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Proliferative and Enzymatic Status of Prostate Glands in Rats as Affected by Dietary Lipid and Energy

by

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

The main objective of this research was to assess the effect of dietary fat and calories on the proliferative status, protein kinase C (PKC) activity and lipid composition of prostate glands in young and old rats. F334 male rats, 3 and 12 weeks old, were allocated to four dietary groups, high fat ad libitum and caloric restricted (HFAL and HFCR) and low fat ad libitum and caloric restricted (LFAL and LFCR) groups. All diets were formulated based on the AIN-76A diet. The low and high fat diet contained 5% and 23% corn oil, respectively, and caloric restriction was maintained at 20% of the ad libitum group. After 12 weeks of feeding, rats were killed and the prostate glands were processed for histological and biochemical analyses. Proliferative status of the prostate glands was assessed by enumerating cells exhibiting proliferating cell nuclear antigen (PCNA). Analysis of the prostate glands for PKC was carried out by quantifying the transfer of *Gama*-P^{32} from ATP to PKC specific substrate.

With respect to PCNA labelling index, in the young animals, significant difference ($p < 0.05$) between the following pairs were found in both the ventral and lateral lobes: HFAL>HFCR, LFAL>LFCR, HFAL>LFAL. In the old animals, significant differences were also found as HFAL>LFAL-HFCR-LFCR in the ventral lobe, and HFAL-LFAL>HFCR-LFCR in the lateral lobe.

In young rats, PKC activity of prostatic tissues, was significantly different between AL and CR or HF and LF diet groups. In the old animals, a significant difference was found only between the AL and CR groups. In both young and old animals, the activity of PKC in membrane was significantly affected by the level of fat and energy ($p < 0.05$),

and the treatment effects were as follows: HFAL>LFAL, HFAL>HFCR, LFAL>LFCR, HFCR>LFCR.

Dietary treatments did not alter the phospholipid composition of prostate glands in any of the animals. These findings demonstrated that dietary fat and caloric intake affected the proliferative and enzymatic status of prostate glands more profoundly in young rats than in old rats. A high fat and energy rich diet increased the proliferative status and was associated with increased PKC activity of prostate glands.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
DAG	diacylglycerol
HFAL	high fat ad libitum
HFCR	high fat caloric restriction
LFAL	low fat ad libitum
LFCR	low fat caloric restriction
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCNA	proliferating cell nuclear antigen
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PKC	protein kinase C
PS	phosphatidylserine
SEM	standard error of the mean

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I. Introduction, Hypothesis and Objective

It is now well accepted that animal models provide unique opportunities to investigate the pathogenesis of a variety of diseases including cancer. An understanding of the pathogenesis of cancer in the whole organism is fundamental to the concept of cancer prevention. There are limited approaches one can take to study prostatic cancer in animal models. In the past, a majority of the studies pertaining to prostatic carcinogenesis have been focused on the incidence and pathology of the disease. In addition, the thrust to understand the role of dietary factors in the pathogenesis of prostatic cancer is increasing. A review of the existing literature pointed to a complete lack of information on the relationship between diet and developmental features of normal prostate glands. There is limited understanding on the prostate glands responding to the dietary treatments on the different stages of life span. The growth and endocrinological features of normal prostate glands influenced by the diet may alter its susceptibility to carcinogenesis. These information is essential because endocrine and proliferative status of prostate glands may contribute significantly to the risk for developing the disease. It is suggested that dietary factors affect these parameters presumably during early childhood and that nutrition during early stage is of the risk of developing a variety of chronic illnesses including hormone dependent cancers.

The **hypothesis**, which formed the basis of this research, is the dietary habits formed during early life is important in modulating individual risk for developing cancer. Dietary factors known to enhance cancer development will also enhance the cancer risk

markers more profoundly in the prostate glands of young than in that of the old rats.

The impetus to formulate and test this hypothesis was provided by the finding of a preliminary study conducted by the author. In this study, effect of a high vitamin E diet on the proliferative status of colon and prostate glands was studied. It was noted that the proliferative status of the prostate glands was affected by a high vitamin E diet much more than that of the colon (Appendix 1). In old rats, prostate glands exhibited negligible proliferation (unpublished observation).

In view of these findings, it was deemed essential to assess whether dietary factors known to modulate the risk of cancer development would influence the proliferative and enzymatic status of prostate glands in both young and old rats.

The main objective of this dissertation was to **assess how malleable the growth and enzymatic parameters of prostate glands are to the amount of dietary lipid and energy restriction in young and old rats**. The proliferating cell nuclear antigen (PCNA) labelling index, protein kinase C (PKC) activity and the lipid composition of prostate glands were investigated.

Information about the role of nutrients and energy in modulating the growth of prostate glands is limited. Therefore, to put the objectives and experimental approaches of the present research in proper perspective, a review of the literature is presented which consists of prostate cancer and dietary factors, histological features of the prostate glands, and the role and importance of protein kinase C and proliferative cell nuclear antigen in cell growth, in differentiation and in carcinogenesis.

II. LITERATURE REVIEW

2.1. Introduction

Prostate cancer is becoming an increasingly important public health problem in the western world and is now the most commonly diagnosed cancer as well as the second leading cause of cancer death in men in the United States (Carter, 1990; Silverberg & Lubera, 1988). It is projected that in 1992 there will be 132,000 new cases of prostate cancer diagnosed as well as 34,000 deaths from prostate cancer; these numbers are expected to continue to increase as the population ages (Carter et al., 1990; Meikle & Smith, 1990; Carter & Coffey, 1990).

Epidemiologic and screening studies performed in the past several decades have raised several important questions about the pathogenesis of this disease, but a definitive cause for prostate cancer has not been established. The ultimate goal of epidemiologic studies is to identify risk factors to guide disease prevention strategies. These risk factors include family history, race, socioeconomic factors, occupation, vitamin A, cadmium exposure, smoking, infectious agents, sexual behaviour, vasectomy, benign prostatic hyperplasia, dietary fat, and hormones (Pienta & Esper, 1993; Pienta & Esper, 1993). Prostate cancer, as most cancers, develops as the result of an interplay of genetic and epigenetic events, both of which may be affected by environmental risk factors (Carter et al., 1990; Yatani et al., 1988; Pienta et al., 1989; Feinberg & Coffey, 1982). Understanding how these factors interact with the prostate will facilitate the investigation

of potential sites of therapeutic intervention as well as present opportunities for prevention. This understanding is especially important in the prostate where the involvement of a specific oncogene in the development of human prostate cancer has not been clearly elucidated.

Screening studies have shown that the age-adjusted incidence rates as well as death rates from clinical prostate cancer vary dramatically from country to country, even after adjustment for differences in screening techniques (Waranabe et al., 1984). For example, they found a 25-fold difference between incidence rates in American black men living in San Francisco and in Japanese men. In 1988, the age-adjusted death rates per 100,000 population were 15.7 for men in the United States and 3.5 for men in Japan (Boring et al., 1992).

Concurrently, autopsy studies from around the world have demonstrated that 15%-30% of men over the age of 50 have histologic evidence of prostate cancer, suggesting that the incidence of histologically evident prostate cancer does not vary dramatically (Yatani et al., 1982). The presence of histologic cancer increases with age so that by age 80 approximately 60% to 70% of men have evidence of histologic carcinoma at autopsy (Dhom, 1983; Holund, 1980; Guileyardo et al., 1980).

It is now generally accepted that the development of a fully malignant cancer cell requires multiple malignant genetic events, including those that initiate cell transformation as well as those that promote or encourage the transformation process (Pienta et al., 1989; Feinberg & Coffey, 1982; Vogelstein et al., 1988). If histologic cancer represents a step

in the development of clinically evident prostate cancer, then the data suggest that the initiating event of prostate cancer appears to occur at approximately the same rate independent of race or place of birth of the individual (Carter & Coffey, 1990). Carter and colleagues have shown that although the age-specific prevalence of histologic prostate cancer is similar in Japan and the United States, there is a marked difference in the age-specific prevalence of clinical prostate cancer between Japanese and American men. These data suggest that the initiation rate of prostate cancer may be the same in both groups but that there appears to be differences in the rate of promotion of progression to clinically evident prostate cancer. This interpretation is further supported by the observation that immigrants who move from low-risk areas to the United States gradually assume the higher risk of the U.S. population (Meikle & Smith, 1990; Dunn, 1975; Haenszel & Kurihara, 1968; Yu et al., 1991; Shimizu et al., 1991; Muir et al., 1991; Flanders, 1984). Therefore, whereas the presence of histologic cancer appears to be related to age, other risk factors that increase the development of prostate cancer probably affect the "promotion" steps of the transformation pathway.

2.2. Dietary factors and prostate cancer

2.2.1. Prostate cancer and dietary fat

Three basic types of studies contribute to the investigation of the association between dietary fat intake and the risk of developing prostate cancer: cross-cultural studies, analytical epidemiologic studies, and carcinogenesis models (Prentice et al., 1989).

Cross-cultural studies, especially those comparing prostate cancer rates between countries, have revealed differences between the fat content of diets in high- and low-risk areas (Wynder et al., 1971; Berg, 1975). Armstrong and Doll (1975) found that prostate cancer mortality in 32 countries was highly associated with total fat consumption, a finding similar to that for breast cancer. Rose et al. (1986) subsequently confirmed these data in population and determined that this association was limited to animal fat intake and did not include vegetable fat intake. Studies of migrant populations (Dunn, 1975; Muir et al., 1991; Flanders, 1984), furthermore, have implicated environmental factors such as diet in the development of prostate cancer. These results may explain why latent cancers found at autopsy occur at a similar frequency around the world but the incidence of cancers that manifest themselves clinically is higher in western Europe, the United States, and Canada, where the fat content of the diet is higher than in developing countries. Migrants who move from low-risk areas to the United States, for example, gradually assume the higher risk of the U.S. population (Meikle & Smith, 1990).

Analytical epidemiologic studies in the form of case-control and cohort studies have examined the association between dietary fat and the risk of developing prostate cancer. In a prospective study of 122,261 men, Hirayama (1979) found a lower mortality rate for men with prostate cancer who consumed green and yellow vegetables daily, i.e., a diet low in fat and high in fibre. Several other studies appear to confirm this relationship between high dietary fat intake and increased prostate cancer risk. In a case-control study of 452 patients with prostate cancer and 899 age-matched population control subjects, Kolonel et

al. (1983) found a positive association between high saturated fat intake and prostate cancer development in men 70 years age or older (relative risk[RR]=1.7), but not in men under the age of 70 (RR=1.0). These investigators reported that eating meat more than five times per week was associated with an RR of 1.43 and consuming milk and dairy products more than five times per week was associated with an RR of 2.58. In 181 Black American men studied as part of a case-control study, Hestmat et al. (1985) demonstrated an increased risk of prostate cancer with increased intakes of total and saturated fats. A large cohort study (Snowdon et al., 1984) of Seventh-day Adventists found that overweight men had an RR of 2.5 for the development of prostate cancer, compared with men of normal weight. In addition, this study found that men who consumed large amounts of milk, cheese, eggs, and meat had an RR of 3.6 for the development of prostate cancer.

Many studies have been conducted on the role of fats in promoting breast and colon cancers, but few have been completed on the role of fats in promoting prostate tumorigenesis. Using the Dunning rat prostate adenocarcinoma model, Clinton et al. (1988) reported that a fat-free diet inhibited the growth of hormone-sensitive, well-differentiated tumours but had no effect on hormone-insensitive, poorly differentiated tumours. These data are consistent with the hypothesis that fat may have a significant effect on prostate cancer development at an early stage where hormone sensitivity would be expected to be predominate. In 1985, Pollard and Luckert found that dietary fat had a promotional effect in a rat prostate carcinogenesis model. In a study of androgen-insensitive human prostate cancer cell lines, Rose and Connolly (1991) demonstrated that

the PC-3 line, but not the DU-145 line, was stimulated to grow in vitro by the presence of linoleic acid, a polyunsaturated fatty acid. This stimulation was parallelled by an inhibition of PC-3 growth in the presence of the fish oil omega-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid. Karmali et al. (1987) reported similar results in vivo, demonstrating that omega-3 fatty acids inhibit the growth of human DU-145 cells in nude mice. Also utilizing DU-145 cells, Rose and Cohen (1988) demonstrated an inhibition of tumour growth in mice fed a diet high in omega-3 fatty acids.

The relationship between fat intake and the promotion of prostate cancer is complex (Pienta & Eesper, 1993). It is very difficult to separate the effect of a given nutrient from the effects of other constituents of the diet and to ascertain association with a given cancer. However, the data demonstrating the similar rates of histologically evident prostate cancer across populations, coupled with the data demonstrating the wide variation in clinically evident prostate cancer, point to a difference in the promotion of this disease around the world. This promotional event appears to be linked to an environmental cause, and dietary fat currently seems to be the most likely environmental culprit.

2.2.2. Prostate cancer and energy intake

Caloric restriction (CR) without essential nutrient deficiency retards the rate of biologic aging and development of cancer and other late-life diseases in mice and rats (Hocman, 1988; Holehan & Merry, 1986; Masoro, 1985; Weindruch, 1989). In 1940, Tannenbaum discovered that underfeeding retarded the appearance and reduced the incidence of spontaneous breast and lung tumours in mice from susceptible strains. These

results were soon confirmed and extended to CR diets (Saxton et al., 1944; Tannenbaum, 1942; Visscher et al., 1942). In the 1960s and 1970s, Ross (1976) characterized effects of CR on spontaneous tumours and life span in male Sprague-Dawley rats. The incidence of the most common neoplasms was reduced by CR, whereas the incidence of the much rarer tumours was either unaffected or increased by CR. Ross and Bras (1971) tested during both a long and a short term period of CR, on the life span and tumour incidence in rats. Rats subjected to severe, long-term CR (one third of ad libitum) lived up to 1400 days, compared to 1000 days in ad libitum group, and had 90% fewer tumours than their ad libitum counterparts. In a study by Weindruch et al. (1989), female mice from a long-lived hybrid strain were fed either 40 kcal/week (restricted) or 85 kcal/week (control) diets from 3 weeks of age. The overall incidence of tumours was 78% for the control group and 38% for CR mice. Lymphoma (the most common neoplasm) was found in 46% of the control group and in only 13% of the CR mice.

The relative importance of calorie versus fat intake on cancer process has been investigated in a limited number of studies. Kritchevsky et al. (1989) showed that when rats whose caloric intake was restricted by 40% were compared to ad libitum-fed controls, they exhibited significantly fewer DMBA-induced mammary tumours, even when their daily fat intake was double that of the control group. Such severe CR also inhibited growth of DMH-induced colon tumours. Rats fed a calorie-restricted high-fat diet exhibited a 90% lower incidence of DMBA-induced mammary tumours than rats fed the same high-fat diet ad libitum and an 84% lower incidence of tumours than rats fed a low-fat diet ad libitum.

There are several plausible mechanisms, by which CR acts to reduce tumour incidence and delay tumour onset. CR might reduce initiation through one or more of the following less activation of carcinogens, more efficient detoxification or removal of activated carcinogens (Pegram et al., 1989), fewer ingested dietary carcinogens, reduced expression of tumour virus genes or protooncogenes (Chen et al., 1990; Nakamura et al., 1989), and enhanced DNA repair (Weraarchakul et al., 1989). The anticancer actions of CR might also relate to other possibilities: lowered basal rates of cell proliferation (Albanes & Winick, 1990), perhaps due to reductions in plasma insulin and related growth factors (Ruggeri et al., 1989; Ruggeri et al., 1989); reduced production of free radicals (believed to be involved in promotion (Ruggeri et al., 1989)); increased rate of free radical removal resulting from increased activities of the free radical scavenging enzymes catalase and superoxide dismutase (Ruggeri et al., 1989; Koizumi et al., 1987; Semsei et al., 1989); more vigorous immune responses (Weindruch & Warfold, 1988); and less energy for tumour growth. Which of these postulated mechanisms underlie the antineoplastic actions of CR is at present unknown.

2.2.3. Biological significance of lipid composition

International comparisons suggest a relationship between prostate cancer incidence and dietary fat (Kroes et al., 1986). The type of dietary fat is of interest to the respective risk of cancer occurrence (Pienta & Esper, 1993). Not only the high fat intake was associated with the higher relative risk of prostate cancer, but the animal products, which were rich in saturated fatty acids, showed greater relative risk than the unsaturated fatty

acid (Rose & Connolly, 1992).

The laboratory studies have demonstrated that the fatty acid composition of the diet, as well as the absolute amounts of fat consumed may influence the prostate cancer risk. Experimentation has shown that n-6 fatty acids stimulated and n-3 fatty acids inhibit human prostate cancer cells in culture. The reason was drawn to that the n-3 fatty acids compete with linoleic acid for enzymes which regulate eicosanoid biosynthesis. By using pharmacological inhibitors of prostaglandins and leukotriene synthesis, it has been demonstrated a requirement for both families of eicosanoids for normal human prostate cancer cell growth in vitro, and its stimulation by linoleic acid (Rose & Connolly, 1992).

