CHAPTER IV

DISCUSSION

Cardiovascular disease (CVD) constitutes one of the most important health problems in almost developed countries and some developing countries like Thailand and accounts not only for the most deaths, but also for the greatest proportion of lost future earnings, the largest number of hospital days, and the greatest number of pharmaceutical prescriptions per year of any major diseases (McPherson and Spiller, 1996). In the past the curation and prevention of CVD was emphasized on lowering hypercholesterolemia of the patients. The dietatic tool was n-6 PUFA's of vegetable oils and their hypocholesterolemic effects. Today we know more about lipid metabolism and etiology of CVD. It has been proven that n-6 PUFA's provide disadvantageous effect for preventing CVD in some circumstances, i.e. lower HDL, produce proatherogenic and prothrombogenic eicosanoids (Grimminger et al, 1995). Now the important functions of n-3 PUFA's, particularly EPA and DHA from fish or fish oil, in atherogenesis, inflammation, thrombus formation, gene expression, and cell-to-cell communication have taken central stage and have led to intervention studies and clinical trials in CVD and many diseases (McPherson and Spiller, 1996).

The current interest in n-3 PUFA was governed from studies of Greenland Eskimos (Inuit) of Bang and Dyerberg (1971). They reported that the Eskimos had lower serum levels of total cholesterol, triacylglycerols (TG), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and higher serum level of

high density lipoproteins (HDL) compared with Danish controls despite the similarly high intake of animal's fat among them. More importantly, the incidence of heart disease especially atherosclerosis and coronary artery disease (CAD) is low in Greenland Eskimos (Kromann & Green, 1980). The conclusion was that Eskimos consumed high amount of marine fat which contained a great proportion of n-3 PUFA (Simopoulos, 1997).

A low occurrence of atherosclerosis and CAD has also been reported in Alaskan Eskimos (Newman et al., 1993). In studies from Japan, which in this respect may be considered a population with an intermediate consumption of seafood, the incidence of cardiovascular disease (CVD) was lower in fishing villages than in farming villages that had a lower intake of fish (Hirai et al., 1989). Several intrapopulation studies have suggested beneficial effects of fish consumption on risk factors for CVD.

Up till now, it is absolutely no doubt that dietary n-3 fatty acids prevents CVD in population with high n-3 PUFA intake derived from high marine animal consumption. These special fatty acids act by a variety of actions (Connor, 1997). EPA and DHA, derived from fish or fish oil, have a strong antiarrhythmic action on the heart (Kang and Leaf, 1996). Both fatty acids also prevent the development of ventricular fibrillation as demonstrated in animals. Sudden death has been prevented in CVD patients who consumed one or two fish meals per week (Siscovick et al., 1995) or, in lieu of fish consumption, took one to two fish-oil capsules per day (Burr et al., 1989). Those who consumed fish had 70% less likelihood of cardiac arrest. Over all mortality was decreased by 29% in men with overt CVD given n-3 PUFA from fish, probably by the reduction in cardial arrests (Burr et al.,

1989). The antiarrhythmic effect of n-3 PUFA is a discovery that fish consumption has great relavance to the prevention of sudden death from ventricular fibrillation (Connor, 1997)

Diet rich in n-3 PUFA can alter various macrophage functions. One possible mechanism by which this occurs is through modulation of the physicochemical properties of the cell membrane and the signal transduction pathways associated with macrophage activation. The role of n-3 PUFA on controling macrophage function is via their presentation on membranes of macrophage. They probably increase the activation potential of membranes' phospholipase C, without affecting platelet-activating-factor (PAF) receptor number and affinity of enzymes and finally enhancing the PAF signaling pathway in macrophage (Chakrabati et al., 1997). In another study, Siafaka-Kapadai and Hanahan (1993) also confirmed that an endogenous inhibitor of PAF-induced platelet aggregation, isolated from rat liver, was identified as free fatty acid.

How PAF plays an important role in thrombogenesis, Sun and Gilboe (1994) investigated the effect of PAF antagonsit BN 50739 and its diluents on mitochondrial respiration and membrane lipids during and following cerebral ischemia employing Pulsinelli-Brierley four-vessel occlusion model in rats. The overall effect of BN 50739 on mitochondrial structure and energy metabolism made them conclude that PAF played a key role in ischemia-induced cerebral injury. Delay the activity of PAF in macrophage, platelets and any other cells with increased free- or esterified n-3 PUFA may reduce the occurrence of thrombogenesis and atherogenesis.

Actually, the n-3 PUFA's from fish oil have many antithrombotic actions of clinical significance. The EPA of fish oil inhibits the synthesis of thromboxane A_2 from AA in platelets (Goodnight et al., 1982). This prostaglandin activates platelet aggregation and vasoconstriction. The n-3 PUFA's exclusively EPA also act as lone precursor of thromboxane A_3 as well as prostaglandin E_3 which delay platelet aggregation and vasoconstriction (Dahlan, 1997). As a result, fish oil increases the bleeding time and decreases the stickiness of the platelets for aggregation to glass beads (Goodnight et al 1981). The administration of fish oil enhances the production of prostacyclin, which produces vasodilation and less sticky platelets.

