AN ABSTRACT OF THE THESIS OF

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David E. Williams

Peroxisome proliferators (PPs) are nongenotoxic carcinogens that produce a well-characterized response in rodents including hepatomegaly, proliferation of peroxisomes, dramatic induction of fatty acid metabolizing enzymes, and hepatocarcinogenesis. The response is species-specific, with rats and mice being sensitive to both peroxisome proliferation and carcinogenesis. Humans appear relatively insensitive to peroxisome proliferation, but their susceptibility to PP-induced carcinogenicity is unknown. An initial goal of this research was to determine if PPs are carcinogenic in rainbow trout, a species that, like humans, has a limited peroxisome proliferating response. Three PPs were tested for modulation of carcinogenesis. The adrenal steroid dehydroepiandrosterone (DHEA) was a complete carcinogen and both DHEA and perfluorooctanoic acid enhanced aflatoxin B1 (AFB_1)-initiated hepatocarcinogenesis. Peroxisomal enzymes were not induced indicating that carcinogenicity is independent of peroxisome proliferation. Additional studies focused on three alternate mechanisms of DHEA carcinogenesis: genotoxicity, intracrine effects, and enhancement of cell proliferation. Mutations of the Ki-ras gene are rare in spontaneous tumors in trout; however, thirty percent of tumors examined from DHEA-treated trout contained ras mutations. Some effects of DHEA may be mediated through conversion into other hormones. A fluorinated DHEA analog, lacking the steroid precursor properties of DHEA, was not carcinogenic and only slightly enhanced tumor

incidence in AFB_1-initiated trout. Finally, cell proliferation could be important in DHEA carcinogenesis. Treatment with DHEA causes dramatic increases in the percentage of body weight made up by liver. Histological examination indicates increases in liver somatic indices are due to both biliary cell proliferation and hepatocyte enlargement. Levels of proliferating cell nuclear antigen (PCNA), an index of DNA replication, were increased and levels of p53, a tumor suppresser protein involved in cell cycle regulation, were decreased. Tumor enhancement by DHEA was not limited to AFB_1-initiated hepatocarcinogenesis. DHEA treatment of N-methyl-N'-nitro-nitrosoguanidine-initiated trout enhanced renal and hepatocarcinogenesis, but inhibited stomach and swim-bladder carcinogenesis. This research demonstrates that the carcinogenicity and tumor enhancing effects of DHEA are not limited to rodents and may occur through mechanisms independent of peroxisome proliferation.

DEHYDROEPIANDROSTERONE CARCINOGENESIS AND TUMOR MODULATING EFFECTS IN TROUT

by

Gayle A. Orner

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Gayle A. Orner, Author

DEDICATION

This thesis is dedicated to the memory of my parents.

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CONTRIBUTION OF AUTHORS

Dan Arbogast coordinated personal and facilities, developed the computerized data acquisition systems, and conducted the gross examination of kidneys and swim bladders in chapter 6. The ras oncogene studies were conducted in the laboratory of Dr. George Bailey, director of the Marine Freshwater Biomedical Center who assisted in study design and interpretation of the results. Dr Hillary Carpenter assisted in the design of the peroxisome proliferator experiments. He conducted the peroxisomal ß-oxidation and catalase assays for the preliminary study described in chapter 1 and for the short-term study described in chapter 4, and instructed me in the methods for conducting these assays. The vitellogenin assays of chapters 4 and 5 were conducted in the laboratory of Dr. Lawrence Curtis by Regina Donohoe. Julie Duimstra and Dr. Olaf Hedstrom performed the electron microscopy described in chapter 4. Dr. Jerry Hendricks did the gross examinations of all livers, the histological classification of tumors described in chapters 2 and 3, and will be classifying tumors from the remainder of the studies. He also assisted in the design of each of the tumor experiments. Kate Mathews conducted the primer mismatch analysis for the initial DHEA experiment and taught me the PCR techniques used in chapter 3. Dr. David Williams was involved in the design and analysis of all experiments and in the preparation of manuscripts.

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Chapter 1

INTRODUCTION

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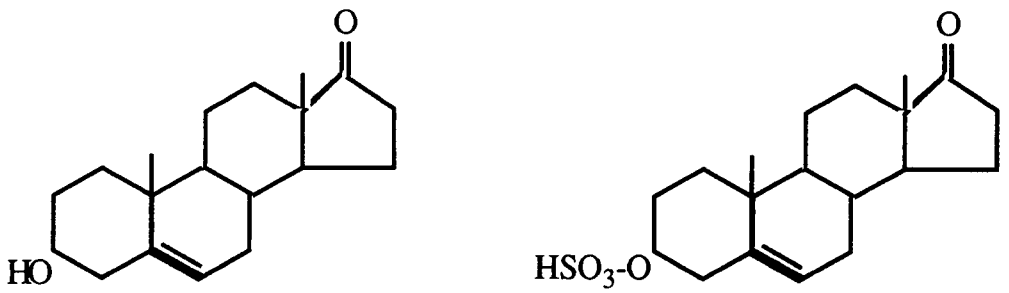
DEHYDROEPIANDROSTERONE SECRETION AND REGULATION

Dehydroepiandrosterone (DHEA, 5-androsten-3ß-ol-17-one) is an adrenal steroid which, along with its sulfated ester (DHEAS) occur in extremely high levels in human plasma (Figure 1.1) (1). The secretion of DHEA, like cortisol is regulated by adrenocorticotropic hormone (ACTH) (2), however under some circumstances there are distinct differences in cortisol and DHEA secretion.



DHEA DHEAS

Figure 1.1. Structures of DHEA and DHEAS.

The functional role of DHEA is still unclear except that it serves as a precursor of other steroids. Adrenalectomy does not result in any particular symptoms of DHEA deficiency (3). During pregnancy, DHEAS secretion by the fetal adrenal gland followed by conversion to estrogens in the placenta provides considerable estrogen production (4). DHEA also serves as a precursor for both estrogens and androgens throughout life; adrenal steroids are an important source of estrogens in most postmenopausal women (5).

DHEA and DHEAS are secreted in a pattern which varies according to age. Levels are low in children, but increase at puberty and peak during the second decade of life. The levels of DHEA and its sulfonated, glucuronidated, and fatty ester derivitives then decline with age (at a rate of about 6 mg DHEAS/dL/year). In both men and women, DHEAS levels of individuals in their 70's are about one-fifth those of people in their 20's and 30's

(1, 6, 7). In men, DHEAS continues to decline after the age of 85, however in elderly women there is no further decrease (6).

The reasons for the differences in DHEAS levels in populations of different ages is unknown. One possible explanation for lower DHEAS levels in older individuals is that DHEAS could be associated with higher mortality, leading to survival of individuals with low DHEAS levels. DHEAS has not been found to be associated with higher mortality, however (6). In addition, long term longitudinal studies in both men and women revealed decines in DHEAS with age in people studied for up to 13 years (8, 9). Therefore, the age-associated decline in DHEAS appears to represent changes in individuals over time rather than the selection of individuals with low DHEAS levels. Although DHEA and DHEAS decline with age, serum cortisol remains relatively unchanged. Upon stimulation with ACTH, the adrenals produce less DHEA and DHEAS in elderly than in younger subjects, but the cortisol and androstenedione responses are similar in both groups (10). Nestler *et al.* proposed that the decrease in DHEA/DHEAS may be the result of hyperinsulinemia which occurs in aging individuals due to increased insulin resistance and decreased insulin clearance (11). Insulin inhibits the activity of 17,20-lyase, the enzyme which converts 17α-hydroxypregnenolone into DHEA, therefore insulin could decrease the production of these steroids (12).

In addition to these age- and sex- differences, DHEA and DHEAS values are also highly variable within age groups (13). Several studies have been conducted to determine if lifestyles may affect these levels. Smokers were found to have higher levels of both DHEA (18% higher) and DHEAS (13% higher) than nonsmokers (14). Alcohol intake was also positively associated with serum DHEA levels in middle-aged men (14). In this study, dietary factors other than alcohol intake had little influence on the levels of serum hormones (14), however an association between vegetarian low fat diets and high DHEAS levels has been reported (15). Low levels of DHEAS have been found to correlate with obesity (16). Serum DHEAS levels were also reported to be higher in men over 45 and

women of all ages who are practitioners of trancendental meditation (17). Obese men and women on low calorie diets excreted lower amounts of DHEA (18).

Even after correcting for smoking and body mass index, DHEA was 58% and DHEAS was 45% lower in men over 64 years of age compared to men between 38 and 45 (14).

Most laboratory animals do not share such high levels of DHEA and DHEAS. It is only in humans and other primates that plasma levels of these adrenal steroids exceed those of all other hormones (19). Rabbits and dogs have an increase in DHEA and DHEAS upon reaching sexual maturity but the levels are still an order of magnitude lower than normal adult human values (19). Rat and mouse adrenals do not convert pregnenolone and progesterone into their 17-hydroxylated metabolites, leading van Weerden *et al.* (20) to conclude that rats and mice are not suitable models for research involving endogenous adrenal androgens.

Like rodents, trout do not have high circulating levels of DHEA. A trout 3ß-hydroxysteroid dehydrogenase Δ^{5-4}-isomerase (3ß-HSD) has recently been cloned. When expressed in a mammalian expression system, this enzyme has high activity for the conversion of DHEA to androstenedione. This is in contrast to the rodent and human 3ß-HSD which favor 17α-hydroxypregnenolone and pregnenolone as substrates. (21)

CHEMOPROTECTIVE PROPERTIES OF DHEA

Anticarcinogenicity

It is well documented that DHEA and DHEAS decrease markedly with age. Since cancer incidence rises during this same period, it is fascinating to speculate that the two events might be related. Considerable epidemiological evidence exists which suggests an association between low DHEA levels and certain cancers. Low levels of DHEA (and/or DHEAS) are associated with cancers of the stomach (22, 23), bladder (24), lung (25), and breast (26-29). Although most of these studies measured hormone levels after the clinical onset of cancer, there were several prospective studies as well. Prediagnostic levels of DHEA in serum of 13 individuals who subsequently developed gastric cancer (median time to diagnosis was 6.5 years) were 38% lower than in matched controls (22). Bulbrook *et al.* (26) measured urinary levels of adrenal androgen metabolites of 5000 healthy women from the island of Guernsey, Great Britain and then followed them clinically for up to nine years. In this study, the 27 patients who subsequently developed breast cancer were found to have had significantly lower prediagnosis adrenal androgen levels than matched controls. The association between low levels of DHEA and breast cancer may be limited to premenopausal breast cancer, however, as Zumoff *et al.* (29) showed that although premenopausal breast cancer patients had low plasma levels of DHEA and DHEAS, these steroids were higher than normal in postmenopausal breast cancer patients. These apparently contradictory findings may be explained by DHEA's dual role as a precursor of androgens or estrogens. In premenopausal women who normally have high estrogen and low androgen levels, DHEA may act to decrease the effects of estradiol by competing for binding for the estrogen receptor (directly or via estrogenic metabolites) (30). If DHEA levels are low, the risk of breast cancer in premenopausal women may be increased because the estradiol effect is unopposed. In postmenopausal women, however, the estrogenic

effects of DHEA and its metabolites could lead to increased risk of breast cancer in women with high levels of DHEA (30).

Breast cancer incidence varies considerably according to geographical location with a seven-fold higher incidence in certain Western countries compared to low incidence areas (31). Wang *et al* (32), examined plasma hormone levels in order to determine if the low incidence of breast cancer in Japanese women, compared to women in Western countries might be related to differences in endocrine function. In contrast to what would be expected if DHEA were having a protective effect, DHEAS levels were statistically higher in British than in Japanese women in all age groups examined (32).

In animal models, DHEA has been shown to be protective towards a wide variety of cancers (see Table 1.1) including spontaneous, chemically induced, and virally induced cancers.

Table 1.1. Prevention of carcinogenesis in animal models by DHEA[a]

Species Strain(s)	Carcinogen[b]	DHEA Treatment[c]	Target Organ(s)	Reference(s)
Mouse C3H-A/A C3H-A^{vy}/A (obese)	None	450 mg/kg p.o. 3x/week	Breast	(33, 34)
Mouse A/J	DMBA	0.6% dietary	Lung	(33, 35)
Mouse A/J	urethan	0.6% dietary	Lung	(35)
Mouse Balb/c (female)	DMH	0.6% dietary	Colon	(36, 37)
Mouse CD-1	DMBA	100, 400 mg, applied to skin 1 hr. prior to DMBA	Skin	(38)
Mouse Swiss Albino	MCA	0.1% dietary	Uterus	(39)
Mouse CD-1	DMBA + TPA	100, 400 mg, applied to skin 1 hr. prior to DMBA	Skin	(40)
Rat F344	DEN + partial hepatectomy	1% dietary	Liver (preneoplastic foci)	(41)
Mouse CD-1	DMBA	100, 400 mg, applied to skin 1 hr. prior to DMBA	Skin	(38)

Table 1.1. (continued)

Mouse Swiss Albino	MCA	0.1% dietary	Uterus	(39)
Mouse CD-1	DMBA + TPA	100, 400 mg, applied to skin 1 hr. prior to DMBA	Skin	(40)
Rat F344	DEN + partial hepatectomy	1% dietary	Liver (preneoplastic foci)	(41)
Rat F344	DHPN	0. 6% dietary	Liver (preneoplastic foci) Thyroid	(42)
Rat	DMBA	Silastic implants	Breast	(43)
Rat	DEN+ AAF partial hepatectomy	0.6% dietary	Liver (preneoplastic foci)	(44)
Rat Sprague-Dawley	MNU	120, 400 or 800 ppm dietary	Breast	(45, 46)
Rat Wistar	MNU+ testosterone	0.1% or 0.2% dietary	Prostate	(47)
Rat F344	DEN, MNU, BBN, DMH, DHPN	0.3% dietary	thyroid, bladder, seminal vesicles (enhanced liver)	(48)
Rat Sprague Dawley (male)	NNM	0.25% dietary (51 wks)	Adrenal (focal lesions)	(49)
Rat F344 (male)	DHPN	0.6% dietary (starting 8 wks after end of carcinogen)	Liver (GST-P positive foci)	(50)
Rat Sprague Dawley (male)	azoxymethane	0.5% dietary	Colon (aberrant crypts)	(51)

[a] Modified from (52)

[b] Abbreviations: 1,2-dimethylhydrazine (DMH), 7,12-dimethylbenz(a)anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), Diethylnitrosamine (DEN), dihydroxy-di-*n*-propyl-nitrosamine (DHPN), methylcholanthrene (MCA), N-acetylamino-fluorine (AAF), N-methyl-N-nitrosourea (MNU), N-nitrosomorpholine (NNM).

[c] DHEA was administered during and subsequent to carcinogen exposure unless otherwise specified.

DHEA has also been shown to be protective towards a number of *in vitro* cell transformation assays. DHEA blocks the conversion of 3T3 fibroblasts to adipocytes (53), and protects against aflatoxin B_1 (AFB_1) and DMBA-induced transformation of cultured embryonic hamster fibroblasts (54).

Mechanisms of DHEA protection towards carcinogenesis

DHEA is a powerful inhibitor of mammalian glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (G6PD) (52, 55, 56). It has been proposed that the cancer chemoprotective effects of DHEA result from G6PD inhibition and subsequent depletion of NADPH and ribose-5-phosphate (37, 57, 58). This could inhibit carcinogenesis in a number of ways. NADPH is required for the function of cytochrome P450s, microsomal enzymes which metabolically activate many carcinogens. Lower NADPH levels could inhibit P450s and result in decreased metabolic activation of carcinogens. Ribose-5-phosphate is necessary for synthesis of deoxyribonucleotides and cell replication. Decreased cell proliferation and anti-promotional activity could result from the lower availability of deoxyribonucleosides (37). Evidence that G6PD is involved in the DHEA chemoprevention is provided by studies in which chemoprotective effects are reversed by supplementation with deoxyribonucleotides. Growth inhibition by DHEA of cultured HeLa TCRC-2 cells was reversed by supplementing the medium with deoxyribonucleosides (59). Treatment of rats with deoxyribonucleosides prevented the inhibition of DNA synthesis in liver preneoplastic tissues of DHEA-treated rats (60). Administration of ribonucleosides and deoxyribonucleosides prevented the DHEA-inhibition of enzyme altered foci in livers of rats initiated with DEN, treated with 2-AAF, and given a partial hepatectomy (60).

Another potential chemoprotective effect of DHEA may also be dependent on G6PD inhibition. DHEA has been shown to inhibit the isoprenylation of *ras* p21 *in vitro* (61). Isoprenylation is one of a series of post-translational modifications necessary for membrane association and oncogenic activity of this protein. DHEA may act through depletion of intracellular pools of mevalonate, a precursor of both cholesterol and farnesyl-PPi and geranylgeranyl-PPi; which participate in isoprenylation reactions (61). The enzyme which catalyzes the formation of mevalonate, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase requires 2 mol of NADPH for each mole of product produced (61). Inhibition of G6PD could result in decreased HMG-CoA reductase activity, depletion of mevalonate,

and inhibition of isoprenylation (61). Exposure of HT-29 cells to DHEA resulted in growth inhibition and cell cycle arrest (at G_1), effects which could be partially prevented by the addition of mevalonate to the culture medium (62). A recent study however, indicates that DHEAS may act to inhibit isoprenylation at a point in the mevalonate pathway subsequent to HMG-CoA reductase, possibly at the level of protein farnesyltransferase (63).

Although G6PD inhibition by DHEA is readily demonstrated *in vitro*, several investigators have questioned its importance in the anticarcingenic process. G6PD activity was not inhibited in male Sprague-Dawley rats fed 0.6% DHEA for 16 days compared to pair-fed controls (64). Malic enzyme and isocitrate dehydrogenase activities were both increased by DHEA treatment, and cytoplasmic NADPH was not depleted (64). G6PD and ribulose-5-phosphate production were inhibited in female rats fed 0.6% DHEA for 15 days, however NADPH levels were not decreased due to increases in isocitrate dehydrogenase and malic enzyme activity (65). Increased activity of malic enzyme has been demonstrated by several investigators (66, 67) and appears to compensate for any decreases in NADPH produced by G6PD inhibition. Increased malic enzyme activity may prevent the reduction of NADPH but does not counteract the decrease in ribose-5-phosphate (65). In addition, certain cellular compartments may still experience a decrease in NADPH.

A series of studies by Prasanna *et al.* (68-75), demonstrates that, even though there is decreased DNA binding of dimethylnitrosamine (NDMA), aflatoxin B_1 (AFB_1), and 7,12-dimethylbenz(a)anthracene (DMBA) in livers of DHEA-fed animals, this decrease is not due to inhibition of metabolic activation. Instead both *in vivo* and *in vitro* studies suggest that DHEA enhances the metabolic activation of these compounds. Binding of metabolites to increased levels of hepatic protein may account for the decreased DNA binding in DHEA-fed animals.

Other possible mechanisms of DHEA cancer chemoprevention include induction of glutathione-S-transferase (GST), inhibition of sulfotransferases, alterations in cytochrome P450 profiles, and inhibition of nitric oxide generation. Dietary DHEA induces a mouse GST (subtype GT-8.7) (76). This is the GST isozyme induced in mouse liver by antioxidants (77). GST induction is a common mechanism of cancer chemoprevention in rodents, although its relevance in humans is not yet known. Sulfation can be an activation pathway for certain carcinogens such as 7,12-dihydroxymethylbenz[a]anthracene and 6-methylbenzo[a]pyrene which can form electrophilic sulfuric acid ester metabolites (78, 79). DHEA is a potent inhibitor of hepatic sulfotransferase activity and may be protective towards carcinogens which are activated in this manner. In addition to its possible effects on cytochrome P450s through NADPH depletion, DHEA may also modify the profile of P450s. DHEA is a peroxisome proliferator and induces the activity of cytochromes P4504A (80). DHEA may reduce the formation of reactive metabolites through alterations in cytochrome P450s (81). Recently it was reported that DHEA and its structural analog inhibit nitric oxide generation in response to lipopolysaccharide and interferon gamma in macrophages (82). There are also some additional properties of DHEA such as hormonal effects which may be important in certain cancers. DHEA's cancer chemoprotective properties may also be closely linked to its effects on other diseases.

Immunoprotection

In addition to DHEA's chemoprotective effects towards cancer, this compound has been shown to enhance the immune response in a variety of animal models. DHEAS treatment prevented spontaneous interleukin-6 (IL-6) production in aged mice (83). IL-6 is a cytokine involved in a wide variety of responses including the immune response, the acute phase response, osteoclastogenesis and hematopoiesis. Unregulated production of IL-6 may be involved in many chronic inflammatory conditions and infectious processes (3) and may be a consequence of aging as plasma levels are increased in the elderly and in

numerous diseases of old age (84). DHEA treatment of burned mice allowed them to maintain resistance to Listeria infection and prevented a prolonged IL-6 response (85). Some of DHEA's immunoprotective effects may be due to its antiglucocorticoid effects. DHEA stimulated T cell proliferation and IL-2 production in animal models and prevented dexamethasone-induced thymic involution in mice (86). DHEA provided protection towards lethal viral and bacterial infections in C57BL/6J mice challenged with herpes simplex type 2 virus (HSV2), human diabetogenic coxsackievirus B4 (CVB4), or *Streptococcus faecallis* (87). A single dose of DHEA (100 mg/kg) reduced mortality from 95% to 24% in CD-1 mice treated with a lethal dose of lipopolysaccharide (endotoxin) (88). The dramatic effects of DHEA on immune function in animals led to interest in the use of DHEA for disorders of the human immune system including lupus, multiple sclerosis, and HIV.

DHEA had mixed effects on cell-mediated immune function in healthy postmenopausal women treated for three weeks with 50 mg/kg DHEA. Subjects had enhanced natural killer cell activity and decreased IL-6 production, however $CD4^+$ (helper) T-cells were decreased (89).

An open-labeled noncontrolled study was conducted in which ten patients with systemic lupus erythematosus were treated with 200 mg DHEA/day. DHEA was well tolerated with the exception of some acneiform dermatitis and appeared to provide some benefits to the patients (90). The improvements may have been the result of increased androgens (testosterone levels were increased about 8-fold). Alternatively they could be due to alterations in cytokine secretion (91).

Multiple sclerosis (MS) is another disease for which DHEA treatment is being examined. The etiology of this neurological disorder is still unclear, however an altered immune response may be involved. Ten of 21 MS patients treated with 90 mg DHEA/day for 14 weeks reported benefits including increases in energy, sexual performance, and mental alertness although there were no significant improvements noted on the Kurtzke

numerical scale (92). In another uncontrolled study, 17 MS patients were given 40 mg/kg/day DHEA for up to three months. Hirsutism and voice changes occurred in some patients. No improvements in motor or sensory symptoms occurred, however relief from fatigue was experienced by 64% of the patients (93).

Plasma levels of DHEA are reduced in individuals infected with human immunodeficiency virus (HIV) (94, 95). The levels of DHEA can be used to predict progression from an asymptomatic stage to autoimmunodeficiency syndrome (AIDS) (96). In AIDS and other chronic infections, there are high circulating levels of cytokines including tumor necrosis factor-alpha (TNFα) and IFNα (97). Both TNFα and IFNα inhibit conversion of DHEAS to DHEA in mouse macrophages (therefore cellular activation results in down-regulation of macrophage metabolism of DHEA to DHEAS) (98). It has been suggested that the usefulness of thalidomide in the treatment of leprosy and HIV infections could be through inhibition of TNFα and subsequent restoration of DHEAS metabolism (98). DHEA itself, however, decreases macrophage production of TNFα (88), therefore the lower circulating levels of DHEA could either result from, or be the cause of the increases in TNFα.

In vitro, DHEA has been shown to inhibit both wild-type and 3' azido-3' deoxythymidine (AZT)-resistant HIV-1 (99). DHEA inhibits HIV-1 replication in human lymphocytes and macrophages (100). One mechanism through which DHEA is believed to act against HIV is similar to that proposed for cancer chemoprevention, that is through inhibition of G6PD and reduction of ribonucleosides necessary for virus replication (99). DHEA has also been shown to interfere with the action of NF-kB binding protein, a transcription factor which up-regulates HIV-1 gene expression (101) .

Clinical trials are in progress to test the effectiveness of DHEA in the treatment of AIDS. A phase 1 clinical trial has already been conducted to evaluate safety and pharmacokinetics. Thirty-one patients with mild symptomatic HIV were treated with up to 2,250 mg DHEA/day for 16 weeks. The subjects are reported to have tolerated these doses

well, however the limited immunological evidence collected in this study was mainly negative (102) .

Antiaging

Declining levels of DHEA and its sulfate ester are among the physiological changes which accompany aging in humans (1). There is considerable interest in the possibility that DHEA might reverse the aging process and allow people to age well and live longer.

Some evidence supports these claims. A large cross-sectional epidemiological study sponsored by the MacArthur Foundation Research Network on Successful Aging, compared a group of 1192 high-functioning[1] elderly men and women with medium and low functioning age- and gender-matched groups (103). DHEAS levels in the high-functioning group were 44% higher than in the low-functioning group ($p \leq 0.001$).

In contrast, however a large prospective study in which baseline DHEAS levels were measured 16 years prior to a series of cognitive tests, failed to find any association between DHEAS levels and cognitive function in old age (104). There were also no significant difference in DHEAS levels between 16 individuals suffering from Alzheimer's disease and 53 with intact cognative function in elderly subjects, aged 85 and over (6).

Several studies have administered DHEA to older adults. In one, DHEA was administered at 50 mg/day for six months to men and women ranging from 40 to 70 years of age. This treatment restored DHEA and DHEAS levels to the levels of young adults and increased serum androgens about 2-fold in women subjects. The most significant finding was that DHEA treatment resulted in increases in perceived physical and psychological well-being (105).

[1] Individuals were classified into functional groups based on both physical (gait, balance, and muscular strength) and cognitive (a battery of memory and language tasks) tests.

Cardiovascular

The reported effects of DHEA on cardiovascular function are mixed with distinct sex differences. A strong age-independent inverse correlation between DHEAS levels and mortality from cardiovascular and ischemic heart disease in men was reported by Barrett-Connor *et al.*, in 1986 (106). In this prospective study, an increase of 100 mg/dL in plasma DHEAS was associated with a 48% reduction in mortality from cardiovascular disease after adjusting for age, blood pressure, cholesterol, smoking status, etc. A followup study confirmed these results in men, but found markedly different effects in women in which those with the highest levels of DHEAS had the highest rates of mortality from cardiovascular disease (107, 108). The reason for the sex-related differences is unknown, but may (as appears to be the case with breast cancer) be dependent on the hormonal environment. In men, DHEA may be acting as an estrogen and protect against cardiovascuar disease, while in women, its conversion into androgens may increase the risk (30).