Besides the fatty acids composition in the diet, the class of phospholipids in cell membrane has also been found related to cell proliferation and carcinogenesis (Lafave et al., 1994). As previously discussed, the hydrolysis of phosphatidyl inositol results in producing DG which in turn activates the PKC in membrane. The increasing activity of PKC positively feedback to enhance the hydrolysis of phosphatidyl choline, by releasing the free fatty acids (mainly arachidonic acid) and lysoPC. Arachidonic acid itself involves in the post receptor pathway of cell signal transition to further increase the PKC activity (Nishizuka, 1992).

This suggests that the amount and the type of phospholipids in membrane may relate to PKC stimulation which control the cell proliferation. The level of the arachidonic acid in cell membrane is of importance in cell signal transition and also in the production of eicosanoids, also regulation of cell growth and differentiation.

2.3. Animal models of prostate carcinogenesis

2.3.1. Animal models

Attempts to develop animal models for prostate cancer have met with only limited success and have not really provided a stimulus for experimental studies on the relationship of dietary factors to prostatic carcinogenesis on a par with those seen in breast cancer research (Carroll & Noble, 1987). Pollard and Luckert (1985) observed the spontaneous development of prostate cancers in genetically susceptible strain of Wistar rats and obtained some indication for a promotional effect of diet supplemented with linoleic acid(LA)-rich corn oil so as to bring its total fat content to 20% (w/w). When the chemical carcinogen N-nitrosomethylurea (NMU) was administered, the prostatic tumour yield was increased, an effect which was enhanced by testosterone administration, and also by a high-fat diet (Pollard et al., 1989). However, in another NMU-induced prostate cancer model developed by Bosland et al. (1983), different levels or types of dietary fat had no promotional effects on tumorigenesis (Kroes, 1986). It is possible that testosterone administration exerts a marked tumour enhancing effect and thus obscures the effect of dietary constituents. Development of an animal model which will allow assessment of the tumour modulating of a nutrient must be considered in future studies.

2.3.2. The structure of rat prostate glands

Rat prostatic tissue is tubuloalveolar and consists of epithelium-lined acini surrounded by a stromal matrix. Each prostatic acinus is surrounded by a single cell layer

of smooth muscle (myoepithelium) (Jesik et al., 1982). There are three distinct lobes consisting of the prostate glands, by the relative locations in reference to the urethra, and are designated the ventral, dorsal and lateral lobes.

The ventral prostate consists of two discrete lobes that are attached to the urethra by a layer of connective tissue and by a series of ducts. Acini of the ventral lobes have a large degree of infolding, particularly of the peripheral acini, which are smaller and more convoluted than the central ones. The epithelium of these acini is basophilic and mostly columnar with occasional cuboidal cells that have basally located nuclei and a prominent supranuclear clear area that corresponds to the Golgi apparatus location (Brandes, 1966). The acini are rather tightly packed in the stroma of the ventral lobes. The secretions in the acini of these lobes are pale and slightly eosinophilic.

The lateral lobes are likewise bound to the urethra by connective tissue and a series of ducts. The acini are generally larger and more variable in size and have walls that are highly infolded. They are also loosely arranged within the supporting stroma. Cuboidal or columnar epithelium lines these acini; cells contain a centrally located nucleus and also a supranuclear clear area. Secretions found in the acini of the lateral lobe stain very intensely with eosin.

The dorsal lobes are separable from the lateral lobes and are bound to the urethra by connective tissue and by many ducts. The acini of the dorsal lobes are quite large and less convoluted than either the ventral or dorsal lobes and are loosely distributed within the stromal tissue. They are lined mainly with cuboidal cells that have centrally placed nuclei

and supranuclear clear area as described previously for the ventral and lateral epithelium. Secretions of dorsal lobe acini stain with an intensity between that of the lateral and ventral lobes.

The stromal tissue of all prostatic lobes consists of an assortment of tissue elements that include extracellular material, small nerve endings, blood vessels, fibromuscular material, and fibroblasts (Flickinger, 1972).

2.4. Biomarkers in prostate carcinogenesis study

It is proposed that, within any one species, cancer risk is proportional to both cell number and the rate of cell division. Carcinogenesis can be increased by either increasing the number of cells at risk for exposure to the relevant transforming factors or increasing the mitotic activity of a tissue. The increasing cell number and rate in growth can be reflected by changes of some enzymes and proteins in cells which are considered as biomarkers to indicate the proliferation level associated with the carcinogenesis risk.

2.4.1. Protein kinase C

2.4.1.1. Chemical structure of PKC

The various PKC family members isolated thus far all exhibit a common primary structural organization consisting of two major domains. The amino terminal domain consists of amino acid residues 1-330 and appears to mediate the binding of all of the known co-factors and activators of PKC including phorbol esters, diacylglycerol, Ca^{2+} and phospholipid. This domain also exhibits a pattern of conserved cysteine residues. Similar

cysteine repeat regions have been found both in proteins which chelate divalent cations and in proteins which possess DNA-binding activity. Thus, either of these properties may be shared by the amino terminal domains of the PKC family members as well.

The carboxyl terminal domain of PKC, from residues 331-671, possesses its serine/threonine protein kinase activity. Interestingly, two consensus ATP-binding sites are present in this domain. The first of these, beginning at G_{349}, is most likely to be the primary ATP binding site since it is conserved in all of the PKC family members and in virtually all of the protein kinases as well. However, we can not rule out the possibility that the second consensus ATP binding site, beginning at G_{585}, also functions in some manner to regulate PKC activity. Site-directed metagenesis studies of this domain will facilitate an understanding of the relative importance of each of these regions.

2.4.1.2. The role of PKC in carcinogenesis

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent serine/threonine protein kinase of fundamental importance in cellular growth control. PKC is activated endogenously by a wide variety of growth factors, hormones, and neurotransmitters and has been shown to be a high affinity receptor for the phorbol ester tumour promoters as well as other agents possessing tumour-promoting activity (Nishizuka, 1984; Nishizuka, 1986; Ashendel, 1984). PKC has been shown to phosphorylate several intracellular protein substrates, including the epidermal growth factor (EGF) receptor (Hunter et al., 1984), $pp60^{src}$ (Gould et al., 1985), the insulin receptor (Bollag et al., 1986), p21 ras (Jeng et al., 1987), and many others (Nishizuka, 1986). Protein phosphorylation plays a central role

in the signal transduction process. Membrane receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and colony-stimulating factor (CSF) have tyrosine kinase activity (Hunter & Cooper, 1985). The kinase activity of these receptors is activated by binding of the respective growth factor to the receptor. In addition, the response of cells to several mitogens is mediated, at least in part, by the stimulation of phospholipid turnover, through the activation of the membrane-associated enzyme phospholipase C. This results in the hydrolysis of phosphatidylinositol 4,5-diphosphate to generate diacylglycerol (DAG) and inositol triphosphate (IP_3) (Berridge & Irvine, 1984). DAG is an activator of PKC. The positive feedback of PKC activates the phospholipase A2 and in turn enhances the release the free fatty acid and lyso phosphatidyl choline. This released free fatty acid is mainly arachidonic acid and consequently increases the PKC activity by involving in the post receptor pathway of cell signal transition (Nishizuka, 1992). PKC plays a central role in numerous cellular processes, including neurotransmitter release, hormone secretion, the action of several growth factors, and tumour promotion. PKC also has a wide range of substrates, including growth factor receptors, ion channel proteins, and cytoskeletal proteins (Nishizuka, 1984). Thus, the activation of PKC can produce a highly pleiotropic response on cells.

Studies have demonstrated that PKC represents a complex gene family of related kinases (isozymes), based on the isolation of distinct cDNA clones and the purification of distinct forms of PKC proteins (Housey et al., 1987). The enzyme has two functional domains, a carboxyl terminal region with consensus sequences for ATP binding and for

kinase activity, and an amino terminal regulatory domain, which contains the putative sites for lipid, Ca^{2+}, and phorbol ester binding. The deduced amino acid sequences from PKC cDNA clones reveal considerable homology to other serine and threonine protein kinases, especially the catalytic regions of the cyclic AMP-dependent protein kinase (protein kinase A) and the cyclic GMP-dependent protein kinase (protein kinase G) (Housey et al., 1987).

Since the essential role of the PKC pathway in tumour promotion and cellular transformation, studies have been conducted to investigate the relationship between the production & the distribution of PKC and the cell growth control & carcinogenesis.

From cell cultural studies, the activity of the phospholipid-dependent enzyme PKC was enhanced by chemical carcinogens treatment, associated with the cell transformation (Weinstein, 1985; Weinstein et al., 1985). Results also suggest the role for PKC in neuronal growth following cell proliferation and in synaptic function (Girard et al., 1988). PKC may be involved in the promotion and maintenance of the differentiate states of neurons. Chakravarthy et al. (1992) found that the activation is associated with erythroleukemia cell differentiation. Overproduction of protein kinase C causes disordered control of growth control in rat fibroblasts (Housey et al., 1988). Also, the inhibition of PKC mediated signal transduction by tamoxifen shows importance for antitumour activity (Horgan et al., 1986), and this result was confirmed by another study which the activity of PKC was increased 15 fold in a highly resistant human breast cancer line (Palayoor et al., 1987).

Furthermore, not only the total activity of PKC can be associated with the growth

state, but also the subcellular amount of the enzyme can be redistributed, in percentage, in endothelial cells culture studies (Uratsuji & DiCorleto, 1988). Treatment of cells with active tumour promoters results in time- and dose-dependent translocation of cytosolic PKC to membrane fractions (Regazzi et al., 1986). Adamo et al. (1986) reported that proliferating cells displayed higher PKC activity than quiescent cells , and in both normal and transformed cells, PKC is preferentially associated with the particulate fraction when the cells are proliferating. These data suggest that PKC activity and translocation is associated with the proliferative state of the cells. Cell membrane associated PKC seems to play an important role as the receptor of tumour promoters (Hecker, 1985).

2.4.2. Proliferating cell nuclear antigen

2.4.2.1. Chemical structure

As determined by 3A resolution with protein crystallographic methods, it is found that the enzyme subunit is built up of two domains, an Alfa/Beta domain consisting of a seven-stranded mixed Beta-sheet with helices on both sides of the sheet and a smaller a domain. There are two disulfides bridges between residues 129-340 and 315-319. Someone eluded that electron density at two of the glycosylation sites for parts of the carbohydrate moieties. The dimer of acid phosphatase is formed through two-fold interactions of edge strand 3 from one subunit with strand 3 from the second subunit, thus extending the Beta-sheet from seven to 14 strands. Other subunit-subunit interactions involve conserved residues from loops between helices and Beta-strands. The fold of the Alfa/Beta domain is similar to the fold observed in phosphoglycerate mutase. The active site is at the

carboxyl end of the parallel strands of the Alfa/Beta domain. There is a strong residual electron density at the phosphate binding site which probably represents a bound chloride ion. Biochemical properties and results from site-directed metagenesis experiments of acid phosphatase are correlated to the three-dimensional structure.

2.4.2.2. The role of PCNA analysis in carcinogenesis

It is proposed that, within any one species, cancer risk is proportional to both cell number and the rate of cell division (Albanes & Winick, 1988). Carcinogenesis can be increased by either increasing the number of cells at risk for exposure to the relevant transformation or cause cellular DNA to be made vulnerable (e.g., during DNA replication) to carcinogenic factors. This idea is supported by observations showing that tumour incidence increased when carcinogen exposure takes place during periods of rapid cell division (Frei & Ritchie, 1964; Shinozuka & Ritchie, 1967) and decreased when DNA synthesis is inhibited by dactinomycin (Bates et al., 1968). Alternatively, shorter cell cycles may lessen the degree of DNA repair occurring prior to the next division, thereby permitting greater inheritance of genomic effects. The early screening tests based on the level of cell proliferation are of interest. Work in this area has focused on the colon (Lipkin, 1987) in humans and in rodents (Goettler et al., 1987). Measures that have the potential to slow the rate of cell division (e.g., reducing body weight or energy intake relative to requirement) may lower the risk of cancer.

Laboratory studies have been dedicated to the identification of cell cycle specific proteins that may participate in the control of cell proliferation in human cells (Celis et al.,

1984). Two S-phase specific proliferation sensitive nuclear proteins (PCNA or cyclin, and dividin) (Celis et al., 1984) have been identified. Increased synthesis of these proteins is first detected in G_1 near the G_1/S transition border of the cell cycle and reaches a maximum in mid to late S-phase (Mathews et al., 1984). Cyclin/PCNA is present in all proliferating cells so far studied (of both normal and transformed origin), and their levels are very low in non-proliferating cells. The precise function of these proteins is unknown although there is firm evidence mainly from immunofluorescence (PCNA antibodies) and autoradiographic studies ([^{3}H]thymidine incorporation) (Celis et al., 1986) indicating that PCNA may play an essential role in DNA replication. Furthermore, early S-phase patterns of PCNA antigen distribution resemble very much the granular pattern observed in cultured cells reacted with 5-bromodeoxyuridine (BrdU) antibodies (Bravo, 1987); these structures are believed to correspond to replicating domains (Celis & Celis, 1985). Experiments using anti-sense oligonucleotides and microinjection of antibodies strongly suggest that PCNA is also essential for cellular DNA synthesis (Madsen & Celis, 1985). It has been hypothesized that there are two forms of PCNA: a soluble form lost on organic solvent fixation and not involved in replication, and an insoluble form associated with the sites of on-going DNA synthesis (Celis & Neilsen, 1986). The total concentration of PCNA varies at the most by only two- to three fold during the cell cycle but there is a greater fraction of PCNA that is insoluble due to chromatin association in S-phase than in other phases of the cell cycle (Nakamura et al., 1986).

2.5 A critical assessment of the existing literature

It is aberrant from the preceding review of the literature that there is considerable interest in understanding the role of diet in the etiology and prevention of prostate cancer. Furthermore, existing literature alluded to the fact that hormone status especially the level of the testosterone of the animals may affect the pathogenesis of prostate cancer. It is well known that the level of testosterone declines with ageing whereas prostate cancer appears in old age. It is generally believed that hormone dependent cancers are probably initiated during early stages of life cycle.

Animal models can be used meaningfully to assess the influence of diet on the growth and development of prostate glands and in the study of pathogenesis of prostate cancer at different stages of life cycle. The review of the literature alluded to the fact that animal models must be developed to study the pathogenesis of prostate cancer and that there is a complete lack of information pertaining to the role of diet in modulating the growth and susceptibility of prostate gland to carcinogenesis.

III. Materials and Methods

Animals

Eighty male F334 rats (3 and 12 week old) were obtained from Charles River Canada Inc., Montreal, Quebec, Canada. Animals were housed in wire cages, 3 rats/cage, with a 12-h light and 12-h dark cycling, temperature and humidity controlled room. Laboratory chow and water were given ad libitum until initiation of the experiment. Animal care was in accordance with the guidelines of the Canadian Council of Animal care.

Diets

Four dietary regimes were formulated, the AIN-76A high fat ad libitum (HFAL) and high fat caloric restricted (HFCR) diets which contain 23% (w/w) of corn oil as the dietary fat, low fat ad libitum (LFAL) and the low fat caloric restricted (LFCR) diets which contain 5% (w/w) of corn oil. The restricted diets were adjusted to ensure equal daily consumption of vitamins, minerals and nonnutritive fibre in relation to ad libitum counterparts with calories at the expense of cornstarch alone creating isocaloric diets within the same fat level classification (Table 1).

Study design

Within each age category, rats were randomly allocated to four groups

Table 1. Nutrient Composition (g/100g) of Experimental Diets[1]

Ingredient	HFAL	HFCR	LFAL	LFCR
Casein	23.0	23.0	20.0	20.0
D-L Methionine	0.3	0.3	0.3	0.3
Corn Starch	33.8	31.5	52.0	50.1
Dextrose	8.5	8.5	13.0	13.0
Corn Oil	23.0	23.0	5.0	5.0
Cellufil	5.9	7.1	5.0	6.0
AIN-76 Minerals Mix	4.1	4.9	3.5	4.2
AIN-76 Vitamins Mix	1.2	1.4	1.0	1.2
Choline Bit	0.2	0.3	0.2	0.2
Energy Density(kcal/g)	4.0	4.0	4.0	4.0
% Energy From Fat	50.3	50.3	11.5	11.5

[1] AIN-76 Semisynthetic Diet;
* HFAL = High fat ab libitum
  HFCR = High fat caloric restriction
  LFAL = Low fat ab libitum
  LFCR = Low fat caloric restriction

(n=10/group), which corresponded to high fat ad libitum and low fat ad libitum (HFAL and LFAL), and caloric restricted groups (HFCR and LFCR), on the 8th day. Animals in the CR groups were pair-fed the HFCR and LFCR diets with food consumption measured and calculated every two days in order to achieve a 20% caloric restricted of ad libitum level. Body weights were determined every week starting at the time of feeding formulated diets on the 8th day until the end of the study. All animals were terminated at week 12, by CO_2 asphyxiation. Liver and prostate glands were obtained from animals right after the termination, and were weighed and processed for biochemical and histological procedures.

