

## **Influence of conjugated linoleic acid enriched ghee feeding on cancer incidence and histopathological changes in 7,12-dimethyl-benz[a]anthracene induced mammary gland carcinogenesis in rats**

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**CHINNADURAI, K., A. K. TYAGI, P. KRISHNAMOORTHY: Influence of conjugated linoleic acid enriched ghee feeding on cancer incidence and histopathological changes in 7,12-dimethyl-benz[a]anthracene induced mammary gland carcinogenesis in rats. Vet. arhiv 78, 511-520, 2008.**

### **ABSTRACT**

A study was conducted to investigate the influence of Conjugated Linoleic Acid (CLA) feeding on cancer incidence and histopathological changes in DMBA induced mammary gland carcinogenesis rats. Female Wistar rats of 21 days were taken for the study and the animals were randomly divided into three groups of 30 animals each. Group I animals were fed with a soybean based diet, whereas group II and group III animals were fed with a low CLA and high CLA ghee based diet, respectively. Soybean oil, low CLA and high CLA ghee were included at the level of 20 per cent in the animal diet. Feeding with test diets started on the day of weaning. The animals were given 7,12 dimethyl-benz[a]anthracene (DMBA) at 5 mg per animal as a single dose by oral intubation at the age of 55 days and the test diets were continued up to 32 weeks after DMBA administration. At the end of 32 weeks all the animals were sacrificed, tumour data recorded and histopathology performed. The tumour incidence in groups I, II and III was 83.33, 63.33 and 46.07 per cent respectively. Histopathology revealed fibroma, adenoma, fibroadenoma in all the groups whereas in group I showed adenocarcinoma. Therefore, the results of the present study showed CLA not only inhibited benign type tumours but malignant tumours as well.

**Key words:** mammary cancer, linoleic acid, dimethyl-benz[a]anthracene, histopathology

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## Introduction

Diet as a mean of controlling and reducing the incidence of cancer has received considerable attention. Interest is growing among consumers about natural nutrients and nutraceuticals present in foods with health benefits in humans. One such natural nutrient is Conjugated Linoleic Acid (CLA). CLA occurs naturally in many foods, however the principle dietary sources are dairy products and other foods derived from ruminant animals (CHIN et al., 1992).

Conjugated Linoleic Acid (CLA) is a collective term describing a mixture of positional and geometric isomers of linoleic acid (C18:2). Each of these positional conjugated diene isomers can occur in *cis-trans*, *trans-cis*, *cis-cis* or *trans-trans* geometrical configurations. At least 8 different CLA isomers have been identified. Of these the *cis-9*, *trans-11* and *trans-10*, *cis-12* have so far been proven to have biological activity (BANNI and MARTIN, 1994). Of the two physically important isomers *cis-9*, *trans-11* is the most prevalent comprising 80-90% of the total CLA in food products from ruminants, whereas *trans-10*, *cis-12* is present in small amounts at 3-5% of all CLA (PARODI, 2003). The predominant CLA in ruminant fat is the *cis-9*, *trans-11* isomer which accounts for more than 80% of total CLA isomers in dairy products. CLA is formed as an intermediate during biohydrogenation of linoleic acid by rumen bacteria or from tissue synthesis of CLA by  $\Delta^9$ -desaturase conversion of *trans-11* fatty acids (BAUMGARD et al., 2000)

There is increasing research interest in CLA and its potential health benefits such as its anticarcinogenic, antiatherogenic, antidiabetic and antiadipogenic effects in animals as well as humans (BELURY, 2002). CLA has received considerable attention as a chemo preventive agent in the past few years since being shown to inhibit rat mammary tumourigenesis, mouse fore stomach neoplasia, and mouse skin carcinogenesis. Additional evidence of carcinogenesis inhibition by CLA has been found in dimethyl-benz[a]anthracene - induced tumourigenesis of the skin, mammary glands and fore stomach neoplasia (HA et al., 1990). Feeding CLA enriched butter fat to rats inhibited mammary tumors by 53% compared with those fed butter fat with normal levels of CLA (IP et al., 1999). Feeding as little as 0.1g CLA /100 g of diet resulted in a reduction in the number of mammary tumors in rats (IP et al., 1994). Rats fed with 1% CLA from weaning at 21 days of age to 55 days of age and then treated with a chemical carcinogen would have long lasting protection from tumor development. Therefore, the aim of our experiment was to study conjugated linoleic acid enriched ghee feeding on cancer incidence and histopathological changes in 7,12 dimethyl-benz[a]anthracene - induced breast cancer in rats.

