NUTRITIONAL PROPERTIES OF BRASSICA CAMPESTRIS VAR. SPAN AND ITS FRACTIONS WHEN FED TO RATS AND SWINE

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Numerous reports indicate rats and other rodents show increased incidence of myocardial lesions when fed high levels rapeseed oils (20 % by weight of diet or 40 calorie per cent). A simple correlation with erucic acid intake has been suggested. The early lipidosis unquestionably correlates with erucic acid concentration but this correlation is much less apparent with the secondary or long term lesion. Let us put this nutritional problem in perspective at the outset. This is not an "all or none" problem - a low or background incidence of myocardial necrosis or fibrosis occurs in male rats irrespective of diet - high RSO intake merely exacerbates something which is present in any case. In the studies reported here, all rat hearts were sectioned at 6 μ and stained with H and E. Three sections were cut per heart, central-extending from apex to base and including interventricular septum, atrial and ventricular walls, two additional sections made parallel to and equidistant from the central section in the middle of each half of the heart. The incidence shown here is quite typical i.e. 20 % to 30 % with control diets i.e. chow, corn oil or lard with the average number of lesions per affected rat (3 longitudinal sections) from 1.0 to 4.5. Rapeseed oils increase this incidence from 30 % to 67 % with lesions per rats from 5.3 to 10.8.

The next experiment deals with our pig studies. 222 Yorkshire pigs weaned at 6 weeks of age from 55 sows were put on test at 8-9 weeks of age. The experiment was designed for 3 replicates with 5 pens of boars and 5 pens of gilts. Each pen had 7 pigs (one was a spare). Replications were started at 3 week intervals. Kill periods were at 1, 4 and 16 weeks on test diets. At that time mean weights were 280 lbs. (replicate 1) 273 lbs. (replicate 2) 310 lbs. (replicate 3).

Each group represents 6 pigs. Five sections were made from each heart. One from the right ventricle, one from the left ventricle, one from the interventricular septum and one from each of the atria. No pig showed the frank necrotic lesions observed with rats. The lesions recorded here are minute lesions of cell infiltration and fiber degeneration. No necrosis was observed in any pig. The random distribution of lesions across all diets makes it obvious that these are not related to soybean oil or span oil addition.

From the same pig hearts, frozen sections were cut and stained with oil red 0. In all sections, fat accumulation, where it occurred was only a trace and in no way comparable to that observed with rats. There is a trend to oil red 0 positive results in pigs fed 20 % of either soybean or rapeseed oil.

The relative incidence of fat accumulation in soy oil and rape oil fed groups for boars and gilts is as follows: 7 days 2:9 and 4:5; 28 days 3:4 and 3:5; 112 days 3:1 and 1:2. Thorough post mortem examinations of these pigs failed to reveal any other lesions in any tissue that could be attributed to diet. Finally, the body weight gains of these 210 pigs were as follows: Diets were 15 % CP; 0.9 % Ca; 0.8 % P 3500 K cal DE/kg control diet; 3800 K cal/kg diet for the 10 % oil containing diet; 4100 K cal/kg for the 20 % oil containing diet. In all cases boars grew faster than gilts.

For the first 4 weeks of experiment, control pigs grew at the same rate as the soy 10 % and span 10 % fed groups which was a better growth rate than in the soy 20 % and span 20 % fed groups. From 4 to 16 weeks growth rates were not statistically different for any of the pigs. Other data showed that efficiency of feed utilization was greater for soy oil and span oil fed pigs than for control pigs and greater for oils fed at the 20 % level than at the 10 % level.

Since the problem of most concern to research workers is the occurrence of myocardial necrosis in male rats we have concentrated much of our research efforts in this area. We have assumed from the beginning that this problem is one of fat metabolism and with this in mind have studied the lipid composition of male rat hearts and changes that occur with time and different diets. This extends our previously published work which indicated that the fatty acid composition of rat liver and rat heart reflected dietary fatty acids particularly during the first week of feeding.

