MOLECULAR MECHANISM OF PROTECTIVE EFFECT OF MILK FAT IN BREAST CANCER

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRYING
(ANIMAL BIOCHEMISTRY)

BY
RITA RANI

ANIMAL BIOCHEMISTRY DIVISION
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL- 132 001 (HARYANA), INDIA

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Regn. No. 1050402

Dedicated to

My Mummy and Papa

for their

Encouragement and Love

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This is to certify that the thesis entitled, **" MOLECULAR MECHAINSM OF PROTECTIVE EFFECT OF MILK FAT IN BREAST CANCER "** submitted by **Ms. RITA RANI** towards the partial fulfilment of the award of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL BIOCHEMISTRY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 14/11/08

Kansal

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MAJOR ADVISOR & CHAIRMAN
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Date: 14 November, 2008

(Rita Rani)

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LIST OF ABBREVIATION

ACF	=	Aberrant crypt foci
ANIT	=	alpha- Naphthyl isothiocyanate
ANOVA	=	Analysis of variance
AOAC	=	Association of official analytical chemist
AOM	=	Azoxymethane
AP-1	=	Activator protein-1
APC	=	Adenomatous polyposis coli
BHT	=	Butylated hydroxytoluene
B(a)P	=	Benzo(a)pyrene
BSA	=	Bovine serum albumin
CDK	=	Cyclin dependent kinase
cDNA	=	Complementary deoxyribonucleic acid
CLA	=	Conjugated linolic acid
CDNB	=	1-chloro-2,4-dinitrobenezene
COX	=	Cyclooxygenease
CYP450	=	Cytochrome P450
DCIS	=	Ductal carcinoma *in situ*
DCPIP	=	Dichlorophenolindophenol
DEPC	=	Diethyl pyrocarbonate
DMBA	=	7,12-dimethylbenz(a)anthracene
DMH	=	Dimethylhydrazine dihydrochloride
DMSO	=	Dimethyl sulphoxide
dNTP	=	Deoxynucleotide triphosphate
EDTA	=	Ethylenediamine tetra-acetic acid
FAD	=	Flavin adenine dinucleotide
GST	=	Glutathione-S-trasferase
GGTP	=	γ-Glutamyltranspeptidase
HAN	=	Hyperplastic alveolar nodules
HMG CoA	=	3-Hydroxy-3-methyl glutaryl coenzymeA
IDPs	=	Intraductal proliferation(s)
IGF-1	=	Insulin like growth factor

IQ	=	2-Amino-3-methylimidazo(4,5-f)quinoline
MFOs	=	Mixed function oxygenase
MOPS	=	3-Morpholinopropanesulfonic acid
M-MuLV	=	Murine-myeloma leukemia virus
MNU	=	N-methyl nitrosourea
NADPH	=	Nicotinamide adenine dinucleotide phosphate reduced
β-NF	=	β-Naphthoflavone
NKK	=	4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone
ODC	=	Ornithine decarboxylase
PAH	=	Polycyclic aromatic hydrocarbon
PCB1	=	Polychlorinated biphenyls
PCR	=	Polymerase chain reaction
PhIP	=	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PG	=	Prostaglandin
15d-PGJ2	=	15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2
PUFA	=	Polyunsaturated fatty acid
p-NP	=	para- Nitrophenol
PPAR-γ	=	Peroxisome proliferator activated receptor-gamma
p- XSC	=	para- 1,4-Phenylene-bis(methylene)selenocyanate
QR	=	Quinone reductase
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse transcription- polymerase chain reaction
TCA	=	Trichloroacetic acid
SCID	=	Severe combined immunodeficient
SD	=	Sprague Dawley
SE	=	Standard error
TARS	=	Thiobarbituric acid reactive substances
TCA	=	Trichloroacetic acid
TZD	=	Thiazolidinedione
TPA	=	12-O-Tetra-decanoyl-phorbol-13-acetate
UDPGT	=	Uridinediphosphoglucuronosyl transferase
UDPGA	=	Uridinediphosphoglucuronic acid

*****

सारांश

स्तन कैंसर महिलाओं में पाया जाने वाला प्रमुख कैंसर है तथा विश्व में महिलाओं में कैंसर से होने वाली मृत्यु का प्रमुख कारण है। विभिन्न महामारी अध्ययन खाद्य वसा तथा स्तन कैंसर उत्पत्ति में परस्पर संबंध दर्शातें है। दुग्ध वसा, प्रतिकर्कट घटकों से युक्त होने के बावजूद कैंसर की वृद्धि में इसकी भूमिका संदेहात्मक है। वर्तमान अध्ययन में घी सोयाबीन तेल की तुलना में, 7, 12 डाईमिथाइल बैंजल एंथरासीन (डी एम बी ए) द्वारा प्रवृत स्तन कैंसर के विरूद्ध सुरक्षात्मक प्रभाव तथा इसकी कार्यविधि का अध्ययन किया गया। स्तन कैंसर को, 55 दिन की आयु पर 30 मिलीग्राम / शारीरिक भार की दर से मुख द्वारा डी एम बी ए खिलाकर प्रवृत किया गया। घी की प्रतिकर्कट क्षमता का उत्तकीय, जैव रसायनिक तथा आण्विक मापदण्डों द्वारा मूल्यांकन किया गया। सोयाबीन तेल पोषित चूहों में ट्यूमर उत्पत्ति का अव्यक्त काल कम था तथा ट्यूमर उत्पत्ति (65.4.1), ट्यूमर भार (6.18 ग्राम) तथा ट्यूमर घनत्व (6285 मिली मीटर^3), गाय घी पोषित चूहों से (क्रमश: 26.6 प्रतिशत, 1.67 ग्राम तथा 1925 मिली मीटर^3) अधिक थे। ट्यूमर के उत्तकीय उध्ययन ने दर्शाया कि कैंसर की वृद्धि सोयाबीन तेल पोषित चूहों में घी पोषित चूहों से अधिक थी तथा केवल सोयाबीन तेल पोषित चूहों में ही एडीनोकारसीनोमा देखा गया। गाय घी ने दोनों कैंसर रहित तथा कैंसर जनित चूहों में, सोयाबीन तेल के विपरित यकृत में फेस-I साइटोक्रोम पी-450 जैव उत्प्रेरकों (साइप 1 ए 1, साइप 1 ए 2, साईप 1 बी 1 तथा साईप 2 बी 1) की सक्रियता को कम किया तथा यकृत एवं स्तन उत्तक में फेस-II (गामा ग्लूटामीलट्रासपैपटीडेस, यू डी पी - ग्लूकूटोनोसिलट्रासफ्रेस तथा क्यूनोन रिडक्टेस) की सक्रियता को बढ़ाया। चूहों की स्तन ग्रंथियों में विभिन्न जीनों की अभिव्यक्ति का अध्ययन किया गया। जिसमें नियंत्रक चूहे, कैंसर रहित चूहे, कैंसर ग्रस्त चूहों के टयूमर तथा टयूमर के समीपवर्ती उत्तक शामिल थे। सोयाबीन तेल पोषित चूहों में साइक्लोआक्सीजीनेस-2 (कॉक्स-2), साइक्लीन-ए तथा साइक्लीन-डी की अभिव्यक्ति गाय घी पोषित चूहों से अधिक थी जो सोयाबीन तेल पोषित चूहों में उच्च कोशिकीय प्रयुरोद् भाव को दर्शाती है परऑक्सीजोम प्रोलीफ्रेटर एक्टीवेटिड रिसेप्टर-गामा की जीन अभिव्यक्ति गाय घी पोषित चूहों में सोयाबीन तेल पोषित चूहों से अधिक थी। अत: यह निष्कर्ष निकाला जा सकता है कि घी, सोयाबीन तेल के विपरित डी एम बी ए द्वारा प्रवृत स्तन कैंसर को बाधित करता है। यह प्रभाव घी समूह के यकृत तथा स्तन उत्तक में, सोयाबीन तेल की तुलना में कैंसरजन की क्रियाशीलता को कम करने तथा कैंसरजन के निराविषीकरण क्रियाओं की वृद्धि से संबंधित था। इसके अतिरिक्त यह कॉक्स-2, साइक्लीन-ए तथा साइक्लीन-डी की घटती अभिव्यक्ति तथा पी पी ए आर-गामा की बढ़ती अभिव्यक्ति से भी संबंधित था।

ABSTRACT

Breast cancer is the most commonly diagnosed cancer in women and is the leading cause of cancer mortality in female in the world. Strong correlation between dietary fat and incidence of breast cancer has been suggested by epidemiological studies. Milk fat, which despite of containing potential anticarcinogenic components, has received the adverse publicity for its suspected role in promotion in cancer. In the present study, the protective effect of ghee vs soybean oil and the possible mechanism behind this was studied in the 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis. Mammary carcinogenesis was induced by oral intubation of 30 mg DMBA/kg body weight at 55 day of age. The antcarcinogenic potential of ghee was evaluated in terms of histological, biochemical and molecular parameters. The DMBA induced animals fed on soybean oil had smaller tumor latency period and higher tumor incidence (65.4%), tumor weight (6.18 g) and tumor volume (6285 mm^3) compared to animals fed on cow ghee (26.6%, 1.67 g, 1925 mm^3, respectively). Histological analysis of tumors showed that the progression of carcinogenesis was more rapid on soybean oil than on cow ghee and adenocarcinoma was found only in soybean oil group. Cow ghee opposed to soybean oil decreased the phase I cytochrome P450 enzymes (CYP1A1, CYP1A2, CYP1B1 and CYP2B1) activities in liver and increased the phase II (γ-glutamyltranspeptidase, UDP-glucuronosyltransferases and quinone reductase) activities in liver and mammary tissue of both carcinogen treated and untreated control rats. The expression of various putative genes was studied in mammary gland of control rats, and in non tumor bearing, uninvolved tissue of tumor bearing and tumor tissue of DMBA treated rats. Expression of cyclooxygenase-2 (COX-2), cyclin A and cyclin D was more on soybean oil than on cow ghee indicating the greater cell proliferation in former group. Peroxisome proliferators activated receptor-γ (PPAR-γ) expression was more on cow ghee group than on soybean oil group. It is concluded that ghee opposed to soybean oil, attenuated the mammary carcinogenesis induced by DMBA. It was associated with the decreased activation of carcinogen and increased carcinogen detoxification activities in liver and mammary tissue on cow ghee compared to soybean oil. Further, it was also associated with decreased expression of COX-2, cyclin A and cyclin D and increased expression of PPAR-γ on cow ghee than on soybean oil.

CHAPTER – 1

Introduction

1. INTRODUCTION

In today's nutritional world, fat has become a dirty word. Animal fats such as butter and ghee have taken a terrible beating in the media over the past few decades and have been blamed for heart disease and cancer. Accordingly, Western people have been virtually brainwashed into thinking that butter and butter oil are unhealthy. So-called safe substitutes like margarine and various vegetable oils have been heavily promoted and advertised with the result being that the public associates these things with health and well being. However, there is no experimental evidence for the claims. When it comes to breast cancer prevention, the so-called 'bad fats' are actually the good guys.

Epidemiological studies supporting the vegetable oil and discrediting saturated fats especially milk fat are subjected to potential biases due to several factors. Most importantly, total energy intake, which is a stronger predicator in pathogenesis of cancer, is not generally taken into account. Even when total energy intake controlled, it may be impossible to completely separate the effects of milk fat from that of other dietary factor that alters cancer risk. Person with a high consumption of dairy product may also be likely to consume large amount of meat and other fat foods that could also contribute to an increased risk of cancer. Further, other factors such as lifestyle, physical activity cannot be controlled in epidemiological studies.

Unlike epidemiological studies, experimental studies suggest that breast cancer is strongly influenced by type of dietary fat. For instance n-6 polyunsaturated fatty acids have strong tumor enhancing effect, whereas n-3 polyunsaturated fatty acids and milk fat have the protective effects in carcinogenesis. Milk fat contains several components such as conjugated linoleic acid (CLA), sphingomyelin, butyric acid, ether lipids, vitamin A, ß-carotene and vitamin D, which have the potential to inhibit the process of carcinogenesis.

Ip *et al.* (1999) and Yanagi *et al.* (1992) showed that the CLA enriched butterfat and milk cream inhibits the development of DMBA induced mammary tumor in rats. Recently our laboratory has reported that ghee (clarified butter fat), cow ghee in particular, inhibited the gastrointestinal carcinogenesis induced by DMH in male rats. The tumor incidence in gastrointestinal track was considerably higher in rats on soybean oil (73.30%) than on cow ghee (55%) or buffalo ghee (40%). Cow ghee also attenuated the toxicity induced by DMBA by decreasing incidence of mortality, tumor multiplicity, tumor weight, tumor volume and non-neoplastic disorders in female rats (Bhatia, 2005).

Recently, Kathirvelan (2007) reported that feeding of low CLA and high CLA ghee decreased the mammary tumor incidence compared to soybean oil fed group. The tumor incidence of DMBA induced mammary tumor was significantly higher in soybean oil fed group (83.33%) than on low CLA (63.33%) and high CLA (46.70%) ghee fed groups.

The biochemical and molecular mechanism by which cow ghee inhibits carcinogenesis is not known. The possible mechanism might involve modulations in enzyme activities involved in drug metabolism and prostaglandin synthesis, influencing expression of transcription factor peroxisome proliferator activated receptor-gamma (PPAR-γ), influencing the cell replication by interfering with cell cycle regulatory proteins or enhancing cell death by way of apoptosis. Following studies are proposed to understand the mechanism by which cow ghee confer protection against mammary carcinogenesis induced by DMBA:

- Effect of cow ghee vs soybean oil on mammary carcinogenesis induced by DMBA.
- Effect of cow ghee vs soybean oil on drug metabolizing enzymes.
- Effect of cow ghee vs soybean oil on expression of COX-2 and transcription factor PPAR-γ.
- Effect of cow ghee vs soybean oil on the expression of genes involved in cell cycle regulation.

CHAPTER – 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 DIETARY FAT AND CANCER

During the past several years, epidemiological studies have indicated the influence of environment and life-styles on the development of certain forms of cancer. About 35 % of all cancer mortality in US may be attributable to dietary factors (Wynder and Gori, 1977; Doll and Peto, 1981). The nutritional factors may relate to the development of cancer in several ways: (a) food additives, contaminants or a particular dietary component may act as carcinogens, and / or promoters, b) dietary constituents act as anticarcinogens, c) nutrient deficiencies and excesses may lead to biochemical alterations that promote neoplastic processes, and d) changes in the intake of selected macronutrients may induce metabolic and biochemical abnormalities, which increase the risk for cancer (Reddy, 1994).

The association between dietary fat and cancer in animals was first identified by Tannenbaum in the 1950s, and has been consistently supported by experimental evidence. The World Cancer Research Fund, in association with the American Institute for Cancer Research, published an extensive review of diet and cancer research in human populations, including dietary recommendations to reduce cancer risk as proposed by international government and professional organizations (WCRF, 1997). American Cancer Society has recommended to limit the intake of fats and oils to reduce risk of cancers (ACSSR, 1984). This was recently expanded to limit saturated fat intake through reduction of animal fat intake.

Data on some epidemiologic evidence that demonstrates associations between dietary fat intake and cancer (breast, prostate, colon, and lung cancers) in humans (Kuller, 1997) is highly controversial. Some of this controversy stems from the limited ability to accurately assess total energy and fat consumption, and the difficulty in assessing the effects of dietary fat

independent of total energy or micronutrient intake and other environmental factors such as physical activity (Greenwald *et al.*, 1997).

A chorus of establishment voices, including the American Cancer Society, the National Cancer Institute and the Senate Committee on Nutrition and Human Needs, claim that animal fat is linked not only with heart disease, but also with cancers of various types. However, when researchers from the University of Maryland analyzed the data, they found that vegetable fat consumption was correlated with cancer, not the animal fat (Enig *et al.*, 1978).

Bartsch *et al.* (1999) and Wynder *et al.* (1997) summarized that mechanisms supporting a relationship between dietary fat and cancer can be classified as either direct or indirect. Potential direct mechanisms include: 1) peroxidation of double bond in PUFAs, leading to persistant oxidative stress and generation of reactive lipid peroxidation products (malondialdehyde, 4-hydroxyalkenals), which induce DNA damage; 2) conversion of essential fatty acids to eicosanoids (short lived hormone synthesized from n-6 unsaturated fatty acids); and 3) interaction between fatty acids with signal transduction pathways leading to altered gene expression. Potential indirect mechanisms include: 1) effect on membrane bound enzymes such as cytochrome P450 (CYP) that regulate xenobiotic and estrogen metabolism; 2) structural and functional changes in cell membranes that can alter the hormone activity and growth factor receptors; and 3) effects on immune function.

2.1.1 Dietary fat and breast cancer

Breast cancer is the most commonly diagnosed cancer in women and is the leading cause of cancer mortality in females in the world (Parkin *et al.*, 1999; Parkin, 2001). Of those cancers with a putative link to dietary fat intake, breast cancer has been the most extensively studied. Analysis of international data has shown a strong positive correlation between per capita fat intake and age adjus ed incidence and mortality from breast cancer (Corroll *et al.*, 1968; Wynder *et al.*, 1986; Prentice *et al.*, 1988). It is likely that sex hormones

especially estrogen, play a promotional role in breast carcinogenesis, stimulating mitotic division of initiated cells and proliferation (AICR, 1997).

It has been shown that an increased amount of both vegetable and animal fat accelerates mammary tumor growth. Different types of fat also have different effects on mammary tumorigenesis. Fay *et al.* (1997) conducted a meta-analysis of 97 reports that studied the effects of dietary fatty acids on mammary tumor incidence and found: 1) n-6 polyunsaturated fatty acids have a strong tumor-enhancing effect; 2) saturated fats have weaker tumor enhancing effects; 3) n-3 polyunsaturated fatty acids have a small non-significant protective effect; 4) the effects of n-6 polyunsaturated fats are stronger than that of saturated fats even at low levels; and 5) there is no effect of monounsaturated fats on mammary carcinogenesis. Literature reviewed by Rose *et al.* (1991) suggest that a high fat diet rich in n-6 polyunsaturated fatty acid in animal models could enhance metastasis of human breast cancer cells.

2.1.1.1 Dairy products and breast cancer

There are several postulated mechanisms through which dairy products could influence breast cancer risk in either positive or negative fashion (Moorman and Terry, 2004 and Terry *et al.*, 2001). The major hypotheses that have been put forth to suggest an increased risk of breast cancer risk associated with the consumption of dairy product include: 1) a high consumption of dairy products may reflect on overall high dietary fat intake particularly saturated fat which in turn has been associated with breast cancer risk; 2) milk product may contain contaminants, such as pesticide that are potentially carcinogenic; and 3) milk may contain growth factors, such insulin like growth factor 1 (IGF-1), which have been shown to promote breast cancer cell growth (Yu and Rohan, 2000).

Other hypotheses suggest an inverse relation between dairy product consumption and breast cancer risk. These hypotheses have focused on the anticarcinogenic effects of vitamin D and calcium, conjugated linoleic acid and

butyric acid. Dairy products have high calcium content and are also a major dietary source of vitamin D in countries where milk and other dairy products are fortified, such as the United States. It has been shown in breast cancer cell lines that vitamin D exerts antiproliferative effects by causing arrest in phase G0 / G1 of the cell cycle (Narvaez *et al.*, 2001; Colston and Hansen, 2002). This lead to down-regulation of several growth promoting factors, such as IGF-1 and the up-regulation of negative growth factor regulators such as transforming growth factor ß.

The cellular functions of vitamin D are closely linked to calcium. Calcium is a pivotal regulator of a wide variety of cellular functions, including cellular proliferation and differentiation (Rasmussen H. 1986a,b). Several investigators have shown that animals fed diet deficient in calcium and vitamin D develops mammary hyperplasia and hyperproliferation (Lipkin and Newmark, 1999). Furthermore, animal studies have shown that supplementation with calcium and vitamin D reduces the risk of mammary tumors in animals fed a high fat diet and prevents the development of mammary tumors in animals induced with the carcinogen 7,12-dimethylbenz (a)anthracene (DMBA) (Jacobson *et al.*, 1989; Newmark, 1994; Mehta *et al.*, 2000).

A third potential mechanism to suggest that dairy products may reduce breast cancer risk involves CLA. Animal studies suggest that CLA confers protection against the development of mammary tumors (Ip *et al.*, 1996). It is interesting to note that tumor formation was inhibited in animals fed CLA, regardless of the type or amount of fat in their diets. Another compound found in dairy products, known to have protective effect against mammary carcinogenesis is butyric acid.

2.1.1.2 Epidemiological studies

Most of the epidemiological studies showed no consistent pattern of increased or decreased breast cancer risk with a high consumption of dairy products (Moorman and Terry, 2004). Two of the cohort studies and 10 of the

case-control studies investigated the association between breast cancer and butter consumption and no consistent pattern was observed with reported butter intake. In a cohort study conducted in Finland (Knekt *et al.*, 1996), an inverse association that was not statistically significant was reported; whereas a slight positive association was reported in a cohort study in the Netherlands (Voorrips *et al.*, 2002). In the case-control studies, odds ratios both > and < 1.0 were reported, but generally differences between cases and controls were not statistically significant. Persons with a high consumption of butter, cheese and other high-fat dairy products may also be more likely to consume large amounts of meat or other high fat-foods that could also contribute to an increased risk of breast cancer. Further, milk fat is rarely used in isolation from other dietary items, and other milk components (milk protein, calcium, lactic acid bacteria) also have anticarcinogenic properties; hence it is not possible to separate the effect of milk fat as such.

2.1.1.3 Animal studies

There were a few studies in which milk fat or butter was compared with vegetable oils or margarines in animal models of carcinogenesis. Carroll and Khor in 1971 reported that vegetable oils (soybean, sunflowers, corn and cotton seed oil) enhanced DMBA induced rat mammary adenocarcinomas more than butter and some saturated fats (coconut oil, tallow, lard). In another study (Klurfeld *et al.*, 1983), female Sprague-Dawley rats were given DMBA to induce mammary tumors and were fed from weaning basal diets containing 15% butter oil or 15% corn oil. The study showed that milk fat exerted a statistically significant protection against mammary tumor induced by DMBA, and it was more effective when introduced in the diet at weaning.

In female mice fed after weaning either with a basal diet (4.6% fat) or the diet enriched with 20% butter, margarine (64 g linoleic acid / 100 g fatty acids) or safflower oil, the incidence of spontaneous mammary tumor, mainly adenocarcinomas, was significantly less on butter fed group (21%) than on the margarine (43%) or safflower oil (44%) fed groups (Yanagi *et al.*, 1989). Similar diets when fed to female rats from one week before tumor induction

with DMBA, the percentage of mammary tumor incidences were 44% on basal diet (4.6% fat), 36% on butter, 63% on margarine, and 46% on safflower oil. To determine if the inhibitory effect of butter on mammary tumor development was due to milk lipids or some other constituent used for the preparation of commercial butter, Yanagi *et al.* (1992) fed rats under similar conditions either a basal diet (4.6% fat) or the basal diet supplemented with dried whole milk (8.9% fat), skim milk (3.9% fat) or milk cream (20.8% fat). The rats fed milk cream diet showed no enhanced tumor development (42.3%) compared with those fed the basal diet (42.35), dried whole milk diet (60%) or the skim milk diet (52%). Further (Yanagi *et al.*, 1994) feeding rats with diet supplemented with increasing level of margarine resulted in subsequently increased incidence of DMBA induced mammary tumor. When butter (20%) was replaced with margarine (20%) in the diet, rats had a non-significantly lower tumor incidence. However, total tumor numbers (99 vs. 48), average tumor numbers (6.19 vs. 3.42) and average tumor diameter (11.6 vs. 9.6 mm) were significantly lower in the butter group. Cope and Reeve (1994) demonstrated that, compared with butter or milk fat, polyunsaturated margarine or sunflower oil enhanced both ultraviolet (UV) light and UV light / DMBA-induced photocarcinogenesis in a hairless mouse model.

Recently work done in this laboratory (Bhatia, 2005) showed that in gastrointestinal carcinogenesis induced by DMH in weanling male rats, the incidence was considerably higher in animal on soybean oil (73.30%) than on cow ghee (55%) or buffalo ghee (40%). Tumor multiplicity and tumor volume were also less on ghee diets than on soybean oil, and cow ghee was more efficacious than buffalo ghee in reducing tumor volume. Increased accumulation of CLA and decreased lipid peroxidation in liver and colorectal tissue on ghee opposed to soybean oil correlated with decreased tumor incidence, tumor multiplicity and tumor volume on ghee diets. Similarly mammary carcinogenesis induced by DMBA in female rats, the mortality incidence was greater on soybean oil than on ghee groups. Cow ghee opposed to soybean oil decreased tumor multiplicity, tumor volume and non-neoplastic disorders. Katherevalin in 2007 showed that the tumor incidence of

DMBA induced mammary tumor was significantly higher in soybean oil fed group (83.33%) than on low CLA (63.33%) and high CLA (46.70%) ghee fed groups. Tumor weight was also less on ghee diet than on soybean oil diet.

These animal model studies wherein high fat intake itself is a risk factor for colon (Reddy, 1992) and mammary (Welsch, 1992) cancer, clearly demonstrate that milk fat based diets produce fewer tumors than polyunsaturated vegetable oil based diets. These studies, however, were of insufficient design to determine if the differences were due to the anticarcinogenic components of milk fat or to the known potential of linoleic acid to promote carcinogenesis in animal models of colon (Reddy, 1992), mammary (Ip *et al.*, 1985; Welsch, 1992) and skin (Reeve *et al.*, 1988) cancer.

2.2 ANTICARCINOGENIC AGENTS IN MILK FAT

Milk fat contains several compounds such as conjugated linoleic acid (CLA), sphingomyelins, butyric acid, ß-carotenes, vitamin A and vaccenic acid, which have the potential to inhibit the process of carcinogenesis.

2.2.1 Conjugated linoleic acid (CLA)

The acronym CLA refers to various positional and geometrical isomers of linoleic acid in which the two double bonds have a conjugated arrangement instead of methylene interruption. CLA is produced in ruminants as an intermediate in the biohydrogenation of dietary linoleic acid by a linoleic acid isomerase from the rumen bacteria *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1966). The animal foods are richer in CLA than the plant foods, and the foods from ruminants contain more CLA than non-ruminants (Chin *et al.*, 1992). The dairy products are the major source of CLA in the human diet. The CLA content of dairy products vary from 0.6 to 30 mg/g fat (Parodi, 1994). The biological properties of dietary CLA are currently attracting considerable interest. CLA is not only a powerful anticarcinogen, it also has antiatherogenic, immunomodulating, growth promoting and lean body mass enhancing properties (Pariza, 1997).

Pariza *et al.* (1979) were first to report the anticarcinogenic property of CLA, when one fraction of the extract of heated ground beef containing a mixture of four isomers of CLA, the c-9, t-11; t-9, t-11; t-10, c-12 and t-10, t-12 inhibited mutagenesis. Subsequently, it was tested in the benzo(a)pyrene-induced mouse forestomach neoplasia model, wherein it significantly reduced tumor incidence as well as tumor multiplicity in mice (Ha *et al.*, 1990). The c-9, t-11 CLA isomer was suggested to be the active form because only this isomer was incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers (Ha *et al.*, 1990). After this, a considerable number of studies were conducted to test the inhibitory potential of CLA in mammary, colon and skin tumorigenesis.

Dietary CLA inhibited mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) in rats in a dose dependent manner up to 1.0 % CLA in the diet (Ip *et al.*, 1991). A lower dose of DMBA (5 mg/animal), as little as 0.1 % CLA in diet was sufficient to reduce significantly the number of tumors (Ip *et al.*,1994). When CLA was removed from the diet, CLA in neutral lipids and phospholipids returned to basal levels in about 4 and 8 weeks, respectively The rate of disappearance of neutral lipid CLA (rather than phospholipid CLA) subsequent to CLA withdrawal paralleled more closely the rate of occurrence of new tumors in the target tissue, thus suggesting that neutral lipid CLA might be more sensitive marker of tumor protection than phospholipid CLA (Ip *et al.*, 1999). The study further showed that when CLA feeding commenced later in life, after tumor promotion has already been initiated, the lifetime CLA supplementation was required to obtain significant tumor protection.

Feeding CLA when started at weaning and continued for 6 months until the end of experiment, it produced essentially the same magnitude of mammary tumor inhibition in DMBA model as that produced in rats fed CLA for 1 month from weaning till tumor induction or in rats fed CLA for 5 months (starting 5 days past tumor induction till the end of experiment), which showed that dietary CLA was effective in suppressing mammary tumor development

during pre-promotion, promotion and progression phases of carcinogensis (Thompson *et al.*,1997). The protective effect of CLA on DMBA induced mammary carcinogenesis plateaued at 1% CLA in diet (Ip and Scimeca, 1997).

Ip *et al.* (1994) showed that CLA was equally effective against the action of a direct acting carcinogen methylnitrosourea, as was against DMBA (a carcinogen which requires metabolic activation); thus suggesting that it may have a direct modulating effect on susceptibility of the target organ to neoplastic transformation. Comparison of CLA fed as triacylglycerol or as the free fatty acid in methylnitrosourea treated rats gave similar results (Ip *et al.*, 1995). This study also showed that CLA feeding for only 3 weeks post-weanling was sufficient to reduce significantly tumor incidence and total tumor yield.

The efficacy of CLA inhibition of mammary tumorigenesis is independent of the amount or type of dietary fat. Since linoleic acid enhances experimental mammary carcinogenesis (Welsch, 1992), the possibility that higher levels of this acid might swamp the CLA effect was addressed (Ip *et al.*, 1996). Inhibition of DMBA-induced mammary tumors by 1% dietary CLA was virtually the same in rats fed either 10 or 20 % fat in the diet. The extent of tumor inhibition by CLA was also the same in rats fed 20 % maize oil diet or 8 % maize oil plus 12 % lard diet. Also, the amount of linoleate present in the diet (2 or 12%) did not affect the inhibitory potential of CLA (Ip and Scimeca, 1997).

Conjugated linoleic acid enriched butter provided the same magnitude of protection against mammary tumorigenesis (induced by methylnitrosourea) in rats as provided by the mixture of free CLA isomers (Ip *et al.*,1999). The accumulation of total CLA in the mammary gland and other tissues was greater in rats consuming the CLA enriched butter fat compared with those consuming mixture of free CLA isomers and was perhaps due to conversion of vaccenic acid (t11-18:1) present in butter fat into CLA via the Δ^9-desaturase. Hubbard *et al.* (2000) showed that CLA significantly decreased

not only the number of tumor cells that spread to other parts of the body but the degree to which they formed new tumors. Moreover, when mice were fed CLA, it took longer for tumors to establish at their primary site compared to mice that were fed diets containing no CLA.

The severe combined immunodeficient (SCID) mouse provides a model for studying the growth of human tumor cells. SCID mice were fed a diet containing 10 g CLA/kg for 2 weeks and then inoculated with 10^7 human breast adenocarcinomas cells (MDA-MB468), and CLA feeding continued for 14 wk. The tumor weight and volume were significantly reduced in CLA treated mice and systemic spread of the tumor into the lungs, peripheral blood and bone marrow was abrogated completely (Visonneau *et al.*, 1997). In another study. the effect of CLA and linoleic acid were compared wherein SCID mice were inoculated subcutaneously 5 x 10^6 human prostate cancer cells (DU145). Linoleic acid or CLA was fed (1%) in diet beginning 2 weeks before inoculation of the DU145 cells, and the mice were observed for another 12 weeks. Tumor volume was significantly higher in linoleic acid fed mice than in CLA fed mice and tumor mass followed the same pattern. Metastatic spread of the tumor to the lungs was observed in 80 to 100% of the control (linoleic acid fed mice) and in only 10% of the mice fed on CLA (Cesano *et al.*, 1998). Also, CLA has been shown to inhibit the growth of a number of human tumor cell lines including colon cancer cells *in vitro* (Shultz *et al.*, 1992a,b; Schonberg and Krokan, 1995; Cunningham *et al.*, 1997).

Inhibitory potential of CLA in human breast cancer is not well established. Postmenopausal breast cancer cases reported lower dietary intakes of CLA and had lower serum CLA concentrations than did the controls (Aro *et al.*, 2000). Other studies, however, failed to confirm this association. Voorrips *et al.* (2002) reported a weak positive association between CLA intake and breast cancer. Chajes *et al.* (2002) compared CLA concentrations in breast adipose tissue between breast cancer patients and women with benign breast conditions. The concentrations of CLA were higher in the breast

cancer patients, but were not significantly different from those in women with benign conditions.

2.2.2 Sphingomyelins

Sphingomyelin (N-acetyl sphingosine-1-phosphocholine or ceramide phosphocholine) predominantly resides in the outer leaflet of the plasma membrane in most animal tissues, where these regulate the membrane fluidity. It is now recognized that in addition to its structural function in membranes, sphingomyelin through its biologically active metabolites, ceramide and sphingosine play an important role in transmembrane signal transduction and cell regulation (Merrill, 1991; Hannun and Bell, 1993; Hannun and Linardic, 1993).

Dillehay *et al.* (1994), utilizing the DMH model of colonic adenocarcinoma, showed that sphingomyelin, isolated from commercially available non-fat dry milk, when fed to CF1 mice inhibited the colonic adenocarcinoma. Mice were fed the diet supplemented with sphingomyelin for 28 weeks and then fed unsupplemented diet for 24 weeks. Mice fed the control diet had a 47% incidence of colon tumours compared with 20% in the sphingomyelin-fed animals. The reduction in tumour incidence was similar in mice fed diets supplemented with either 0.025, 0.05 or 0.1 g sphingomyelin/100g diet. In another experiment of shorter duration (7 weeks), the number of colonic ACF, precancerous lesions, induced by DMH was significantly reduced in mice fed 0.05 % sphingomyelin diet. Using murine model, Schmelz *et al.* (1996) confirmed that dietary sphingomyelin suppressed colonic ACF formation, but the incidence or the number of tumours was not reduced. Sphingomyelin supplementation, however, produced more adenomas than the advanced malignant adenocarcinomas, compared with control animals.

2.2.3 Butyric acid

Butyric acid is an inhibitor of cell proliferation and a potent inducer of differentiation *in vitro* for a wide variety of neoplastic cells, including leukemia,

lymphoma, breast, colon, rectum, liver, cervix, ovarian and neural (Prasad, 1980; Chen and Breitman, 1994). Milk fat contains from 7.5 to 13.0 mol% of butyric acid. Chen and Breitman (1994) observed that butyric acid acted synergistically with all trans-retinoic acid to induce differentiation in HL-60 cells. Velazquez *et al.* (1996) observed that a 3-hydroxy-3-methyl glutaryl coenzymeA (HMG CoA) reductase inhibitor had a synergistic anti-proliferative effect on a murine colon cancer cell line, when combined with butyrate. Perrin *et al.* (1994) succeeded in reversing a late stage of carcinogenesis (carcinomatosis) in a rat model of colon cancer using a combination of butyrate and interleukin-2, whereas neither of these substances alone proved effective. Yanagi *et al.* (1993) reported that the inclusion of sodium butyrate at 6% level in the diet containing 20% margarine significantly reduced the incidence of DMBA-induced rat mammary carcinomas and adenocarcinomas.

2.2.4 β-Carotene and vitamin A

Vitamin A and β-carotene are widely investigated natural anticarcinogens. Epidemiological studies consistently report that people eating more fruits and vegetables rich in β-carotene or having higher blood concentrations of β-carotene had a lower risk of developing several types of cancer, especially lung cancer (Van Poppel, 1993; Petru *et al.*, 1995; Toma *et al.*, 1995). There is a strong epidemiological evidence for an inverse association between vitamin A (retinol) intake and cancer, particularly skin, urinary and aerodigestive tract cancer (Lotan, 1997; Petru *et al.*, 1995). In animals, β-carotene suppresses chemically induced tumours at some sites. The effect is probably enhanced, when combined with other micro nutrients, such as vitamin E, vitamin C, glutathione and selenium (Toma *et al.*, 1995).

2.2.5 Vaccenic acid

Vaccenic acid refers specifically to the trans-monoene fatty acid and is produced as an intermediate in the rumen during microbial biohydrogenation of linoleic acid to stearic acid (Griinari and Bauman, 1999). Vaccenic acid, major trans monoene in milk fat (Bauman *et al.*, 2000), accounts for as much

as 1% of total fatty acids and this amount can be increased up to 10 times by dietary manipulations (Ip *et al.*, 1999). Based on the finding (Ip *et al.*, 1999) that rats fed the CLA-enriched butter accumulated considerably more CLA in their tissues than those fed the same level of a synthetic preparation of c-9, t-11 CLA, Banni *et al.* (2001) speculated that vaccenic acid in butter may be an important precursor for the endogenous formation of CLA and thus could act as anticarcinogen. They tested the efficacy of vaccenic acid in cancer prevention in rats injected with methylnitrosourea (50 mg/kg body wt) and observed that vaccenic acid at 2 % level in diet reduced the development of premalignant lesions to the same extent as did CLA at 1% level. They favoured the hypothesis that the effect of vaccenic acid is mediated primarily through its conversion to CLA.