## Materials and methods

*Ghee preparation.* Low (6.92 mg/g fat) and high CLA (19.54 mg/g fat) ghee was prepared from milk from buffaloes fed on groundnut oil cake and mustard cake plus

2% mustard oil based concentrates, respectively. By creamery method the cream was separated by centrifugation and ghee was prepared directly from the cream. The milk was heated to 40 °C and the cream was separated in a cream separator. The cream was heated to 115 °C in a stainless steel jacket ghee kettle fitted with an agitator, steam control valve, pressure and temperature gauges and a movable, hollow, stainless steel tube centrally bored for emptying out the contents. Heating was discontinued as soon the color of the ghee residue turned to golden yellow. The level of Total CLA was estimated by using HPLC as described by (WERNER et al., 1992)

*Experimental Animals.* Female Wistar rats, 21 days of age (32 to 38 g of body mass) were housed in Polypropylene cages and maintained in a Small Animal House, NDRI. Room temperature was  $24 \pm 1$  °C with 12:12 h light/dark cycle. The animals were randomly divided into 3 groups of thirty animals each and group I animals fed with a soybean based diet whereas group II and group III animals were fed with low CLA and high CLA ghee based diets, respectively. The composition of the diets offered to the animals is listed in Table 1. The feeding experiment was conducted for a period of 32 weeks after a 7, 12 DMBA injection. Water and the diet were given to the animals on an *ad libitum* basis. The study was approved by the Institutional Animal Ethics Committee (IAEC) and the rats were maintained in accordance with the National Institute of Nutrition, India guidelines for care and use of laboratory animals.

Table 1. Composition of three experimental diets (g/kg of diet)

Component	Soybean oil based diet	Low CLA ghee diet	High CLA ghee based diet
Bengal gram	540.0	540.0	540.0
Wheat	130.0	130.0	130.0
GNC	60.0	60.0	60.0
Soybean oil	200.0	-	-
Low CLA ghee	-	200	-
High CLA ghee	-	-	200
Skimmed milk powder	44.4	44.4	44.4
Mineral mixture*	21.6	21.6	21.6
Vitamin mixture*	2.0	2.0	2.0
Choline chloride*	2.0	2.0	2.0

\* Prepared and mixed according to the method of Association of Official Analytical Chemist (1995)

*Tumour induction.* At 55 days of age, each animal was administered with 5 mg 7,12-dimethyl-benz[a]anthracene (DMBA) in soybean oil by oral intubation as a single dose. The animals were palpated weekly to determine the time of appearance and location of tumors. At the end of the experimentation period, the animals were sacrificed by cervical

dislocation. At Necropsy, mammary glands were exposed and the tumors were excised and measured. Tumour incidence and tumour mass were calculated. A portion of the mammary gland was preserved in 10% formal saline for histopathological study. The washed tissue was dehydrated in the ascending grades of isopropanol and finally cleared in xylene. The tissue was then embedded in molten paraffin wax. Sections were cut at 5  $\mu$ m thickness and stained with hematoxylin and eosin (BANCROFT and STEVENS, 1996).

## Results

The rats showed incidences of diarrhoea, particularly those given the CLA ghee diets at the start of experimental feeding, however, the rats adjusted to the respective diets and at the time of DMBA administration all rats were free from diarrhea, irrespective of dietary treatment. After DMBA administration, the rats appeared dull, off feed and started losing mass in all the groups as compared to those not exposed to the carcinogen. Rats fed on CLA ghee based diets started gaining body mass after 2 months from the carcinogen injection, and appeared more active than those fed on the soybean oil based diet.

