EFFECTS OF OLEIC AND STEARIC ACIDS ON DEVELOPMENT OF AZASERINE-INDUCED PANCREATIC CARCINOGENESIS IN RATS: CHANGES IN FATTY ACID COMPOSITION AND DEVELOPMENT OF ATYPICAL ACINAR CELL FOCI

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SUMMARY : Dietary fat has been shown to enhance pancreatic carcinogenesis, but little is known of the effect of individual fatty acids. Administration of diet with altered fatty acid contents appears to effect the composition of rat pancreatic acinar cells. If administration of fatty acids can indeed alter the tissue fatty acid composition of pancreas, and can effect the development of preneoplastic foci, then it is possible that this may reflect in the target cells. In this study, in order to examine the particular effects of unsaturated (oleic) and saturated (stearic) fatty acids in development of pancreatic carcinogenesis, the fatty acid profiles of pancreatic tissues have been analyzed at a single time period (12 months). The findings of this study showed that in azaserine initiated oleic acid fed rats (AzOl Group) oleic acid caused an enrichment of tissue oleic acid content which was accompanied by a decrease in polyunsaturated linoleic, arachidonic and saturated stearic fatty acid levels. The results of this study may suggest that oleic acid can block the desaturation/elongation reactions leading from linoleic to arachidonic acid that polyunsaturated fatty acids may promote pancreatic carcinogenesis in this manner. The possibility remains that oleic acid may enhance tumorigenesis by a mechanism independent of prostaglandin production.

Key Words: Pancreas, carcinogenesis, oleic acid, stearic acid, linoleic acid.

INTRODUCTION

It has long been known that low concentrations of unsaturated fatty acids in the diet can promote tumor development in azaserine initiated rat pancreas. The promotive effects appear to be related to increased fatty acid contents of pancreatic cells (1). Previous studies have shown that dietary fatty acids can change fatty acid composition of tissue lipid levels (2). It has been suggested that this may alter various properties of cellular membranes such as fluidity, permeability and distribution of receptor sites. Any such changes could exert an influence on fatty acid composition of various tumors and subsequently to tumor development (3,4). From epidemiological and experimental studies it appears that oils rich in polyunsaturated fatty acids (PUFAs), in contrast to those rich in saturated fatty acids (SFAs) may enhance pancreatic carcinogenesis

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(5,6). Several studies have shown that unsaturated fatty acids may increase the size and number of atypical acinar cell foci (AACF) in the pancreas of rats (1,7). *In vitro* studies suggest that oleic acid may increase the proliferation of normal and preneoplastic mammary cells, whereas saturated stearic acid does not (8). Apoptolov *et. al.* (9) reported that there is a consistent increase in oleic acid content relative to stearic acid in transferred membrane cells that may reflect protein kinase activity known as a tumor marker in cell membranes (10,11). However, the roles of oleic and stearic acids seem to be more complex since, oleic acid blocks metabolic cooperation and intercellular communication between cells (12). There are some indications that in the azaserine rat model, pancreatic lesions may increase both in incidence and number by feeding with unsaturated fatty acids (13). However, no study has been carried out so far on the effects of pure dietary stearic and oleic acids on pancreatic carcinogenesis. The aim of the present investigation was to determine whether a diet with 20% stearic or oleic acid can inhibit/promote development of putative precursor lesions in the azaserine-initiated rats. It has been suggested that linoleic acid is responsible for the promoting effect of dietary polyunsaturated fatty acids on pancreatic carcinogenesis via an accelerated prostaglandin synthesis, caused by metabolism of linoleic acid derived arachidonic acid in preneoplastic tissues. The purpose of the present study was to investigate whether saturated or unsaturated fatty acids change in the pancreatic tissues can cause tumor promotion. The present study carried out by using fatty acids rather than olive oil and animal fats in order to reduce any complicating influence of other fatty acids or impurities.

MATERIALS AND METHODS

Animals

Male inbred Leeds strain rats were obtained from our breeding colony and were housed and kept five animals to a cage under standard conditions (room temperature 23°C, lighting 7am-7pm), on sawdust bedding. Standard diet (Paterson and Christopher Hill Group, Porton-Rat diet PRD) and tap water were supplied *ad libitum*.