A slight inverse association between DHEAS and coronary artery disease (CAD) in men was also observed by Contoreggi *et al.* (109) ($p = 0.06$), however it was quite weak compared to the strong relationship between cholesterol or systolic blood pressure and CAD . The difference might be due to the fact that the Barrett-Connor studies were limited to cardiovascular disease resulting in mortality while this study looked at non-fatal cases as well. A third large scale longitudinal study found lower age-adjusted DHEAS levels in individuals who subsequently developed fatal coronary heart disease but no differences between cases of nonfatal myocardial infarction and controls (110).

In the Helsinki Heart Study, treatment of dyslipidemic middle-aged men with gemfibrozil produced favorable changes in plasma lipoproteins and reduced the incidence of coronary heart disease (111). A nonsmoking subset of this population was examined and found to have increased serum DHEA, DHEAS, androstenedione, androstanediol glucuronide, and cortisol compared to placebo-treated controls (112). It is possible that the

effects of fibrate drugs on lipid metabolism are mediated through pituitary-adrenocortical function and androgen metabolism (112).

Population studies, however, do not support the idea of an inverse association between DHEAS and coronary heart disease (110). Of three populations reported in the literature, DHEAS levels were highest in white Californian men (106), intermediate in Japanese men living in Honolulu (110), and lowest in Japanese men residing in Japan (113). Coronary disease rates follow the same pattern with Californian males having the highest levels of heart disease and Japanese males the lowest. This is the opposite of what would be expected if DHEAS were protective towards heart disease.

Animal studies provide some evidence that DHEA may protect against atherosclerosis. In New Zealand White rabbits which had atherosclerosis induced by aortic balloon catherization and a high cholesterol diet (2%) atherosclerotic plaque size was reduced by almost 50% by DHEA feeding (114). In another study, dietary DHEA inhibited fatty streak formation in cholesterol-fed rabbits by 30 to 40% independent of plasma cholesterol levels (115).

Antiobesity

DHEA's effects on energy metabolism have been extensively studied (reviewed in (116, 117)). Studies in mice (118), rats (119), and dogs (120) all demonstrated that DHEA reduced body fat without affecting caloric intake. Most of the weight difference in mice was from lower triacylglyerol content, possibly through decreased lipogenesis (118).

Weight loss also occurred in the obese Zucker rat, however in this model differences in food consumption were noted between lean and obese animals. In lean animals, caloric intake increased with DHEA treatment, but in obese animals, food intake was lower in DHEA-treated animals, indicating that DHEA's effects on obesity may be due in part to appetite suppression (121). At high (0.3-0.6%), but not at low doses, energy intake appears to be altered by taste aversion. Non-oral DHEA also altered energy intake

however, indicating that effects of DHEA on obesity are not simply due to taste aversion (122).

Several clinical trials have been conducted in humans. In normal men given high doses of DHEA (1600 mg/day) for 4 wks, a remarkable 31% decrease in body fat was reported (123). When administered to obese males for the same length of time, however there were no effects on total weight, body fat mass, fat distribution, or lipid status (124). Other researchers failed to find any differences in body weights, lean body mass, metabolic rate, leucine flux, or rate of incorporation of leucine into muscle protein in normal males given 1600 mg/day for 4 weeks (125). This study utilized a double blind crossover study in which subjects were all treated with the drug for four weeks, either in the first or second phase of the study as compared to the design in the Nestler, 1988 paper where subjects were assigned to either a DHEA or placebo group but no crossover was conducted (123).

Diabetes

Lower levels of DHEAS have been found in men who subsequently were treated for diabetes (110). Treatment of obese Zucker rats with 6000 ppm DHEA for 5 weeks decreased serum insulin levels without altering insulin resistance or changing pancreatic insulin levels (126). Dietary DHEA had marked therapeutic effects in mutant mice with diabetic-obesity syndromes,including reducing blood sugar levels to normal, preventing islet atrophy, and sustaining insulin levels (127). It has recently been shown, however, that mice with this mutation also express a unique sulfotransferase with considerably different kinetic properties than the enzyme in control mice (128). This model, therefore may not be appropriate for studies on DHEA and diabetes.

Adult human males (normal or obese) treated with 1600 mg DHEA/day did not have any changes in tissue sensitivity to insulin (123, 124).

ADVERSE EFFECTS OF DHEA

DHEA, however, has several properties that limit its use as a chemopreventive agent. It is a precursor for androgens and estrogens and in human clinical trials has been shown to increase androgen levels in female subjects (105, 129). In the rat, DHEA stimulates androgen dependent gene expression and increases ventral prostate weight probably through its conversion into dihydrotestosterone (DHT) (130). Growth of implanted prostate carcinomas in castrated rats was greatly enhanced by DHEAS treatment (131) (Table 1.2). This contrasts with a study listed in Table 1.1 in which DHEA protected towards prostate cancer in rats (47). In that study, however prostate cancer growth was promoted with testosterone. In the presence of high levels of androgens, DHEA probably would function as an estrogen and be protective. In the absence of testosterone, however, the androgenic effects of DHEA prevail and prostate cancer is enhanced. The contribution of adrenal androgens to prostate cancer growth has important clinical implications for human prostate cancer patients. Because 5-10% of circulating androgens are of adrenal origin, therapy which inhibits only androgens of testicular origin may not be effective (131).

In addition to being a steroid precursor, DHEA is also a peroxisome proliferator (80, 132, 133) and rodent carcinogen (134) (Table 1.2). Peroxisome proliferators (PPs) are a class of nongenotoxic carcinogens which produce a well characterized response in rodents including increases in size and numbers of the cellular organelles known as peroxisomes, dramatic induction of peroxisomal and microsomal enzymes involved in lipid metabolism, and liver enlargement as well as hepatocarcinogenicity (135).There is considerable human exposure to peroxisome proliferators as many common chemicals produce this response. Some of the agents which induce peroxisome proliferation include hypolipidemic drugs such as clofibrate (CLOF), phenoxyacetic acid herbicides, plasticizers such as diethylhexylphthalate (DEHP), perfluronated fatty acids, and nonsteroidal

Table 1.2. Enhancement or mixed effects towards carcinogenesis by DHEA

Species Strain(s)	Carcinogen	DHEA Treatment	Target Organ(s)	Reference(s)
Rat F344	BBN +BHA	0.6% dietary	Forestomach (Bladder cancer was inhibited)	(136)
Mouse SWXJ-9	None	0.4% dietary or silastic capsules of 10 mg	Ovaries	(137)
Rat Lewis	AZA	0.6% dietary (acetate)	Pancreas	(138, 139)
Rat Wistar	DMN	0.6% dietary	Kidneys	(140)
Rat Fisher/ Copenhagen	R3327 prostate carcinoma implant	22.5, 16.8, 11.2 mg/wk Sub. Q DHEAS	Prostate	(131)
Rat F344	None	0.5 % 1% dietary (78wks)	Liver	(134)
Rat F344	DHPN	0.6% (during initiation phase only)	Lung (Liver foci inhibited)	(141)
Hamster Syrian golden	DHPN + *Opisthorchis* infestation		Liver (GST-P foci)	(42)
Rainbow Trout Mt. Shasta	None	222, 444, 888 ppm dietary (7 months)	Liver	Chapt. 3,5,
Rainbow Trout Mt. Shasta	AFB1	55-888 ppm dietary	Liver	Chapt. 3,5,6
Rainbow Trout Mt. Shasta	MNNG	55-888 ppm dietary	Liver Kidney (Stom. and Swim Bladder inhibited)	Chapt. 7

Abbreviations: N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), butylated hydroxyanisole (BHA), azaserine (AZA), dimethylnitrosamine (DMN), aflatoxin B_1 (AFB1), N-methyl-N'-nitro-nitrosoguanidine (MNNG), dihydroxy-d-n-propylnitrosamine (DHPN)

antiinflammatory agents (142). Most of the effects of peroxisome proliferators are believed to be mediated through peroxisome proliferator activated receptors (PPARs). PPARs are nuclear hormone receptors belonging to the same class of receptors as the vitamin D, thyroid hormone, and retinoic acid receptors as well as several orphan receptors (143). Like other nuclear hormone receptors, PPARs are zinc finger proteins that bind to specific response elements on genes such as the acyl CoA oxidase gene. PPARs appear to bind as a heterodimer with the retinoic acid receptor (144). Three PPARs (α, β, and γ) have been

cloned from Xenopus, one from mouse, one from rat, and one from human (145), The protein sequences of these share a high degree of homology, particularly in the DNA-binding domain.

In addition to enzymes involved in lipid metabolism, malic enzyme has also been shown to be regulated by the PPAR, as a PPRE has recently been characterized for this enzyme (146). This is the first example of a gene regulated by PPAR which is not directly involved in fatty acid oxidation. Peroxisome proliferators may also have effects which are independent of the PPAR. Certain peroxisome proliferators (fibrates) down regulate transcription of apoA-1 in a manner which appears to be independent of the PPAR (147).

DHEA, like most other peroxisome proliferators is nongenotoxic in short-term mutagenicity assays. DHEA was negative in the Ames *Salmonella* mutagenicity (spot test) (148), sister chromatid exchange (149), and rat primary hepatocyte unscheduled DNA synthesis assays (150). In an *in vitro* DNA binding assay, however, DHEA was metabolically activated to a form which bound to DNA and protein (74).

Treatment of rats and mice with DHEA produced the characteristic response of peroxisome proliferators including increases in size and number of peroxisomes, levels and activity of P4504As, peroxisomal ß-oxidation activity, levels of the peroxisomal bifunctional enzyme, catalase activity, and liver weight (67, 80, 132, 133, 151-157).

Although DHEA produces the same spectrum of responses as other peroxisome proliferators, DHEA has some important differences. DHEA does not appear to be able to activate the PPAR (158, 159). In addition, DHEA lacks the carboxylic acid group common to most peroxisome proliferators (160). There is some evidence that the sulfate conjugate of DHEA is the active peroxisome proliferator (161), however this compound is also unable to activate the PPAR (158, 159). DHEA and DHEAS appear to bind specifically to certain cytosolic proteins (162, 163). It is possible that DHEA is acting through a separate signal transduction pathway than other PPs. The differences between DHEA and other

peroxisome proliferators may aid in the determination of the mechanism of peroxisome proliferator induced carcingenesis.

There are dramatic species difference in the response to peroxisome proliferation (164, 165). Rats and mice are highly responsive, whereas, primates are relatively resistant. The mechanism through which these nongenotoxic carcinogens produce liver tumors is unknown, but may involve the formation of a prooxidant state (166), stimulation of cell proliferation (167-169), or alterations in signal transduction (170, 171). If the carcinogenicity is directly related to peroxisome proliferation, then these compounds may pose little risk to humans because primates are fairly resistant to this response. The carcinogenicity may be due to mechanisms independent of peroxisome proliferation, however. Rainbow trout may prove to be appropriate models for the study of peroxisome proliferators. Like primates, trout appear to be weak responders to peroxisome proliferation (172-175).

Several different approaches have been used to minimize the adverse side effects of DHEA treatment. A DHEA formulation has been developed which places micronized DHEA within a lipophilic matrix (176). The authors suggest this delivery method should decrease conversion of DHEA to more potent steroids by minimizing the hepatic first-pass effects. Postmenopausal women administered a single 150 or 300 mg dose of oral micronized DHEA had 4- and 7-fold higher serum testosterone levels than placebo controls, however these values were still within the normal range of testosterone in premenopausal women. Serum DHEA was induced 7- and 12-fold by these same doses (176).

One of the eight individuals paticipating in this study had elevated liver enzymes at the conclusion of the study and developed clinical hepatitis 2 weeks later (176). The antinuclear antibody titers were present prior to the experiment, however this does not eliminate the possibility that DHEA could have enhanced the development of the condition . Considering what is known about DHEA effects on the livers of laboratory animals, this is

a rather disturbing side effect and should not be dismissed lightly, especially since DHEA is being considered for long-term clinical trials.

Another approach to decrease the toxic side effects of DHEA treatment has been the development of structural analogs lacking these adverse effects. 3ß-methylandrost-5-en-16-one (DE-7) is a synthetic steroid in which the 3ß-hydroxyl group is replaced by a methyl group, a modification which minimizes the conversion to androstenedione (37). DE-7 is not estrogenic (according to the rat uterotropic assay) and has enhanced chemoprotective properties towards skin cancer (40). DE-7 is also considerably more effective than DHEA as an antiobesity and antidiabetic agent in laboratory animals (38). 16α-fluoro-5-androsten-17-one (8354) and 16α-fluoro-5α-androstan-17-one (8356) are fluorinated DHEA analogs that are more potent than DHEA as inhibitors of G6PD but which lack the peroxisome proliferating, androgenic, and estrogenic effects of DHEA (177). Compound 8354 is considerably more effective than DHEA as an antiobesity agent in mice (177). DHEA analogs 8354 and 8356 are more effective than DHEA in blocking 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulation of prostaglandin E_2 in mouse epidermis (177). Analog 8354 is a stronger inhibitor of HIV-1 latency reactivation in ACH-2 cells stimulated by TPA than DHEA (101). These structural analogs show considerable promise as chemopreventive agents. Certain chemopreventive properties of DHEA, however may be due to its conversion into androgens and estrogens. For these conditions, the analogs will probably not be beneficial.

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Chapter 2

PEROXISOME PROLIFERATORS AS MODULATORS OF HEPATOCARCINOGENESIS IN TROUT

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ABSTRACT

Three structurally diverse peroxisome proliferators, clofibrate (CLOF), dehydroepiandrosterone (DHEA) and perfluorooctanoic acid (PFOA), were tested in the trout tumor model as complete carcinogens or enhancers of aflatoxin B_1 (AFB_1)-initiated hepatocarcinogenesis. Dietary exposure of trout for seven months demonstrated that only DHEA (0.18%) was a complete carcinogen. The effects of these compounds on AFB_1 carcinogenesis were mixed and dependent on dose. Dietary DHEA or PFOA at 1800 ppm significantly enhanced the incidence, multiplicity and size of liver tumors in AFB_1-initiated trout, the predominant type of tumor being either mixed carcinoma or hepatocellular carcinoma. Lower doses of PFOA (222-888 ppm), however, did not enhance carcinogenesis, instead they provided some protection. High doses of CLOF (1800-4400 ppm) inhibited AFB_1-initiated hepatocarcingenesis while lower doses (111-444 ppm) of CLOF had no effect on tumor incidence. The highest doses of CLOF produced a shift in tumor type to predominantly cholangiocellular carcinomas. The maximal induction of peroxisomal ß-oxidation was less than two-fold, confirming previous observations that trout are relatively insensitive to peroxisome proliferators. Therefore, the tumorigenic properties of DHEA and the tumor promoting activities of DHEA and high doses of PFOA in trout appear to be independent of peroxisome proliferation.

INTRODUCTION

Peroxisome proliferators comprise a set of structurally diverse chemicals which elicit a similar spectrum of biochemical and pathological alterations including hepatomegaly, increased numbers of peroxisomes, induction of enzymes involved in the ß- and ω-oxidation of fatty acids in peroxisomes and microsomes, respectively and hepatocarcinogenesis in rodents upon prolonged exposure (1-4). The mechanism of enzyme induction involves activation of a specific receptor, the peroxisome proliferator-activated receptor (PPAR), which binds to specific response elements in 5'-flanking regions of inducible genes (5).

A marked species difference in sensitivity to peroxisome proliferation has been documented (6, 7). Rats and mice are highly responsive, whereas, primates are relatively resistant. The mechanism through which these nongenotoxic carcinogens produce liver tumors is unknown, but may involve the formation of a prooxidant state (8), stimulation of cell proliferation (9, 10), or alterations in signal transduction (11, 12). If the carcinogenicity is directly related to peroxisome proliferation, then these compounds may pose little risk to humans because primates are fairly resistant to this response. The carcinogenicity may be due to mechanisms independent of peroxisome proliferation, however.

One way to determine if peroxisome proliferators pose a risk to human health is to test them for carcinogenicity in species that resemble humans in their response to these compounds. Rainbow trout may prove to be appropriate non-rodent models for the study of peroxisome proliferators. Ciprofibrate treatment of rainbow trout induced peroxisome proliferation, causing increases in peroxisomal volume, peroxisomal bifunctional enzyme and catalase levels, and acyl-CoA oxidase activity (13). The response was minimal, however, compared to rodents with doses that produce 10-fold increases in peroxisomal ß-oxidation in rats causing less than a 2-fold increase in trout (14). Trout appear to be similar to primates in their response to peroxisome proliferators (13).

We selected three representative peroxisome proliferators for study in the trout. Clofibrate (CLOF) is a hypolipidemic drug, which has been shown to be a complete hepatocarcinogen in rodents (15, 16). Perfluorooctanoic acid (PFOA) is one of a group of perfluorinated fatty acids widely used in hydraulic fluids, corrosion inhibitors, heat exchangers and water repellents (17). PFOA has been found to increase the incidence of malignant hepatocellular carcinomas in diethylnitrosamine-initiated rats (18). Dehydroepiandrosterone (DHEA) has been classified as a peroxisome proliferator but differs structurally and functionally and is the only peroxisome proliferator which does not appear to activate the PPAR (19-21). DHEA is a C_{19} steroid produced by the adrenal of humans in a pattern characterized by rising plasma levels (predominantly as the sulfate) early in life, peaking at 25 years of age with subsequent declines (22). Plasma levels of DHEA-sulfate in humans appear to be inversely correlated to the risk of developing some cancers (23). Animal studies have documented chemopreventive properties of DHEA toward atherosclerosis, diabetes, obesity and cancer (24-26). The anticarcinogenesis properties of DHEA are still being actively investigated, but may be due to inhibition of glucose-6-phosphate dehydrogenase and NADPH depletion (25). In contrast to its anticarcinogenic properties, however, prolonged administration of DHEA to rodents results in hepatocarcinogenesis (27). The phenotypic expression of various enzymatic markers in tumors of DHEA-treated rats differs from tumors initiated by genotoxic agents and resembles tumors produced by other peroxisome proliferators (28).

The studies reported here examine tumor modulation by these three peroxisome proliferators in the trout. Both PFOA and DHEA, when fed at 1800 ppm significantly enhanced the incidence and multiplicity of tumors in aflatoxin B_1 (AFB_1)-initiated trout. In addition, DHEA was a complete hepatocarcinogen in sham-initiated trout. At high doses, CLOF inhibited hepatocarcinogenesis in AFB_1-initiated trout and altered the predominant tumor type from mixed carcinoma to cholangiocellular carcinoma.

MATERIALS AND METHODS

Chemicals

DHEA, NAD^+, palmitoyl CoA, FAD, dithiotreitol, and coenzyme A were purchased from Sigma Chemical Co. (St. Louis, MO). CLOF and PFOA were from Fluka Chemika (Switzerland). Potassium cyanide was purchased from Mallinckrodt (Paris, Kentucky).

Animals and diets

Two separate tumor experiments are described in this chapter. In each, Mt. Shasta rainbow trout (*Oncorhynchus mykiss*) were spawned and raised in our laboratory, then were initiated as fry (10 weeks post-hatch) by immersion in 0.01 ppm AFB_1 for 30 min. Control trout were sham-exposed to vehicle (0.01% ethanol) alone. The trout were fed modified Oregon Test Diet (OTD) (29) containing 100 ppm vitamin E for 3 months, following which the experimental diets were fed for 30 weeks until the termination of the study. In the first experiment, five different experimental diets were fed to AFB_1-initiated or control trout. CLOF was fed at either 0.18 or 0.44% (wet weight). The high dose of dietary CLOF was initially 0.72% but had to be reduced after 2 weeks due to food refusal. PFOA was fed at either 0.02 or 0.18%, and DHEA was fed at a single dietary dose of 0.18%. In the second experiment, trout were fed 0, 111, 222, or 444 ppm CLOF; 222, 444, 888, or 1108 ppm PFOA; or 55, 111, 222, 444, or 888 ppm DHEA. The diets were made up in batches of two kg and stored at -20°C until used. Trout were killed after thirty weeks on experimental diets by an overdose of tricaine methanesulfonate anesthetic (MS222) and livers removed, weighed, examined grossly and stored in fixative (Bouin's solution) until tumors could be confirmed and classified histologically as described by Hendricks (30).

Enzyme Assays

Peroxisomal enzyme activities were measured after 29 (experiment 1) or 27 (experiment 2) weeks on experimental diets. Livers were removed, weighed, and placed into ice cold homogenization buffer (60 mM tris, 0.25 M sucrose buffer, pH 8.3). They were homogenized, centrifuged at 600 g for 20 min (4°C) to pellet cell debris and nuclei, and the supernatant centrifuged at 12,000 g (4°C) for 30 min to obtain the mitochondrial/peroxisomal fraction. This fraction was resuspended in homogenization buffer and protein concentration determined by the method of Lowry *et al.*, using bovine serum albumin as a standard (31). Peroxisomal ß-oxidation was assayed by spectrophotometrically monitoring the reduction of NAD^+ to NADPH in the presence of palmitoyl CoA and cyanide (32, 33). Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm (34).

Statistical analysis

The effect of dietary peroxisome proliferators on tumor incidence was analyzed by logistic regression analysis using SAS (Sas Institute, Cary NC). Tumor multiplicity data was analyzed by the Kruskal-Wallis test with p-values based on the exact permutation distribution (StatXact). The average log tumor size was compared using one-way analysis of variance (ANOVA) followed by Dunnett's test using both weighted and unweighted analyses (with identical conclusions). The effects of peroxisome proliferators on liver weight and ß-oxidation were analyzed with SAS using one-way ANOVA followed by the Scheffe test for establishing significant differences between control and experimental treatments.

RESULTS

Experiment 1

In the preliminary study, diets containing 0.18% DHEA or PFOA significantly ($p < 0.0001$) enhanced the incidence of liver tumors in AFB_1-initiated trout (Fig. 1). The enhanced tumor yield was especially striking in the DHEA-fed trout, as 100% of these animals had tumors. In addition, the multiplicity increased significantly ($p < 0.0001$) in both DHEA- and PFOA-fed trout (Fig. 1). The majority of DHEA-treated trout had six or more tumors per liver compared to one or two tumors per liver in tumor-bearing control animals. Tumors in the DHEA-fed group were also larger ($p < 0.0001$) than in the control AFB_1-initiated group (data not shown).

The results of post-initiation feeding with CLOF were markedly different than with DHEA or PFOA, i.e., a reduction in tumor incidence ($p< 0.08$) and multiplicity ($p< 0.004$) was observed (Fig. 2.1). These results might be attributed to depressed growth as the clofibrate-fed trout were much smaller than controls (Table 2.1). However, DHEA also markedly inhibited growth, whereas, PFOA had little effect and both of these compounds yielded higher tumor incidences. Therefore, no consistent relationship between growth and sensitivity to tumor enhancement is obvious in this study.

The ability of these peroxisome proliferators to act as complete carcinogens was also examined by feeding the same diets to sham-initiated trout. Consumption of dietary DHEA at 0.18% for 30 weeks resulted in 20% of the trout developing liver tumors (Table 2.1). No tumors were seen in non-initiated trout fed the control, CLOF or PFOA diets. Historically the spontaneous liver tumor incidence in trout of this age is about 0.1%.

Histological examination of AFB_1-initiated trout confirmed previous findings from our laboratory (35) that mixed carcinomas are the predominant tumor type in AFB_1-initiated trout. Neither PFOA or DHEA appeared to alter the tumor types which occurred in AFB_1-initiated trout, however post-initiation feeding of CLOF produced a unique shift in tumor

type to solely cholangiocellular carcinomas and cholangiomas (Table 2.2). The alterations in tumor type by CLOF, however, may be the result of the toxicity and high mortality which occured in these treatment groups.

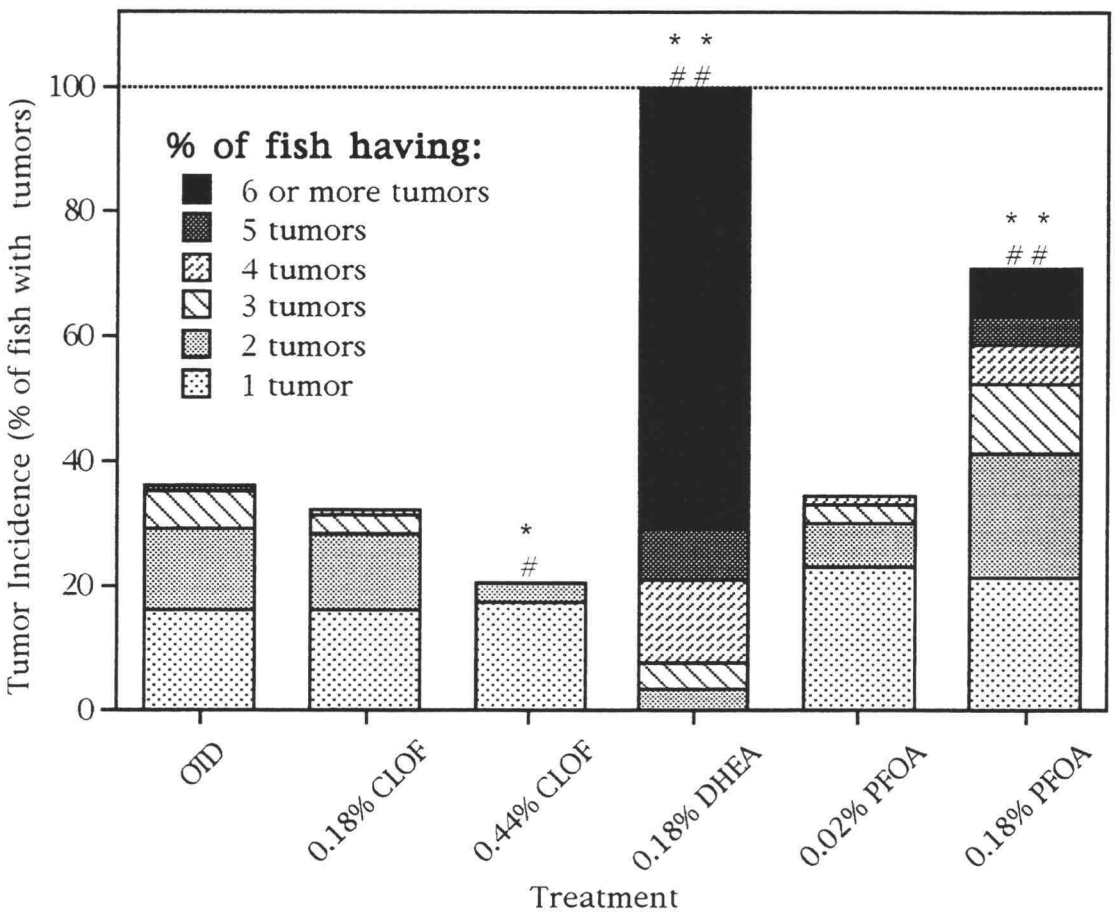

Figure 2.1. Tumor incidence and multiplicity in trout initiated with AFB_1 followed by 30 weeks dietary exposure to peroxisome proliferators (Experiment 1). The values represent one tank/treatment with each tank containing between 68-100 individuals. The trout were initiated as fry by immersion in a solution of 0.01 ppm for 30 min. All groups were fed control diet for the next 3 months, followed by experimental diets for 30 weeks prior to study termination. *Indicates a significant difference in tumor incidence compared to controls at $p<0.08$. **Indicates a significant difference in tumor incidence compared to controls at $p<0.0001$. #Indicates a significant difference in tumor multiplicity at $p<0.004$ compared to controls. ##Indicates a significant difference in tumor multiplicity at $p<0.0001$.