Separation of rat prostatic tissue into subcellular fractions

Prostate glands were removed and homogenized in 25mM Tris buffer (pH=7.5) containing 0.25 mM phenylmethylsulfonyl fluoride, 15 mM ethyleneglycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.25 M sucrose and 10 ug/ml trypsin inhibitor. The homogenized mixture was centrifuged at 33,000xg for 60 min. The supernatant represented the cytosolic fraction. The pellet was resuspended and homogenized in Buffer a with 0.5% Triton-X 100, centrifuged at 33,000xg for 30 min, and its supernatant represented the membrane fraction. All procedures were carried out at 4C° unless otherwise noted.

Assay of PKC in subcellular fractions

Activity was determined by comparing the transfer of P from Gama-[P]adenosine

triphosphate (ATP) (Amersham International plc, Amersham, UK) to a peptide which is specific for PKC. All assay components should be thawed to 25 C before beginning the assay. A 25 ul aliquot of the reaction mixture was added to 25 ul of sample; the reaction was then initiated by addition of 25 ul of magnesium [^{32}P]ATP buffer (10uCI/ml) and allowed to incubate at 25 C for 15 min. Another 100 stop reagent was added to terminate the reaction then 125 ul of the terminated reaction mixture was pipetted onto small squares of P81 phosphocellulose paper. The paper was immersed in 75 mM acetic acid (5% v/v) and the dried squares were counted for radioactivity on a Beckman LS 6000 TA Liquid Scintillation Counter (Beckman Instruments, Fullerton, CA). Protein content was analysed by using the Coomasie Blue protein assay (Sigma).

Measurement of Proliferating Cell Nuclear Antigen (PCNA) indices

Prostatic tissue was embedded in paraffin wax, and 5-um thick section were processed for immunohistochemistry employing the unlabelled antibody bridge method and the Universal Peroxidase kit from Signet Laboratories (ID Labs Inc., London, ON, Canada), the method is similar to that described by Richter et al. (Richter, 1992). Tissue sections were deparaffinized and then flooded with normal goat serum and incubated for 20 min to block nonspecific binding. The anti-PCNA monoclonal antibody (Dimension Laboratories INC., Mississauga, ON, Canada) diluted with antibody diluting buffer (1:40) was applied to tissue sections, and the slides were consequentially incubated for 1 h. Each tissue section was then sequentially incubated with anti-mouse IgG (antibody bridge) and

mouse IgG peroxidase (labelling agent). The peroxidase reaction was initiated by immersing the slide in 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in PBS to which 0.03% H_2O_2 had been added immediately prior to use. Finally, the slide was lightly counterstained with haematoxylin, dehydrated, and mounted with Permount (Fisher, Scientific, Ottawa, ON, Canada). All incubations were carried out in a humidified chamber at room temperature, and between incubations slides were extensively washed with PBS. The PCNA labelling index was determined by identifying ten well-orientated acini in central and lateral lobes. The PCNA labelling indices was calculated at the number of positive cells per acinus divided by the total number of cells per acinus multiplied by 100.

Extraction and Analysis of lipids

Animals were killed by CO_2 asphyxiation and their prostate glands removed rapidly and rinsed in ice-cold PBS. Prostate were homogenized with saline and lipids were extracted as described by Folch et al. (1957) using chloroform/methanol (2:1, v/v). Phospholipids were separated by thin-layer chromatography using Silica Gel Merck 60 (BDH Inc., Toronto, Ontario, Canada) precoated plates. All major phospholipids were clearly separated following development of the plates in chloroform/methanol/acetic acid/water (50:37.5:3:1.5, by v). Lipid bands were visualized with 2''-dichlorofluorescein and identified by comparison of their migration rate with those of standard phospholipids (Serdary Research laboratories Inc., London. Ontario, Canada). Bands corresponding to

phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were scraped from the plates and the lipid fractions were directly transmethylated with 6% sulfuric acid in methanol for 2h at 80 °C in the presence of heptadecenaoic acid (17:0) as internal standard. Following incubation, the reaction mixture was cooled and diluted with water (1ml) and petroleum ether (2ml). The upper phase containing the fatty acid methyl esters was removed, dried under pure nitrogen, and reconstituted in small volumes of hexane. Reconstituted fatty acid methylesters were analysed by gas-liquid chromatography using a fused Omega Wax capillary column (30x0.25 um) held at 160 °C for 1 min, then ramped to 220 °C at 2 °C per min, and then held for 20 min using helium as the carrier gas.

Statistical analysis

SAS statistical software for microcomputers was used for all statistical analyses. Statistical analyses of caloric intake, organ and body weights, total and subcellular fractions PKC activities were performed by analysis of variance (ANOVA) and Duncan's multiple range test. This data was further analysed using a two way ANOVA in order to determine the main effects of fat, calorie and possible fat-calorie interactions (fat x calorie) (Lasko & Bird, 1995). A p value < 0.05 was considered significant in all statistical tests.

IV. MODULATION OF PROLIFERATIVE STATUS OF PROSTATE GLANDS BY DIETS

4.1. Introduction

It is proposed that cancer risk is proportional to both cell number and the rate of cell division (Mathews et al., 1984). The risk of cancer can be increased by either increasing the number of cells for exposure to the relevant transformation or cause cellular DNA to be made vulnerable to carcinogenic factors. Shorter cell cycles may lessen the degree of DNA repair occurring prior to the next division.

PCNA is a proliferation sensitive nuclear antigen which participates in the control of cell proliferation and differentiation (Jaskulski et al., 1988). Immunoflurescence and autoradiographic studies indicate that PCNA may play a role in some specific aspect of DNA replication (Celis, 1985). Different levels of PCNA in cells indicate different proliferating status and have been associated with the high or low risk of carcinogenesis.

Dietary fat and caloric restriction have been reported to significantly relate to human prostate cancer incidence (Bravo & Macdonald-Bravo, 1987). It is suggested a low fat diet and caloric restriction lower the incidence of prostate cancer (Kolonel et al., 1988). In the animal studies, dietary fat has been used as a promoter in chemically induced cancer model and 25% caloric restriction significantly inhibit tumour incidence (Klurfeld et al., 1989). However, for high-fat intake as a tumour promoting agent in prostate carcinogenesis, there was few reports published and only one of five found a positive

relation (Albanes & Winick, 1988). Furthermore, the mechanism by which either dietary fat or caloric restriction affected the prostate glands is not well explained.

In light of the report ability of a high fat diet to promote prostate carcinogenesis and CR to inhibit the process, it was of interest to investigate whether CR and dietary fat affect proliferative status of prostate glands in young and old rats.

The present study was designed to determine if the dietary fat and caloric restriction would significantly alter the proliferation status of prostate glands in young and old rats, as measured by PCNA labelling index.

4.2. Results

The weights of liver and prostate glands were shown in Table 2. Animals with high calorie intake had significantly higher weights of liver and prostate glands in either the young or old rats ($p < 0.05$), compared to their CR counterparts.

The results of PCNA labelling index were shown in Table 3.

4.2.1. PCNA labelling index in the young animals

The effect of the CR on the PCNA labelling index was significantly different ($p < 0.05$) from that of the AL in either the ventral or lateral lobe of the prostate in young rats (Figure 2). However between the HF and LF groups, difference was only observed in AL category in either the ventral or lateral lobe. The LF diet group had lower proliferative status than the HF group ($p < 0.05$).

Significant difference were found in the ventral lobe as well as the lateral lobe in

following pairs: HFAL>HFCR, LFAL>LFCR, HFAL>LFAL ($p<0.05$).

4.2.2. PCNA labelling index in the old animals

In the old rats, the effects of dietary fat and CR were evident on the PCNA labelling index in both the ventral and lateral lobes ($p<0.05$). Either the LF or CR group had decreased the proliferative index compared to the respective AL counterpart in both ventral and lateral lobes (Figure 3).

Significant differences ($p<0.05$) were found in following pairs: HFAL>HFCR, LFAL>LFCR for the ventral and HFCR>LFCR, HFAL>HFCR, LFAL>LFCR for the lateral lobes.

4.2.3. PCNA labelling index in young vs old rat prostate glands

The PCNA labelling index of each dietary group in old rats were lower than that of respective dietary group in young animals ($p<0.05$), which indicated the different level of prostate growth in the life span of rats.

The effects of dietary fat and CR were more pronounced in young rats than in old rats. The effect of CR on PCNA labelling indices was significantly higher in both ventral and lateral lobes of young than in the old rats ($p<0.05$).

4.3. Discussion

The amount of dietary fat and calories significantly altered the proliferative pattern of the prostatic acinus in either young or old animals. Generally, assessment of S-phase cells has been advocated to estimate the risk of cancer development (Albanes & Winick,

1988). Cell replication is a complex multistep process that can be interrupted or stagnated at several stages by endogenous and exogenous factors. Therefore, depending on the method of assessment, different stages of the cell cycle are enumerated. The assessment of cells exhibiting PCNA allows the identification of cycling cells. The antigens detected include various naturally occurring cell cycle related proteins (Coffey, 1993) and several studies have suggested that PCNA immunohistochemistry may be a superior technique for determining proliferative status (Lafave et al., 1994).

In the present study, it is apparent that the prostate glands in young rats were proliferating more rapidly than the prostate glands of old rats. Therefore, young rats will be more sensitive to the initiating events than old rats.

Among the three lobes in the prostate glands, the ventral lobe is the most sensitive to neoplastic changes than the lateral and dorsal lobes (Coffey, 1993). Interestingly, among the young rats of HFAL and LFAL groups, the ventral lobes exhibited higher proliferative status than the lateral lobes of the groups. The higher sensitivity of the ventral lobe to carcinogenesis compared to lateral lobes could be related to their proliferative status as well. These findings also support the notion that dietary lipids and energy intake profoundly affect the proliferative status of the prostate glands, especially among the young rats and allude to the fact that fat and energy intake during early status in the life cycle may be inportant in one's risk for developing prostate cancer.

In the present study, dietary fat and calories significantly altered the proliferative status of ventral or lateral lobe of prostate glands in young and old animals. However the

change of alternation was obviously different in the young than in the old animals. Highly proliferating glands is probably accompanied by a high testosterone level. Whether an energy rich high fat diet accentuated the level of testosterone remains to be seen.

The findings of the present study demonstrated that the high level of fat and energy exerted marked growth stimulatory effect on rat prostate glands and these effect were more significant in young developing organ than the organ which had reached maturity.

Table 2. Weights of liver and prostate glands

Group	Diet	Liver(g)	Prostate(g)
Young	HFAL	13.08 ± 0.67[a]	0.62 ± 0.06[a]
	HFCR	9.64 ± 0.21[b]	0.42 ± 0.05[b]
	LFAL	14.36 ± 0.85[a]	0.68 ± 0.05[a]
	LFCR	9.75 ± 0.61[b]	0.41 ± 0.04[b]
Old	HFAL	13.90 ± 0.51[a]	0.80 ± 0.04[a]
	HFCR	11.98 ± 0.50[b]	0.58 ± 0.01[b]
	LFAL	15.88 ± 0.82[c]	0.74 ± 0.04[a]
	LFCR	11.80 ± 0.53[b]	0.52 ± 0.04[b]

* All values shown are the mean ± SEM (n = 10/group);
* Different letter in the column represents the significant difference ($p < 0.05$);
* HFAL = High fat ad libitum
  HFCR = High fat caloric restriction
  LFAL = Low fat ad libitum
  LFCR = Low fat caloric restriction

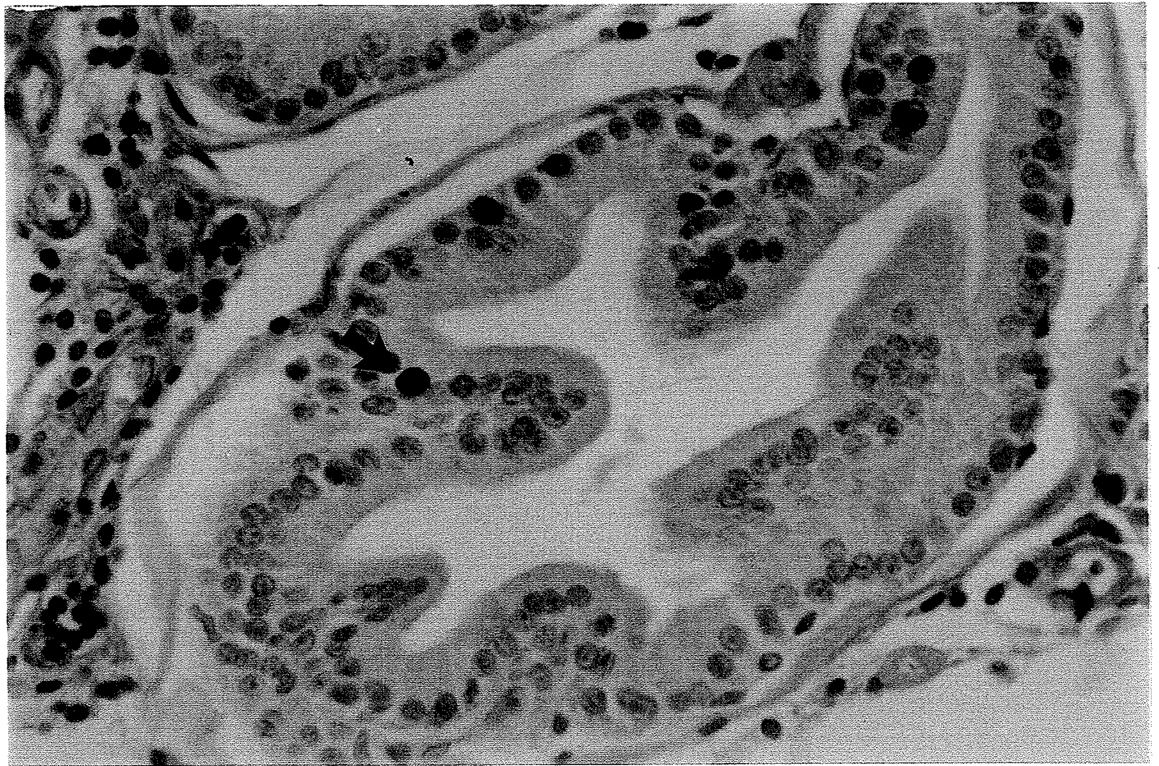
Table 3. Proliferating Cell Nuclear Antigen Labelling Index[1] in Ventral and Lateral Lobes of Prostate Glands

Group	Diet	Ventral(%)	Lateral(%)
Young	HFAL	18.8 ± 1.1[a]	11.0 ± 0.8[a]
	HFCR	4.1 ± 0.4[b]	4.2 ± 0.4[b]
	LFAL	11.9 ± 0.9[c]	8.8 ± 0.9[c]
	LFCR	4.0 ± 0.3[b]	5.0 ± 0.5[b]
Old	HFAL	7.3 ± 1.0[a]	4.8 ± 0.9[a]
	HFCR	2.7 ± 0.6[b]	2.3 ± 0.6[b]
	LFAL	3.2 ± 0.5[b]	3.3 ± 0.5[a]
	LFCR	1.2 ± 0.1[b]	1.2 ± 0.2[b]

[1] Proliferating Cell Nuclear Antigen Labelling Index = the number of labelled cells/100 accounted cells in prostate glands;
* All values shown are the mean ± SEM;
* Different letter in the column represents the significant difference ($p < 0.05$);
* HFAL = High fat ad libitum
HFCR = High fat caloric restriction
LFAL = Low fat ad libitum
LFCR = Low fat caloric restriction;

Figure 1. The histological sections of rat prostate glands. Note the presence of cells exhibiting immunoperoxidase staining for PCNA x 200.
A, Ventral lobe; B, Lateral lobe.