Materials

Azaserine, oleic acid and stearic acid (98% purity), and fatty acid methyl esters (FAME) were obtained from Sigma Chemical Co. Ltd, UK. Acetone, formalin, HCl, boron trichloride, chloroform, methanol and trimethylpentane were obtained from BDH Chemicals Ltd, UK.

Table 1: Constituents of test diets.

INGREDIENTS	Control Diet %	High - fat Diet %
Ash	5.3	4.24
Crude protein	17.9	14.32
Crude oil	2.4	22.4
Crude fibre	3.6	2.88
Calcium (Ca)	0.82	0.65
Phosphorus (P)	0.73	0.54
Sodium (Na)	0.32	0.25
Potassium (K)	0.72	0.57
Magnesium (Mg)	0.1	0.13
Chloride (Cl)	0.47	0.38
Starch	45	36
Threonine	6.6	5.28
Glycine	9.4	7.52
Valine	9.0	7.20
Cystine	2.8	2.24
Methionine	3.1	2.48
Isoleucine	7.8	6.24
Leucine	14.3	11.44
Tyrosine	6.6	5.28
Phenylalanine	8.5	6.8
Lysine	1.0	0.8
Histidine	4.4	3.52
Arginine	11.1	8.88
Tryptophan	2.1	1.68
Vitamins (per kg)		
Vitamin A (I.U.)	15.000	12.000
Vitamin D (I.U.) (Chalecalciferol)	2.400	1.900
Vitamin E (I.U.) (α - Tocopherol)	88	70.4
Vitamin K mg	150	120
Vitamin B1 mg	12	9.6
Vitamin B2 mg (Riboflavin)	13	10.4
Niacin mg	83	66.4
Panthothenic acid (mg)	20	16
Folic acid (mg)	3.1	2.48
Vitamin B12 (mg) (Cyanocobalamin)	0.08	0.063
Trace elements		
Manganese (Mn)	63.0	50.4
Copper (Cu)	14.0	11.2
Cobalt (Co)	0.40	0.32
Iron (Fe)	106.0	84.8
Iodine (I)	0.28	0.22
Selenium (Se)	0.17	0.13
Chromium (Cr)	0.49	0.39
Zinc (Zn)	6.0	1.0

Table 2: The fatty acid composition of the test diets (expressed as percentages of fatty acids of dietary fat and of the total diet were as follows).

Fatty acid	Standard diet (3% fat)		Oleic acid diet		Stearic acid diet	
	Diet fat	Total diet	Diet fat	Total diet	Diet fat	Total diet
Myristoleic acid (14:0)	0.55	0.002	0.04	0.02	0.08	0.02
Palmitic acid (16:0)	16.56	0.46	1.59	0.37	1.62	0.37
Palmitoleic acid (16:1)	1.24	0.34	0.13	0.27	1.18	0.27
Stearic acid (18:0)	2.11	0.06	0.22	0.05	87.63	20.05
Oleic acid (18:1)	23.44	0.64	90.67	20.5	12.22	0.51
Linoleic acid (18:2)	45.16	1.24	4.37	0.99	4.33	0.99
Linolenic acid (18:3)	3.17	0.09	0.31	0.07	0.31	0.07
Decosahexaenoic acid (22:6)	2.73	0.08	2.65	0.60	2.62	0.60

Preparation of diets

Stearic acid or oleic acid was added to the stock standard diet to a 20% w/w concentration. Despite the stability of oleic and stearic acids, diets were freshly prepared every 2 weeks and stored at 4°C to minimize fatty acid degradation. The stock diet was used as the control diet. The composition of the feeds is given in Table 1. The diets were formulated to ensure that they contained sufficient essential fatty acids and were tested over 12 months period in the groups that did not receive carcinogens. The animals weighed at the end of the test period and this served as a measure of their growth rate (Table 2).

Treatment of animals

Starting at two weeks of age, male rats received a single weekly i.p. (30 mg/kg body weight) dose of azaserine for 3 weeks, dissolved in 0.9% NaCl solution on the day of injection. At 28 days of age, 30 male azaserine-initiated and 30 untreated rats were randomly divided into 6 groups of 10 animals each as follows: Group 1 (UnCt) Untreated control rats; Group 2 (AzCt) Azaserine-initiated control rats; Group 3 (AzOl) Azaserine-initiated 20% oleic acid-fed rats; Group 4 (AzSt) Azaserine-initiated 20% stearic acid-fed rats; Group 5 (OlCt) Normal rats without azaserine injection fed 20% oleic acid; Group 6 (StCt) Normal rats without azaserine injection fed 20% stearic acid. The rats of all groups were sacrificed at the end of 12 months after starting feeding.