Table 2.1. The effect of dietary peroxisome proliferators on body weight, mortality, and tumor incidence (Experiment 1).

Treatment Initiation/Promotion	Body Wt.[a] (g)	Mortality[b] (%)	Tumor Incidence (%)
None/OTD	107 ± 29	15	0/85 (0)
None/0.18% CLOF	44* ± 18	40	0/60 (0)
None/0.44% CLOF	23* ± 8	29	0/71 (0)
None/0.18% DHEA	28* ± 10	5	19/95 (20)
None/0.02% PFOA	75 ± 18	0	0/100 (0)
None/0.18% PFOA	78 ± 28	25	0/75 (0)
AFB_1/OTD	92 ± 25	0	36/100 (36)
AFB_1/0.18% CLOF	33* ± 12	0	32/100 (32)
AFB_1/0.44% CLOF	23* ± 8	32	14/68 (21)
AFB_1/0.18% DHEA	31* ± 9	10	90/90 (100)
AFB_1/0.02% PFOA	76 ± 17	0	34/100 (34)
AFB_1/0.18% PFOA	75 ± 22	10	64/90 (71)

[a] Values are average body weights ± S.D. at the conclusion of the tumor study.
[b] These values are based on the number of animals remaining at the conclusion of the tumor study.
* indicates significant difference in body weight compared to control ($p < 0.05$).

Table 2.2. The effect of dietary peroxisome proliferators on tumor types in AFB_1-initiated trout (Experiment 1).

Treatment	Classification of Tumors (%)[a]						
Initiation/Promotion	MC	HCC	CCC	MA	HCA	Ch	BF
None/None	0	0	0	0	0	0	0
None/0.18% CLOF	0	0	0	0	0	0	0
None/0.44% CLOF	0	0	0	0	0	0	0
None/0.18% DHEA	67	25	0	4	4	0	0
None/0.02% PFOA	0	0	0	0	0	0	0
None/0.18% PFOA	0	0	0	0	0	0	0
AFB_1/None	54	10	10	5	3	5	12
AFB_1/0.18% CLOF	15	0	74	0	2	7	2
AFB_1/0.44% CLOF	0	0	81	0	0	19	0
AFB_1/0.18% DHEA	72	25	<1	0	1	1	<1
AFB_1/0.02% PFOA	50	11	16	5	0	14	5
AFB_1/0.18% PFOA	37	46	1	2	8	1	5

[a]Abbreviations: MC = mixed carcinoma, HCC = hepatocellular carcinoma, CCC = cholangiocellular carcinoma, MA = mixed adenoma, HCA = hepatocellular adenoma, Ch = cholangioma, BF = basophilic foci

The analysis of liver palmitoyl CoA oxidation in trout fed peroxisome proliferators for 27 weeks, shows no induction of peroxisomal enzyme activities by CLOF, DHEA or PFOA (Fig. 2.2). In fact, peroxisomal ß-oxidation activity was significantly reduced by the highest dose level of each compound. These results indicate that trout are relatively insensitive to peroxisome proliferation even at doses which cause considerable toxicity.

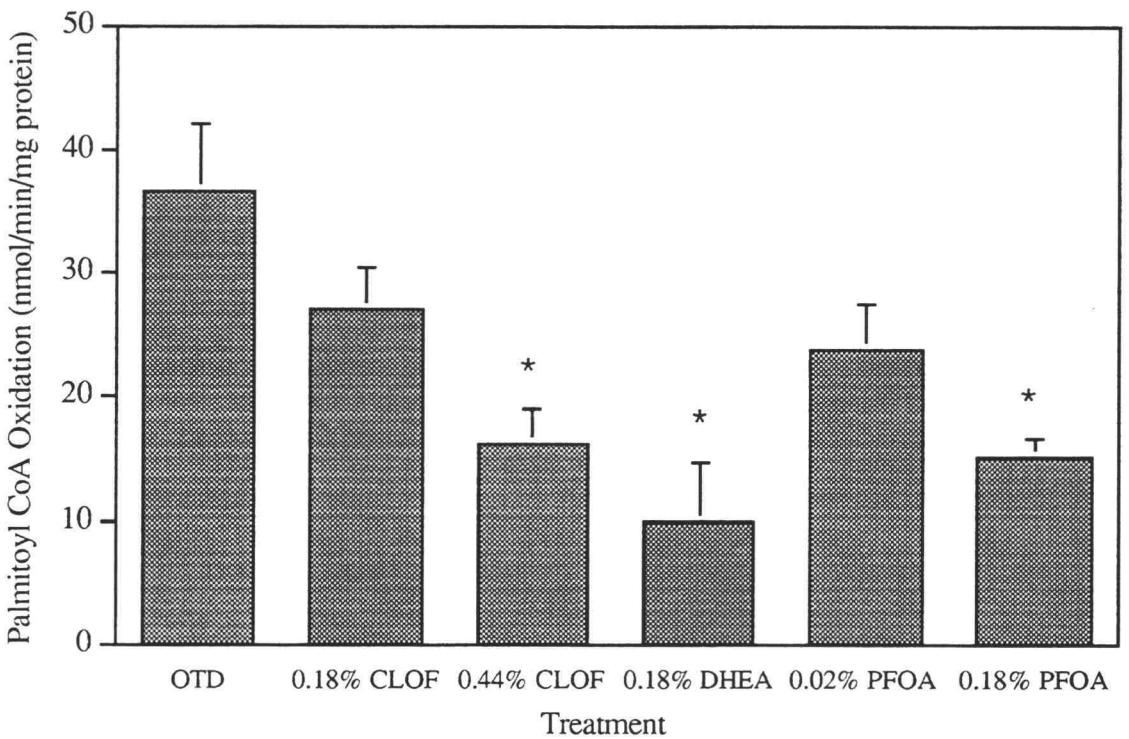

Figure 2.2. Peroxisomal ß-oxidation activity after 27 weeks of dietary peroxisome proliferators (Experiment 1). Six trout were sampled from the noninitiated treatment groups, livers removed and assayed for palmitoyl CoA oxidation activity as described in Materials and Methods. Values represent means of three measurements per treatment plus SEM. A * indicates significantly different from controls ($p < 0.05$).

Experiment 2

The dose levels used in the first experiment clearly exceeded the maximum tolerated doses. Therefore, a followup tumor study was conducted to examine a lower range of doses of CLOF, PFOA, and DHEA. The DHEA portion of this study is presented

separately in chapter 3. The CLOF and PFOA doses used in the second experiment were well tolerated as there was little mortality, and body weights were not significantly different from controls except in the AFB_1-initiated, high PFOA treatment group (Table 2.3.).

Table 2.3. The effect of dietary peroxisome proliferators on body weight, mortality, and liver somatic index (Experiment 2).

Treatment Initiation/Promotion	Body Wt.[a] (g)	Mortality[b] (%)	Liver Somatic Index[c] (%)
None/OTD	81 ± 29	1	0.68 ± 0.09
None/111 ppm CLOF	73 ± 35	0	0.68 ± 0.11
None/222 ppm CLOF	73 ± 36	0	0.71 ± 0.12
None/444 ppm CLOF	73 ± 32	0	0.71 ± 0.09
None/222 ppm PFOA	76 ± 31	0	0.67 ± 0.10
None/444 ppm PFOA	84 ± 30	0	0.64 ± 0.08
None/888 ppm PFOA	75 ± 33	0	0.88 ± 0.66
AFB_1/OTD	85 ± 36	0	0.68 ± 0.09
AFB_1/111 ppm CLOF	88 ± 36	0	0.70 ± 0.12
AFB_1/222 ppm CLOF	74 ± 30	0	0.69 ± 0.09
AFB_1/444 ppm CLOF	77 ± 34	0	0.70 ± 0.15
AFB_1/222 ppm PFOA	89 ± 32	0	0.61* ± 0.06
AFB_1/444 ppm PFOA	77 ± 30	0	0.67 ± 0.09
AFB_1/888 ppm PFOA	76 ± 32	1	0.76* ± 0.12
AFB_1/1108 ppm PFOA	71* ± 30	0	0.85* ± 0.14

[a]Values are average body weights ± S.D. at the conclusion of the tumor study.
[b]These values are based on the number of animals remaining at the conclusion of the tumor study.
[c]Values are average liver somatic indexes (% of body weight made up by liver) ± S.D. at the conclusion of the tumor study.
* indicates significantly different from the appropriate controls ($p < 0.05$).

CLOF treatment increased peroxisomal palmitoyl CoA oxidation in a dose-dependent manner with the top two treatment groups having significantly higher activity than in controls (fig. 2.3). This enzyme activity was 1.8-fold higher in trout fed 444 ppm CLOF for 29 weeks than in animals fed control diet. There were no significant alterations in peroxisomal ß-oxidation in PFOA-treated trout. Catalase activity was not increased by either CLOF or PFOA (fig. 2.4).

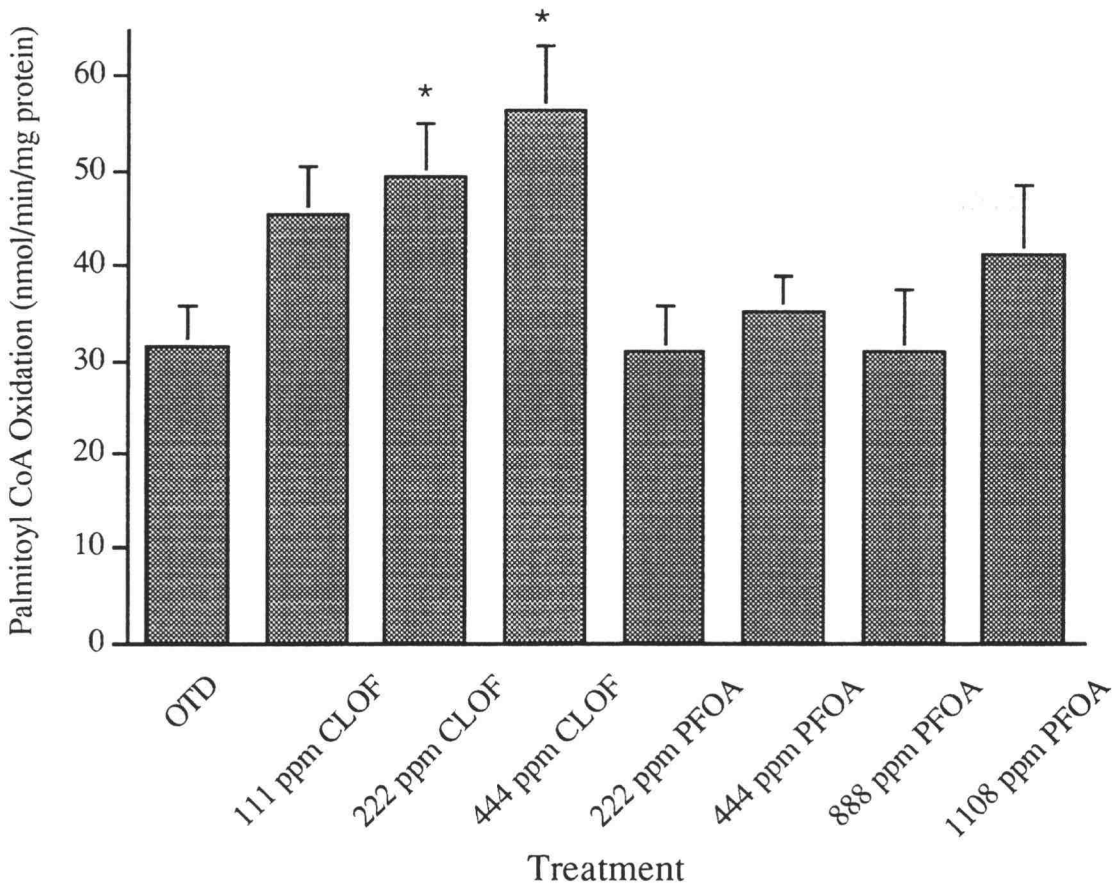

Figure 2.3. Peroxisomal ß-oxidation activity after 29 weeks of dietary peroxisome proliferators (Experiment 2). Twelve trout were sampled from the noninitiated experimental diet groups, livers removed and assayed for palmitoyl CoA oxidation activity as described in Materials and Methods. Values represent means of 12 measurements per treatment plus SEM. A * indicates significantly different from controls ($p < 0.05$).

As observed in the previous study, neither CLOF or PFOA produced tumors in sham-initiated trout (Table 2.4). The results in the AFB_1-initiated trout were considerably different than the preliminary study, however. Treatment with 111-444 ppm CLOF did not significantly alter tumor incidence. No protection towards AFB_1-initiated hepatocarcinogenesis was observed at these doses of CLOF. The tumor types observed in CLOF-treated trout were predominantly mixed carcinomas rather than the cholangiocellular tumors observed in experiment 1. In contrast to the tumor enhancement which occurred when initiated trout were fed 1800 ppm PFOA in the preliminary experiment, doses of 444-

888 ppm PFOA provided significant protection towards AFB_1 carcinogenesis ($p < 0.05$). At a dose of 1108 ppm, however, tumor incidence was slightly increased compared to initiated controls (Table 2.4).

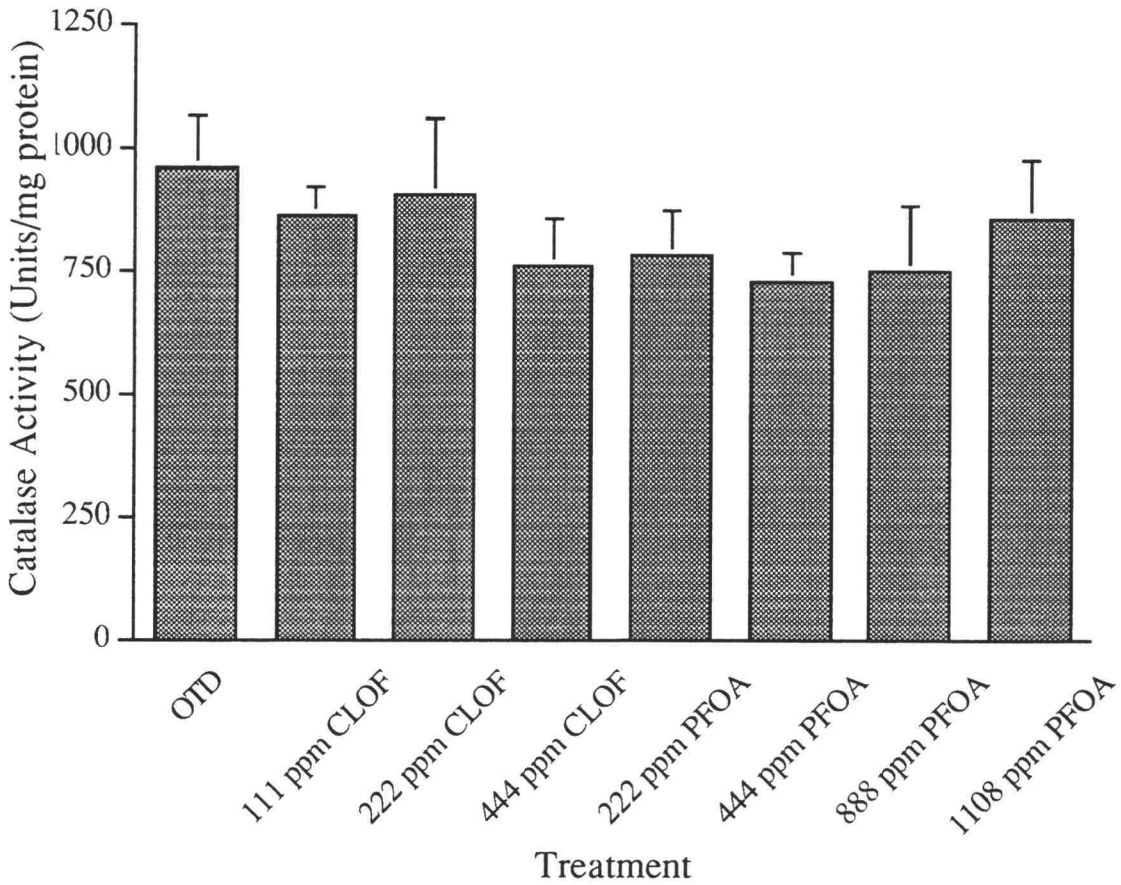

Figure 2.4. Catalase activity in peroxisome proliferator-fed trout (Experiment 2). Twelve trout were sampled from each of the noninitiated groups after 29 weeks on experimental diets, livers removed and assayed for catalase activity as described in Materials and Methods. Values represent means of 12 measurements per treatment plus SEM. A * indicates significantly different from controls ($p < 0.05$).

Table 2.4. Liver tumor incidence and histological classification in AFB1-initiated trout fed peroxisome proliferators for 30 weeks.

Treatment	Tumor Incidence (%)[b]	% of total tumors by tumor type[a]					
		MC	HCC	MA	HCA	CCC	CA
OTD	11/84 (13.1)	61	28	0	11	0	0
111 ppm CLOF	15/78 (19.2)	63	26	0	7	4	0
222 ppm CLOF	12/91 (13.0)	60	27	0	13	0	0
444 ppm CLOF	15/83 (18.1)	53	24	0	18	6	0
222 ppm PFOA	10/75 (13.3)	30	10	30	30	0	0
444 ppm PFOA	7/84 (8.3)*	29	14	0	57	0	0
888 ppm PFOA	4/89 (4.5)*	80	20	0	0	0	0
1108 ppm PFOA	19/85 (22.4)	48	33	7	7	0	3

[a] Abbreviations: MC, mixed carcinoma; HCC, hepatocellular carcinoma; MA, mixed adenoma; HCA, hepatocellular adenoma; CCC, cholangiocellular carcinoma; CA, cholangiocellular adenoma.

[b] * indicates significantly different tumor incidence from initiated trout fed OTD ($p < 0.05$).

DISCUSSION

The compounds examined in this study are representative of a class known as peroxisome proliferators. These agents induce the levels of a number of peroxisomal and microsomal enzymes involved in the ß- and ω-oxidation of fatty acids, respectively (2, 5, 36-38). Prolonged exposure to peroxisome proliferators can be carcinogenic in rodents, however the mechanism of peroxisome proliferator hepatocarcingenesis is still unknown.

A marked species difference has been observed with respect to the peroxisome proliferators, with primates described as resistant, compared to rodents (6, 7). As a result, the risk that these compounds represent to humans is uncertain. In this study, we have examined the carcinogenic properties of three peroxisome proliferators in rainbow trout, a species which, like humans, is relatively resistant to peroxisome proliferation.

Dietary exposure for seven months to the highest dose (0.18%) of PFOA significantly enhanced the yield and multiplicity of tumors in AFB_1-initiated trout. DHEA was even more potent, as 0.18% in the diet raised the incidence to 100%, there were markedly more tumors/liver, and tumors were significantly larger. Somewhat surprisingly, DHEA was carcinogenic by itself, as feeding 0.18% in the diet for seven months produced a 20% incidence of hepatocarcinogenesis. This adrenal steroid has been shown to be chemopreventive toward a number of disease states, including cancer (24, 39, 40), however, prolonged exposure in rats has been demonstrated to produce hepatocarcinogenesis with a phenotype similar to ciprofibrate-induced tumors (27, 28).

CLOF treatments failed to enhance tumorigenesis in control or AFB_1-initiated trout. In fact, the higher dose (0.44%) significantly reduced the incidence and multiplicity of AFB_1-initiated tumors. Furthermore, a shift in tumor type was observed, from predominantly mixed carcinomas to cholangiocellular carcinomas and cholangiomas. These effects are probably due to the toxicity of this dose of CLOF, as lower, less toxic doses of CLOF had no effect on tumor incidence or type. PFOA had mixed dose-

dependent effects on AFB_1-initiated carcinogenesis as well. Although high doses enhanced carcinogenesis, lower doses provided protection. The protective effects of PFOA do not appear to be due to growth inhibition, because these doses of PFOA did not cause reduced weight gain. The potent peroxisome proliferator WY 14,643 has also been shown to inhibit AFB_1-initiated hepatocarcingenesis in rainbow trout (41).

This study confirms previous reports that trout are weak responders to peroxisome proliferators (13, 42). The cacinogenicity of DHEA, and tumor modulating effects of DHEA, PFOA, and CLOF do not appear to require peroxisome proliferation. In fact, the treatment which produced the greatest increase in peroxisomal ß-oxidation had no significant effect on tumor incidence. Liver somatic indexes were increased, however, in treatments which enhanced carcinogenesis. Trout may be useful models for further study of this class of compounds, because they resemble primates in being relatively resistant to peroxisomal proliferation, have a very low liver tumor background, are sensitive to chemically-initiated carcinogenesis and are promoted by agents which induce oxidative stress (43, 44). We have shown that two of the three compounds tested could initiate or enhance hepatocarcinogenesis in trout by mechanisms which are independent of peroxisome proliferation.

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Chapter 3

DEHYDROEPIANDROSTERONE KI-RAS ACTIVATION, HEPATOCARCINOGENESIS, AND POTENT TUMOR PROMOTION WITHOUT PEROXISOME PROLIFERATION IN THE RAINBOW TROUT MODEL

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ABSTRACT

Dehydroepiandrosterone (DHEA), fed for 30 weeks to rainbow trout after initiation with the hepatocarcinogen aflatoxin B_1 (AFB_1), produced a dose-dependent enhancement of carcinogenesis as measured by increased tumor incidence, multiplicity and size. Significant enhancement was observed at 222 ppm, which corresponds to a daily dosage one-half that previously administered to humans in clinical trials. DHEA was also capable of acting as a complete carcinogen in this model, producing liver tumors at doses as low as 222-444 ppm. Tumors isolated from trout treated with DHEA alone contained mutations in K*i-ras*, primarily 12(1)G→A transitions, providing the first evidence that DHEA could be a genotoxic carcinogen. The carcinogenicity of DHEA in trout is independent of peroxisome proliferation, as measurements of peroxisomal ß-oxidation and catalase activity support previous observations that trout, like humans, are weak responders to peroxisome proliferators.

INTRODUCTION

Dehydroepiandrosterone (DHEA), and its sulfate (DHEA-S), produced by the adrenal gland, are the major circulating steroids in human plasma (reviewed in 1). The physiological function of DHEA, other than as a precursor for sex steroids in both males and females (2), is unknown. Human DHEA-S levels peak at about 25 years of age and subsequently decline with age (3) to about 10-20% of maximum.

DHEA has been intensively studied owing to its wide range of chemopreventive properties in animal models toward a number of diseases including atherosclerosis, diabetes, obesity, lupus, and cancer (1, 4-6). DHEA is also being actively studied with respect to its anti-aging properties (5). In animal models DHEA has proven to be chemopreventive against spontaneous, viral, and chemically-induced tumors (4, 5).

The clinical usefulness of DHEA as a chemopreventive agent is compromised by studies showing it to be a peroxisome proliferator and hepatocarcinogen in rodents (7-10). Long-term feeding of DHEA at 0.45% to male Fischer-344 (F-344) rats produces hepatocellular carcinomas (11). DHEA-initiated tumors in rats are phenotypically similar to those initiated by other peroxisome proliferators (11-13). The mechanism of peroxisome proliferator-induced hepatocarcinogenesis is still unclear, however and may not be the direct result of peroxisome proliferation. Trout, like humans are relatively insensitive to peroxisome proliferators (14, 15) and thus provide a useful alternative animal model to study these agents independent of peroxisome proliferation.

Using this model, we now report dietary DHEA to be a complete carcinogen as well as a potent enhancer of aflatoxin B_1 (AFB_1)-initiated hepatocarcinogenesis in the absence of peroxisome proliferation. In addition, evidence is presented for the first time that DHEA may be genotoxic and capable of inducing Ki-*ras* mutations. These findings indicate that caution should be advised in the treatment of humans with DHEA until the mechanisms of DHEA carcinogenesis, and the implications for human cancer risk, are more thoroughly understood.

MATERIALS AND METHODS

Materials

DHEA (5-androsten-3ß-ol-17-one), NADPH, and AFB_1 were purchased from Sigma Chem. Co. (St. Louis, MO). Replitherm™ DNA polymerase was purchased from Epicenter Technologies (Madison, WI). Potassium cyanide was purchased from Mallinckrodt (Paris, Kentucky). All other reagents were purchased from Sigma.

Animals and treatments

Two tumor studies are described in this paper. Rainbow trout (*Oncorhynchus mykiss*) were hatched and reared at the Oregon State University Food Toxicology and Nutrition Laboratory in 12-14°C flowing well water. In the first experiment, fry (75 days post-spawning) were initiated by immersion in 10 ppb AFB_1 for 30 min. Sham-exposed fry were exposed to vehicle alone (0.01% ethanol) and served as non-initiated controls. Trout were fed Oregon Test Diet (OTD), a semi-purified casein-based diet (16), for an additional 16 weeks and then were divided into experimental groups and fed modified OTD diets (100 mg/kg vitamin E; rockfish oil substituted for salmon oil) containing 0, 55, 111, 222, 444, or 888 ppm (w/w) DHEA for 30 weeks. DHEA was dissolved in the lipid component and mixed into the diet. Diets were prepared monthly and stored frozen. Once on experimental diets, trout were fed once daily, five times per week. Animals were fed only what could be consumed in 5 minutes and DHEA exposure by routes other than dietary is believed to be negligible. In the first experiment, each treatment group consisted of 70-100 trout, housed in a single 3 ft diameter (100 gal) continuous flow tank. In a separate experiment, the same five levels of DHEA were fed to noninitiated trout for 42 weeks beginning 111 days after spawning. Duplicate tanks of 120 trout per dose level were used for this study for all doses except the noninitiated controls (single tank). Trout were killed by an overdose of MS-222 after 30 or 42 weeks on experimental diets and the

body weights and liver weights measured. The liver was grossly examined for tumors and the incidence, multiplicity and size recorded. A portion of the tumors observed during necropsy were quick-frozen in liquid nitrogen and stored at -20°C for analysis of Ki-*ras* mutations. Livers were fixed in Bouin's solution for histological examination. After 48 hr tumors were hand sliced to verify previously seen tumors or detect internal, previously undetected, tumors. One slide per tumor-bearing liver, containing as many tumors as possible, was prepared and stained with hematoxylin and eosin for tumor identification. Classification of tumors was on the basis of criteria described previously (17).