Synthesis of CLA via the Δ^9-desaturase enzyme was shown to be the primary source of CLA in bovine milk fat (Griinari *et al.*, 2000) Recently, Mosley *et al.* (2006) reported that in lactating women vaccenic acid was converted in to CLA via Δ^9-desaturase. However, the possibility that vaccenic acid may have an independent effect by itself, cannot be ruled out as vaccenic acid was able to inhibit modestly the growth of HT-29 human colon cancer cells compared with an equimolar concentration of stearic acid (Awad *et al.*, 1995).

2.3 MECHANISM OF ACTION OF DAIRY FAT IN CARCINOGENESIS

The biochemical mechanisms by which dairy fat inhibits the mammary carcinogenesis are not established, however some mechanism for CLA, the major anticarcinogenic agent in dairy products, has been proposed like alteration of lipid peroxidation, tissue fatty acid composition, eicosanoid metabolism, carcinogen metabolizing enzymes, cell cycle regulation, cell proliferation and apoptosis.

2.3.1 Carcinogen metabolizing enzymes

Carcinogenesis is a complex and protracted multistage process, yet the entire course can be initiated by a single event wherein a cellular

macromolecule is damaged by an endogenous or exogenous agent. Strategies for protecting cells from these initiating events include decreasing metabolic enzymes responsible for generating reactive species (phase I enzymes) while increasing phase II enzymes that can deactivate radicals and electrophiles known to intercede in normal cellular processes.

The protective effect of CLA against carcinogenesis may be due to altered metabolism of carcinogen. Most of the carcinogens act as the procarcinogens and in phase I procarcinogen is converted in to active carcinogen. The major reaction in phase I is hydroxylation, catalyzed by class of enzymes referred to as cytochrome P450s (CYPs). In phase II, the hydroxylated and other compounds produced in phase I are converted in to various polar metabolites by conjugation with glutathione, glucuronic acid, sulfate, acetate or certain amino acids by specific enzymes like glutathione-S-transferase (GST), quinone reductase (QR), γ-glutamyltranspeptidase (GGTP) and UDP- glucuronosyltransferase (UDPGT). Overall purpose of phase II of metabolism of carcinogen is to increase its solubility and thus excretion from body (Murray, 2003) Most of the chemopreventive agent protects against cancer by altering the level of either phase I or phase II enzymes (Manson *et al.*, 1997; Sohn *et al.*, 1999; Kleiner *et al.*, 2001; Tepsuwan *et al.*, 2002; Talalay, 1995).

2.3.1.1 Phase I enzymes

Phase I enzymes, cytochromes P-450 (CYPs) comprise a superfamily mixed function oxygenases (MFOs) involved in metabolism of both endogenous compounds, such as steroid hormones, steroids and fatty acids, and exogenous xenobiotics, such as PAH (polycyclic aromatic hydrocarbon), PCB1(polychlorinated biphenyls) and various drugs (Parke, 1990). Many of them are induced following exposure of the animal to a specific substrate or chemical agent (Parke, 1990).

Historically, CYP1A1 had been thought to be responsible for activation of benzo(a)pyrene (B(a)P) and other carcinogenic polycyclic aromatic

hydrocarbons in experimental animals (Conney, 1982; Pelkonen *et al.*, 1982; Ryan and Levin, 1990). CYP1A2 catalyzes activation of variety of aryl- and heterocyclic amines (Shimada *et al.*, 1989). CYP1A2 also catalyzes the activation of PAH-diols to reactive metabolites at much slower rates than CYP1A1 and CYP1B1 (Shimada *et al.*, 2001).

CYP1B1 has been identified to biotransform various xenobiotics, such as ethoxyresorufin, theophylline, and caffeine (Shimada et al., 1997), and shows overlapping catalytic activities with CYP1A1 and CYP1A2 (Crespi *et al.*, 1997; Shimada *et al.*, 1997). CYP1B1 appears to also have an important role in activation of diverse procarcinogens including polycyclic aromatic hydrocarbons, aryl- and heterocyclic amine, and nitroarenes (Shimada *et al.*, 1996; Shimada *et al.*, 2001). CYP1B1 converts estrogens to 4-hydroxylated metablites that may cause initiation of breast cancer in humans (Murray *et al.*, 1997).

Most of the carcinogenic polycyclic aromatic hydrocarbons are mainly metabolized by CYP1A1 and CYP1B1 to reactive metabolites. DMBA is a procarcinogen and requires metabolic conversion to its ultimate carcinogenic metabolite, DMBA-3,4-dihydrodiol-1,2-epoxide, a process that includes two separate oxidations by the microsomal CYP1 enzymes. The first oxidation produces the 3,4-dihydrodiol and is catalyzed by either CYP1A1 or CYP1B1 (Christou *et al.*, 1994). The second oxidation produces the highly mutagenic 3,4-dihydrodiol-1,2-epoxide metabolite and is catalyzed by CYP1B1. CYP1A1 and CYP1B1 are expressed in both the liver and the mammary gland and both enzymes are induced by DMBA (Shimada *et al.*, 1996).

Chemical inhibitors of CYP1A1 and CYP1B1 have been reported to suppress tumor formation caused by carcinogenic PAHs in experimental animals (Chun *et al.*, 2001; El-Bayoumy *et al.*, 1992; Klenier *et al.*, 2002). Chun and Kim, (2003) reported that l, -4-phenylene-bis (methylene)selenocyanate (p-XSC) prevents mammary tumor formation by DMBA and o- and m-XSC inhibit DMBA-DNA adduct formation in rats (El-Bayoumy *et al.*, 1992; Chae *et al.*, 1997). These synthetic organoselenium

compounds inhibit human CYP1B1 more strongly than CYP1A1. Reseveratol, which inhibits CYP1A1 enzymes activities, prevents tumor formation caused by DMBA in mice (Chun *et al.*, 1999; Jang *et al.*, 1997; Potter *et al.*, 2002). Likewise, 1- ethylenepyrene that inhibits the activities of CYP1A1 and CYP1B1, prevents the binding of DMBA and B(a)P to epidermal DNA (Viaje *et al.*, 1990) and prevents tumor formation caused by DMBA and B(a)P in mouse skin (Jang *et al.*, 1997).

Ha *et al.* (1987) showed that CLA inhibited the metabolism of DMBA *in vitro* to its ultimate carcinogen via CYP450. Liew *et al.* (1995) showed that CLA inhibited the IQ induced colon cancer by decreasing the activity of CYP1A1 and CYP1A2.

CYP2B subfamily monooxygenases, on the other hand, are involved in the detoxification of barbiturates, phenytoin, DDT, and other chemicals, and also take part in the metabolic activation of arenes, arylamines, nitrosamines and several hepatotoxic, carcinogenic and teratogenic substances (e.g., bromobenzene, cyclophosphamide) (Gibson and Skett, 1994).

CYP2E1 is vital in catalyzing the activation of nitrosodimethylamine, alkanes, halogenated hydrocarbons, and many other low molecular mass environmental chemicals (Guengerich *et al.*,1991). CYP2E1 metaboloizes rat mammary carcinogen benzene and nitrosamine that are present in tobacco smoke (Maltoni *et al.*, 1989). Inhibition of CYP2E1 is expected to block the toxicity and carcinogenicity of these compounds. Hepatic CYP2E1 is induced by alcohol (Takahashi *et al.*, 1993). Ethanol ingestion has been shown to enhance rat mammary carcinogenesis induced by 7,12-dimethylbenz(a)anthracene (Singletary *et al.*, 1991) and N-methylnitrosourea (Singletary *et al.*, 1995).

2.3.1.2 Phase II enzymes

Induction of Phase II enzymes is an effective and sufficient strategy for achieving protection against the toxic and neoplastic effects of many carcinogens. Phase II enzymes (e.g. glutathione-S-transferase,

NAD(P)H:quinone reductase, UDP-glucuronosyltransferases) and high intracellular levels of glutathione play a prominent role in providing such protection (Talalay *et al.*, 1995). In laboratory animals and cell culture systems, several chemopreventive agents have been identified solely on the basis of their ability to induce phase II enzymes (Lam *et al.*, 1982; Wattenberg *et al.*, 1986; Zhang *et al.*, 1994), and it was observed that induction of phase II detoxification enzymes is a relevant mechanism for cancer chemoprevention.

Glutathione-S-transferase (GST) is enzyme involved in the detoxification of xenobiotics, carcinogen, free radicals and peroxides by conjugating those toxic substances with reduced glutathione to yield products containing exceptionally stable, thioether linkage (Jakoby, 1977). GST catalyze the reaction of compounds with thiol group of GSH, thus neutralize their electrophilic sites and render the product more water soluble (Habig *et al.*, 1974).

γ-glutamyltranspeptidase (GGTP), a glycoprotein, catalyzes the transfer of the γ-glutamyl moiety from glutathione or other γ-glutamyl derivates to hydroxyl, carboxyl or amine groups, or to water. GGTP has been suggested as a putative marker for hepatic neoplasia (Laishes *et al.*, 1975). Enhanced levels of GGTP activity has been reported in chemically induced hepatomas (Fiala and Fiala, 1973). GGTP activity in mammary gland and sera of low fat fed rats were 6 fold higher than in the high fat diet ones, and higher activity of γ-glutamyltranspeptidase was associated with lower tumor incidence (Sachdev *et al.*, 1980).

UDP-glucuronosyltransferases (UDPGT), is the main enzyme of phase II metabolism, catalyzes the conjugation of glucuronic acid to hydroxyl, carboxyl or amine groups. Glucuronidation is a major pathway for detoxification of numerous carcinogens such as polycyclic hydrocarbons, aromatic and heterocyclic amines by the formation of polar conjugates that are readily excreted (Hecht, 2002; Daly, 2003; Tate *et al.*, 1976).

The two-electron reduction of the metabolic products of polycyclic aromatic hydrocarbons such as quinone, catalyzed by quinone reductase (QR), has been considered to be a detoxification pathway through mercapturic acid pathways, since the resulting hydroquinones may be conjugated and excreted through mercapturic acid pathways (Monks *et al.*, 1992).

Lynda *et al.* (1999) reported that chemopreventive activity of 4-bromoflavone in DMBA induced mammary carcinogensis was achieved by modification of carcinogen metabolism (inhibition of cytochrome P4501A1 and induction of phase II detoxification enzymes GST and QR), and decreases in carcinogen-DNA interactions. Singletary (1990) reported that dietary butylated hydroxytoluene inhibited the initiation of DMBA-induced mammary carcinogenesis due to increased liver GST, QR and EH activities, which, increased the metabolism of DMBA to products that do not bind to DNA.

2.3.2 Cellular proliferation

Number of studies shows that the CLA suppresses the cancer by reducing the tumor cell proliferation. Dietary CLA reduced the bromodeoxy labeling index (proliferation index) in lobuloalveolar compartment of mammary gland (Ip *et al.*, 1994) and decreased the proliferation of terminal end bud and lobuloalveolar bud structures, the site at which tumor forms in both rat and human mammary cancer (Thompson *et al.*, 1997). Further, (Ip *et al.*, 1999) showed that CLA and CLA enriched butter fat decreased the terminal end bud density and proliferating cell nuclear antigen (marker of cell proliferation) expression in terminal end bud of mammary gland. Mammary adenocarcinomas induced by PhIP contained significantly fewer proliferating cell nuclear antigen positive cells in rats fed dietary CLA compared with rats fed a control diet without CLA (Futakuchi *et al.*, 2002).

Benjamin *et al.* (1990) reported that CLA reduced the ornithine decarboxylase activity (ODC) in mouse forestomach, induced by the tumor promoter 12-O-tetra-decanoyl-phorbol-13-acetate (TPA). ODC is a marker of cell proliferation and is associated with tumor promotion (Parodi, 1994).

Feeding CLA inhibited TPA activated protein kinase-C, which is implicated in a number of cellular processes including intercellular communication, cell proliferation and tumor promotion.

Number of studies show that CLA could reduce tumor cell proliferation by modifying cell cycle proteins that regulate this process. As reviewed by Belury (2002b), considerable *in vitro* work suggests that incubating tumor cells with CLA alters the expression of key proteins that regulate the cell cycle.

2.3.2.1 Cyclin A

The mammalian cell cycle has been divided into a series of sequential phases. The G1, S, G2, and M phases are sequentially transitioned in response to growth factor or oncogenic stimulation. In G1 (gap 1) phase cell enlarges its size and prepares to copy its DNA, in S (Synthesis) phase synthesis of DNA take place. The G2 (gap 2) phase is a gap phase when the chromosomes prepare to split. In M (mitosis) phase chromosomes separate to produce two daughter cells, each with a complete set of chromosomes. Cyclins are the prime cell cycle regulators and are central to the control of major check points in eukaryotic cells cycle. Cyclins function by forming a complex and activating a family of cyclin-dependent protein kinases (CDKs), at various stages in the cell cycle. The activated kinase starts a complex kinase cascade that directs the cell into DNA synthesis and/or mitosis (Draetta, 1990; Walker and Maller, 1991). The major regulatory events leading to proliferation in animal cells occur in the G1 phase of the cell cycle (Pardee, 1989). The deranged expression of cyclins and CDKs active in G1 may be the key to oncogenesis.

The aberrant expression of two cyclins (cyclin A and D1) has been observed in some cancers, suggesting they may be involved in loss of growth control. Cyclin A and D are key proteins involved in facilitating entry of cells into the cell cycle and progression through S phase, respectively (Johnsin and Walker, 1999).

Increased expression of cyclin A has been reported in human breast cancer, in both primary tumor (Keyomarsi and Pardee, 1993) and in cell lines (Buckley *et al*, 1993; Keyomarsi and Pardee 1993). Sgambato *et al.* (1995) reported that the level of cyclin A and cyclin D was increased in most of the rat mammary tumor, when compared with the normal mammary gland.

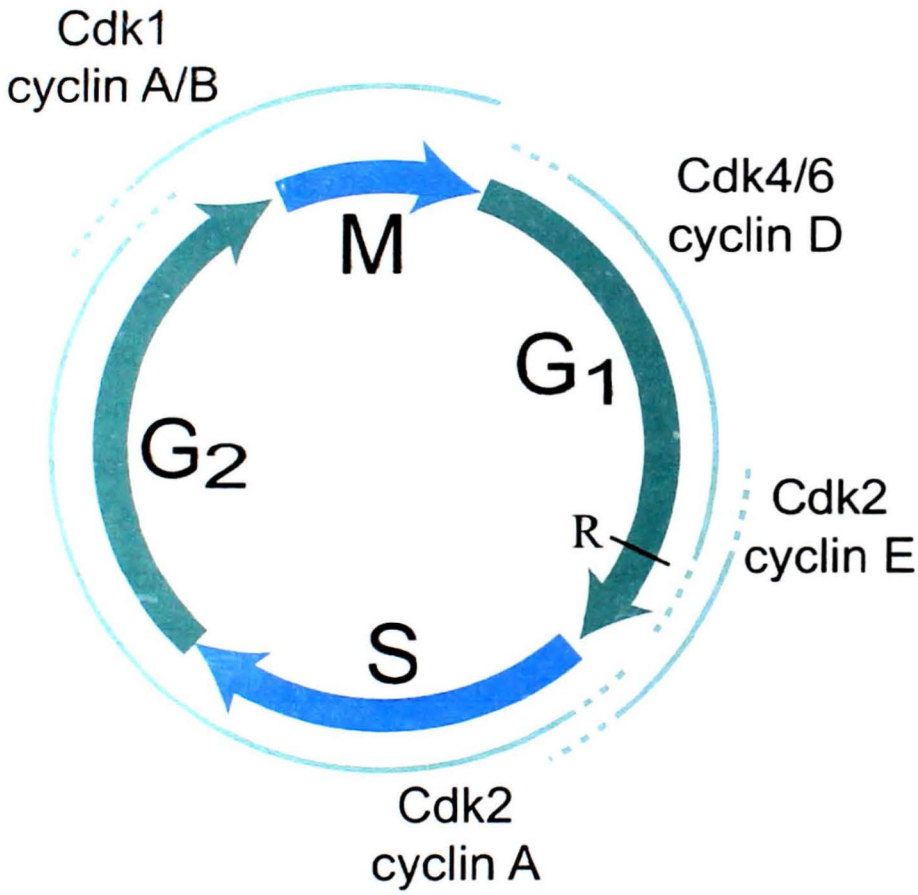


Figure 2: Schematic representation of the mammalian cell cycle. Cyclin-CDK complexes in higher eukaryotes, and their approximate time of activity during the cell cycle.

2.3.2.2 Cyclin D

Cyclin D1 is the product of the *CCND1* gene and was first implicated in tumourigenesis following localisation to chromosome 11q13 (Motokura *et al.*, 1991), a region of the genome that is commonly amplified in a range of human

carcinomas including breast cancer (Fantl *et al.*, 1993). Cyclin D1 plays a pivotal role in regulation of progression from G1 to S phase of cell cycle through the formation of active enzyme complexes with cyclin-dependent kinases Cdk4 and Cdk6. These kinases phosphorylate substrates including the retinoblastoma gene product, pRb, thus relieving pRb's inhibitory function on S phase entry. This rate-limiting step in cell cycle progression is regulated by a number of mechanisms including cyclin D1 abundance; consequently, dysregulation of cyclin D1 gene expression and this is a probable contributor to loss of normal cell cycle control during carcinogenesis (Sherr, 1996).

Cyclin D1 is frequently overexpressed in both human (Devilee *et al.*, 1994) and rat mammary tumors (Sgambato *et al.*, 1995), and is thought to be an important factor in their development. This notion was strengthened by studies that showed that mice engineered to overexpress cyclin D1 in their mammary glands develop hyperplastic lesions and eventually mammary carcinomas (Wang *et al.*, 1994).

It has been shown (Weinstat-Saslow *et al.*, 1995) that cyclin D1 overexpression might be a critical early event in human breast tumor development, because overexpression of this gene is common in early lesions that ultimately form malignant breast cancers, but not in those that form benign tumors. It is thought that rat mammary tumorigenesis occurs through the progression of the early intraductal proliferation(s) (IDPs) to ductal carcinoma *in situ* (DCIS) and eventually to adenocarcinomas (Russo and Russo, 1991). Cyclin D1 expression investigated in normal mammary tissue, preneoplastic lesions, and tumors in a susceptible strain of rat (Hu, *et al.*, 1999) showed that the percentage of cyclin D1-positive cells was very low (approximately 2.4%) in normal mammary tissue. In IDPs, approximately 13.6% of cells were positive, and this value increased to 40% in adenocarcinomas.

Studies on the effects of n-3 and n-6 fatty acids fatty acids showed that n-6 PUFAs increased cyclin D1 mRNA in T47D breast cancer cells (Razanamahefa *et al.* 2000), the n-3 PUFA docosahexaenoic acid reduced

cyclin D1-, E-, and A-associated kinase activity in HT-29 colon cancer cells (Chen and Istfan 2001), and the n-3 PUFA, eicosapentaen-oic acid, reduced cyclin D1 and cyclin E in NIH 3T3 cells (Palakurthi *et al.* 2000).

Besides this, CLA feeding up regulated the expression of p53, a tumor suppressor gene (Belury, 2002b). The p53 protein is involved in monitoring the quality of DNA after G1 phase and, if DNA is damaged, it will block entry of the cell into S phase by altering the expression of genes involved in growth arrest and promotion (Leake, 1996). Ip *et al.* (2001) demonstrated that feeding CLA or c-9, t-11 CLA rich butter fat for 4 weeks reduced the expression of cyclins D and A in the terminal end buds and alveolar clusters of the mammary epithelium.

2.3.3 Apoptosis

Apoptosis or programmed cell death appears to be an important mechanism in deletion of tumor cells rather than increased cell proliferation (Sun *et al.*, 1997; Huang *et al.*, 2000). Feeding CLA has been reported to induce apoptosis in mammary (Ip *et al.*, 2000), colon (Park *et al.*, 2001) and adipose (Tsuboyama-Kasaoka *et al.*, 2000) tissues. CLA *in vitro* induced apoptosis in breast (Majumder *et al.*, 2002), SGC-7901 (Johnson and Walker, 1999) and HT-29 (Cho *et al.*, 2003) tumor cells. It is suggested that a 50:50 mixture of the CLA isomers (c-9, t-11 and t-10, c-12) is more effective than individual isomers in inducing apoptosis in breast cancer cell lines (Majumder *et al.*, 2002). Conjugated linoleic acid may induce apoptosis either by down regulating ErB3 signalling and the phosphoinositide 3-kinase (PI3K) and serine/threonine kinase (Akt) pathway (Cho *et al.*, 2003) or by decreasing expression of bcl-2, a gene involved in suppression of apoptosis (Ip *et al.*, 2000).

2.3.4 Prostaglandins and cyclooxygenase (COX)

Prostaglandins (PGs) are bioactive lipids derived from the metabolites of membrane polyunsaturated fatty acids (PUFAs), and play important roles in a number of biological processes (Herschman *et al.*, 1995). The

anticarcinogenic action of CLA may, in part be explained by its ability to inhibit arachidonic acid derived prostaglandin, since mammary tumors can be inhibited by agents that interfere with the archidonic acid cascade (Rose, 1997). Prostaglandin modulates the tumorigenesis in many tissues including mammary gland, skin, prostate and colon (Fischer, 1995). Conjugated linoleic acid reduces the arachidonic-derived prostaglandin by one of the two mechanisms. First, the CLA displaced the arachidonate incorporation into phospholipid as reported in cultured kertainocytes (Liu *et al.*, 1998). Thompson *et al.* (1997) observed that when CLA was fed to rats, there was 6 and 14-fold increase in conjugated $C_{18:3}$ and $C_{20:3}$ fatty acids in mammary tissue, respectively. These unusual isomers of PUFA may inhibit the metabolism of linoleic acid (Holman *et al.*, 1991) and could also be precursors of unusual prostaglandins or inhibit the synthesis of normal prostaglandins derived from linoleic acid arachidonic acid cascade. These normal prostaglandins have been implicated in mammary tumor development. Belury *et al.* (1997) showed that CLA displaced the arachidonate precursor, linoleate in dose responsive manner in liver of mice fed various dose of CLA (0.5-1.5 g/100 g).

A second explanation for reduction of arachidonate-derived prostaglandins by CLA may be through inhibition of cyclooxygenase (COX) enzyme. Cyclooxygenase catalyzes the rate-limiting step in the synthesis of prostaglandin from arachodonic acid (Smith *et al.*, 2000). Two isoforms of COX are known COX-1 and COX-2. COX-1 is constitutively expressed and COX-2 is up regulated in response to growth factors, tumor promoters and cytokines (reviewed by Herschaman, 1996). Prostaglandins produced by COX-1 mediate various physiological responses, while PGs produced by COX-2 (e.g., PGE2) induce inflammation and are potent mediators of a number of signal transduction pathways that modulate apoptosis, cell adhesion and growth and are implicated in carcinogenesis (Vane *et al.*, 1998).

The literature reviewed by Mazhar *et al.* (2006) shows that there is a clear relationship between tissue prostaglandin levels in human breast

tumours, the development of metastases and survival (Bennett, 1986). Cyclooxygenase-2 is overexpressed in breast cancer cell lines such as the highly invasive, metastatic line MDA-MB-231 (Liu and Rose, 1996) as well as in tumours. Parrett *et al.* (1997) detected the COX-2 expression in 13/13 human breast tumors compared with no detectable expression in normal tissue, and observed a correlation between COX-2 expression and increasing tumor cell density. Cyclooxygenase-2 expression is correlated with prognostic markers that reflect a poor chance for survival, which includes tumour size, axilliary node metastases, tumour grade, ductal histology, receptor negative disease and HER-2 amplification (Ristimaki *et al*, 2002; Boland *et al*, 2004). Moreover, elevated COX-2 expression has recently been shown to correlate with distant metastases in breast cancer (Ranger *et al*, 2004). Liu (2001) reported that COX-2 overexpression in mice is sufficient to induce mammary tumors.

Cyclooxygenase-2 is related to cancer outlook through direct and indirect mechanisms. Prostaglandins may directly stimulate mitogenesis through a direct effect on fibroblasts, osteoblasts, and mammary cells. Cyclo-oxygenase-2 indirectly affects mutagenesis, angiogenesis, and increased cell migration and apoptosis as reviewed by Mazhar *et al.* (2006).

Specific COX-2 inhibitors like nimesulide, celecoxib and ibuprofen can prevent mammary tumours from developing in experimental animals (Nakatsugi *et al.*, 2000; Harris *et al.*, 2000; Robertson *et al*, 1998).

The promotion of mammary carcinogenesis in rats by n-6 PUFAs is associated with enhanced expression of COX-2, whilst its inhibition by n-3 PUFA is associated with diminished production of COX-2 (Badwai *et al.*, 1998). Bulgareela *et al.* (2001) showed that in *in vitro* activity assay, CLA inhibited the rate of oxygenation of arachidonate in the presence of COX. Cheng *et al.* (2004) reported that CLA reduced the COX at the level of mRNA and protein in cultured macrophage cell line.

2.3.5 Peroxisome proliferators activated receptor- gamma (PPAR-γ)

One potential mediator of the effects of specific fatty acids and fatty acid metabolites on mammary tumorigenesis is peroxisome proliferators activated receptor-gamma (PPAR-γ) a ligand activated transcription factor expressed in normal and malignant mammary epithelial cells (Gimble *et al.*, 1998; Mueller *et al.*, 1998; Yee *et al.*, 2003). PPAR-γ is a member of the steroid nuclear hormone receptor superfamily, with several putative natural ligands, including specific fatty acids and eicosanoids, as well as synthetic activators (Lambe *et al.*, 1996; Krey *et al.*, 1997).

PPAR-γ ligands induce cell differentiation and apoptosis in several types of cancer, suggesting its potential application as anticancer agents (Elstner *et al.*, 1998; Rumi *et al.*, 2001; Ohta *et al.*, 2001; Heaney *et al.*, 2003). Postulated mechanisms by which PPAR-γ ligands exert their effects include modulation of the oncogenic Wnt pathway, inhibition of nuclear factor kappaB (NF-kB), and modulation of cell cycle pro- and antiapoptotic proteins. Wnt signaling is a complex pathway in which β-*catenin* binds to transcription factors in the nucleus and plays a role as a central mediator in regulating cell proliferation and differentiation (Bienz and Clevers, 2000). PPAR-γ activation causes a decrease in β-*catenin* expression in adipocytes *in vitro* and in normal intestinal mucosa in mice (Gerhold *et al.*, 2002). PPAR-γ activation also induces the activation of the proapoptotic caspase-3 protein in human liver cancer cell lines and a reduction in antiapoptotic Bcl-2 and Bcl-XL protein level in human colon and gastric cancer cell lines, respectively (Chen *et al.*, 2002; Toyoda *et al.*, 2002; Yoshida *et al.*, 2003).

Elsttner *et al.* (1998) reported that PPAR-γ ligand inhibited the growth and induced the apoptosis in series of breast cancer cell lines both *in vitro* and in xenograft growing in nude mice. Suh *et al.* (1999) showed that PPAR-γ ligand (GW 7854) significantly reduced the tumor incidence, tumor number and tumor weight when fed after the carcinogen administration. PPAR-γ ligand

inhibited the cell cycle progression through repression of cyclin D1 (Wang *et al.*, 2001) and PPAR-γ is reported to promote apoptosis in many tumor cell lines (McCarty, 2000).

Conjugated linoleic acid (CLA) was shown to act as a high affinity ligand and an activator of PPAR-γ (McCarty, 2000). Considerable data support that CLA can increase peroxisome-proliferator activated receptor-γ (PPAR-γ) expression in tissues (Belury, 2002a). Houseknecht *et al.* (1998) reported that CLA activates the PPAR-γ *in vitro* in CV-1 cells transfected with PPAR-γ expression vector. Anticarcinogenic activity of CLA is mediated by PPAR-γ activation in susceptible tumors (McCarty, 2000). When treated with CLA, PPAR-γ expression is increased and adenomatous polyposis coli (APC) and c-myc (cellular- viral oncogene of the avian myelocytomatoris retroviruses) proteins are down-regulated in the human colon cancer cells, and finally proliferation of cancer cells is inhibited (Bozzo *et al.*, 2007; Yasui *et al.*, 2006a; Yasui *et al.*, 2006b).

2.3.6 Lipid peroxidation

Oxidative stress, especially lipid peroxidation, is known to be involved in carcinogenesis (Trush and Kensler, 1991). Increased level of lipid peroxidation products play a role in the early phases of tumor growth (Rice-evans and Burdan, 1993). Bhatia (2005) reported that ghee opposed to soybean oil decreased the level of TARS in liver, colorectal and mammary tissue and this was related with decreased tumor incidence, tumor multiplicity, and tumor volume in ghee fed rats. Recently, Kaithervaen (2007) reported that increased incidence of mammary tumor in soybean oil fed group was related with the incresed level of TARS in liver and mammary gland of soybean oil fed rats as compared to high and low CLA ghee fed rats.

Several reports demonstrate that CLA supplementation can increase oxidative stability. Ha *et al.* (1990) suggested that CLA has ability to suppress peroxide formation from unsaturated fatty acids exposed to air or heated to elevated temperature for a prolonged period of time. Ip *et al.* (1991) observed

that feeding CLA reduced the accumulation TARS in rat mammary glands in parallel with inhibition of mammary carcinogenesis. Devery *et al.* (2001) suggested that the different isomers could have different oxidative properties in healthy tissues, and the proportion of c-9, t-11 isomer to other CLA isomers, particularly t-10, c-12 isomer, could alter the balance between anti- and pro-oxidant activity. Igarashi and Miyazawa (2001) found that growth inhibitory effect of CLA was induced by a change in fatty acid metabolism, not by the facilitation of lipid peroxidation in human hepatoma cell line, HepG2.

Another mechanism through which CLA may suppress cancer cell growth by inhibiting protein and nucleotide biosynthesis (Shultz *et al.*, 1992b). Reduced formation of carcinogen DNA adducts in CLA fed animals have been demonstrated in several studies (Liew *et al.*, 1995; Schut *et al.*, 1997; Josyula and Schut, 1998; Josyula *et al.*, 1998).

2.4 HISTOPATHOLOGY OF MAMMARY GLAND

The primary criteria used for diagnosing hyperplasia is an increase in layers of epithelial cell lining the acini and ducts. In the mild hyperplasia it varies from three to four cells thick and in moderate hyperplasia it is more than four cell thick with an occasional bridge of epithelium across the lumen. Hyperplastic lesions are characteristically round to oval and generally lack nuclolei. Often there is a haphazard arrangement of nuclei (Russo *et al.*, 1977).

Russo *et al.* (1991) reported that the morphology of DMBA induced lesions in mammary gland of Sprague Dawely rats appears to be related to the site of origin. Adenocarcinomas results when the target of the carcinogenic stimulus is the terminal end bud. Benign lesions, such as adenomas, hyperplastic alveolar nodules and cysts, are derived from more differentiated portion of the gland, namely the alveolar buds and lobules. The stromal reaction appears to be closely related to the type of lesion. Benign structures, such as hyperplastic alveolar nodules (HAN) and cysts, do not show modifications in the surrounding connective tissue. Infiltration of round

cells is not seen even in old lesion. Only large carcinomas are associated with lymphocytic infiltrates. The role of lymphocytic infiltration in human breast carcinomas is currently in dispute.

Barros *et al.* (2004) reported that the DMBA induced neoplasm were adenocarcinoma with several morphological types.The most common type encountered was adenoid cystic carcinoma, which is characterized by sheets of tumor cells separated by small cystic spaces. Papillary carcinoma was also very common, which consists of proliferating epithelial cells with delicate cores of connective tissue separated by narrow spaces. A smaller proportion of tumors were of the myoepithelial type, characterized by a myxoid appearance and resembling mixed mammary tumors of dogs.

Ip *et al.* (1991) showed that the feeding of CLA reduced the incidence of adenocarcinoma. Recently, Katherevalin (2007) reported that feeding of low CLA and high CLA ghee decreased the mammary tumor incidence compared to soybean oil fed group and adenocarcinoma (12%) was found only in soybean oil fed group.

CHAPTER – 3

Materials and Methods

3. MATERIAL AND METHODS

3.1 MATERIALS

Sigma-Aldrich (St. Louis, MO, USA) was the source of 7,12-dimethylbenz(a)anthracene (DMBA), benzoxyresorufin, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, resorufin sodium salt, dicumarol, uridinediphosphoglucuronic acid (UDPGA), γ-glutamyl-p-nitroanilide, glycylglycine, calcium chloride, diethylpyrocarbonate (DEPC), 3-morpholinopropanesulfonc acid (MOPS), TRI Reagent, Trizma base, RNA later, chloroform and isopropanol. The source of nicotinamide adenine dinucleotide phosphate reduced (NADPH), flavin adenine dinucleotide (FAD), bovine serum albumin (BSA), para-nitrophenol (p-NP), 1-chloro-2,4-dinitrobenzene (CDNB), isoamyl alcohol, dimethyl sulphoxide was Sisco Research Laboratories (SRL), Mumbai. RevertAid™ first strand cDNA synthesis kit, dNTPs and O'Rangeruler™ 100bp DNA ladder were purchased from Fermentas Life Science (Genetix Biotech Asia Pvt. Ltd., New Delhi). Banglore Genei, Bangalore was the source of Taq DNA polymerase and 6 X gel loading dye. Primers were synthesized from Imperial Life Sciences (P) Ltd, Gurgaon, India. All other chemicals were of analytical grade and obtained from s.d. Fine Chemicals Lid., Mumbai.

3.2 METHODS

3.2.1. Animals and diet

Female weaned (21 d old) albino (Wistar) rats obtained from the Small Animal House of the institute were housed in metal cages in well-ventilated room and were given water and diet *ad libitum*. The experimental diet was comprised of Bengal gram, 56.4%; wheat, 15%; groundnut cake, 10%; cow ghee or soybean oil, 10%; skim milk powder, 6%; salt mixture, 2.16%; vitamin mix, 0.2% and choline chloride, 0.2%.

Salt mixture required for 100 kg diet (2.162 kg) contained sodium floride 1.5 g, copper sulphate, 1.0 g, manganese sulphate, 16.5 g, alum, 0.25 g, sodium chloride, 634 g, calcium carbonate, 1348 g, magnesium sulphate, 50 g, ferric ammonium citrate, 110.665 g, potassium iodide, 0.125 g and zinc sulphate 0.2 g. Vitamin mixture (200 g) comprised of biotin, 0.025 g; folic acid 0.075 g, vitamin B_{12}, 0.003 g, menadione, 0.5 g; para aminobezoic acid, 10 g, meso-inositol 5 g, thiamine, 0.082 g, riboflavin, 0.452 g, pyridoxine, 0.415 g, calcium pentothenate, 3.833 g and starch 172.58 g. The composition of salt and vitamin mixture were so designed that the diet contained the vitamin and minerals (including those derived from other feed intergradients) at level in accordance with AOAC (1975). Vitamin A (2×10^6 IU), vitamin E (104 IU) and vitamin D (2×10^5 IU) were administered to the diet (100 kg) through oil / fat.

3.2.2 Experimental design

Animals randomly divided in to two groups of 35 each were fed on cow ghee or soybean oil diets. After 34 d each animal was administered through oral intubation 7,12-dimethylbenz(a)anthracene (DMBA) (30 mg/ kg body weight) (Fig. 3.1) in soybean oil. Feeding on respective diets was continued until 39 weeks past DMBA administration. Another two groups of 20 rats (21 d) fed cow ghee and soybean oil diets for 44 weeks, served as negative controls. Body weights were recorded biweekly. Animals palpated weekly to determine the time of appearance and location of tumors. After the number of palpable tumors plateaued for at least few weeks, the animals were sacrificed by cervical dislocation.

Five animals from each group were sacrificed at selected time intervals by cervical dislocation and studied for carcinogen metabolism. At conclusion of experiment, a portion of mammary tissue from no tumor bearing and uninvolved tissue of tumor bearing animals and tumor tissue was frozen in RNA later for gene expression studies, and another portion of tumor tissue was fixed in formalin (10%) for histopathological studies.

