Flaxseed Oil and Lipoprotein (a) Significantly Increase Bleeding Time in Type 2 Diabetes Patients in Cape Breton, Nova Scotia, Canada

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Abstract: Platelet hyper-aggregation is a serious manifestation of type 2 diabetes and a precipitating factor in the most frequent cause of death in type 2 diabetes-myocardial infarction. Consumption of flaxseed oil as a dietary supplement containing alpha-linolenic acid (ALA, 18:3 n-3) through its metabolism to eicosapentaenoic acid (EPA, 20:5 n-3) and subsequent production of anti-aggregatory eicosanoids may reduce such aggregation *in vivo*. Lp (a) may also influence platelet aggregation *in vivo*. Furthermore, serum Lp(a) concentrations are increased and bleeding time is decreased in type 2 diabetics presenting an enhanced risk of myocardial infarction. It was hypothesised that Lp(a) and bleeding time would be correlated due to the considerable molecular homology between apolipoprotein (a) and plasminogen which should decrease bleeding time. Bleeding time is an excellent measure of *in vivo* platelet aggregability. One purpose of this study was to determine the impact flaxseed oil consumption on bleeding time compared to those on safflower oil and to determine the impact of Lp(a) on bleeding time. It was a secondary purpose to determine if there were any gender differences pre- or post-treatment in bleeding time. Subjects (N = 40) were randomly divided to take either the treatment, flaxseed oil (N = 20) or the placebo, safflower oil (N = 20). Each of the treatment or placebo groups contained equal numbers of males (N = 10) and females (N = 10). Some subjects dropped from the study due to reasons not related to treatment side effects. Subjects came for 3 visits, each 3 months apart. On each visit age, gender and BMI were recorded and bleeding time was performed. At the completion of visit 2, subjects were randomly assigned to take 1 gram of oil per 10 kg body weight each day for 3 months. Comparing pre- and post-treatment, there was a statistically significant increase in bleeding time in the flaxseed oil group including each of males and females while there was no change in the safflower group in total or by gender. Males had a statistically shorter bleeding time pre-treatment while males and females showed no difference post-treatment with flaxseed oil consumption. Males and females showed a non-significant correlation and statistically significant correlation respectively between pre-treatment values for Lp(a) and bleeding time. The statistically significant correlation also held for the whole population though at a lower value than females. It is concluded that flaxseed oil consumption has a statistically significant effect on slowing bleeding time thus likely reducing the risk of myocardial infarction and that such effect is more profound in males than females suggesting a greater efficacy of flaxseed oil administration for type 2 diabetic males. It is concluded that type

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2 diabetic females take better advantage of elevated Lp(a) concentrations than do males thus, at least in part rendering the impact of flaxseed oil on bleeding time greater in males.

Key words: type 2 diabetes, platelet, lipoprotein (a), bleeding time, gender

1 Introduction

Type 2 diabetics usually die from atherosclerosis, a significant feature of which is the formation of thrombi and/or emboli(1, 2). The ability of platelets to clot is reflected in the bleeding time(3, 4). Type 2 diabetes features platelet hyperaggregation (5, 6). Type 2 diabetics are frequently placed on various therapeutic regimes intended to reduce platelet aggregation (7, 8). Reduced *in vitro* platelet aggregation has not been observed in some persons taking flaxseed oil (9,10) though in other studies the impact of increased dietary ALA suggests a decreased platelet function(11-13); however flaxseed oil has never been tried in type 2 diabetics in an attempt to manipulate platelet aggregation. Flaxseed oil contains alpha-linolenic acid (ALA, 18:3 n-3) which is metabolized in part to eicosapentaenoic acid (EPA, 20:5 n-3) (14, 15). Upon agonist stimulation of platelets, EPA produces prostaglandin E₃ (PGE₃) and prostaglandin I₃ (PGI₃), both of which reduce platelet aggregation and prolong bleeding time(16, 17). EPA also reduces the arachidonic acid (AA, 20:4 n-6) content of platelets thus reducing the AA- derived proaggregatory eicosanoids, thromboxane A₂ (TxA₂) and prostaglandin E₂ (PGE₂) produced upon agonist stimulation of platelets(18, 19). It was hypothesized that a dietary supplement of flaxseed oil rich in ALA would prolong bleeding time in type 2 diabetics. The objective of this study was to determine if a dietary supplement of flaxseed oil delivering an average of 60.0 ± 0.4 mg of ALA per kg body weight/day for 3 months would significantly lengthen bleeding time in type 2 diabetics. It was a secondary objective to determine if there were significant differences in bleeding time between males and females before flaxseed oil administration and if the impact of flaxseed oil was significantly different in male versus female type 2 diabetics.