Table 2. Effect of soybean oil/low CLA ghee/high CLA ghee/ on mammary gland tumour incidence in 7,12 DMBA administered rats

Groups	Percent tumour incidence*	Percent Individual tumour incidence				Mean tumour mass (g)
		Fibroma	Adenoma	Fibro adenoma	Adenoma sarcoma	
Soybean oil	83.33 (25/30)	36.00 (9/25)	24.00 (6/25)	28.00 (7/25)	12.00 (3/25)	3.30 $\pm$ 1.39
Low CLA ghee	63.33 (19/30)	42.11 (8/19)	21.05 (4/19)	36.84 (7/19)	0.00	2.42 $\pm$ 0.88
High CLA ghee	46.70 (14/30)	57.14 (8/14)	14.29 (2/14)	28.57 (4/14)	0.00	1.29 $\pm$ 0.27

Rats were killed 32 weeks after DMBA administration; \*Includes both gross and histopathological examination

*Cancer incidence.* Table 2 summarizes the data on tumour incidence and mean tumour mass from each cancer group. CLA ghee feeding produced a statistically significant ( $P < 0.05$ ) reduction in tumour incidence. High CLA ghee fed rats had lower ( $P < 0.05$ ) mean tumour mass than the other two groups. Fibroma, adenoma, and fibroadenoma were the common types of tumours in all three groups. However, malignant type tumours (adenocarcinoma) were only detected in the rats fed on soybean oil diet.

### *Gross and histopathology*

*Soybean oil fed group.* The diameter of mass ranged from 0.5 to 3.8 cm (Fig. 1). On palpation, tumours were found to be hard and firm in nature. Initially, the growth was

small but it increased in size progressively with the passage of time. The mass of the tumors ranged from 0.3 to 16.14 g with a mean value of 3.30 g.

The histopathological examination (Table. 2) indicated that, the neoplasm types were fibroma, adenoma, fibro adenoma and adenocarcinoma. The most common type cancer was fibroma, which was characterized by increased stromal tissue elements with epithelial cells (Fig. 2). Adenomas were characterized by numerous acini with stromal elements and nucleus of benign type (Fig. 3) whereas fibroadenoma was characterized by a high amount of fibrous stroma along with acini containing secretions. Adenocarcinoma type tumors were only found in the soybean oil fed group. They were characterized by anisocytosis and anisokariosis of nucleus containing one or two nucleoli, and stromal invasions (invasion of basement membrane by the cluster of alveolar cells) with tumor cells. The mitotic nuclei were also seen (Fig. 4). Metastasis stage was not detected despite tumor malignancy in the autopsies. The induced DMBA tumors in this study were multifocal and locally aggressive but no single case of metastasis was identified. Epithelial and myoepithelial cell proliferation were observed in most of the induced tumors in this group.

*Low CLA group.* The diameter of the tumors ranged from 0.3 to 2.4 cm and their mass ranged between 0.24 to 9.6 g with a mean value of 2.42 g. The neoplasm types were fibroma, adenoma, and fibro adenoma. The most common type was fibroma, characterized by spindle shaped fibrous tissue with oval or flattened nuclei, which were benign. The adenomas were characterized by acini containing hyper plastic nuclei and anisokaryosis

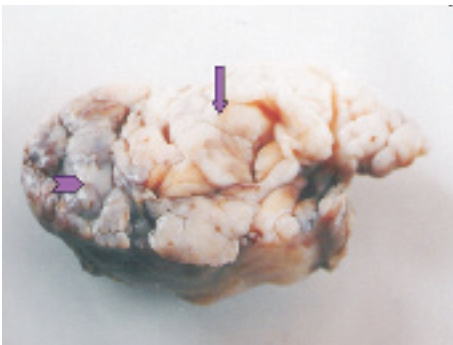


Fig. 1. Soybean oil based diet: fixed tumour size measuring approximately  $4.8 \times 3.2$  cm showing white area (arrow) and necrosed area (arrow head)

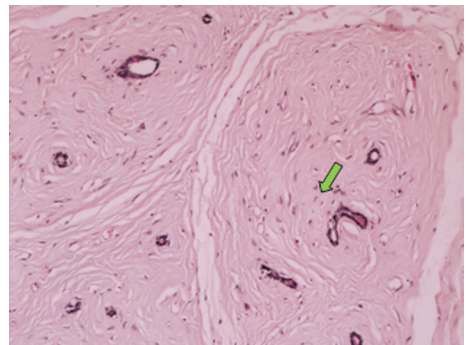


Fig. 2. Soybean oil based diet: fibroma with increased stromal tissue elements with epithelial cells (arrow). H&E;  $\times 200$ .



and fibrous stroma dividing the gland into numerous lobules (Fig. 5) whereas the fibro adenoma showed a moderate amount of fibrous tissue stroma with alveoli containing benign nuclei.