In an *in vivo* baboon model, dietary fish oil prevented platelet deposition in a plastic vascular shunt (Harker et al., 1993). Injury to the intima of the carotid artery of the baboon invariably caused a marked proliferative and inflammatory lesion, greatly thickening the wall. When the animals were fed fish oil, this damage and intimal thickening were completely prevented.

The formation of plaque, the major cause of CVD, is initiated and progressed by two mechanisms: atherogenesis and thrombogenesis (Dahlan, 1997). The role of platelets in preventing thrombogenesis has been established, eg., the delay of bleeding time by producing antithrombogenic eicosanoids and inhibiting prothrombogenic eicosanoids, recently their role in atherogenesis has been discovered. Connor (1997) concluded that EPA and DHA produce several effects to inhibit the development of atherosclerosis. There is direct evidence in both pigs and monkeys that dietary fish oil prevents atherosclerosis by actions other than through lowering plasma cholesterol concentrations. These actions may be associated with the inhibition of

monocyte migration into the plaque, with less cytokine and interleukin 1 α production, and through stimulation of the endothelial production of nitric oxide which was previously known as endothelial-derived relaxing factor (EDRF).

How n-3 PUFA's play very crucial roles in preventing CVD at both major mechanisms of atherogenesis and thrombogenesis is explained by the action of n-3 PUFA's on platelet membranes. Dietary n-3 PUFA's are incorporated into these groups of blood cells (Schmidt and Dyerberg, 1994). Activation of platelets with high n-3 PUFA often leaves less AA available for conversion to proaggretory TXA_2 (Kristensen et al., 1989) and dose-dependently reduces urinary excretion of TXA_2 metabolites (von Schacky et al., 1985). Competition between AA and EPA for the cyclo-oxygenase enzyme may further reduce platelet formation of TXA_2. This is considered an important, but not the sole mechanism by which n-3 PUFA inhibit platelet reactivity (Scheurlen et al., 1993).

Tremoli et al. (1995) confirmed the effect of n-3 PUFA's in platelet PL that prolonged the inhibition of platelet aggregation. They administered to healthy volunteers for 6 wks with low dose of n-3 PUFA ethyl esters and investigated platelet aggregation as well as TXA_2 formation. The result was that the long-lasting impairment in platelet aggregation was accompanied by the retention of n-3 PUFA's in platelet PL.

Vlasic et al. (1993) confirmed that lipid fluidity of cells modulated platelet aggregation as well as agglutination. They treated human platelets with A2C, a cyclopropyl fatty acid ester which was known to enhance fluidity of intrinsic membrane bilayer constituents and increase membrane fluidity. They found that platelet with less

fluidity demonstrated its diminished aggregation/agglutination with several activators. The conclusion was that membrane fluidity was involved in the regulation of platelet function.

Platelet adhesiveness has been reported to decrease in healthy individuals with n-3 PUFA administration by most (Goodnight et al., 1981; Terano et al., 1983). The impression from numerous studies is that n-3 PUFA slightly impair platelet aggregability in a dose-dependent way (Hornstra, 1989; Kristensen et al., 1989). The effect of dosage of less than 4 g/day of n-3 PUFA administration on platelet adhesion need to be studied. The high oral consumption of n-3 PUFA's in order to increase n-3 PUFA's on platelet's membranes is thus inefficient and uncertain.

Another interesting possible function of platelet is in brain physiology and biochemistry of patients with Alzheimer's disease. Rao et al. (1996) reported that in this kind of disease the aggregation response of platelets of patients to thrombin and AA was considerably compromised. In view of the normal biochemistry and signal transduction capabilities, suggested an extrinsic defect. The authors demonstrated that a plasmatic factor was at least in part responsible for the functional abnormalities of the patients' platelets. Whether membrane PUFA involved in this abnormality or not need to be investigated. For other diseases, Vecino et al. (1996) found that platelet hyperactivity in patients with migraine was not due to an altered lipid content of those cells. It is thus confirm that the alteration of fatty acids on platelet membranes involving in many but not all alterations of platelet functions.

It is well recognized that the increase consumption of certain fatty acids induces the rise of proportion of those fatty acids on platelet membranes. Sanikorski et

al. (1996) found AA and EPA on platelet membranes changed after feeding rats with high fat diet. Leece and Allman (1996) found that platelet EPA:AA value of rat increased as the dietary ALA:AA increased.

In order to increase membrane n-3 PUFA composition, in our experiment we employed lecithin-rich fat emulsion or PL liposomes as carrier of n-3 PUFA's and induced the transfer of those PUFA to membranes of platelets by brief incubation.