Enzyme assays

The effects of dietary DHEA on peroxisomal enzyme activities in trout were examined by sampling twelve non-initiated individuals from each dietary dose of DHEA after 29 weeks. Animals were stunned and killed by decapitation. Livers were immediately removed from the animal and placed into ice cold homogenization buffer (60 mM tris, 0.25 M sucrose buffer, pH 8.3). Three livers were pooled, homogenized, centrifuged at 600 g for 20 min (4°C) to pellet cell debris and nuclei, and the supernatant centrifuged at 12,000 g (4°C) for 30 min to obtain the mitochondrial/peroxisomal fraction. The 12,000 g pellet was resuspended and protein concentration determined by the method of Lowry *et al.*, using bovine serum albumin as a standard (18). Cyanide-insensitive ß-oxidation of palmitoyl-CoA was followed spectrophotometrically via the reduction of NAD^+ to NADPH at 340 nm (19, 20). Catalase activity was determined spectrophotometrically as the loss of absorption at 240 nm during the decomposition of hydrogen peroxide (21).

Mutational analysis of Ki-ras

Tumor tissue was digested with proteinase K and mutations in K*i-ras* analyzed by 3'-primer mismatch polymerase chain reaction (PCR) as described previously (22).

Positive mutations were verified by a second primer mismatch analysis. In addition, some of the mutations were confirmed by direct sequencing. The initial PCR products from four tumors were sequenced on an automated cycle sequencer (Applied Biosystems, Inc., Foster City, CA). Predicted mutations were detected in three of the four samples. The ratio of mutant:normal ras alleles in the fourth tumor DNA may have been too low to permit detection by direct sequencing.

Statistical analysis

The effect of DHEA on tumor incidence was assessed by logistic regression. Fisher's exact test (two-tailed) was used to determine which treatment groups had significantly different tumor incidences than controls. DHEA enhancement of tumor size was determined by ANOVA of the log average size, followed by the Scheffe's test for pairwise comparisons. The nonparametric Kruskal-Wallis test was employed to compare the average number of tumors per animal in trout with tumors using chi-square approximation of p-values. In groups with differences, pairwise comparisons were made by the Wilcoxon Rank Sum test. Body and liver weight data were log transformed and analyzed by ANOVA followed by the Scheffe's test for pairwise comparisons. All statistical analysis was conducted using the Statistical Analysis System (SAS Institute, Inc. Cary, NC) version 6.04.

RESULTS AND DISCUSSION

Dietary DHEA produced a dose-dependent decrease in body weight, ($p<0.0001$). AFB_1-initiated trout fed 444 ppm and higher levels of DHEA for 30 weeks had significantly ($p<0.05$) lower body weights at the conclusion of the study than controls in the AFB_1-initiated group (Table 3.1). Non-initiated trout fed 888 ppm DHEA for 30 weeks (Table 3.1) or 55 ppm or higher for 42 weeks (not shown) also had significantly lower body weights than controls. Similar decreases in weight gain have been observed in rodents in long-term DHEA feeding protocols (reviewed in 5). These observations have been the basis for the therapeutic use of DHEA in veterinary medicine as an anti-obesity

Table 3.1. Trout body weights, liver weights, and relative liver weights after 30 weeks of dietary DHEA.[a,b]

Treatment Initiation/DHEA	Body Wt.[b] (g)	Liver Wt. (g)	Liver Somatic Index (%)
Sham/0 ppm (n=77)	$81^{c} \pm 3$	$0.55^{c} \pm 0.02$	$0.68^{c} \pm 0.01$
Sham/55 ppm (n=78)	$81^{c} \pm 4$	$0.62^{c,d} \pm 0.03$	$0.76^{c,d} \pm 0.01$
Sham/111 ppm (n=80)	$76^{c,d} \pm 3$	$0.62^{c,d} \pm 0.03$	$0.80^{d} \pm 0.01$
Sham/222 ppm (n=85)	$70^{c,d} \pm 4$	$0.64^{c,d} \pm 0.03$	$0.92^{e,f} \pm 0.01$
Sham/444 ppm (n=86)	$65^{c,d,e} \pm 3$	$0.72^{c,d} \pm 0.03$	$1.11^{g,h} \pm 0.01$
Sham/888 ppm (n=88)	$40^{f} \pm 2$	$0.54^{c} \pm 0.03$	$1.35^{i} \pm 0.04$
AFB_1/0 ppm (n=84)	$85^{c} \pm 4$	$0.57^{c} \pm 0.03$	$0.68^{c} \pm 0.01$
AFB_1/55 ppm (n=84)	$73^{c,d} \pm 3$	$0.62^{c,d} \pm 0.02$	$0.85^{d,e,f} \pm 0.02$
AFB_1/111 ppm (n=76)	$81^{c} \pm 3$	$0.66^{c,d} \pm 0.03$	$0.81^{d,e,f} \pm 0.01$
AFB_1/222 ppm(n=88)	$66^{c,d,e} \pm 3$	$0.63^{c} \pm 0.03$	$0.96^{f,g} \pm 0.02$
AFB_1/444 ppm (n=100)	$60^{d,e} \pm 2$	$0.76^{c,d} \pm 0.04$	$1.27^{h,i} \pm 0.06$
AFB_1/888 ppm (n=70)	$52^{e,f} \pm 3$	$1.12^{d} \pm 0.16$	$2.11^{j} \pm 0.21$

[a]Values are average body weight, liver weight, or liver somatic index at the conclusion of the study ± SE.
[b]Numbers bearing the same superscripts are not significantly different from one another ($p<0.05$) (within the same column).

agent (23) and investigations into the potential for DHEA use in the treatment of human obesity (24). No effect of AFB_1 treatment on body weight was observed.

DHEA produces hepatomegaly in rodents (8, 25-27) and we present evidence here for liver enlargement in a non-mammalian model which is much less responsive to peroxisome proliferation (14, 15). Liver somatic indices were elevated by dietary DHEA (Table 1) in a dose-dependent manner. Non-initiated and AFB_1-initiated trout treated for 30 weeks with diets $\geq$ 111 ppm and $\geq$ 55 ppm DHEA, respectively, had significantly higher relative liver weights ($p<0.05$) than controls (Table 3.1). Part of this increase is due to the decreased body weights, however, absolute liver weights were also higher in DHEA-fed trout. The markedly higher somatic indices in the AFB_1-initiated groups fed the two highest DHEA diets is largely due to enhanced tumor tissue mass.

In rodents, the DHEA-dependent enhanced liver somatic index is accompanied by marked increases in peroxisome size, number and enzymatic activity (7, 28). In trout, the hepatomegaly does not appear to be due to peroxisomal changes. Instead, electron microscopy reveals enlarged hepatocytes packed with rough endoplasmic reticulum. In addition, there is dramatic proliferation of biliary preductule cells (29). These hepatic changes are similar to those observed in mature female rainbow trout during the period of vitellogenesis (30) and probably are the result of DHEA conversion into androgens and estrogens rather than its peroxisome proliferating effects.

DHEA administered by diet to AFB_1-initiated trout enhanced tumor incidence in a dose-dependent manner ($p=0.0001$) from 13% (11/84 trout) in control diets to 97% (68/70 trout) at 888 ppm DHEA (Fig. 1). The number of tumors per tumor-bearing animal was also significantly ($p<0.0001$) enhanced (Fig. 3.1). Initiated trout fed control diet had an average of 1.4 ± 0.2 tumors per tumor-bearing animal. DHEA, fed at 222, 444 and 888 ppm DHEA increased this value to 1.9 ± 0.2 ($p<0.10$), 3.9 ± 0.3 ($p<0.0005$) and 6.1 ± 0.4 ($p<0.0001$), respectively. The size of tumors was also significantly ($p<0.0001$) enhanced by dietary DHEA in a dose-dependent manner. The average tumor diameter was

1.3 ± 0.1 mm in initiated trout fed control diet. The two highest levels of DHEA (444 and 888 ppm) increased the average tumor size two-(2.8 ± 0.2 mm) and three-(3.9 ± 0.3 mm) fold, respectively.

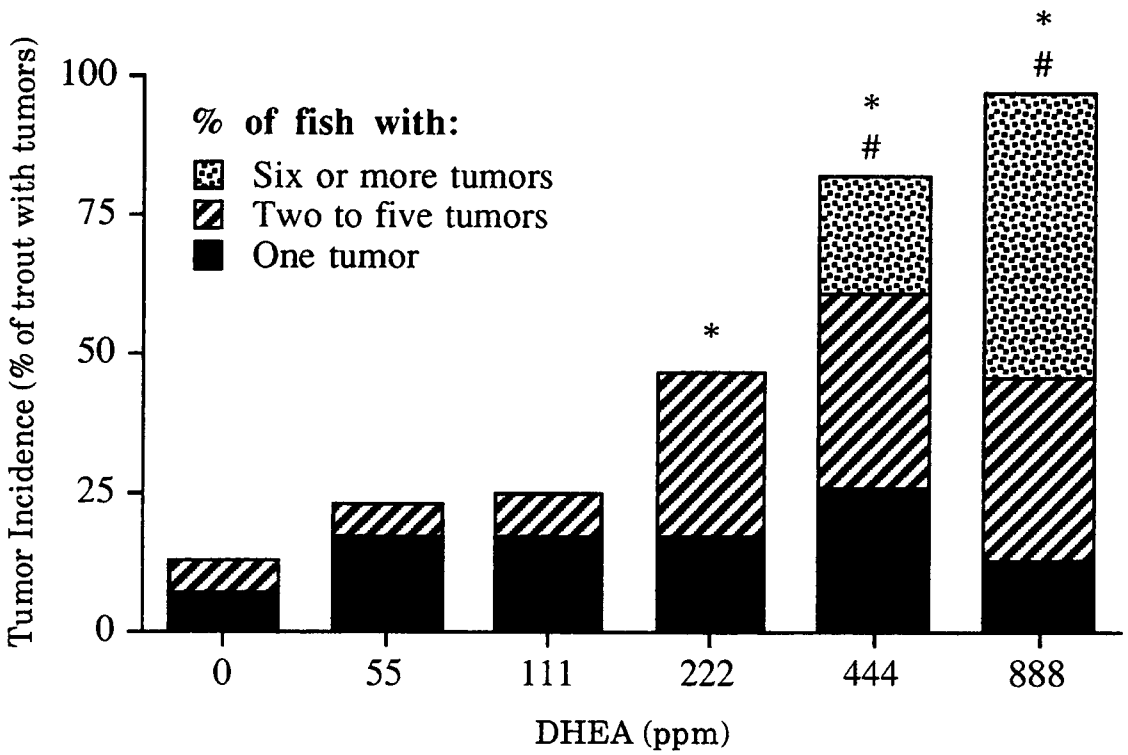

Figure 3.1. Liver tumor incidence and multiplicity in AFB_1-initiated trout fed 0, 55, 111, 222, 444 or 888 ppm DHEA. Each treatment group consisted of a single tank containing between 70 and 100 animals. A * indicates a significantly ($p < 0.05$) higher tumor incidence than controls. A # indicates significantly higher tumor multiplicity than controls.

Dietary DHEA exposure was therefore, a highly effective post-initiation enhancer of AFB_1-initiated hepatocarcinogenesis in trout. Tumor incidence, multiplicity and size were all significantly increased in a dose-dependent manner. The enhancement in incidence was significant at dietary doses as low as 222 ppm. Enhancement of carcingenesis by DHEA has previously been observed in rodent studies, however much higher doses were utilized.

For example, feeding male Lewis rats initiated with azaserine, 6000 ppm DHEA for 4 months post-initiation, produced a 75% increase in the incidence of pancreatic preneoplastic lesions (31). SWXJ-9 Mice fed 4000 ppm DHEA for 50 days exhibited a 6-fold increase in spontaneous ovarian granulosa cell tumors (32).

In addition to enhancing AFB_1-initiated hepatocarcinogenesis, DHEA was a complete carcinogen producing a dose-dependent increase in tumor incidence ($p<0.0001$) in non-initiated trout fed DHEA for 30 or 42 weeks (Fig. 3.2). Sixty-eight percent of animals fed the highest dose of DHEA for 42 weeks developed tumors while no tumors were detected in the non-initiated controls. There were also significantly more tumors per liver in trout fed 444 or 888 ppm DHEA for 42 weeks, or 888 ppm DHEA for 30 weeks compared to the next highest treatment (not shown). Trout appear to be much more susceptible than rats to DHEA as a complete carcinogen. For example, Rao *et al.*, reported that feeding 4500 ppm DHEA to male F-344 rats for 84 weeks was hepatocarcinogenic in 15/16 animals (11). However, Moore *et al.*, found that feeding F-344 rats diets containing 6000 ppm DHEA for 9 months did not produce liver or lung tumors in either sex (33).

Classification of liver tumors was based on a subsample of the total tumors evaluated, i.e., those present in the one slide prepared from each tumor bearing liver. For livers with one to only a few tumors, we would have seen all or most of the tumors, for heavily tumored livers, we would have seen only a part of the total. The same array of hepato- and cholangiocellular tumors were seen as was previously described (17), however, the relative percentages of some tumor types differed from what has been reported with other carcinogen-initiation protocols. In previous studies using AFB_1 (34), N-nitrosodiethylamine (35), and 7,12-dimethylbenz[a]anthracene (22), the mixed hepatocholangiocellular carcinoma (MC) was the predominant tumor type (about 60% of all tumors), with hepatocellular carcinomas (HCC) second in number (20-30%), and all other types [mixed adenomas (MA), hepatocellular adenomas (HCA), cholangiocellular carcinomas (CCC), and cholangiomas (Ch) making up the final 10-20%. In the current

study, AFB_1 alone produced a similar spectrum of tumors (Table 3.2), but DHEA promotion resulted in a shift from MC (down to the 30% range) to HCA (up to the 20-30% range), while HCC remained about the same. DHEA initiation alone, however, produced the more normal pattern of MC>HCC>HCA.

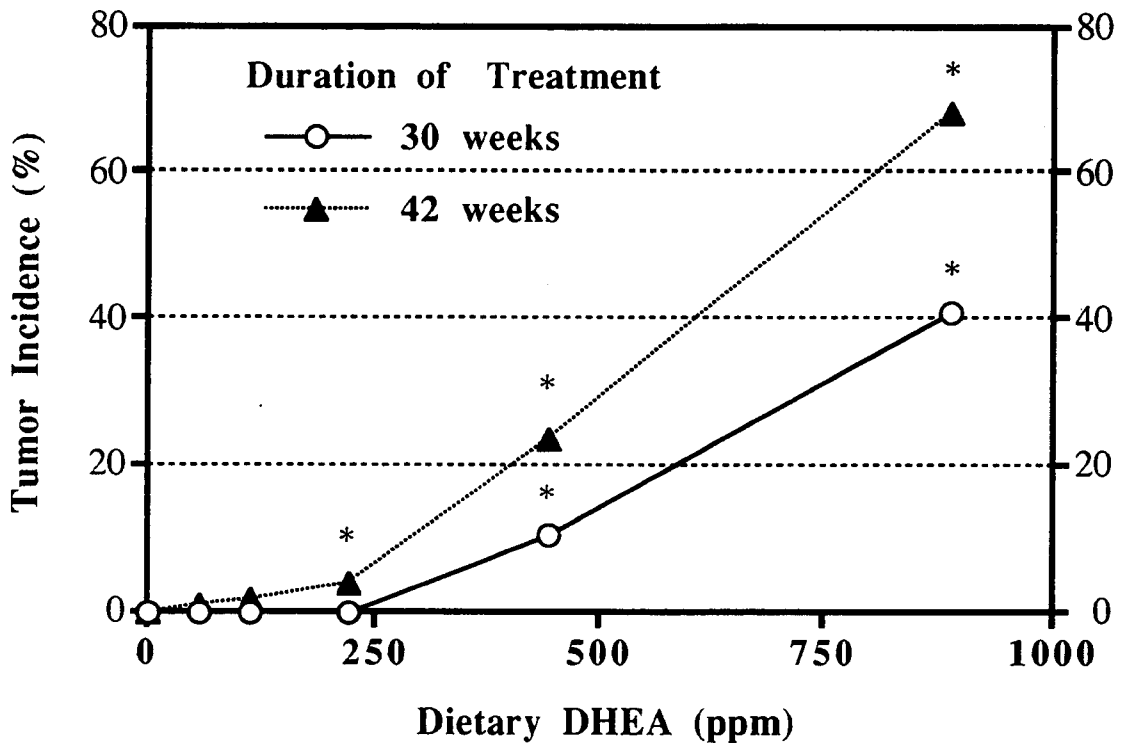

Figure 3.2. Liver tumor incidence and multiplicity in sham and non-initiated trout fed 0, 55, 111, 222, 444, or 888 ppm DHEA for 30 or 42 weeks. For the 30 week experiment, each treatment group consisted of a single tank containing between 77 and 88 animals. The 42 week study utilized duplicate tanks of 100 trout per treatment. Liver tumors in the 42 week experiment have not yet been confirmed by histological examination, however we have found that tumor incidence based on gross examination is an excellent approximation of final tumor incidence. A * indicates a significantly ($p < 0.05$) higher tumor incidence than controls.

The combined effects of AFB_1 and DHEA are clearly more than the additive effects of AFB_1 and DHEA acting as initiators. Thus, it would seem reasonable that DHEA is promoting clones of cells that were initiated by AFB_1 but would not have developed into

recognizable tumors with that treatment alone. We believe that the benign HCA is a progressive step towards the malignant HCC, and in most cases not a terminal lesion. Thus, the increased number of HCA observed with DHEA promotion may represent a late stage promotion of AFB_1-initiated lesions that have not had sufficient time to develop malignancy.

Table 3.2. Histological classification of liver tumors in trout fed DHEA for 30 weeks.

Treatment Initiation/DHEA	% of total tumors by tumor type[a]					
	MC	HCC	MA	HCA	CCC	Ch
Sham/0 ppm	0	0	0	0	0	0
Sham/55 ppm	0	0	0	0	0	0
Sham/111 ppm	0	0	0	0	0	0
Sham/222 ppm	0	0	0	0	0	0
Sham/444 ppm	70	20	0	0	0	10
Sham/888 ppm	53	36	3	9	0	0
AFB_1/0 ppm	61	28	0	11	0	0
AFB_1/55 ppm	50	32	0	18	0	0
AFB_1/111 ppm	36	43	0	18	0	4
AFB_1/222 ppm	39	40	0	22	0	0
AFB_1/444 ppm	30	36	2	32	0	1
AFB_1/888 ppm	39	31	5	25	0.3	0

[a]Abbreviations: MC, mixed carcinoma; HCC, hepatocellular carcinoma; MA, mixed adenoma; HCA, hepatocellular adenoma; CCC, cholangiocellular carcinoma; Ch, cholangiomas.

The potential for DHEA to function as a peroxisome proliferator in trout was determined by assays of palmitoyl CoA ß-oxidation and catalase. Dietary DHEA did not significantly ($p<0.05$) alter palmitoyl CoA oxidation at any dose (Fig 3.3). The group fed 888 ppm DHEA exhibited a mean increase of 40% but this was not significant at $p<0.05$.

Catalase activity was not elevated, and in fact was significantly ($p<0.0005$) decreased by dietary DHEA in a dose-dependent manner (Fig. 3.4). The highest dose of DHEA produced a 2.5-fold decrease in catalase activity.

A clear distinction between rat and trout with respect to DHEA as a hepatocarcinogen, exists not only with respect to potency, but also with the observation that the latter species is relatively insensitive to the peroxisome proliferating properties of DHEA. Long-term feeding of DHEA to trout does not enhance ß-oxidation of palmitoyl CoA and significantly decreases catalase activity at the higher doses.

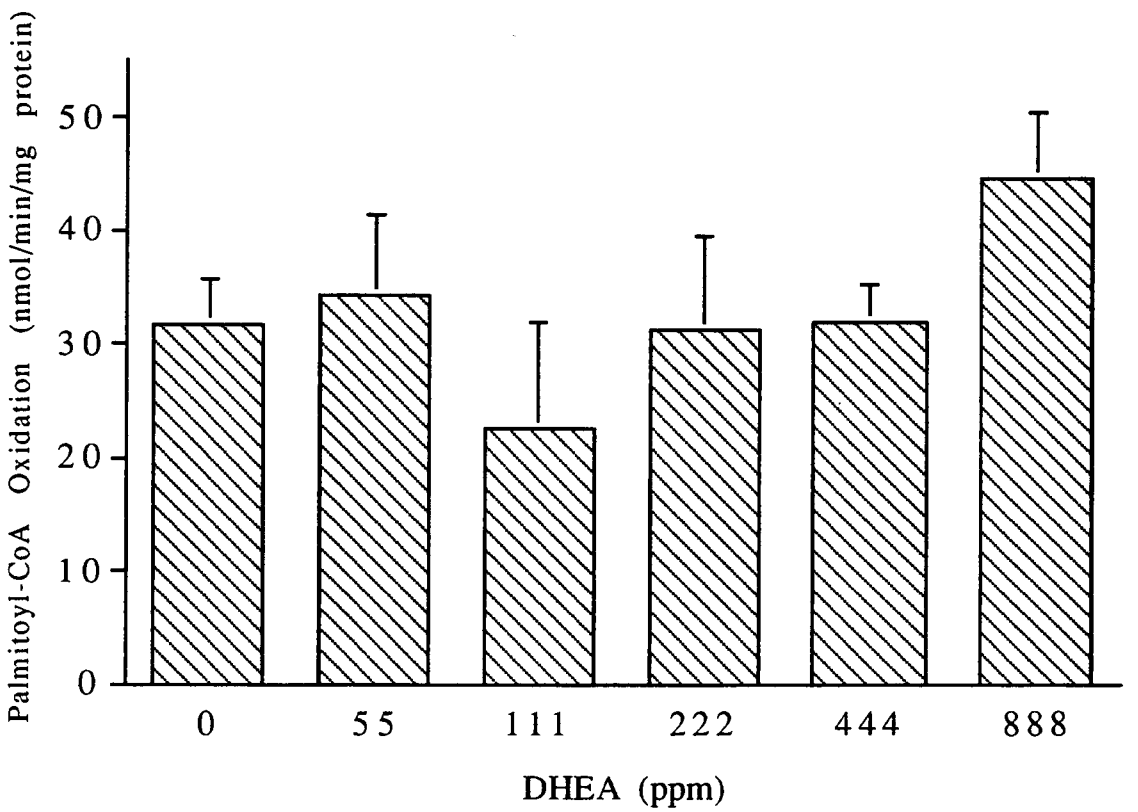

Figure 3.3. Peroxisomal palmitoyl CoA ß-oxidation in non-initiated trout fed diets containing 0, 55, 111, 222, 444 or 888 ppm DHEA for 6 months. Twelve trout from each DHEA dose were removed 1 week prior to the conclusion of the study. Peroxisomal ß-oxidation was measured as described in Materials and Methods. The bars represent the mean ± S.E. of 4 pools of 3 livers. A * indicates significantly ($p < 0.05$) different from controls.

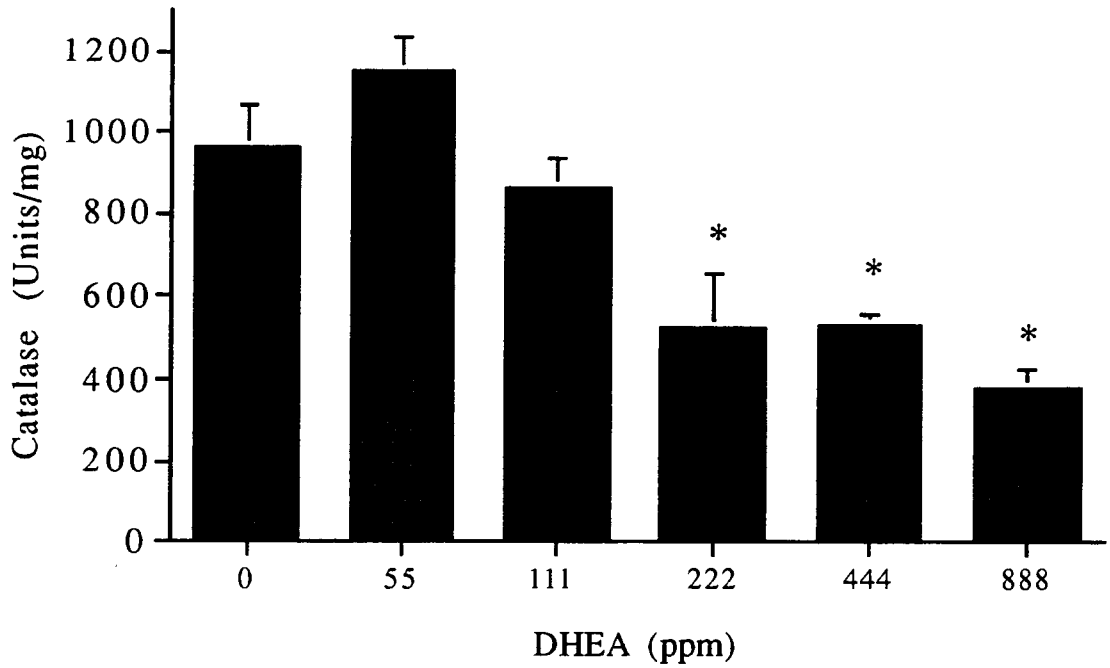

Figure 3.4. Peroxisomal catalase activity in non-initiated trout fed diets containing 0, 55, 111, 222, 444 or 888 ppm DHEA for 6 months. Twelve trout from each DHEA dose were removed 1 week prior to the conclusion of the study. Peroxisomal ß-oxidation and catalase activities were measured as described in Materials and Methods. The bars represent the mean ± S.E. of 4 pools of 3 livers. A * indicates significantly ($p < 0.05$) different from controls.

Ki-*ras* mutations in a significant percentage of DHEA-induced tumors support its action as a complete carcinogen in trout. The presence of mutated Ki-*ras* oncogenes in DNA isolated from hepatic tumors of trout was examined in two seperate experiments using primer mismatch PCR. Tumor DNA from a preliminary experiment in which trout were fed 1800 ppm DHEA for six months was analyzed. Fifty percent (3/6) of the tumors carried activated Ki-*ras* and both 12(1) G→A transitions (1/6) and 13(2) G→T transversions (2/6) were observed (Table 3.3). Tumor DNA from trout initiated with AFB_1 exhibited an incidence of 84 and 86% activated K*i-ras* in groups fed control diet or 1800 ppm DHEA, respectively. In addition, as previously observed in AFB_1-initiated trout, the mutations were predominantly 12(2) G→T transversions (Val_{12} p21) both with

and without DHEA. In the present study, approximately 1/3 of the tumors isolated from sham-initiated trout fed 888 ppm DHEA for 30 weeks contained mutated Ki-*ras* and the mutation was exclusively a G→A transition at the first G of codon 12 (Table 3.3). This mutation would result in expression of a Arg_{12} p21.