This was most striking with an Arlo-Echo mixture of rapeseed oil (22.3 % erucic acid). After 1 week on diet, the heart lipids contained 11.1 % eicosenoic acid and 23.2 % erucic acid. In contrast liver lipids contained 5.1 % eicosenoic acid and 3.0 % erucic acid. This diet in addition decreased the concentration of stearate and palmitate and increased the concentration of oleic and linolenic acids. Of interest was the observation that the ratio of saturated to unsaturated fatty acids, in male rat hearts at one week on test was 1.06 to 0.86 for lard and corn oil diets but dropped to as low as 0.23 for rapeseed oil diets. All changes tended to normalize after 16 weeks of feeding test diets.

Kramer in our group has also shown that after a 16 week feeding trial eicosenoic and erucic acids can be recovered from liver PE and PC in appreciable quantities that again reflect dietary intake. PL are largely membrane lipids and we were also able to show that long chain monoenes accumulate in highly purified rat liver plasma membranes. After 4 weeks on a diet containing 20 % rapeseed oil with 22.3 % erucic acid, the liver plasma membrane contained 5.6 % eicosenoic and 1.7 % erucic acid. It is therefore apparent that dietary fatty acids in the rat not only influence the fatty acid composition of neutral lipids but that of phospholipids and membrane lipids as well. We therefore proceeded to obtain some more detailed information on the fatty acid changes in heart muscle with time when rats were put on several different dietary treatments. Since swine respond differently to rapeseed oil feeding than do rats, these studies are being extended to

include sequential changes in fatty acid composition for pig hearts.

Now to the fatty acid composition of the oils we used. The only part needing explanation is the column headed lard plus free erucic acid and lard plus esterified erucic acid. The former has commercially available erucic acid melted into commercial lard, throughly mixed, to give a final analysis of 5.4 % erucate. The latter was back fat from swine fed 22.3 % containing rapeseed oil for 16 weeks then rendered, in our own plant, to lard. It has the same erucate content but in addition has 10 % eicosenoic acid, 4 % linolenic acid, and a marked drop in palmitic and stearic acid. We note that while lard plus free erucic is definitely without effect on myocardial lesions in rats, in two experiments the lard plus esterified erucic acid shows a clear trend toward increasing the incidence of lesions above control value.

A difference was in the fatty acid composition of rat hearts when 4 different diets are fed, i.e. corn oil, span rapeseed oil, lard plus free erucic acid and lard plus esterified erucic acid which we called rendered pig fat or RPF. The span oil diet as opposed to the corn oil diet produced the expected drop in palmitate concentration in rat hearts. Of interest is the difference in the lard plus free erucic acid and lard plus esterified erucic acid diets (RPF). Clearly the low palmitate content of the latter is reflected in heart fatty acid composition. The same pattern may be noted for linolenic acid. Corn oil and lard diets result in low heart linolenic concentrations, while span and RPF, again reflecting the diet, resulted in higher heart linolenic and concentrations.

The combined results of stearic and eicosenoic acid levels in rat hearts showed that stearic acid tended to be lower in the RPF and span oil fed groups than in the corn or lard fed groups. This is surprising since corn oil is low in C:18 (1.7 %). Eicosenoic acid levels in rat hearts can reflect either dietary erucic acid levels or dietary eicosenoic acid levels. Lard, which contains less than 1 % eicosenoic acid, nevertheless increased rat heart eicosenoic acid levels to $\simeq$ 2 % when 5 % erucic acid was added to it. As has already been suggested (CRAIG and BEARE, 1967) eicosenoic acid is a metabolic intermediate in the oxidation of erucic acid and accumulates in tissues after feeding of 13-cis-docosenoic acid. With lard as the sole source of fat eicosenoic acid levels in hearts remained below 1 %.