Platelet Concentrates

Since high consumption of n-3 PUFA give benefit to the health, supplementation of n-3 PUFA into diet is thus the recommendation for patients with CVD to relief from the disease and may protect them from acute myocardial infarction as well as stroke. However, such a procedure is time-consuming and probably ineffective in some circumstances. Handerson et al. (1994) determined in patients with cystic fibrosis whether they could incorporate n-3 PUFA into their plasma and membrane PL without adverse effects. After spending of 6 wks for supplementing high n-3 PUFA in form of fish oil (3.2 g EPA and 2.2 g DHA), the investigators found significant increment of both n-3 PUFA in plasma and RBC membranes. However, they could not detect any statistically and clinically significant changes in platelet aggregation. The effectiveness of oral supplementation of n-3 PUFA to the patients is thus uncertain after spending plenty of time and budget.

In our experiment, incorporation of n-3 PUFA was directly induced to

the cells employing artificial lipoproteins or fat emulsions as n-3 PUFA donor. Whether newly incorporated n-3 PUFA act as naturally-occurring n-3 PUFA is needed to be elucidated. Dietary enrichment of membrane PL with n-3 PUFA has attracted attention as a putative therapeutic regimen for suppression of inflammatory and coaggulatory events (Kramer et al., 1996). In our experiment we augmented proportion of n-3 PUFA on platelet membranes.

Platelet is one of blood cells represent the fracmented cytoplasm of bone marrow megakaryocytes. Ultrastructurally, three types of secretory granules are present in platelets: α-granules, lysosomes, and dense granules (Robinson, 1994). Platelets help in hemostasis and be the major cell that produce eicosanoids such as prostaglandin (PG) from PUFA's in membranes (Broekman et al., 1976). EPA as a representative of n-3 PUFA and AA of n-6 PUFA presented in membranes are two major precursors of prostanoids in the two series of E_3 and E_2, respectively (Simopoulos, 1991). Thus both AA and EPA have vital functions as precursors of eicosanoids apart from being structurally important for cell membranes.

Eicosanoids such as PG, thromboxane (TX) or leucotrienes (LT) are physiologically essential as locally acting hormone-like mediators which are produced and released upon stimulation. AA-derived mediators reduce immune responsiveness at various levels, enhance platelet aggregation and increase inflammatory responses. It appears that a major role of EPA is to serve as a substrate for mediators which down-regulate exaggerated responses of AA-derived mediators. By competing with AA, EPA and DHA are preferentially incorporated into the phospholipids of membranes of cells especially platelets. The further metabolic transformation of AA and EPA is also a

competitive event in which the relavant enzymes have a preference for EPA. However, enough AA-derived mediators will still be formed to fulfil n-6 functions. EPA-derived mediators just attenuate overreactive and harmful production of AA metabolites (Gerster, 1995).

To increase n-3 PUFA's on platelets and blood cells can be achieved by regular consumption of foods containing certain n-3 PUFA's. It has been demonstrated in man and in animals that the fatty acid composition of plasma, erythrocytes, and platelet phospholipids reflects the fatty acid composition of the dietary fat. Dougherty et al. (1987) conducted a study in rural areas of three countries to study the long-term effects of dietary fats on the lipids of plasma and blood cells of males 40-45 years. They concluded in their study that the fatty acid composition of the glycerophospholipids of plasma, RBC's, and platelets reflect the major dietary fatty acids. The higher consumption of dietary n-3 PUFA's for very long period of time, the higher content of n-3 PUFA's found in membranes of various blood cells. Dyer and Greenwood as mentioned in Greenwood et al. (1989) fed rats with 20% fat diet containing blended oil sources to produce n-6/n-3 fatty acid ratios ranging from 1.8 to 165 for 8 weeks (equivalent to 8 years for human) prior to sacrifice. They found the alterations of 22-carbon fatty acids in rat brain synaptosomal phosphatidylethanolamine corresponding to the consumed dietary fatty acids. The results confirmed the transfer of dietary fatty acids into body tissues and brain after the long period of feeding time. Ferrier et al. (1995) supplemented male volunteers with four hen eggs rich in ALA and DHA for 2 wks and found that total n-3 PUFA as well as n-3 to n-6 PUFA ratio of

platelet PL were increased. It implies that dietary n-3 PUFA from egg influences fatty acid composition in platelet within 2 wks.

The more rapid alterations of blood cell membrane lipids and PUFA's were demonstrated by Dahlan et al. (1992a, 1992b). Fat emulsion with egg yolk lecithin as emulsifier was infused intravenously at high rate (0.3 g/kg.BW/h) into normal subjects for 6 h affecting alteration in red blood cell (RBC) membrane lipid composition: RBC-cholesterol dropped with RBC-phospholipids rose and made RBC-cholesterol to phospholipid ratio markedly decreased (Dahlan et al., 1992a). In another experiment, Dahlan et al. (1992b) reported the alteration of PUFA's of RBC of patients with inflammatory bowel disease receiving intravenous total parenteral nutrition with egg yolk-derived lecithin fat emulsion for the period of 3 months. Actually, liposomes of egg yolk-derived lecithin contained in fat emulsion induced RBC membranes the decrement of DHA whereas augment of AA and LA within a few weeks of experiment.