Table 3.3. Ki-*ras* mutations in DNA from trout liver tumors.[a]

Treatment		**Ki-*ras* mutations**			
Initiation/DHEA	%Tumor[b]	12(1)G→A	12(2)G→A	12(2)G→T	13(2)G→T
		Preliminary Study[c]			
AFB_1/0 ppm	36	2/32	nd[d]	22/32	3/32
AFB_1/1800 ppm	100	1/29	nd	17/29	7/29
Sham/1800 ppm	20	1/6	nd	0/6	2/6
		This Study[e]			
Sham/888 ppm	41	8/25	0/25	0/25	0/25

[a]Mutations were identified by 3' primer mismatch PCR as described previously (22).
[b]The percentage of animals with tumors (number of individuals sampled per group was 87-100).
[c]Trout fry were initiated by 10 ppb AFB_1 for 30 min and fed either control diet or 1800 ppm DHEA for 28 weeks.
[d]nd signifies not determined.
[e]Trout fry were sham initiated and fed OTD containing 888 ppm DHEA for 30 weeks as described in Materials and Methods.

The trout Ki-*ras* oncogene is highly homologous to human Ki-*ras* (36) and is mutated in approximately 80% of hepatic tumors induced by AFB_1, 7,12-dimethylbenz[*a*]anthracene (DMBA) or 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) (22, 37, 38). However, the mutational spectrum differs for each of these initiators. AFB_1 initiation induces primarily codon 12(2) G→T transversions, with smaller amounts of 13(2)

G→T transversions and 12(1) G→A transitions (38). Initiation with DMBA, on the other hand, yielded equal amounts (4/11) of 12(1) G→A transitions and 12(2)G→T transversions, with a single observed 61(2) A→T transversion (22). Hepatocarcinogenesis induced by MNNG produced almost entirely G→A transitions in both codon 12 and 13 (37). Our initial screening of 6 tumors from DHEA-fed trout indicated that 3 carried mutated Ki-*ras*. One mutation was a 12(1) G→A transition and the other 2 were 13(2) G→T transversions. In a subsequent experiment, 8/25 tumors were positive for mutated K*i-ras*, and all were 12(1) G→A transitions. The observation that we have yet to detect a Ki-*ras* mutation in the rare spontaneous liver tumors (0.1%), suggests that activated Ki-*ras* is important in the etiology of DHEA hepatocarcinogenesis in trout, as it appears to be with known genotoxic carcinogens such as AFB_1, DMBA and MNNG. These results can be contrasted with K- and H-*ras* mutations from ciprofibrate-induced mouse liver tumors. In this tumor model the frequency of mutated *ras* in tumors from ciprofibrate-treated animals is lower than found in spontaneous tumors (39). We believe this to be the first report of oncogene activation by DHEA or, in fact, the first report of any genotoxicity associated with this compound. More extensive studies as spontaneous trout liver tumors become available will be needed to substantiate this finding.

Human clinical trials have employed dosages of DHEA as high as 21-27 mg/kg daily for 28 days or a total dose of 600-750 mg/kg (40, 41). In this study, we have found that daily doses (trout were fed at 5.4% of their body weight 5 days per week) of 12 mg/kg for 30 weeks or a total dose of 1800 mg/kg significantly enhanced AFB_1-initiated hepatocarcinogenesis in trout. DHEA was effective as a complete carcinogen at 12 mg/kg/day for 30 weeks, or 6 mg/kg/day for 43 weeks. In the absence of evidence that the mechanism of DHEA-dependent carcinogenesis in trout is not applicable in humans, it would seem prudent to reconsider clinical trials involving supra pharmacological doses of DHEA administered over prolonged periods.

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Chapter 4

SHORT-TERM EFFECTS OF DIETARY DEHYDROEPIANDROSTERONE IN TROUT

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ABSTRACT

Dehydroepiandrosterone (DHEA) is an adrenal steroid which is being studied as a possible therapeutic agent for a wide variety of human diseases including cancer, diabetes, lupus, and AIDS. DHEA, however, is also a peroxisome proliferator and hepatocarcinogen in rats. Rainbow trout are relatively insensitive to the peroxisome proliferating effects of DHEA but are highly sensitive to its carcinogenicity. It is possible that the hepatocarcinogenicity of DHEA is caused by a different mechanism than that causing peroxisome proliferation. This study was designed to examine the time course of the histological and biochemical changes associated with administration of DHEA to trout. Rainbow trout (*Oncorhynchus mykiss*) were fed 0.18% DHEA and sampled after 0, 1, 2, 4, 7, or 14 days of treatment. Significant increases in the liver wt. to body wt. ratio occurred by the fourth day of treatment. The increase in liver size appears to be due to a combination of hypertrophy of hepatocytes and proliferation of biliary preductule cells. Peroxisomal ß-oxidation was significantly increased on the second day of treatment, but decreased on subsequent days. Catalase activity was reduced on days 7 through 14. Serum albumin, cholesterol, and direct bilirubin levels increased over the course of the study. The histological and biochemical changes associated with short-term administration of DHEA may be useful in elucidating the carcinogenic mechanism(s) of this compound.

INTRODUCTION

The adrenal steroid dehydroepiandrosterone (DHEA) has been reported to have chemoprotective effects in a variety of rodent carcinogenesis models (1). DHEA, however, also causes hepatomegaly, proliferation of peroxisomes, and increases in peroxisomal ß-oxidation, characteristics of a group of nongenotoxic rodent carcinogens known as peroxisome proliferators (PPs) (2-5). Recently DHEA was shown to produce hepatocellular carcinomas in rats, possibly through peroxisome proliferation (6).

Rainbow trout may prove to be an appropriate model for studying the mechanisms of PP carcinogenicity because their response to these compounds appears to be similar to that of primates. Rainbow trout treated for three weeks with the hypolipidemic drug ciprofibrate had significantly increased peroxisomal ß-oxidation and peroxisomal volume density, although to a lesser extent than typically seen in rodents exposed to the same levels (7). Our laboratory showed that DHEA was a liver carcinogen and a strong enhancer of aflatoxin B_1 hepatocarcinogenesis in the trout tumor model (8). These effects were not accompanied by increases in peroxisomal ß-oxidation or ω-hydroxylation of fatty acids. DHEA treatment did result in dramatic increases in liver weights and histological changes including hypertrophy of hepatocytes and proliferation of presumptive bile preductule cells (9). This study was designed to examine the time course of the histological and biochemical changes associated with short-term administration of DHEA to trout.

MATERIALS AND METHODS

Materials

DHEA, palmitoyl-CoA, and NAD^+ and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Western blotting reagents were purchased from Biorad (Hercules, CO). [1-^{14}C] Lauric acid was obtained from American Radiolabeled Chemicals, Inc (St. Louis, MO). Antibody to proliferating cell nuclear antigen (PCNA) was purchased from Paracelsian (Ithaca, NY). Vitellogenin antibody and standards were provided by Dr. David R. Idler (Memorial Univ. of Newfoundland, St. John's, Newfoundland, Canada).

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were spawned and raised in our laboratory. Six months after spawning, 500 juvenile trout (average wt 18 g) were fed Oregon test diet (OTD) (10) containing 0.18% (w/w) DHEA for up to 18 days. Animals were housed in a single 4 ft. tank with continuously flowing, 12° well water and a 12 hour light dark cycle and were fed 2% of their body weight once per day. Fish were sampled prior to beginning experimental diets and after 1, 2, 4, 7, and 14 days of treatment between 8 and 10 am. In addition, some trout were kept on experimental diets for 4 additional days (until day 18), then were sampled on day 21 for light and electron microscopy. On the day of sampling, trout were anesthetized with MS-222, weighed, blood was drawn from the caudal vein, and livers removed, weighed, and placed into the appropriate fixative or buffer.

Clinical Chemistry

Serum was obtained by centrifugation of pooled blood samples (6 pools of 10 fish each). Bilirubin, albumin, cholesterol, and glutamic-oxaloacetic transaminase (SGOT) were measured using a Ciba Corning 550 Express automated analyzer according to

manufacturer's instructions. Serum DHEA and DHEA-sulfate were determined by radioimmunoassay (Wien Laboratories, Succasunna, NJ) and vitellogenin with an enzyme linked immunosorbent assay (11).

Enzyme Assays

Sixty fish were sampled at each time point. Pools of ten livers were homogenized (20% w/v) in 60 mM Tris, 0.25 M sucrose buffer (pH 8.3) and separated by differential centrifugation into nuclear (600 g.), mitochondrial (12,000 g.), and microsomal (105,000 g.) fractions. The mitochondrial/peroxisomal fraction was used to measure peroxisomal ß-oxidation and catalase activity. Cyanide insensitive ß-oxidation was monitored by spectrophotometrically measuring the reduction of NAD^+ to NADH in the presence of palmitoyl-CoA (12). Catalase activity was measured by monitoring the decomposition of hydrogen peroxide (13). Protein was measured using the Bradford method (14). Microsomes were incubated with [1-^{14}C] lauric acid, then hydroxylated products extracted with ethyl acetate and separated by HPLC (15). Total P450 was measured by spectrophotometrically comparing the reduced versus the oxidized carbon monoxide difference spectrum (16). The protein concentration of the microsomal fractions was determined according to the method of Lowry *et al.*, using bovine serum albumin as a standard (17).

Protein Separation and Western Blotting

Proteins were separated using polyacrylamide gel electrophoresis (10%) in the presence of sodium dodecyl sulfate (SDS-PAGE) (18) . They were blotted onto nitrocellulose (Buchler semi-dry blotter, Lenexa, KS); and blots probed with a mouse monoclonal antibody to rat PCNA followed by a horseradish peroxidase linked secondary antibody. PCNA was detected by chemiluminescence (Amersham Corp., Arlington Heights, IL). The blots were scanned on a flatbed scanner (HP ScanJet IIcx) and relative

densities of the bands analyzed with the public domain software NIH Image, version 1.54 (written by Wayne Rasband at the National Institute of Health and available by anonymous ftp from zippy.nimh.nih.gov over the internet) on a Power Macintosh 7100.

Light Microscopy

Livers were fixed in Bouin's solution, processed to paraffin sections via conventional methods, and stained with hematoxylin and eosin (H&E).

Transmission Electron Microscopy

Livers from control and DHEA-treated fish were sampled at ten weeks and processed for electron microscopy. One mm thick slices were excised and immediatly fixed by immersion in phosphate buffered 4% formaldehyde-1% glutaraldehyde fixative at 4°C for two hours. Tissues were then post fixed in 1.0% OsO_4 for one hour, dehydrated and embedded in epoxy resin. Ultrathin sections of 60 to 80 nm were cut and subsequently stained with uranyl acetate and bismuth subnitrate. Specimens were examined in a Zeiss EM 10/A transmission electron microscope at an accelerating voltage of 60 kV.

Statistical Analysis

Data were analyzed using one-way ANOVA (with and without log transformation) followed by the Fisher PLSD test. Data analysis was performed using StatView 512+ (Brainpower, Calabasas, CA) on a Macintosh computer. P-values of 0.05 or less were considered significant.

RESULTS

Dietary administration of 0.18% DHEA caused increases in the percent of body weight made up by liver (Fig. 4.1). Relative liver weight increased in a linear manner and on day 14 was 90% higher than on day 0. The increase in the percent of body weight made up by livers was not due to decreased animal weights as DHEA-treated trout continued to gain weight (average weight gain during the 14 days was 7.8 g) (Table 4.1).

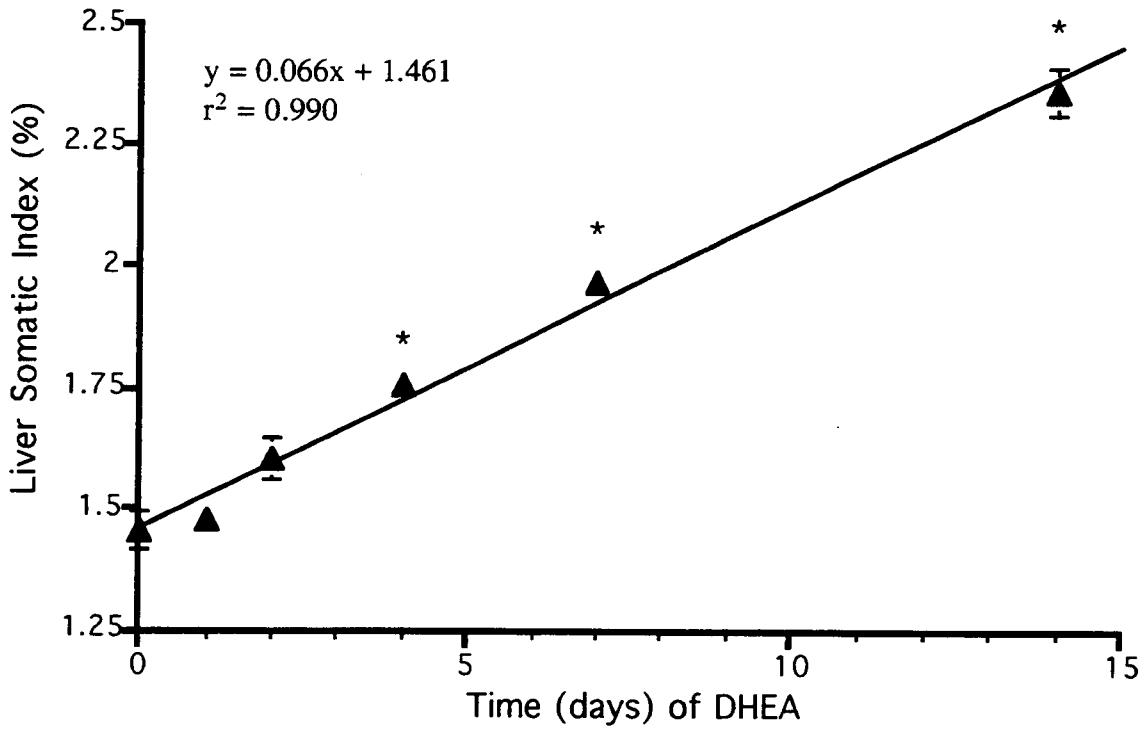

Figure 4.1. The effect of dietary DHEA on percent liver weights (g liver/100 g body weight). * = significantly different from day 0 ($p < 0.05$).

Dietary DHEA produced rapid increases in serum DHEA from nondetectable levels in untreated animals to more than 90 mg/dl on the second day of treatment (Table 4.1). This is within the normal human range for DHEAS, however in trout, the steroid appears

to be present primarily as DHEA rather than the sulfated form. Serum DHEA levels then declined to 6.3 mg/dl on the 14th day of dietary DHEA. This may represent increased metabolism of DHEA or binding to serum proteins or lipids. Serum cholesterol was significantly elevated on the 4th, 7th, and 14th days of treatment. SGOT was elevated on days 1, 2, and 14 (Table 4.1).

Table 4.1. Body weights, and serum DHEA, cholesterol, and SGOT in trout fed 0.18% DHEA for up to 14 days.

Time[a] (days)	Fish Wt.[b,c] (g)	Serum DHEA[b,d] (μg/dL)	Serum Cholesterol[b,e] (mg/dL)	Serum SGOT[b,e] (mg/dL)
0	18.21 ± 0.81	not detected	125 ± 1	290 ± 29
1	17.98 ± 0.68	76.0[f] ± 6.4	120 ± 3	489[f] ± 26
2	19.40 ± 0.65	93.3[f] ± 7.3	130 ± 5	458[f] ± 29
4	19.16 ± 0.63	53.0[f] ± 3.5	216[f] ± 12	390 ± 26
7	22.58[f] ± 0.79	39.7[f] ± 10.4	181[f] ± 11	345 ± 10
14	26.08[f] ± 0.76	6.3 ± 3.5	221[f] ± 6	473[f] ± 28

[a] Number of days of treatment with 0.18% DHEA
[b] Values are means ± SEM
[c] Number of animals (n) = 67
[d] Values are means ± SEM of three measurements
[e] Values are means ± SEM of four measurements
[f] Significant differences from day 0 values were determined by one-way ANOVA followed by Fisher PLSD on log transformed data. ($p < 0.05$)

Peroxisomal and microsomal enzymes were measured to determine if DHEA causes peroxisome proliferation in trout as in rodents (Table 4.2). Palmitoyl CoA oxidation was increased by 2-fold on the fourth day of treatment. The induction was transient, however, and after 14 days of treatment activity was significantly lower than controls. Peroxisomal catalase activity was significantly decreased beginning on the fourth day of dietary DHEA. Total microsomal P450 content was reduced at all time-points compared to controls.

Hydroxylation of lauric acid was not significantly enhanced by DHEA treatment. No hydroxylation was observed at the ω position. Recently Buhler et al. demonstrated that, although the ω-1 hydroxylated product is the major metabolite of lauric acid produced by juvenile trout microsomes, hydroxylation at the ω-2, ω-3, ω-4, ω-5, and ω-6 positions also occurs (19). The HPLC conditions used in this experiment did not separate ω-1 from ω-2 through ω-6. There were no differences in the overall hydroxylation of lauric acid throughout the 14 days of DHEA treatment, however, we cannot rule out the possibility that the ratio of ω-1 to other hydroxylated products was changed. Overall, the enzyme data support the classification of trout as weak responders to peroxisome proliferators.

Table 4.2. Peroxisomal and microsomal enzymes in DHEA-treated trout.

Time[a] (days)	Palmitoyl CoA Oxidation[b,c] (nmol/min/mg)	Catalase[b] (Units/min/mg)	Lauric Acid hydrox.[b] (nmol/ω-1 /min/nmol P450)	Total P450[b] (nmol/mg)
0	56.6 ± 6.4	622 ± 48	0.945 ± 0.411	0.42 ± 0.03
1	52.5 ± 4.9	632 ± 47	0.856 ± 0.374	0.20[c] ± 0.02
2	105.9[c] ± 8.3	432[c] ± 36	1.455 ± 0.560	0.30[c] ± 0.03
4	45.5 ± 4.4	482 ± 30	2.654 ± 1.861	0.27[c] ± 0.03
7	65.2 ± 5.4	340[c] ± 28	1.186 ± 0.169	0.31[c] ± 0.03
14	17.1[c] ± 1.8	206[c] ± 7	1.271 ± 0.170	0.27[c] ± 0.02

[a] Number of days of treatment with 0.18% DHEA
[b] Values are means ± SEM of six measurements
[c] Significant differences from day 0 values were determined by one-way ANOVA followed by Fisher PLSD on log transformed data. ($p < 0.05$)

In addition to being enlarged, livers of DHEA-treated fish were yellow and animals appeared jaundiced. Serum levels of bilirubin were elevated, primarily in the direct (conjugated) form (Figure 4.2).

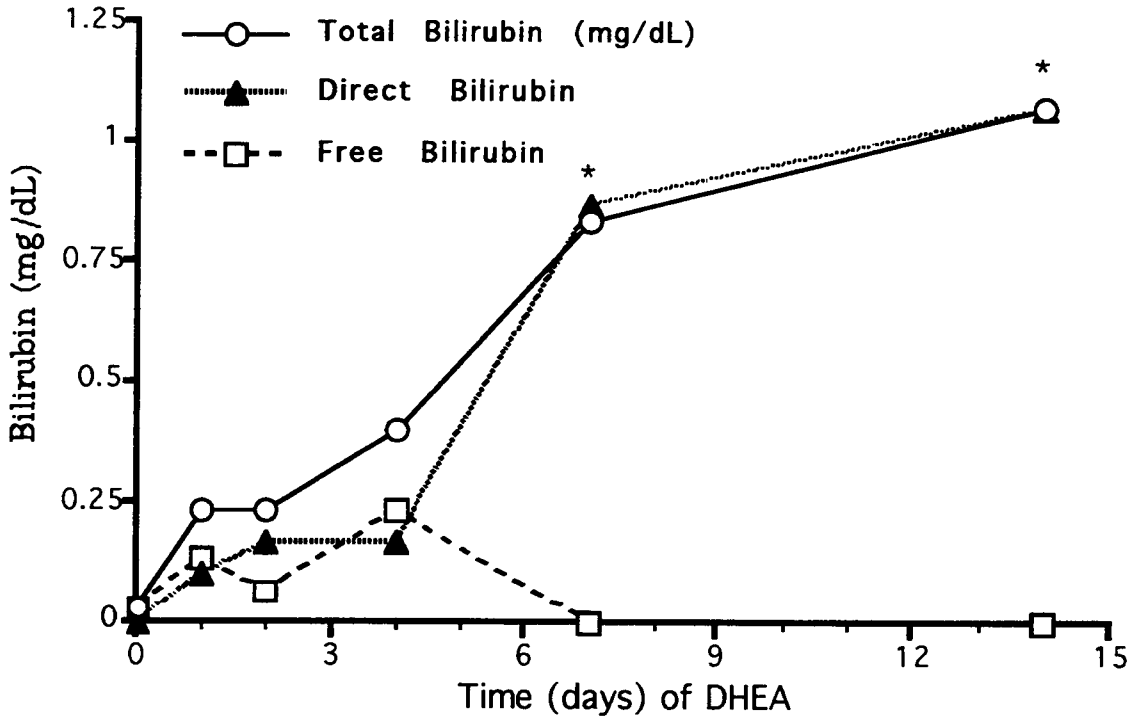

Figure 4.2. Serum bilirubin levels in trout fed 0.18% DHEA for up to 14 days. * = significantly different from day 0 ($p < 0.05$).

Light Microscopy

Livers of DHEA-treated trout were examined by light and electron microscopy. The first four days of treatment resulted in increased mitotic activity, decreased glycogen levels, nuclear swelling, and hepatocyte enlargement (Fig. 4.3-4.5). Proliferation of small biliary preductule cells within the hepatic tubules was observed on days seven through twenty-one, hepatocytes were swollen, and mitotic figures were common (Fig. 4.6-4.8).

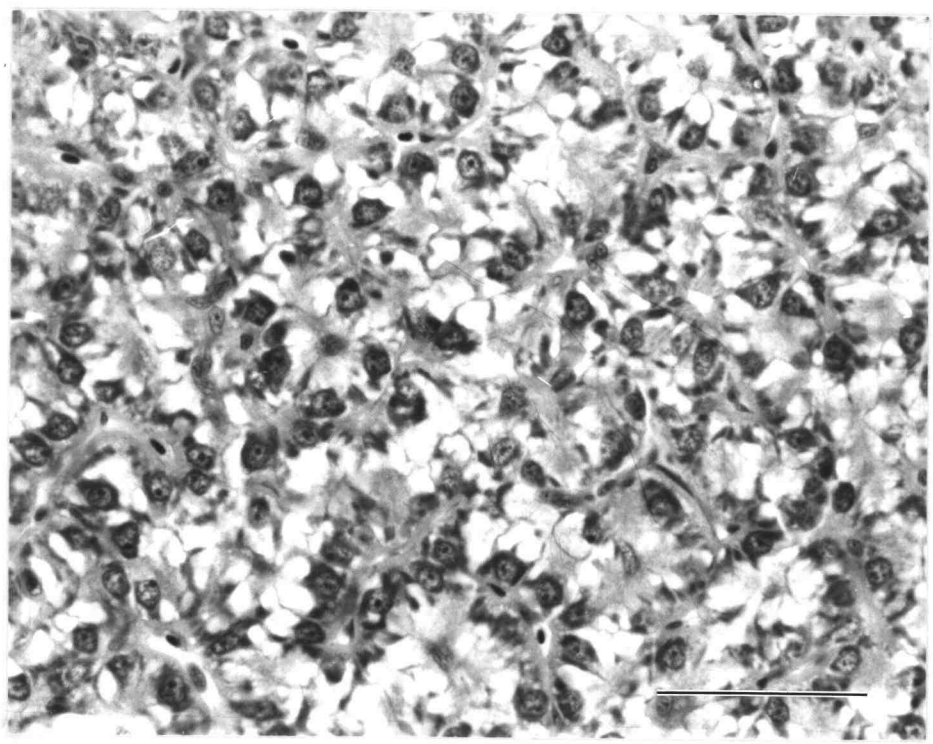

Figure 4.3. Control liver from a six-month old rainbow trout, demonstrating tubular architecture, abundant glycogen, and no mitotic figures. Bar, 50 mm; X 563.

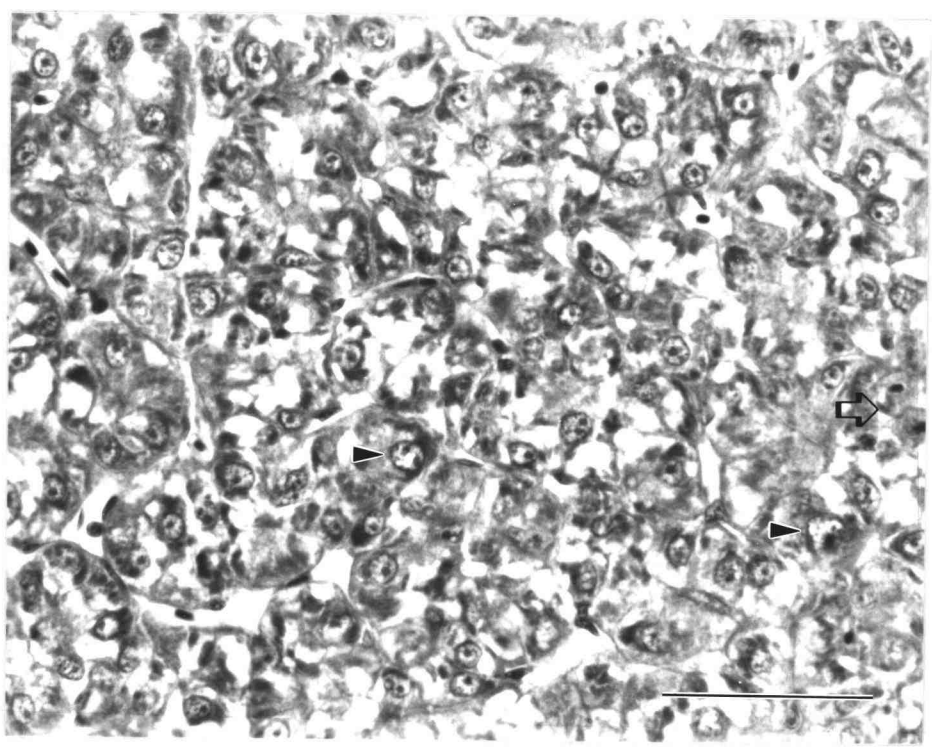

Figure 4.4. Section of liver from a rainbow trout after receiving 0.18% dietary DHEA for one day. Some hepatocyte nuclei appear enlarged (arrowheads), glycogen vacuolation is slightly reduced and a mitotic figure (arrow) is present. Bar, 50 mm; X 563.