A



B

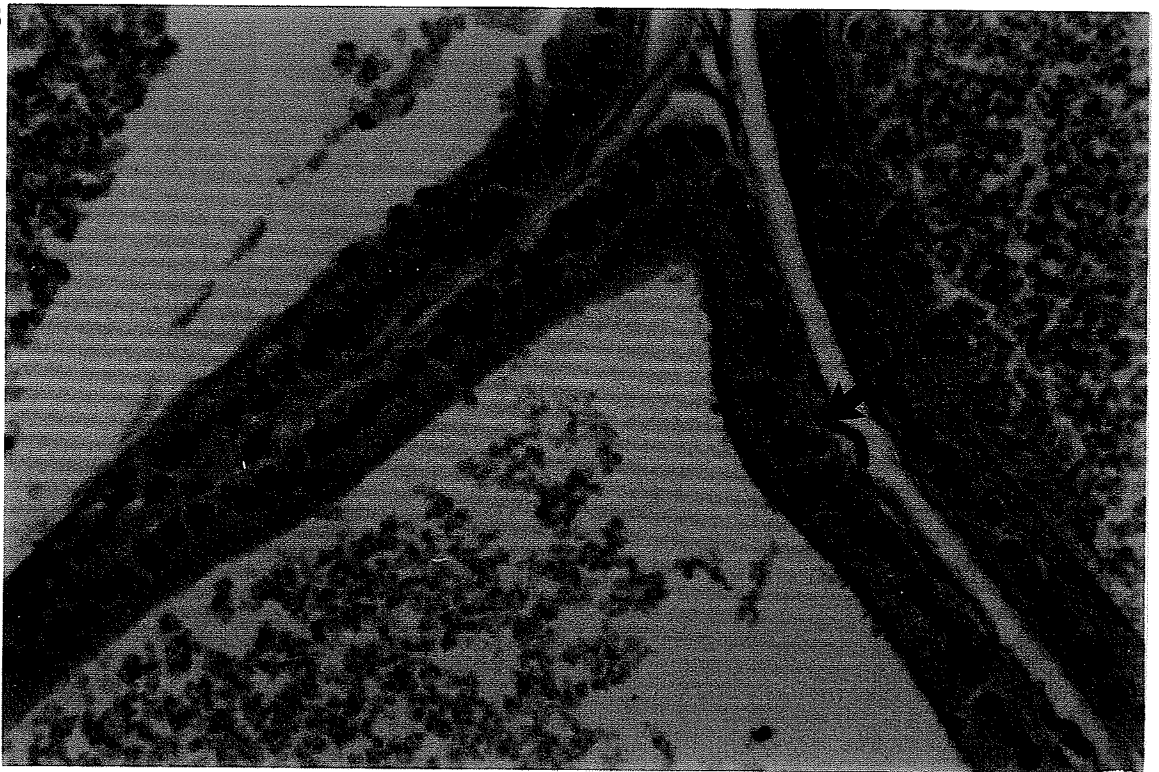
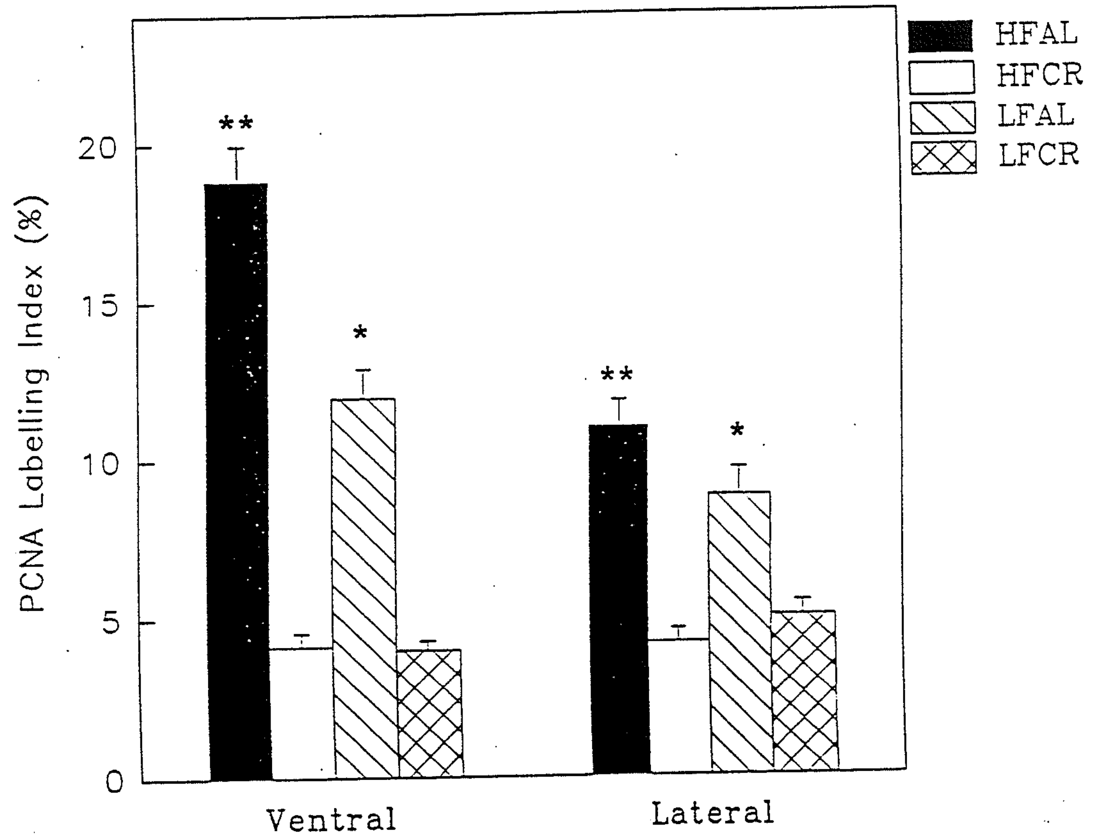
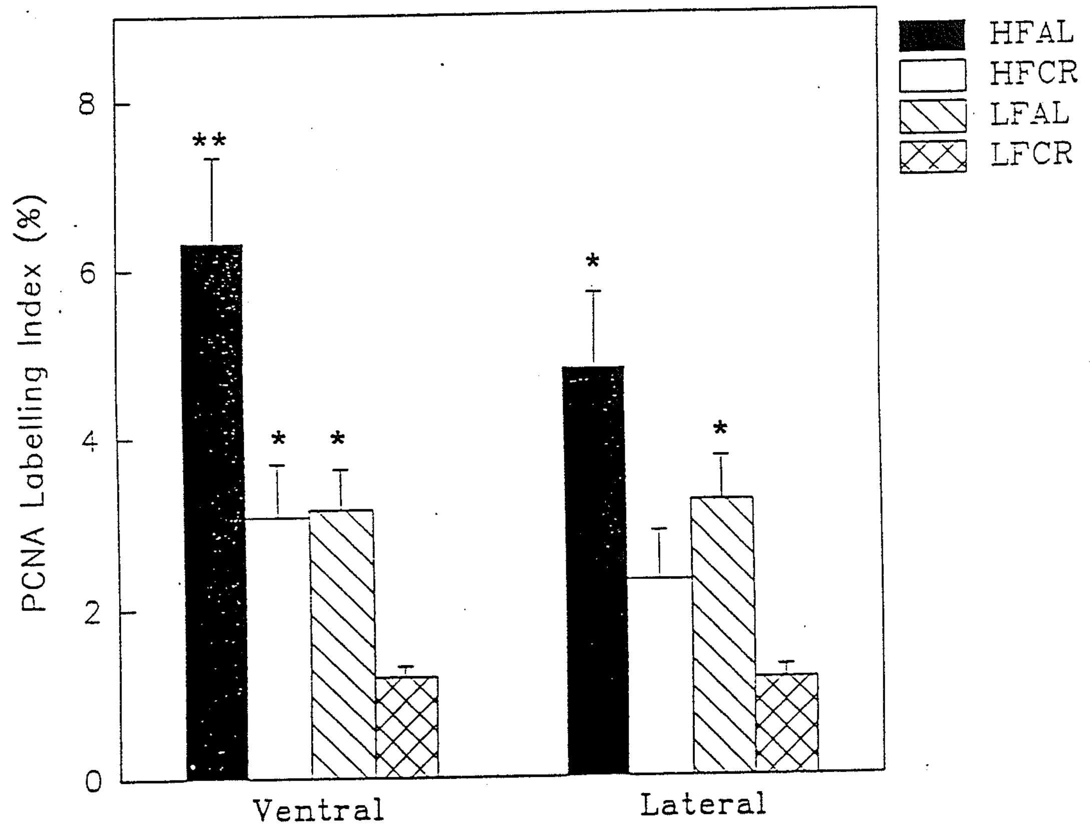


Figure 2. Proliferating cell nuclear antigen labelling index in the ventral and lateral lobes of prostate glands in young rats as affected by various dietary treatments (n=10/group). Columns represent the mean + SEM.



PCNA Labelling Index (%)
0
5
10
15
20
**
*
**
*
Ventral
Lateral
HFAL
HFCR
LFAL
LFCR

Figure 3. Proliferating cell nuclear antigen labelling index in the ventral and lateral lobes of prostate glands in old rats as affected by various dietary treatments (n=10/group). Columns represent the mean + SEM.



PCNA Labelling Index (%)
0
2
4
6
8
**
*
*
*
*
Ventral
Lateral
HFAL
HFCR
LFAL
LFCR

V. PROTEIN KINASE C ACTIVITY OF RAT PROSTATE GLANDS AS AFFECTED BY AGE AND DIETS

5.1. Introduction

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent serine/threonine protein kinase, fundamentally important to cellular growth control. PKC takes part in cellular responses to various agonists including hormones, neurotransmitters, and some growth factors. The enzyme is activated by increased amounts of diacylglycerol in membranes that result from agonist-induced hydrolysis of inositol phospholipid (Kroes et al., 1986). Sustained activation of PKC is essential for subsequent response such as cell proliferation and differentiation (Housey et al., 1988). Overproduction of PKC is implicated with disordered growth control in rat fibroblasts (Gould et al., 1985).

The subcellular distribution of PKC in proliferative and quiescent cells has been studied (Housey et al., 1988). Results indicated that proliferating cells displayed higher PKC activity than quiescent cells. Furthermore, in both normal and transformed cells, PKC is preferentially associated with the particulate (membrane) fraction when the cells are proliferating, while in mitotically quiescent cells the majority of the enzyme activity is found in the soluble (cytosolic) fraction.

Epidemiological studies have indicated that dietary fat and caloric restriction are closely related to prostate cancer occurrence and mortality (Giovannucci et al., 1993). In the study reported here (Chapter IV), we observed that, a high fat diet fed ad libitum significantly increased the proliferating status by increasing the number of cycling cells.

A caloric restricted diet lowered the number of cycling cells compared to the AL group, regardless of fat intake.

The study reported here on the effect of dietary fat and calorie on protein kinase C activity of prostate glands was investigated at different stages of animals life span. It was reasoned that increased proliferative status of the prostate glands was mediated via increased PKC activity in the membrane fraction, the active site of this enzyme.

5.2. Results

The results of protein kinase C in total cell and subcellular fractions were shown in Table 4.

5.2.1. PKC in young animals

In the young animals, the cellular activity of PKC was significantly higher in the HF than in the LF, and in the AL than in the CR groups ($p<0.05$). Among four dietary groups, HFAL had the highest cellular PKC activity in prostate, while LFCR had the lowest (Figure 4).

In the cytosolic fraction, the effect of dietary treatments on PKC activity was evident (Figure 5). The HF groups had markedly higher cytosolic PKC activities than their LF counterparts ($p<0.05$). The effect of CR was only observed in HF fed rats, with HFAL having significant higher PKC activity than HFCR ($p<0.05$). There was no difference in PKC activity between the LFAL and LFCR groups.

In the membrane fraction, the effect of dietary treatments on PKC activity was

pronounced with the respect to either dietary fat or CR (Figure 5). Significant differences were found in all following pairs: HFAL-LFAL, HFCR-LFCR, HFAL-HFCR, LFAL-LFCR.

5.2.2. PKC in old animals

In the old animals, the difference between the groups seems more obvious with the respect of dietary calorie than the dietary fat (Figure 4). The HF and AL group had higher total PKC activity than the LF and CR group, respectively, however only the difference between the HFAL to HFCR reached the level of significance ($p<0.05$).

In the cytosolic fraction, either HF or AL groups had lower PKC activity than the LF or CR groups respectively ($p<0.05$). Among four groups, HFAL group had the lowest cytosolic PKC activity in prostate, while the LFCR had the highest (Figure 6).

In the membrane fraction, HF or AL group had significantly higher PKC activity than their respective LF or CR group ($p<0.05$). Among the four groups, HFAL had the highest while the LFCR had the lowest activity (Figure 6).

5.2.3. PKC activity in young and old rats as affected by fat and caloric intake

The activities of total PKC in young rats were significantly higher than its respective group in old animals. These trends were also found in the activities of membrane PKC between the young and old rats.

The dietary treatment effects were more pronounced in young animals than in old rats. The difference between HF and LF or AL and CR groups was more evident in young animals than those in old animals, with the respect to the total as well as membrane

fraction of PKC activity.

5.3. Discussion

PKC is known to exist in various isoforms which exhibit differences in co-factor dependence and activator specificity (Albanes & Winick, 1988). Higher membrane associated PKC activity in the prostate glands of animals fed the high-fat or ad libitum diet apparently suggest that these diets may have increased the translocation of PKC from cytosolic to membrane as was evident in the lower level of cytosolic PKC activity. This observation is consistent with the reports of others in which tumour promoters appear to increase the translocation of cytosolic PKC to the membrane fraction and that a high fat diet is considered to be tumour promoter. Furthermore, a study investigating the role of dietary fat and PKC in skin carcinogenesis demonstrated that the activity in the particulate fraction increased two-fold, and the cytosolic PKC activity decreased dramatically in animals consuming a high fat diet. PKC has been recognized as having an important role in the regulation of cell growth and differentiation (Housey et al., 1988). Protein kinase C is a receptor for phorbol ester tumour promoters, and overproduction of this enzyme has been associated with disordered growth in rat fibroblasts (Nishizuka, 1984). The subcellular redistribution of PKC has been found to be growth-dependent in cells in culture. The mechanisms by which PKC and its isoforms are involved in cell growth and differentiation is complex and are being explored in various model systems. A variety of additional biological role has been attributed to the PKC family of isoenzymes. Also, this

enzyme has been implicated in imparting drug resistance and is thought to act as a tumour suppressor gene (Palayoor et al., 1987). These findings taken together illustrate the possible importance of this enzyme in the growth and proliferation of prostate glands.

In the present study, AL groups had more particulate PKC activity in the prostate glands compared to their CR counter parts and that young animals exhibited higher particulate bound PKC in their prostate glands compared to the old animals. These findings are noteworthy and suggest that age and diet are both important factors affecting the growth and development of prostate glands. The presence of an increased amount of PKC in the particulate fraction in the young animals may be a reflection of the amount of PKC being translocated by the cells. In young rats, the dietary influences on PKC activity in soluble and particulate fractions were parallel to each other and a diet related reduction in both the cytosolic and particulate fraction was noted. In old rats, the effect of CR and low fat appear to increase the cytosolic PKC compared to HFAL group and in contrast, to decrease the membrane bound PKC.

In the cytosolic fractions of prostate glands, different trends have been found between the young and old rats. Higher PKC activity in cytosolic fraction in young rats associated with higher fat and calorie intake might be due to the effect of diet on the synthesis of the enzyme. High fat and calorie diets not only enhance the translocation of PKC from cytosolic to membrane fraction, as higher PKC activity in membrane were observed in HF and AL groups, but also stimulate the synthesis that more PKC has been found in cytoplasma in HF and AL groups. However, in the old rats, no obvious clue

supports that high fat and calorie intake significantly increase the synthesis of PKC. These findings suggest that PKC was being regulated quite differently in the young vs old rats. In the old rats, the effect of CR was to reduce the translocation of PKC from the cytosolic to membrane whereas in the young rats dietary effects was also on the synthesis of and turnover rate of cytosolic PKC.

The findings of the study have demonstrated that by lowering the fat and energy in the diet, possibly the cellular production of PKC was affected as well as the translocation of the enzyme to active site. The mechanisms by which diet exerts its regulating effect on PKC remains to be investigated.

Table 4. Protein kinase C activity in total cell and subcellular fractions of prostate glands in rats

group	diet	total	cytosolic	membrane
young	HFAL	7089.4±881.2[a]	1980.8±317.9[a]	4196.4±790.9[a]
	HFCR	4596.7±167.3[b]	1077.8±110.2[b]	2854.5±167.8[b]
	LFAL	4354.5±307.2[b]	209.6± 31.0[c]	2941.6±689.6[b]
	LFCR	3840.0±242.2[c]	271.5± 68.0[c]	1541.3±470.5[c]
Old	HFAL	2039.7±129.2[d]	339.0± 37.8[d]	1317.4± 95.5[c]
	HFCR	1578.0±155.2[e]	418.6± 30.3[d]	839.1± 57.9[d]
	LFAL	1877.5±328.1[d]	546.7± 84.6[e]	986.4±179.0[d]
	LFCR	1545.1±163.0[e]	678.1±130.0[e]	597.1± 88.5[e]

* All values shown are the mean ± SEM;
* Different letter in the column represent the significant difference ($p<0.05$).