Dahlan (1995) produced a fat emulsion by employing lecithins extracted from fish meal (Dahlan et al., 1996) and demonstrated that this novel fat emulsion with lecithin derived from G-1 Thai fish meal could alter major lipid composition of RBC membrane after brief incubation by raising n-3 PUFA's especially EPA and DHA and lowering n-6 PUFA's. It was then confirmed in our previous study that lecithin rich fat emulsions especially that with n-3 PUFA in moiety of PL could induce alteration of fatty acids on blood cell membranes. The results is extrapolated for the alteration of membranes of platelets so as to the membrane-modified platelets would be utilized as abrupt precursor of antithrombogenic eicosanoids.

In the present experiment, we prepared fat emulsion rich in n-3 PUFA's especially EPA and DHA in PL surface aiming to utilize such an emulsion for improving n-3 PUFA status of platelet membranes. The exchanges of fatty acids between platelets and our novel emulsion during the incubation were studied in *in vitro* and the results will be discussed and summarized herein. The effectiveness of n-3 PUFA rich fat emulsion on modifying platelet membranes as well as the magnitude of modification was studied in comparison to fat emulsions produced from other sources of emulsifier, i.e., egg yolk-derived lecithins as well as soya derived lecithins.

It is widely known among hematologist that freshly prepared platelet concentrates (PC) are prone to easily self aggregate. According to the long experience as pro-hand of Thai Red Cross's technician, the horizonally continuous shake under cold temperature prevents platelets from adhesion and clumping. During the incubation, platelets were subjected in cold atmosphere and the aggregation of platelets were effectively inhibited. Hawker et al. (1996) used PGE_1 as anti-aggregatory substances during the preparation of PC. What they found was that despite the ability of PGE_1 to facilitate platelet resuspension and inhibit platelet aggregation and activation during the preparation of PC, the reduced *in vivo* survival time might preclude the use of PGE_1 during routine PC preparation.

Effects of Gender and Blood Group on Platelet Concentrates

PC were prepared and provided for the present experiment by aseptic procedure under handling of keen technicians of Thai Red Cross. All specimen were

obtained from healthy volunteers with HIV-free and viral hepatitic-free. Male volunteers were vast majority contributing for 82% (100 in 122) in the subjects recruited in our study. However, the gender of the subjects showed no effect on the preparation of PC either considering in various blood cell content or specific gravity of PC (**Table 2**). When PC was grouped according to blood group, it was found that B group was the majority contributing for 79.5% (97 in 122). Again, blood group provided no effect to the characteristics of the PC specimen (**Table 3**).

It is concluded in this circumstance that neither gender nor blood groups of the blood donors could provide any pronounced effect to the PC preparation as well as to the experiment. PC's were then randomized and sampling liberally from gender and blood group for the incubation with freshly prepared fat emulsions.

Sources of Lecithin

Fish meal has a privilege of marine animal origin which widely accepted as rich source of n-3 PUFA (Simopoulos, 1991). Our preliminary survey showed that fish meal had prominent characteristics in its fatty acid constituents, e.g. very long chain with high unsaturation especially n-3 PUFA (Dahlan, 1996). Fish meal is one of the major marine by-products upon which the aquaculture feed industry depends. Chile, Peru, Denmark, Iceland, Norway and South Africa are actually counted as the major fish meal manufacturing countries in the world and originated in 1989 approximately 97% of the world trade of fish meal (Bololanik, 1989).

In our previous experiment as reported in M.Sc. dissertation of Miss Sopana Chatnilbandhu (1996), 4 grades of Thai fish meal were screened for their fat and lecithin contents. It was found that G-1 Thai fish meal was superior to other rivals according to its significant DHA content of 18.8%. However, the obstacles for selecting G-1 Thai fish meal for our present experiment were the availability, the stability of n-3 PUFA and the consistency of the production obtained from manufacturer. We finallly decided to select Danish fish meal as source of lecithin in our present experiment according to its higher contents of polyenes, EPA, DHA, total n-3 PUFA's and the ratio of n-3/n-6 than those of G-1 Thai fish meal as well as to the reliability of regular supply from the Department of Marine Sciences. All above mentioned results comparing fatty acid composition between Danish fish meal and G-1 Thai fish meal are shown in **Table 4**.

In addition, grade A Danish fish meal used in the present experiment was found to contain fat as high as 17.4 % of weight with 10.2 % lecithin in its lipid composition. Fish meal lecithin prepared from our study constituted high content of n-3 PUFA especially of DHA (average in PL-FA of 38 and 28% for n-3 PUFA and DHA, respectively).

Actually, lecithins can be obtained from all kinds of living matter, as their constituents are essential components of cell membranes (Devlin, 1993). This means that lecithins can be produced from vegetable, animal or microbial sources. But in practical commercial terms, they are made basically from vegetable products like soybean, sunflower, rapeseed, corn and groundnuts. Typically, vegetable lecithins are obtained as a by-product of the oil refining process, where substances containing

phosphorus have to be removed to give the oil better stability and/or facilitate further refining. Composition of typical vegetable lecithins make it obvious that there are no crucial differences between them (Pardun, 1982).