Plate 3.1 7,12-dimethylbenz(a)anthracene (DMBA) administration through oral intubation

3.2.3 Tumor examination and data recording

At necropsy, mammary glands were exposed and the tumors were excised. After removing, the length (L), width (W) and depth (D) of each tumor were measured with caliper, and tumor volume calculated (Rasic and Kurmann, 1983).

$$V = L \times W \times D \times \pi/6$$

Tumor incidence, tumor multiplicity and average tumor volume were calculated as follows:

$$\text{Tumor incidence} = \frac{\text{Number of animals with tumor}}{\text{Number of animals in the group}} \times 100$$

$$\text{Tumor yield} = \text{Number of tumors in the group}$$

$$\text{Tumor multiplicity (average number of tumors per tumor bearing animal)} = \frac{\text{Tumor yield}}{\text{Number of tumor bearing animals}}$$

Average tumor volume:

i) $$\text{Per tumor} = \frac{\text{Total volume of tumors}}{\text{Tumor number}}$$

ii) $$\text{Per tumor bearing animals} = \frac{\text{Total volume of tumors}}{\text{Number of tumor bearing animals}}$$

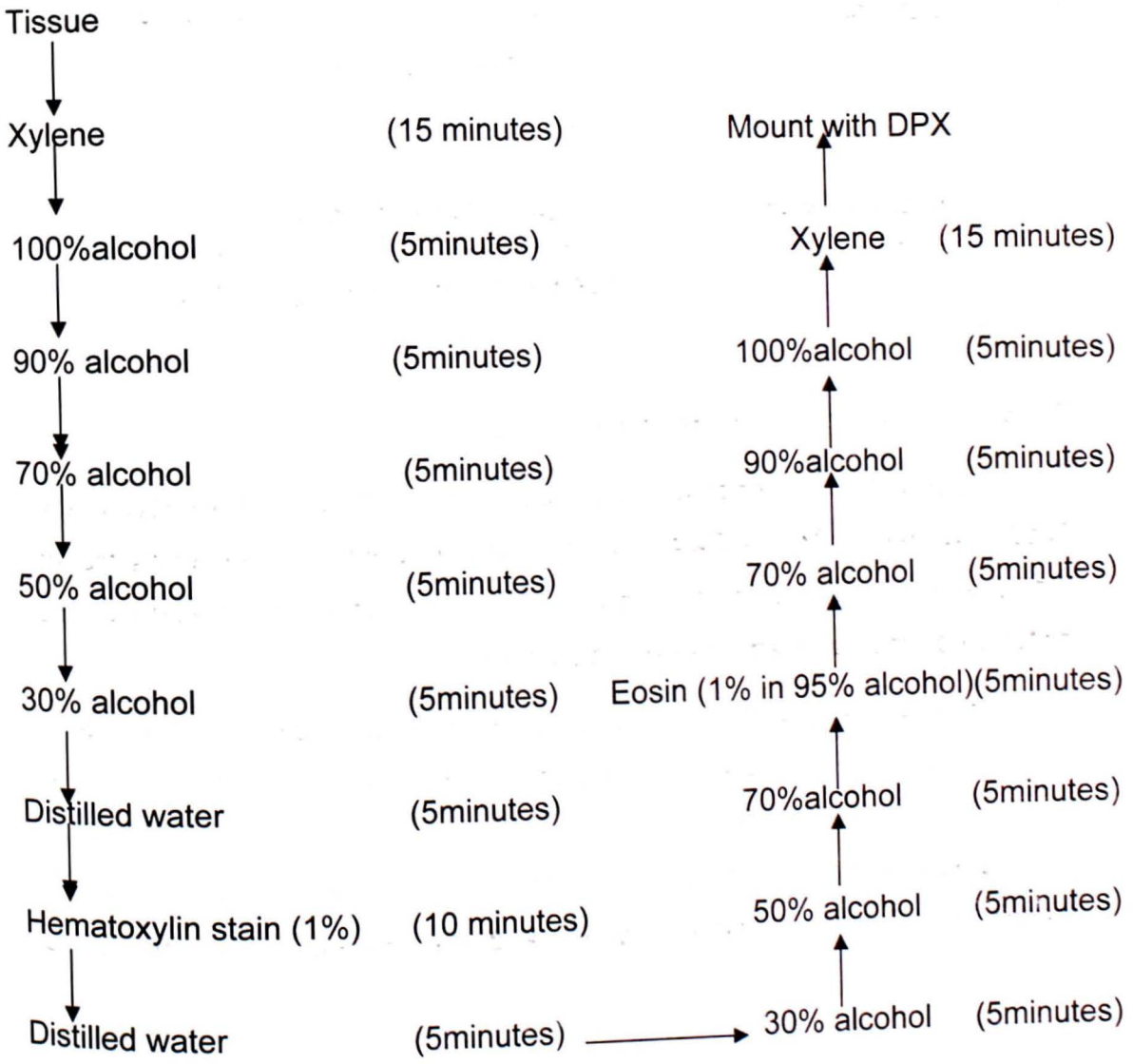
3.2.4 Histopathological analysis of tumors

A portion of tumor tissue larger than 1 mm diameter was excised and fixed in formalin (10%) for 24-48 h at room temperature. This process hardens the tissue by coagulating proteins, prevents autolysis, preserves the histological structures and prevents shrinkage.

Before cutting sections, fixative was removed in running tap water for 1 h. Tissue was dehydrated in ascending grades of alcohol: 70% alcohol for overnight, 90% alcohol for 15 min, and then absolute alcohol for 10 min. The tissue was then treated with xylene in following order: xylene-absolute alcohol (50:50) for 15 min, xylene for 15 min and then xylene - wax (50:50) for overnight.

For embedding, tissue was impregnated in pure molten wax (melting point 50-60°C) for 5 h. Wax blocks were prepared using two "L" shaped moulds arranged in the form of a rectangle over the porcelain slab. Molten paraffin wax was poured in to the mould and the tissue was oriented such that the cutting surface of tissue faces the porcelain slab. The moulds were removed as soon as paraffin wax sets and the section cutting were done after 24 h. The block was trimmed by removing the excess wax and 7μm thick sections were cut and then stained with hematoxylin and eosin as under:

Hematoxylin and eosin staining protocol



3.3 Biochemical assays

3.3.1 Preparation of microsome

Microsomes are small vesicles derived from smooth endoplasmic reticulum during homogenization of tissue. Drug metabolizing enzymes (like cytochrome P450 enzymes, involved in oxidative metabolism) are present in microsomes.

Reagents

Sucrose (0.25 M) in potassium phosphate buffer (0.01 M, pH 7.4)

Potassium phosphate buffer (0.1 M, pH 7.0)

$CaCl_2$ (80 mM)

KCl (0.15 M)

NaCl (0.9%)

Procedure

Liver excised immediately after sacrifice was washed with NaCl solution and microsomes were prepared by differential centrifugation (Cinti *et al.*, 1972). Liver homogenized in 3 volumes of ice cold sucrose was centrifuged (12,000 x g) for 20 min at 4°C. The supernatant (3.6 ml) was mixed with 0.4 ml of $CaCl_2$ and centrifuged (27,000 x g) for 15 min at 4°C. The microsomal pellet was resuspended in 4 ml KCl solution, and recentrifuged (27,000 x g) for 15 min at 4°C. Finally, the pellet was dissolved in 1.0 ml of potassium phosphate buffer (pH 7.0) and stored at 4°C until assayed.

3.3.2 Assays for CYP1A1, CYP1A2, CYP1B1 and CYP2B1 enzymes

Reagents

Tris-HCl buffer (0.1 M, pH 7.4) containing $MgCl_2$ (50 mM) and BSA (3.2 mg/ml): Tris (1.221 g) and $MgCl_2$ (1.016 g) dissolved in 80 ml water, pH was adjusted to 7.4 and volume made to 100 ml. BSA was added (3.2 mg/ml) before use.

NADPH (625 µM)

Ethoxyresorufin (42.5 µM in dimethyl sulfoxide, DMSO)

Methoxyresorufin (125 µM in DMSO)

Benzoxyresorufin (62.5 µM in DMSO)

Pentoxyresorufin (250 µM in DMSO)

Resorufin (0.2 µM in DMSO)

Methanol

Procedure

Fenaxazone ethers (*e.g.* methoxyresorufin, ethoxyresorufin, benzoxyresorufin and pentoxyresorufin) are substrates for *O*-dealkylation reactions mediated by cytochrome P450 (CYP450) enzymes. These substrates are metabolized by different CYP450 isoforms (*e.g.* ethoxyresorufin is metabolized by CYP450 subfamily 1A1, methoxyresorufin by CYP450 1A2, benzoxyresorufin by CYP450 1B1 and pentoxyresorufin CYP450 2B1), but have in common, a final step, the hydroxylation of the fenaxazone ring which generates hydroxifenaxazone (resorufin).

Ethoxyresorufin-*O*-dealkylase (CYP1A1) activity was assayed according to procedure described by Burke *et al.* (1985) and modified by Teel and Huynh (1998). The mixture comprised of tris-HCl buffer (0.5 ml) containing 50 mM $MgCl_2$ and 3.2 mg/ml BSA, ethoxyresorufin (40 µl), microsomal preparation (50 µl) (section 3.3.1) and water to a volume of 0.8 ml was preincubated at 37°C for 5 min. The reaction initiated with NADPH (200 µl) was carried at 37°C for 10 min and then stopped with methanol (2 ml). The mixture was centrifuged (6000 rpm, 4670 g) for 10 min and the fluorescence in the supernatant was measured by using excitation wavelength 550 nm and emission wavelength 585 nm in Cary1. UV-Visible spectroflorometer (Varian,California USA). Blank contained all the reagents except NADPH. Resorufin released was calculated using resorufin standard curve (Fig 3.1), prepared using 0.02 to 0.30 nmole resorufin in tris-HCl buffer (3.0 ml). The enzyme activity was expressed as pmoles of resorufin released per min per mg of microsomal protein.

Methoxyresorufin O-dealkylase (CYP1A2), benzoxyresorufin O-dealkylase (CYP1B1) and pentoxyresorufin O-dealkylase (CYP2B1) activities were measured using 40 µl methoxyresorufin, benzoxyresorufin and pentoxyresorufin, respectively in the above said reaction mixture.

3.3.3 Assay of cytochrome P4502E1 (CYP450 2E1)

Reagents

Potassium phosphate buffer (0.2 M, pH 6.8)

para-Nitrophenol (p-NP, 2 mM)

NADPH (5 mM)

Perchloric acid (0.6 M)

NaOH (10 M)

Procedure

CYP2E1 activity in liver microsomes was determined spectrophotometrically as described by Reinke and Moyer (1985). The mixture containing potassium phosphate buffer (250 µl), p-NP (50 µl) and microsomal preparation (100 µl) (section 3.3.1) was preincubated at 37°C for 3 min. The reaction initiated with NADPH (100 µl) was carried at 37°C for 10 min and then stopped with 250 µl of perchloric acid (0.6 M). Blank contained all the reagents except NADPH. The contents were centrifuged (6000 rpm, 4670 x g) for 10 min. The supernatant (0.5 ml) mixed with NaOH (50 µl), and the product (p-nitrocatechol) was measured spectrophotomertrically at 526 nm (using Specord 200 double beam UV/visible Spectrophotometer, Germany). Enzyme activity was calculated using molar extinction coefficient of 9.53 mM^{-1} cm^{-1} and expressed as units/mg protein, where one unit is defined as the amount of enzyme that catalyzed formation of 1 nmole of product per min.

$$\text{Enzyme units/mg protein} = \frac{\Delta OD/min \times 1000 \times 0.825 \text{ (Dilution factor)}}{\text{Volume of sample (ml)} \times 9.53 \times \text{Protein concentration of sample (mg/ml)}}$$

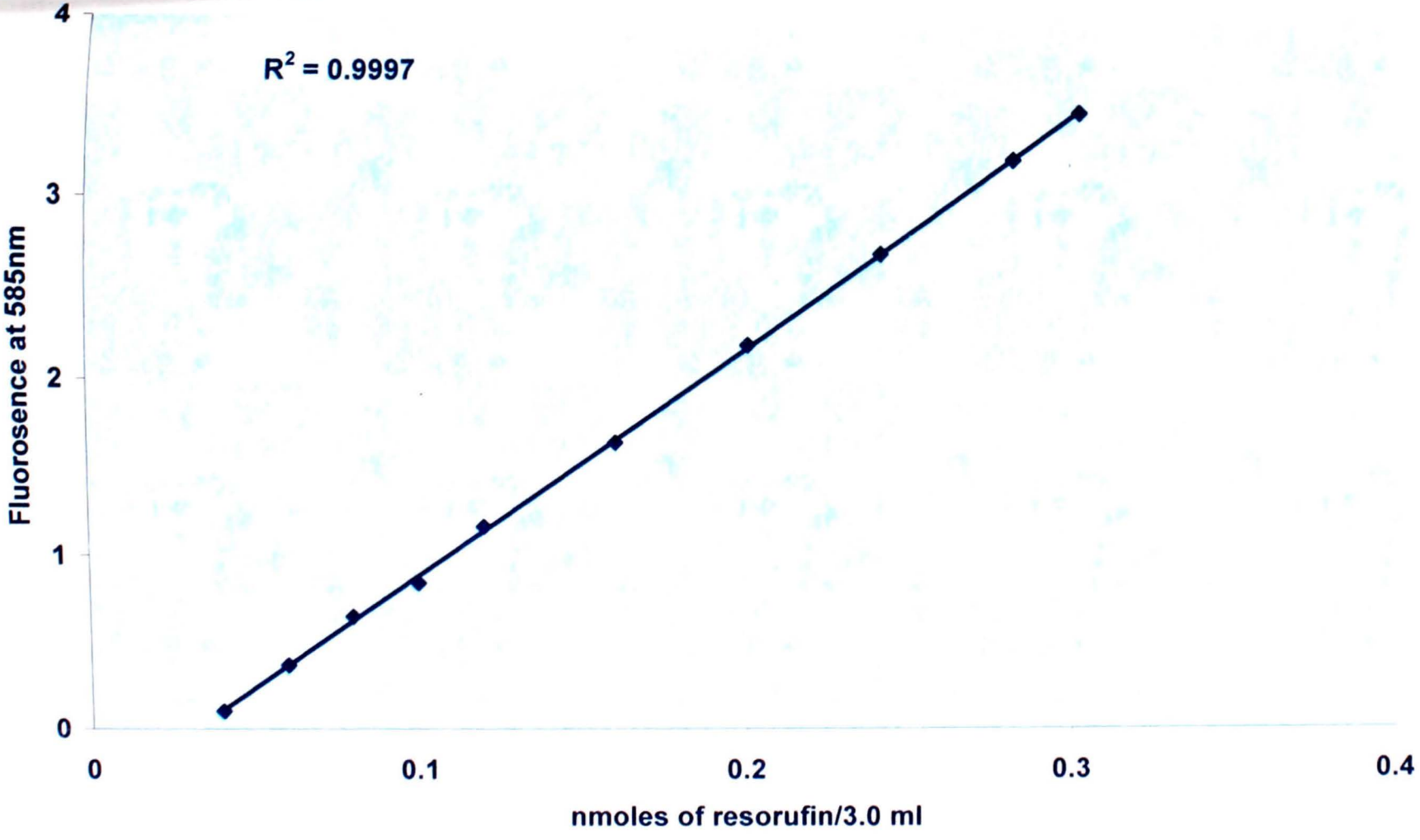


Fig. 3.1 Resorufin standard curve

3.3.4 Assay of glutathione-S-transferase (GST)

Reagents

Potassium phosphate buffer (0.1 M, pH 6.5)

Potassium phosphate buffer (0.1 M, pH 7.0)

Glutathione (10 mM) in 0.1 M potassium phosphate buffer (pH 6.5)

1-Chloro-2,4-dinitrobenzene (CDNB, 10 mM) : CDNB (20.26 mg) was dissolved in 5 ml ethanol and added to it 5 ml potassium phosphate buffer (0.1 M, pH 6.5)

EDTA (10 mM) in potassium phosphate buffer (0.1 M, pH 6.5)

Procedure

Tissue homogenates (10%) prepared in phosphate were centrifuged (10,000 x g) for 30 min, and the supernatants filtered through whatman filter paper to remove fat. Glutathione-S-transferase assay (Habig *et al.*, 1974) follows the increase in absorbance at 340 nm due to the formation of thioether (conjugation of substrate with glutathione). The reaction mixture contained 0.6 ml phosphate buffer (pH 6.5), 0.1 ml glutathione, 0.1 ml CDNB, 0.1 ml EDTA and appropriately diluted tissue homogenate (50-100 µl of 50 time diluted 10% liver homogenate or 50 µl of 4 time diluted mammary homogenate) to a total volume of 1 ml. Blank cuvette contained all above reagent but sample. The change in absorbance was recorded for 3 min during which reaction rate was linear, and the enzyme concentration was adjusted such that the rate of change in absorbance was less than 0.05 per min. The GST activity was calculated using molar extinction coefficient of 9.6 mM^{-1} cm^{-1} and expressed as units/mg protein, where one unit is defined as the amount of enzyme catalyzing the formation of 1 nmole of product per min.

$$\text{Enzyme units/mg protein} = \frac{\Delta OD/min \times 1000}{\text{Volume of sample (ml)} \times 9.6 \times \text{Protein concentration of sample (mg/ml)}}$$

3.3.5 Assay of quinone reductase (QR)

Reagents

Sucrose (0.25 M) in potassium phosphate buffer (0.01 M, pH 7.4)

Tris-HCl buffer (50 mM, pH 7.4) containing 0.046% BSA and 0.02% Tween20: Tris (605.70 mg) and tween 20 (20 µl) were dissolved in 80 ml water and pH adjusted to 7.4, and final volume was made to 100 ml. BSA was added (46 mg/100 ml) to buffer before use.

FAD (50 µM)

NADPH (2 mM)

Dichlorophenolindophenol (DCPIP, 1.2 mM)

Dicumarol (100 µM) in 0.1 N NaOH

Procedure

Tissue homogenate (10%) prepared in sucrose potassium phosphate buffer was centrifuged (12,000 x g) for 20 min, and the supernatant was filtered through whatman filter paper to remove fat. Quinone reductase assay (Ernster, 1967) follows the decrease in absorbance at 600 nm due to the reduction of DCPIP. The reaction mixture contained Tris-HCl buffer (0.5 ml), FAD (0.1 ml), NADPH (0.1 ml), DCPIP (0.1 ml) and appropriately diluted tissue homogenate (50-100 µl of 50 times diluted 10% liver homogenate or 50 µl of 4 times diluted mammary homogenate) to a total volume of 1 ml. The reaction was initiated with DCPIP. Blank cuvette contained all above reagents but NADPH and DCPIP. Another set of blanks containing all above reagents along with of dicumarol (0.1ml) was used to correct the nonenzymatic reduction of DCPIP. The dicumarol sensitive part of the activity was taken as a measure of quinone reductase activity. Assay was performed at 25°C and the change of absorbance was recorded for 5 min during which reaction rate was linear. The QR activity was calculated using molar extinction coefficient of 21 mM^{-1} cm^{-1} and expressed as units/mg protein, where one unit is defined as the amount of enzyme catalyzing the formation of 1 nmole of product per min.

$$\text{Enzyme units/mg protein} = \frac{\text{(Difference in } \Delta\text{OD in presence or absence of dicumarol /min)} \times 1000}{\text{Volume of sample (ml)} \times 21 \times \text{Protein concentration of sample (mg/ml)}}$$

3.3.6 Assay of γ-glutamyltranspeptidase (GGTP)

Reagents

Sucrose (0.25 M) in potassium phosphate buffer (0.01 M, pH 7.4)

Tris-HCl buffer (0.2 M, pH 8.2)

Glycylglycine (300 mM)

Acetic acid (1.5 N)

Mixture 1: Tris-HCl buffer, glycylglycine and water in 5:1:1 ratio

Reaction mixture: γ-glutamyl-p-nitroanilide (2.5 mM) in mixture 1

Procedure

γ-glutamyltranspeptidase was assayed in liver microsomes and mammary tissue homogenate as described by Tate and Meister (1974). Liver microsome was prepared as described in section 3.3.1. Mammary tissue homogenate (10%) was prepared in sucrose potassium phosphate buffer was centrifuged (12,000 x g) for 20 min at 4^0C. The supernatant was filtered through whatman filter paper to remove fat. For assay of GGTP in liver, 0.8 ml reaction mixture was incubated with 0.2 ml of liver microsomal preparation at 37^0C for 5 min. For assay of GGTP in mammary tissue, 0.7 ml reaction mixture was incubated with 0.3 ml tissue homogenate at 37^0C for 5 min. The reaction was then terminated with 2 ml acetic acid (1.5 N). For blank 2 ml acetic acid (1.5 N) was added to the 0.8/0.7 ml reaction mixture followed by sample (0.2/0.3 ml). The sample was then centrifuged at 3000 rpm (2000 x g) for 15 min and supernatants transferred to correspondingly numbered test

tube. The yellow color p-nitroaniline released was measured at 405 nm. The enzyme activity was calculated using molar extinction coefficient of 10.2 mM^{-1} cm^{-1} and expressed as units/mg protein, where one unit is defined as the amount of enzyme catalyzing the formation of 1 nmole of product per min.

$$\text{Enzyme units/mg protein} = \frac{\Delta OD/min \times 1000}{\text{Volume of sample (ml)} \times 10.2 \times \text{Protein concentration of sample (mg/ml)}}$$

3.3.7 Assay of uridinediphosphoglucuronosyl transferase (UDPGT)

Reagents

Tris-HCl buffer (0.2 M, pH 7.4) containing 10 mM $MgCl_2$ and 0.2% Triton X-100: Tris (1.2114 g), 0.101 g $MgCl_2$ and 0.1 g triton X-100 were dissolved in 40 ml water, pH adjusted (1 N HCl) to 7.4, and final volume made to 50 ml.

p-Nitrophenol (p-NP, 5 mM)

Uridinediphosphoglucuronic acid (UDPGA, 7.5 mM)

Trichloroacetic acid (TCA, 5%)

NaOH (2 M)

Procedure

Uridinediphosphoglucuronosyl transferase activity was determined as reported by Bock *et al*, (1983) with modification made by Martin and Black, (1994) spectrophotometrically using UDP-glucuronic acid (UDPGA) and p-nitrophenol as substrates. Liver microsomes (50 µl) (section 3.3.1) were preincubated at 37°C for 3 min with tris-HCl buffer (250 µl), UDPGA (100 µl) and water to make volume 0.45 ml. The reference was prepared containing all reagents, except UDPGA. The reaction started with p-NP (50 µl) was carried at 37°C for 10 min and then stopped with TCA (1 ml). It was then centrifuged

(6000 rpm; 4670 x g) for 10 min to precipitate glucuronidated products. The supernatants (1 ml) of reference as well as sample were separately mixed with NaOH (250 µl), and the absorbance was read at 412 nm using water as blank (Specord 200 double beam UV/visible Spectrophotometer). The UDPGT activity was calculated from difference in p-nitrophenol concentration between the reference and sample tubes, using molar extinction coefficient of 14.9 mM^{-1} cm^{-1} and expressed as units/mg protein, where one unit is defined as the amount of enzyme catalyzing the formation of 1 nmole of product per min.

$$\text{Enzyme units/mg protein} = \frac{\Delta OD/min \times 1000 \times 1.875 \text{ (Dilution factor)}}{\text{Volume of sample (ml)} \times 14.9 \times \text{Protein concentration of sample (mg/ml)}}$$

3.3.8 Protein estimation

Protein was estimated according to the method of Lowry *et al.* (1951).

Reagents

Lowry's reagent: 48 ml of 2 percent Na_2CO_3 (in 0.1N NaOH) was mixed with one ml each of $CuSO_4.5H_2O$ (0.5%) and sodium potassium tartarate (1%).

Folin-Ciocalteau reagent: Diluted 1:1 before use

Procedure

Sample was mixed with Lowry's reagent (5 ml) to make a volume of 6 ml and incubated for 10 min at room temperature, followed by addition of 0.5 ml of Folin-Ciocalteau reagent. The contents were mixed and incubated for 30 min at room temperature and OD was read at 660 nm using Specord 200 double beam UV/visible Spectrophotometer. The protein content of sample was calculated from the standard curve drawn taking 10 to 200 µg of bovine serum albumin.

3.3.9 Estimation of conjugated linoleic acid (CLA) in ghee

Reagents

Methanolic NaOH (1M): Sodium hydroxide (40%) prepared in distilled water diluted ten times with methanol.

Standard CLA: Stock solution of CLA (0.6 mg/ml) was prepared in acetonitrile containing 0.14 percent acetic acid. The working standard was prepared diluting stock solution 5 times. Stock solution or working CLA solutions were stored at 4°C in dark bottles.

Eluting solvent (acetonitrile/H_2O/acetic acid, 70:30:12): All solvents were of HPLC grade and filtered through 0.45 µm polyvinlidiene fluoride (HVHP) membrane and degassed after mixing.

Procedure

Hydrolysis of fat

The fat was hydrolyzed to free fatty acids according to the method of Werner *et al.* (1992). Fat (15 mg) was heated with methanolic NaOH (1 ml) in a screw capped test tube in a boiling water bath for 15 min and then cooled to room temperature. The reaction mixture was acidified with 2 N HCl (1 ml) and the free fatty acids extracted with chloroform (2 X 2 ml) vortexing and centrifuging at 2000 rpm for 10 min. Pooled extract was evaporated to dryness in vacuum oven, and the traces of solvent removed by passing a stream of nitrogen gas.

High performance liquid chromatography (HPLC)

The hydrolyzed fat was dissolved in acetonitrile containing acetic acid (0.14%). Separation of CLA was carried out on Waters HPLC System equipped with dual λ absorbance 2487 detector. A C-18 Water Spherisorb® ODS2 column (250 X 4.6 mm, 5 µm spherical particle) was used with a mobile phase of CH_3CN / H_2O / CH_3COOH (70:30:12) at wavelength 234 nm and flow rate of 1.5 ml/min (Banni *et al.*, 1999). Sample size injected was 20 µl containing 0.5 to 8 µg of CLA. The peaks were identified comparing with

reference CLA standard peaks, one major peak eluting at 13 to 16 min and the minor peak at 16 to 18 min.

CLA was determined in 8 lots of cow ghee used for diet preparation, and the average value of CLA was 5.47mg/g fat.

3.4 Evaluation of gene expression in mammary tissue

3.4.1 Isolation of total RNA

Mammary tissue collected aseptically was immediately transferred to RNA later (4 ml/g tissue) in a sterile tube at 4°C.The samples were stored at -70°C until used.

Reagents and materials:

Ribonuclease-free glass wares and plastic wares: The sterile, disposable plastic wares essentially free of RNase were used for isolation and storage of RNA. General laboratory glass wares were baked at 250°C for 8 h. The plastic wares treated with 0.1% diethylpyrocarbonate (DEPC) for 24 h at room temperature were dried autoclaved and dried and again autoclaved before use. DEPC is an efficient, strong, and nonspecific RNase inhibitor. Since the main source of RNase contamination is from the hands of workers, care was taken to use gloves during work.

Liquid nitrogen

TRI reagent

Chloroform

Isopropanol

Ethanol (75 % in DEPC treated water)

Nuclease free water

Procedure

Total RNA was isolated (using DEPC treated and sterilized apparatus) with TRI reagent as per the manufacturer's instructions according to the method described by Chomczynski and Sacchi (1987). The tissue

(approximately 100 mg) was homogenized in liquid N_2 by using sterile pestle and mortar (dry sterilized at 150°C for 1-2 h followed by autoclaving). The tissue powder was transferred to eppendorf tube with the help of DEPC treated and sterile spatula and added to it TRI reagent (1.0 ml), and vortexed for 5 min until a clear homogenate was obtained. The homogenate was centrifuged (12,000 x g) at 4°C for 12 min to remove the insoluble material (extracellular membranes, polysaccharides, and high molecular weight DNA). The supernatant contains RNA, proteins and a layer of fatty material on the surface. The fatty layer was removed and the clear supernatant was transferred to a fresh sterile eppendorf tube. The supernatant was allowed to stand for 5 to 10 min at room temperature, and then added to it 0.2 ml chloroform per ml TRI reagent. The mixture mixed vigorously for 15 s, allowed to stand at room temperature for 15 min was centrifuged (12,000 x g) for 12 min at 4°C. The upper clear aqueous phase (avoiding interphase that contain DNA) was transferred to a fresh eppendorf tube and added to it 0.5 ml isopropanol per ml TRI reagent. The resulting solution was mixed gently and allowed to stand at room temperature for 10 min prior to centrifugation (12,000 x g) for 12 min at 4°C. The supernatant was discarded and the RNA pellet obtained was washed with 1.0 ml of ethanol (75 %) per ml TRI reagent and then centrifuged (7500 x g) for 5 min at 4°C. The supernatant was discarded and the RNA pellet was air dried (standing at room temperature for 20-30 min), finally resuspended in nuclease free water (20 µl) and stored at –70°C.

3.4.1.1 RNA quantitation and purity

RNA stock (5 µl) was diluted with 995 µl of sterile DEPC-treated water (pH 8.0), and read at 260 nm and 280 nm using Specord 200 double beam UV/visible Spectrophotometer, and the RNA content was calculated using the following equation:

$$\text{Total RNA } (\mu g/\mu l) = (A_{260} \times 40 \times 200) / 1000$$

Where, A260 is absorbance at 260 nm, 40 is a constant for RNA quantitation, 200 is dilution factor and division by 1000 yields µg/µl. RNA purity was

determined by evaluation of 260:280 ratio, a value greater than 1.7 or 1.8 is acceptable.

3.4.1.2 Integrity or quality of RNA

Reagents

10 X MOPS buffer (0.2 M; pH 7.0) containing sodium acetate (20 mM), EDTA (10 mM): MOPS (4.18 g) was dissolved in 70 ml sterile DEPC treated water and pH adjusted up to 7.0 with 2 N NaOH. 2.0 ml of 1 M sodium acetate and 2.0 ml of 0.5 M EDTA (pH 8.0) was added to this and final volume (100 ml) made with DEPC treated water. The buffer was stored in dark color bottle at 4°C.

10 X formaldehyde gel loading dye containing glycerol (50%), EDTA (10 mM; pH 8.0), bromophenol blue (0. 25 %), xylene cyanol FF (0.25 %): Mixed 5.0 ml glycerol, 0.2 ml 0.5 M EDTA (pH 8.0), 25 mg bromophenol blue and 25 mg xylene cyanol FF and final volume (10 ml) was made with DEPC treated water.

Ethidium bromide (200 µg/ml)

Formaldehyde (molecular biology grade)

Agarose

Procedure

The quality or integrity of RNA was tested by electrophoresis on denaturing agarose gel. Agarose (1.5 g) in 72 ml DEPC treated water taken in a conical flask was melted on a hot plate (or oven at 100°C) to obtain clear solution. The content was allowed to cool (50-60°C) and then added to it 10 ml of 10 X MOPS buffer and 18 ml of formaldehyde. The content was transferred to a horizontal gel casting apparatus and gel was allowed to polymerize for about 40 min. RNA (4 µg) mixed with 10 X MOPS buffer (2 µl), formaldehyde (4 µl) and of ethidium bromide (2 µl) taken in an eppendorf tube was denatured by incubating at 65-70°C for 10-15 min. The mixture was

cooled and added to it 10 X formaldehyde gel loading dye (2 μl) and loaded to 1.5 percent agarose gel. The gel was run in a mini-submarine gel electrophoresis system in approximately 250 ml 1 x MOPS buffer at 100 v for approximately 1 h. The products visualized using an UV transilluminator.

3.4.2 Primer designing

The primer sequence of cyclin D (Hur *et al.*, 2000), COX-2 and GADPH (Badawi *et al.*, 1998) were taken from literature. Cyclin A and PPAR-γ primers were designed using Primer 3 output software, and got synthesized from Imperial Life Sciences (P) Lid. (Gurgaon., India). The sequence of primers, product size and annealing temperature are given in table-3.1.

3.4.3 Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction was performed using Fermentas RevertAid™ first strand cDNA synthesis kit. RT-PCR reaction was carried out in two steps, first strand cDNA synthesis (RT reaction) and then PCR reaction (amplification of cDNA).

3.4.3.1 Reverse transcription-first strand cDNA synthesis

Reagents

Oligo $(dT)_{18}$ primer

dNTP mix (10 mM)

5 X RT buffer

RNase Inhibitor

M-MuLV Reverse Transcriptase

DEPC-treated water

Procedure

Taken in a sterile RNase free microcentrifuge tube were total RNA (2 μg), oligo $(dT)_{18}$ primer (1 μl) and DEPC-treated water to make volume up to 12 μl. Secondary structure of RNA was denatured by incubating at 70^0C for 5

Table: 3.1 Primer sequence, annealing temperature and expected size of PCR product of various genes

Gene	Primer sequence	Annealing temperature	Product size
COX-2	5'- CTGTATCCCGCCCTGCTGGTG 3'- CTTGCGTTGATGGTGGCTGTCTT	55°C	279
PPAR-γ	5'- CATCGAGGACATCCAAGACAAC 3'- TGAAGGCTCATATCTGTCTCC	55°C	161
Cyclin A	5'- AAGACGAAAAGCCAGTGAATG 3'- GGTGCTCCATTCTCAGAACC	58°C	396
Cyclin D	5'- TGGAGCCCCTGAAGAAGAG 3'- AAGTGCGTTGTGCGGTAGC	60°C	405
GAPDH	5'- CCTTCATTGACCTCAACTAC 3'- GGAAGGCCATGCCAGTGAGC	60°C	574

min. Added to each tube were 2 μl of dNTP mix (10 mM), 4 μl of 5 x RT buffer and 1 μl RNase Inhibitor. The content were mixed, spun gently and briefly, and incubated in a thermocycler at 37°C for 5min. Finally 1 μl of M-MuLV reverse transcriptase (200 IU) was added and the mixture was again briefly spun. It was then incubated for 1h at 42°C in a thermocycler followed by 10 min incubation at 70°C and 4°C pause. cDNA was stored at –70°C. RT –ve control was prepared similarly excluding M-MLV reverse transcriptase in the incubation mixture.

3.4.3.2 Optimization of PCR for annealing temperature

The primers are jiggling around, hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. More stable bonds last a little bit longer (primers that fit exactly) and on that little double-stranded piece, the DNA polymerase can attach and start copying the template. Annealing temperatures is important to optimize signal and to minimize non-specific binding of primers to the template. Thus, optimum annealing temperature was determined for cyclin A and PPAR-γ. For cyclin A, 55° C, 58° C, 60° C, 62° C and 65° C were tested and for PPAR-γ, 52° C, 55° C, 58° C, 60° C and 62° C were tested. The amplified products were analyzed on agarose gel to select the optimum annealing temperature for high yield and specificity. For COX-2 and GADPH (Badawi *et al.*, 1998) and cyclin D (Kim *et al.*, 2000), annealing temperature was selected from literature.

3.4.3.3 Optimization of PCR for magnesium chloride ($MgCl_2$) concentration:

All thermostable DNA polymerase require free divalent cations usually Mg^{2+} for activity. Because dNTPs and oilgonucleotides bind Mg^{2+}, the molar concentration of the cations must exceed that of phosphate groups contributed by dNTPs and primers. Thus, determination of optimal concentration for cyclin A and PPAR-γ was performed using different $MgCl_2$ concentration (0.5 to 2.5 mM) in standard PCR reaction using $MgCl_2$ free 10 X

Taq buffer. The amplified products were analyzed on agarose gel to select the optimum $MgCl_2$ concentration that gives the high yield and specificity.

3.4.3.4 Polymerase chain reaction (PCR)

Reagents

10 X Taq buffer (containing 15 mM $MgCl_2$)

Forward & reverse primer (10 µM)

dNTP mix (2 mM)

Taq DNA polymerase

Nuclease free water

Procedure

For the amplification of cDNA, the following components were added to a PCR tube: 4 µl of RT product, 0.5 µl each of forward and reverse gene specific primer (10 µM), 2.5 µl of dNTP mix (2 mM), 2.5 µl of 10 x *Taq* buffer containing 15 mM $MgCl_2$, 0.5U *Taq* DNA polymerase (1U/µl) and final volume made up to 25 µl with nuclease-free water. PCR –ve control was prepared similarly excluding cDNA. After brief spin, PCR reaction was performed in a thermocycler (Biometra, Analtik GmbH, Germany). The reaction mixture was incubated at 94°C for 4 min (denaturation) followed by 30 cycle of PCR reaction in following steps:

Incubation at 94°C for 1 min (denaturation)

↓

Incubation at annealing temperature (different annealing temperature or different gene as given in table. 3.1) for min

↓

Incubation at 72°C for 1 min (extension)

After 30 cycles of above three steps the mixture was incubated at 72°C for 7 min (final extension) and then pause at 4°C.

3.4.3.5 Agarose gel electrophoresis of PCR products

Reagents

50 X tris-acetate-EDTA (TAE) buffer : Tris base (121 g) was dissolved in 200 ml water and added to it 28.55 ml glacial acetic acid and 50 ml 0.5 M EDTA, and volume made to 500 ml.