Figure 4. Total protein kinase C activity of prostate glands in the young and old rats as affected by different dietary treatments (n=10/group). Columns represent the mean + SEM.

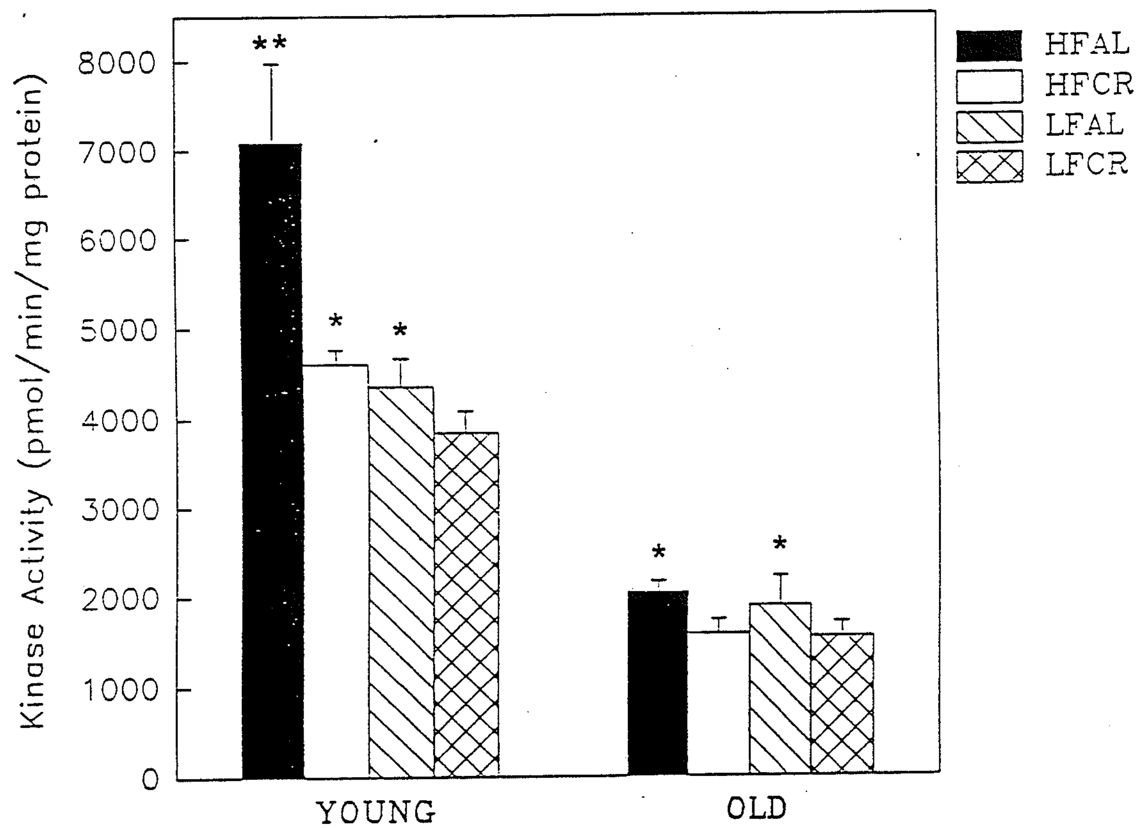
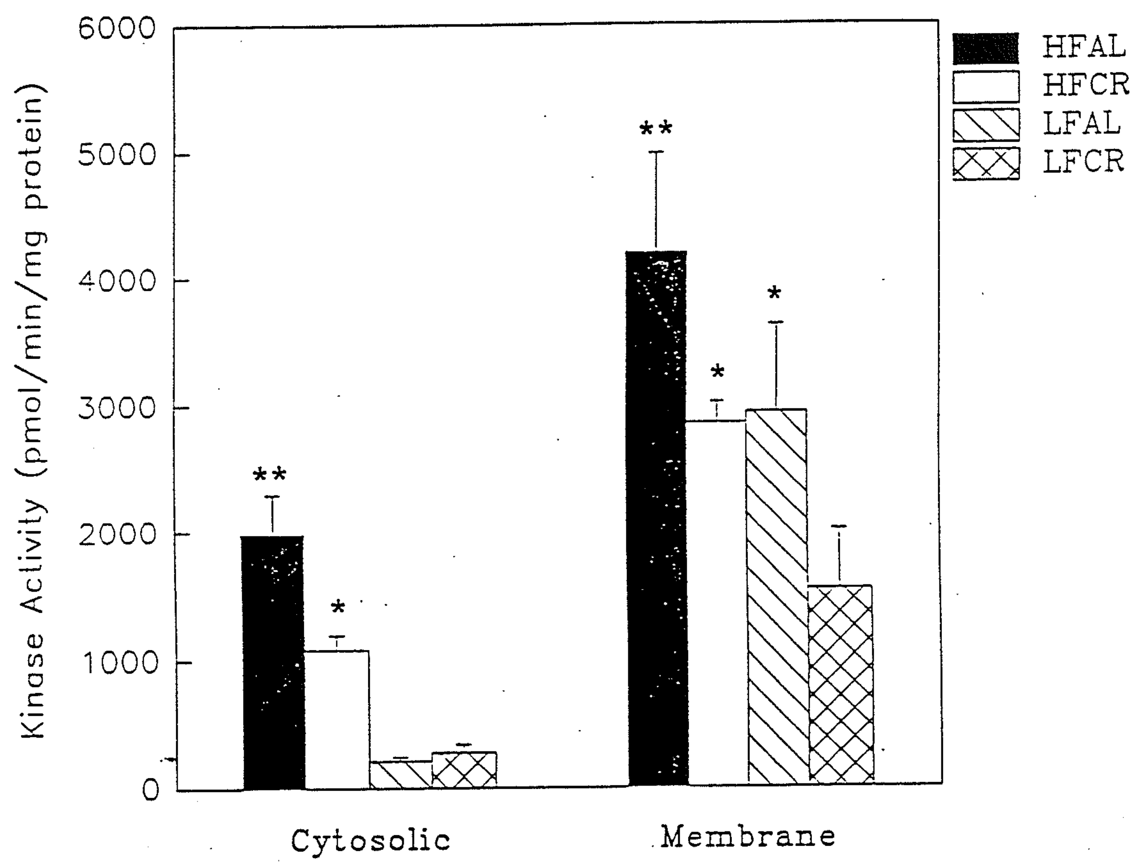
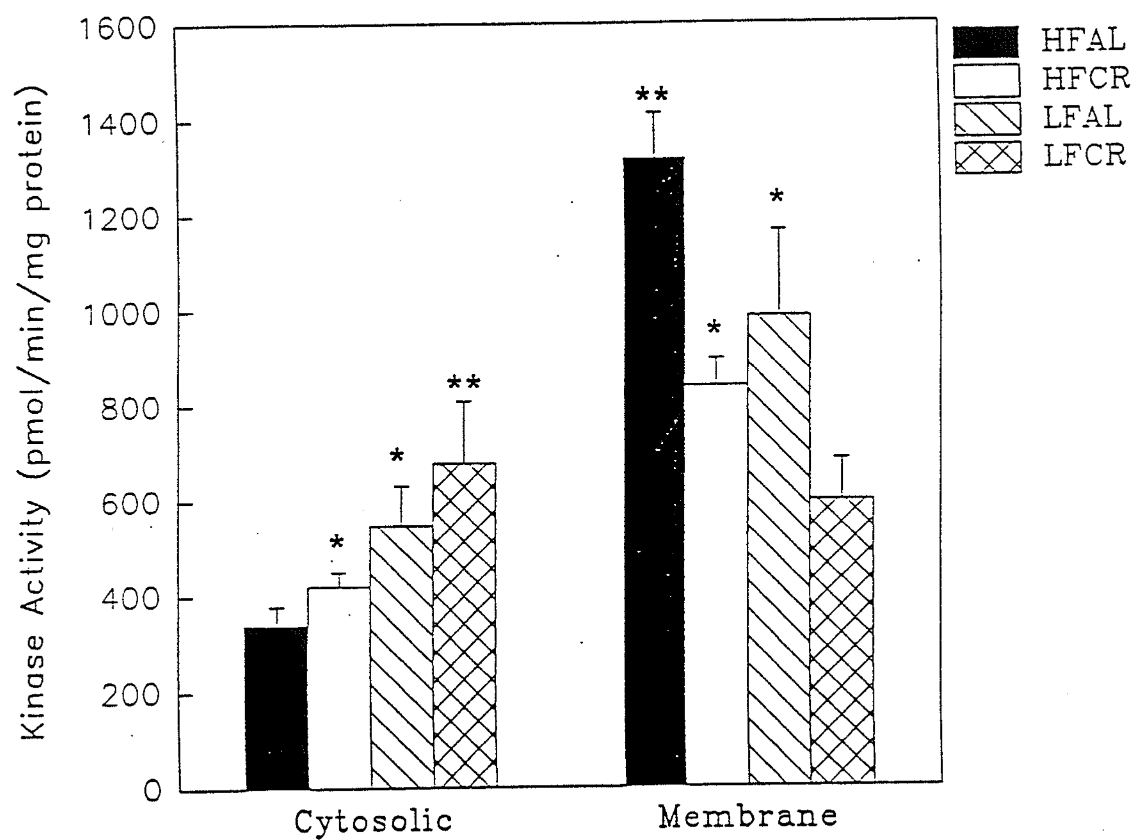

Kinase Activity (pmol/min/mg protein)
8000
7000
6000
5000
4000
3000
2000
1000
0
**
*
*
*
*
YOUNG
OLD
HFAL
HFCR
LFAL
LFCR

Figure 5. Protein kinase C activity in subcellular fractions of prostate glands in the young rats as affected by different dietary treatments (n=10/group). Columns represent the mean + SEM.



6000
5000
4000
3000
2000
1000
0
Kinase Activity (pmol/min/mg protein)
HFAL
HFCR
LFAL
LFCR
**
*
Cytosolic
Membrane

Figure 6. Protein kinase C activity in subcellular fractions of prostate glands in the old rats as affected by different dietary treatments (n=10/group). Columns represent the mean + SEM.


Kinase Activity (pmol/min/mg protein)
0
200
400
600
800
1000
1200
1400
1600
Cytosolic
Membrane
HFAL
HFCR
LFAL
LFCR
*
**

VI. LIPIDS COMPOSITION OF PROSTATE GLANDS AS AFFECTED BY DIETS

6.1. Introduction

Exploration of the composition of phospholipids in membrane and their role in modulating biological responses including cell growth control has drawn interests in recent years. Hydrolysis of phospholipids produces diacylglycerol and consequently activated the PKC, the enzyme of much interests, may play an important role in cell proliferation and differentiation control (Nishizuka, 1992). It was demonstrated that dietary fat and caloric restriction significantly affect the cellular activity and distribution of PKC. It has been suggested (Lafave et al., 1994) that dietary lipids affect membrane lipid composition which in turn profoundly affects the membrane function and its involvement in signal transduction pathway, including PKC activity. However, whether the dietary factors affect PKC by altering lipid composition of the membrane is not clear.

The concept that dietary fat may also be important in influencing prostate cancer development has been proposed (Kroes et al., 1986). Animal fat, which consists of saturated fatty acids mainly, has been associated positively with the high incidence of prostate cancer. Furthermore, a high fat diet was found having a promotional effect in a rat prostate carcinogenesis model (Pienta & Esper, 1993). It can be hypothesized that the dietary fat and caloric restriction may also change the lipids composition in the target organ and consequently provides a favourable or unfavourable environment for the cell proliferation and differentiation. However, the relationship between the dietary fat and

caloric restriction and the lipid composition in prostate glands is not well understood.

In light of the previous discussion, the objective of the present study was to investigate membrane phospholipids composition of the prostate glands of the young and old rats as affected by the different dietary treatment.

6.2. Results

6.2.1. Phospholipids composition

The amount of different phospholipids in the prostate tissue of the young and old animals were shown in Table 5.

The level of PC was similar among the dietary groups in either young or old rats. However, in the old animals, consistently higher levels of PC were found in the two groups in CR compared to their AL counterparts, and HFAL and HFCR were higher than LFAL and LFCR respectively ($p > 0.05$).

There was no difference among the dietary groups in either young or old rats for PS. However, when dietary fat was considered as the main variable (Table 6), the HF groups had significantly higher level of PS than the LF group ($p \leq 0.05$). Furthermore, significant interaction between fat and age was found in PS fraction, and between calorie and age in PE fraction ($p < 0.05$) (Table 6).

There was no difference in the level of PI among the dietary groups, when calorie or age was regarded as the main variable. Significant higher of PI was found in HF fed animals ($p < 0.05$), compared to their LF counterparts (Table 6).

The level of PE was not significantly affected by the dietary treatments in the young animals. In the old animals, a significant effect on the level of PE was noted when energy intake was considered as the main variable. It was noted that the CR groups had higher values than their AL counterparts ($p \leq 0.05$). In all animals, both young and old, high fat fed animals had higher PE than the low fat fed rats ($p < 0.05$) (Table 6).

6.2.3. Fatty acids composition

Fatty acid composition as amount (n moles) as well as the percentage of total fatty acids were determined for each phospholipids. Generally, there was no significant difference among the dietary groups, regardless of the age of the animals. The amount of saturated, monounsaturated and polyunsaturated fatty acids were similar among the groups (Table 7,8 & 9).

When individual fatty acid was considered, fat and energy were ineffective in changing the composition. Among the fatty acids, the level of arachidonic acid appeared to be affected by dietary variables. The level of arachidonic acid as the percentage of total fatty acid was not affected by dietary treatments (Table 10 & 11). However, when the amount of arachidonic acid in different phospholipid classes was analysed, high fat diet was found significantly increasing the arachidonic acid in the PE fraction ($p < 0.05$) (Table 12 & 13). Age was an important variable in affecting the level of arachidonic acid in PC and PE. There were also significant higher amount of arachidonic acid in either PC ro PE fraction of old animals, compared to their young counterparts ($p < 0.05$) (Table). There

was also a significant interaction between fat and age in modulating the level of arachidonic acid in the PE fraction ($p < 0.05$) (Table 13).

6.3. Discussion

Analysis of phospholipids composition of tissue is of special interests in carcinogenesis because of their roles in the PKC pathway (Niskizuka, 1986). Nishizuka demonstrated that alternation of the phospholipids was associated with the stimulation of PKC and the amount DAG production and PKC activity. It was noted that dietary fat was an important variable in affecting the level of PS, PI and PE. A decrease in the level of phospholipid may indicate an increased turnover or decreased synthesis. Conversely, an increase in the level of phospholipids could be due to an increase in the synthesis or a decrease in the turnover rate. Clearly, the findings on the phsopholipid composition is interesting and suggest that effect of diet on the synthesis and turnover of phospholipids should be further investigated.

Fatty acid composition of different phospholipid classes was studied to see if there were alternations due to level of dietary lipid, age or caloric restriction. The composition of n-6 family of fatty acids was of interest due to their direct roles in mediating signal transduction and as precursors of bioactive eicosanoids. It was interesting to note that older animals accumulated arachidonic in the two major phospholipid, PC and PE. These findings suggest that age dependent alteration in the metabolism of n-6 fatty acids occurred and that increased level of arachidonic acid may have been due to decreased utilization.

This may explain a lower protein kinase C activity in old animals. These findings suggest that lipids compositional changes are more detectable in stable organs than those which are rapidly growing as one would expect for the prostate glands in old than in young rats.

Many studies have demonstrated that there is a strong relationship between fat consumption and prostate cancer incidence and mortality (Giovannucci et al., 1993; Pienta & Esper, 1993). The case-control studies have found that the high prostate cancer occurrence is associated with high fat intake. In animal studies, high fat diet has shown to promote chemically induced carcinogenesis (Pollard & Luckert, 1985). Caloric intake is positively related to human prostatic cancer incidence whereas CR significantly inhibit the chemically induced carcinogenesis (Weidruch et al., 1991).

It has been shown that the type of dietary fat is important in modulating prostatic carcinogenesis. Giovannussi et al. (1993) found that the saturated fat, monounsaturated fat, and a-linolenic acid, but not linoleic acid, were associated with advanced prostate cancer risk. In another study, n-6 fatty acids stimulated and n-3 inhibited human prostate cancer cells in culture (Rose & Connolly, 1992). These findings suggest that the type of dietary fat may be important during different stages of carcinogenesis. It can be hypothesized that different dietary treatments change the inner-environment of the target organ which may be more or less beneficial for the growth of prostate glands.

It was concluded that although some differences were seen among different groups, with respect to phospholipid and fatty acid composition, these changes were insufficient to explain the effect of fat and caloric restriction on PKC and proliferative status of

prostate glands. Further studies must be conducted to elucidate diet mediated alteration in the phospholipid synthesis and turnover and its implication to signal transduction pathway.