Though in the U.S. only vegetable lecithins are considered to be GRAS (generally recommened as safe), egg lecithin (historically the first lecithin to be discovered) also plays an important role, though mainly in pharmaceuticals because of their relative high price (Schneider, 1992). It is well recognized that the composition of polar lipids in egg lecithin is remarkeably different from vegetable lipids. Clear differences are also seen in the fatty acid breakdown. This fact is also confirmed in the present study as one can see in **Tables 10** and **14** of this text.

In the present study fish meal not egg yolk or any vegetables was used as source of lecithin due to its high content of n-3 PUFA. The rich presentation of n-3 PUFA in the surface of PL of our novel fat emulsion was proven in our previous study that it was an excellent supplier of n-3 PUFA to blood cells during their incubation with blood cells (Dahlan et al., 1997; Chatnilbandhu, 1996). We then seek for a good source of n-3 PUFA rich lecithin. This purpose brought fats of marine origin which had been widely known as mostly exclusive source of n-3 PUFA into our consideration. As mentioned in our previous investigation (Chatnilbandhu, 1996), fish meal especially from Danish manufacturer was an accessible and plentiful marine product. It was finally selected as raw materials for the preparation of crude lecithins.

In fact, fish meal has been used in feeds of poultry, pigs ruminants, fish, crustaceans, pets and fur-bearing animals for long time since it increases productivity and improves feed efficiency. As good source of many nutrients, fish meal provides a

unique balance of essential amino acids, energy, vitamins, minerals and trace elements, which implement other feed ingredients by correcting their deficiencies (Bimbo and Crowther, 1992). Hitherto, there are at least five supportive reasons for us to select fish meal as source of lecithin employed in the present study (previously mentioned in Chatnilbandhu, 1996).

Firstly, fish meal has a privilege of marine animal origin which widely accepted as rich source of n-3 PUFA (Simopoulos, 1991). Our preliminary survey showed that fish meal had prominent characteristics in its fatty acid constituents, e.g. very long chain with high unsaturation especially n-3 PUFA (Dahlan, 1996). Grade A Danish fish meal used in the experiment was found to contain fat as high as 17.4 % of weight with extraordinary high as 10.2 % lecithin in its lipid composition. Fish meal lecithin prepared from our study constituted high content of n-3 PUFA (38%) especially of DHA (28%) as shown in **Figure 12**.

Secondly, fish meal is the principal ingredients in feeds of many important species of farmed fish and shrimps. It is well established that fish meal is categorized in most countries based on its protein content, with higher value going to fish meals with higher than average protein contents. In contrary to protein, fat content of fish meal is neglected and unneeded since it facilitates ease oxidation and rancidity to the products (Hardy and Masumoto, 1991). Despite of the fact that fat obtained from fish meal is a good source of AA utilized for cat nutrition and also a rich source of n-3 PUFAs which possibly represent over 30% of the total fatty acid present, however, it has been eliminated along with fish meal manufacturing process in order to diminish origin causing rancidity and oxidation of the end product and not to invest too

much for the addition of antioxidant (Bimbo, 1990). According to the Department of Fisheries and Oceans in Canada for Atlantic salmon diets and the United States federal hatcheries, fat content in fish meal was limited not exceed to 10% (Hardy and Masumoto, 1991). This figure was also defined for the limitation of fat content in fish meal used as raw material for the preparation of quality and standard animal feeds in Thailand in the past but not for the present time.

According to Thailand's Ministry of Agriculture and Cooperatives (TMAC), limit point of fat content was ruled out from specification of fish meal raw materials in 1995. At the middle of 1995, TMAC announced the Act No. 8/1995 as a new regulation for standard of fish meals utilized for animal feed (TMAC 1995 and Chatnilbandhu, 1996). In this regard, the limitation of fish meal fat content was totally skipped out and the present fish meal hence contains significant amount of fats approximately closes to its natural origin. Accordingly, fish meal was considered in our study as advantageous for this aspect. In addition, Danish fish meal with full fat content was also introduced into our experiment.

Thirdly, apart from the advantage of new regulation as mentioned above, fish meal is also considered as a natural not processed source of fish lipids. It has been widely accepted that lipids especially PUFA well kept in its original sources especially in cell's fat vacuoles are more stable to oxidation than lipids released from their natural source, e.g. fish oils in crude fat wastes and in fish oils stocks (Li, 1991). Therefore, fish meal as a natural source of fish lipids was utilized with its full benefits in our experiment in comparison to crude or refined fish oils. The latters were

pretreated and refined under the process causing n-3 PUFA delayed in atmospheric oxygen for a certain period of time.

Fourthly, fish meal is one of the major marine by-products upon which the aquaculture feed industry depends. Chile, Peru, Denmark, Iceland, Norway and South Africa are actually counted as the major fish meal manufacturing countries in the world and originated in 1989 approximately 97% of the world trade of fish meal (Bololanik, 1989). Thus fish meal is plentiful and accessible for us to select unanimously as raw materials in the experiment.