The oleic and erucic acid levels in rat hears showed reflecting diet, that oleic acid is low in the corn oil fed group, high in the span oil fed group and intermediate in the lard and RPF fed groups. Erucic acid levels in heart also reflect dietary intakes. From almost zero level in the corn oil fed groups, erucic acid concentration rose to approximately 4 % in the span, lard, and RPF fed groups. There are two points of interest. First, irrespective of dietary C22:1 intake, the C22:1 levels in rat heart decreased to $\simeq$ 1 % with time (i.e. 16 weeks). Second, 13-cis-docosenoic acid, even when added to lard as the free acid is readily absorbed and incorporated into the TG's and PL fraction. This quite clearly shows that C22:1 by itself i.e. when incorporated into lard does not cause myocardial le-

sions in rats. Clearly, myocardial necrosis in rats does not occur when 5 % docosenoic acid is added to the lard containing diet. The incidence of myocardial necrosis does increase when back fat from pigs fed a 22 % erucic acid containing rapeseed oil is rendered to lard and refed to rats. There are a number of fatty acid compositional changes in this RPF fraction. In RPF, docosenoic acid is in ester linkage not the free acid; RPF contains 10 % ester linked eicosenoic acid; in RPF, the linoleic and linolenic acid levels are increased and the palmitic and stearic acid levels are decreased.

Again, linoleic acid levels in rat heart myocardium reflect dietary intake. The highest levels were found in the corn oil fed group. The docosatetraenoic acids of the W^2 and W^6 series, appear to follow a general pattern in rat hearts. These are highest in the corn oil and lard plus free erucic fed group and lower in the RPF and span oil fed groups. These differences become more pronounced with time. Conversely C22:5 and C22:6 (W^6 and W^3 series) decrease with time in the corn oil and lard fed groups but remain elevated in the RPF and span oil fed groups. The arachidonic acid levels in the four dietary treatments are all well within the normal range i.e. that of the corn oil fed group.

Next, the question arose whether the triglyceride fraction in the oils or a minor constituent present in the oils was responsible for myocardial lesions in rats. To answer this, we decided to fractionate the oils in order to obtain a relatively pure triglyceride fraction, as free of sterols, sterol esters, hydrocarbons and trace contaminants as possible. Since the bioassay can only be carried out with the male Sprague Dawley rat, sufficient quantities of each fraction were required to feed a minimum of 20 rats for 16 weeks.

To do this, we initially opted for a molecular distillation procedure which was done for us by Distillation Products Industries, Rochester, New York, in a Molecular Still, Model CMS-36. 2100 lbs. were placed in 600 gal. holding a tank for molecular distillation. Operating conditions of the CMS-36 were 2 μ vacuum, temp. from 276° dropping with successive cycles to 248° and distillation rats of 240 to 480 ml per min. The original 2100 lbs. of oil were recycled through the still and distillation cuts of approximately 10 % were collected. The distillates were cooled to 80° and collected in drums. N_2 was flushed continually during collection.

The first distillate, D_1 contained all the free sterols and tocopherols and a high concentration of sterol esters. D_2 contained a trace of sterols and a high concentration of sterol esters. D_3 and D_4 contained no free sterol and lesser amounts of sterol esters. D_5, D_6 and D_7 contained virtually no sterol esters.

Distillates 1 and 2 were selected for redistillation to concentrate the volatile non-saponifiable components of span oil. The CMS-36 was purged with D_3. D_1 plus D_2 (742 lbs.) were distilled and yielded 222 lbs. of Light-Fraction 28 (L.F., distillate) and 510 lbs. residue. L.F. 28 was redistilled to yield 71 lbs. L.F. 29 and 150 lbs. residue.

Sterol analyses were carried out by the 0-phthaldehyde procedure of RUDEL and MORRIS (1973). Span oil had a total sterol content of 0.57 %. D_5, D_6 and D_7 showed progressively less sterol ester content. No free sterols were left. L.F. 29 showed a high sterol plus sterol ester enrichment over original span oil, i.e. 8.18 %. Span contained 8.7 % Brassica sterol; 36.6 % campesterol and 54.7 % B. sitosterol. With successive distillations with D_4, these values change to B. sterol 2.5 %; campesterol 28.7 % and B. sitosterol 68.9 %.