Table 5. The Amount of Different Phospholipids in Prostate Glands (n moles/g)

Group	Diet	PC	PS	PI	PE
Young	HFAL	3189.1 ± 425.7	467.9 ± 44.0	479.2 ± 70.2	1786.5 ± 312.0[a]
	HFCR	3922.9 ± 806.2	455.4 ± 8.6	489.4 ± 89.7	1589.9 ± 67.0
	LFAL	3420.0 ± 949.2	453.0 ± 48.0	557.8 ± 227.0	1233.5 ± 114.2
	LFCR	3465.1 ± 732.5	446.5 ± 73.5	496.7 ± 201.3	1194.6 ± 53.3
Old	HFAL	3700.6 ± 790.6	507.6 ± 45.5	463.5 ± 96.6	1299.3 ± 120.7[a]
	HFCR	4294.9 ± 851.7	500.1 ± 18.3	487.2 ± 122.1	1915.5 ± 208.2
	LFAL	2992.1 ± 703.5	383.8 ± 21.1	502.1 ± 189.9	1244.3 ± 220.9[a]
	LFCR	3602.5 ± 615.4	410.1 ± 76.7	475.7 ± 198.7	1698.7 ± 102.1

* All values shown are the mean ± SEM;
* Different letter in the column represents the significant difference ($p < 0.05$), otherwise none of values within that column for individual phospholipids were found to be significantly different;
* HFAL =High fat ad libitum
  HFCR = High fat caloric restriction
  LFAL = Low fat ad libitum
  LFCR = Low fat caloric restriction.

Table 6. The main and interactive effects of fat, calorie and age on the phospholipids in membrane: 2-way ANOVA.

factor	PC	PS	PI	PE
Fat	0.8525	0.0037	0.0086	0.0189
Cal	0.1037	0.2596	0.5100	0.2321
Age	0.3565	0.9593	0.3823	0.8164
Fat*Cal	0.5306	0.3308	0.9887	0.8623
Fat*Age	0.2831	0.0622	0.6612	0.3405
Cal*Age	0.4753	0.6422	0.5515	0.0317
Fat*Cal*Age	0.9876	0.8370	0.6169	0.5354

* All values shown are the p values.

Table 7. The Profiles of Saturated Fatty Acid as Percentage of Total Fatty Acids in Different Phospholipid Classes

Group	Diet	PC	PS	PI	PE
Young	HFAL	50.4 ± 1.5	43.8 ± 0.2	51.8 ± 0.7	22.1 ± 1.9
	HFCR	49.5 ± 2.3	41.1 ± 3.5	54.4 ± 2.2	27.7 ± 3.2
	LFAL	49.3 ± 2.1	43.1 ± 1.6	54.3 ± 2.8	26.0 ± 2.1
	LFCR	50.3 ± 2.5	44.5 ± 1.3	53.0 ± 1.7	29.8 ± 3.7
Old	HFAL	51.4 ± 1.5	42.1 ± 0.2	50.4 ± 1.2	25.9 ± 2.3
	HFCR	49.0 ± 3.5	46.8 ± 1.2	56.7 ± 4.5	22.9 ± 2.4
	LFAL	51.8 ± 1.6	44.4 ± 1.7	56.8 ± 1.8	20.1 ± 2.6
	LFCR	42.1 ± 7.5	41.8 ± 4.1	52.9 ± 1.2	21.3 ± 0.6

* All values shown are the mean ± SEM;
* None of values within each column for individual phospholipids were found to be significantly different.

Table 8. The Profiles of Monounsaturated Fatty Acid as Percentage Of Total Fatty Acids in Different Phospholipid Classes

Group	Diet	PC	PS	PI	PE
Young	HFAL	19.3 ± 0.6	29.5 ± 0.9	6.1 ± 0.2	15.3 ± 0.7
	HFCR	20.9 ± 0.9	31.1 ± 0.6	5.3 ± 0.7	16.6 ± 0.4
	LFAL	21.5 ± 1.4	32.1 ± 1.3	6.4 ± 0.9	17.8 ± 0.3
	LFCR	20.4 ± 1.4	32.2 ± 0.7	6.3 ± 0.4	15.6 ± 1.9
Old	HFAL	18.2 ± 2.4	32.4 ± 1.0	4.4 ± 0.4	16.1 ± 0.4
	HFCR	19.0 ± 0.8	30.7 ± 1.1	4.8 ± 0.2	14.4 ± 1.0
	LFAL	24.7 ± 1.4	33.3 ± 0.2	6.1 ± 0.4	17.5 ± 1.7
	LFCR	19.7 ± 1.4	31.1 ± 2.1	5.5 ± 0.3	17.8 ± 0.3

* All values shown are the mean ± SEM;
* None of values within each column for individual phospholipids were found to be significantly different;
* HFAL = High fat ad libitum
HFCR = High fat caloric restriction
LFAL = Low fat ad libitum
LFCR = Low fat caloric restriction.

Table 9. The Profiles of Polyunsaturated Fatty Acid as Percentage of Total Fatty Acids in Different Phospholipid Classes

Group	Diet	PC	PS	PI	PE
Young	HFAL	28.3 ± 1.6	27.1 ± 1.2	39.0 ± 3.0	61.8 ± 2.5
	HFCR	30.7 ± 6.6	24.4 ± 0.6	36.6 ± 2.9	55.6 ± 3.3
	LFAL	31.4 ± 3.5	22.9 ± 1.8	35.9 ± 3.2	56.3 ± 2.1
	LFCR	26.3 ± 2.2	27.3 ± 4.8	36.7 ± 3.3	54.2 ± 2.3
Old	HFAL	33.3 ± 2.3	26.2 ± 1.0	40.8 ± 3.8	56.0 ± 3.1
	HFCR	35.5 ± 4.6	24.1 ± 2.1	34.7 ± 4.4	60.8 ± 2.9
	LFAL	31.7 ± 4.2	23.7 ± 1.8	33.6 ± 3.7	60.5 ± 2.1
	LFCR	33.3 ± 4.5	21.1 ± 2.4	36.6 ± 4.5	58.6 ± 1.8

* All values shown are the mean ± SEM;
* None of values within each column for individual phospholipids were found to be significantly different;
* HFAL = High fat ad libitum
  HFCR = High fat caloric restriction
  LFAL = Low fat ad libitum
  LFCR = Low fat caloric restriction.

Table 10. The Profiles of Arachidonic Acid as Percentage of Total Fatty Acid in Different Phospholipid Classes (%)

Group	Diet	PC	PS	PI	PE
Young	HFAL	13.3 ± 1.5	7.9 ± 0.5	29.5 ± 1.3	37.7 ± 1.2
	HFCR	13.9 ± 0.9	7.2 ± 0.4	28.2 ± 1.8	35.3 ± 1.4
	LFAL	17.4 ± 0.8	7.8 ± 0.8	29.2 ± 2.5	39.9 ± 0.8
	LFCR	15.9 ± 1.8	7.3 ± 0.4	29.7 ± 1.7	37.7 ± 0.9
Old	HFAL	16.9 ± 1.4	8.1 ± 0.1	33.3 ± 1.0	38.6 ± 2.7
	HFCR	18.1 ± 4.1	6.0 ± 1.2	31.7 ± 5.8	40.3 ± 0.9
	LFAL	15.8 ± 2.3	8.0 ± 1.1	27.1 ± 2.6	40.1 ± 1.8
	LFCR	19.9 ± 2.7	6.1 ± 1.3	30.2 ± 1.9	37.5 ± 1.3

* All values shown are the mean ± SEM;

* None of values within each column for individual phospholipids were found to ve significantly different;

* HFAL = High fat ad libitum
HFCR = Hifh fat caloric restriction
LFAL = Low fat ad libitum
LFCR = Low fat ad libitum.

Table 11. The main and interactive effects of fat, calorie and age on the 20:4 as the percentage to total fatty acid (%) in phospholipids classes: 2-way ANOVA

factor	PC	PS	PI	PE
Fat	0.8631	0.7055	0.5043	0.8203
Cal	0.7895	0.1860	0.8197	0.8912
Age	0.1977	0.9291	0.5745	0.0576
Fat*Cal	0.7033	0.8887	0.1913	0.7853
Fat*Age	0.3323	0.8980	0.3363	0.8256
Cal*Age	0.2897	0.6402	0.6536	0.9972
Fat*Cal*Age	0.6692	0.7644	0.2140	0.8024

* All values shown are the p values.

Table 12. The Levels of Arachidonic Acid in Different Phospholipids Classes

Group/Diet	PC	PS	PI	PE
Young				
HFAL	943.7 ± 25.9	69.1 ± 4.9	229.1 ± 26.9	1281.0 ± 187.7
HFCR	1060.5 ± 101.7	61.2 ± 3.6	206.4 ± 20.0	1042.4 ± 80.0
LFAL	1096.7 ± 104.6	80.4 ± 4.2	183.3 ± 27.5	934.8 ± 116.2
LFCR	800.8 ± 69.8	50.2 ± 6.1	173.7 ± 20.3	757.5 ± 54.4
Old				
HFAL	1271.6 ± 174.1	74.9 ± 6.3	226.9 ± 14.7	1020.8 ± 48.4
HFCR	1312.9 ± 96.7	71.9 ± 4.2	168.9 ± 32.5	1215.5 ± 79.7
LFAL	1228.5 ± 289.8	65.9 ± 4.0	158.2 ± 19.4	1123.4 ± 68.5
LFCR	1486.8 ± 68.9	50.1 ± 19.6	199.4 ± 34.3	1191.2 ± 51.5

* All values shown are the mean ± SEM; N moles/g of tissues

* None of values within each column for individual phospholipids were found to be significantly different;

* HFAL = High fat ad libitum
HFCR = High fat caloric restriction
LFAL = Low fat ad libitum
LFCR = Low fat caloric restriction.

Table 13. The main and interactive effects of fat, calorie and age on the amount of 20:4 in phospholipids classes: 2-way ANOVA

factor	PC	PS	PI	PE
Fat	0.9897	0.1690	0.1830	0.0364
Cal	0.8814	0.1149	0.5933	0.4200
Age	0.0060	0.4510	0.9540	0.0056
Fat*Cal	0.5819	0.3525	0.1785	0.4907
Fat*Age	0.6256	0.3333	0.9033	0.0064
Cal*Age	0.2895	0.8389	0.9370	0.1528
Fat*Cal*Age	0.1345	0.9987	0.1556	0.5936

* All values shown are the p values.

VII. General Discussions

A systematic approach was taken to investigate the effect of dietary fat and energy intake on prostate glands proliferative status, protein kinase C activity and lipid composition.

Several studies have provided evidence in support of the concept that cell proliferation and differentiation events are intimately involved with many of the protein kinases some of which serve as oncogenes and some as growth factor receptor kinases. Many of the protein kinases can phosphorylate each other and thus mitigate their biological responses. In the present research it was noted that prostatic tissue exhibiting increased PCNA labelling index also exhibited increased membrane associated PKC activity. Whether, a high fat energy rich diet enhances the proliferative status by altering the receptors of the cell membrane so that the cells are more responsive to exogenous growth regulators or whether the diet is exerting a direct effect on the expression of genes involved in growth regulation remains to be determined. Several studies have demonstrated that dietary lipids modify lipid composition of cell membranes and the function of specific receptors and associated enzymes.

In previous studies we have seen increased PKC activity in colonic tissue without marked increases in the proliferative status. The observation in prostate glands of increased PKC activity in the tissue with increased proliferative status suggest that these events may be related. More convincing evidence to support this conjecture must be explored in future studies. For instance, there are several known inhibitors of PKC activity. It would be important to demonstrate that in high fat fed animals, PKC inhibitors reduced PKC activity along with a reduction in the

proliferative state of the prostatic tissue. In several organ systems, the activation of PKC in normal appearing tissue has been used to assess the risk of cancer development. Chemicals known to activate PKC are also tumour promoters. In the majority of studies, PKC has been measured within minutes to hours after an acute exposure of cells or tissues to known tumour promoters. A limited number of studies have explored PKC activity as affected by chronic exposure to a known tumour promoting environment such as high fat diet.

Protein kinase C activity has been linked to phospholipids and their catalytic products. Present research has demonstrated that the analysis of prostatic tissue prospholipids and their fatty acid composition was not as informative and did not support the notion that marked changes in phospholipids composition is associated with altered PKC activity.

It is noteworthy that the differences among the dietary groups with respect to lipids composition of prostate glands were more evident in old than in young rats. For example, a consistent increase in the amount of PC fraction was noted in the old rats in the CR groups, compared to their AL counterparts. It is expected that prostatic tissue in young rats would be active and that lipids in the membrane would be rapidly turning over and therefore may not accumulate changes induced by dietary fat and energy restriction. Conversely, in old animals, prostatic tissue was more stable and therefore exhibited changes in the membrane lipid composition. The increased level of PC among the CR groups compared to their corresponding AL groups may suggest a slowing down of the turnover of PC resulting in an increase in its level and/or an increase in the conversion of PE to PC thus increasing the level of PC.

However, one important observation was that older animals had higher level of arachidonic

acid in their phospholipids than the younger animals. These findings suggest that altered metabolism of n-6 fatty acids may have occurred with ageing. The present research also addressed the issue that the age of the animals may be an important variable in assessing dietary influences on specific enzymatic and growth features of prostate glands. It was evident that young animals were more sensitive to dietary effects than older animals when proliferative and enzymatic status of prostate glands were considered. This brings to light the importance of early childhood nutrition in modulating an organ's susceptibility to chronic illnesses. A concept which seems to be gaining attention recently.

Findings of the present research have provided evidence in support of the notion that diet and age exerts marked influence on the growth and enzymatic status of developing prostate glands. The present research has provided fundamental information on the growth regulation of prostate glands as affected by age and dietary factors. Future studies should be directed to understand the role of nutrients and energy in modulating the normal growth and development of prostate glands and their potential role in affecting the preneoplastic and neoplastic prostatic tissue.

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Appendix 1. Modulation of Colonic Aberrant Crypt Foci and Proliferative Indices in Colon and in Prostate Glands of Rats by Vitamin E

Abstract

The effect of a high vitamin E diet on the early stages of colon carcinogenesis and on the proliferative indices in the colon and in the prostate glands was investigated in rats. F344 male rats were injected with azoxymethane (AOM), 15 mg/kg sc.. One week later, animals were randomly allocated into two dietary groups (n=8 rats/group), normal vitamin E (50 IU/kg diet) or high vitamin E diet (200 IU/kg diet). The basal diet was the AIN-76 diet, modified to contain high corn oil (23% w/w). After 8 weeks of feeding, concentrations of vitamin E in plasma, liver and prostate were analyzed. Enumeration of aberrant crypt foci (ACF) in colons and proliferative indices of colons and prostate glands were determined. The total number of ACF and the number of AC/focus were similar in both dietary groups. ACF were defined as as small (1-3 crypts/focus), medium (4-6 crypts/focus), or large (> 7 crypts/focus) ACF. Only the small ACF category showed a significant treatment effect with values being lower in the high vitamin E group than in the control group ($p < 0.05$). No significant difference was observed in colonic proliferative indices assessed by enumeration of metaphase cells, S-phase cells or cells exhibiting proliferative cell nuclear antigen (PCNA). The PCNA labelling index in the prostate glands and the activity of prostatic acid phosphatase in plasma were higher in high vitamin E fed rats ($p < 0.05$) than control animals. The present study demonstrate that additional vitamin E does not inhibit the induction and growth of ACF, but it enhances the proliferative status of the prostate glands.

Introduction

An abundance of epidemiologic evidence supports the concept that micronutrients may play important roles in human cancer prevention (1-5). Stahelin et al. (1) found that serum vitamin E level was lower in colon cancer patients than in healthy individuals and this has been confirmed by others (2,5).

The potential cancer preventive effect of vitamin E has been investigated in animals as well as in cancer cell in vitro (3.6.7.8.12.13.14). Vitamin E has been found to exert cancer proventive effect in CD-1 (ICD) BR mice with colitis. However, vitamin E deficiency did not accelerate the growth of colonic tumour when rats were fed a high polyunsaturated fat diet (11). It has been reported that vitamin E inhibited the growth of DU-15 human prostatic carcinoma cells in vitro (12,13,14). Interestingly, an opposite effect of nitamin E on prostate cancer development has been also reported. Vitamin E increased the level of testosterone which altered the proliferative status of the prostate and actually was used as a promoter in chemically induced model of prostatic cancer (15,16). Although the effect of vitamin E in combination with other vitamins and minerals on cancer prevention has been documented, the exact role vitamin E plays in colon cancer and its effect on prostate glands remains poorly understood and requires further investigations.