Fifthly, it is believed generally that depot fats exist in cells consist largely of TG while the total lipids of various body organs and muscle tissues can by comparison contain large proportion of PL (Stansby et al. 1990). Actually, fish oil as well as crude fat waste obtained mainly from crude oil released after steaming during fish canning manufacturing process are good examples of fat from cell depot. As mentioned earlier, our previous study exhibited that lecithin content in such oils, i.e. crude fat waste from tuna canning manufacturer and refined tuna oil, was much less content than that found in fish meal. This result can be explained by the fact that fish meal is produced from whole marine fish not from any released fat hence it yields high proportion of PL and probably less free radical content.

Lecithin-Rich Fat Emulsions

Use of n-3 PUFA rich fat infusions for parenteral nutrition results in micromolar concentrations of EPA and DHA in the plasma free fatty acid fraction.

Kramer et al. (1996) investigated the influence of free EPA and free DHA on platelet TXA_2 and TXA_3 formation. They reported that enrichment of the plasma free fatty acid fraction with n-3 PUFA's might offer a therapeutic regimen to suppress the synthesis of the potent proaggregatory and vasoconstrictory agent TXA_2. However, the presentations of free EPA and DHA around platelets and blood cells are consequently incorporated those fatty acids into cell membranes and n-3 PUFA in moiety of PL provides higher effect on aggregation and vasoconstriction.

Jenski et al. (1995) invented liposomes containing DHA at *sn*-2 position of phosphatidylcholines (18:0, 22:6 PhC) and exposed them with murine leukemia T27A tumor cells. They found that cells increased permeability and altered surface protein expression, indicating a direct effect of DHA on plasma membrane structure and function. The authors predicted that, compared with other PL, 18:0, 22:6 PhC might inhibit tumor growth and viability and extend host longivity *in vivo*. They tested their prediction in male BALB/c mice inoculated intraperitoneally with a T27A tumor dose known to cause 100% mortality of syngeneic (BALB/c) mice in less than 2 weeks. The authors found that DHA-containing lipid vesicles caused a statistically significant increase in survival of the tumor-bearing mice when compared with 18:0, 18:1 PhC. The achievement of their experiment encouraged our study to utilize DHA-rich liposomes or fat emulsions as n-3 PUFA supplier to membranes of cells.

The PL subclasses residencing for DHA and other n-3 PUFA's is also significance for membrane function. Zerouga et al. (1996) cultured T27A cells in DHA-supplemented medium. They found that DHA incorporated rapidly and preferentially into phosphatidylethanolamine (PE), with lesser and slower incorporation

into phosphatidylcholine (PhC). They also found that DHA-containing PhC vesicles produced a dose-dependent decrease in cell viability, whereas PE-containing vesicles had little effect although they appeared more fusogenic.

The advantages of intravenous alimentation with fish oil derived fat emulsions have been reported regularly. Grimminger et al. (1996) investigated profound changes in fatty acid profiles and lipid mediator generation in plasma and corresponded prolong heart transplant survival of rats perfused with fish oil emulsion in excess to n-6 lipids.

In our present experiment, FM-LRFE was produced and the presentation of DHA in PL moiety was as high as 28%. They were majorily PhC-DHA as mentioned in our previous experiment (Dahlan et al., 1995). We employed this lecithin-rich fat emulsion with high DHA on emulsion PL surface as carrier of n-3 PUFA's providing to blood cells and predicted that blood cells could consume n-3 PUFA to their membranes and yielded the benefit of having n-3 PUFA presentation as earlier mentioned.

Conditioning the Incubation of Platelets

In 1995, Bayon and colleaques reported an interesting experiment on the selective modifications of the PL-FA composition in human platelet membranes using nonspecific and specific lipid transfer proteins (Bayon et al., 1995a). According to their objective, the fatty acid composition of cell membranes were specifically modified. PhC and PE subclasses containing LA and DHA at the *sn-2* position were

incorporated into human platelet membranes using the endogenous phosphatidylinositol (PI)/PhC transfer protein and the PL transfer protein from maize. The result showed that specific transfer protein catalyzed a strict exchange of PL between platelet membranes and unilamellar vesicles. Their experiment confirmed the possibility to employ PL liposomes as supplier of n-3 PUFA for incorporating those PUFA into membrane PL. In our experiment, we demonstrated that in the presence of plasma (reasonably containing lipid transfer proteins) the magnitude of n-3 PUFA incorporation into membranes was limited. The direct contact and exchange of fatty acids occurred between platelets and vesicles of fat emulsion due to unexplainable mechanisms were higher and faster with the absence of plasma proteins (**Tables 17, 18, Figures 14, 15**). Furthermore, all fatty acids incorporated in the membranes were maintained in platelets for at least 5 h after leaving the newly-washed incubated platelets in NSS (**Table 19, Figure 16**).

