76±3.32	28.99±3.81	32.52±0.92
Total UFA	33.45±5.62	67.24±3.32	71.01±3.81	67.48±0.92
Total MUFA	13.43±7.04	27.72±1.14	27.64±1.32	29.04±4.06
Total PUFA n-3	0.38±0.01	13.43±1.39	6.52±0.07	0.63±0.06
Total PUFA n-6	19.64±1.42	26.09±2.99	36.86±2.57	37.82±4.24
n-6:n-3 ratio	51.82±4.37	1.94±0.06	5.67±0.45	61.69±9.73
UFA: SFA ratio	0.53±0.14	2.11±0.29	2.56±0.41	2.08±0.09
PUFA: SFA ratio	0.30±0.13	1.25±0.24	1.56±0.26	1.19±0.14

ND: Not Detected; Total SFA: Sum of 16:0 and 18:0; Total UFA: Sum of 18:1n-9, 24:1, 18:2 n-6, 20:4 n-6, 18:3 n-3, 20:5 n-3, 22:6 n-3; Total MUFA: Sum of 18:1 n-9; Total PUFA n-3: Sum of 18:3 n-3, 20:5 n-3 and 22:6 n-3; Total PUFA n-6: Sum of 18:2 n-6, 20:4 n-6. n-6: n-3: Total PUFAn-6 (Sum of 18:2 n-6, 20:4 n-6): Total PUFA n-3 (Sum of 18:3 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3)

Table 4: Plasma fatty acid composition of samples from treatment groups (mean±SE; n = 10)

Fatty acid composition	BCD	MCD	SCD	CD	p-value
Palmitic acid (16:0)	25.34±1.82	21.09±0.83	21.89±1.21	23.57±0.76	0.1200
Stearic acid (18:0)	13.76±0.40	13.20±0.74	10.96±0.93	13.24±0.82	0.4400
Oleic acid (18:1)	17.20±1.47	17.67±2.07	17.66±1.81	18.76±1.50	0.7400
Linoleic acid (18:2 n-6)	22.03±1.45	23.59±1.10	29.18±1.84	23.33±0.55	0.3800
Alpha-linolenic acid (18:3 n-3)	$0.48±0.05^{b}$	$0.81±0.16^{b}$	$3.03±0.14^{a}$	$0.46±0.03^{b}$	<0.0001
Arachidonic acid (20:4 n-6)	$21.18±2.13^{a}$	$11.99±1.67^{b}$	$16.72±1.34^{ab}$	$20.63±2.11^{ab}$	0.0400
Ecosapentanoic acid (20:5 n-3)	ND	$5.99±0.54^{a}$	$0.19±0.04^{b}$	ND	0.0003
Docosahexanoic acid (22:6 n-3)	ND	$5.66±0.54^{a}$	$0.37±0.05^{b}$	ND	0.0010
Total SFA	$39.10±1.76^{a}$	$34.29±1.19^{b}$	$32.85±1.45^{ab}$	$36.81±1.28^{ab}$	0.0400
Total UFA	$60.90±1.76^{b}$	$65.71±1.19^{ab}$	$67.15±1.45^{a}$	$63.19±1.28^{ab}$	0.0400
Total MUFA	17.20±1.47	17.67±2.07	17.66±1.81	18.76±1.50	0.7400
Total PUFA n-3	$0.48±0.05^{c}$	$12.46±0.85^{a}$	$3.58±0.17^{b}$	$0.46±0.03^{c}$	<0.0001
Total PUFA n-6	43.22±3.03	35.58±2.24	45.90±2.32	43.97±2.13	0.5600
n-6: n-3 ratio	$97.26±9.08^{a}$	$2.95±0.24^{b}$	$12.96±0.71^{b}$	$97.86±4.01^{a}$	<0.0001
UFA: SFA ratio	$1.59±0.10^{b}$	$1.95±0.10^{ab}$	$2.09±0.14^{a}$	$1.75±0.09^{b}$	0.0300
PUFA: SFA ratio	1.16±0.11	1.43±0.11	1.55±0.13	1. 23±0.09	-

[a-c]Means within rows with different superscript differ significantly (p<0.05); Total SFA: Sum of 16:0 and 18:0; Total UFA: Sum of 18:1 n-9, 24:1, 18:2 n-6, 20:4 n-6, 18:3 n-3, 20:5 n-3, 22:6 n-3; Total MUFA: Sum of 18:1 n-9; Total PUFA n-3: Sum of 18:3 n-3, 20:5 n-3 and 22:6 n-3; Total PUFA n-6: Sum of 18:2 n-6, 20:4 n-6. n-6: n-3: Total PUFAn-6 (Sum of 18:2 n-6, 20:4n-6): Total PUFA n-3 (Sum of 18:3 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3)

The highest proportion of LC-PUFA n-3 including DHA and EPA was found in the MCD group which menhaden oil was used in the dietary treatment. In the MCD group, the plasma total n-3 was the highest at 12.8% (p<0.05) resulting in the lowest n-6:n-3 ratio at 2.95. This clearly reflects the contribution of LC-PUFA n-3 from menhaden oil in the diet. The plasma from SCD rats had n-6:n-3 ratio of 12.96 with the majority of n-6 dominated by LA. Both BCD and CD rats had very unfavourable n-6:n-3 ratio exceeding 51.82 in both groups. There was no significant (p>0.05) different in P:S ratio for all treatment groups.