Stereological analysis

The entire rat pancreas was excised at autopsy (only a very small portion, 100 μg was taken for fatty acid analysis), and all adherent fat, mesentery and lymph nodes were carefully trimmed off. The wet weight of each pancreas was recorded before fixation in 10% buffered neutral formalin. Before immersion in the fixative solution, each pancreas was spread out on a piece of porous paper to ensure maximal transectional area for subsequent sectioning. Thus, a single section of maximal area was obtained for stereology from each pancreas. Histological sections were stained with haematoxylin and eosin and were examined by light microscopy. Foci and tumors in the sections were identified and classified according to the established criteria (14,15). The total area of exocrine pancreatic tissue was measured directly in a single histological section from each pancreas by means of a VIDS III video image analyzer (Analytical Measuring Systems, Cambridge). This system included microscope, video camera, and data capture software. The same instrument was used to count acidophilic and basophilic AACF and to measure their transectional area. The observed data were processed numerically by a computer software package (Volugen), which uses an algorithm based upon the mathematical formula of Campbell *et. al.* (16), as modified by Pugh and his co-workers (17). In this model foci with areas below reliably detectable values are subtracted from the total number of focal intersections counted. The actual numerical lower limits adopted were 0.005 mm^2 for basophilic AACF and 0.01 mm^2 for the acidophilic variety. These values correspond to those chosen by Rao's group (14) in quantitative studies of pancreatic carcinogenesis.

Fatty acid analysis of pancreatic tissues

For fatty acid analysis, samples of pancreatic tissue from each of the rats killed at the 12 months stage of the fatty acid feeding experiment were taken. These were kept at -70°C under nitrogen gas to prevent oxidation. Lipids were extracted using methanol and chloroform-methanol as described by Folch (18).

The lipid extract was dried down under nitrogen and was saponified by incubation at 65°C for 30 minutes to release the free fatty acids. Following acidification with HCl, the free fatty acids were taken up into benzene. Fatty acid methyl esters (FAME) were derived by incubation with 15% boron trichloride in methanol at 95°C for 10 minutes (19). Unwanted salts were taken up and removed with distilled water and the FAME was dried down and dissolved in trimehylpentane. The FAME were analyzed by temperature-programmed (160°C to 260°C at 4 per minute) gas-liquid chromatography, using Philips PU4550 gas chromatography, with a 2.1 mm x 2 m ID glass column packed with 3% SP-2310/2% SP-2300 on 100/200 mesh Chromosorb W (Superco Ins.). The carrier gas was nitrogen at a flow rate of 20 ml/minute and individual FAME standards were used against which the chromatogram peaks were calibrated. A sample control was also run as a check on extraction efficiency. The percentage concentrations of fatty acids were calculated from the absolute fatty acid concentrations derived from the areas under the chromatogram peaks.

Statistical analysis

Numbers of pancreatic foci, mean values and either standard errors of means or ranges were determined for all data. Non-parametric statistical analyses were performed using the Kruskal-Wallis one-way analysis of variance, the Mann-Whitney U-test and F-test. The differences between groups were considered significant where $p < 0.05$, comparisons with the appropriate controls are reported.

RESULTS

By the end of experimental period AzCt (Group 2), AzOl (Group 3) and AzSt (Group 4) rats presented with pancreatic nodules of various sizes. However, no metastases were observed in the lungs, livers or other intestinal organs of the tumor-bearing animals.

Body weight

After 12 months, the mean body weight of AzOl (Group 3) was considerable higher than that of AzSt rats (Group 4) (Table 3). The difference between AzOl (Group 3) and AzSt (Group 4) rats was statistically significant, but differences between AzCt (Group 2) and AzOl (Group 3) failed to reach significance level. The OlCt (Group 5) and StCt (Group 6) animals were significantly lighter than the UnCt controls (Group 1).