Fatty Acid Transfer from Fat Emulsions to Platelets

For years, intravenous lipid supply has been considered as a means of providing an efficient fuel to many tissues of the body and of preventing or correcting fatty acid deficiency. The potential for lipid emulsions to modulate cell function via their content of specific fatty acids transferring from both PL surface and TG core to membranes of tissues has not received much attention yet. Carpentier et al. (1997) reported their studies both *in vitro* and *in vivo* the metabolism of emulsions made of a mixture of MCT, soybean LCT and fish oil TG's. What they found was that plasma

elimination of such preparations appeared to be very fast, and their infusion was not associated with a prolonged residence of emulsion particles. In addition, uptake of remnants enriched with n-3 PUFA was fairly fast and occurred in several types of cells, leading to an efficient incorporation of n-3 PUFA's in cell membranes within a few hours (6 h). However, the mechanism of n-3 PUFA transferring abruptly from emulsion's core TG to membrane PL are needed to be elucidated. The questions are whether the increased n-3 PUFA on platelets are PL-PUFA not free PUFA and whether platelets have receptors for uptaking particle remnants.

How fast n-3 PUFA's travel from ingested dietary fat until appearing on platelet membranes is still unclear. Cukier and Waitzberg (1996) explained that after 72 h of n-3 PUFA intake there were changes of membrane composition and decrease of synthesis of PG, LT, and TX of the 2 and 4 series production and substitution for PG, TX and LT of 3 and 5 series, respectively. These alterations could modulate the inflammatory response in some diseases.

Morlion et al. (1996) measured the incorporation of n-3 and n-6 PUFA's into leukocyte membranes and the LTB_4-, LTB_5-, LTC_4- and LTC_5-synthesizing capacity in stimulated leukocytes following parenteral n-3 PUFA nutrition in 20 postoperative patients. Following 5 days of fish oil emulsion administration, the content of EPA was increased 2.5 fold, LTB_5 1.5 fold, and LTC_5 7 fold without any alterations of LTB_4 and LTC_4. They concluded that five day parenteral infusion of fish oil supplementation had an immunodulatory effect on lipid-mediator generation in human leukocytes in postoperative trauma.

The time delay for dietary n-3 PUFA spent prior to incorporate into platelet membranes have been studied. Terano et al. (1994) investigated the effect of administration of low doses of fish oil concentrate on platelet function in elderly subjects in comparison to younger subjects. Fish oil concentrates were administered in subjects daily for more than 1 month. In elderly subjects, the authors found the plasma EPA content had increased dose depedently, with suppression of platelet aggregation and improvement of RBC funtion. In younger subjects receieving the same amount of EPA, the elevation of plasma EPA was less than that observed in the elderly. They summarized that low dose EPA administration could improve the function of platelets and RBC to an anti-thrombotic state and would be useful to prevent the occurrence of cerebrovascular diseases in elderly subjects without any side effects. However, the effective period for such an administration was not less than 1 month.

Porta et al. (1994) administered two fat emulsions: long-chain TG (LCT) and medium-chain TG (MCT)/LCT into 23 adult, critically ill, non septic patients, who were in need of TPN for at least 7 days. Before beginning lipid perfusion, platelet activation evaluated by beta TG and PF_4 levels was notable, 6-keto-$PGF_1 \alpha$ production was elevated and no hyperaggregation of platelets was observed in patients. The high content of n-6 PUFA's in TG core made the investigator expecting the alteration of n-6 PUFA-derived eicosanoids which consequently affect platelet function. However, the studies of platelet function at 4 and 7 days of TPN did not show significant changes with respect to the basal data or between the 2 groups. The results implied that PUFA's present in TG core migrated slowly with several factors influence before entering platelet membranes.

In stead of wasting time for manipulating membrane n-3 PUFA's by exposing with long-term oral dietary marine fat intake and/or intermediate-term intravenous n-3 PUFA containing fat emulsion administration, we prepared fat emulsion with n-3 PUFA rich lecithins and incubated intimately with platelets in order to induce direct and abrupt transfer of n-3 PUFA from emulsion's surface to platelet membranes.

Membrane PLs and their fatty acids are also renewed by several mechanism (Dahlan, 1989). Membrane fatty acids are turn over either by deacylation-reacylation in situ where plasma albumin-FFA complexes play a crucial roles as fatty acid donor and receptor. Other mechanisms are the renewal of membrane PLs by acylation and incorporation of plasma lysophospholipids, and by passive exchange or net transfer of PL and cholesterol with lipoproteins. Additionally, both the content and composition of membrane PL may be altered by the incorporation or fusion of exogenous PL as demonstrated for erythrocyte membranes during intravenous infusion of Intralipid emulsion and incubation of RBC with lipid liposomes as demonstrated by Dahlan et al (1997).