6 X Gel loading dye

Ethidium bromide (10 mg/ml)

Procedure

The PCR products were separated on 2% agarose gel. The amount of agarose gel was prepared depending upon the size of gel casting apparatus. Agarose (0.5 g) was melted in 1 x TAE buffer (25 ml) on a hot plate. The content was cooled to 50-55 °C then added to it 1.5 µl of ethidium bromide. The gel was casted on horizontal gel casting apparatus (Bangalore Genei, Bangalore, India) and was allowed to polymerize for about 40 min prior to use. The PCR product (2 µl) mixed with 6 x gel loading dye (2.0 µl) in an eppendorf tube was loaded to the agarose gel. The experimental samples, PCR-minus, RT-minus controls and 100bp DNA ladder loaded in separate wells were run in a mini-submarine gel electrophoresis system in approximately 250 ml of 1 x TAE buffer at 100 V for approximately 1 h. The products were visualized using an UV transilluminator. The gel photographs were taken with a gel documentation apparatus (Alpha Innotech Corporation, USA).

3.5 Statistical analysis

The results were expressed as mean ± SE. Statistical analysis for enzymes activities and gene expression studies was done through analysis of variance (ANOVA) and comparisons between means were tested by Turkey: compare all pair of column using Prism 3.0 software. Statistical analysis for tumor incidence was done by Chi-square test using Systat 7.0 software. Unpaired student t-test was used for the feed intake, body weight, tumor weight, tumor volume and tumor multiplicity using Prism 3.0 software. A

probability level of $P<0.05$ was used to determine the statistical significance. Tabulation and graphical presentation of data was done using Microsoft Excel.

CHAPTER – 4

Results

4. RESULTS

4.1 Effect of cow ghee vs soybean oil on phase I and phase II pathways of xenobiotic metabolism in normal rats

Effect of cow ghee on carcinogen metabolizing activities was compared with that of soybean oil in female rats fed for 44 weeks. The phase I activities were determined at 0 d, 5 and 44 weeks in liver microsomes. Glutathione-S-transferase (GST) and quinone reductase (QR) activities were determined in 10000 x g liver and mammary tissue homogenates. γ- glutamyl transpeptidase (GGTP) and UDP- glucuronosyl transferase (UDPGT) activities were determined in liver microsomes, and the former was also determined in 10000 x g mammary tissue homogenate. The mammary tissue was examined At 44 weeks for the expression of various genes involved in mammary carcinogenesis. The feed intake (Table 4.1) and growth rate (Table 4.2, Fig. 4.1) were similar in two dietary groups.

4.1.1 Phase I Pathway Activities in Liver Microsomes

Cytochrome P450s activities were determined in liver microsomes in order to evaluate the effect of dietary fat on carcinogen activating status in rats.

4.1.1.1 Cytochrome P4501A1 activity in rat liver

Cytochrome P4501A1 activity increased up to 5 weeks on both soybean oil and cow ghee diets (Fig. 4.2), and the magnitude of increase was greater on soybean oil (60%) than on cow ghee (28%). The Cyp1A1 activity on cow ghee opposed to soybean oil decreased by 20.1 and 11.4% at 5 and 44 weeks of dietary treatment, respectively (Table 4.3; Fig. 4.2).

4.1.1.2 Cytochrome P4501A2 activity in rat liver

Cytochrome P4501A2 activity was not affected significantly on soybean oil, while it decreased on cow ghee by 41.5% at 5 weeks, and thereafter it did not change significantly in either group. In cow ghee group, Cyp1A2 activity was decreased by 47.4 and 44.2%, in comparison with soybean oil group, at 5 and 44 weeks of dietary treatment, respectively (Table 4.3, Fig. 4.3).

4.1.1.3 Cytochrome P4501B1 activity in rat liver

Cytochrome P4501B1 activity remained unaffected on soybean oil throughout the experimental period, while on cow ghee it decreased by 55 and 37% at 5 and 44 weeks, respectively. The Cyp1B1 activity on cow ghee decreased by 54.6 and 37.2%, in comparison with soybean oil group, at 5 and 44 weeks of dietary treatment, respectively (Table 4.3, Fig. 4.4).

4.1.1.4 Cytochrome P4502B1 activity in rat liver

Cytochrome P4502B1 activity was not affected on soybean oil, while on cow ghee it decreased by 44% at 5 weeks. The Cyp2B1 activity on cow ghee decreased by 45.9 and 36.9%, in comparison with soybean oil group, at 5 and 44 weeks of dietary treatment, respectively (Table 4.3, Fig. 4.5).

4.1.1.5 Cytochrome P4502E1 activity in rat liver

Cytochrome P4502E1 activity in liver microsomes was not affected by feeding either cow ghee or soybean oil, and no difference was observed between the two dietary treatments throughout the experimental period (Table 4.3; Fig 4.6).

4.1.2 Phase II pathway activities in rat liver and mammary tissue

Phase II activities was determined in liver and mammary tissue in order to evaluate the effect of dietary fat on carcinogen detoxifying status in rats.

Table 4.1 Feed intake (g) of rats fed soybean oil or cow ghee

Days	Soybean oil	Cow ghee
0-30	6.89 ± 0.40	7.26 ± 0.44
31-60	9.65 ± 0.22	9.47 ± 0.30
61-90	7.80 ± 0.14	7.83 ± 0.17
91-120	8.13 ± 0.21	8.19 ± 0.25
121-150	9.96 ± 0.21	10.47 ± 0.20
151-180	9.58 ± 0.20	10.76 ± 0.19
181-210	9.98 ± 0.23	10.52 ± 0.24
211-240	10.44 ± 0.16	10.39 ± 0.23
241-270	12.90 ± 0.19	12.66 ± 0.15
271-300	13.15 ± 0.17	13.53 ± 0.22
Overall mean	9.85 ± 0.63	10.11 ± 0.64

Values (g/day) are mean ± SE for n= 5

Table 4.2 Body weight (g) of rats fed soybean oil or cow ghee

Weeks	Soybean oil	Cow ghee
0	27.7 ± 1.6	29.5 ± 0.6
2	61.0 ± 4.4	67.7 ± 2.2
4	90.7 ± 6.1	104.2 ± 2.2
6	117.2 ± 6.6	134.7 ± 1.1
8	140.7 ± 7.4	147.2 ± 1.6
10	158.5 ± 9.2	164.5 ± 2.6
12	167.5 ± 8.3	169.0 ± 4.0
14	170.5 ± 6.4	178.7 ± 4.0
16	174.5 ± 8.7	184.0 ± 3.2
18	181.2 ± 9.0	199.7 ± 3.1
20	190.7 ± 7.4	210.7 ± 3.7
22	197.5 ± 9.6	219.0 ± 4.5
24	203.7 ± 10.4	227.7 ± 7.3
26	201.7 ± 9.2	230.2 ± 6.3
28	213.5 ± 10.4	226.5 ± 5.4
30	216.7 ± 9.6	232.7 ± 9.4
32	220.4 ± 9.1	238.7 ± 7.8
34	224.5 ± 11.7	245.2 ± 8.7
36	227.5 ± 12.7	251.7 ± 10.6
38	233.3 ± 16.4	256.2 ± 9.7
40	239.2 ± 13.1	265.2 ± 8.5
42	246.2 ± 11.6	276.4 ± 9.1
44	251.2 ± 13.2	280.9 ± 8.7

Values are mean ± SE for n=8

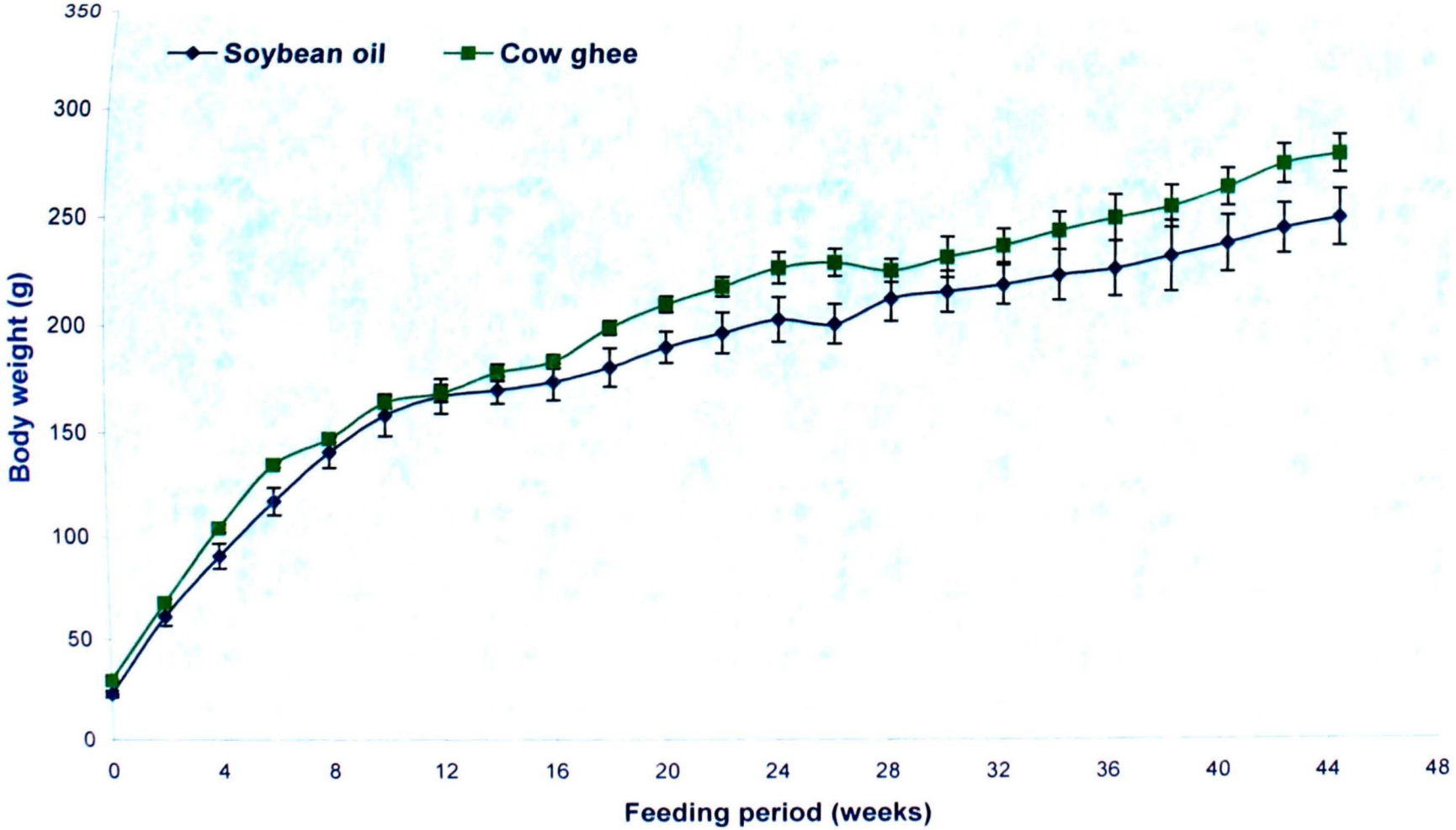


Fig. 4.1 Body weight of rats fed soybean oil or cow ghee

Values are mean± SE for n=8

Table 4.3 Effect of cow ghee vs soybean oil on carcinogen activating enzymes in rat liver

	Dietary fat	0 day	5 weeks	44 weeks
CYP4501A1 (units*/mg protein)	Soybean oil	$22.1^{a} \pm 0.8$	$35.4^{b} \pm 2.3$	$33.2^{b} \pm 1.0$
	Cow ghee	$22.1^{a} \pm 0.8$	$28.3^{c} \pm 1.1$	$29.4^{c} \pm 1.1$
CYP4501A2 (units*/mg protein)	Soybean oil	$19.7^{a} \pm 1.2$	$21.7^{a} \pm 1.6$	$24.0^{a} \pm 1.4$
	Cow ghee	$19.7^{a} \pm 1.2$	$11.4^{b} \pm 0.7$	$13.4^{b} \pm 0.5$
CYP4501B1 (units*/mg protein)	Soybean oil	$5.29^{a} \pm 0.07$	$5.24^{a} \pm 0.68$	$5.32^{a} \pm 0.23$
	Cow ghee	$5.29^{a} \pm 0.07$	$2.38^{b} \pm 0.09$	$3.34^{c} \pm 0.22$
CYP4502B1 (units*/mg protein)	Soybean oil	$4.46^{a} \pm 0.29$	$4.64^{a} \pm 0.59$	$4.12^{a} \pm 0.12$
	Cow ghee	$4.46^{a} \pm 0.29$	$2.51^{b} \pm 0.22$	$2.60^{b} \pm 0.15$
CYP2E1 (units#/mg protein)	Soybean oil	1.16 ± 0.06	1.07 ± 0.06	1.06 ± 0.04
	Cow ghee	1.16 ± 0.06	1.07 ± 0.04	1.06 ± 0.05

Values (mean ± SE for n=5) for the said activity with different superscripts are significantly different ($P<0.05$).

* One unit is equal to one pmole of product formed/min.

\# One unit is equal to one nmole of product formed/min.

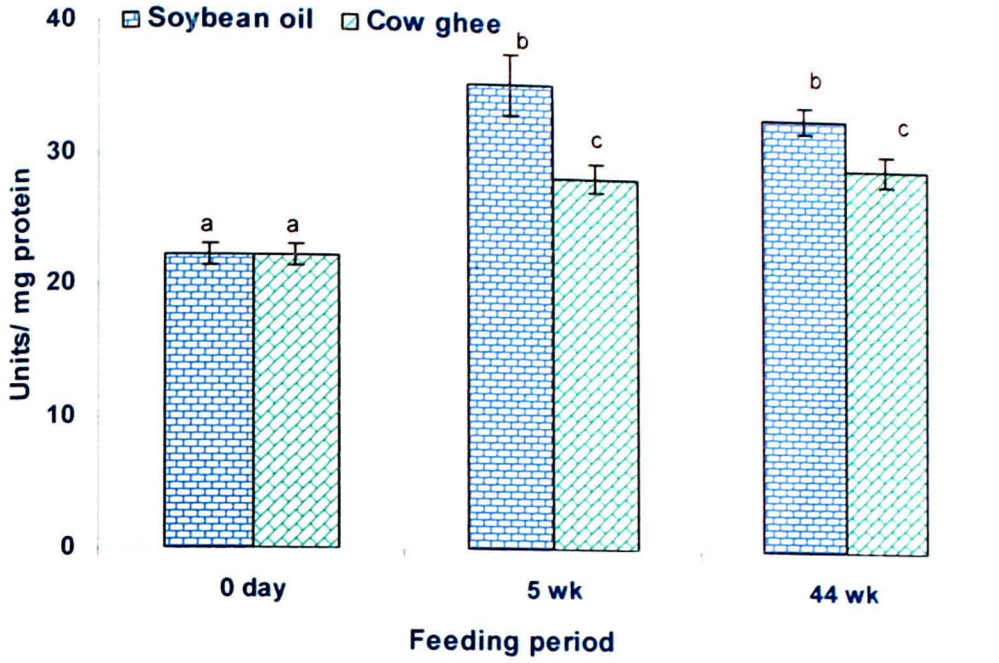


Fig. 4.2 Effect of feeding cow ghee vs soybean oil on CYP1A1 activity in rat liver

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of the product per min.

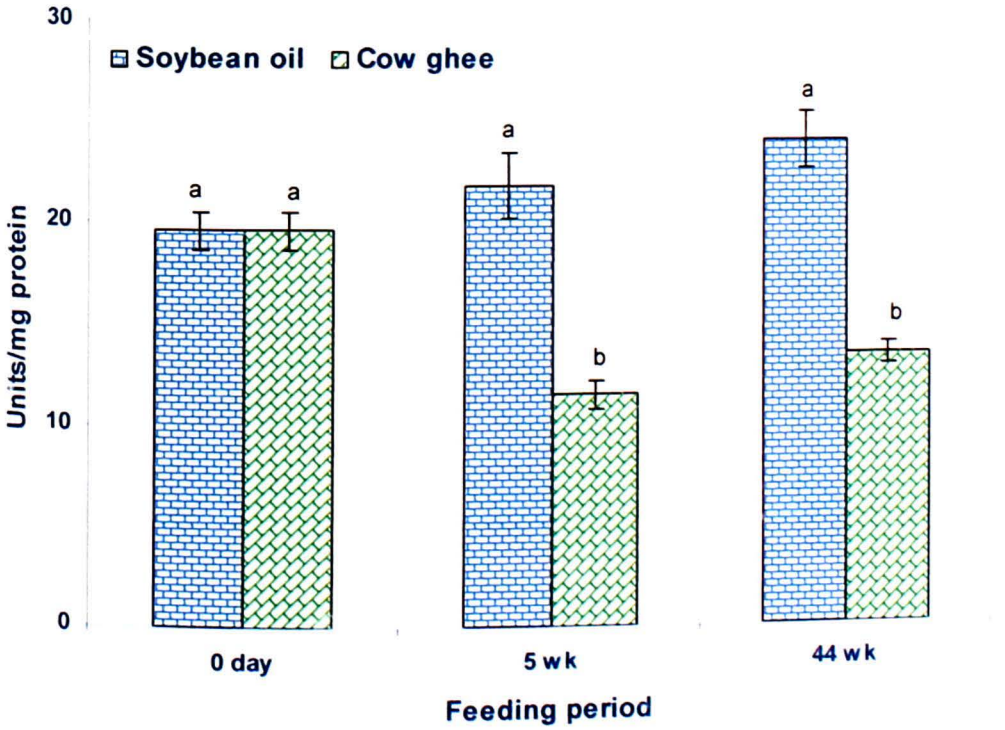


Fig. 4.3 Effect of feeding cow ghee vs soybean oil on CYP1A2 activity in rat liver

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of product per min.

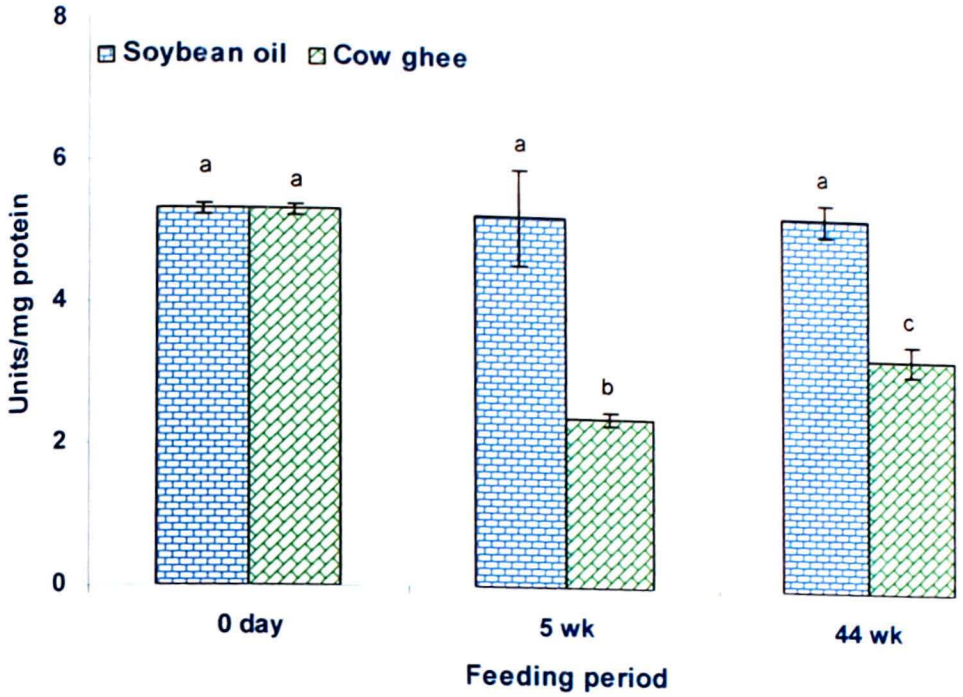


Fig. 4.4 Effect of feeding soybean oil vs cow ghee on CYP1B1 activity in rat liver

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of product

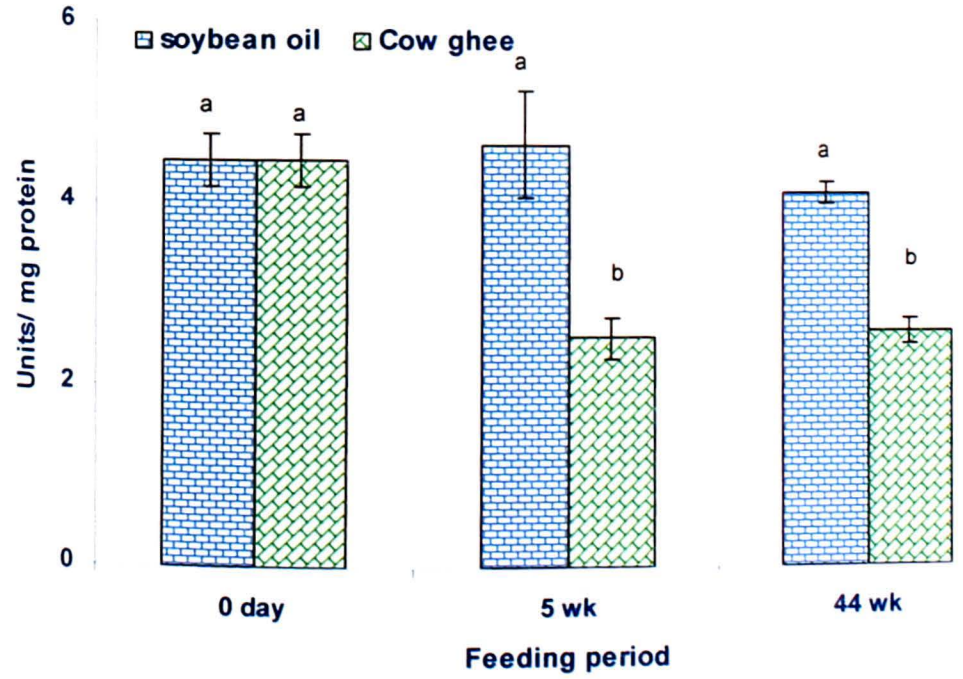


Fig. 4.5 Effect of feeding cow ghee vs soybean oil on CYP2B1 activity in rat liver

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of product per min.

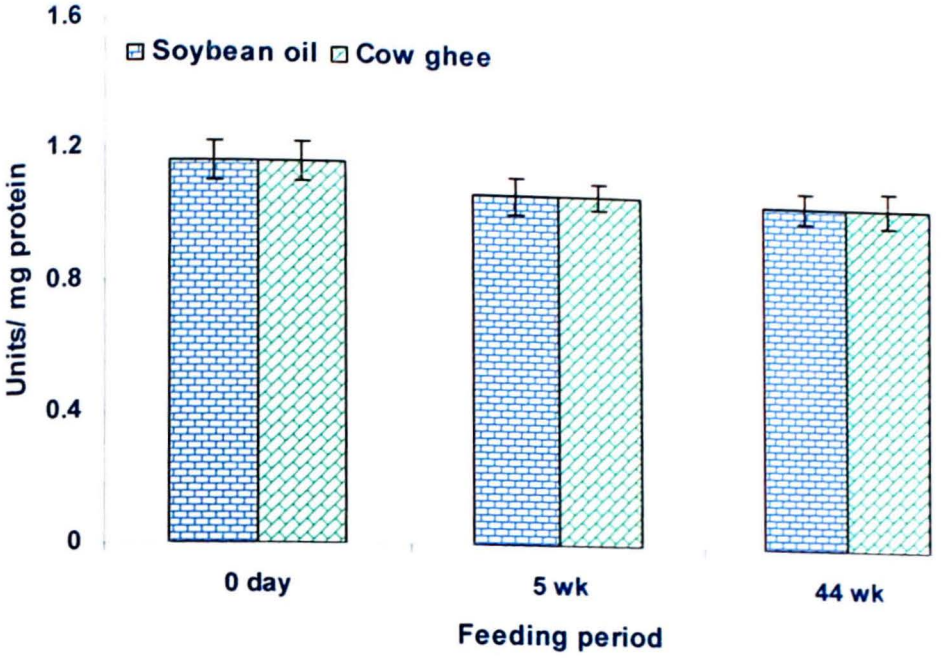


Fig. 4.6 Effect of feeding cow ghee vs soybean oil on CYP2E1 activity in rat liver

Values are mean ± SE for n=5; Values are not statistically different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

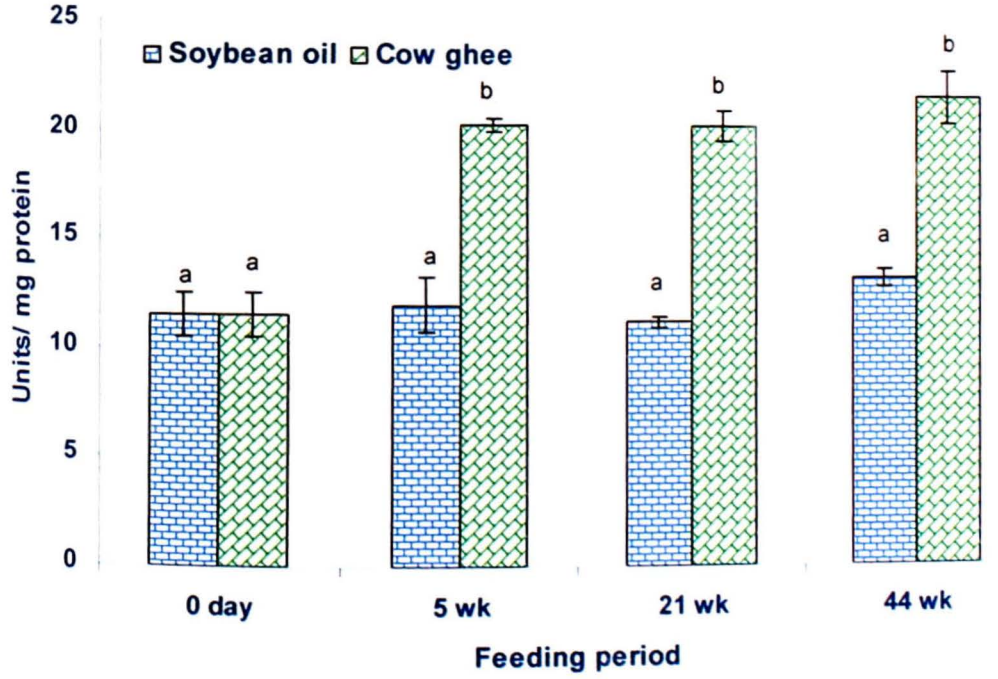


Fig. 4.7 Effect of feeding cow ghee vs soybean oil on UDPGT activity in rat liver

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

4.1.2.1 UDP- glucuronosyl transferase activity in rat liver

UDP- glucuronosyl transferase (UDPGT) activity in liver microsomes was not significantly affected on soybean oil, while on cow ghee diet, it increased by about 76% at 5 weeks, and thereafter remained stable. The UDPGT activity in ghee group was 63 - 69% higher than in soybean oil group during 5 to 44 weeks (Table 4.4; Fig 4.7) of dietary treatment.

4.1.2.2 Glutathione-S- transferase activity in rat liver and mammary tissue

Glutathione-S-transferase (GST) activity increased similarly in both soybean oil and cow ghee groups during first 5 weeks. The rise in GST activity after 5 weeks was not significant in soybean oil group, while in cow ghee group, the rise in activity was statistically significant. The difference between two dietary groups was, however, not significant at any stage (Fig. 4.8; Table 4.4).

In mammary tissue, GST activity was not affected by either of the two dietary treatments, and the difference between two dietary groups was also not statistically significant at any stage (Fig. 4.9; Table 4.5).

4.1.2.3 γ- glutamyl transpeptidase activity in rat liver and mammary tissue

γ- glutamyl transpeptidase (GGTP) activity in liver microsome was not affected by dietary treatment with cow ghee, while it decreased significantly on soybean oil diet. The activity of GGTP in soybean oil fed rats was decreased by 34.0, 27.2 and 22.2% at 5, 21 and 44 weeks, respectively, compared to age-matched cow ghee fed rats (Table 4.4, Fig. 4.10).

In mammary tissue homogenate (10000 x g), the GGTP activity increased in both soybean oil and cow ghee groups. The magnitude of increase in GGTP activity was not statistically significant on soybean oil, while on cow ghee it rose significantly by 125% at 44 weeks compared to o day controls (Table 4.5, Fig. 4.11). The GGTP activity increased significantly on

cow ghee by about 131% at 5 and 21 weeks and by 285% at 44 weeks, compared to age-matched soybean oil fed rats (Fig. 4.11; Table 4.5).

4.1.2.4 Quinone reductase activity in rat liver and mammary tissue

The QR activity in liver homogenate on soybean oil diet was not affected up to 21 weeks, and it increased by 62.6% at 44 weeks compared to 0 day controls. The QR activity in cow ghee groups increased by 60, 128 and [illegible]% at 5, 21 and 44 weeks, respectively, compared to 0 day control (Fig. 4.12). The QR activity in cow ghee group compared to soybean oil group, increased by 48, 110 and 61% at 5, 21 and 44 weeks, respectively (Table 4.6). The QR activity in mammary tissue increased in cow ghee groups by over [illegible]% compared to age-matched soybean oil groups (Fig. 4.13; Table [illegible]).

4.2 Effect of dietary cow ghee vs soybean oil on mammary carcinogenesis

Effect of cow ghee on mammary carcinogenesis was studied and compared with that of soybean oil using two groups of female rats fed for 44 weeks diets containing cow ghee or soybean oil. Mammary tumors were induced by administered DMBA at 55 d of age, when mammary tissue was in rapid developing phase. Five animals from each group were sacrificed 18 weeks post carcinogen administration for the analysis of phase I carcinogen metabolizing activities. Rest of the animals were sacrificed [illegible] weeks post carcinogen administration for examination of tumor, phase I and phase II enzymes of carcinogen metabolism and expression of various putative genes ([illegible], cyclin A, cyclin D1 and [illegible]) involved in mammary carcinogenesis.

[illegible]

Table 4.4 Effect of cow ghee vs soybean oil on carcinogen detoxifying activities (units*/mg protein) in rat liver

	Dietary fat	0 day	5 weeks	21 weeks	44 weeks
UDP-glucouronsyl transferase	Soybean oil	$11.5^{a} \pm 1.0$	$11.9^{a} \pm 1.3$	$11.1^{a} \pm 0.2$	$13.2^{a} \pm 0.4$
	Cow ghee	$11.5^{a} \pm 1.0$	$20.1^{b} \pm 0.3$	$20.2^{b} \pm 0.7$	$21.6^{b} \pm 1.2$
Glutathione-S-transferase	Soybean oil	$97.7^{a} \pm 3.5$	$134.4^{b} \pm 8.3$	$141.5^{b} \pm 4.7$	$157.1^{bc} \pm 5.0$
	Cow ghee	$97.7^{a} \pm 3.5$	$134.1^{b} \pm 5.1$	$147.3^{bc} \pm 6.0$	$177.4^{c} \pm 10.0$
γ-glutamyl transpeptidase	Soybean oil	$4.75^{a} \pm 0.17$	$3.26^{b} \pm 0.36$	$3.11^{b} \pm 0.50$	$3.68^{b} \pm 0.12$
	Cow ghee	$4.75^{a} \pm 0.17$	$4.94^{a} \pm 0.22$	$4.27^{a} \pm 0.17$	$4.73^{a} \pm 0.12$
Quinone reductase	Soybean oil	$60.7^{a} \pm 5.2$	$65.5^{a} \pm 3.1$	$65.7^{a} \pm 4.9$	$98.6^{b} \pm 3.3$
	Cow ghee	$60.7^{a} \pm 5.2$	$96.7^{b} \pm 6.8$	$138.3^{c} \pm 8.4$	$158.3^{c} \pm 4.7$

Values (mean ± SE for n=5) for the said activity with different superscripts are significantly different ($P<0.05$).

* One unit is equal to one nmole of product formed/min

Table 4.5 Effect of cow ghee vs soybean oil on carcinogen detoxifying activities (units*/mg protein) in rat mammary tissue

	Dietary fat	5 weeks	21 weeks	44 weeks
Glutathione-S-transferase	Soybean oil	20.1 ± 1.2	20.6 ± 0.8	23.6 ± 2.3
	Cow ghee	22.1 ± 1.9	22.0 ± 1.2	23.4 ± 1.6
γ-glutamyl transpeptidase	Soybean oil	0.98^{a} ± 0.07	1.31^{a} ± 0.07	1.53^{a} ± 0.21
	Cow ghee	2.61^{b} ± 0.21	3.02^{b} ± 0.11	5.89^{c} ± 0.36
Quinone reductase	Soybean oil	13.2^{a} ± 1.7	14.6^{a} ± 0.8	16.4^{a} ± 0.8
	Cow ghee	21.3^{b} ± 1.9	22.2^{b} ± 0.8	25.0^{b} ± 1.4

Values (mean ± SE for n=5) for the sald activity with different superscripts are significantly different ($P<0.05$).