Pancreatic weight

At 12 months, AzOl rats (Group 3) had a greater mean pancreatic weight than did AzSt rats (Group 4) (Table 3). At the same time, the mean pancreatic weights of AzCt rats (Group 2) were also greater than that of AzSt rats (Group 4), but neither of the differences reached a significant level. However, OlCt rats (Group 5) had significantly smaller pancreatic weights than UnCt rats (Group 1).

Basophilic AACF and their quantitative analysis

The end of period only the azaserine-initiated groups (AzCt, AzOl and AzSt) showed the presence of pancreatic basophilic AACF in pancreata (Table 4). The quantitative parameters of basophilic foci in AzOl rats (Group 3) were found to be lower than in AzCt rats (Group 2). AzCt rats had significantly higher numbers of foci per unit area (0.0074 vs. 0.0024), unit volume (0.0213 vs. 0.0066) per pancreas (31.901 vs. 8.304) and volume of foci as a per cent of the pancreas (0.047 vs. 0.013) than AzOl (Group 3) rats. The mean diameter and mean volume of the foci

Table 3: Effect of 12 months of feeding oleic or stearic acid, following initiation with azaserine, on rat body and pancreatic weights (Mean value ± standard error of mean).

Group	(1) UnCt	(2) AzCt	(3) AzOl	(4) AzSt	(5) OlCt	(6) StCt
No of rats	10	10	10	10	10	10
Body weight (g)	351.4 ± 35.2	390.5 ± 24.4 *vs. UnCt	393.4 ± 28.8 *vs UnCt	331.0 ± 25.5 ***vs. AzCt ***vs. AzOl	313.8 ± 15.9 **vs. UnCt	295.3 ± 16.0 ***vs. UnCt
Pancreatic weight (g)	1.06 ± 0.23	1.21 ± 0.36	1.15 ± 0.16	0.93 ± 0.23	0.77 ± 0.18 *vs. UnCt	1.078 ± 0.17
Pancreas as % of body weight	0.30	0.31	0.29	0.28	0.21	0.32

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 4: Effect of 12 months of feeding oleic or stearic acid, following initiation with azaserine, on induction of pancreatic basophilic atypical acinar cell foci (AACF) (Mean value ± standard error of mean).

Group	(1) UnCt	(2) AzCt	(3) AzOl	(4) AzSt	(5) OlCt	(6) StCt
No of rats	10	10	10	10	10	10
No of AACF per (mm^2)	0	0.0074 ± 0.0045	0.0024 ± 0.0018 * vs AzCt	0.0039 ± 0.0026 * vs. AzCt	0	0
No of AACF per (mm^3)	0	0.0213 ± 0.0208	0.0066 ± 0.0054 **vs. AzCt *vs. AzSt	0.0133 ± 0.0102	0	0
No of AACF per pancreas	0	31.90 ± 30.75	8.30 ± 7.87 *vs. AzCt	11.82 ± 7.86 * vs UnCt	0	0
Volume of AACF as % of pancreas	0	0.047 ± 0.034	0.013 ± 0.011 * vs AzCt	0.077 ± 0.016 * vs. AzCt	0	0
Mean focal diameter (mm)	0	0.370 ± 0.213	0.280 ± 0.208	0.281 ± 0.179	0	0
Mean focal volume (mm^3)	0	0.039 ± 0.053	0.020 ± 0.017	0.018 ± 0.020	0	0

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

were also larger in the AzCt (Group 2) rats, when compared with AzOl (Group 3) rats, but the differences did not reach significant levels. In AzSt rats (Group 4) the basophilic AACF exhibited higher quantitative parameters in comparison with those of the AzOl group, except for the focal diameter and volume, but these differences were not statistically significant. Compared with AzSt rats the AzCt rats (Group 2) had higher numbers of basophilic AACF per unit area (0.0074 vs. 0.0039), per unit volume (0.0213 vs. 0.0133) and per pancreas (31.901 vs. 11.82). The mean diameter (0.370 vs 0.281) and mean volume of foci (0.039 vs. 0.018) were also greater in AzCt animals. Only the volume of foci as a per cent of the pancreas (0.047 vs. 0.077) was smaller than that of AzSt rats (Group 4).