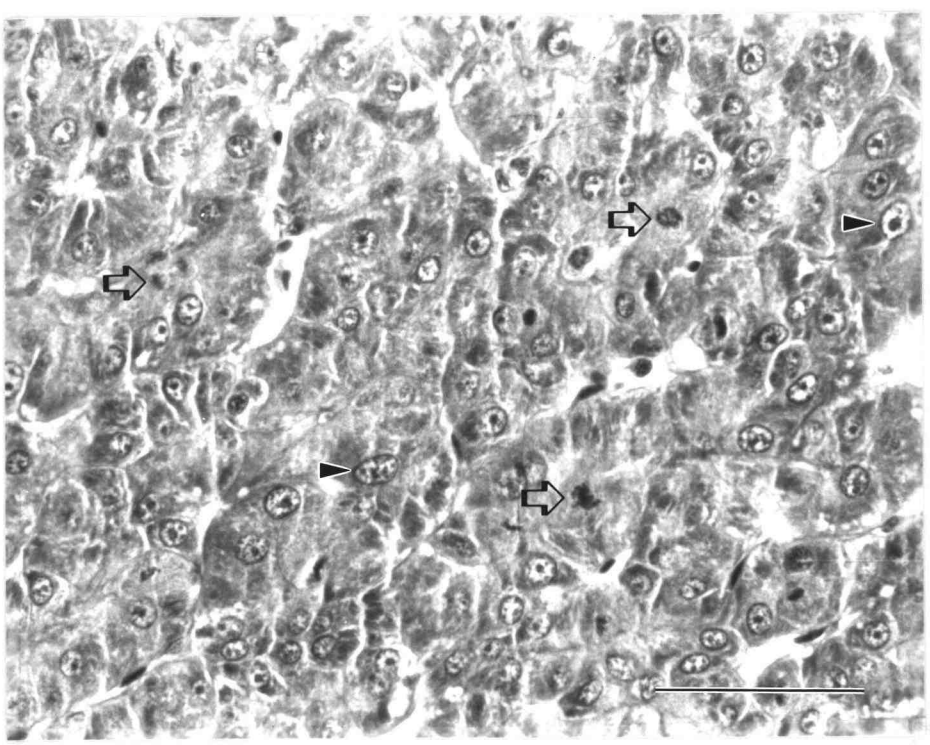

Figure 4.5. Trout liver section after four days on 0.18% dietary DHEA. Glycogen levels are greatly reduced, mitotic figures (arrows) are numerous, nuclei are enlarged (arrowheads), and hepatocytes appear swollen. Bar, 50 mm; X 563.

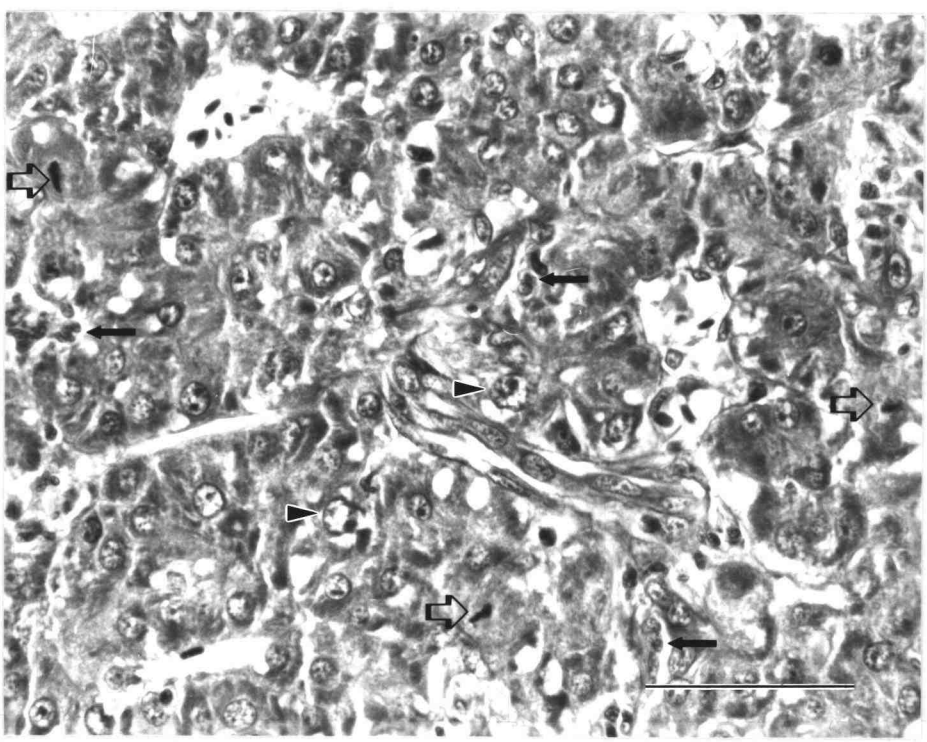

Figure 4.6. Trout liver section after seven days on 0.18% dietary DHEA. Mitotic activity (open arrows) remains high, nuclear atypia (arrowheads) are prominent, increased numbers of small cells (nuclei) (solid arrows) appear in centrotubular locations, and a biliary ductule is present. Bar, 50 mm; X 563.

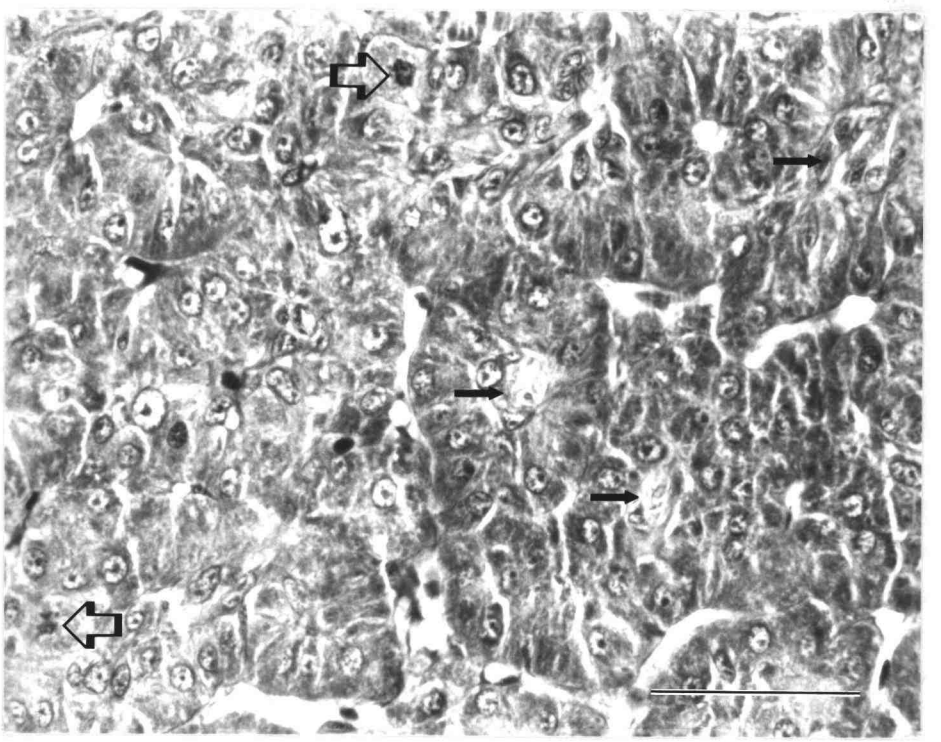

Figure 4.7. Trout liver section after 14 days on 0.18% dietary DHEA. Most hepatic tubules contain numerous biliary cells (solid arrows) forming bile ductules. Residual hepatocytes are swollen, mitotic figures (open arrows) still common. Bar, 50 mm; X 563.

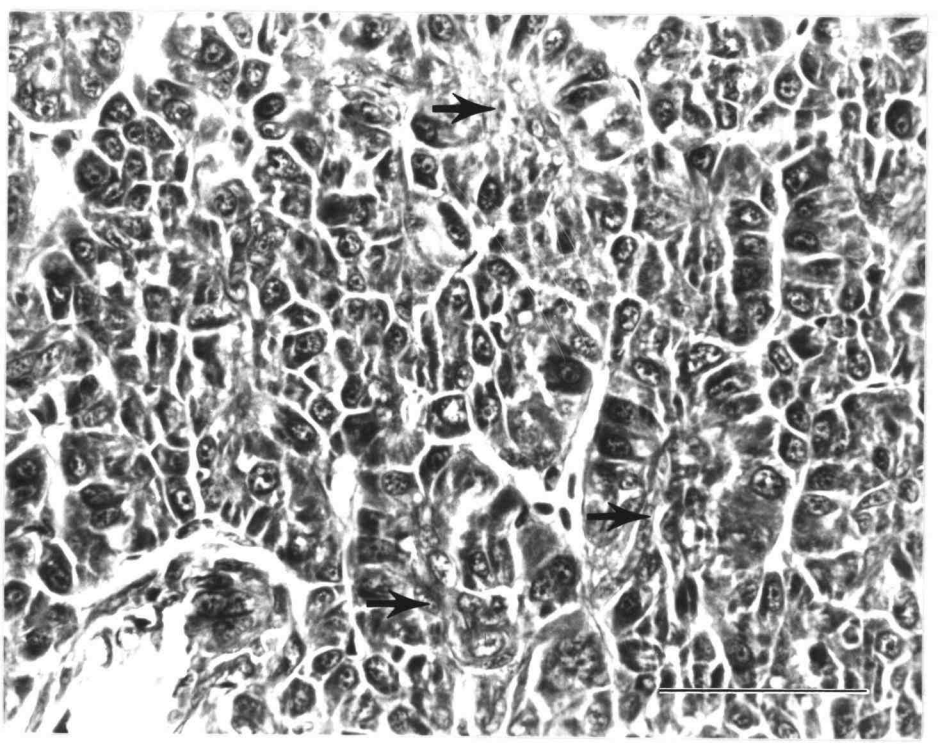

Figure 4.8. Trout liver section 21 days after onset of dietary 0.18% DHEA. Residual hepatocytes have prominent intercellular spaces, nearly all tubular profiles have biliary cells (solid arrows) streaming from the central region. Bar, 50 mm; X 563.

Ultrastructural Changes

Electron microscopy revealed dramatic cellular changes in livers of DHEA-treated animals (Fig. 4.10-4.16). Cytoplasmic clefts, possibly resulting from crystalline inclusion bodies are present in both hepatocytes and biliary cells. The hepatomegaly does not appear to be due to peroxisome proliferation, instead hepatocytes are packed with rough endoplasmic reticulum (RER). Vacuoles containing very low density lipoprotein (VLDL) -like material associated with golgi and areas of vesiculation of RER indicate that hepatocytes are producing a secretory protein. Other changes include nuclear folding and cellular swelling.

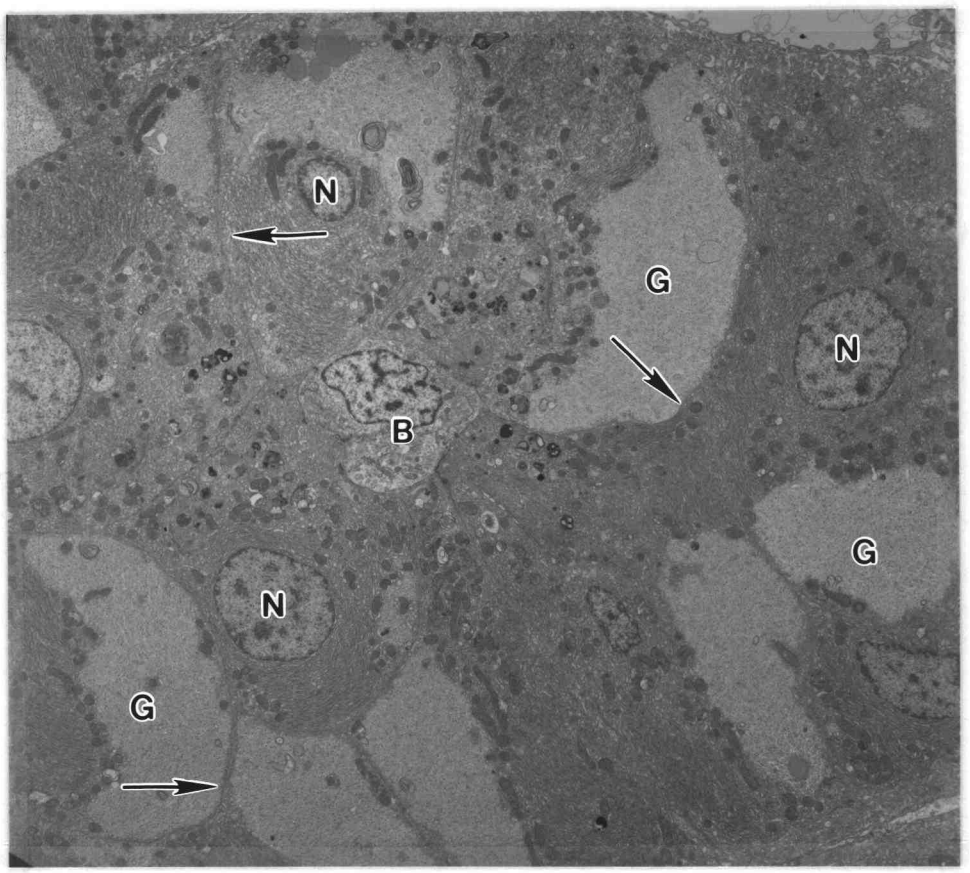

Figure 4.9. Transmission electron micrograph of liver obtained from 6-month old rainbow trout. Hepatocytes are polygonal shaped with distinct cell boundaries (arrows) and round-to-oval nuclei (N). Cytoplasmic organelles usually have a perinuclear compartmentalized orientation and large peripheral deposits of glycogen (G). Biliary cells (B) have indented nuclei and sparse cytoplasmic organelles. X 2600.

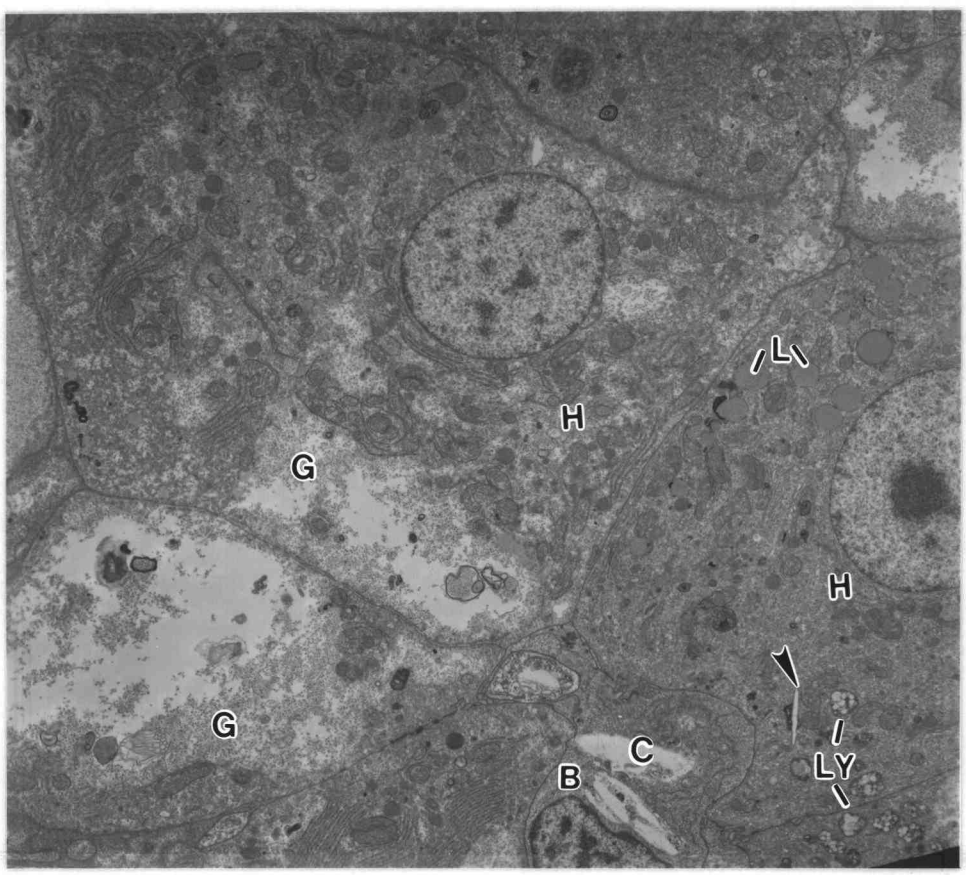

Figure 4.10. Micrograph of trout liver following treatment with 0.18% dietary DHEA for two days. Hepatocyte (H) demonstrates a clear cleft (arrowhead) within a lysosome, which is suggestive of a cholesterol crystalline inclusion, adjacent lysosomes (Ly), and numerous lipid droplets (L). Hepatocytes also appear to have slightly decreased amounts of glycogen (G) deposits. Biliary cells (B) have clear cytoplasmic clefts (C). X 3300.

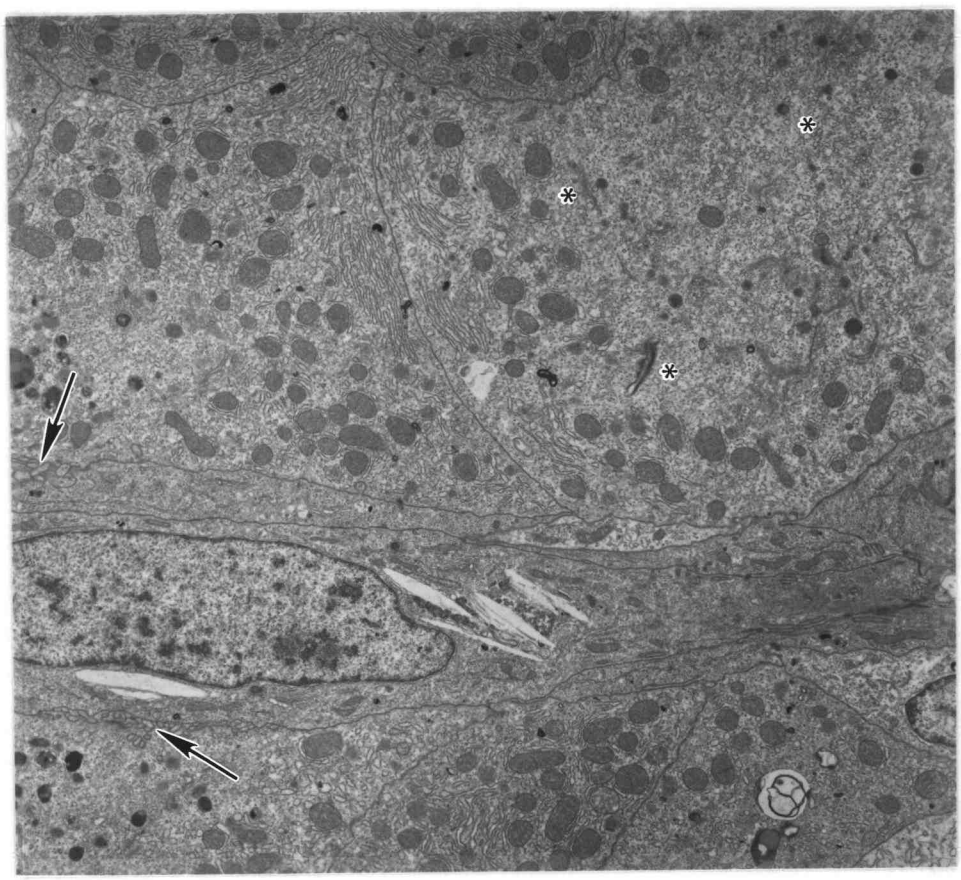

Figure 4.11. Micrograph of trout after four days on 0.18% dietary DHEA. Hepatocytes show evidence of toxicity because of the marked variation in cell shape (elongated to swollen), areas of vesiculation of RER (*) and reduplication of basement membranes (arrows). X 4100.

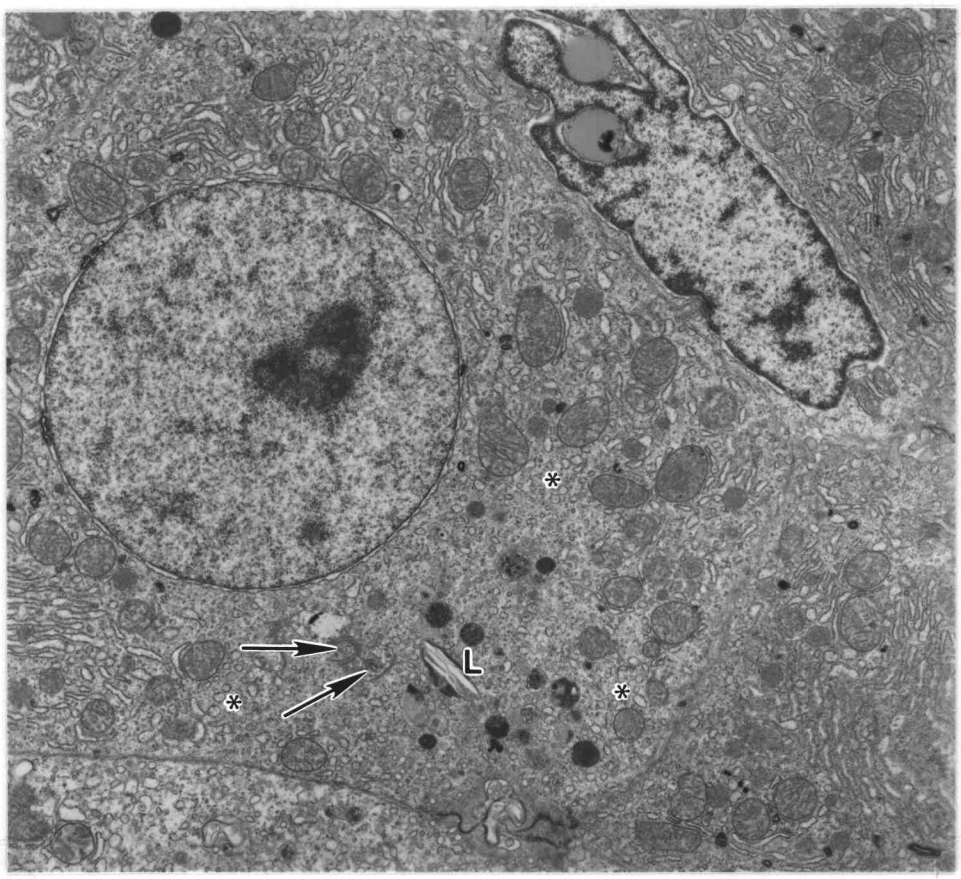

Figure 4.12. Micrograph of trout after four days on 0.18% dietary DHEA.. Note vacuoles (arrows) containing VLDL protein-like material associated with golgi, areas of vesiculation of RER (*) and a lysosome containing clear clefts. X 6500.

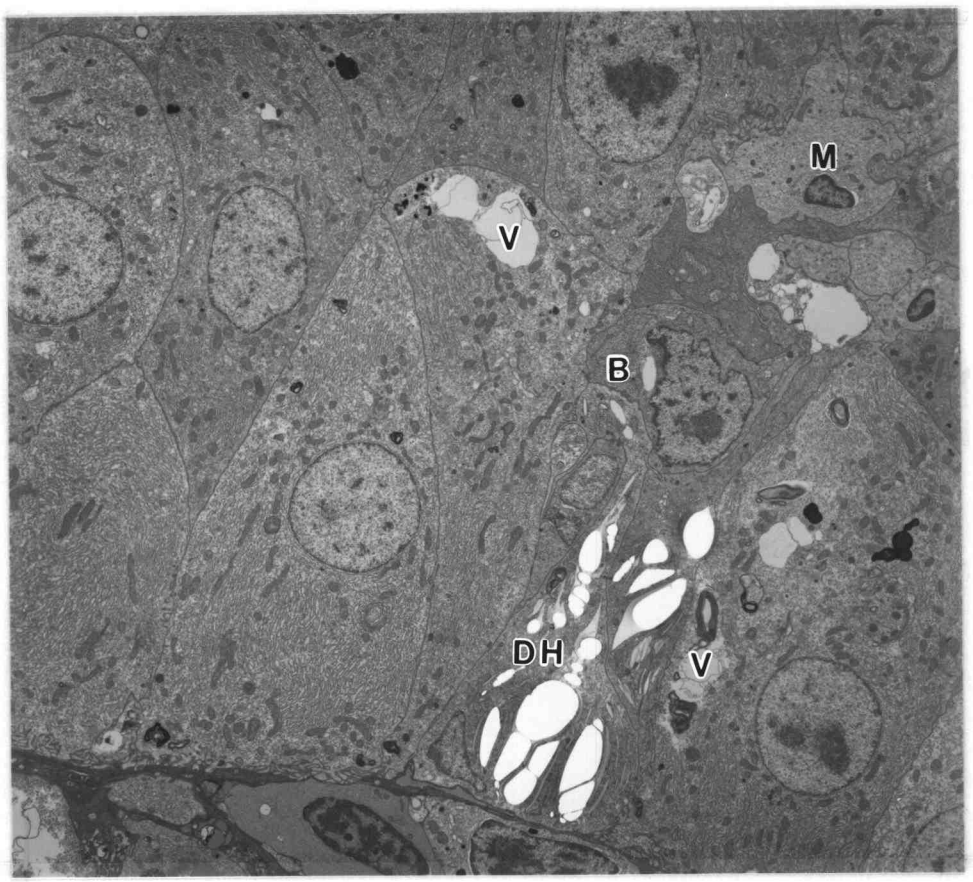

Figure 4.13. This low power micrograph of trout liver after seven days on 0.18% DHEA shows destruction of liver architecture because of the marked variation in hepatocyte shape, absence of glycogen, large cytoplasmic vacuoles, and filling of the cytoplasm with RER. Degenerate hepatocytes (DH) appear to contain numerous clear clefts Biliary cell (B). X 2600.