*One unit is equal to one nmole of product formed/min

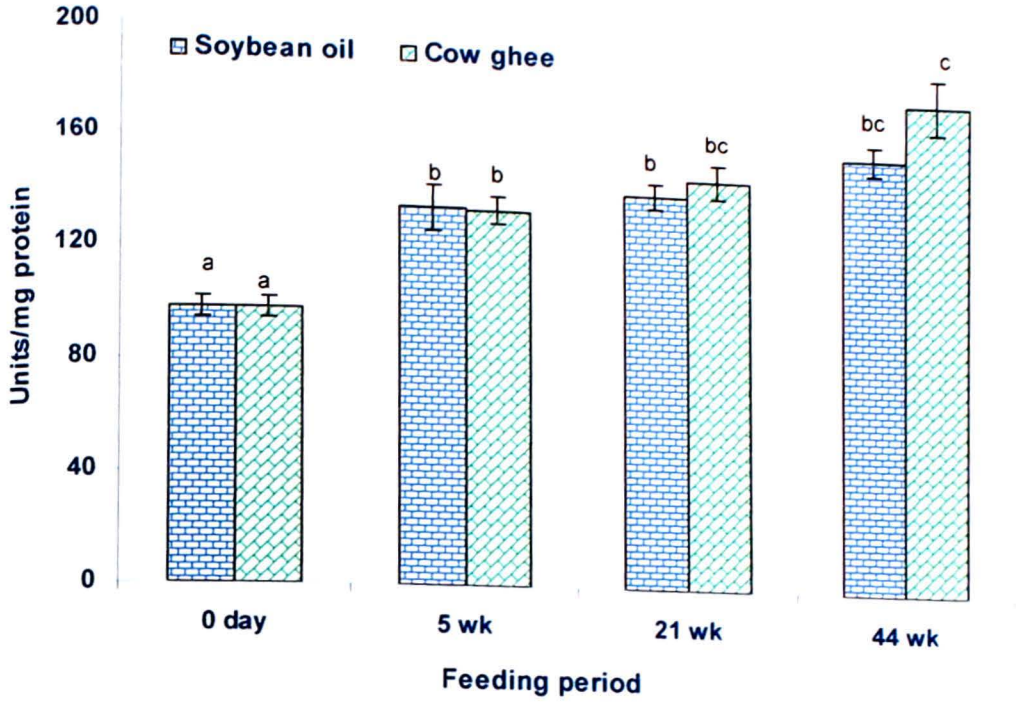


Fig. 4.8 Effect of feeding cow ghee vs soybean oil on GST activity in rat liver

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

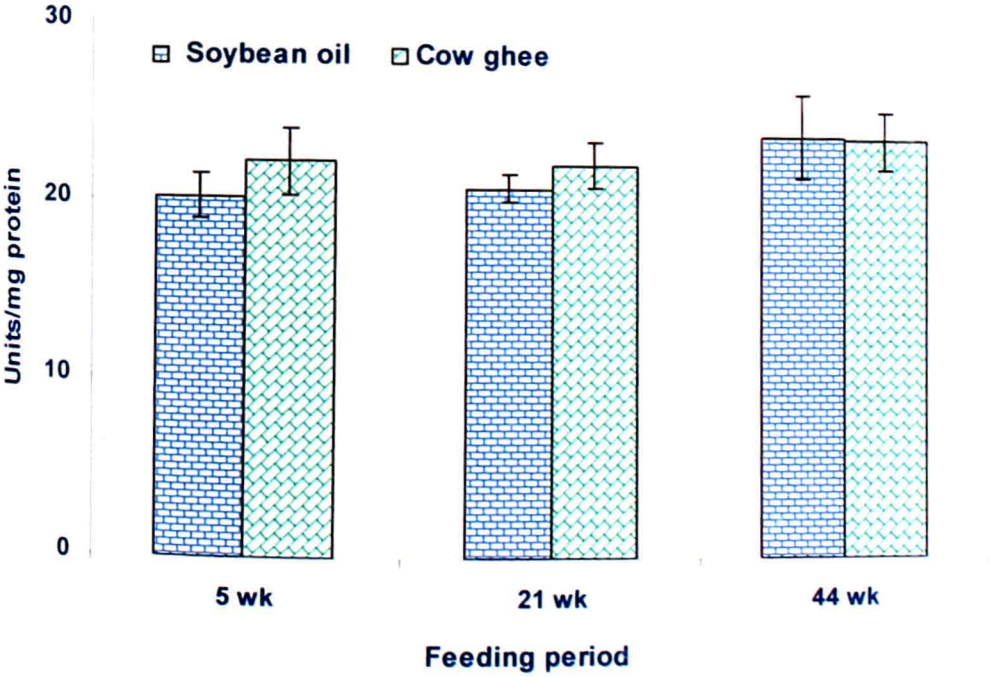


Fig. 4.9 Effect of feeding cow ghee vs soybean oil on GST activity in rat mammary tissue

Values are mean ± SE for n=5; Values are not statistically different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

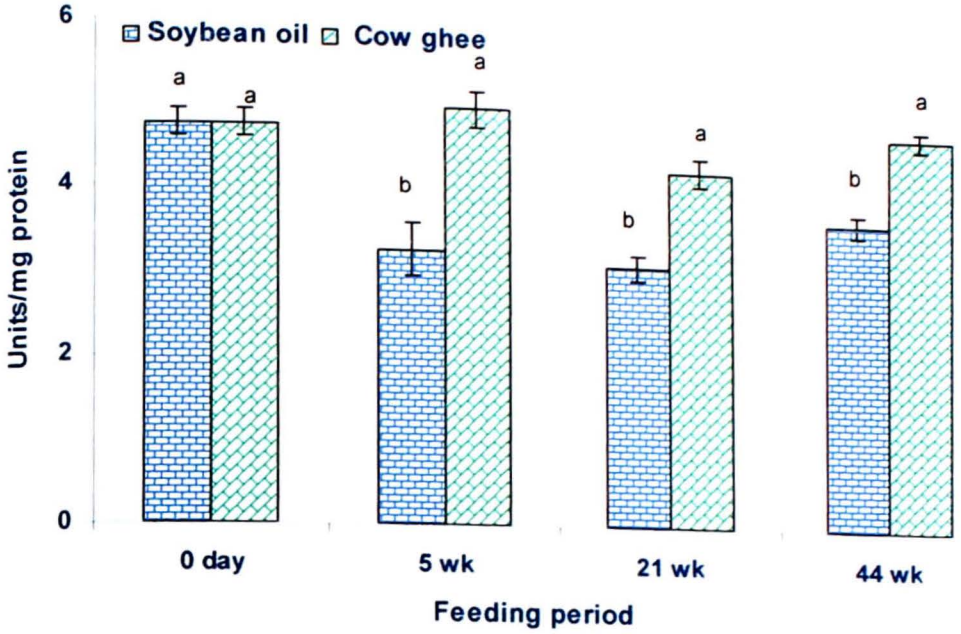


Fig. 4.10 Effect of feeding cow ghee vs soybean oil vs on GGTP activity in rat liver

Values(mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

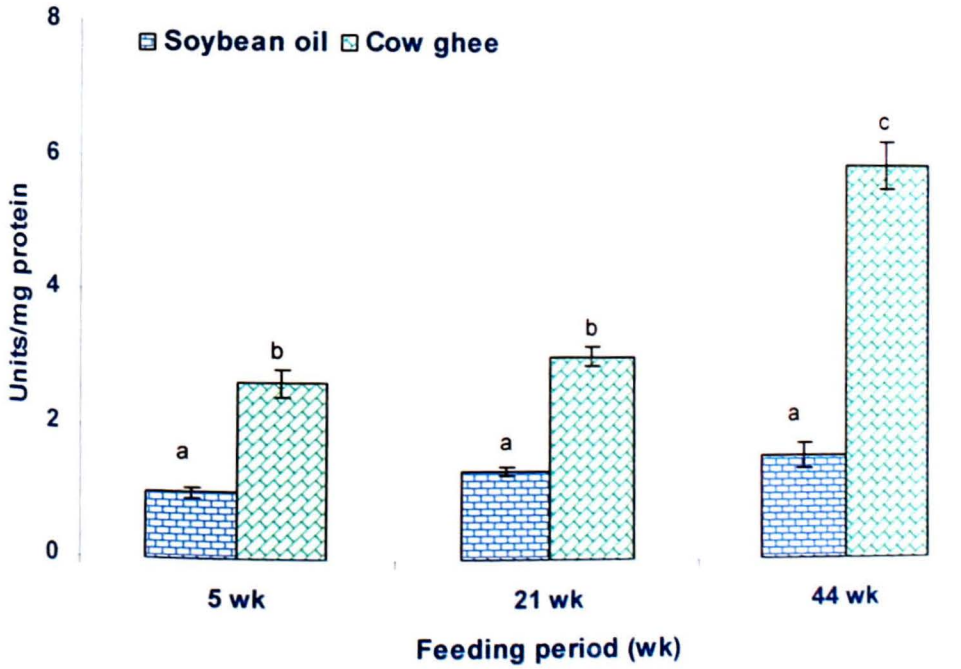


Fig. 4.11 Effect of feeding cow ghee vs soybean oil on GGTP activity in rat mammary tissue

Values(mean ± SE for n=5) with different letters are significanlyt different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

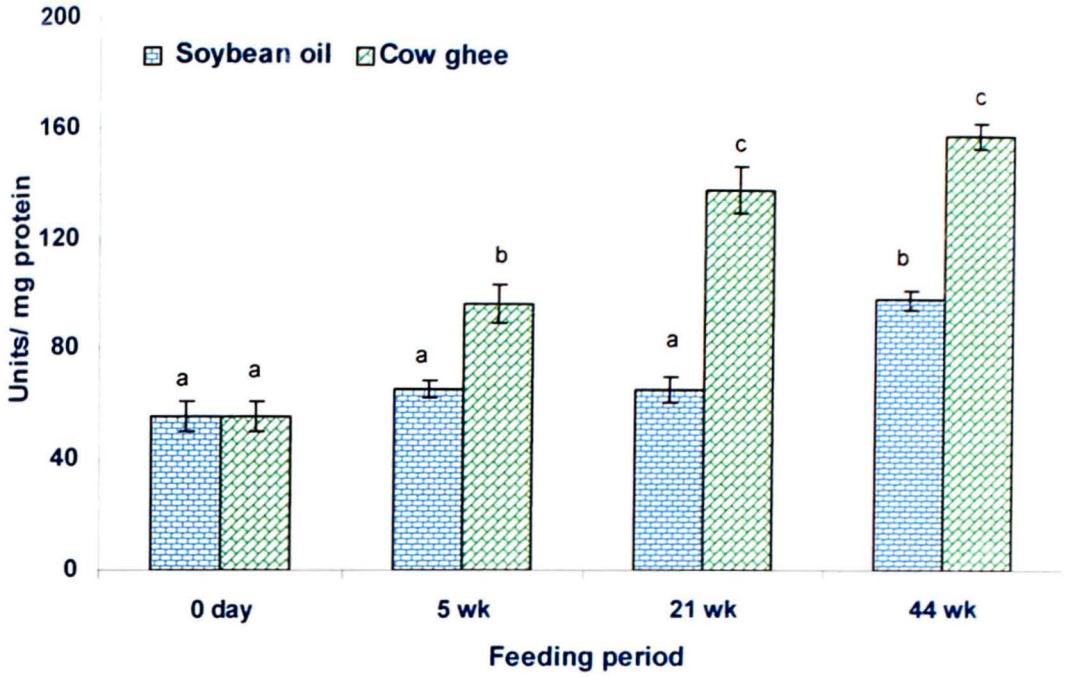


Fig. 4.12 Effect of feeding cow ghee vs soybean oil on QR activity in rat liver

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

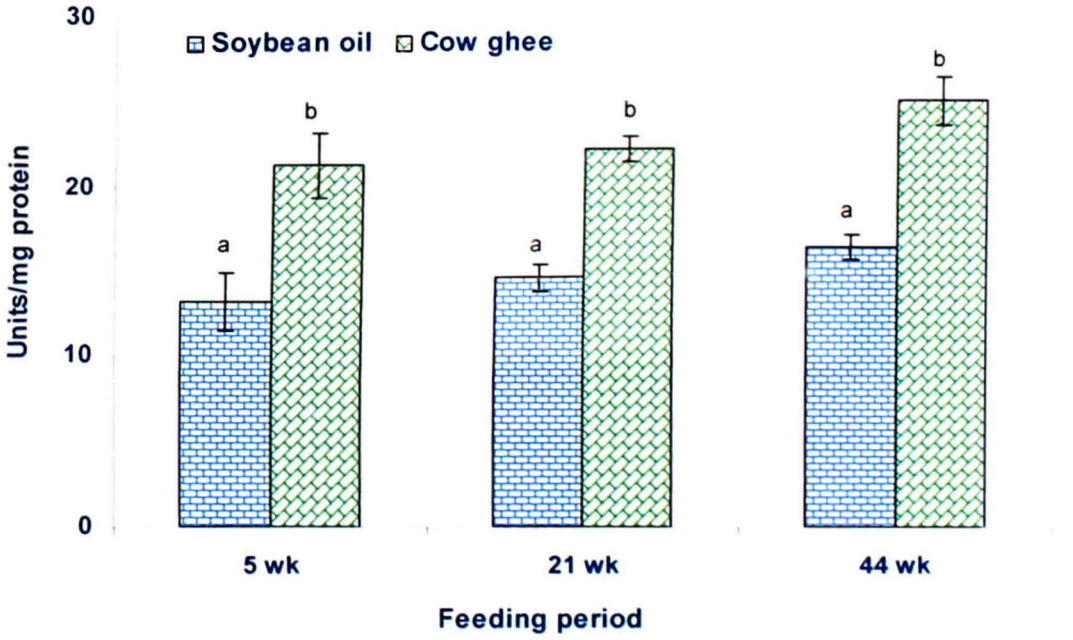


Fig. 4.13 Effect of feeding cow ghee vs soybean oil on QR activity in rat mammary tissue

Values(mean ± SE for n=5)with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

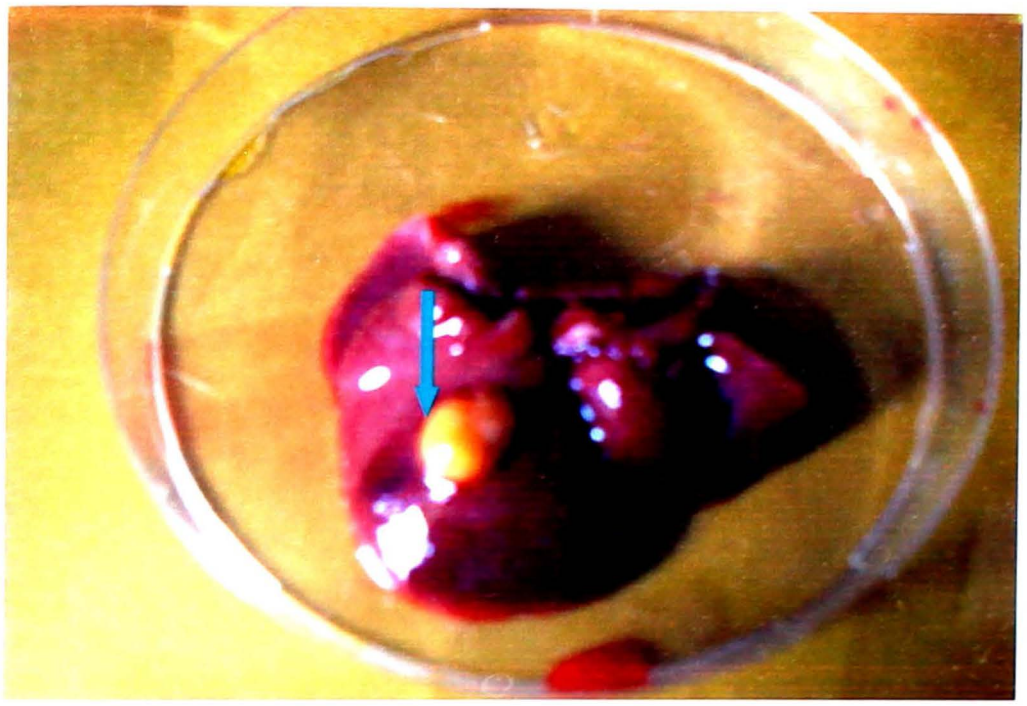


Plate 4.1 Liquifactive necrosis observed in DMBA treated soybean oil fed rats

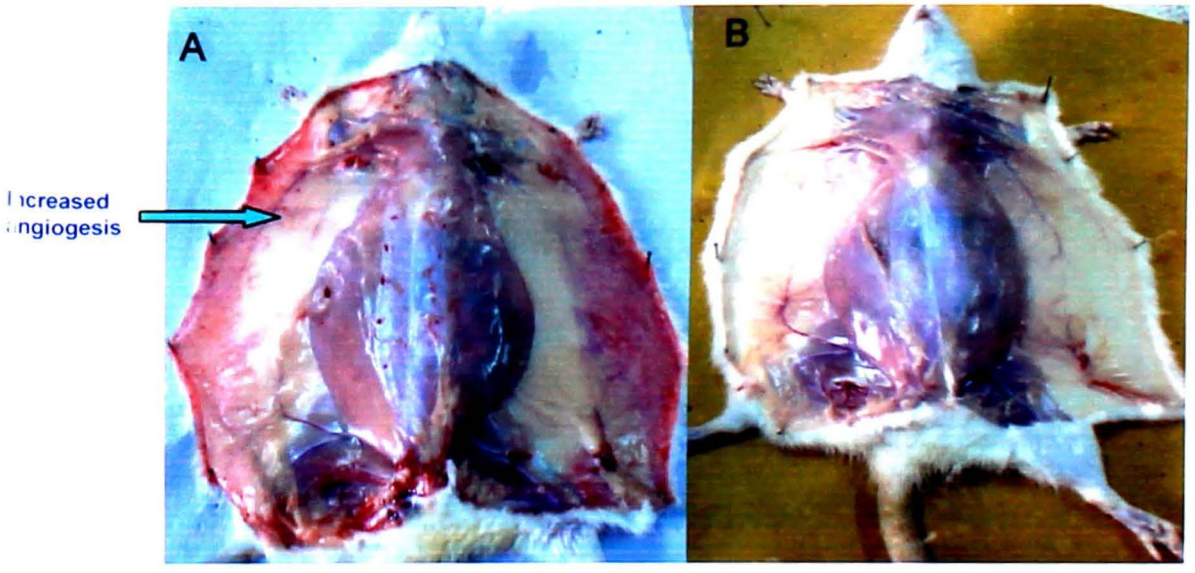


Plate 4.2 A) Rats fed on soybean oil showing increased angiogenesis
B) Rats fed on cow ghee diet

4.6) and gain in body weights (Table 4.7; Fig 4.14) were similar in two dietary groups.

4.2.1 Development of tumor in mammary gland

Table 4.8 summarizes the observation on incidence, latency period, multiplicity, weight and volume of tumor in mammary gland. The incidence of tumors on soybean oil (65.4%) was significantly higher than on cow ghee (26.6%). The tumor latency period was 27 weeks on cow ghee compared to 23 weeks on soybean oil. The average size of tumor was generally bigger in soybean oil group than in cow ghee group (Plate 4.3 and 4.4). The average tumor weight per tumor bearing animals or per tumor in soybean oil group (9.2 g and 6.18 g, respectively) was significantly higher than in cow ghee group (3.9 g and 1.67 g, respectively). Similarly, average tumor volume was significantly less on cow ghee (1925 mm^3) than on soybean oil (6285 mm^3). Tumor multiplicity (tumor number / tumor bearing rat) was, however, similar in two dietary groups (Table 4.8). Further the animals, which did not develop tumor showed greater degree of angiogenesis in soybean oil group than in cow ghee group (Plate 4.2).

4.2.2 Histopathology of tumor and progression of mammary tumorigenesis

Based on histopathological analysis, tumors were classified in to 5 types: papilloma, fibroma, adenoma, fibroadenoma and adenocarcinoma. The papilloma was characterized by 4-5 layers of epithelial cells. The epithelial cells were, however, homogeneous in size and shape (Plate 4.5). The fibroma was characterized by increased stromal tissue element with detachment of epithelial cells, and the epithelium was composed of several layers of cells (Plate 4.6). Adenoma was characterized by numerous acini with stromal elements and nucleus of benign type (Plate 4.7). The fibroadenoma was characterized by high amount of fibrous stromal element along with acini (Plate 4.8). Adenocarcinoma type tumors were found only in soybean oil fed

group. It is a malignant type of tumor characterized by solid sheets of neoplastic epithelial cells with loss of tubular alveolar pattern (Plate 4.9).

Table 4.9 shows that there was no adenocarcinoma in cow ghee group, while 8 % of tumors recorded in soybean oil group were adenocarcinoma type. The majority of tumors on cow ghee group were of beingn type (fibroma). Therefore progression of carcinogenesis was slower on cow ghee than on soybean oil.

Table 4.9 Effect of cow ghee vs soybean oil on mammary tumor in DMBA administered rats

Dietary fat	Papilloma	Fibroma	Adenoma	Fibro-adenoma	Adeno-carcinoma
Soybean Oil	20.0 5/26	16.0 4/26	24.0 6/26	32.0 8/26	8.0 2/26
Cow ghee	16.7 2/30	50.0 6/30	25.0 3/30	8.33^{a} 1/30	0.0

Values (expressed as percent of total tumor number) with different superscripts are significantly different ($P<0.05$).

4.2.3 Effect of dietary fat on carcinogen activating enzymes in liver microsomes of DMBA treated rats

4.2.3.1 Cytochrome P4501A1

The CYP1A1 activity in liver tissue was similar in tumor bearing and no tumor bearing rats in both cow ghee and soybean oil groups. Further, no

Table 4.6 Feed intake (g) of carcinogen treated rats fed soybean oil or cow ghee

Period (days)	Soybean oil	Cow ghee
0-30	6.51 ± 0.4	6.49 ± 0.3
31-60	10.1 ± 0.2	9.81 ± 0.1
61-90	8.35 ± 0.2	7.86 ± 0.1
91-120	8.05 ± 1.0	9.78 ± 0.2
121-150	10.10 ± 0.2	9.64 ± 0.2
151-180	10.16 ± 0.1	9.85 ± 0.1
181-210	9.66 ± 0.1	10.8 ± 0.1
211-240	10.74 ± 0.1	11.17 ± 0.2
241-270	12.48 ± 0.1	12.95 ± 0.2
271-300	13.37 ± 0.1	13.68 ± 0.2
Overall mean	9.95 ± 0.6	10.20 ± 0.7

Values (g/day) are mean ± SE for n=8

Table 4.7 Body weight (g) of carcinogen treated rats fed soybean oil or cow ghee

Weeks	Soybean oil	Cow ghee
0	22.0 ± 0.5	22.4 ± 1.5
2	53.2 ± 1.7	57.2 ± 3.8
4	79.6 ± 3.1	84.1 ± 4.1
6	91.1 ± 5.0	112.0 ± 3.9
8	122.0 ± 5.5	131.1 ± 3.1
10	132.0 ± 7.0	151.7 ± 2.3
12	143.6 ± 6.8	156.6 ± 3.0
14	157.4 ± 6.9	169.9 ± 3.6
16	166.1 ± 8.6	176.9 ± 4.3
18	171.9 ± 6.8	185.0 ± 3.7
20	186.2 ± 8.0	200.5 ± 5.1
22	191.5 ± 9.1	204.9 ± 5.3
24	199.7 ± 8.9	212.2 ± 4.2
26	200.6 ± 9.8	218.2 ± 2.5
28	206.7 ± 10.3	222.9 ± 2.4
30	213.7 ± 9.3	226.2 ± 1.6
32	219.1 ± 9.9	231.7 ± 2.2
34	220.0 ± 11.3	235.4 ± 3.6
36	224.7 ± 10.5	239.7 ±3.7
38	229.9 ± 11.8	244.7 ± 4.6
40	235.1 ± 13.4	246.9 ± 6.1
42	230.1 ± 13.9	248.1 ± 4.4
44	235.8 ± 12.9	252.6 ± 3.6

Values are mean ± SE for n=8

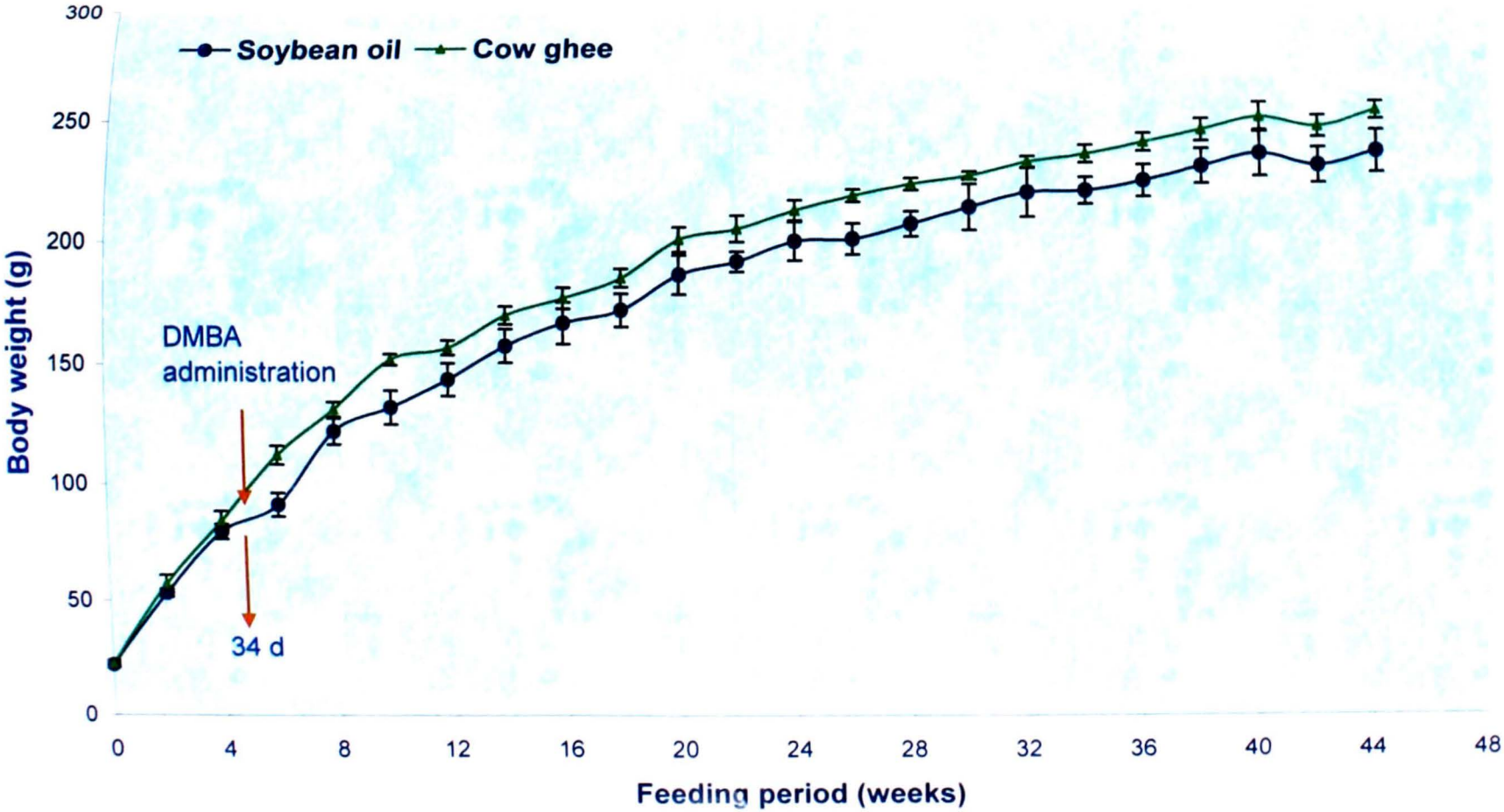


Fig. 4.14 Body weight of DMBA administered rats fed soybean oil or cow ghee

Values are mean ± SE for n=8; DMBA, 7,12-dimethylbenz(a)anthracene

Table 4.8 Effect of feeding cow ghee vs soybean oil on mammary tumor in 7,12-dimethylbenz(a)anthracene (DMBA) administered rats

	Soybean oil	Cow ghee
Tumor latency period	23 week	27 week
Tumor Number	25	12
Palpable tumor	15	11
Nonpalpable tumor	10	1
Tumor incidence (%)	65.4 (17/26)	26.6[a] (8/30)
Tumor multiplicity	1.47 ± 0.15	1.5 ± 2.67
Tumor wt (g)/ tumor bearing rats	9.21 ± 1.38	3.86[a] ± 1.22
Tumor wt (g)/ tumor	6.18 ± 1.76	1.67[a] ± 0.60
Tumor volume (mm^3) / tumor bearing rats	6285 ± 2674	1925[a] ± 852

[a] Values (mean ± SE) are significantly different from soybean oil fed rats ($P<0.05$)

Data for tumor multiplicity, tumor weight and tumor volume were compared by unpaired student t-test and tumor incidence by ψ^2 analysis.

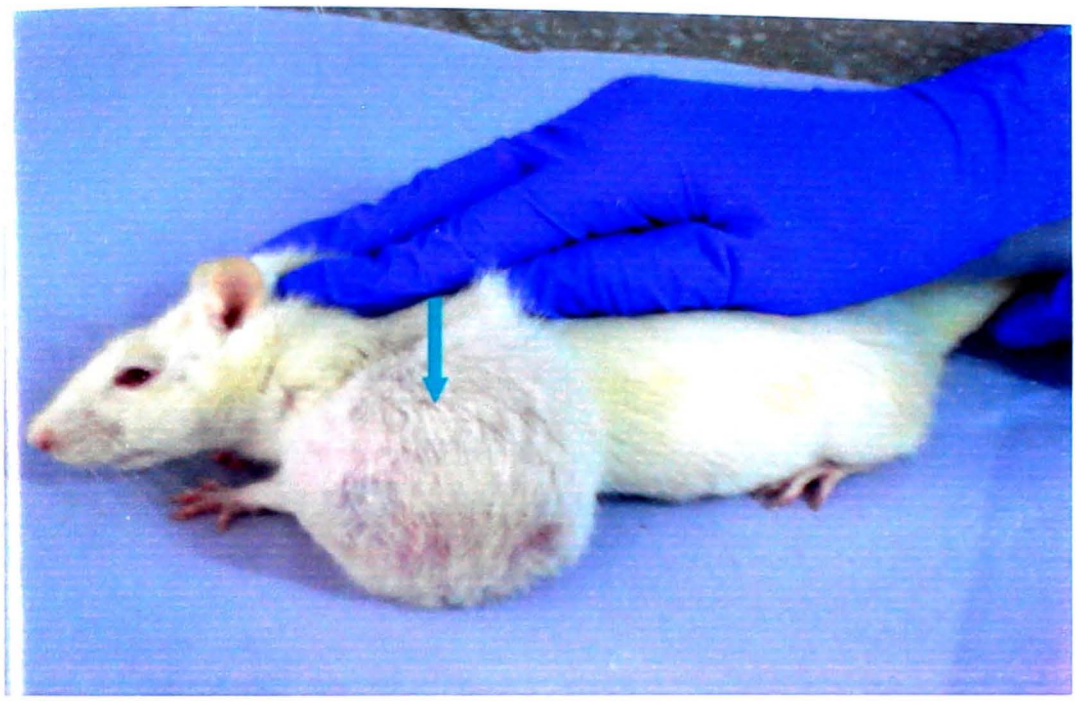


Plate 4.3 External view of mammary tumor

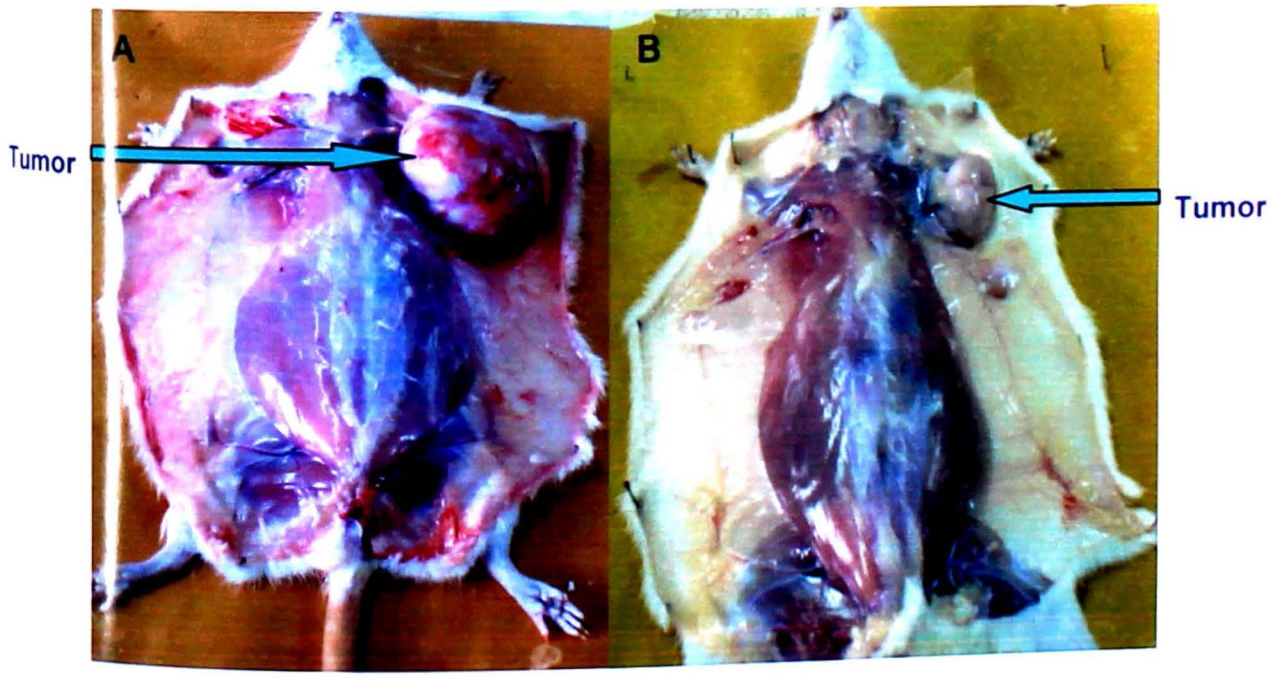


Plate 4.4 Internal view of mammary tumor. A) Rats fed with soybean oil diet showing large tumor, B) Rats fed with cow ghee diet showing small tumor

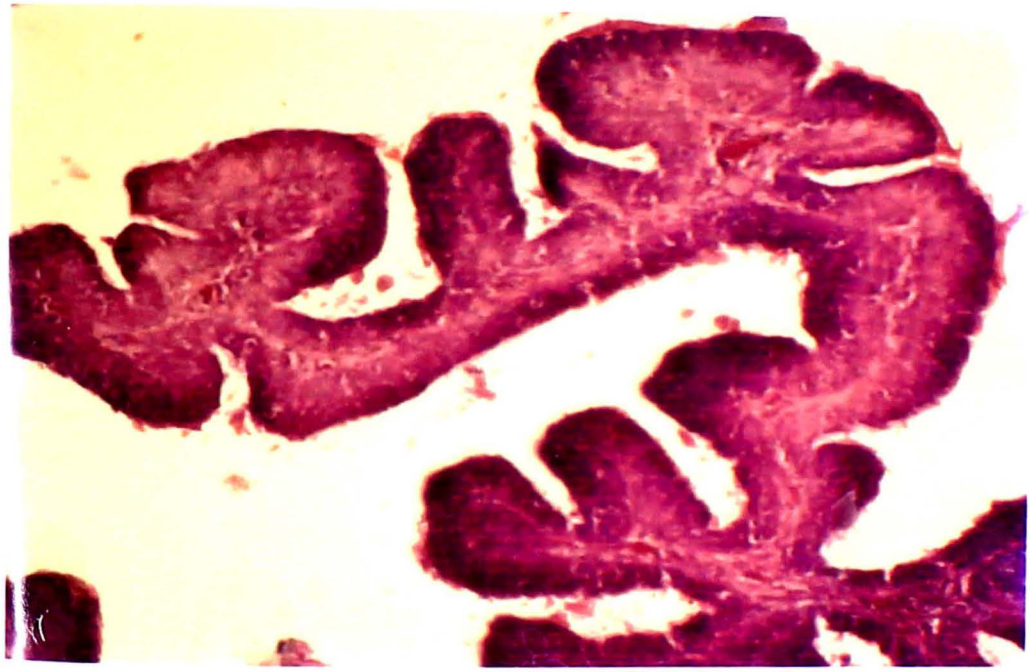


Plate 4.5 Mammary papilloma showing 2-4 layers of epithelial cells (400 x)

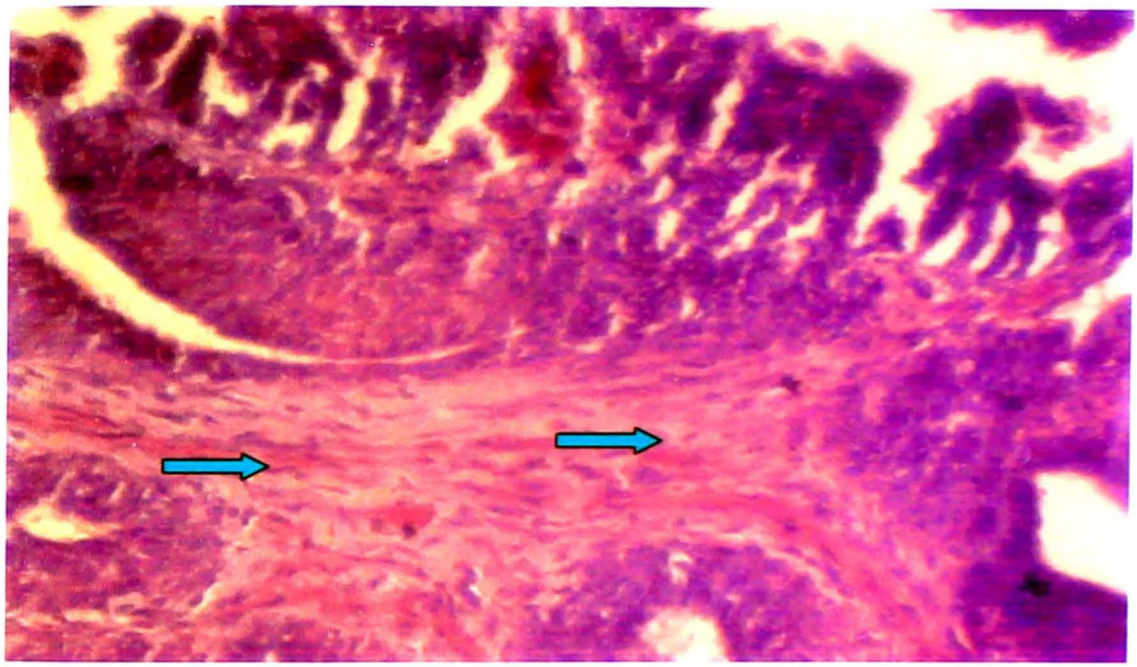


Plate 4.6 Mammary fibroma showing spindle shaped fibrous tissue with flattened nuclei (arrow) detachment of epithelial cells (400 x)