In our study, we demonstrated that n-3 PUFA's especially DHA and EPA were augmented to 135 and 205%, respectively, after incubation with FM-LRFE at the concentration of 600 mg PL/dl. **Figure 17** shows the percentage of changes of all major fatty acids of platelets but not for EPA which has too high alteration (205%). With SY-LRFE we can induce the increment of n-6 PUFA especially LA for 154% and decrement of DHA for 5% at the incubation condition of 600 mg PL/dl (**Figure 18**). We also confirmed for the first time that fish oil rich in DHA and EPA in TG core

cannot influence the exchange of fatty acids between emulsion's surface and platelet membranes (**Figure 19**) except for the correlation of n-3 to n-6 PUFA ratio ($r = 0.38$, $p > 0.05$ and $r = 0.65$, $p < 0.005$ for SL-FOFE and SY-LRFE, respectively, shown in **Tables 31-32**). As shown in **Figures 18 & 19**, incubations of platelets with soya-lecithin rich emulsions cored inside with either soya oil and fihs oil induce LA accumulation on membranes of platelets reached to 145-154% and DHA dropped for 4.6-6% in contrary to the results obtained from the incubation of FM-LRFE as shown in **Figure 17**.

EY-lecithin fat emulsion or 20% Lipofundin induced the least alteration of fatty acids in platelet membranes (**Figure 20**). LA raised only 25% in platelet incubated with commercial EY emulsion in comparison to 155% incubated with our novel SY-LRFE (**Figure 18**). The results of PUFA alteration on membranes of platelet in this study as well as on RBC membranes in our previous study (Dahlan et al., 1997) imply the implementation of the experiment on tailoring PUFA composition of platelet membranes so as to adjust platelet function appropriated for the condition or the disease of the patients. We have proven that fish oil in core of fat emulsion cannot provide ample changes of PUFA on membranes in brief incubation between vesicles of fat emulsion and blood cells both *in vivo* and *in vitro*. The restructure of PUFA composition in membranes if required is recommended by brief contaction with liposomes or emulsion rich in lecithins with high in specific PUFA's. However, the protection of those PUFA's fragile for peroxidation was not well prepared and should be further investigated. The question in mind for our next experiment is likely that

which form of antioxidants are appropriate for maintaining the stability of transferred PUFA and whether antioxidants are transferred together with PUFA or not.

Preparation of n-3 PUFA rich emulsion without the addition of antioxidant may provoke the autooxidation of PUFA. Lipid peroxidation of n-3 PUFA may take place, with good and bad consequences. As the number of double bonds is high, n-3 PUFA may easily react with oxygen radicals. Drevon et al. (1995) performed a studies where 5 g/day of n-3 PUFA was given as a supplement for four months along with vitamin E. They found that oxidized LDL occurred much less in vitamin E supplement group than in control.

After incorporating n-3 PUFA's into membranes of platelets, a raised question is whether the altered ratio of those PUFA affect membrane function. Bayon et al. (1995b) studied the effect of specific PL molecular species incorporated in human platelet membranes on TXA_2/PGH_2 receptors. They confirmed in their previous experiment that more than 80% of DHA incorporated in platelet membranes was esterified in the various endogenous PL classes as well as in neutral lipids and in the nonesterified pool (Bayon et al., 1995a). After modifying membrane platelet n-3 PUFA 's, they found that fluidity parameters measured by electron spin resonance of 5- and 16-nitroxy-stearic acids were not significantly different in membrane enriched with C16:0/DHA-PL relative to those enriched with C16:0/LA-PL, arguing against a generalized perturbation of the membrane due to DHA incorporation. However, they still concluded after investigating the specific binding of anlogues of TXA_2 and PGH_2 to the modified mambrane that molecular species of PC with DHA at the *sn-2* position

could affect TXA_2/PGH_2 receptors. This is confirmed that newly incorporated DHA to membrane can influence its function.

How to improve the survival time and to increase the activity of prepared platelets *in vivo* has to be further investigated. Gawaz et al (1996) found that the platelet function altered in symtomatic CVD could be modulated by the administration of intravenous Mg^{2+}. Furthermore, the supplementation of antioxidants especially fat-soluble vitamin E has to be considered so as to prevent n-3 PUFA on membranes from peroxidation. Other substances involved to improve platelet funtion as reported by Knight et al. (1997) are nitrates which cause inhibition of aggregation by binding with fibrinogen, and degrenulation by expressing P-selectin, calcium antagonists enhancing degranulation, and beta-blockers enhancing aggregation.

The discovery of apoprotein E that inhibits platelet aggregation through the L-arginine:nitric oxide pathway implicating for vascular disease as reported by Riddell et al. (1997) is also interesting topic for the research. Increment of antioxidant content on membranes of platelets by natural way is also important. Pellegrini et al. (1996) found that the non-alcoholic components in red wine consumed by healthy volunteers could be incorporated into platelet membranes and act as antioxidant.

In our experiment, we could augment n-3 to n-6 PUFA ratio of platelets after brief incubation with FM-LRFE employing the specific condition as described earlier. The alteration of platelet PUFA are speculated to influence their membrane function as well as eicosanoid production. How magnitude and mechanism of alterred membrane funtions are still needed for further investigation.