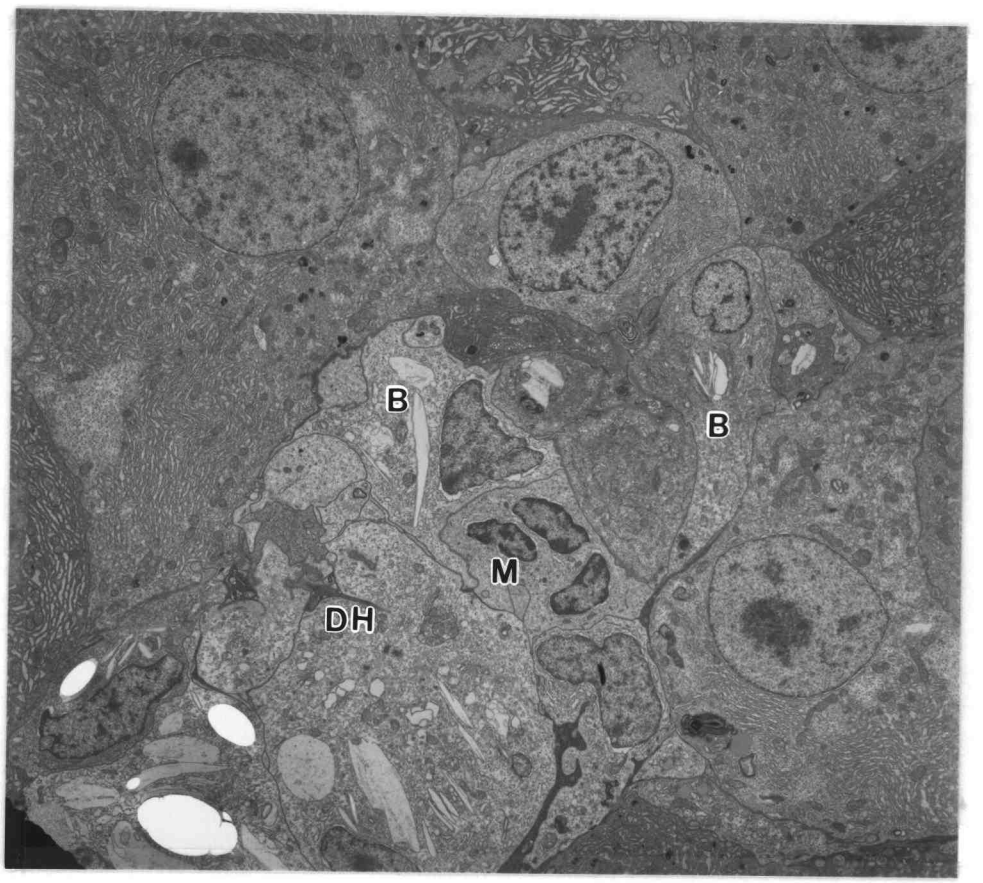

Figure 4.14. Micrograph of trout liver after seven days on 0.18% DHEA. Note the atypical cellular centers composed of biliary cells (B), macrophages (M) and degenerate hepatocytes (DH). X 2600.

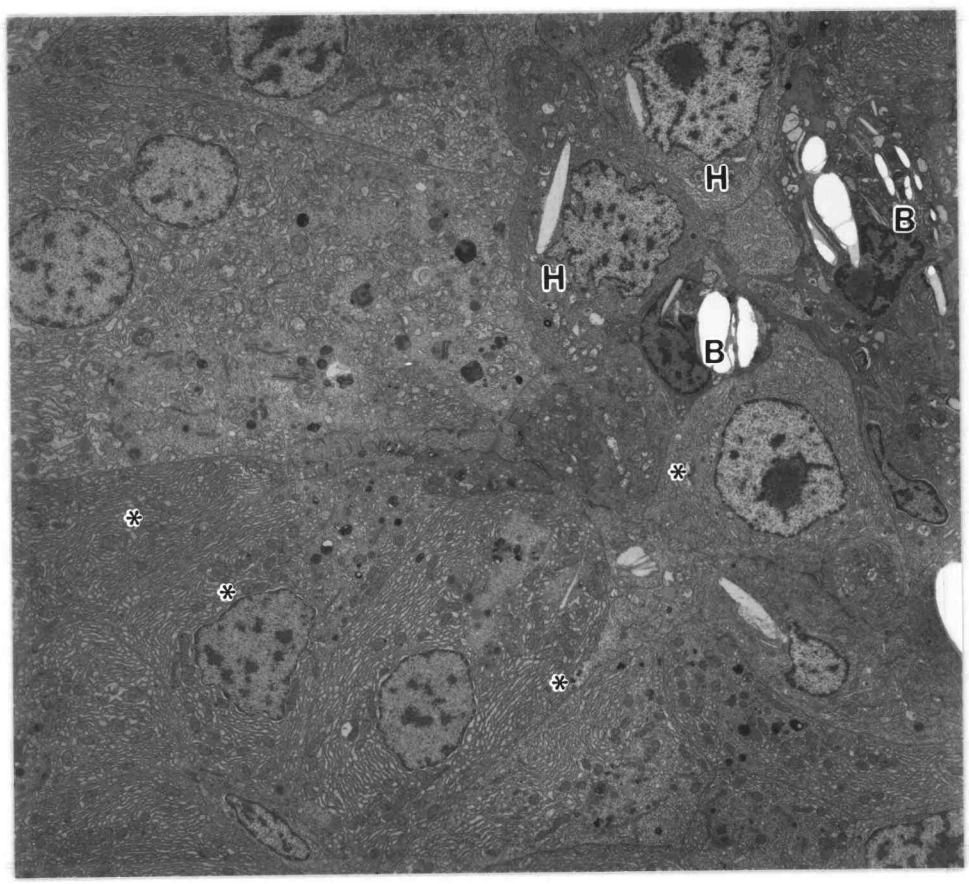

Figure 4.15. Micrograph of trout liver following 14 days of dietary exposure to 0.18% DHEA. The nuclear envelopes of hepatocytes (H) and biliary cells (B) are invaginated or indented. The cytoplasm of hepatocytes are usually filled with RER (*). X 2600.

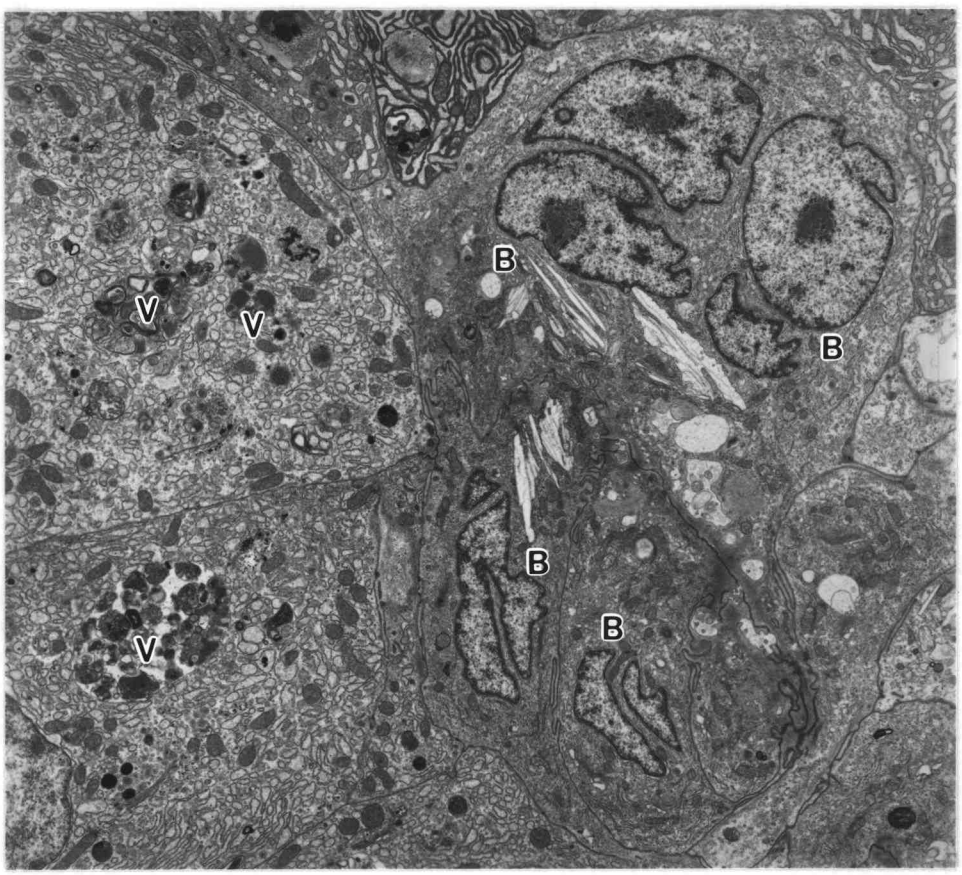

Figure 4.16. Micrograph of trout liver after 21 days of exposure to 0.18% DHEA. Note the cluster of degenerate hepatocytes (DH) that have marked folding and invagination of their nuclear envelopes. These hepatocytes also display large cytoplasmic clear clefts and have distorted, irregular shapes. Adjacent hepatocytes display large autophagocytic vacuoles (V). X 6500.

The histological and ultrastructural changes indicate that the liver enlargement produced by DHEA treatment does not result from peroxisome proliferation. Instead, a combination of proliferation of biliary preductule cells and hepatocyte swelling (due to increases in RER) appears to be responsible. PCNA was used as an additional measure of cell proliferation. Levels of PCNA in liver homogenates were increased by 4, 7, or 14 days of DHEA treatment (Fig. 4.17).

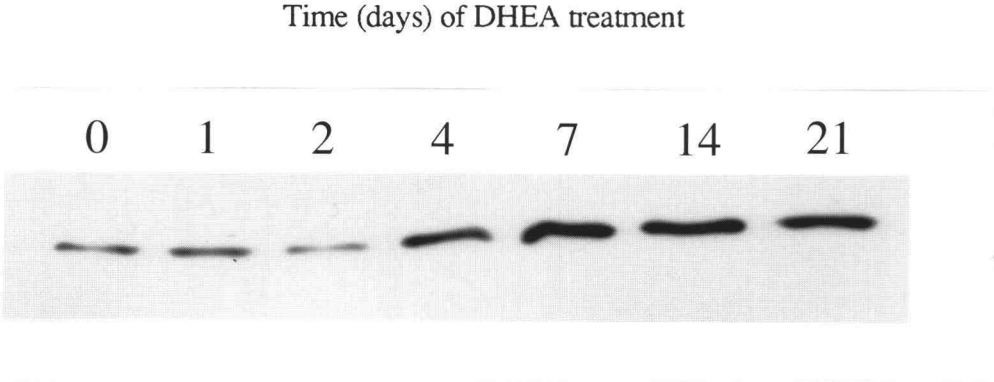

Figure 4.17. Western blot analysis of liver homogenates probed with antibody to proliferating cell nuclear antigen (PCNA).

Ultrastructural examination of hepatocytes indicates that these cells are producing a secretory protein. Serum vitellogenin levels were measured to determine if this yolk protein precursor was the protein being secreted by the liver. Vitellogenin levels increased from nondetectable in serum of control trout (limit of detection = 2.5 μg/ml) to greater than 3 mg/ml in serum of trout fed 0.18% DHEA for 14 days.

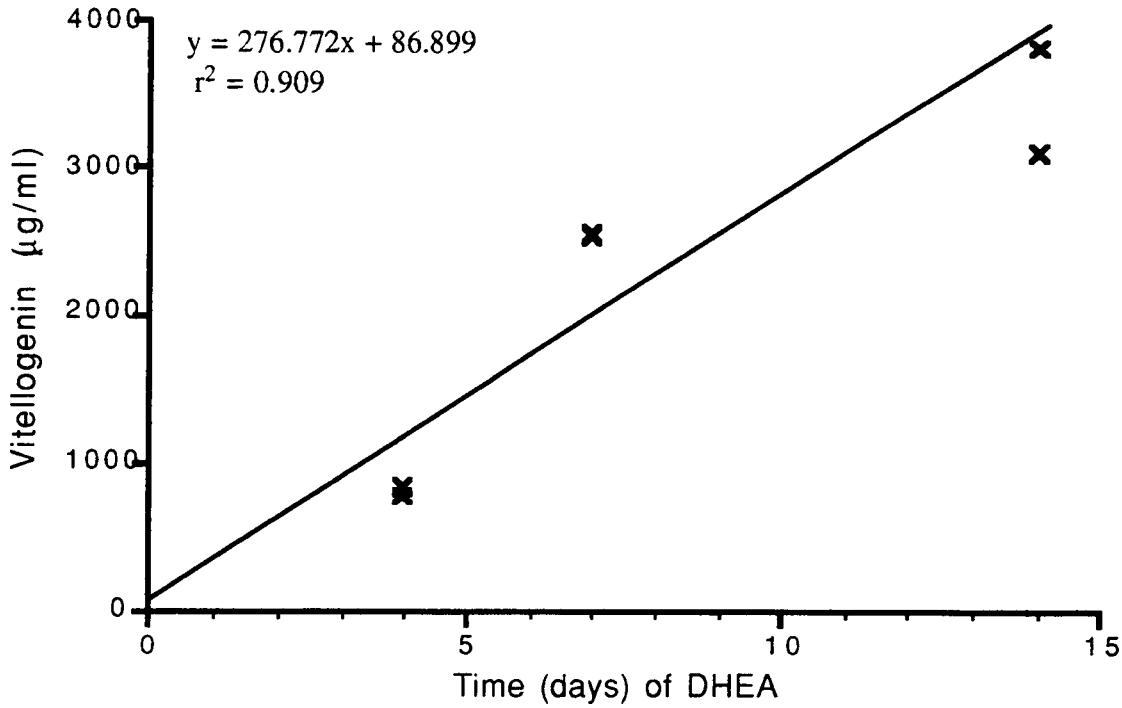

Figure 4.18. Serum vitellogenin levels in trout fed 0.18% DHEA. Symbols represent vitellogenin levels in each of two pooled blood samples per time point.

DISCUSSION

Dietary DHEA effectively raised serum DHEA to levels comparable to human DHEAS levels (20). In DHEA-fed trout, however, circulating levels of DHEA and DHEAS are approximately equal, while in humans the ratio of DHEAS to DHEA is about 100:1 (21). Serum DHEA levels rose sharply for the first two days of treatment then decreased. This decline may represent enhanced metabolism of the steroid or could be due to increased binding to serum proteins.

A transient increase in palmitoyl CoA oxidation (1.9-fold) occurred with this treatment, but to a lesser extent than that reported with rodents. Male Wistar rats treated with 300 mg DHEA/kg body weight for 14 days had a 7.9-fold increase in peroxisomal ß-oxidation and a 1.6-fold increase in catalase activity (22). In contrast to the response in rats, peroxisomal catalase activity was significantly decreased in trout fed DHEA.

In rodents, peroxisome proliferators also induce levels of cytochrome P450s responsible for ω-hydroxylation of fatty acids. In male Sprague-Dawley rats, dietary DHEA (0.45% for 7 days) increased ω-hydroxylation of lauric acid 17-fold (4). DHEA at 100 mg/kg/day for four days increased cytochromes P4504A protein and mRNA levels by 20-fold (5). In this experiment, we did not observe any ω-hydroxylation of lauric acid and hydroxylation at other positions was not significantly increased at any timepoint. The separation conditions used for this experiment did not distinguish between the ω-1 through ω-6 hydroxylated products, so we cannot rule out the possibility of changes in the ratios of these hydroxylated products.

Total cytochrome P450 content was decreased by dietary DHEA treatment. In trout, as in mammals, treatment with androgens and estrogens have opposite effects on hepatic cytochrome P450 levels, with estrogens increasing and androgens depressing specific content (23, 24). In mammals, DHEA serves as a precursor of both androgens

and estrogens (25). The decrease in total P450 levels in trout may indicate that DHEA has more of an androgenic than estrogenic effect in this model.

A third marker of peroxisome proliferation in rodents is hepatomegaly, the result of increased numbers and size of peroxisomes. Trout fed DHEA for 4-14 days had significantly elevated liver somatic indices compared to controls. The hepatomegaly observed with DHEA treatment of trout, however, does not appear to be due to peroxisome proliferation. Instead, it results from a combination of cell proliferation and hepatocyte enlargement. The proliferating cells appear to be biliary preductule cells, although the tumors produced by DHEA treatment are primarily mixed carcinomas and hepatocellular carcinomas. The existence of a liver stem cell, capable of differentiating into either hepatocytes or bile ductule cells has been proposed (26, 27). Recently it was demonstrated that cultured oval cells derived from carcinogen-treated rats exhibit properties of both hepatocyte and biliary cells (28). If the cells induced to proliferate by DHEA treatment are actually undifferentiated precursor or stem cells, then this could explain the tumor outcome. This preductule hyperplasia may also be the cause of the cholestasis and increases in serum bilirubin levels observed in DHEA treated trout.

The ultrastructural changes, including proliferation of rough endoplasmic reticulum, vesiculation of endoplasmic reticulum, mitochondrial swelling, and decreased glycogen indicate that there is production of a secretory protein. Similar changes have been reported as part of the normal reproductive cycle in mature female rainbow trout during the period of exogenous vitellogenesis (29). We found that DHEA treatment dramatically induced serum levels of the glycolipophosphoprotein vitellogenin in juvenile trout. Induction of this yolk protein precursor is mediated by the estrogen receptor, however, high doses of androgens have also been shown to increase its production in fish (30, 31). Ultrastructural examination of DHEA-treated fish revealed cytoplasmic clefts, possibly resulting from crystalline inclusion bodies. Secretory granules containing yolk-like crystalline structures have been reported in the liver of estradiol-treated goldfish (30). Many of the

ultrastructural changes observed with DHEA treatment appear to be due to the conversion of DHEA into androgens and/or estrogens.

Multilamellar lipid-like structures were observed in some hepatocytes from DHEA-fed trout. Myelin figures have been reported in cultured human epithelial cells treated with DHEA (32, 33). These structures may be related to DHEA's effects on lipid metabolism. Although DHEA has been shown to have anti-atherogenic and anti-obesity properties, its effects on serum cholesterol are mixed. In this experiment, cholesterol levels were increased by DHEA treatment. Loria *et al.* (34), found that the effects of dietary DHEA on cholesterol in C57BL/6J mice were dependent on diet, with DHEA lowering plasma cholesterol in mice fed a standard semipurifed diet and raising it in animals fed a hyperlipemic diet (1% cholesterol and 0.5% cholic acid added). Our diet resembles the hyperlipemic diet in that it contains 10% salmon oil and 0.35% choline (10). In addition, the observed increases in serum cholesterol might be related to the increases in vitellogenin because about 10% of the lipid content of rainbow trout vitellogenin is cholesterol (35).

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Chapter 5

COMPARISON OF THE ENHANCING EFFECTS OF DEHYDROEPIANDROSTERONE WITH THE STRUCTURAL ANALOG 16α-FLUORO-5-ANDROSTEN-17-ONE ON AFLATOXIN B_1 HEPATOCARCINOGENESIS IN RAINBOW TROUT

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ABSTRACT

Dehydroepiandrosterone (DHEA) is an adrenal steroid with chemoprotective effects against a wide variety of conditions including cancer, obesity, diabetes, and cardiovascular disease. However, DHEA is also a carcinogen in laboratory animals, possibly through its function as a precursor of sex steroids or through peroxisome proliferation. The structural analog, 16α-fluoro-5-androsten-17-one (8354) has been reported to have enhanced chemopreventive activity without the steroid precursor and peroxisome proliferating effects of DHEA. This study compares DHEA and 8354 in rainbow trout, a species that is resistant to peroxisome proliferation but is highly susceptible to the carcinogenic and tumor enhancing effects of DHEA. Trout were exposed as fry to aflatoxin B_1 (AFB_1) or given a sham exposure, then were fed diets containing 444 ppm DHEA or 8354 for six months. Post-initiation treatment with DHEA significantly increased liver tumor incidence, multiplicity, and size compared to initiated controls. The analog 8354 slightly increased tumor incidence ($p<0.06$) but had no effect on multiplicity or size. Six percent of trout treated with DHEA alone developed tumors, while no tumors occurred in non-initiated trout fed control or 8354 containing diets. Serum levels of androstenedione were elevated by DHEA- (48-fold) or 8354- (6-fold) treatment. Serum ß-estradiol titers were increased in DHEA- but not 8354-treated trout. Vitellogenin was induced significantly by either DHEA (434-fold) or 8354 (21-fold). Peroxisomal ß-oxidation was not increased by either compound and catalase activity was decreased in DHEA-treated animals. This research suggests that in rainbow trout the tumor enhancing effects of DHEA may be due to its function as a sex steroid precursor and are unrelated to peroxisome proliferation. These carcinogenic properties are reduced in the analog 8354 which has been advocated as an alternative to DHEA for chemoprevention.

INTRODUCTION

Dehydroepiandrosterone (DHEA), is the major circulating hormone in human plasma. This adrenal steroid is secreted in a pattern that peaks in early adulthood, then declines with age (1). The function of DHEA is largely unknown except as a precursor of other hormones. Low levels of DHEA have been associated with a number of disease conditions including breast cancer (2), bladder cancer (3), lung cancer (4), HIV, and Alzheimer's disease (5). In animal models, DHEA has been shown to be protective towards a wide variety of conditions including atherosclerosis, diabetes, obesity, and cancer (6). The cancer chemoprotective effects of this compound may be due to its action as an inhibitor of glucose-6-phosphate dehydrogenase (G6PD) with subsequent depletion of NADPH and ribose-5-phosphate intermediates (7).

DHEA, however, has several properties that limit its use as a chemopreventive agent. DHEA is a precursor for androgens and estrogens and, in human clinical trials has been shown to increase androgen levels in female subjects (8, 9). In addition, DHEA is a peroxisome proliferator (10-12) and rodent carcinogen (13). Exposure to peroxisome proliferators leads to increases in the number and size of peroxisomes, hepatomegaly, and induction of peroxisomal and microsomal enzymes involved in fatty acid metabolism (14, 15). Prolonged exposure to peroxisome proliferators results in hepatocarcinogenesis in some species. Rats and mice are particularly sensitive to the peroxisome proliferating effects of these compounds, whereas, primates appear to be relatively resistant (16). A recent review concluded that peroxisome proliferators are a discreet class of non-genotoxic rodent-specific carcinogens with little potential as hepatocarcinogens in humans, and therefore, the risk to humans was judged to be insignificant (17).

We have recently shown, however, that DHEA is also carcinogenic in rainbow trout (18), a species that resembles humans in being relatively insensitive to peroxisome proliferation (19, 20). Trout are particularly sensitive to the carcinogenicity and tumor

enhancing effects of DHEA, despite being insensitive to its peroxisome proliferating effects. DHEA is carcinogenic in trout at doses 20-fold lower than the dose which is hepatocarcinogenic in rats. It is possible that the carcinogenicity of DHEA is related to the capacity of this compound to be metabolized to androgens and estrogens, rather than as a peroxisome proliferator.

The DHEA structural analog 16α-fluoro-5-androsten-17-one (8354) is reported to maintain many of the chemoprotective effects of DHEA with minimal side effects (21). This analog is a stronger inhibitor of G6PD activity than DHEA but does not appear to be readily metabolized to androgens and estrogens, or cause peroxisome proliferation in rodents (22). DHEA analog 8354 had protective effects similar to DHEA against N-methyl-N-nitrosourea-induced mammary carcinogenesis (23), was protective towards colon and small intestine cancer in azoxymethane-initiated male F344 rats (24), and was more potent as an inhibitor of dimethylbenz[a]anthracene-initiated, tetradecanoylphorbol-acetate-promoted skin papillomas than was DHEA (25). In addition to its cancer chemoprotective effects, 8354 also reduced hyperglycemia without causing any increase in seminal vesicle weight in insulin-resistant diabetic mice (26).

This study compares the effects of dietary 8354 with those of DHEA towards AFB_1-initiated hepatocarcinogenesis in rainbow trout. As in mammals, 8354 had a much lower capacity to be metabolized to androgens and estrogens than DHEA. The analog 8354 was not hepatocarcinogenic at the dose level used in this study and had only slight enhancing effects on AFB_1-initiated carcinogenesis. DHEA, as previously reported was a complete carcinogen and dramatically enhanced AFB_1 hepatocarcinogenesis.

MATERIALS AND METHODS

Materials

DHEA analog 8354 was provided by Dr. Arthur G. Schwartz (Temple Univ., Philadelphia, PA). The vitellogenin antibody and standards were provided by Dr. David R. Idler (Memorial Univ. of Newfoundland; St. John's, Newfoundland, Canada). Proliferating cell nuclear antigen (PCNA) antibody was purchased from Paracelsian (Ithaca, NY). Western blotting reagents were obtained from Biorad (Hercules, CA). DHEA, AFB_1 and other reagents were purchased from Sigma Chem. Co. (St. Louis, MO).

Animals and treatments

This paper describes the results of two studies, a nine month tumor study and a two-week short-term experiment. Rainbow trout (*Oncorhynchus mykiss*) were hatched and reared at the Oregon State University Food Toxicology and Nutrition Laboratory. The tumor study used 800 animals; 500 of these were initiated as fry (75 days post-spawning) by a 30 min water bath exposure to 10 ppb AFB_1. The remainder were given a sham exposure (30 min in 0.01% ethanol). For the first three months after initiation, trout were fed Oregon Test Diet (OTD), a semi-purified, casein-based diet (27). Trout were then divided into experimental groups and fed modified OTD diets containing 444 ppm (w/w) DHEA or 8254 until the conclusion of the study 42 weeks after initiation. The modified OTD contained less vitamin E (100 mg/kg) than standard OTD but was still above the levels required for normal growth (28). Once on experimental diets, trout were fed *ad lib* (2.8-5.6% body wt.), five times per week. Each treatment group contained 100-120 trout, housed 50-60 per tank in duplicate 100 gal continuous flow tanks (4 tanks of initiated controls were used). Trout were deeply anaesthetized by an overdose of MS-222 nine months post-initiation while still sexually immature. Blood was drawn from the caudal vein and then livers were removed and examined. Livers were fixed in Bouin's solution

for future histological examination. In the short-term study, 36 fish, each weighing approximately 100 g were distributed into three two-foot tanks and fed one of the above experimental diets daily for 14 days.

Enzyme assays

Trout liver peroxisomal ß-oxidation and catalase activities were measured after two weeks of dietary DHEA or 8354. Animals were stunned by a blow to the head and killed by decapitation. This is an acceptable method of euthanasia according to the 1993 report of the AVMA.panal on euthanasia (29). Livers from four fish per diet were immediately removed and placed in 60 mM Tris, 0.25 M sucrose buffer (pH 8.3). Livers were homogenized, centrifuged at 600 g for 20 min (4°C), and the supernatant was centrifuged at 12,000 g (4°C) for 30 min to obtain the mitochondrial/peroxisomal fraction. The 12,000 g pellet was resuspended and protein concentration determined by the method of Lowry *et al.*, using bovine serum albumin as a standard (30). Peroxisomal ß-oxidation of palmitoyl-CoA was measured (in triplicate) by spectrophotometrically monitoring the reduction of NAD^+ to NADPH at 340 nm in the presence of cyanide (31, 32). Catalase activity was determined by measuring the decomposition of hydrogen peroxide spectrophotometrically at 240 nm (33).