*High CLA group.* The diameter of the tumors ranged from 0.2 to 1.6 cm and their mass ranged from 0.26 g to 3.56 g with a mean value of 1.29 g. The most common type of tumor was fibroma, which was characterized by fibrous tissue stroma with no epithelial

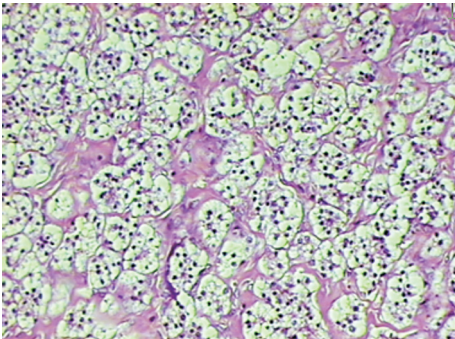


Fig. 3. Soybean oil based diet: adenoma showing numerous acini with stromal elements and nuclei

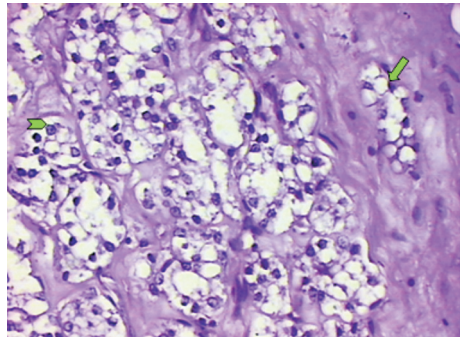


Fig. 4. Soybean oil based diet: adenocarcinoma showing anisokariosis and anisocytosis of nucleus containing one or two nucleoli (arrow head) and stromal invasions (arrow) with tumor cells. H&E;  $\times 400$ .

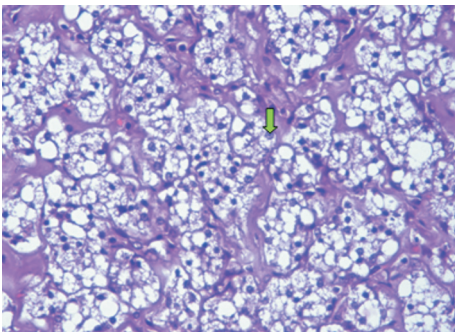


Fig. 5. Low CLA ghee based diet: adenoma showing acini containing hyperplastic nucleus and anisocaryosis (arrow), fibrous stroma dividing the gland into numerous lobules. H&E;  $\times 400$ .

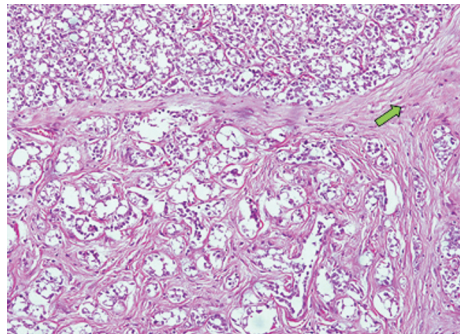


Fig. 6. High CLA ghee based diet: adenoma showing acinar pattern with moderate stromal elements (arrow). H&E;  $\times 200$ .

cells. Adenoma showed acinar pattern with moderate stromal elements (Fig. 6). Mild amount of stromal elements with numerous acini and few acini were filled with secretions in fibrous adenoma.

## Discussion

Malignant tumour (adenocarcinoma) was only detected in the soybean oil fed group, which showed that CLA inhibited not only benign types of tumours but also malignant tumours. IP et al (1991) reported that feeding CLA at 0.5, 1.0 and 1.5 percent lowered cancer incidences by 32, 56 and 60 percent respectively against the control group. BENJAMIN et al. (2003) injected single doses of methyl nitrosourea (50 mg/kg b wt) intra peritoneally at 52 days of age in female Sprague-Dawley rats, and detected 93 percent cancer incidences in the control group whereas the CLA fed groups (at the level of 10% in diet contains CLA of 37 mg/g fat) had only 47 percent cancer incidence and concurred with other findings. CLA feeding by cow ghee/ buffalo ghee (10% in diet) showed a 25 percent lower cancer incidence than in soybean oil fed rats (BHATIA, 2005)