Acidophilic AACF

The 12 months acidophilic AACF quantitative data are shown in Table 5. AzOl rats (Group 3) had slightly higher quantitative parameters than those of AzCt (Group 2) rats, except for the number of foci per mm^3 in pancreas. The differences between these two groups, except for the mean focal diameter and mean focal volume, were statistically significant. However, the difference between AzSt rats (Group 4) and AzOl rats (Group 3) was striking at 12 months. Thus, acidophilic AACF in AzOl rats were about three times larger than those of AzSt rats (1.766 vs. 0.642), they occupied 16 times more of the pancreatic volume (7.868 vs 0.488) and the number of foci per pancreas was four times higher (23.77 vs. 5.94). Interestingly, AzSt (Group 4) showed significantly decreased quantitative AACF parameters when compared with AzCt rats (Group 2), in all respects: the foci in AzSt rats were about half in size, occupied only one fifth as much as pancreatic volume (0.448 vs. 2.161) and there were half as many per pancreas (5.94 vs. 12.03) (Table 5).

Pancreatic fatty acid profiles

The fatty acid profiles of the pancreas reflected the different diets administered. The fatty acid profiles of the pancreatic tissues at 12 months showed that feeding either oleic or stearic acid produced relatively selective alterations in the fatty acid content of pancreatic tissue(Table 6). The changes reflected the diet being fed; enriching the diet with stearic acid or oleic acid increased the tissue content of the same fatty acid within the pancreas, with or without carcinogen treatment. In addition, the linoleic and arachidonic acids amounts were reduced by oleic acid feeding, also linoleic acid was significantly lower in AzOl rats ($p<0.001$).

Table 5: Effect of 12 months of feeding oleic or stearic acid, following initiation with azaserine, on induction of pancreatic acidophilic atypical acinar cell foci (AACF) (Mean value ± standard error of mean).

Group	(1) UnCt	(2) AzCt	(3) AzOl	(4) AzSt	(5) OlCt	(6) StCt
No of rats	10	10	10	10	10	10
No of AACF per (mm^2)	0	0.015 ± 0.006	0.037 ± 0.017 *** vs. AzCt *** vs. AzSt	0.007 ± 0.005 * vs AzCt	0	0
No of AACF per (mm^3)	0	0.0240 ± 0.0450	0.0220 ± 0.0106 *vs. AzCt **vs. AzSt	0.0050 ± 0.0061 *vs. AzCt	0	0
No of AACF per pancreas	0	12.03 ± 5.71	23.27 ± 10.95 *vs. AzCt **vs. AzSt	5.94 ± 6.39 *vs. AzCt	0	0
Volume of AACF as % of pancreas	0	2.161 ± 1.759	7.868 ± 3.249 *** vs AzCt ***vs. AzSt	0.448 ± 0.477 ** vs AzCt	0	0
Mean focal diameter (mm)	0	1.622 ± 0.464	1.766 ± 0.297 **vs. AzSt	0.642 ± 0.717 **vs. AzCt	0	0
Mean focal volume (mm^3)	0	0.271 ± 1.765	3.636 ± 2.209 **vs. AzSt	1.221 ± 2.489	0	0

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

DISCUSSION

Dietary fats have been shown to modulate incidence of chemically induced pancreatic cancer in rats: in azaserine initiated rats, a high unsaturated fat diet had been shown to elevate the incidence of pancreatic neoplasia whereas a high saturated fat did not (1). The effect of oleic acid feeding on the body and pancreatic weights of azaserine-initiated rats in this study corresponded closely to those reported in previous findings (1,20,21), in which feeding unsaturated fats increased the body and pancreatic weight of rats. However, the decrease in body weight caused by feeding stearic acid to azaserine-initiated rats in the present study appears to be consistently less than that found in parallel studies in which similarly initiated animals were fed saturated fats (1,20,21). Azaserine initiation alone caused an increase in body weight, feeding oleic acid to azaserine-initiated rats did not diminish this influence. It is generally considered that stearic acid is poorly absorbed (22,23) which could explain the observed decline in animal body weights, but another research claimed that stearic acid absorbed as effectively as oleic acid (24), so that the differences observed in this study are unlikely to be due to differences of absorption. The markedly increased pancreatic weights of AzOl rats probably reflects the promoting effects of oleic acid in azaserine initiated rats but, there was no indication of any such effect by stearic acid feeding on azaserine initiated rats. The data observed in the present study show that basophilic foci were more numerous in AzCt rats than in AzOl or AzSt rats. It has been claimed that basophilic AACF (25) seem to have only a low potential for growth and progression to neoplasms, whereas acidophilic foci seem to have the potential for such progression (14). It has been postulated that basophilic foci may be capable of progression to acidophilic foci and become involved in tumor development (15). The results of the present study point to an enhancing effect of monounsaturated oleic acid in azaserine initiated rats that the diameter of acidophilic foci, number of foci per pancreas and per cent volume of foci in the pancreas were significantly greater in AzOl rats compared with AzSt fed rats. This suggests that the type of dietary fat is a significant factor in the induction of pancreatic foci during the post-initiation stage in azaserine initiated rats. In the light of the foregoing, this decrease in focal parameters of AzSt rat pancreata may indicate that stearic acid can not be a promoter of acidophilic foci. These results are in agreement with those of Tinsley (2) who found that increasing the level of