Figure 1 shown the body weight of the rats during the 20 weeks of experimental period. The BCD group showed significantly higher (p<0.05) body weight in compared to other treatment groups throughout the 20 weeks of treatment. The CD group have the lowest body weight gain in compare to the other groups. The bodyweight was followed the trend of BCD>MCD> SCD>CD in all time of the experiment.

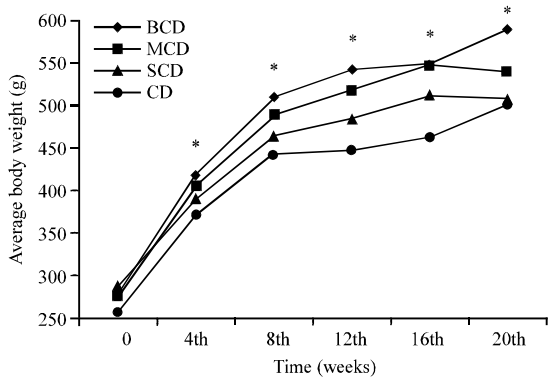

Fig. 1: Average body weight of rats for all treatment groups after 20 weeks of feeding trial; *significant difference at p<0.05

The fatty acid composition of plasma and tissue can be changed by many factors such as food intake, metabolism and absorption in the body (Rise *et al*., 2007). Dietary fatty acid profile strongly influences the fatty acid

composition of plasma (Boden *et al*., 2008; Amira *et al*., 2010). Dietary fat can alter the membrane fatty acid profile leading to change in physicochemical properties including fluidity and modulation of membrane associated physiological processes (Hulbert *et al*., 2005; Sonnino *et al*., 2008). Furthermore, several studies showed that dietary fatty acid composition was able to change the plasma fatty acid profile in rats (Winters *et al*., 1994; Mohamed *et al*., 2002; Garcia-Roves *et al*., 2007). In this research, it was revealed that the dietary fatty acid manipulation resulted significant changes in blood plasma fatty acid composition. These changes were directly reflecting the dietary fatty acid of the treatment diet.

The total SFA in BCD group was significantly higher when compared with other treatment groups due to BCD group diet composed mainly of butter. The major constituent of butter was palmitic acid (16:0). This showed that there was a correlation between the fatty acid supplemented to the plasma fatty composition. While for the total UFA, SCD group leads the highest concentration for the treatment oil, treatment diet and plasma where it was mostly consist of PUFA n-6. SCD group was a mixture of higher ratio of soybean oil and lower ratio of menhaden oil where its n-6:n-3 ratio was 5.67:1 hence, the high composition of PUFA n-6 was due to the presence of LA. In MCD group, the n-6:n-3 ratio was 1.94:1 due to higher ratio of menhaden and lower ratio of soybean oil thus giving it a significantly higher ($p<0.05$) PUFA n-3 when compared to other treatment groups. This was due to the presence of EPA and DHA which was rich in fish oil (Friedman and Moe, 2006).

The amount of fat supplemented on the diet influenced the interaction between the fatty acids where LA and ALA uses the same Δ-6 desaturase, the first enzyme for the elongation and desaturation of LA to AA (20:4, n-6) and for ALA to EPA, respectively (Mohrhauer and Holman, 1963; Rahm and Holman, 1964; Simopoulos, 2003; Anderson and Ma, 2009). Both LA and ALA worked antagonistically in the conversion of LA to AA and ALA to EPA and DHA by a series of desaturation, elongation and oxidation steps (Sprecher, 2000; Nakamura and Nara, 2003; Jump *et al*., 2005). Raz *et al*. (1998) found that feeding rat with LC-PUFA n-3 can inhibit the Δ-6 desaturase activities and subsequent decrease in AA level in the plasma of rat supplemented with fish oil. Amira *et al*. (2010) also showed that by increasing the fish oil in rat diet the AA significantly decreased which is in line with current finding.

The body weight of the rats showed a rapid increment on the first 8 weeks due to growth phase and slows down after week 8 when the animals had matured (Adams and Boice, 1983). The body weight of the BCD group was high due to the high amount of SFA in the diet. This result is agreed with the findings of Bell *et al*. (1997), Larson *et al*. (1996) and Westerterp and Goran (1997) which fed rat with high SFA diet resulted heavier body weight in compared to other types of fatty acids. Whereas, PUFA like soybean oil had been observed to have negative correlation to body weight gained in rats (Hill *et al*., 1992; Ikemoto *et al*., 1996). This supports the current finding where SCD group had an intermediate body weight when compared with other treatment groups. SCD group showed a better control of body weight due to n-6:n-3 ratio of 5.67:1 which is in range of recommended daily dietary intake of n-6:n-3 (Krauss *et al*., 2000; Kris-Etherton *et al*., 2002).

CONCLUSION

In this study, diets with lower PUFA n-6:n-3 ratios resulted in lower AA levels in plasma. This could be a result of down-regulated AA production or mobilization. This study also successfully demonstrated the changes induced by varying levels of dietary n-6:n-3 PUFA ratio on tissue and plasma fatty acid contents.

The long term observation had showed that animals fed higher amounts of saturated fat had greater weight gain even though the dietary energy from both unsaturated and saturated fats are the same. These are potentially promising tools for the control and prevention of the obesity as body weight changes can be achieved by manipulating the intake of the types of fatty acid in the diet.

ACKNOWLEDGEMENT

This study was supported by the University Putra Malaysia Research University Grant Scheme (Vote No. 91734).

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