It was also of interest to know what impact if any occurred on the bleeding time as the result of Lp(a) potentially mediated gender differences before treatment. Lipoprotein (a) blood plasma concentrations are elevated in type 2 diabetics (20, 21). Such elevation results in an enhanced risk of atherosclerosis (22, 23).

This elevated risk is due to increased lipid influx into the arterial wall (24), perturbation of the arterial endothelium (25) and a considerable homology between apolipoprotein (a) in Lp(a) and plasminogen (26, 27). This homology is believed to interfere with the binding of plasminogen to the fibrin holding the embolus or thrombus together (28, 29), thus reducing the activity of plasmin (30, 31). Plasmin breaks down the fibrin thus reducing the residency time of embolus or thrombus and consequently decreasing the risk of a myocardial infarction(32, 33). However, previous studies (34-38) suggest that there is a negative correlation between platelet reactivity *in vitro* and Lp(a) concentrations based on competitive displacement of fibrinogen from the IIb protein on the fibrinogen (GPIIb/IIIa) receptor on agonist-stimulated platelets and manipulation of platelet c-AMP concentrations by Lp(a). On the contrary, a number of authors have shown an increased (39, 40) or no change (41) in *in vitro* platelet reactivity upon incubation with increasing concentration of Lp(a). However the correlation between blood serum concentrations of Lp(a) and bleeding time has never been published. It was hypothesized that while elevated Lp(a) concentrations would present a enhanced risk of atherosclerosis in terms of increased lipid influx into the arterial wall and potential interference with plasmin activity, they may also modify platelet reactivity *in vivo* type 2 diabetics. Such modification may help offset the impact of elevated Lp(a) concentrations in terms of increased embolus or thrombus residence time.

2 Methods

Subjects (n = 20 male, 20 female) were randomly chosen from among 84 Caucasians responding in approximately equal sex numbers to a Sydney, Nova Scotia newspaper advertisement and two area physicians. The inclusion criteria were 18 years of age or older, diagnosed type 2 diabetic for longer than six months and with well controlled blood glucose levels, while the exclusion criteria were: not meeting the inclusion criteria, pregnant or planning on becoming pregnant during the clinical trial, involved in an athletic

training programme and/or taking insulin or any omega 3 supplement. Subjects were randomly assigned in equal gender numbers to receive flaxseed oil or the placebo, safflower oil. Regarding hypolipidaemic medications, subjects were evenly distributed among the four groups. This study received approval from the University College of Cape Breton Human Ethics Review Committee.

Subjects came for 3 visits, each 3 months apart. On visit 1 their age, sex and body mass index were recorded and bleeding time were performed. On visit 2, measures identical to visit 1 was performed. At the completion of visit 2 subjects were randomly assigned to receive 1 gram of oil(flaxseed(treatment) or safflower(placebo) per 10 kg body weight and instructed to consume the oil for 3 months until the evening before visit 3. Fatty acid composition of the oils was determined by gas liquid chromatography. Bleeding time was measured via the Ivy bleeding time (42) using the Simplate II R device and following the manufacturer's (Organon Teknika, Durham, North Carolina, USA) directions.

3 Statistical Analyses

The data in **Tables 2** and **3** was analysed by a one way analysis of variance while the data in **Table 4** was analysed by a two way analysis of variance. Individual differences were determined by a Tukey's test. Pearson correlations were done to determine the degree of relation between serum Lp(a) concentrations and bleeding time (**Table 5**). Statistical significance was $p < 0.05$.

4 Results

The composition of the oils is shown in **Table 1**. Levels of oil and alpha-linolenic acid consumption are listed in **Table 2**. Subject baseline characteristics (visits 1 and 2) are contained in **Table 3**. Pre-treatment bleeding time data is found in **Table 4** and **5**. Males had statistically significant shorter bleeding time before treatment than females. For all subjects or for males or females comparing visits 1 and 2 there was no significant difference in bleeding time. At the end of flaxseed oil treatment males did not have significantly different bleeding time compared to females. Safflower oil was without effect on bleeding time for males and females combined or considered separately (**Table 6**). Conse-

quently males were still significantly lower in bleeding time compared to females when both had completed the

Table 1 Fatty Acid Composition (Weight Percent i.e. mg of an Individual Fatty Acid per 100 mg of Fatty Acids) of Flaxseed Oil (Treatment) and Safflower Oil (placebo). Data is reported as mean for five selected capsules of each oil type.