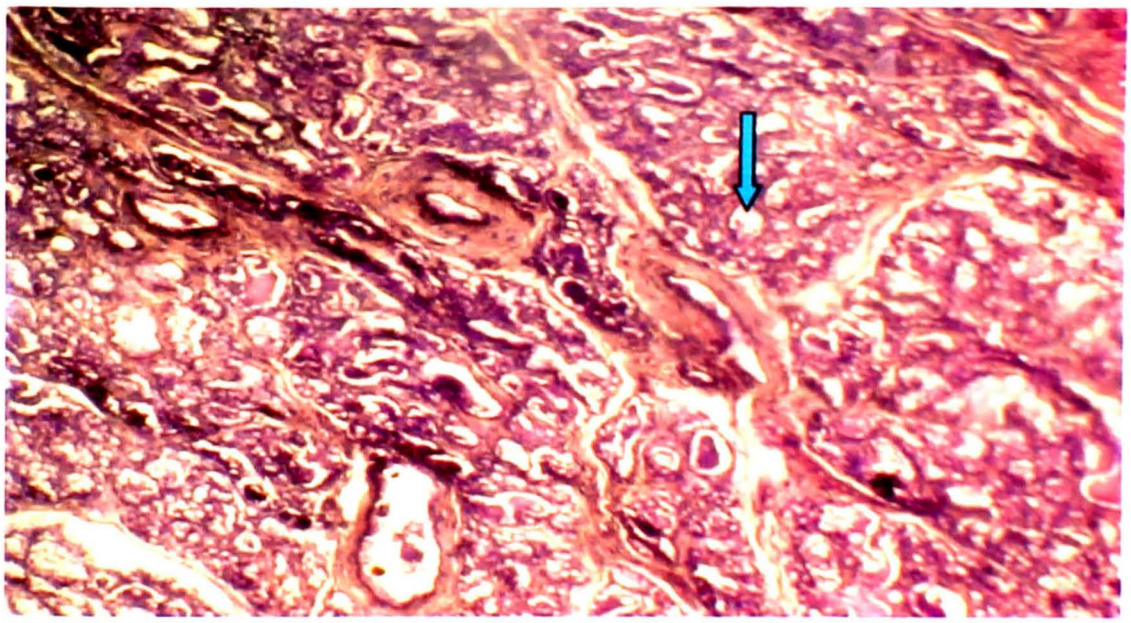


Plate 4.7 Mammary adenoma showing numerous acini (arrow) with moderate stromal element (400 x)

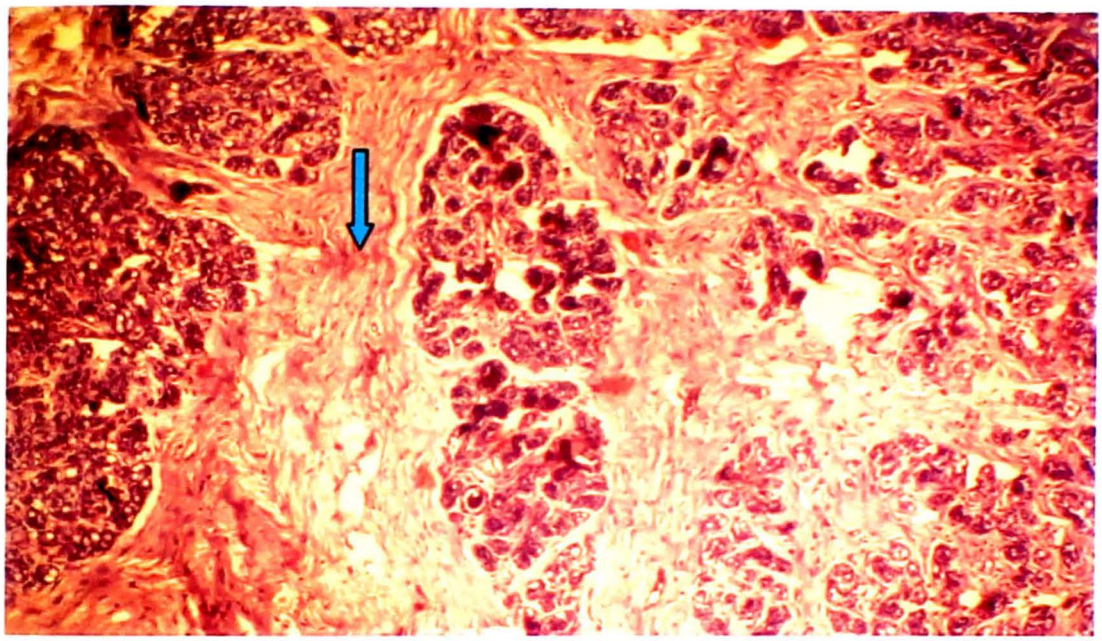


Plate 4.8 Mammary fibroadenoma tumor showing high amount of fibrous tissue (arrow) (400 x)

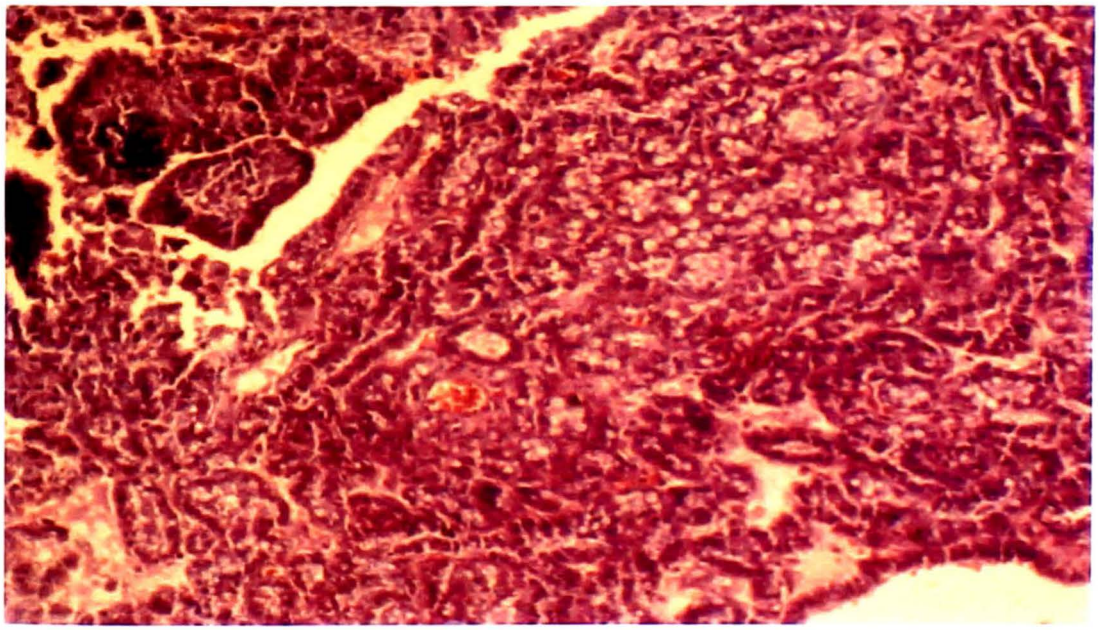


Plate 4.9 Mammary adenocarcinoma with solid sheet of neoplastic epithelial cell (400 x)

significant difference was observed in CYP1A1 activity between soybean oil and cow ghee groups (Fig. 4.15; Table 4.10).

4.2.3.2 Cytochrome P4501A2

Cytochrome P450 1A2 activity was significantly higher in tumor bearing than in no tumor bearing rats in both soybean oil and cow ghee groups. Though CYP1A2 activity on soybean oil was higher than on cow ghee group in both tumor bearing and no tumor bearing rats, the difference could not reach to the level of statistically significant (Fig. 4.16; Table 4.10).

4.2.3.3 Cytochrome P4501B1

The CYP1B1 activity on cow ghee was significantly higher in tumor bearing than in no tumor bearing rats. In animals fed on soybean oil, the difference in CYP1B1 activity between tumor bearing and no tumor bearing rats was statistically not significant. The CYP1B1 activity on cow ghee decreased by 45.3 and by 29.6% in no tumor bearing and tumor bearing rats, respectively, compared to respective soybean oil group (Fig.4.17; Table 4.10)

4.2.3.4 Cytochrome P4502B1

The CYP2B1 activity on cow ghee decreased by 32% in no tumor bearing and by 41% in tumor bearing rats, compared to respective soybean oil group. The CYP2B1 activity in soybean oil group was significantly higher in tumor bearing than in no tumor bearing rats, while in ghee group the difference was statistically not significant between tumor bearing and no tumor bearing rats (Fig. 4.18; Table 4.10).

4.2.3.5 Cytochrome P4502E1 activity

There was no significant difference in CYP2E1 activity between tumor bearing and no tumor bearing rats in both cow ghee or soybean oil groups. Further, the difference in CYP2E1 activity between cow ghee and soybean oil groups was also statistically not significant (Fig. 4.19; Table 4.10).

4.2.4 Effect of dietary fat on carcinogen detoxifying activities in liver and mammary tissue of DMBA treated rats

4.2.4.1 UDP- glucuronosyl transferase (UDPGT) activity in liver tissue

The UDPGT activity in liver microsome at 16 weeks in cow ghee group increased by 47%, compared to age-matched soybean oil group. At 39 weeks, it increased in cow ghee groups by 88 % in no tumor bearing and by 98% in tumor bearing rats, compared to respective soybean oil group (Fig. 4.20). The difference in UDPGT activity between tumor bearing and no tumor bearing rats was statistically not significant in both soybean oil and cow ghee groups (Fig. 4.20; Table 4.11).

4.2.4.2 Glutathione-S- transferase activity in liver and mammary tissue

Their was no significant difference in liver GST activity between soybean oil and cow ghee groups, at both 16 and 39 weeks post DMBA administration. Further, there was no difference in liver GST activity between tumor bearing and no tumor bearing rats in both soybean oil and cow ghee groups (Fig. 4.21; Table 4.11).

In mammary tissue, no difference was observed in GST activity in soybean oil group between 16 and 39 weeks, while in cow ghee group, rats bearing tumor exhibited 37% higher GST activity at 39 than at 16 weeks post DMBA administration. The difference in GST activity was statistically not significant between soybean oil and cow ghee groups at 16 weeks and in no tumor bearing rats at 39 weeks. In tumor bearing rats, however, the GST activity was 33% higher on cow ghee than on soybean oil (Fig. 4.22; Table 4.12).

4.2.4.3 γ- glutamyl transpeptidase (GGTP) activity in liver and mammary tissue

The GGTP activity in liver microsomes of DMBA treated rats increased significantly with age on cow ghee as well as on soybean oil, and the magnitude of increase was higher on cow ghee than on soybean oil. The liver

Table: 4.10 Effect of cow ghee vs soybean oil on carcinogen activating enzymes* in liver of carcinogen treated rats

	Dietary fat	No tumor bearing rats	Tumor bearing rats
CYP4501A1 (units†/mg protein)	Soybean oil	39.9 ± 3.3	44.9 ± 4.2
	Cow ghee	41.1 ± 1.8	47.8 ± 1.8
CYP4501A2 (units†/mg protein)	Soybean oil	35.7^{ac} ± 4.1	55.3^{b} ± 6.5
	Cow ghee	25.4^{a} ± 1.7	45.01^{bc} ± 6.0
CYP4501B1 (units†/mg protein)	Soybean oil	9.87^{ac} ± 0.35	11.39^{a} ± 0.61
	Cow ghee	5.40^{b} ± 0.34	8.02^{c} ± 0.20
CYP4502B1 (units†/mg protein)	Soybean oil	5.80^{a} ± 0.30	8.45^{c} ± 0.39
	Cow ghee	3.95^{b} ± 0.57	4.97^{ab} ± 0.62
CYP2E1 (units#/mg protein)	Soybean oil	1.10 ± 0.06	1.08 ± 0.15
	Cow ghee	1.25 ± 0.02	1.12 ± 0.07

*The activities were determined after 39 weeks of carcinogen administration.

Values (mean ± SE for n=5) of the said activity with different letters are significantly different ($P<0.05$).

† One unit is equal to one pmole of product formed/min.

One unit is equal to one nmole of product formed/min.

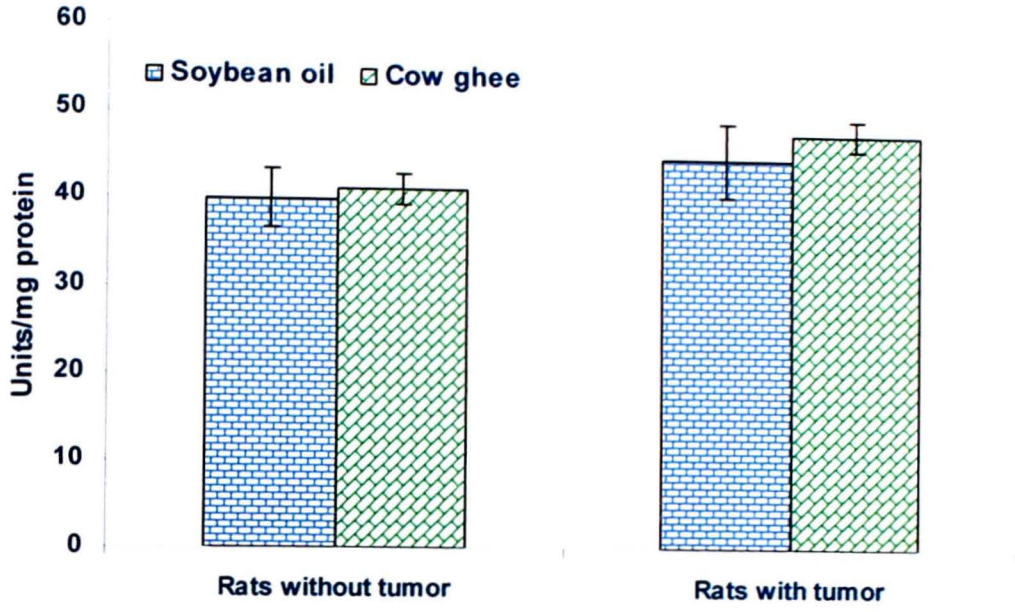


Fig. 4.15 Effect of feeding cow ghee vs soybean oil on CYP1A1 activity in liver of carcinogen treated rat

Values (mean ± SE for n=5) determined 39 weeks following carcinogen administration. Values are not statistically different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of product per min.

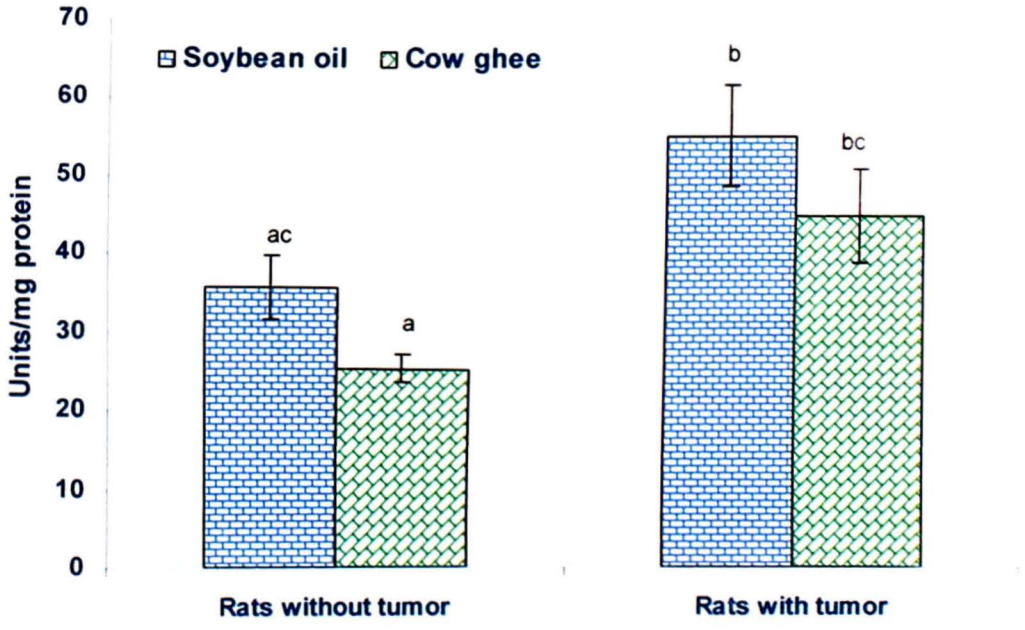


Fig. 4.16 Effect of feeding cow ghee vs soybean oil on CYP1A2 activity in liver of carcinogen treated rat

Values(mean ± SE for n=5) determined 39 weeks following carcinogen administration; Values with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of product per min.

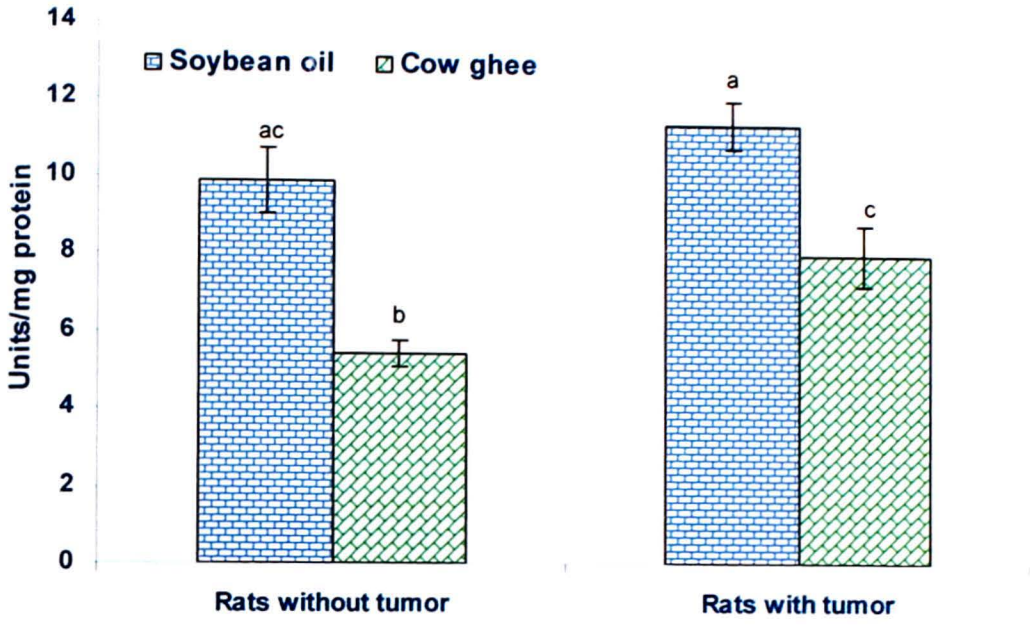


Fig. 4.17 Effect of feeding cow ghee vs soybean oil on CYP1B1 activity liver of carcinogen treated rat

Values (mean ± SE for n=5) determined 39 weeks following carcinogen administration; Values with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of product per min.

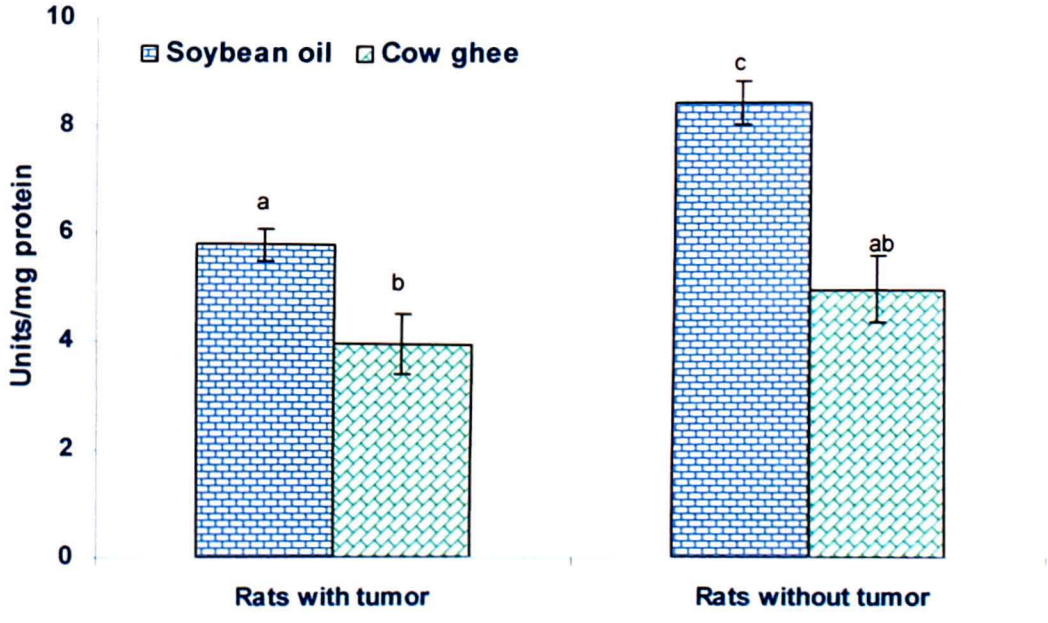


Fig. 18 Effect of feeding cow ghee vs soybean oil on CYP2B1 activity in liver of carcinogen treated rat

Values (mean ± SE for n=5) determined 39 weeks following carcinogen administration; Values with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 pmole of resorufin per min.

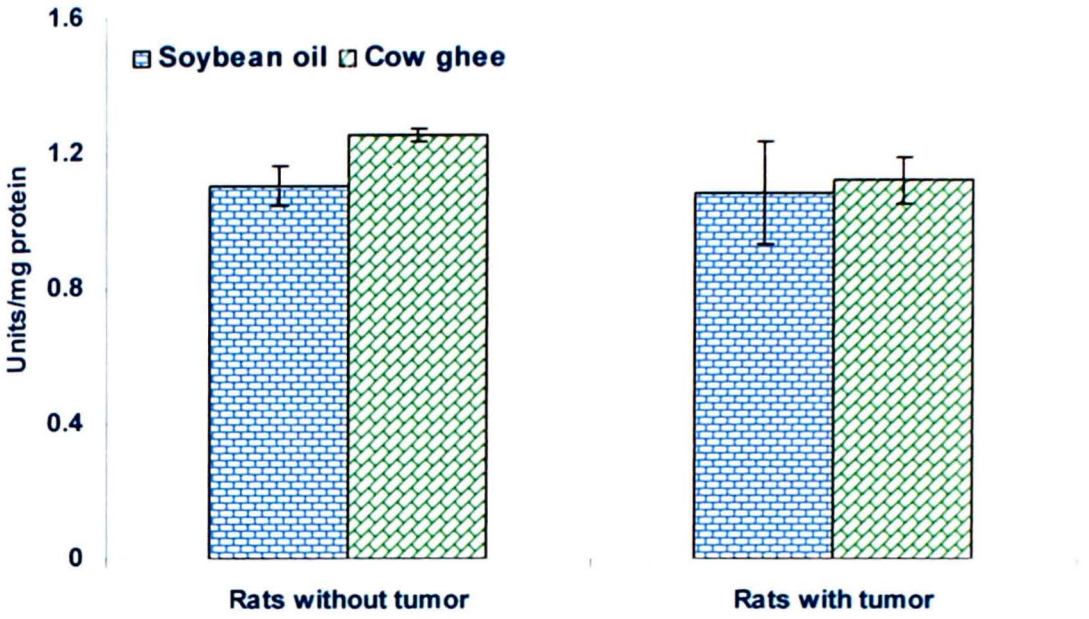


Fig. 4.19 Effect of feeding cow ghee vs soybean oil on CYP2E1 activity in liver of carcinogen treated rat

Values (mean ± SE for n=5) detemined 39 weeks following carcinogen administration; Values are not statistically different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product formed per min.

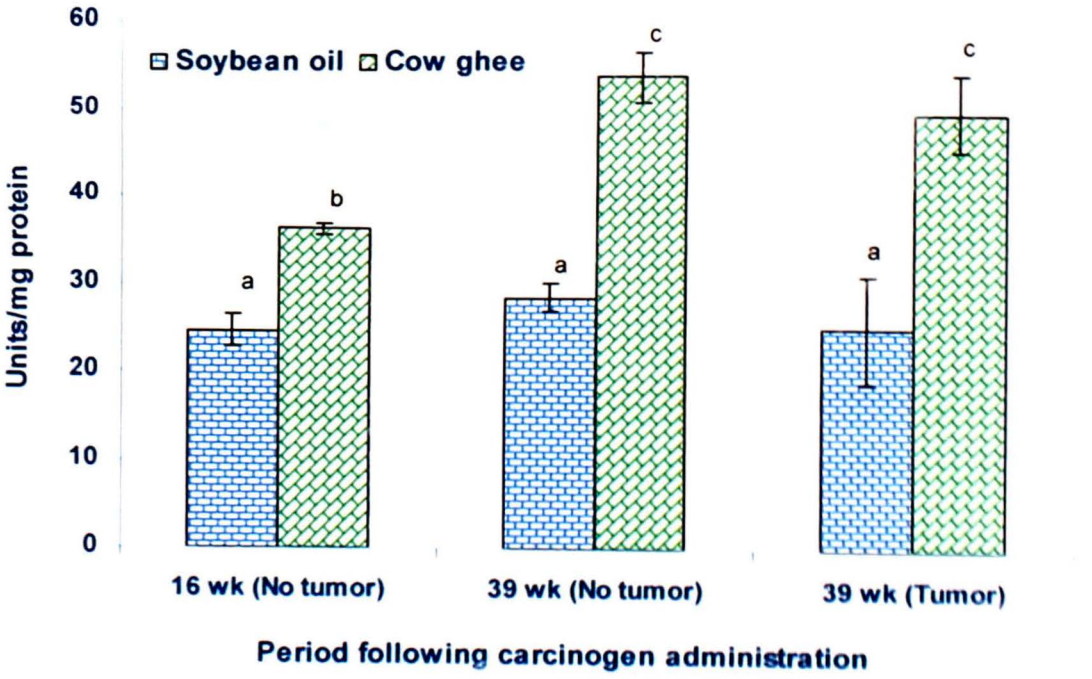


Fig. 4.20 Effect of feeding cow ghee vs soybean oil on UDPGT activity in liver carcinogen treated rat

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05). One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min

Table 4.11 Effect of cow ghee vs soybean oil on carcinogen detoxifying activities (units*/mg protein) in liver of carcinogen (DMBA) treated rats

	Dietary fat	After 16 weeks of carcinogen treatment	After 39 weeks of carcinogen treatment	
			No tumor bearing rats	Tumor bearing rats
Glutathione-S-transferase	Soybean oil	194.8 ± 6.5	193.0 ± 13.9	180.0 ± 5.5
	Cow ghee	211.6 ± 3.6	195.9 ± 7.4	202.0 ± 14.6
γ-glutamyl transpeptidase	Soybean oil	3.91^{a} ± 0.12	5.64^{b} ± 0.52	4.75^{b} ± 0.44
	Cow ghee	5.51^{b} ± 0.21	8.40^{c} ± 0.40	8.66^{c} ± 0.59
UDP-glucouronsyl transferase	Soybean oil	24.6^{a} ± 1.7	28.5^{a} ± 1.6	24.8^{a} ± 6.1
	Cow ghee	36.2^{b} ± 0.6	53.5^{c} ± 2.8	49.2^{c} ± 4.3
Quinone reductase	Soybean oil	84.8^{a} ± 9.2	109.3^{a} ± 7.6	92.9^{a} ± 3.3
	Cow ghee	153.6^{b} ± 5.8	160.4^{b} ± 5.9	150.3^{b} ± 3.9

Values (mean ± SE for n=5) of the said activity with different superscripts are significantly different ($P<0.05$).

* One unit is equal to one nmole of product formed/min

Table 4.12 Effect of cow ghee vs soybean oil on carcinogen detoxifying activities (units*/mg protein) in mammary tissue of carcinogen (DMBA) treated rats

	Dietary fat	After 16 weeks of carcinogen treatment	After 39 weeks of carcinogen treatment	
			No tumor bearing rats	Tumor bearing rats#
Glutathione-S-transferase	Soybean oil	$25.0^{a} \pm 0.5$	$24.3^{a} \pm 1.7$	$27.3^{a} \pm 2.1$
	Cow ghee	$26.4^{a} \pm 1.7$	$28.1^{a} \pm 2.9$	$36.3^{b} \pm 1.1$
γ-glutamyl transpeptidase	Soybean oil	$1.32^{a} \pm 0.03$	$3.25^{b} \pm 0.36$	$2.48^{ab} \pm 0.56$
	Cow ghee	$4.07^{bc} \pm 0.12$	$5.49^{c} \pm 0.48$	$7.25^{d} \pm 0.31$
Quinone reductase	Soybean oil	$11.7^{a} \pm 0.5$	$27.3^{c} \pm 0.4$	$25.0^{c} \pm 1.3$
	Cow ghee	$15.5^{b} \pm 1.1$	$27.9^{c} \pm 1.1$	$27.8^{c} \pm 3.8$

Values (mean ± SE for n=5) with different superscripts are significantly different ($P<0.05$).

* One unit equal to one nmole of product formed/min

The activities were determined in uninvolved tissue

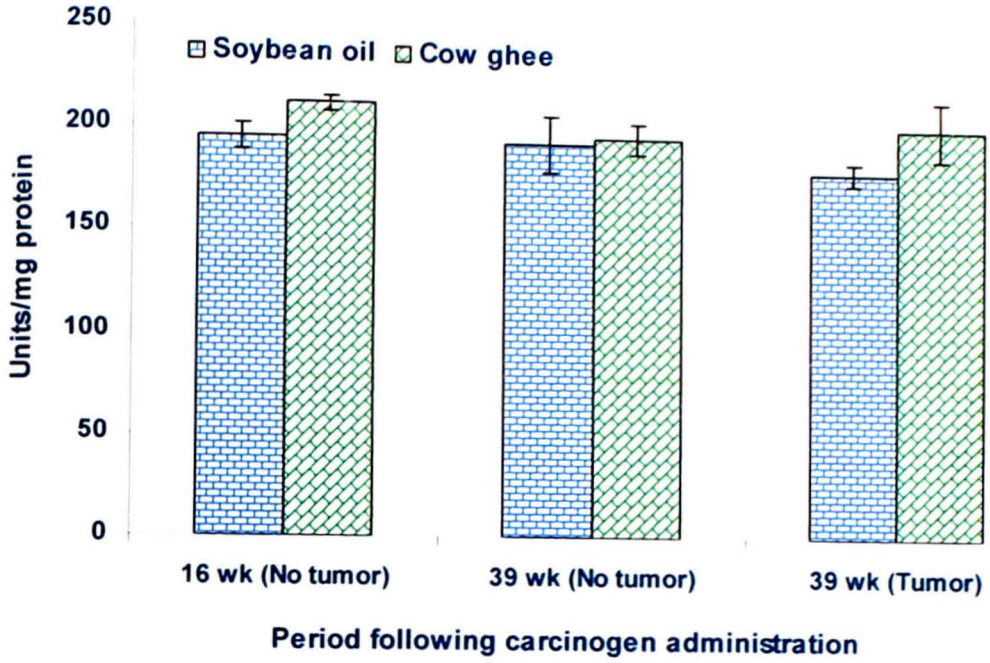


Fig. 4.21 Effect of feeding cow ghee vs soybean oil on GST activity in liver of carcinogen treated rat

Values are mean ± SE for n=5; Values are not statistically different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

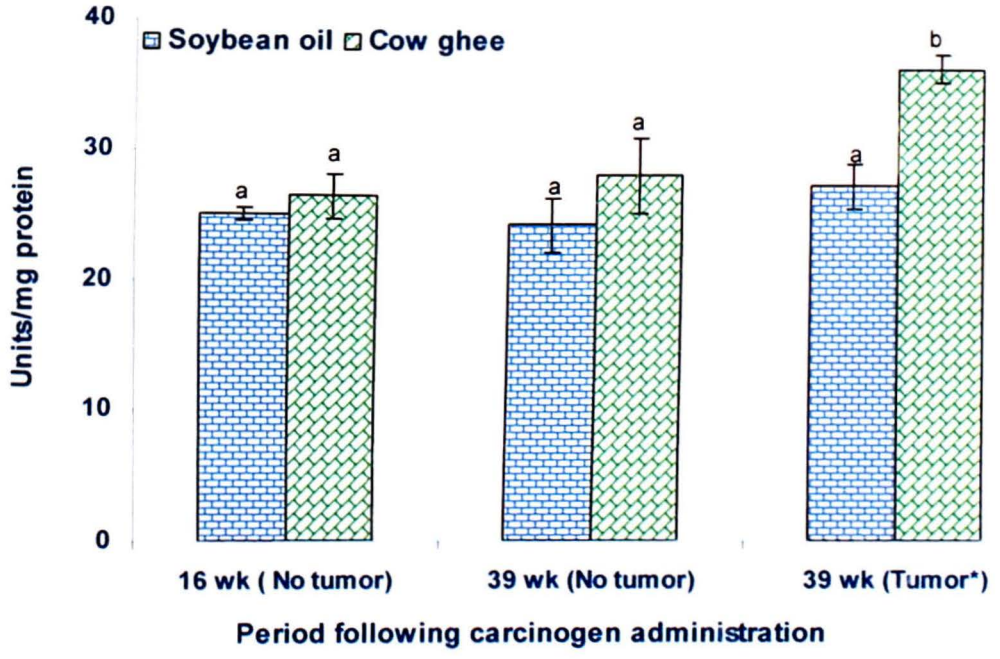


Fig. 4.22 Effect of feeding cow ghee vs soybean oil on GST activity in mammary tissue of carcinogen treated rat

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

* The activity in tumor beraing rats was determined in uninvolved mammary tissue

GGTP activity on cow ghee increased by 41% at 16 weeks and by 49 and 82% at 39 weeks in no tumor bearing and tumor bearing rats, respectively, compared to age-matched respective soybean oil groups (Fig. 4.23; Table 4.11).

In mammary tissue also GGTP activity increased significantly with age on cow ghee as well as on soybean oil. The GGTP activity on ghee increased by 208% at 16 weeks and by 69 and 192% at 39 weeks in no tumor bearing and tumor bearing rats, respectively, compared to age-matched respective soybean oil groups. In ghee group, the GGTP activity was significantly higher in tumor bearing than in no tumor bearing rats (Fig. 4.24; Table 4.12).

4.2.4.4 Quinone reductase (QR) activity in liver and mammary tissue

The QR activity in liver homogenate (10000 x g) increased on cow ghee by 81% at 16 weeks, and by 47 and 62% at 39 weeks in no tumor bearing and tumor bearing rats, respectively, compared to age-matched respective soybean oil groups. There was no significant difference in liver QR activity between tumor bearing and no tumor bearing animals in both cow ghee and soybean oil groups (Fig. 4.25; Table 4.11).

In mammary tissue, there was no difference in QR activity between tumor bearing and no tumor bearing animals in cow ghee as well as in soybean oil groups. At 16 weeks post DMBA administration the QR activity was 32% higher on cow ghee than on soybean oil. At 39 weeks, however, there was no difference between two dietary groups in both tumor bearing and no tumor bearing rats (Fig. 4.26; Table 4.12).

4.3 Effect of Dietary fat on expression of putative genes involved in mammary carcinogenesis

In order to elucidate the molecular mechanism by which dietary fat modulates mammary carcinogenesis, the effect of dietary fat on the expression of the COX-2, cyclin A, cyclin D and PPAR-γ genes was examined in female rats.

4.3.1 RNA Integrity and Standardization of PCR Condition for Cyclin A and PPAR-γ

RNA was isolated as described in section 3.4.2 and the purity was evaluated by taking the ratio of absorbance at 260 and 280 nm, which in range of 1.8 to 2.0 is acceptable for pure RNA preparation. Two major bands with high intensity corresponding to 28S and 18S RNA observed in the gel suggest the good integrity of RNA isolated (Plate 4.10).

The $MgCl_2$ concentration of 1.5 mM is optimum for amplification (PCR) of cyclin A and PPAR-γ cDNA (Plate 4.11 and Plate 4.11). The optimum temperature for amplification of cyclin A and PPAR-γ cDNA is 58°C and 55°C, respectively (Plate 4.13 and 4.14).

4.3.2 Expression of cyclooxygenase-2 (COX-2) in mammary tissue of normal and carcinogen treated rats

In control rats fed either cow ghee or soybean oil, no expression of COX-2 was observed. In DMBA treated rats, the expression of COX-2 was significantly greater in tumor bearing than in no tumor bearing rats on both cow ghee and soybean oil diets. Within tumor bearing animals in cow ghee as well as in soybean oil group, the expression of COX-2 was greater in tumor tissue than in uninvolved adjoining tissue. The expression of COX-2 in no tumor bearing animals on soybean oil diet was 2.8 fold of that on cow ghee diet. Similarly, the expression of COX-2 in tumor bearing rats on soybean oil diet was 1.7 to 1.8 fold of that on cow ghee diet in both tumor bearing as well as in uninvolved adjoining mammary tissue (Table 4.13; Fig. 4.27; Plate 4.15).