Now to the TLC of fractions isolated by molecular distillation. Samples (200 μ g per sample) were applied to Silica Gel and plates developed in Hexane: Diethyl ether: Acetic acid, 85:15:1. From origin to solvent front the standard separate as follows: 1, monoglycerides and P~lipids at origin; 2, free sterol; 3, free fatty acids; 4, triglycerides; 5, methyl esters; 6, sterol esters.

Samples are: 1, standard; 2, span oil; 3, D_1; 4, D_2; 5, D_3; 6, D_4; 7, D_5; 8, D_6; 9, D_{7a}; 10, D_{7b}.

The fatty acid composition of the distillate is as follows: The separation by weight of the TG fatty acids is apparent. Palmitate, oleate and linoleate tend to be in higher concentrations in L.F. 29, and D_5 than in D_7. In contrast, D_7 contains more docosenoic and eicosenoic acids than either D_5 or L.F. 29. Of particular value to us was the fact that D_5 is almost identical in fatty acid composition to the original span oil.

Feeding these fractions to rats, each fraction was fed at 20 % by weight of the diet. Purina chow and olive oil showed 35 % and 20 % incidence respectively with an average of 1.7 lesions per affected rat heart. Oro and span oils showed a 44 % and 48 % incidence respectively with an average of 3.8 and 5.1 lesions per affected rat heart.

In our experiments Distillates 5, 6 and 7 showed increasing number of lesions and number of lesions per affected rat heart. The severity was outstanding with D_7. Fourty-one out of 50 rats showed heart lesions with a total of 539 lesions or 13.2 lesions per affected rat heart. Up to this point we appear to have a correlation of lesions with per cent of eicosenoic and docosenoic acids since the concentrations of these increase from D_5 to D_6 to D_7.

Unfortunately this relationship breaks down when we look at L.F. 29 in which 20:1 and 22:1 is less than in the D fractions. Half, of the L.F. 29 fed rats were killed at 12 weeks because insufficient material was left to complete 16 week feeding trial for all 50 rats. If we look at the L.F. 29 fed rats kept the full 16 weeks the incidence is 80 % with 10.1 lesions per affected rat heart.

Finally, we have started experiments to test span rapeseed oil fractionated by silicic acid adsorption chromatography. We contracted with Applied Science Laboratories of State College Pennsylvania to undertake the separation of 200 kg span rapeseed oil. They used 30" I.D. columns packed to

a heigt of 36" with silicic acid to give a ratio of 3.5:1 silicic acid to oil. The oil was dissolved in a minimal amount petroleum ether and passed through a dry packed column. Elution with petroleum ether continued until the eluate, continually monitored by TLC, showed no further sterol esters. This fraction was called F_1. Pure triglycerides were then eluted with 2 % diethyl ether in petroleum ether until polar lipids emerged. This was called fraction F_2. This was followed by chloroform:methanol 1:1 and this fraction (F_3) contained a mixture of TG, mono- and diglycerides, FFA, free sterols and P~ lipids. About 41 kg of pure triglyceride (F_2) was obtained. About 84 % of the original lipid was recovered, the remainder was left on the silicic acid. The silicic acid (≃1000 lbs.) was placed in 40 gal. steel drums, put under N_2 and is being stored at -15°. Our analyses indicate that the fatty acid composition of F_2 is essentially that of the original span oil and even heavily overloaded TLC plates fail to show any signs of lipid other than pure TG. The results of a preliminary 16 week experiment (10 rats per treatment) were as follows: The control diets (Purina chow and olive oil) showed the usual 10 to 20 % incidence of myocardial necrosis. Span gave a 60 % incidence of lesions with 4.8 lesions per affected rat heart while the highly purified TG showed 80 % of the rats affected with 7.9 lesions per affected rat heart.