Fatty acid	Flaxseed oil	Safflower oil
14:0	-	0.2
16:0	5.6	6.3
18:0	-	2.2
18:1 n-9	15.1	14.1
18:2 n-6	14.6	74.2
18:3 n-3	57.2	< 0.1
20:4 n-6	-	-
20:5 n-3	-	-
22:6 n-3	-	-

Table 2 Average Oil Consumption (grams/day and mg/kg body weight/day) and Alpha-Linolenic Acid Consumption (grams/day and mg/kg body weight/day).

Average oil consumption (grams/day)

	Flaxseed oil	Safflower oil
Male	9.8 $\pm$ 0.3	9.7 $\pm$ 0.4
Female	9.4 $\pm$ 0.3	9.2 $\pm$ 0.1

Average oil consumption (mg/kg body weight/day)

	Flaxseed oil	Safflower oil
Male	103.8 $\pm$ 0.7	103.0 $\pm$ 1.5
Female	106.7 $\pm$ 0.9	103.4 $\pm$ 0.8

Average alpha-linolenic acid consumption (grams/day)

	Flaxseed oil	Safflower oil
Male	5.3 $\pm$ 0.2	< 0.01
Female	5.5 $\pm$ 0.2	< 0.01

Average alpha-linolenic acid consumption (mg/kg body weight/day)

	Flaxseed oil	Safflower oil
Male	59.2 $\pm$ 0.4	< 0.01
Female	60.8 $\pm$ 0.5	< 0.01

Table 3 Pre-Treatment Characteristics of Subjects (all Caucasian). Data (N = 32) is reported as mean $\pm$ standard error of the mean (S.E.M.) for the subjects who completed the trial. Statistically significant differences are marked with a superscript letter with different letters representing a statistically significant difference across a row.

	All subjects	All males	All females	Flaxseed males	Flaxseed females	Safflower males	Safflower females
Age (years)	59.5 $\pm$ 1.7	60.7 $\pm$ 2.9	58.3 $\pm$ 1.7	57.9 $\pm$ 4.4	56.6 $\pm$ 2.6	64.6 $\pm$ 3.0	60.8 $\pm$ 1.1
Sex (M/F) N=	32	18	14	10	8	8	6
Body mass index (BMI) kg/m ² visit 1	32.4 $\pm$ 0.9	30.3 $\pm$ 0.7	33.7 $\pm$ 1.6	30.9 $\pm$ 1.2	33.5 $\pm$ 1.8	29.5 $\pm$ 0.5	33.0 $\pm$ 2.6
Body mass index (BMI) kg/m ² visit 2	32.2 $\pm$ 1.0	30.3 $\pm$ 0.8	33.4 $\pm$ 1.7	30.6 $\pm$ 1.2	33.6 $\pm$ 2.0	29.8 $\pm$ 0.8	33.4 $\pm$ 2.6

Table 4 Bleeding Time in Seconds Measured at Visits 1 and 2 (Prior to Treatment or Placebo Administration). Data (N = 32) is reported as mean $\pm$ standard error of the mean (S.E.M.) for the subjects who completed the trial. Statistically significant ($p < 0.05$) differences are marked with a superscript representing a statistically significant difference across a row. One or more shared superscripts signify a lack of significant difference.

All subjects visit 1	All subjects visit 2	All males visit 1	All males visit 2	All females visit 1	All females visit 2
N = 32	N = 32	N = 18	N = 18	N = 14	N = 14
219.3 $\pm$ 25.6	225.4 $\pm$ 27.2	204.3 $\pm$ 17.2	208.5 $\pm$ 18.2	234.7 $\pm$ 21.3 ^a	243.6 $\pm$ 23.3 ^a

Table 5 Average Bleeding Time in Seconds Measured for Visits 1 and 2 (Prior to Treatment or Placebo Administration) and Visit 3. Data (N = 32) is reported as mean $\pm$ standard error of the mean (S.E.M.) for the subjects who completed the trial. Statistically significant ($p < 0.05$) differences are marked with a superscript representing a statistically significant difference across a row or down a column. One or more shared superscripts signify a lack of significant difference.