4.3.3 Expression of cyclin A in mammary tissue of normal and carcinogen treated rats

The expression of cyclin A in mammary tissue was similar in control soybean oil and cow ghee control groups. In soybean oil groups, the DMBA treated rats, where no tumor appeared, the expression of Cyclin A was 49% higher than in DMBA minus control. On the other hand on cow ghee, no significant difference in expression of cyclin A was observed between DMBA

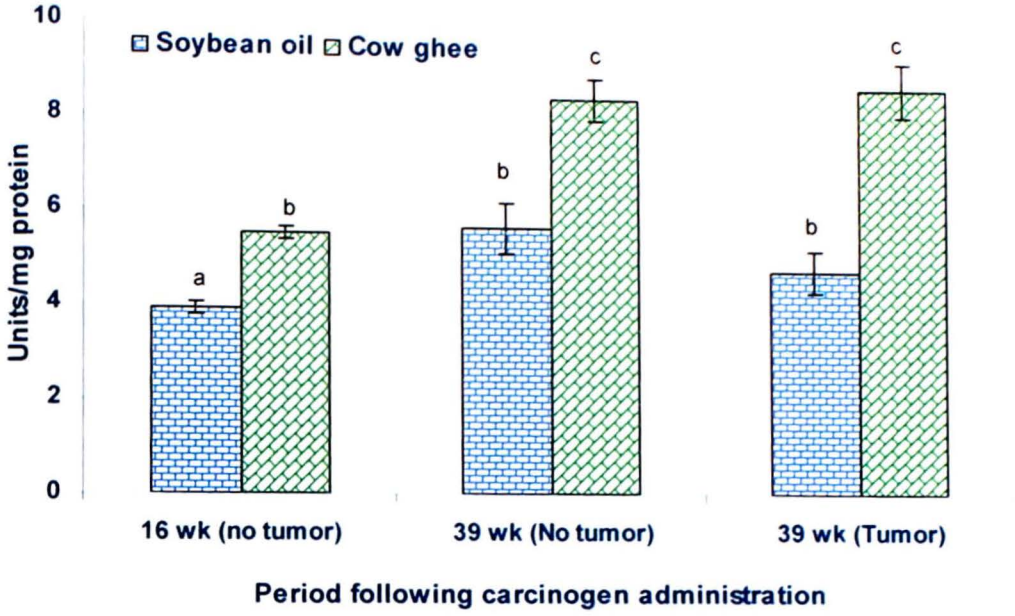


Fig. 4.23 Effect of feeding cow ghee vs soybean oil on GGTP activity in liver of carcinogen treated rat

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

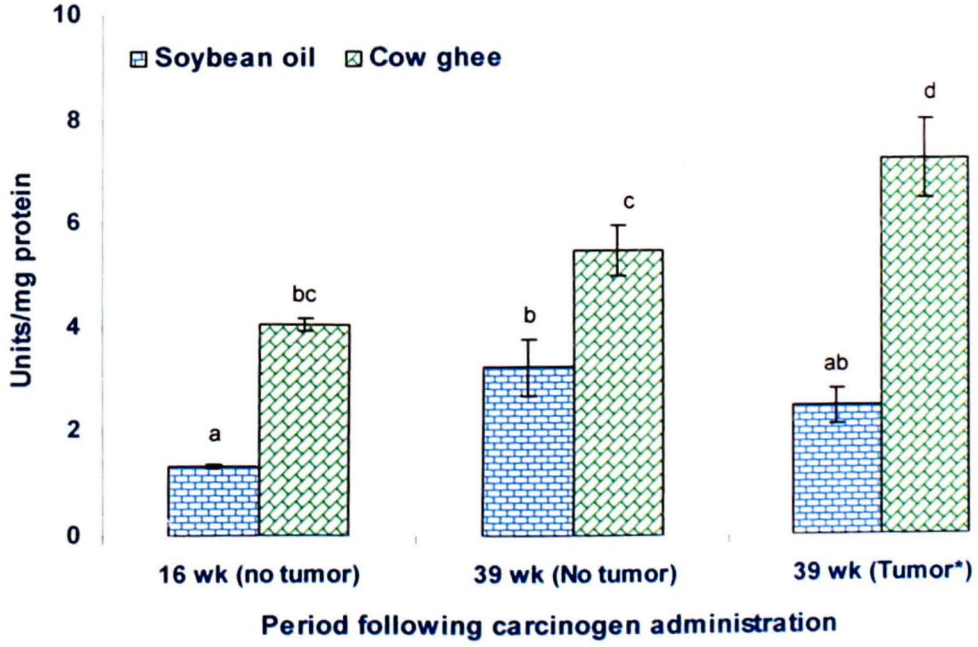


Fig. 4.24 Effect of feeding cow ghee vs soybean oil on GGTP activity in mammary tissue of carcinogen treated rat

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.
* The activity in tumor bearing rats was determined in uninvolved mammary tissue

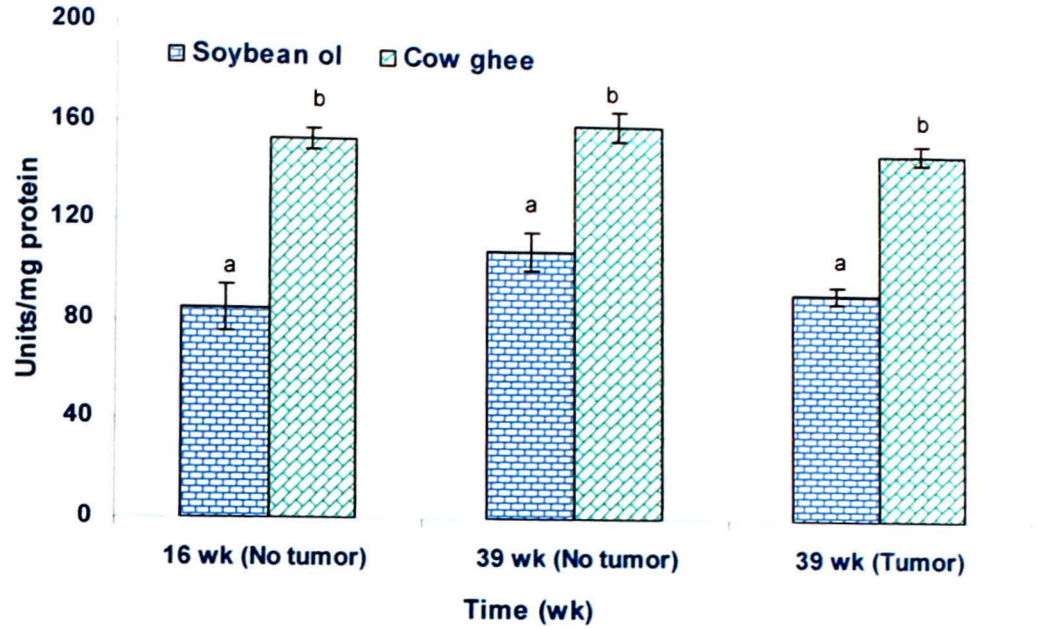


Fig. 4.25 Effect of feeding cow ghee vs soybean oil on QR activity in liver of carcinogen treated rat

Values (mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.

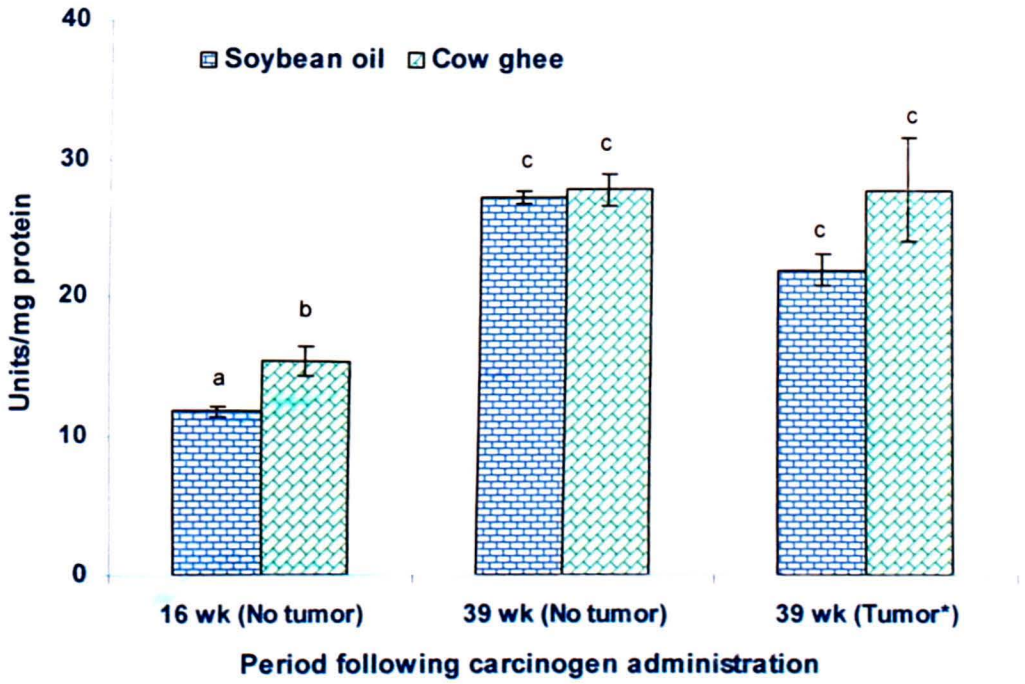


Fig. 4.26 Effect of feeding cow ghee vs soybean oil on QR activity in mammary tissue tissue of carcinogen treated rat

Values(mean ± SE for n=5) with different letters are significantly different (P<0.05); One unit is the amount of enzyme that catalyzes the formation of 1 nmole of product per min.
* The activity in tumor bearing rats was determined in uninvolved mammary tissue

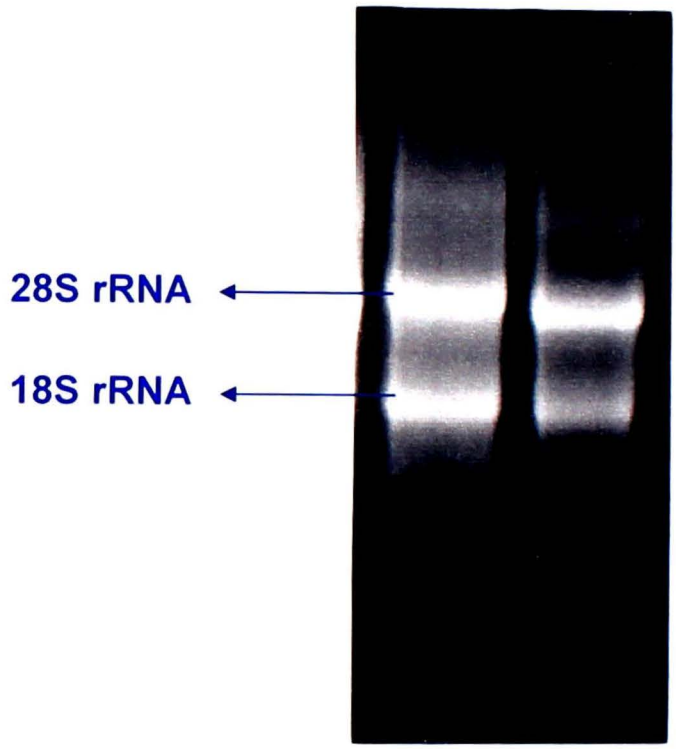


Plate 4.1 RNA integrity after its denaturation with formamide in formaldehyde agarose gel.

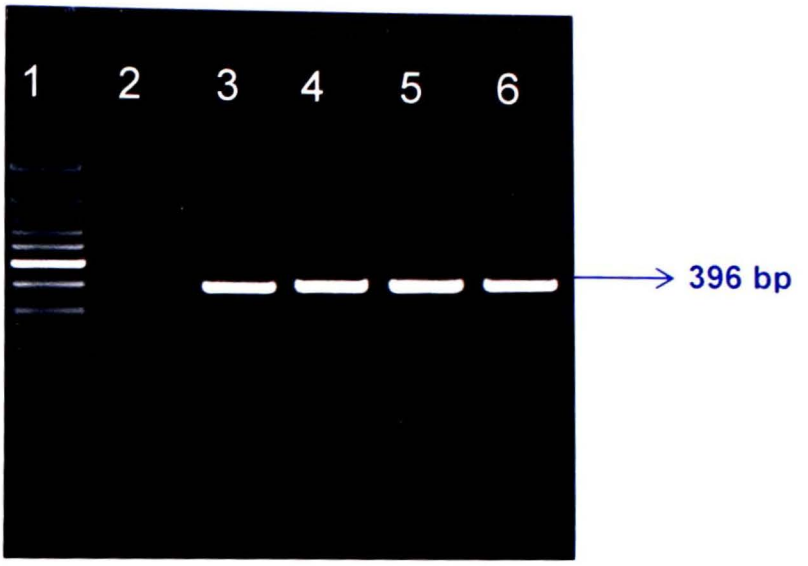


Plate 4.11 Standardization of $MgCl_2$ concentration for cyclin A. Lane-1, DNA Ladder; Lane-2, 0.5 mM $MgCl_2$; Lane-3, 1.0 mM $MgCl_2$, Lane-4, 1.5 mM $MgCl_2$; Lane-5, 2.0 mM $MgCl_2$ and Lane-6, 2.5 mM $MgCl_2$.

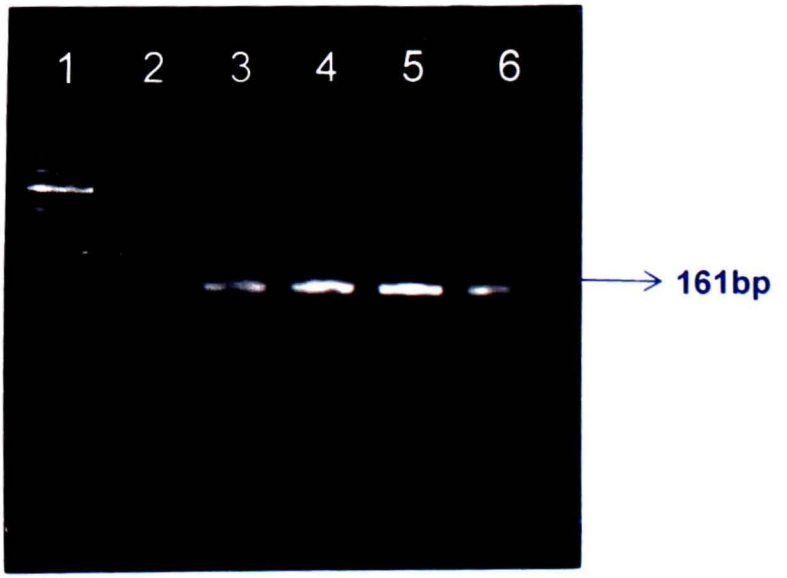


Plate 4.12 Standardization of $MgCl_2$ concentration for PPAR-γ. Lane-1, DNA Ladder; Lane-2, 0.5 mM $MgCl_2$; Lane-3, 1.0 mM $MgCl_2$, Lane-4, 1.5 mM $MgCl_2$; Lane-5, 2.0 mM $MgCl_2$ and Lane-6, 2.5 mM $MgCl_2$.

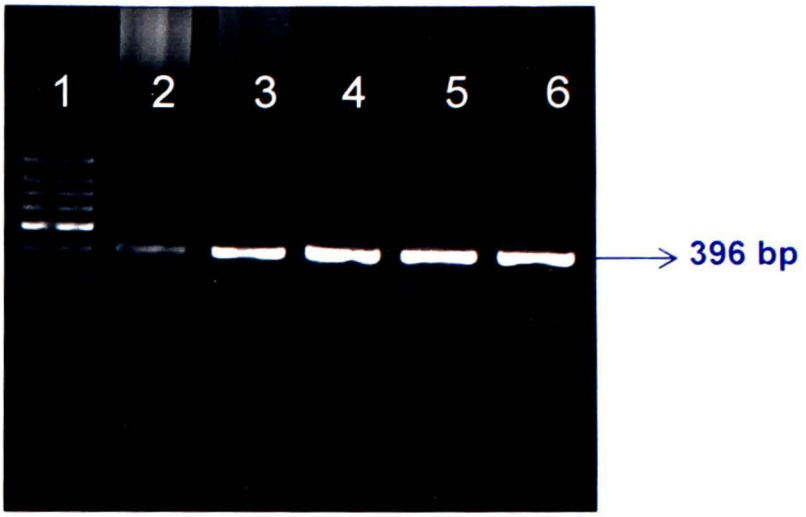


Plate 4.13 Standardization of annealing temperature for cyclin A. Lane-1, DNA Ladder; Lane-2, 55^{0}C; Lane-3, 58^{0}C; Lane-4, 60^{0}C; Lane-5, 62^{0}C and Lane- 6, 64^{0}C.

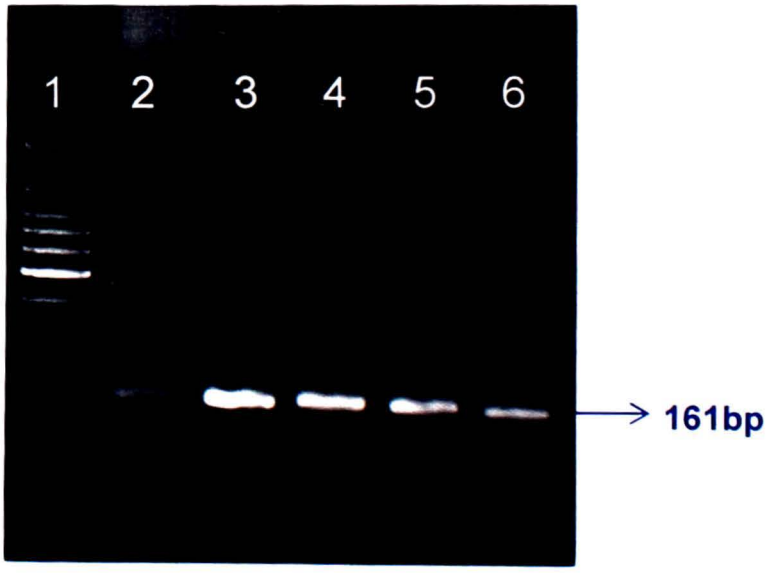


Plate 4.14 Standardization of annealing temperature for PPAR-γ. Lane-1, DNA Ladder; Lane-2, 52^{0}C; Lane-3, 55^{0}C; Lane-4, 58^{0}C; Lane-5, 60^{0}C and Lane-6, 62^{0}C.

Table 4.13 Effect of cow ghee vs soybean oil on expression of genes influencing mammary carcinogenesis in rat

	Dietary fat	Control	Carcinogen treated		
			No tumor bearing rats	Tumor bearing rats	
				Uninvolved tissue	Tumor tissue
COX-2	Soybean oil	-	$34.6^{a} \pm 4.5$	$65.6^{c} \pm 5.5$	$85.3^{c} \pm 7.4$
	Cow ghee	-	$12.3^{b} \pm 3.7$	$38.0^{a} \pm 4.6$	$46.5^{a} \pm 5.1$
Cyclin A	Soybean oil	$22.2^{a} \pm 0.1$	$33.1^{b} \pm 1.6$	$94.7^{c} \pm 5.0$	$181.3^{d} \pm 5.2$
	Cow ghee	$18.8^{a} \pm 1.8$	$20.0^{a} \pm 0.7$	$38.3^{b} \pm 2.0$	$130.4^{e} \pm 5.6$
Cyclin D	Soybean oil	$44.9^{a} \pm 2.3$	$53.8^{a} \pm 1.7$	$118.0^{c} \pm 2.6$	$143.2^{d} \pm 8.8$
	Cow ghee	$19.6^{b} \pm 1.7$	$18.4^{b} \pm 1.3$	$50.8^{a} \pm 4.7$	$92.8^{e} \pm 4.6$
PPAR-γ	Soybean oil	$58.7^{a} \pm 1.6$	$53.5^{ad} \pm 3.3$	$31.0^{c} \pm 3.0$	$46.5^{cd} \pm 1.4$
	Cow ghee	$129.0^{b} \pm 3.2$	$122.5^{b} \pm 6.1$	$136.8^{b} \pm 3.6$	$65.6^{a} \pm 3.6$

Values (mean ± SE for n=4) are in percent relative to house keeping gene (GADPH).

Values for the said gene with different superscripts are significantly different ($P<0.05$).

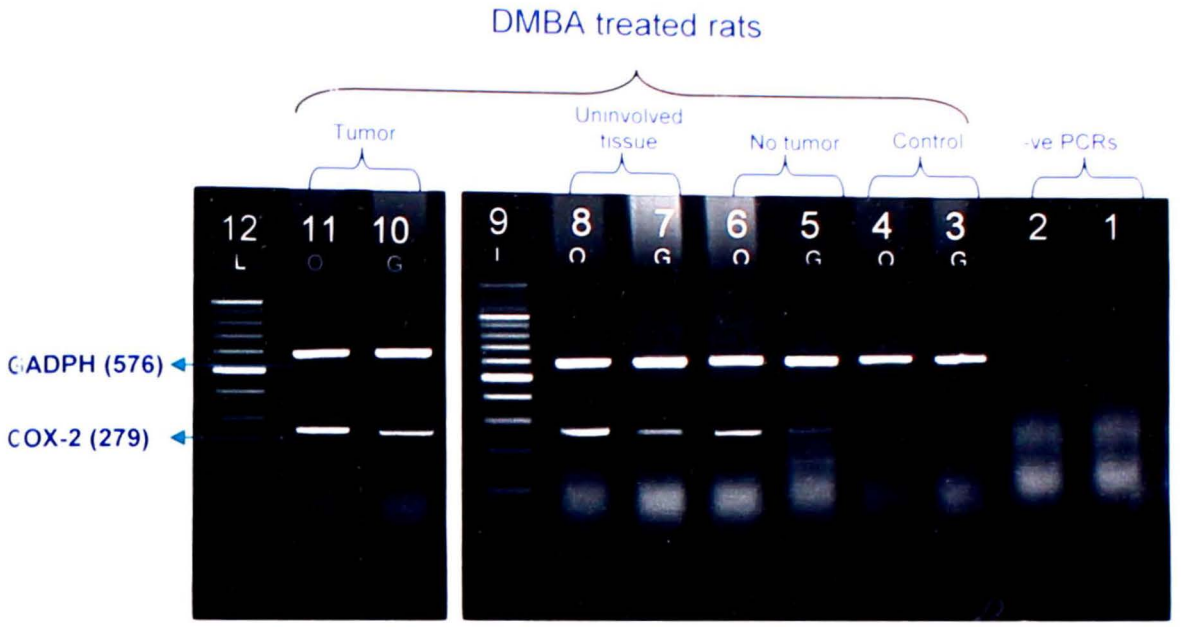


Plate: 4.15 Effect of feeding cow ghee vs soybean oil on expreession of COX-2 in mammary tissue of control and DMBA treated rats. Lane-1, -ve RT-PCR, Lane-2, -ve PCR; Lane-3, cow ghee control; Lane-4, soybean oil control, Lane-5, cow ghee + DMBA (no tumor); Lane- 6, soybean oil + DMBA (no tumor), Lane-7, cow ghee + DMBA (uninvolved tissue); Lane-8, soybean oil + DMBA (uninvolved tissue), Lane-9, DNA ladder, Lane-10, cow ghee + DMBA (tumor), Lane-11, soybean oil + DMBA (tumor), Lane-12, DNA ladder, (G – Ghee, O - Oil, L- DNA ladder)

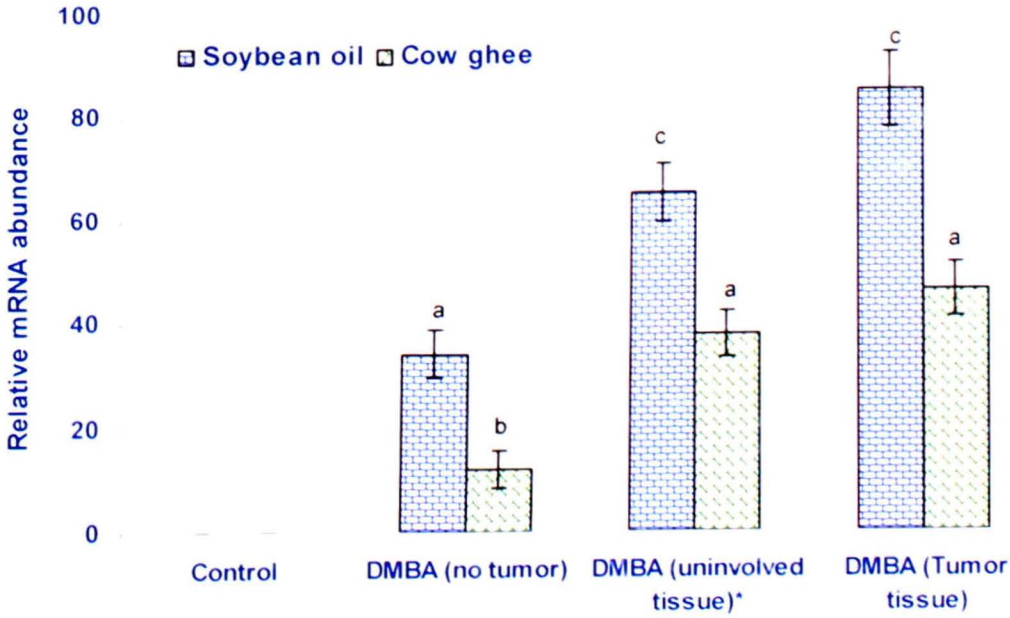


Fig. 4.27 Effect of feeding cow ghee vs soybean oil on expreession of COX-2 in mammary tissue of control and DMBA treated rats

Values (mean ± SE for n=4) with different letters are significantly different (P<0 05)
* Uninvoved tissue of tumor bearing rats

treated, no tumor bearing and DMBA minus control rats. The expression of cyclin A in tumor bearing rats on cow ghee diet increased by 104 and 594% in uninvolved tissue and tumor tissue, respectively, compared to cow ghee fed DMBA minus control. In tumor bearing animals on soybean oil, the expression of cyclin A increased by 327 and 717% in uninvolved mammary tissue and tumor tissue, respectively, compared to soybean oil DMBA minus control (Table 4.13; Plate 4.16).

When compared with cow ghee, the expression of cyclin A on soybean oil diet was 1.7, 2.5 and 1.4 fold in mammary tissue of no tumor bearing, uninvolved tissue of tumor bearing and tumor tissue on DMBA treated rats, respectively, compared to cow ghee fed DMBA treated rats (Fig.4.28; Table 4.13).

4.3.4 Expression of cyclin D in mammary tissue of normal and carcinogen treated rats

The expression of cyclin D in soybean oil fed control rats was about 2 fold of that in cow ghee fed control rats. In carcinogen treated, but no tumor bearing rats on both cow ghee and soybean oil diets, the expression of cyclin D was almost of the same magnitude as observed in their age-matched DMBA minus controls. The expression of cyclin D in tumor bearing rats on soybean oil diet increased by 162 and 219 % in uninvolved tissue and tumor tissue, respectively, compared to age-matched DMBA minus soybean oil control. Similarly, in DMBA treated cow ghee group the expression of cyclin D in tumor bearing rats increased by 160 and 374%, in uninvolved tissue and tumor tissue, respectively, compared to age-matched DMBA minus cow ghee control (Table 4.13; Plate 4.17).

The expression of cyclin D on soybean oil was 2.9, 2.3 and 1.5 fold in no tumor bearing, uninvolved tissue of tumor bearing and tumor tissue of DMBA treated rats (Table 4.13; Fig. 4.29), respectively, compared to age-matched DMBA minus cow ghee controls.

4.3.5 Expression of peroxisome proliferator activated receptor-γ (PPAR-γ) in mammary tissue of normal and carcinogen treated rats

The expression of PPAR-γ in cow ghee fed control rats was 2.2 fold of that in soybean oil control rats. In carcinogen treated but no tumor bearing rats on both cow ghee and soybean oil diets, the expression of PPAR-γ was almost of the same magnitude as observed in their respective DMBA minus controls. The expression of PPAR-γ in tumor bearing rats on soybean oil diet decreased by 47.2 and 20.8 %, in uninvolved tissue and tumor tissue, respectively, compared to DMBA minus soybean oil control. In cow ghee group, no decline in PPAR-γ expression was observed in uninvolved tissue of tumor bearing rats, compared to cow ghee fed DMBA minus control. In tumor tissue, however PPAR-γ expression decreased significantly in comparison with DMBA minus cow ghee control (Table 4.13; Plate 4.18).

The expression of PPAR-γ on cow ghee diet was 2.3, 4.4 and 1.4 fold in no tumor bearing, uninvolved tissue of tumor bearing and tumor tissue of DMBA treated rats (Fig. 4.30; Table 4.13), respectively, compared soybean oil fed DMBA treated rats.

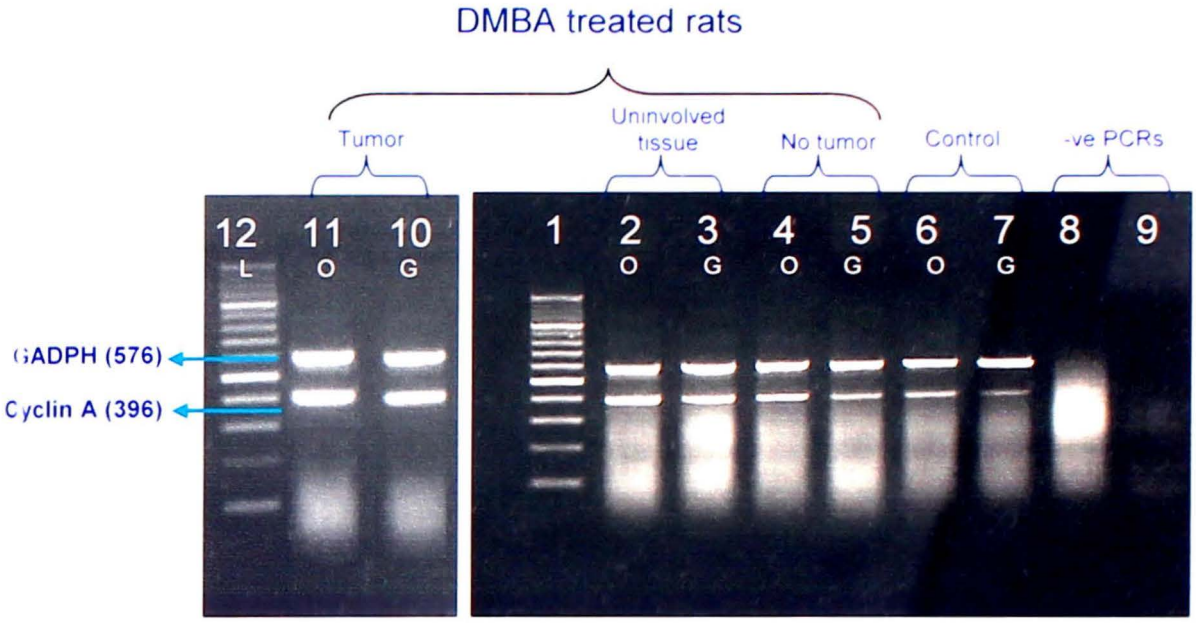


Plate 1 Effect of feeding soybean oil vs cow ghee on expreession of cyclin A in mammary tissue of control and DMBA treated rats. Lane-1, -ve RT-PCR; Lane-2, -ve PCR; Lane-3, cow ghee control; Lane-4, soybean oil control; Lane-5, cow ghee + DMBA (no tumor); Lane- 6, soybean oil + DMBA (no tumor); Lane-7, cow ghee + DMBA (uninvolved tissue); Lane-8, soybean oil + DMBA (uninvolved tissue); Lane-9, DNA ladder; Lane-10, cow ghee + DMBA (tumor); Lane-11, soybean oil + DMBA (tumor); Lane-12, DNA ladder, (G – Ghee, O - Oil, L- DNA ladder)

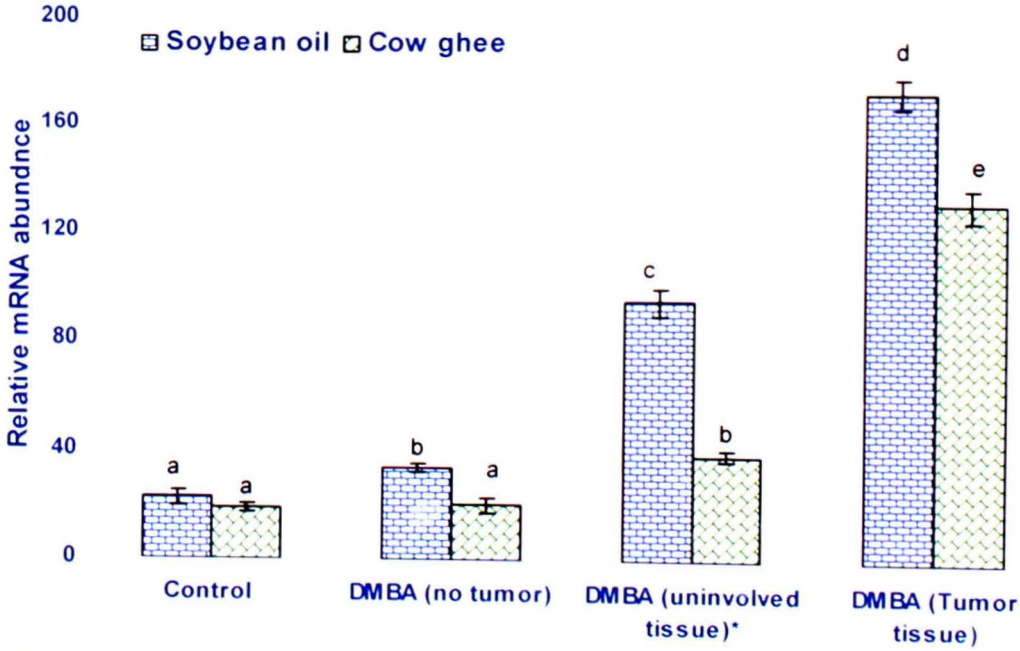


Fig. 4.28 Effect of feeding cow ghee vs soybean oil on expreession of cyclin A in mammary tissue of control and DMBA treated rats

Values (mean ± SE for n=5) with different letters are significantly different ($P<0.05$)
* Uninvolved tissue of tumor bearing rats

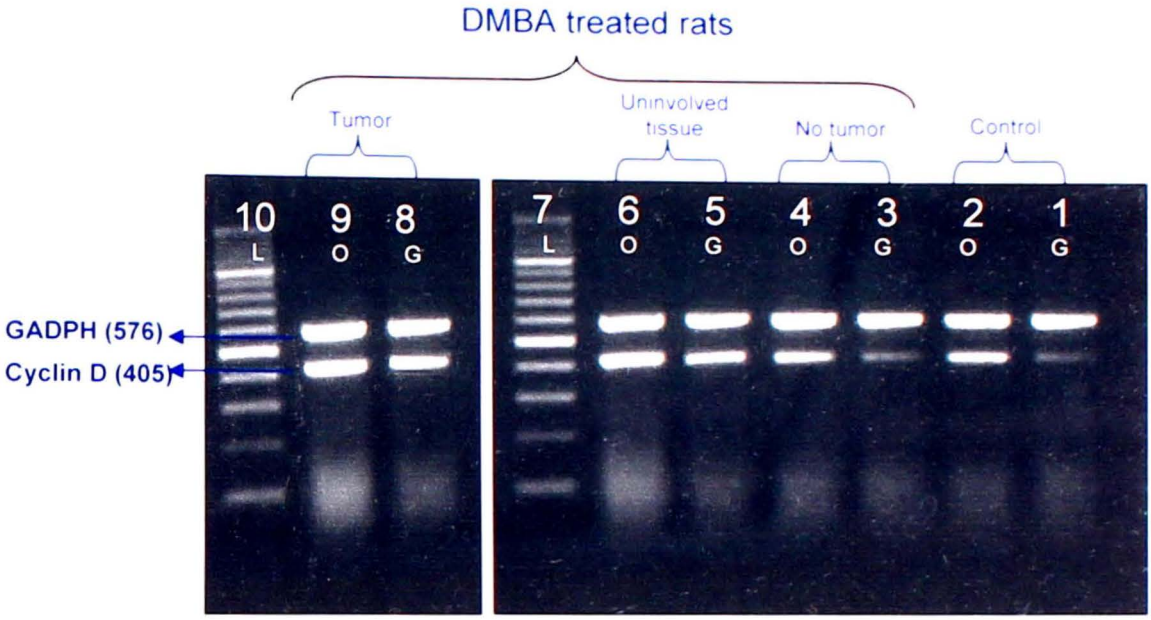


Plate: 4.13 Effect of feeding cow ghee vs soybean oil on expreession of cyclin D in mammary tissue of control and DMBA treated rats. Lane-1, cow ghee control, Lane-2, soybean oil control, Lane-3, cow ghee + DMBA (no tumor), Lane-4, soybean oil + DMBA (no tumor), Lane-5, cow ghee + DMBA (uninvolved tissue), Lane-6, soybean oil + DMBA (uninvolved tissue), Lane-7, DNA ladder, Lane-8, cow ghee + DMBA (tumor), Lane-9, soybean oil + DMBA (tumor) and Lane 9, DNA ladder (G – Ghee, O - Oil, L- Ladder)