Summary

222 Yorkshire boars and gilts were fed low fat, 10 % and 20 % soybean oil and span oil diets for periods up to 112 days. No necrotic lesions were found in any pig hearts examined although areas of minute vacuolation of myocardial fibres and cell infiltration were observed randomly distributed across all groups. An experiment with a high erucic oil (Arlo Echo mixture, 22 % erucic acid) is currently underway and preliminary results again indicate no evidence of lesions attributable to the oil.

The lack of lesions found in swine fed rapeseed oil shows there is a marked species variation in susceptibility. The reasons for this are no clear but it is noted that in swine hearts we normally find from 1 to 2 mole % eicosenoic acid as well as about 0.1 % docosenoic acid. Therefore, this species may have adequate enzyme activities for metabolism and oxidation of these long chain fatty acids. By contrast for rat heart, the long chain acyl-CoA synthetase and triglyceride lipase were relatively inactive with docosenoic acid and trierucin as substrate. Consistent with this hypothesis is the finding that pig myocardium does not show the rapid accumulation of erucic acid found in rats and other species. This was shown by our results as well as by results from TRUDELL and FLANZY quoted by ROCQUELIN (1973).

Our rat data is summarized as follows: Our studies were all done with Sprague Dawley rats. Previous results showed female rats do not get cardiac necrotic lesions to the extent male rats do and the incidence is randomly distributed across all dietary treatments. Again, the present results show, and this must be emphasized repeatedly, rapeseed oils fed to male rats do not cause novel or hitherto unrecognized heart lesions, but simply increase the incidence of necrotic myocardial lesions that are commonly

found in rats on socalled control diets, i.e. Purina Chow, Corn Oil, Olive Oil or Soybean Oil. Our pathologists are adamant on this point. The lesions I just presented are clear cut necrotic myocardial lesions and they occur with all dietary treatments. Only the frequency of incidence increases with rapeseed oils.

It is tempting to postulate the existence of a micro constituent in these oils which may directly or indirectly relate to myocardial necrosis. Naturally occurring catecholamines, K deficiency, severe muscular exertion, cold exposure, digitalis intoxication or administration of the drug isoproterenol are known to cause myocardial necrosis in rats. Selye suggests a transcellular electrolytic imbalance as the final common pathway. Calcium accumulates after isoproterenol treatment. The suggestion is made that this may interfere with ATP production and in turn cause cellular disruption. In any case, this drug causes ultrastructural changes in mitochondria i.e. swelling, ruptured cristae and rupture of outer membrane.

Three experiments suggest that the triglyceride fraction of the oil is responsible for myocardial necrosis. TG isolated by either molecular distillation or adsorption chromatography produced lesions and there was no evidence that fewer lesions occur with decreasing quantities of sterol concentration. Lard prepared from swine fed rapeseed oil produced heart lesions and the presence of appreciable quantities of plant sterols or glycosides in lard seems improbable. Since erucic acid added to lard did not result in myocardial necrosis then it appears that we are dealing with a problem in addition to erucic acid in the rapeseed oil triglyceride. The presence of eicosenoic acid, linolenic acid should be considered as well as the low levels of palmitic and stearic acids. Other questions yet to be answered are:

1) the position of long chain monoenes in the triglyceride molecule of the oils and in the fat of animals to which these oils are fed;
2) the position of the double bonds in the long chain fatty acids isolated from animal tissues after feeding rapeseed oils containing eicosenoic and docosenoic acids.

Finally it should be kept in mind that triglyceride metabolism in the rat is probably quite different from triglyceride metabolism in the pig and that any changes in rapeseed oil TG's we introduce to decrease the incidence of myocardial necrosis in the rat may not improve the nutritional properties of these oils fed to other species such as the pig.

References

1. CRAIG, B. M. and J. L. BEARE (1967):
Can. J. Biochem. 45, 1075

2. ROCQUELIN, G. (1973):
Can. Nutrit. Diet. VIII 2, 103-116

3. RUDEL, L. L. and M. D. J. MORRIS (1973):
Lipid Res. 14, 364