Flaxseed males -visit 1 N = 10	206.2 $\pm$ 13.7	Safflower males-visit 1 N = 8	203.7 $\pm$ 40.7
Flaxseed males-visit 2 N = 10	211.4 $\pm$ 17.1	Safflower males-visit 2 N = 8	204.6 $\pm$ 37.2
Flaxseed females-visit 1 N = 8	238.3 $\pm$ 18.4 ^a	Safflower females-visit 1 N = 6	230.0 $\pm$ 29.8 ^a
Flaxseed females-visit 2 N = 8	249.2 $\pm$ 22.6 ^a	Safflower females-visit 2 N = 6	238.0 $\pm$ 30.8 ^a

Table 6 Bleeding Time (seconds) Average of Visits 1 and 2 versus Visit 3 to Treatment or Placebo. Data (N = 32) is reported as mean $\pm$ standard error of the mean (S.E.M.) for the subjects who completed the trial. Statistically significant differences are marked with a superscript letter with different letters representing a statistically significant difference across a row or column. Values are statistically identical if they share one or more superscripts across a row or column. One or more shared superscripts signify a lack of significant difference.

Flaxseed-all subjects average of visits 1 and 2 N = 18	226.3 $\pm$ 9.9 ^a	Safflower-all subjects average of visits 1 and 2 N = 14	219.7 $\pm$ 17.7 ^a
Flaxseed-all subjects visit 3 N = 18	311.2 $\pm$ 16.6 ^b	Safflower-all subjects visit 3 N = 14	230.3 $\pm$ 24.1 ^a
Flaxseed males average of visits 1 and 2 N = 10	208.8 $\pm$ 10.7 ^c	Safflower males average of visits 1 and 2 N = 8	204.2 $\pm$ 21.4 ^c
Flaxseed males visit 3 N = 10	298.3 $\pm$ 19.7 ^{b, d}	Safflower males visit 3 N = 8	219.3 $\pm$ 20.4 ^a
Flaxseed females average of visits 1 and 2 N = 8	243.4 $\pm$ 14.4 ^c	Safflower females average of visits 1 and 2 N = 6	234.3 $\pm$ 20.4 ^{a, c}
Flaxseed females visit 3 N = 8	327.3 $\pm$ 28.5 ^{b, f}	Safflower females visit 3 N = 6	241.2 $\pm$ 23.2 ^{a, c}

safflower placebo. Bleeding time and Lp(a) data and their respective correlations are found in **Table 7**. There were no statistically significant differences in Lp(a) concentrations or bleeding time between visits 1 and 2 for a given gender or for all subjects combined. In terms of gender, only females showed a statistically significant correlation between Lp(a) concentrations and bleeding time, pre-treatment. However, when all subjects were assessed there was still a statistically significant correlation between Lp(a) concentrations and bleeding time.

5 Discussion and Conclusion

Bleeding time is an accurate measure of *in vivo* platelet aggregation (3, 4). Platelet hyper-aggregation plays a significant role in myocardial infarction in type 2 diabetics (5, 6). Such aggregation results in the formation of thrombi and/or emboli which contribute significantly to the reduction of blood flow to the

myocardium. Such reductions in blood flow result in decreased oxygen tension in the myocardium. When the myocardium is compromised, tissue death (myocardial infarction) results. Males had a much shorter bleeding time compared to females before the administration of either oil. This is the first comparison of male versus female bleeding time in type 2 diabetics. However, the overall bleeding time data are similar to the literature (43, 44). The absence of significant differences in males and females in Lp(a) concentrations suggest that the longer pre-treatment bleeding time seen in females are due to a stronger effect of Lp(a) than in males (**Table 5**). In females about 50 percent of the variability in platelet aggregation may be attributed to Lp(a) concentrations while in males it is only about one percent. The reason for this is not clear but it would appear that other factors affecting platelet aggregation, such as platelet membrane fatty acid composition and subsequent balance between fatty acid derived pro- and anti-aggregatory eicosanoids basally or in the platelet-stimulated

Table 7 Bleeding Time in Seconds, Serum Lp(a) Concentrations in mg/dl and Pearson Correlations between Bleeding Time and Lp(a) Concentrations. Data (N = 32) is reported as mean $\pm$ standard error of the mean (S.E.M.) for the subjects who completed the trial. Statistically significant differences are marked with a superscript letters with different letters representing a statistically significant difference across a row. One or more shared superscripts signify a lack of significant difference.