Aberrant crypt foci (ACF) have been identified in the colons of carcinogen-treated animals and are proposed to represent putative preneoplastic lesions (17,18). Various studies have supported the concept that ACF are preneoplastic lesions which can be used as biological end points in the study of modulation of colon carcinogenesis (19-21). The objective of the present investigation was to assess the effect of diets containing normal or a high level of vitamin E on the growth features of ACF. We also assessed the effect of these diets on proliferative indices in the colon and the prostate glands.

Materials and Methods

Animals

Sixteen male Sprague Dawley male F344 rats were obtained from Campus Breeding, Department of Animal Care, University of Manitoba, Canada. Animals were housed in wire cages, 3 rats/cage, with a 12-h light and 12-h dark cycle. Temperature and humidity were controlled at 22°C and 50%, respectively. Animal care was in accordance with the guidelines of the Canadian Council of Animal Care.

Carcinogen

Azoxymethane (Sigma Chemical Co., St. Louis, MO) in sterile saline was injected s.c. at a dose of 15 mg/kg body weight. Animals were given one dosage of the carcinogen.

Diets

The formulated diets were based on a modified AIN-76 diet (22,23). The high vitamin E diet had an additional 150 IU of vitamin E/kg diet, a level of vitamin E 4 times that in the control diet.

Study design

On the 8th day after injection, rats were randomly allocated into diet groups (n = 8) and were fed either the high vitamin E or control diet for 8 weeks. Initial and biweekly body weights were recorded. Two hours and one hour prior to termination by carbon dioxide asphyxiation, animals received an i.p. injection of colchicine (Sigma), 1 mg/kg body weight or 5'-bromo-2'-deoxyuridine (BudR) (Sigma), 30 mg/kg body weight. Blood, liver, colon and prostate glands were obtained immediately after termination.

Concentration of vitamin E in plasma

The procedure to analyze the vitamin E concentration in plasma was similar to that of

Driskell et al. (24), except that tocopherol acetate was used as the internal standard instead of retinol acetate and methanol was used instead of ethanol to dissolve samples prior to injection. In brief, a 100 ul of internal standard in ethanol was mixed with 100 ul of plasma on a vortex for 20 seconds, then 200 ul of hexane was added and the sample was mixed for 45 seconds. After centrifugation for 30 seconds at 12,000 RPM, 100 ul of the hexane layer was transferred to a 1.5 ml microcentrifuge tube and evaporated under nitrogen. Methanol, 200 ul, was used to wash down sides of tubes, tubes were capped and mixed on vortex for 30 seconds and placed in the refrigerator until HPLC analysis onto a Beckman System Gold HPLC (with a model 116 pump) was equipped with a 250 mm x 4.6 mm Beckman Ultrasphere ODS C-18 column, 5 um. Peaks were detected with a Beckman Model 166 UV detector at the wavelength of 280 + 0.01 nm.

Concentration of vitamin E in tissues

Tissues analysis was as described by Ingold et al. (25). Tocopherol acetate was used as an internal standard. In brief, liver and prostate samples were homogenized in water at a ratio of 1:6 (w/v). 2.0 ml of the homogenate was placed in a centrifuge tube. Internal standard (100 ul), 0.1 M sodium dodecyl sulfate (2.0 ml), ethanol (4.0 ml) and hexane (2.5 ml) were each added to the homogenate in succession and mixed with a vortex. After centrifugation, 200 ul of the hexane extract was transferred to a vial, evaporated under nitrogen and washed down with 200 ul of methanol. Vials were capped and mixed on a vortex and refrigerated till injected onto the HPLC.

Quantification of ACF

ACF were quantified following the protocol established by Bird (26). Fixed colons were stained in a 0.02% methylene blue (Sigma), and the number and growth of ACF were assessed under the light microscope. Criteria used to identify ACF included: (i) increased size, (ii) thicker epithelial cell lining and (iii) increased pericryptal zone relative to normal crypts (27).

Visualization and quantification of the number and crypt multiplicity of ACF in the entire colon were done as previously described (26,28). In order to determine crypt multiplicity, the number of crypts in each focus was recorded. Multiplicity was further analyzed by categorizing ACF in small (1-3 crypts/focus), medium (4-6 crypts/focus) and large (>7 crypts/focus). ACF size was determined using an ocular grid to measure the approximate area occupied by the ACF as viewed at 100 x magnification.

Measurement of mitotic and BudR indices in colon

Colons were removed immediately after termination, flushed with phosphate buffered saline (PBS), slit open from caecum to anus, and fixed in 70% ethanol. Histology and mitotic proliferation as assessed by scoring crypt sections for colchicine (Sigma) arrested mitotic figures. BudR immunohistochemistry was performed as described for proliferating cell nuclear antigen (PCNA) immunohistochemistry with the additional step of DNA digestion by 2N HCl treatment for 1 h following rehydration (29). The anti-BudR monoclonal antibody (Becton-Dickinson, San Jose, CA) 1:40 dilution in antibody diluting buffer was applied to tissues for 1 h. For determination of the BudR labelling index or mitotic index, ten well orientated crypts were evaluated in which the base, the lumen, and the top of crypts could be seen displaying a U-shaped configuration. The number and the position of the positively identified cells in each crypt column were recorded in terms of serial position counting upwards from position 1, at the base of the crypt up to the mouth of the crypt. The BudR labelling index and mitotic index were calculated as the number of cells per crypt multiplied by 100.

Measurement of PCNA indices in colons and prostate glands

Colonic and prostatic tissues were embedded in paraffin wax, and 5-um thick sections were processed for immunohistochemistry employing the unlabelled antibody bridge method and the

Universal peroxidase kit from Signet Laboratories (ID labs Inc., London, ON, Canada); the method is similar to that described by Richter et al. (29). Tissue sections were deparaffinized and then flooded with normal goat serum and incubated for 20 min to block nonspecific binding. The anti-PCNA monoclonal antibody (Dimension Laboratories, Inc., Mississauga, ON, Canada) diluted with antibody diluting buffer (1:40) was applied to tissue sections, and the slides were subsequently incubated for 1 h. Each tissue section was then sequentially incubated with anti-mouse IgG (antibody bridge) and mouse IgG peroxidase (labelling agent). The peroxidase reaction was initiated by immersing the slides in 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in PBS to which 0.03% H_2O_2 had been added immediately prior to use. Finally, the slides were lightly counterstained with haematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Ottawa, ON, Canada). All incubations were carried out in a humidified chamber at room temperature, and between incubations slides were extensively washed with PBS.

In colon, the PCNA labelling index was determined by identifying ten well-orientated crypts in which the base, the lumen and the top of crypts could be seen displaying a U-shaped configuration. The number and the position of labelled cells in each crypt column were recorded in terms of serial position counting upward from position 1, at the base of the crypt, up to the mouth of the crypt. The PCNA labelling index was calculated as the number of positive cells per crypt divided by the total number of cells per crypt multiplied by 100. In prostate, the PCNA labelling index was determined by identifying ten well-continued acini in which the wall of the acinus was not broken. The number of the labelled cells and the total cells in each acinus were recorded and the PCNA labelling index was calculated as the number of positive cells per acinus divided by the total number of cells per acinus multiplied by 100.

Plasma prostatic acid and alkaline phosphatases

The acid and alkaline phosphatases were determined by commercially available kits (Sigma Chemical Co. Ltd., St. Louis) (30,31). Enzyme activity was calculated based on the change in absorption per minute at 405 nm.

Statistical analysis

Statistical analysis of data was conducted by Student t-test to compare group means using the SAS statistical software for microcomputers (SAS Institute, Inc., Carry, NC). A p value of < 0.05 was considered significant.

Results

The body weights of animals fed the control diet and the high vitamin E diet were similar throughout the experiment (Figure 1).

The concentrations of vitamin E (Figure 2) in liver, plasma and prostate were markedly higher in the high vitamin E group than the control group (plasma: 1.28 + 0.07 IU/100ml vs. 0.80 + 0.08 IU/100ml; liver: 0.29 + 0.02 IU/g vs. 0.14 + 0.02 IU/g; prostate: 0.18 + 0.02 IU/g vs. 0.10 + 0.01 IU/g, respectively).

The total number of ACF per colon (No. of foci/colon) and the mean multiplicity (No. of AC/focus) did not significantly differ between dietary groups (Table 1). Neither did the size of foci in the two groups (Figure 3). Significant differences occured in ACF categorized as small (1-3 crypts/focus) with the high vitamin E group having a lower percentage of small ACF (72.74 + 2.36) than the control group (79.80 + 1.38) (Figure 4). In medium (4-6 crypts/focus) or large (>7 crypts/focus) categories, differences between the high vitamin E group (medium: 24.32 + 2.09, large: 3.01 + 0.57) and control group (medium: 18.40 + 2.53, large: 2.16 + 0.55) did not reach statistical significance. The mitotic index (MI), the S-phase labelling index and PCNA

labelling index were lower in the high vitamin E group than the control group, but the difference was not statistically significant (Figure 5).

In prostate glands, the proliferative features were investigated in the ventral and lateral lobes by quantifying the PCNA labelling index (Table 2). The number of labelled acini/100 acini and the number of multilayered acini/100 acini were compared in respective lobes between dietary groups. The high vitamin E group had a significantly higher PCNA labelling index in the ventral lobe ($p < 0.05$). The high vitamin E group also had a higher number of labelled acini and mutlilayered acini compared to the control group, although these differences did not reach statistical significance.

Values for prostatic acid and alkaline phosphatases in plasma were higher ($p < 0.05$) in the high vitamin E group (11.60 + 0.77 vs. 8.70 + 0.51) than those of the normal vitamin E group, respectively (6.50 + 0.34 vs. 5.60 + 0.30), (Figure 6).

Discussion

Many studies indicate that vitamin E, as an antioxidant, exerts an anticancer effect when it is combined with some other vitamins and minerals. However, the use of vitamin E as a single agent has produced contradictory results (32,33) and the effect of vitamin E on the process of carcinogenesis and proliferation is still unclear. In the present investigation, we explored potential biological effects of a high vitamin E diet on the number and growth of ACF, putative preneoplastic lesions, and the enzymatic and proliferative features of prostate glands. The results indicated that additional vitamin E in the diet did not exert an inhibitory effect on the development of ACF or the proliferative indices of the colonic epithelium measured by three different procedures. The high vitamin E diet exerted a more pronounced effect on the prostate glands.

Vitamin E supplementation increased the proliferative status in the ventral ($p < 0.05$) and lateral lobes, and also increased the levels of prostatic acid and alkaline phosphatases.

As expected, increasing the level of vitamin E in the diet increased tissue concentrations of vitamin E. The ability of a high vitamin E diet to moderately increase the number of ACF with high crypt multiplicity could be interpreted as the high vitamin E diet was favouring the growth and stability of ACF that are purposed preneoplastic lesions. Therefore, in a long term study, vitamin E may not inhibit tumour growth. This suggestion is based on the assumption that a high vitamin E diet does not affect the later stages of colon carcinogenesis (34). It is recognized that colon carcinogenesis is a multistep process which includes sequential selection and propagation of preneoplastic lesions. Aberrant crypt foci are present in carcinogen-treated rodent colons as well as in humans at high risk for colon cancer development and in patients with colon cancer (17,27,35-37). Several studies aimed at investigating the genotypic, morphologic and growth features of ACF have supported the contention that ACF are preneoplastic lesions (27,35,36,38). Moreover, a colon harbouring greater number of ACF with advanced growth features is at greater risk for developing the disease than one with fewer advanced ACF (19,39,40). The ACF-system is commonly used to identify and study the modulation of colon carcinogenesis (17,34,36). The ability of the high vitamin E diet to increase the number of ACF with higher crypt multiplicity corroborates work in which vitamin E deficiency did not increase tumour incidence (11) and a high vitamin E intake was unable to retard the development of tumours (41). It is possible that additional vitamin E is also providing a growth stability to preneoplastic lesions. Whether vitamin E affects later stages of colon carcinogenesis must be assessed in future studies.

The high vitamin E diet did not significantly alter the proliferative activity of the colonic epithelium as compared to the normal vitamin E diet. It is known that cell replication is a complex

multistep process that can be interrupted or stagnated at several stages by endogenous and exogenous factors (42,43) and that, depending on the method of assessment, different stages of the cell cycle are enumerated. We employed three different methods to evaluate proliferation of the colonic mucosa. The BudR labelling index assesses the percentage of cells engaged in DNA synthesis, whereas the metaphase arrest technique allows enumeration of cells actually undergoing metaphase (44,45). The assessment of cells exhibiting PCNA allows the identification of cycling cells. This method detects various naturally occurring cell cycle related proteins (44). As a result, PCNA labelling indices tend to be higher than the BudR labelling indices. It has been suggested that this may be due to the gradient in PCNA expression throughout the cycle which enlarges the window of reactivity (46). In our experiments all three parameters were slightly decreased in high vitamin E fed animals, however the difference did not reach statistical significance.

Vitamin E deficiency has been associated with reproductive failure in rats (47). The possibility that vitamin E may a exert growth modulating effect in rat prostate glands was also studied. In contrast to the effect of a high vitamin E diet on colonic epithelium, the epithelial cells of the prostate glands were profoundly affected by increased vitamin E intake. The ventral lobe of the gland, which is more sensitive to carcinogenesis and is functionally active, showed a significant response to the high vitamin E diet. More cells in this lobe were engaged in cell division than in the lateral lobe. It has been reported that the growth of the prostate glands is androgen-dependent and testosterone, produced in testes, has been used as promoter in prostate carcinogenesis studies (48). It may be hypothesized that the higher vitamin E increased the production of testosterone, which in turn stimulated prostate proliferation. In the present study, we found that cell proliferation measured by PCNA labelling index was increased in high vitamin E fed animals, measured by PCNA labelling index. The possibility that the increasing effect of

vitamin E on the prostate glands was mediated via testosterone is corroborated by data on prostatic acid phosphatase in plasma of rats. Prostatic acid phosphatase has been used as a biomarker in clinical diagnosis and chemotherapy (49,50). The increasing enzyme activity in plasma is associated with the active growth of glands (51). In our study, the activity of prostatic acid phosphatase in plasma increased in high vitamin E fed animals. How and if any of these changes relate to potentially altering the risk of prostate cancer development remains to be evaluated.

In conclusion we have demonstrated that, in a short term study employing ACF as a model system, a high vitamin E diet did not exert a cancer preventative effect in rat colon. Prostate glands responded differently to a high vitamin E diet; proliferative and enzymatic status of prostate glands were significantly affected. A systematic examination of the effect of vitamin E on the multisteps process of colon and prostate carcinogenesis is warranted.

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Table 1. Effect of Vitamin E on AOM-induced ACF in Rat Colons

	High Vit. E	Control
Incidence	8/8[1]	8/8
No. of foci/colon[2,3]	168.50 ± 13.62	188.25 ± 8.91
No. of AC/colon[2,3]	433.50 ± 40.17	491.38 ± 22.91
Mean no. of AC/foci[2]	2.67 ± 0.46	2.62 ± 0.21

1. No. of rats with ACF/total rats.
2. The total number of foci and AC in all regions examined.
3. Values are mean ± SE (n=8 rats/group).

Table 2. Proliferation Features of Prostate Lobes

	Ventral Lobe		Lateral Lobe	
	High VE	Control	High VE	Control
No. of labelled cells/100 cells[1]	5.39±0.80[a]	2.69±1.23	1.28±1.09[a]	0.87±0.39
No. of labelled acini/100 acini[1]	25.44±5.29[a]	16.37±6.85	12.84±3.59[a]	9.36±2.07
No. of multi-layered/100 acini[1]	37.84±6.95[a]	28.51±7.34	16.67±5.32	14.45±3.72

1. Values are mean ± SE (n=8 rats/group).

a. Significant difference between dietary groups ($p<0.05$).

Figure 1. Body weights of rats fed high vitamin E or control diets (n=8/diet) 8 weeks after azoxymethane injection. Values are the mean $\pm$ standard error (SE).

Figure 2. The concentration of vitamin E in plasma, liver and prostate glands of rats fed high vitamin E or control diets (n=8/diet) at week 8, after azoxymethane injection. Values are the mean $\pm$ SE, an asterisk indicates a significant difference ($p \leq 0.05$).

Figure 3. Average sizes (area in mm^2) of ACF in colons of rats fed high vitamin E or control diets (n=8/diet) at week 8 after azoxymethane injection. Values are the mean $\pm$ SE.

Figure 4. Percentages of total ACF categorized as small, medium or large at week 8 after azoxymethane injection in colons of rats fed high vitamin E or control diets (n=8/diet). Values are the mean $\pm$ SE, where an asterisk indicates a significant difference ($p \leq 0.05$).
Small = ACF with 1-3 crypts/focus;
Medium = ACF with 4-6 crypts/focus;
Large = ACF with $\geq$ 7 crypts/focus.