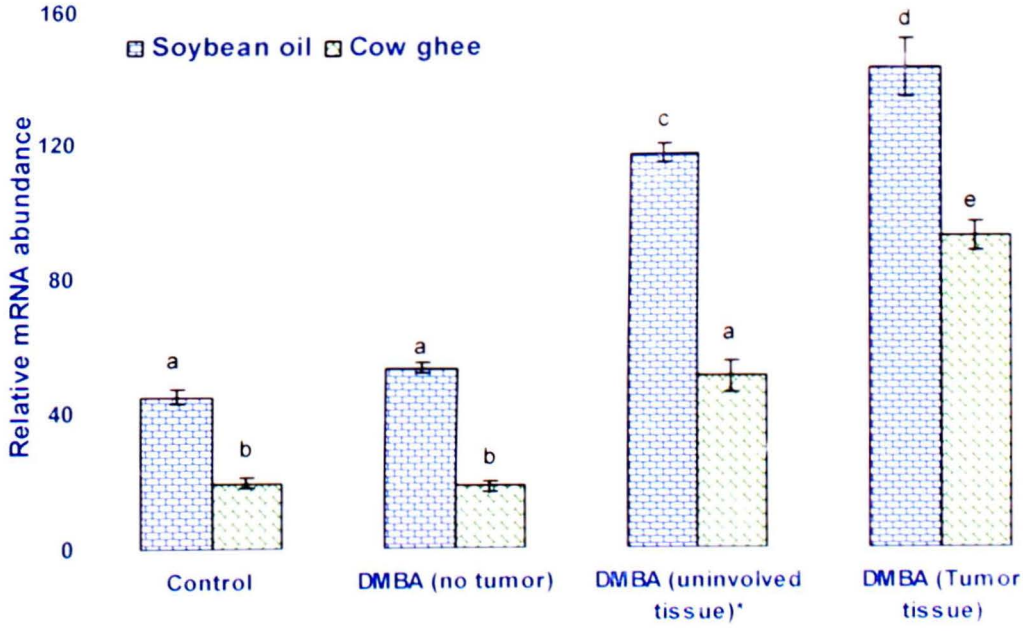


Fig. 4.29 Effect of feeding cow ghee vs soybean oil on expreession of cyclin D in mammary tissue of control and DMBA treated rats

Values (mean ± SE for n=4) with different letters are significantly different (P<0 05)

* Uninvolved tissue of tumor bearing rats

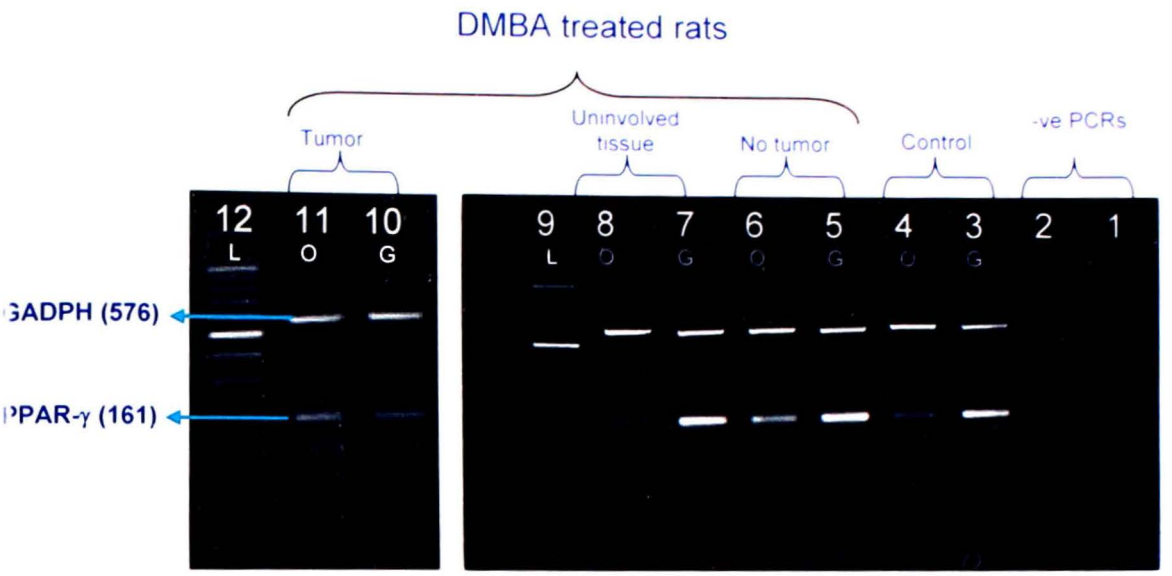


Plate 13 Effect of feeding cow ghee vs soybean oil on the expression of PPAR-γ in rat mammary tissue of control and DMBA treated rats Lane-1, -ve RT-PCR; Lane-2, -ve PCR; Lane-3, cow ghee control; Lane-4, soybean oil control; Lane-5, cow ghee + DMBA (no tumor); Lane- 6, soybean oil + DMBA (no tumor); Lane-7, cow ghee + DMBA (uninvolved tissue); Lane-8, soybean oil + DMBA (uninvolved tissue); Lane-9, DNA ladder; Lane-10, cow ghee + DMBA (tumor); Lane-11, soybean oil + DMBA (tumor); Lane-12, DNA ladder. (G – Ghee, O - Oil, L- DNA ladder)

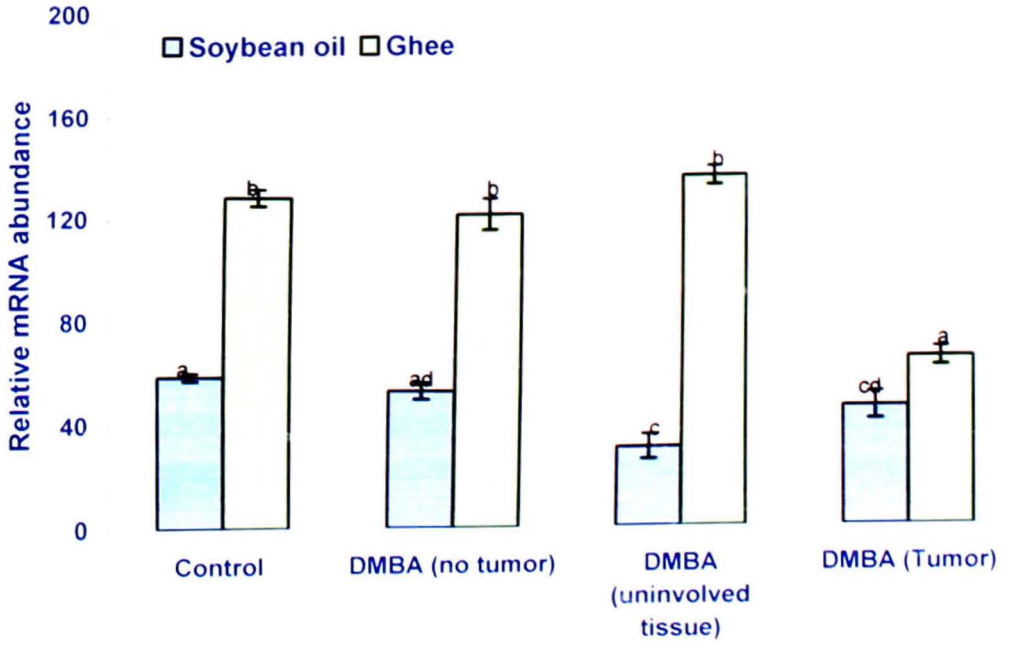


Fig. 4.30 Effect of feeding cow ghee vs soybean oil on the expression of PPAR-γ in mammary tissue of control and DMBA treated rats

Values (mean±SE for n=4) with different letters are significantly different (P<0.05)

* Uninvolved tissue of tumor bearing rats

CHAPTER – 5

Discussion

5. DISCUSSION

Breast cancer is a leading killer among cancers that affect women around the world. It is widely accepted that environmental and dietary factors play a role in determining the risk of breast cancer. There is an extensive and growing amount of work devoted to the possible links between dietary fat and risk of breast cancer, and the type of fat that may be responsible for increased breast cancer susceptibility in women. Milk fat, besides having a unique combination of fatty acids, contains a number of micronutrients (CLA, vaccenic acid, sphingolipids, butyric acid, β-carotene etc.), which have the potential of nutraceuticals and therapeutic agents. Contrary to milk fat, vegetable oils contain large amount of linoleic acid known to have promotional role in carcinogenesis (Ip *et al.*, 1985). High intake of PUFA has been shown to contribute to large number of diseases including cancer, immune system dysfunction, damage to liver, reproductive organs and lungs, digestive disorders, etc. (Pinckney and Pinckney, 1973; Harmon *et al.*, 1976; Meerson et al., 1983; Felton *et al.*, 1994; Valero-Garrido *et al*; 1990).

Ayurevedic literature has held cow ghee in high esteem in terms of health benefits, while very little has been said about buffalo ghee, and the former has been used for the treatment of various ailments. However, there is no much scientific literature to explain the role of milk fat in treatment of these diseases. Pervious work done in this laboratory shows that that ghee (clarified butter fat) opposed to soybean oil attenuates gastrointestinal carcinogenesis. Cow ghee also attenuates the toxicity induced by DMBA by decreasing incidence of mortality, tumor multiplicity, tumor weight, tumor volume and non-neoplastic disorders (Bhatia, 2005). Kathirvelan (2007) also reported that low CLA and high CLA ghee decreased the mammary tumor incidence compared to soybean oil. The present study has attempted to explore the biochemical

and molecular basis of anticarcinogenic effect of cow ghee opposed to soybean oil in mammary cancer.

Intervention of dietary fat in carcinogenesis was evaluated in mammary tumor model, with tumor induced by DMBA. Since the dietary treatment started 34 d prior to carcinogen administration and continued for 39 weeks, the results are net outcome of dietary fat intervention on all phase of carcinogenesis, i.e., initiation, promotion and progression.

In the present study, no significant difference was observed in body weight between cow ghee and soybean oil fed rats in carcinogen treated as well as in untreated groups. Similar observation was made earlier by Bhatia (2005) and Kathirvelan (2007). In general, carcinogen (DMBA) administration depresses growth in the animals (Cawein and Syndor, 1968; Ready et al., 1976; Bhatia, 2005). The present study also confirms this observation in that the body weight of carcinogen treated rats was less although statistically not significant than the untreated rats. At necropsy, animals on cow ghee revealed reduced abdominal fat as compared to animals on soybean oil diet and similar observation was also made by Bhatia (2005). The fat reducing effect of cow ghee could be due to the presence of CLA, which is known to reduce body fat in rats (West et al., 1998) and mice (Sagwal, 2006).

In studies evaluating the intervention of dietary fat on mammary carcinogenesis, fewer incidence of tumors were observed on ghee than on soybean oil diet. The greater tumor latency period on cow ghee than on soybean oil is suggestive of protection conferred by ghee during initiation phase of carcinogenesis. Further, smaller size of tumors on cow ghee opposed to soybean oil suggests cow ghee confers protection during promotional and progression phase of carcinogenesis. Malignant tumor (adenocarcinoma) was detected only in soybean oil fed group, which shows that ghee also protects from the progression of tumor to malignancy. Similarly, Kathirvelan (2007), observed that number of adenoma and adenocarcinoma was significantly higher in soybean oil fed rats compared to low and high CLA ghee rats.

A number of animal's studies have demonstrated differential effects of milk fat and vegetable oil in mammary carcinogenesis. Carroll and Khor (1971) reported that vegetable oils (soybean, sunflowers, corn, and cotton seed oil) enhanced DMBA induced rat mammary adenocarcinomas more than butter and some saturated fats (coconut oil, tallow, lard). In another study, Klurfeld *et al.* (1983) observed that when dietary treatment was started from weaning, mammary tumor incidence was 20 to 26 percent on butter oil opposed to 56 to 58 percent on corn oil in DMBA induced female Sprague – Dawley rats. When the experimental diets were introduced after administration of carcinogen, the tumor incidence was 56 to 70 percent on butter oil and 70 to 100 percent on corn oil. Similarly, Yanagi *et al.* (1989) reported that in mice, mammary tumor incidence was significantly less in butter fed group than in margarine (containing 64% linolic acid) or safflower oil fed groups.

Conjugated linoleic acid, for which milk fat is the richest natural source, has been shown unequivocally to inhibit mammary carcinogenesis in animal model. As little as 0.1 percent CLA in the diet given for 3 weeks from weaning reduced the number of tumors significantly in DMBA induced rats (Ip.*et al.*, 1994). The authors suggested that feeding CLA during early part of life before initiation of tumors by carcinogen would provide a life long protection for mammary cancer. When CLA feeding commences later in life, after tumor development has already been initiated, the lifetime CLA supplementation would be required to obtain significant protection from tumorigenesis (Ip *et al.*, 1997). Thompson *et al.* (1997) observed that when CLA feeding started at weaning and continued for six months until the termination of the experiment, it produced essentially the same magnitude of tumor inhibition in DMBA model as that produced in rats fed CLA for one month from weaning till tumor induction or in rats fed CLA for five months starting 5 d past tumor induction till the termination of the experiment.

CLA fed as triacylglycerol or as the free fatty acid in methylnitrosourea treated rats produced similar results (Ip *et al.*, 1995), and the efficacy of CLA was independent of the amount or type of dietary fat (Ip *et al.*, 1996; Ip and

Scimeca, 1997). CLA enriched butter provided the same magnitude of protection against mammary tumorigenesis in methylnitrosourea induced rats as provided by the mixture of free CLA isomers.

Bhatia, (2005) reported that ghee opposed to soybean oil, attenuated gastrointenstinal carcinogenesis induced by DMH, and cow ghee was more efficacious than buffalo ghee in reducing tumor volume. Further she observed that cow ghee opposed to soybean oil, attenuated the toxicity induced by DMBA by decreasing incidence of mortality, tumor multiplicity, tumor weight, tumor volume and non-neoplastic disorders. Katherevalin (2007) observed that incidence of DMBA induced mammary tumors was significantly higher in soybean oil fed group (83.33%) than in low CLA (63.33%) and high CLA (46.70%) ghee fed groups. Tumor weight was also less on ghee diet than on soybean oil diet.

The mechanism through which dietary fat provides protection against tumorigenesis is not known. It may involve altered metabolism of the carcinogen resulting in accelerated excretion of its reactive intermediates or to an inhibition of its activation, or both. The metabolism of chemical carcinogen involves two steps; in the first step carcinogen is metabolized to a reactive molecule by phase-I enzymes and in the second step, active metabolite gets detoxified by several phase-II enzymes. Thus the relative activity of phase-I and phase-II enzymes would determines the extent of tumorigenesis. Many of the metabolic enzymes responsible for the metabolic activation and detoxification of carcinogens are membrane-bound. Since these enzymes (e.g., the CYP superfamily) actually require the lipid membrane for activity, it is hypothesized that altering membrane lipid composition by feeding animals on singular source of fat might affect carcinogen metabolism (Talaska *et al.*, 2006).

Cytochrome P450 enzymes (phase I enzymes) play a critical role in the activation of carcinogens to electrophiles that bind to DNA, producing DNA adducts. The formation of DNA adducts is a necessary step for cancer induction by many carcinogens. Inhibition of P450s involved in carcinogen

activation to DNA adducts frequently results in inhibition of tumor formation. DMBA being lipophilic in nature requires metabolic activation for its carcinogenicity by CYP1A1, CYP1A2 and CYP1B1 enzymes.

In the present study, the activities of CYP1A1, CYP1A2 and CYP1B1 were significantly higher on soybean oil diet than on cow ghee diet, in untreated rats. In DMBA treated rats, the activity of CYP1B1 was higher on soybean oil than on cow ghee diet. This metabolic alteration might have contributed to increased latency period and reduced progression of mammary carcinogenesis on cow ghee diet.

Similarly, Rowlands *et al.* (2001) observed that feeding of soy protein isolate decreased the activity and expression of CYP1A1, CYP1A2 and CYP1B1 in liver and mammary tissue of rats, and suggested this as the potential mechanism by which soy protein isolate reduced DMBA induced tumor incidence (Hakkak *et al.*, 2000). Resveratrol, inhibited the CYP1A1 enzymes activity, and prevented tumor formation by DMBA in mice (Chun *et al.*, 1999; Jang *et al.*, 1997). Likewise, 1-ethynylpyrene prevented the tumor formation and inhibited CYP1A1 and CYP1B1 activity in DMBA treated mice (Viaje *et al.*, 1990). Ha *et al.* (1987) showed that CLA inhibited the metabolism of DMBA *in vitro* to its ultimate carcinogen via CYP450 activity. Liew *et al.* (1995) showed that CLA inhibited the IQ induced colon cancer by decreasing the activity of CYP1A1 and CYP1A2.

Rowlands *et al.* (2001) observed that the activity of CYP1A1 and CYP1A2 in liver tissue of DMBA treated rats was significantly more as compared to untreated rats. Shimada *et al.* (1996) also reported that CYP 1A1 and CYP 1B1 enzymes are induced by the DMBA. Our study also supports the fact that DMBA induces the activity of CYP1A1, CYP1A2 and CYP1B1 in liver tissue.

We further observed that cow ghee opposed to soybean oil feeding decreased activity of CYP2B1 in carcinogen treated as well untreated rats. Similar results have been reported by Sohn *et al.* (1999) wherein p- XSC,

inhibited CYP 2B1 activity in DMBA treated rats, and prevented mammary tumor development.

Many chemopreventive agents modulate the process of carcinogenesis by decreasing hepatic CYP2E1 activity. Disulfiran, a colon cancer chemopreventive agent, inhibits CYP2E1 activity (Brady *et al.*, 1991). Trans-reseveratrol inhibits the DMBA induced preneoplastic lesion formation in mouse (Jang et al., 1997) and it is a potent inhibitor of CYP2E1 (Mikstacka *et al.*, 2002). Likewise, quinacrine protects against the 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced toxicity by inhibiting the CYP2E1 activity (Karamanakos *et al.*, 2008). In the present study, CYP2E1 activity in liver tissue did not vary between soybean oil and cow ghee groups both in carcinogen treated as well as untreated rats, therefore, dietary fats do not modulate mammary carcinogenesis by way of CYP2E1. Similarly, indole-3-carbinol lowers the colon cancer risk without involving the reduction of carcinogen activation by CYP2E1 (Plate and Gallahar, 2006)

Glutathione-S-transferase is involved in the detoxification of xenobiotics, free radicals and peroxides by conjugating these with thiol group of reduced glutarhione, thus neutralize their electrophilic sites and render the product more water soluble (Habig *et al.*, 1974). In the present study, GST activity in liver and mammary tissue did not vary between soybean oil and cow ghee groups. Similarly, Bhatia (2005) reported glutathione-S- transferase in rat liver and colorectal tissue did not vary between soybean oil and ghee fed rats. Dietary CLA also does not modulate GST activity in rat tissue (Ip *et al.*, 1991). Hence, dietary fats do not modulate mammary carcinogenesis by way of glutathione-S-transferase.

γ-Glutamyltranspeptidase (GGTP), a glycoprotein which catalyzes the transfer of the γ-glutamyl moiety from glutathione and several other γ-glutamyl derivates to various amino acid and peptide acceptors, or to water, is known to play an important role in the detoxification of compounds such as carcinogens by the formation of mercapturic acid derivatives (Tate *et al.*, 1976).

Several studies have shown higher tumor incidence on high fat diet than on low fat diet (Carroll and Hopkins, 1979; Kollmorgen *et al.*, 1979). Sachdev *et al.*, (1980) suggested a reverse correlation between GGTP activity and tumor incidence. Taniguchi (1974) showed that in contrast to normal adult rat liver, the chemically induced hepatomas contained considerably higher GGTP activity. Sulakhe (1987) reported that the activity of GGTP was increased in the liver of rats bearing DMBA-induced mammary adenocarcinomas relative to age-matched controls. These reports corroborate our finding on increased GGTP activity in liver and mammary tissue of rats treated with DMBA. The increased GGTP activity, observed in present study, in liver and mammary tissue on cow ghee compared to soybean oil diet, might be the contributing factor for low tumor incidence on cow ghee diet.

Feeding cow ghee significantly increased UDPGT activity in liver of carcinogen treated as well as in untreated rats, which might have caused increased rate of glucuronidation of DMBA dihydrodiols leading to carcinogen detoxification, and may explain, the decreased tumor incidence on cow ghee group than on soybean oil diet. Elegbede *et al.* (1993) reported that the anticarcinogenic activity of monotrepene was mediated through induction of hepatic detoxification enzymes GST and UDPGT. The 1,4-phenylenebis(methylene)selenocyante (p-XSC) treatment significantly increased UDPGT activity in liver and it correlated with the decreased DMBA-DNA adduct formation in mammary tissue and decreased mammary tumor incidence in rats (El-Bayoumy *et al.*, 1992; Chae *et al.*, 1997; Sohn *et al.*, 1999). Similarly, Malejka-Giganti *et al.* (2005) reported that treatment with β-naphthoflavone (β-NF) increased the hepatic UDPGT activity and it led to suppression of DMBA-induced mammary carcinogenesis in Sprague Dawley rats.

Quinone reductase catalyzes the two-electron reduction of the metabolic products of polycyclic aromatic hydrocarbons such as quinone to inactive product such as hydroquinines and it has been considered to be a detoxification step through mercapturic acid pathways. Singletary (1990)

showed that rat fed 0.6% dietary butylated hydroxytoluene (BHT) had increased liver GST and QR activities, which resulted in decreased binding of DMBA to mammary DNA. Similarly, Lynda *et al.* (1999) reported that increase in the GST and QR activities in 4-bromoflavone treated rats was correlated with the chemopreventive activity of 4-bromoflavone in DMBA induced mammary carcinogensis. The alpha-naphthyl isothiocyanate (ANIT) treatment significantly increased the activities of GST and QR in rat liver and it shows potent inhibitory effects on mammary carcinogenesis induced by PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) in female Sprague Dawley rats (Sugie *et al.*, 2005). In the present investigation, feeding on cow ghee increased QR activity in liver of carcinogens treated rats, and it was associated with the decreased tumor incidence on cow ghee opposed to soybean oil diet.

Foregoing discussion shows that cow ghee stimulates the activities of phase II enzymes GGTP, UDPGT and QR in liver and mammary tissue, and decreases the activities of the phase I enzymes, CYP1A1, CYP1A2, CYP1B1 and CYP 2B1 in liver. Therefore, the decrease in incidence of mammary tumors on cow ghee could partly have been a consequence of decreased metabolic activation in liver and increased detoxification of DMBA in liver and mammary tissue.

The anticarcinogenic potential of cow ghee may, in part be explained by its ability to modulate pathway of prostaglandin (PG) synthesis. Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin synthesis, of which two isoforms were identified: the constitutive COX-1 and the inducible COX-2. Prostaglandins produced by COX-1 mediate various physiological responses, while prostaglandins produced by COX-2 (e.g., PGE2) induce inflammation and are potent mediators of a number of signal transduction pathways that modulate apoptosis, cell adhesion and growth and are implicated in carcinogenesis (Vane *et al.*, 1998).

Mammary carcinogenesis is triggered by inappropriate induction and up-regulation of COX-2 due to omega-6 fatty acids (e.g. linoleic acid) in diet.

Harris *et al.* (1999) hypothesed that the expression of normally silent COX-2 gene results in excess production of PGE2 and increase in local estrogen biosynthesis by aromatse. This results in to three major forces that drive the process of mammary carcinogenesis: (a) mutagenesis by creation of free radical involved in sustained prostaglandin biosynthesis; (b) angiogenesis by stimulation of vascular endothelial growth factor by PGE2; and c) mitogenesis without natural apoptosis due to estrogen production by aromatase.

In the present study, as expected, COX-2 was undetectable in normal mammary tissue but its expression was induced in DMBA treated rats and, the expression of COX-2 was significantly higher in tumor tissue as compared to uninvolved mammary tissue. Similar observations were made earlier by Badawi *et al.* (2004), wherein COX-2 was not expressed in normal mammary tissue but its expression was induced in response to methylnitrosourea treatment (mammary carcinogen), and the tumor exhibited the 2 fold higher COX-2 expression compared with uninvolved tissue.

Badawi *et al.* (1998) showed that the promotion of mammary carcinogenesis in rats by n-6 PUFA (safflower oil) was associated with enhanced expression of COX-2, whilst its inhibition by n-3 PUFA (menhaden oil) was associated with diminished production of COX-2. Olivo and Clarke (2005) showed that low fat (16 % energy) n-3 PUFA diet (from menhaden oil) reduced the risk of carcinogen induced breast cancer compared to high fat (39 % energy) n-3 PUFA diet and it was associated with the increased epithelial differentiation and apoptosis, reduced cell proliferation and COX-2 activity in former group. Conjugated linoleic acid, the potent anticarcinogen present in milk fat, reduced COX-2 mRNA expression and COX-2 protein in cultured macrophage cell line (Chenge *et al.*, 2004). Celecoxib (COX-2 inhibitor) down regulated COX-2 expression by 3-fold in tumor tissue and it was associated with the decreased in tumor incidence (Badawi *et al.*, 2004)

In present study, the reduced expression of COX-2 in mammary tissue in cow ghee fed rats compared to soybean oil fed rats was associated with decreased tumor incidence in the former group. Overexpression of COX-2 in

mice induced the mammary tumors (Liu *et al.*, 2001) and specific COX-2 inhibitors such as nimesulide (Nakatsugi *et al*, 2000), and celecoxib (Harris *et al.*, 2000) prevented mammary tumor from developing in experimental animals. Increased level of COX-2 was observed in HER2/neu-transformed human mammary epithelial cells (Subbaramaiah *et al.*, 2003) and celecoxib delayed the onset of HER2/neu induced tumor (Howe *et al.*, 2002). HER2/neu-induced mammary tumors and angiogenesis have been shown to be reduced in COX-2 knockout mice (Howe *et al.*, 2005).

One potential mediator of the effects of specific fatty acids and fatty acid metabolites on mammary tumorigenesis is peroxisome proliferators activated receptor-gamma (PPAR-γ). PPAR-γ is a ligand activated transcription factor expressed in normal and malignant mammary epithelial cells (Gimble *et al.*, 1998; Mueller *et al.*, 1998; Yee *et al.*, 2003). In breast cancer cell lines, PPAR-γ activation by ligands like thiazolidinedione (TZDs) or 15d-PGJ2 (15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2) induced cellular differentiation (Elstner *et al.*, 1998; Mueller *et al.*, 1998). Both TZDs and 15d-PGJ2 inhibited cell cycle progression through direct repression of cyclin D1 gene expression (Wang *et al.*, 2001).

McCarty, (2000) proposed that the anticarcinogenic activity of CLA is mediated by PPAR-γ activation. When treated with CLA, PPAR-γ expression was increased and APC and c-myc proteins were down-regulated in the human colon cancer cells, and finally proliferation of cancer cells was inhibited (Bozzo *et al.*, 2007; Yasui *et al.*, 2005; Yasui *et al.*, 2006a; Yasui *et al.*, 2006b). Moreover, feeding with seed oils (like bitter melon and pomegranate seed oil) containing 9*c*,11*t*,13*t*-, 9*c*,11*t*,13*c*-, and 9*t*,11*t*,13*c*-conjugated linolenic acid, which are converted to 9*c*,11*t*- and 9*t*,11*t*-CLA within colonic and liver cells, suppressed AOM-induced colon carcinogenesis by increased expression of PPAR-γ protein in the colon mucosa (Kohno *et al.*, 2004a; Kohno *et al.*, 2004b; Suzuki *et al.*, 2006; Yasui *et al.*, 2007). Similarly, in the present investigation, the expression of PPAR-γ was significantly higher in

cow ghee fed than in soybean oil fed rats, and it was associated with decreased tumor incidence in the former group.

In the present study, the expression of PPAR-γ was significantly less in carcinogen treated than in untreated rats and furthermore, the expression was more in uninvolved tissue than the tumor tissue in DMBA treated rats. These finding corroborates with those of Badawi *et al.* (2004) who observed greater expression of PPAR-γ in normal mammary tissue than in uninvolved and tumor tissue of carcinogen treated rats and up-regulation of PPAR-γ expression was associated with decrease in tumor incidence.

Several studies show an interrelationship between COX-2 and PPAR-γ pathways. Konstantinopoulos *et al.* (2007) reported an inverse correlation between PPAR-γ and COX-2 expression in colon adeocarcinomas. The PPAR-γ down-regulation in colon adenocarcinomas enhances AP-1 (activator protein-1) transcriptional activity leading to up-regulation of COX-2 expression. In the genesis of breast cancer, evidences suggest that induction of COX-2 and down-regulation of PPAR-γ can be the key component (Badawi *et al.*, 2002; Badawi *et al.*, 2003). Badawi *et al.* (2004) reported that simultaneous targeting with COX-2 inhibitor (celecoxib) and PPAR-γ agonist (*N*-(9-fluorenyl-methyloxycarbonyl)-L-leucine) inhibited the rat mammary gland carcinogenesis. Moreover, activation of PPAR-γ by cigiltazone (PPAR-γ ligand) decreased the COX-2 expression (Yang and Frucht, 2001), whereas inhibition of COX-2 activated PPAR-γ (Clay *et al.*, 1999). In the present study also, an inverse relationship between COX-2 and PPAR-γ expression was observed and increased expression of PPAR-γ and decreased expression of COX-2 was associated with decreased mammary tumor incidence in cow ghee fed rats compared to soybean oil fed rats.

A number of studies show that CLA could reduce tumor cell proliferation by modifying the cell cycle proteins. Cyclins are the prime cell cycle regulators and are central to the control of major check points in eukaryotic cells cycle. Cyclin A and D are key proteins involved in facilitating

entry of cells into the cell cycle and progression through S phase, respectively (Johnsin and Walker, 1999), and the over expression of cyclins and activation of CDKs in G1 may be the key to oncogenesis.

Cyclin A is significantly over-expressed in human breast tumor sample (Kayomarsi and Pardee, 1993). Said and Medina (1995) reported 4 to 6 fold higher level of cyclin A in hyperplastic cell lines and tumor sample compared to normal mammary cells. Sgambato *et al.* (1995) observed a 2 to 6 fold increase in the level of cyclin A protein in NMU induced, mammary tumors, compared to normal rat mammary gland. Similarly, we observed 2 to 8 fold increase in expression of cyclin A in mammary gland and in tumor samples in DMBA treated rats, compared to normal mammary gland. It is suggested that overexpression of cyclin A, caused decontrolled cell division and transformation and this resulted in increased tumor incidence in soybean oil fed rats compared to cow ghee fed rats.

We also observed increased expression of cyclin D1 in uninvolved mammary tissue and in mammary tumor tissue of DMBA treated rats when compared with age matched normal mammary tissue. These findings concur with previous studies reporting an increased expression of cyclin D1 in DMBA induced mammary carcinomas (Sgambato *et al.*, 1995: Jang *et al.*, 2000; Shan *et al.*, 2002), and studies showing an increased expression and increased protein level of cyclin D1 and CDK4 in NMU-induced carcinomas, compared to normal mammary gland (Wang *et al.*, 2001). In human breast cancer, cyclin D1 gene is amplified in approximately 20% of mammary carcinomas, and the protein is over expressed in >50% of cases (Barnes and Gillett, 1998).

Razanamahefa *et al.* (2000) reported that n-6 PUFAs increased the cyclin D1 mRNA expression in T47D breast cancer cells and it is associated with tumor stimulatory effect of n-6 PUFA. The n-3 PUFA docosahexaenoic acid reduced cyclin D1-, E-, and A associated kinase activity in HT-29 colon cancer cells (Chen and Istfan, 2001), and the n-3 PUFA, eicosapentaenoic acid, reduced cyclin D1 and cyclin E in NIH 3T3 cells (Palakurthi *et al.* 2000)

and this effect was associated with the anticancer potential of n-3 PUFA. Similarly in present study, feeding of cow ghee decreased the expression of cyclin D1 in mammary tissue of carcinogen treated rats, compared to soybean oil fed rats, and it was associated with decreased tumor incidence in cow ghee fed rats.

Recent studies showed that PPAR-γ ligand inhibited the cell cycle progression through repression of cyclin D1 (Wang *et al.*, 2001). Wang *et al.* (2003) reported that relative abundance of cyclin D1 was increased and that of PPAR-γ was decreased in mammary tumor, compared with normal mammary tissue, and cyclin D1 abundance was also increased in mammary tumor compared with that in the adjacent mammary epithelium in the same animal, with reciprocal changes in PPAR-γ expression. Similarly in the present study, the expression of cyclin D1 was increased and that of PPAR-γ was decreased in tumor tissue compared to age-matched controls. These studies show an antagonism between cyclin D1 gene (involved in cell proliferation) and tumor suppressor PPAR-γ.

We suggest that the reduced PPAR-γ expression, together with increased COX-2, cyclin A and cyclin D1 expression, are the genetic feature of transition from normal mammary tissue to mammary tumor state, and cow ghee protects against DMBA induced mammary carcinogenesis by modulating the expression of these genes.

CHAPTER – 6

Summary and Conclusions

6. SUMMARY AND CONCLUSIONS

SUMMARY

Breast cancer is the most commonly diagnosed cancer in women and is the leading cause of cancer mortality in female around the world. Strong correlation between dietary fat and incidence of breast cancer has been suggested by epidemiological studies. Milk fat because of its saturated fatty acids has adverse publicity for its suspected role in promotion of cancer, although there is no experimental evidence to support this contention. Recent work in our laboratory showed that dairy ghee has a role in attenuation of carcinogenesis in mammary gland and gastrointestinal tract. The present study was, therefore, undertaken to investigate the biochemical and molecular mechanism of mammary carcinogenesis influenced by dietary intervention with cow ghee versus soybean oil.

Two groups of 21 d old female albino (Wistar) rats, each consisted of 35 animals, were fed for 44 weeks diet containing cow ghee or soybean oil (10% level). Mammary carcinogenesis was induced with 7,12-dimethylbenz(a)anthracene (DMBA) (30 mg/ kg body weight) given through oral intubation at 55th d of age. Carcinogen activating and detoxifying activities were studied in liver and mammary tissue at 21 and 44 weeks of dietary treatment. Development of tumor and expression of putative genes involved in mammary carcinogenesis were studied at 44 weeks of dietary treatment. Another two groups of 21 d old female rats, each consisted of 20 animals, fed for 44 weeks diet containing cow ghee or soybean oil served as negative controls. The carcinogen metabolizing activities were studied at 0 d and 5, 21 and 44 weeks of dietary treatment.

Effect of cow ghee versus soybean oil on mammary Carcinogenesis

- The tumor latency period was greater on cow ghee (27 weeks) than on soybean oil diet (23 weeks).
- The tumor incidence was considerably higher in animals on soybean oil (65.4%) than on cow ghee diet (26.6%).
- The tumor volume and tumor weight were significantly less on cow ghee (1925 mm^3, 1.67 g) than on soybean oil diet (6285 mm^3, 6.18 g).
- The progression of carcinogenesis was more rapid on soybean oil than on cow ghee diet. While no adenocarcinoma was observed in cow ghee group, 8% of tumors in soybean oil group were adenocarcinoma.

Effect of cow ghee vs soybean oil on the enzymes involved in carcinogen metabolism

- Cow ghee opposed to soybean oil decreased cytochrome P4501A1 (CYP1A1) and CYP1A2 activities in liver of control rats. However, in DMBA treated rats no difference was observed in these activities between two dietary groups.
- Cow ghee opposed to soybean oil decreased CYP1B1 and CYP2B1 activities in liver of both control and DMBA treated rats.
- The CYP2E1 activity in liver was not affected by dietary cow ghee or soybean oil in control as well as in DMBA treated rats.
- Cow ghee opposed to soybean oil increased carcinogen detoxifying activities γ-glutamyltranspeptidase (GGTP), uridinediphospho-glucuronosyl transferase (UDPGT) and quinone reductase (QR) in liver and mammary gland tissue of control as well as DMBA treated rats.
- The glutathione-S-transferase (GST) was not affected by dietary cow ghee or soybean oil in both control and DMBA treated rats.

Effect of cow ghee vs soybean oil on the expression on gene involved in mammary carcinogenesis

- Cyclooxygenase-2 (COX-2) expression was observed in DMBA treated rats, but not in control rats, in both cow ghee and soybean oil dietary groups.
- Expression of COX-2 was significantly greater on soybean oil diet than on cow ghee diet in DMBA treated rats.
- Expression of cyclin A and D was significantly greater on soybean oil diet than on cow ghee diet.
- Expression of peroxisome proliferators activated receptor-γ (PPAR-γ) was significantly more on cow ghee diet than on soybean oil diet.

CONCLUSIONS

- Ghee opposed to soybean oil attenuates the DMBA induced mammary carcinogenesis.
- The decreased carcinogen activating and increased carcinogen detoxifying activities on cow ghee than on soybean oil diet correlate with increased tumor latency period and decreased tumor incidence in the former group.
- The increased tumor latency period and decreased tumor incidence in cow ghee than on soybean oil fed rats was associated with decreased expression of cyclooxygenase-2 (COX-2), cyclin A, cyclin D and increased expression of peroxisome proliferators activated receptor-γ (PPAR-γ) in mammary tissue in former group.

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