Parameter	All subjects visit 1	All subjects visit 2	All males visit 1	All males visit 2	All females visit 1	All females visit 2
N	N = 32	N = 32	N = 18	N = 18	N = 14	N = 14
Bleeding time (sec)	242.3 $\pm$ 25.6 ^a	247.4 $\pm$ 27.2 ^a	220.3 $\pm$ 17.2 ^b	223.5 $\pm$ 18.2 ^b	264.7 $\pm$ 21.3 ^{a,c}	271.6 $\pm$ 23.3 ^{a,c}
Lp(a) concentrations mg/dl	11.3 $\pm$ 4.1	12.5 $\pm$ 4.5	9.5 $\pm$ 3.7	9.1 $\pm$ 3.9	13.1 $\pm$ 7.4	8.9 $\pm$ 8.6
Pearson correlation between bleeding time and Lp(a) concentration	0.51 ^{a,b}	0.54 ^{a,b}	0.09 ^c	0.11 ^c	0.74 ^{a,d}	0.72 ^{a,d}

^acorrelation is significantly different from zero ($p < 0.05$)

^{b,c,d} correlations are significantly different from other correlations not sharing the superscript ($p < 0.05$)

^c correlation is not significantly different from zero ($p > 0.05$)

state (45) may provide an explanation. In females, the current data is consistent with previous work (34-38) suggesting reduced platelet reactivity *in vitro* with increasing Lp(a) concentrations based on competitive displacement of fibrinogen from the IIb protein on the fibrinogen (GPIIb/IIIa) receptor on agonist-stimulated platelets and subsequent elevation of platelet c-AMP concentrations by Lp(a). This data is validated by its consistency between visits 1 and 2 for males or females alone or the genders combined.

The difference in bleeding time made males more amenable to changed bleeding time. Reasons for shorter bleeding time in males may have included higher levels of AA content in platelet phospholipid membranes and/or a greater efficiency of conversion of AA to AA-derived pro-aggregatory eicosanoids. It could also be that other factors favouring platelet aggregation predominate in males.

The increase in bleeding time was most likely due to the fact that flaxseed oil contains ALA which is metabolized in part, upon consumption by type 2 diabetic humans, to EPA (14, 15). ALA is the only fatty acid present in such large amounts in flaxseed oil compared to only the trace amounts found in safflower oil. Fur-

thermore when platelets are stimulated by agonists, EPA in the platelet membrane produces PGE₃ and PGI₃, both of which reduce platelet aggregation and prolong bleeding time(16, 17). Increases in platelet membrane EPA also reduces the AA, content of platelets thus reducing the AA-derived proaggregatory eicosanoids, TxA₂ and PGE₂ produced upon agonist stimulation of platelets(18,19). It may be that males are more amenable to the accumulation of EPA in the phospholipids of the platelet membrane and/or EPA's metabolism to anti-aggregatory eicosanoids. It may also be that if males have a greater EPA accumulation in platelet membrane phospholipids, then AA levels in the same are more dramatically reduced thus more significantly reducing the AA-derived pro-aggregatory eicosanoids. It could also be that flaxseed oil administration slows the molar efficiency of AA transformation into AA derived pro-aggregatory eicosanoids. The effect of the flaxseed oil must have been due its ALA content since that was the only fatty acid which is not present in the safflower oil.

The results are valid as there was no statistically difference between visits 1 and 2 for all subjects or for females or males separately when comparing visits 1

and 2. Furthermore, the results are valid because male bleeding times were not statistically significantly different comparing flaxseed versus safflower groups with the same applying to females.

The effects of the flaxseed oil were without reported side-effects. All patients completing the trial were compliant with requests made of them.

It is concluded that male bleeding time is shorter than female bleeding time prior to treatment. The shorter bleeding time for males is more amenable to lengthening than with female subjects though both sexes significantly lengthened their bleeding time. Thus the usefulness of flaxseed oil may be greater in males than in females in terms of reducing the ability of platelets to contribute to the risk of myocardial infarction. It is also concluded that the effect of the flaxseed oil on bleeding is due to the alpha-linolenic content of flaxseed oil. It is further concluded that Lp(a) significantly reduces bleeding time in female but not male type 2 diabetics with a significant correlation existing in the entire population. The most likely explanation is a dominating effect of competitive displacement of fibrinogen from the IIb protein of the GPIIb/IIIa receptor by Lp(a) in females and subsequent elevation of platelet c-AMP concentrations but not so with significant impact in males. Thus the impact of alpha-linolenic acid impact on platelet aggregation in males is greater due, at least in part as the result of the insignificant impact of Lp(a) on bleeding time.

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