Figure 5. Proliferative indices of crypts in colons of azoxymethane-treated rats fed high vitamin E or control diets (n=8/diet) at week 8. Values are the mean $\pm$ SE.
MI = mitotic index;
S-LI = S-phase labelling index;
PCNA-LI = PCNA labelling index;
(See Materials and Methods for details).

Figure 6. The activity of prostatic acid and alkaline phosphatases in plasma of rats fed high vitamin E or control diets (n=8/diet). Values are the mean $\pm$ SE, an asterisk indicates a significant difference ($p \leq 0.05$).

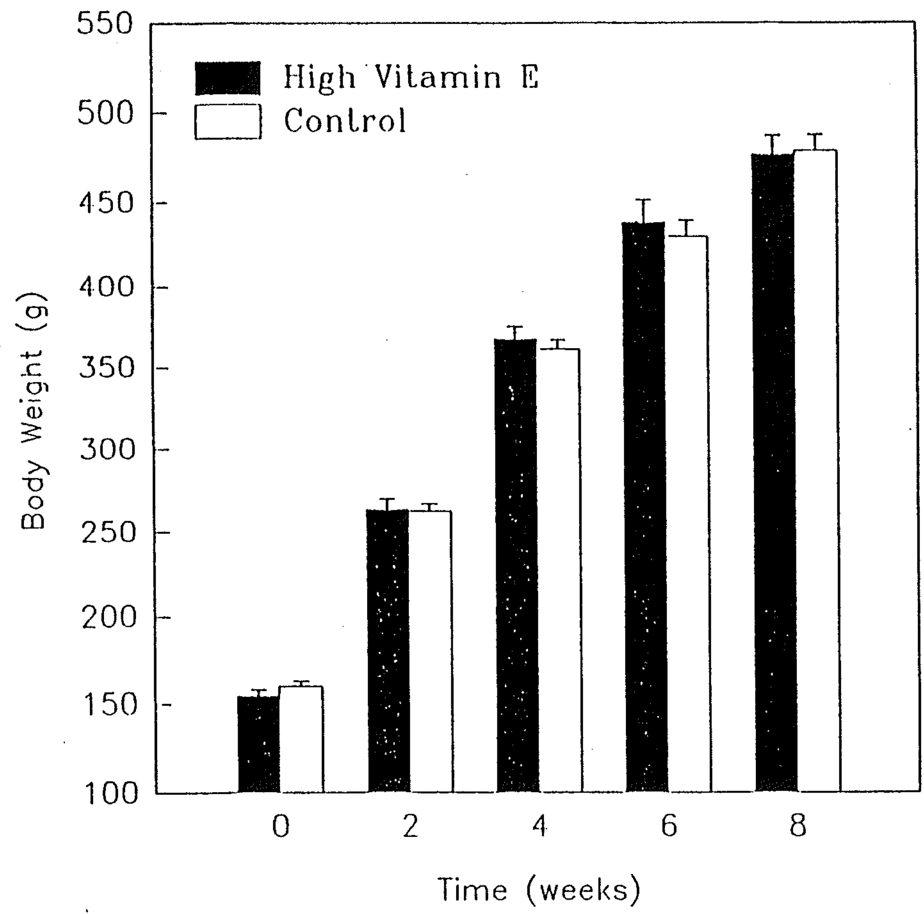

Figure 1

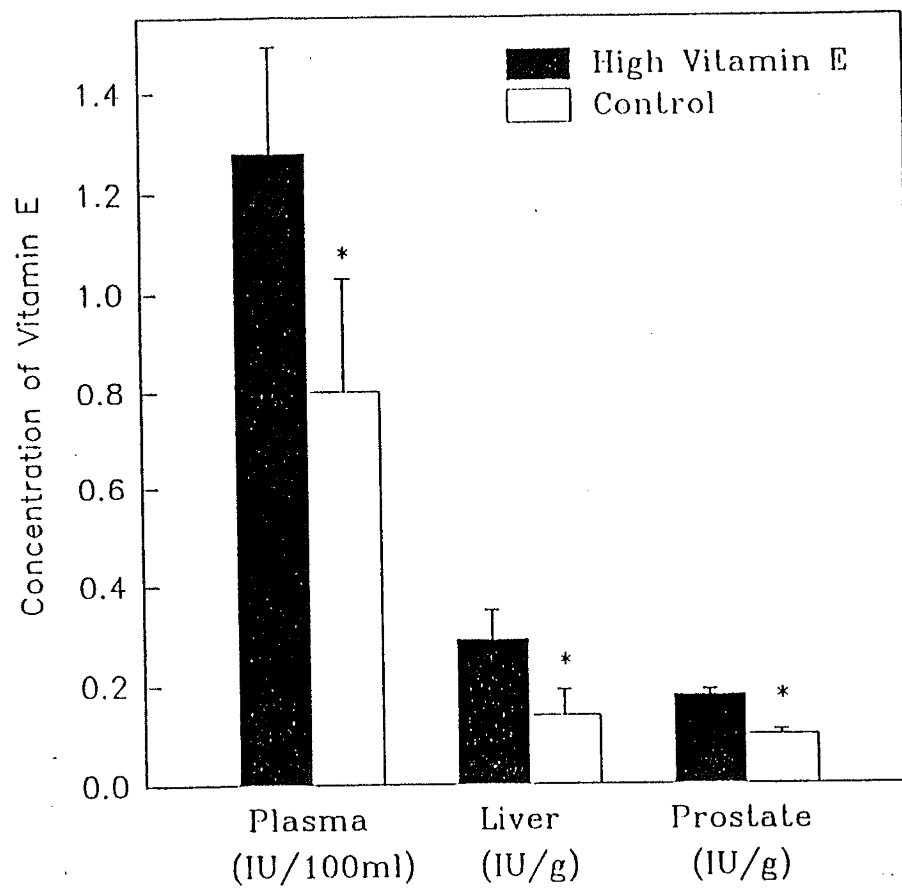

Figure 2

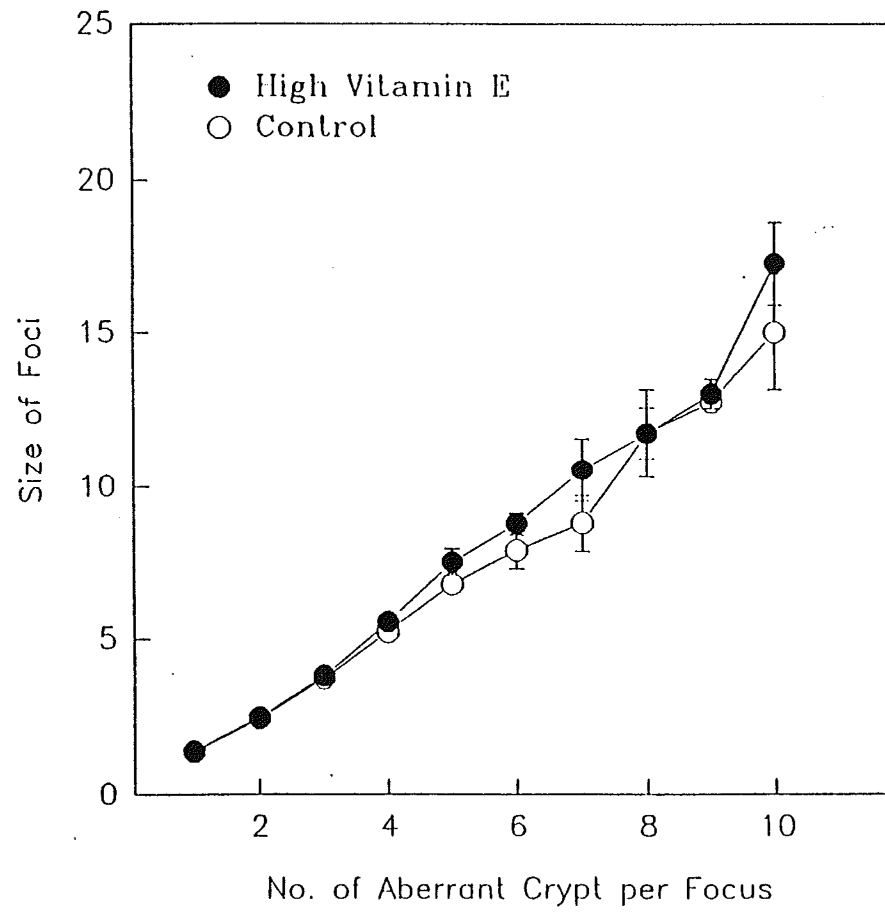

Figure 3

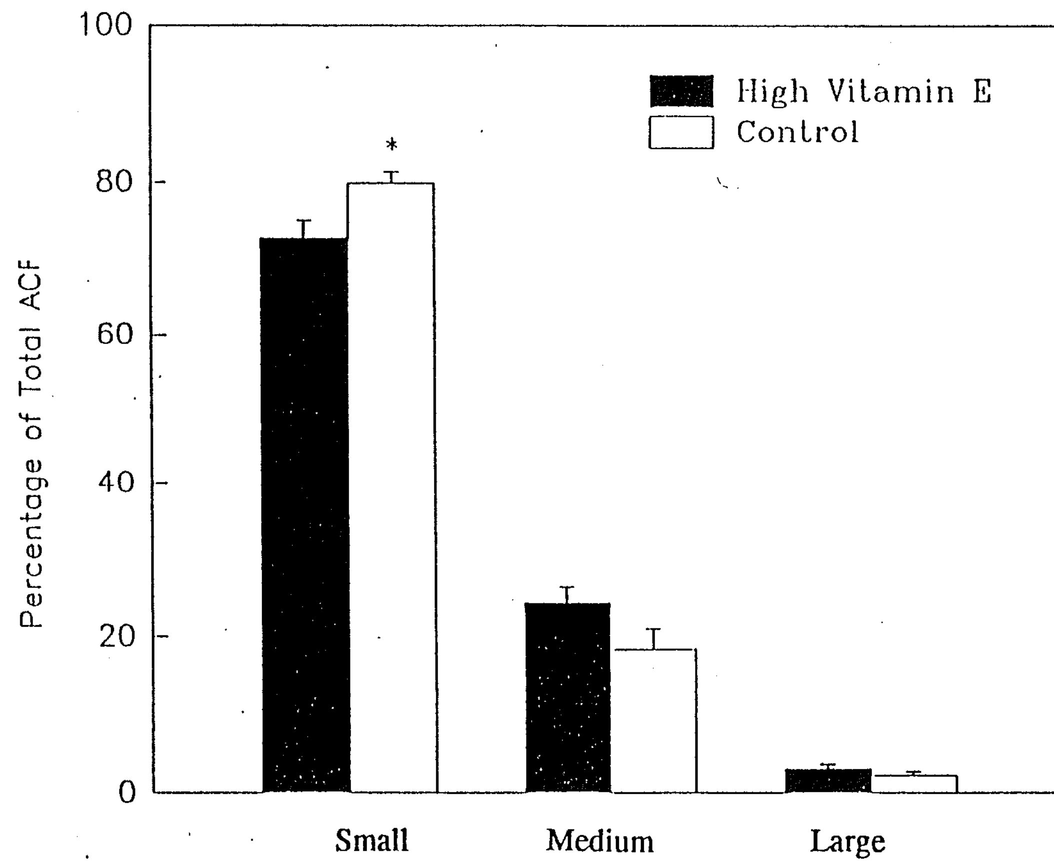

Figure 4

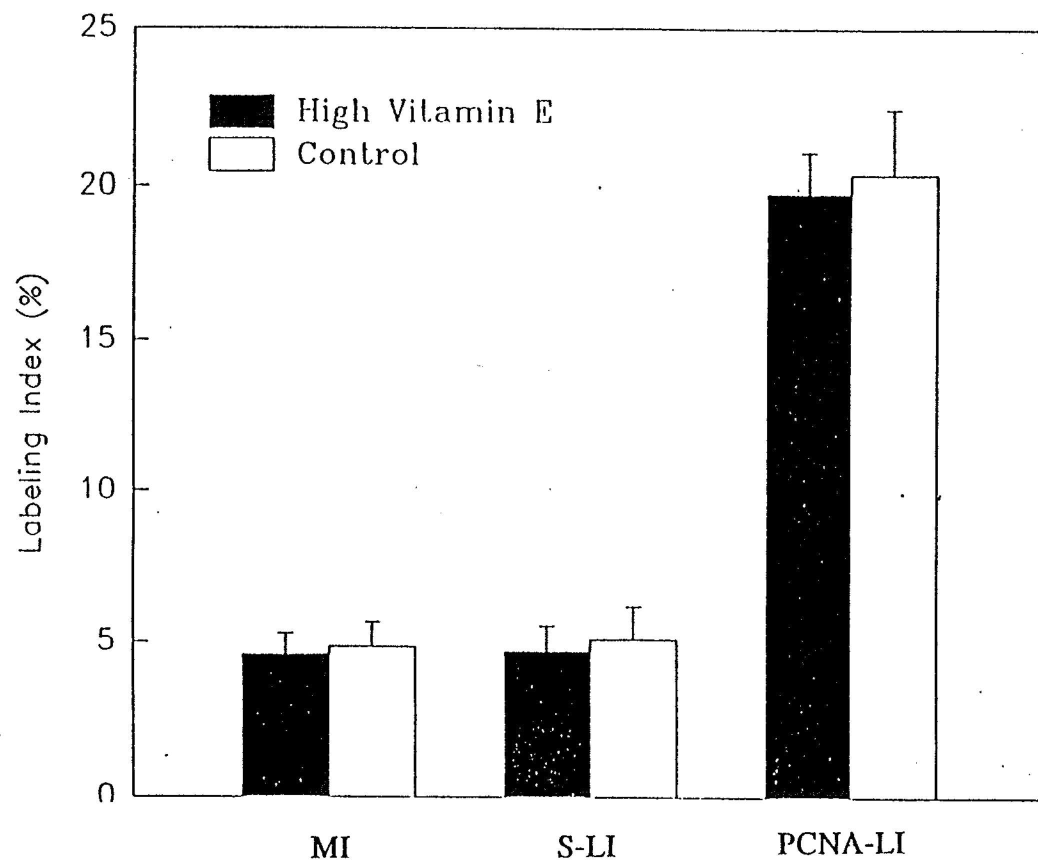

Figure 5

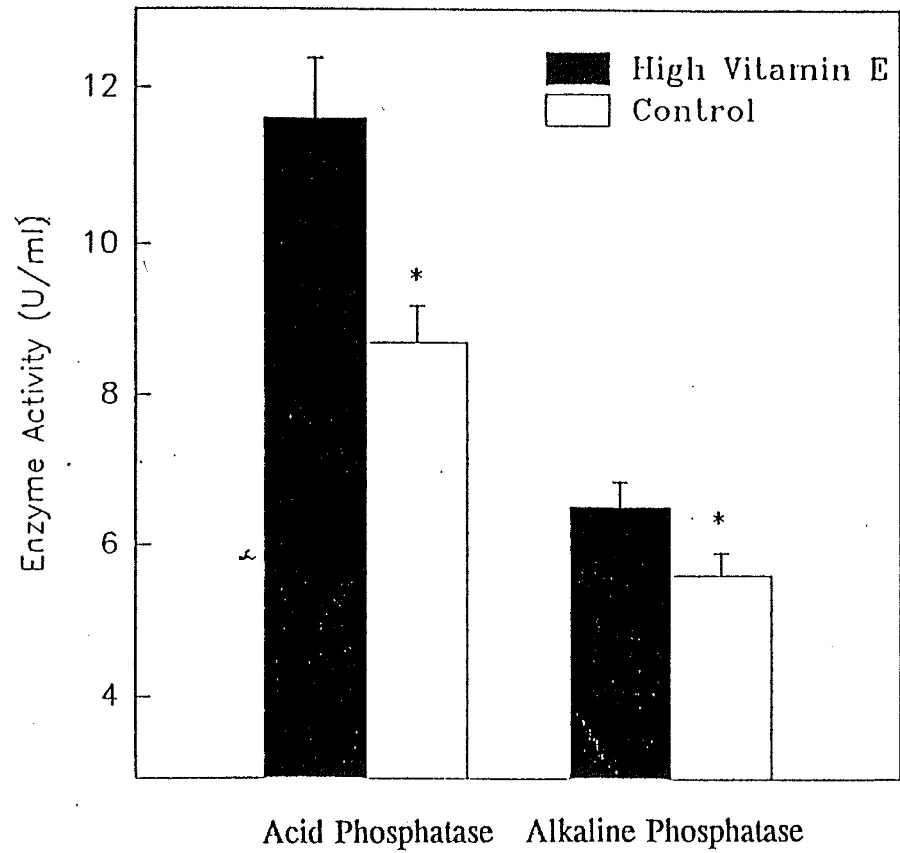

Figure 6