In another study IP et al. (1999) reported that feeding butter fat CLA (0.8 %) to rats during the time of pubescent mammary gland development lowered ( $P < 0.05$ ) cancer incidence (53%) compared to the control group (93%). They also suggested that CLA feeding during pubescent mammary gland development lowered the population and proliferate activity of the target Terminal End Buds (TEB) cells, and therefore reduced mammary cancer risk when the rats are challenged with a carcinogen. TEB cells are the target cells for mammary chemical carcinogenesis. In the present study feeding of CLA ghee also started during the pubescent period of mammary gland development and led to 37 percent lower cancer incidences than in the control group. Feeding CLA during the early part of life before the initiation of tumour by carcinogen would provide life long protection against mammary cancer and later in life after the initiation of tumour development, life time CLA supplementation would be required to obtain significant protection from carcinogenesis (IP et al., 1997).

RUSSO et al. (1977) reported that 20 mg DMBA administration in virgin female Sprague dawley rats by oral intubation produced both benign and malignant type tumors and numerous lesions in the rats' mammary glands. In adenocarcinomas the target of carcinogenic stimulus is the terminal end buds (TEB) but benign lesions such as adenomas, hyper plastic alveolar nodules and cysts are derived from more differentiated portions of the gland, namely the alveolar buds and lobules. In the present study, adenocarcinoma observed in soybean oil fed rats showed the stromal infiltration of tumour cells which was indicative of a low degree of malignancy, however adenocarcinomas were not detected in CLA fed rats. CLA triggers apoptosis through up regulation of cell signal systems at the level of gene expression, both mRNA and protein (WAHLE and HEYS, 2002) or through

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oxidative stress mediated by protein kinase C and NADPH oxidase pathways (BERGANO et al., 2004). CLA also down regulates cyclooxygenase-2 activity related to carcinogenesis and inflammation (WATKINS et al., 1999; IWAKIRI et al., 2002).

### **Conclusion**

This study showed that CLA supplemented diets significantly lowered cancer incidence. CLA not only inhibited benign type tumours but malignant tumours as well. Prevention, rather than therapy, must be an important strategy for conquering cancer and CLA could well do that, although CLA is not a cancer drug but it would be useful in addition to other cancer treatments. The successful therapy of breast cancer will have to identify natural products that have significant therapeutic and preventive potential without the toxic side effects of the common anticancer drugs. Further study has to be conducted on molecular levels to know the exact action of CLA on cancer control.

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**CHINNADURAI, K., A. K. TYAGI, P. KRISHNAMOORTHY: Utjecaj davanja maslaca obogaćenoga konjugiranom linolnom kiselinom na incidenciju i patohistološke promjene raka mliječne žlijezde štakorica uzrokovanoga 7,12-dimetilbenz[a]anthracenom. Vet. arhiv 78, 511-520, 2008.**

**SAŽETAK**

Istraživan je utjecaj davanja maslaca od bivoljega mlijeka obogaćenoga konjugiranom linolnom kiselinom na incidenciju i patohistološke promjene u nastanku raka mliječne žlijezde štakorica uzrokovanoga 7,12-dimetilbenz[a]anthracenom. Wistar štakorice u dobi od 21 dana bile su nasumce podijeljene u tri skupine po 30 životinja. Prva skupina bila je hranjena hranom na bazi soje, dok je druga dobivala hranu na bazi bivoljega mlijeka s malom koncentracijom konjugirane linolne kiseline, a treća hranu s dodatkom veće količine konjugirane linolne kiseline. Sojino ulje i linolna kiselina dodavani su u hranu u količini od 20%. Hranidba navedenom hranom započela je na dan zalučenja. Svakoj je životinji peroralno dan 7,12-dimetilbenz[a]antracen (DMBA) u količini od 5 mg u dobi od 55 dana, a testirana hrana davana je do 32 tjedna nakon primjene DMBA. Sve su životinje bile žrvovane nakon 32 tjedna te pretražene na pojavnost i patohistološke značajke tumora. Incidencija tumora u I skupini bila je 83,33%, u II skupini 63,33% i u III skupini 46,07%. Patohistološki je ustanovljen fibrom, adenom i fibroadenom u štakorica svih skupina, dok je u štakorica I skupine ustanovljen i adenokarcinom. Rezultati su pokazali da je konjugirana linolna kiselina imala inhibirajući učinak i na dobroćudni i na zloćudni tumor.

**Cljučne riječi:** tumor mliječne žlijezde, konjugirana linolna kiselina, dimetilbenz[a]antracen

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