Table 6: Effect of 12 months of feeding oleic or stearic acid on the fatty acid profiles of total lipid extracts of rat pancreatic tissue (Mean value ± standard deviation).

Group	(1) UnCt	(2) AzCt	(3) AzOl	(4) AzSt	(5) OlCt	(6) StCt
No of rats	10	10	10	10	10	10
Stearic acid	8.73 ± 1.07	8.34 ± 2.51	9.34 ± 0.32	10.46 ± 0.76	9.16 ± 0.17	18.03 ± 1.06 ** vs. UnCt
Oleic acid	8.20 ± 1.70	8.95 ± 1.75	36.86 ± 1.40 ***vs. UnCt	6.85 ± 2.06	41.05 ± 1.81 ***vs. UnCt	12.63 ± 6.38 * vs. UnCt
Stearic / Oleic Ratio	1.12 ± 0.06	0.89 ± 0.06	0.293 ± 0.01 ***vs. UnCt	1.56 ± 0.11 **vs. UnCt	0.22 ± 0.01 **vs. UnCt	1.43 ± 0.12 **vs. UnCt
Linoleic acid	25.13 ± 1.15	21.03 ± 1.52	11.72 ± 0.58 *** vs. UnCt	22.35 ± 1.15 *** vs. UnCt	11.73 ± 0.38 *** vs. UnCt	24.15 ± 2.69
Arachidic acid	0.08 ± 0.03	0	70.32 ± 0.13 ***vs. UnCt	0.04 ± 0.03	78.03 ± 0.05 *** vs. UnCt	0.12 ± 0.08
Arachidonic acid	32.71 ± 1.53	33.91 ± 3.83	16.2 ± 1.54 **vs. UnCt	33.61 ± 0.58	17.1 ± 0.77 ** vs. UnCt	24.16 ± 1.15

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

stearate in high-fat diets was associated with decreased tumor incidence and increased time to tumor development in C3H mice.

The pancreatic fatty acid content reflected the diet administered, and magnitude of fatty acid change suggests that this may have been an important factor in the promotion of preneoplastic foci. The fall of the linoleic and arachidonic acid fractions on high fat diets was more pronounced when rats fed with 20% oleic acid.

The stimulation of growth by oleic acid, whose metabolites are not converted to prostaglandins and linoleic acid, suggests that an additional mechanism besides that of prostaglandin production should be responsible for the stimulatory effect of unsaturated fatty acids (27). In this study, enrichment of tissues oleic acid was accompanied by a decrease in polyunsaturated and saturated fatty acids. This may suggest that oleic acid may replace both unsaturated and saturated fatty acids. Since oleic acid can compete with the reactions leading from linoleic acid to arachidonic that it can limit prostaglandins synthesis (28). The possibility remains that oleic acid may enhance. As a result, in the present study quantitative stereological assessment of azaserine-initiated and oleic or stearic acid fed rats has demonstrated that 20% oleic acid in diet promotes development of acidophilic AACF in rat pancreas however, stearic acid has no such promoting effect. In order to analyze further the mechanism of action of oleic/stearic acids on pancreatic carcinogenesis it would be useful to evaluate relative ingestion rates of oleic and stearic acids by rats.

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