G6PD activity was measured in whole blood obtained from trout fed control diet, DHEA, or 8354 for two weeks. The assay, which measures the production of NADPH from NADP in the presence of glucose-6-phosphate, was conducted using a Sigma diagnostic kit according to manufacturer's instructions except that incubations were performed at 30 rather than 37° C. Results were normalized to hemoglobin concentrations.

Electrophoresis and Immunoblotting

PCNA levels were determined in liver homogenates from the two-week experiment (prepared in 60 mM tris, 0.25 M sucrose buffer as described above). Proteins were

separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels (34) and electrophoretically transfered to nitrocellulose (Biorad Trans-Blot®). Blots were probed with a mouse monoclonal antibody to rat PCNA followed by a horseradish peroxidase-linked secondary antibody and proteins detected by chemiluminescence (Amersham Corp., Arlington Heights, IL). Western blots were scanned on a flatbed scanner (HP ScanJet IIcx) and analyzed on a Power Macintosh 7100 with the public domain software NIH Image version 1.54 (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov).

Serum Hormones

Serum ß-estradiol and androstenedione were determined by radioimmunoassay (Wein Laboratories, Succasunna, NJ) according to manufacturer's instructions except that, for the ß-estradiol assay, extractions were performed in ethyl acetate: hexane (3:2), isotope concentrations were halved, and antibody concentrations were decreased by three-quarters in order to increase sensitivity. Under these conditions, the lower limit of detection was 10 pg/ml.

Clinical Chemistry

Serum cholesterol, albumin, and serum glutamic-oxaloacetic transaminase (SGOT) were analyzed on a Ciba Corning 550 Express Clinical Chemistry Analyzer using manufacturer's protocols and reagents. Packed cell volume was determined by centrifugation. Serum vitellogenin was measured with an enzyme linked immunosorbent assay (35).

Statistical analysis

Differences in tumor incidence were assessed using Fisher's exact test (two-tailed). Body weights, liver weights, clinical values, and tumor size were log transformed and compared by ANOVA followed by the Scheffe's test for pairwise comparisons. The nonparametric Kruskal-Wallis test was employed to compare the average number of tumors per animal in trout with tumors using chi-square approximation of p-values. All statistical analyses were performed in SAS version 6.04 (SAS Institute, Cary, NC).

RESULTS

Dietary DHEA was a complete carcinogen in trout. Six percent of the sham-initiated trout fed 444 ppm DHEA developed liver tumors. No tumors occurred in sham-initiated animals given either control diet or 444 ppm 8354. Tumor incidence was ten-fold higher in AFB_1-initiated trout fed DHEA than in initiated trout fed control diet ($p < 0.0001$) (Fig. 5.1).

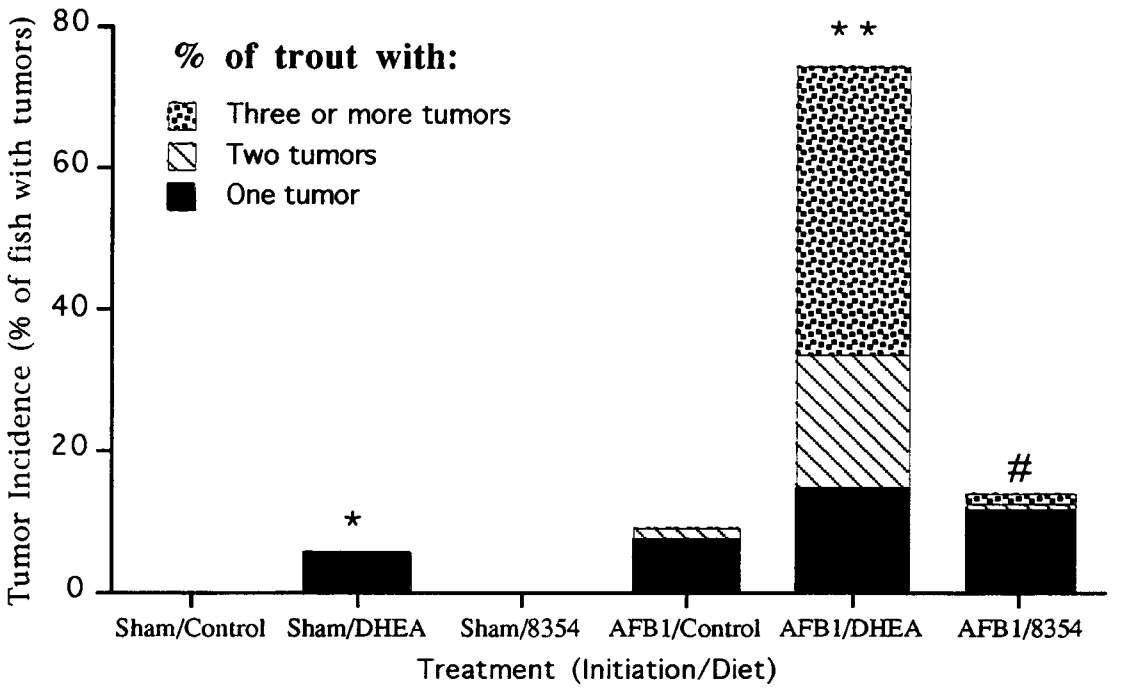

Figure 5.1. Liver tumor incidence and multiplicity in sham and AFB1-initiated trout fed control, 444 ppm DHEA, or 444 ppm 8354. Tumor incidence (bar height) and multiplicity (patterns) were determined by gross examination at the time of euthanasia and confirmed by hand slicing of fixed tissues. * indicates tumor incidence significantly different from noninitiated controls ($p < 0.05$). ** indicates tumor incidence and multiplicity significantly different from initiated controls ($p < 0.0001$). # indicates trend towards significance ($p = 0.06$) in tumor incidence.

Tumor size and multiplicity were also enhanced by DHEA treatment. The average tumor size (± SEM) in initiated controls was 1.7 ± 0.4 mm and the average number of tumors per tumor bearing animal was 1.2 ± 0.1. In initiated trout treated with DHEA the average tumor size was 2.7 ± 1.7 and multiplicity was 2.9 ± 0.2. Tumor incidence was slightly higher in animals treated with 444 ppm 8354 ($p < 0.06$). The analog had no significant effects on tumor size (1.7 ± 0.2) or multiplicity (1.3 ± 0.2).

Peroxisomal ß-oxidation and catalase activity were measured after two weeks on experimental diets to determine the extent of peroxisome proliferation (Fig. 5.2). Neither compound altered cyanide insensitive palmitoyl CoA oxidation. In contrast to the enhancing effects of DHEA on catalase activity in rodents (36), DHEA treatment in trout decreased peroxisomal catalase activity. We have also observed dose-dependent decreases in catalase activity in trout fed DHEA for 29 weeks (Chapter 2). Analog 8354, as in rodents, had no effect on this enzyme. These results are consistent with previous studies indicating that trout are relatively insensitive to peroxisome proliferation (19, 20).

G6PD activity was measured in red blood cells of trout fed experimental diets for 14 days. In contrast to what has been reported in rodents, G6PD activity was not inhibited by DHEA or 8354 (Fig. 5.3).

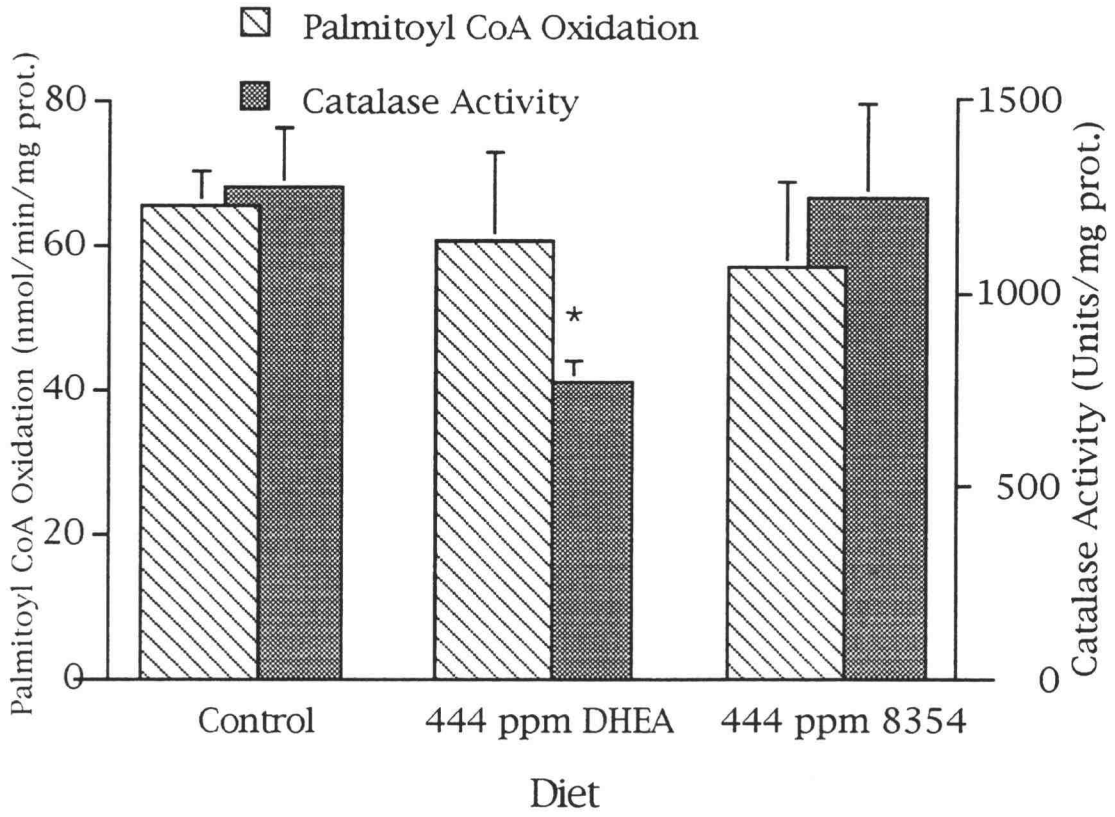

Figure 5.2. Peroxisomal ß-oxidation (left axis) and catalase activity (right axis) after two weeks of experimental diets. Each bar represents the mean (+ SEM) of palmitoyl CoA oxidation or catalase activity in four trout. Activities were measured as described in Materials and Methods.

Serum androstenedione and ß-estradiol levels were measured at the conclusion of the tumor study in order to monitor the potential for DHEA and the analog to be converted into other steroids (Fig. 5.4). Male and female trout of this age have very low titers of androgens or estrogens (37). Levels of androstenedione were determined to be elevated over 40-fold by DHEA treatment, however because the antibody used for this RIA has some cross reactivity with DHEA, part of this increase may represent serum DHEA levels (1 ng DHEA = 55 pg androstenedione equivalent). Androstenedione was also elevated by 8354 treatment (6-fold). ß-estradiol levels were significantly elevated by DHEA (3-fold)

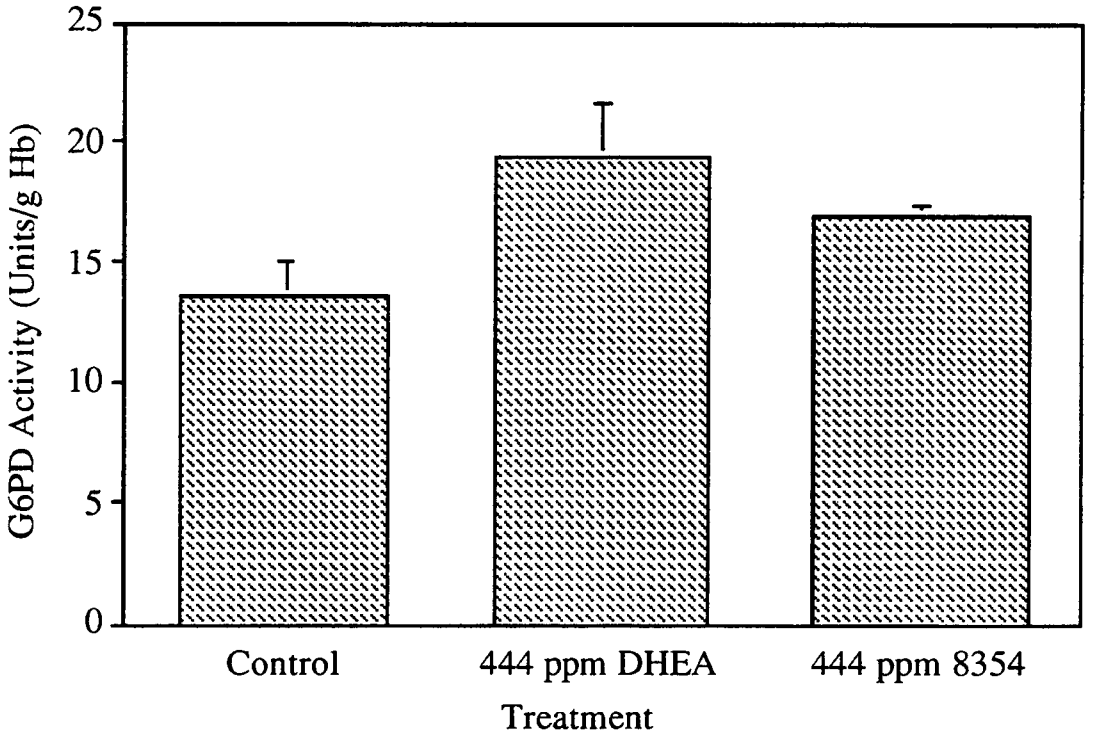

Figure 5.3. G6PD activity after two weeks of experimental diets. Each bar represents the mean (+ SEM) of G6PD activity in four trout. Activity was measured as described in Materials and Methods.

but not by compound 8354. Levels of vitellogenin, a yolk protein induced by ß-estradiol were increased from nondetectable (limit of detection of 2.5 µg/ml) to 50 µg/ml in the 8354-treated trout and to more than 1 mg/ml in the DHEA-treated animals. Both treatments resulted in a higher incidence of maturing males than in the control group (data not shown).

Some toxicity was observed in animals fed either DHEA or 8354 (Table 5.1). The average body weight at the conclusion of the tumor study was significantly lower in animals fed either DHEA or 8354 than in those fed control diets. There were no significant differences in body weights between sham and AFB1-initiated trout fed the same diets (therefore, only the data from sham-initiated trout are shown). The percentage of body weight made up by liver was twice as high in the DHEA-fed compared to control fish.

Liver somatic indexes in 8354-fed trout were 25% higher than in control animals. The increases in relative liver weight are partly due to the lower body weights, however absolute liver weights were also significantly higher in the DHEA-fed trout despite the lower body weights (Table 5.1). Livers of animals fed DHEA or 8354 for two weeks had higher levels of PCNA than untreated animals (6-fold and 3-fold respectively) (Fig. 5.5) indicating that cell proliferation is involved in the liver enlargement.

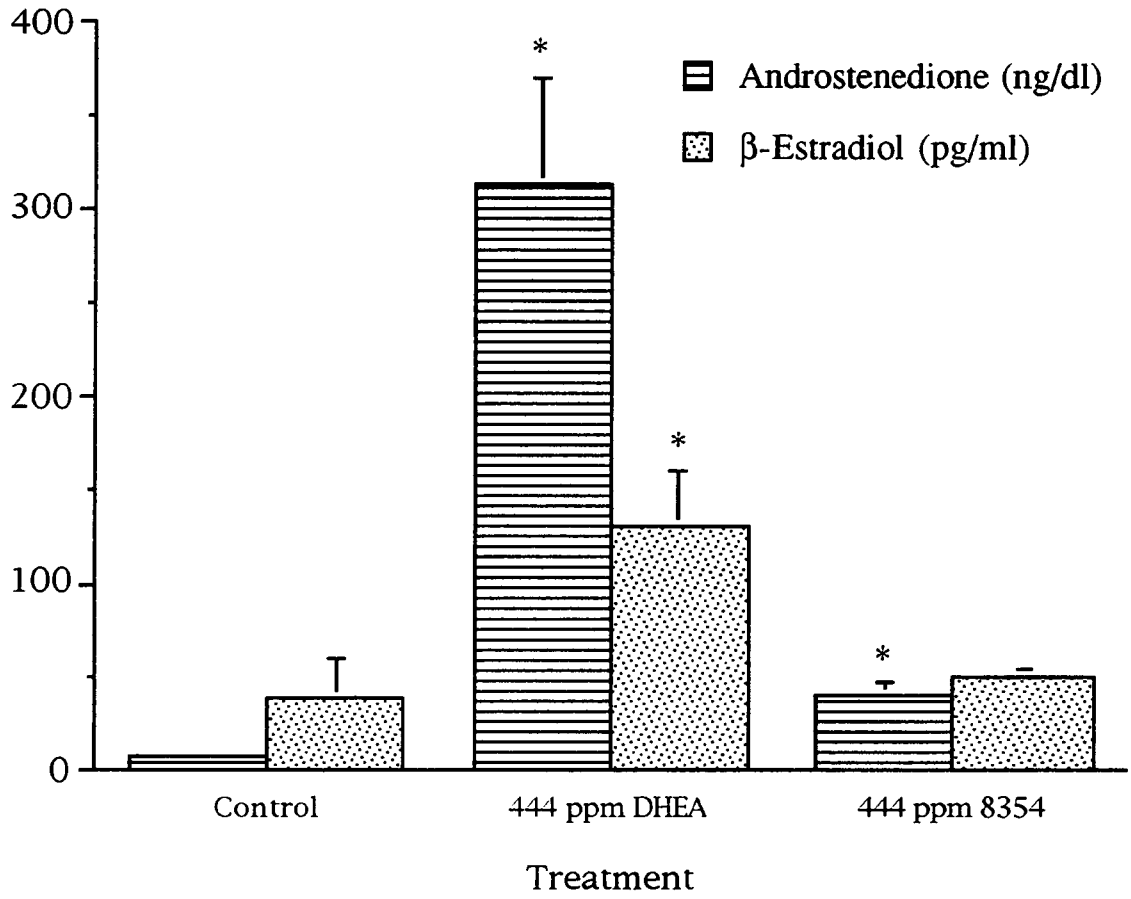

Figure 5.4. Serum androstenedione (ng/dl) and ß-estradiol levels after 26 weeks on experimental diets. Each bar represents the mean (+ SEM) of the serum hormone levels of four individual animals. * indicates significantly different from controls

Animals treated with DHEA or 8354 for 26 weeks had decreased packed blood cell volumes (Table 5.1). Hematocrits were lowest in the 8354-fed animals but were also significantly decreased by DHEA treatment. Serum cholesterol, albumin, and SGOT were all increased by DHEA treatment with values in the 8354-treated animals intermediate between control and DHEA-treated animals. No significant mortality was associated with any of the treatment groups.

Table 5.1. Clinical Parameters in Non-inititated Trout Fed Control, DHEA or 8354 Diets.

	Treatment		
	Control	**444 ppm DHEA**	**444 ppm 8354**
Number of Animals	98	101	92
Body Weight[a] (g)	$94.84^{b} \pm 2.90$	$80.89^{c} \pm 2.61$	$70.17^{b} \pm 2.97$
Liver Weight (g)	$0.69^{b} \pm 0.02$	$1.33^{c} \pm 0.04$	$0.73^{b} \pm 0.03$
Liver Somatic Index (%)	$0.74^{b} \pm 0.01$	$1.66^{c} \pm 0.03$	$1.04^{d} \pm 0.02$
Hematocrit[e] (%)	$34.50^{b} \pm 1.19$	$27.25^{c} \pm 1.32$	$20.75^{d} \pm 1.11$
Cholesterol (mg/dl)[e]	$88^{b} \pm 27$	$383^{c} \pm 31$	$248^{d} \pm 9$
Albumin (g/dl)[e]	$0.98^{b} \pm 0.33$	$2.98^{c} \pm 0.15$	$2.10^{b,c} \pm 0.40$
SGOT (IU/L)[e]	243 ± 86	462 ± 38	359 ± 70
Vitellogenin (µg/ml)[e]	$<2.5^{b}$	$1086^{c} \pm 88$	$53^{d} \pm 14$

[a]Values are means ± SEM. Numbers in the same row bearing different superscripts are significantly different from one another ($p < 0.05$).
[e]Values are means ± SEM of 4 samples per treatment.

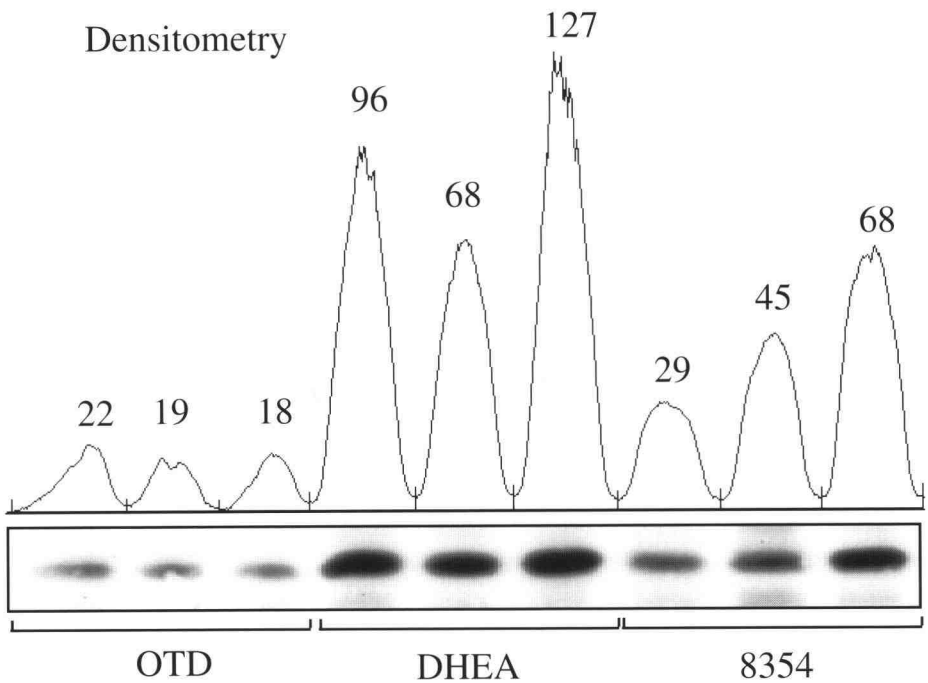

Figure 5.5. Western blot of trout liver homogenates probed with antibody against rat PCNA. Lanes 1-3 are from control animals, 4-6 from animals treated with 444 ppm dietary DHEA for 14 days, and 7-9 from trout treated with 444 ppm dietary 8354 for 14 days. Each lane contains 15 μg protein from liver homogenates.

DISCUSSION

DHEA is a promising chemopreventive agent, however the potential of this compound for human use is limited by its role as a precursor for sex steroids and its carcinogenic effects. In rats, the hepatocarcinogenicity of DHEA has been attributed to peroxisome proliferation (13), a mechanism which may have little or no relevance towards humans (17). However, we have demonstrated that DHEA is also a carcinogen in rainbow trout, a species which responds only weakly to peroxisome proliferators. Trout are quite sensitive to the carcinogenic and tumor promoting effects of DHEA with dietary doses as low as 222 ppm for 30 weeks significantly enhancing AFB_1-initiated hepatocarcinogenesis (18). In the current study we use this highly sensitive model to compare the tumor modulating effects of DHEA with those of its structural analog 8354.

Limited quantities of 8354 were available, therefore only a single dose level was tested. We selected a DHEA dose which had previously been shown to produce a moderate incidence of tumors in animals treated with DHEA alone, and to markedly enhance AFB_1-initiated hepatocarcinogenesis. This dose, however did have some toxicity associated with it as hematocrits were decreased, and cholesterol and albumin levels increased by these experimental diets. The decreased hematocrits do not appear to be due to inhibition of G6PD, as the activity of this enzyme in red blood cells was not decreased by DHEA or 8354 treatment. Both DHEA and 8354 decreased trout weight gain compared to animals on control diet. This was not unexpected because DHEA has received considerable attention for its antiobesity properties (38). Compound 8354 has previously been shown to be more effective than DHEA in reducing weight gain in BALB/cJ mice (22) and this appears to be true in trout as well.

The dose of DHEA employed in this study strongly enhanced AFB_1-initiated carcinogenesis, producing increases in tumor incidence, tumor size, and tumor multiplicity, and also was a complete carcinogen in this model. In contrast, the analog did not produce

tumors when fed to sham-initiated trout. In AFB_1-initiated trout, tumor incidence was slightly higher in the 8354-treated animals than in fish fed control diet, however this difference was not statistically significant ($p = 0.06$). Tumor size and multiplicity were not enhanced by treatment with the analog. The markedly different effects of these two compounds on carcinogenesis in trout supports the hypothesis that conversion of DHEA to sex steroids plays an important role in its biological effects.

Although trout are weak responders to peroxisome proliferators, we cannot rule out the possibility that DHEA carcinogenicity in trout is linked to its peroxisomal effects. In trout, dietary DHEA did not change peroxisomal ß-oxidation but decreased peroxisomal catalase. Treatment with 8354 had no effect on either activity. One proposed mechanism for the carcinogenicity of peroxisome proliferators is based on the differential induction of hydrogen peroxide generating and degrading enzymes and resultant oxidative stress (39, 40). Even though the hydrogen peroxide generating pathway of peroxisomal ß-oxidation was not increased in trout, DHEA treatment decreased catalase activity, a situation that may have resulted in a pro-oxidant condition. Hepatomegaly is one of the classic markers of peroxisome proliferation in rats and mice. DHEA, and to a lesser extent 8354, caused enlarged livers in trout as well. Histological and ultrastructural studies of livers of DHEA-treated trout in a previous study suggested that the liver enlargement was not due to peroxisome proliferation, but instead may be due to androgenic or estrogenic effects (41). In the current study, the liver somatic indices were significantly higher in DHEA- than in 8354-treated animals and correlated well with the intracrine and tumor enhancing effects of the two compounds.

In mammals, DHEA serves as a precursor of both androgens and estrogens (42). Although circulating levels of DHEA and the sulfate are quite low in trout, a trout 3ß-hydroxysteroid dehydrogenase Δ^{5-4}-isomerase has recently been cloned which, *in vitro*, has high activity for the conversion of DHEA to androstenedione (43). We found that dietary DHEA-administration significantly elevated serum androstenedione and ß-estradiol.

These steroids were only slightly elevated in trout fed 8354, so it appears that in trout, as in mammals, 8354 is considerably less effective as a sex steroid precursor than DHEA. Further support for this comes from serum levels of the estrogen-inducible yolk protein vitellogenin. Vitellogenin was induced over 400-fold in animals treated with DHEA, but only about 20-fold in 8354-treated animals. Vitellogenin levels in DHEA-treated fish were higher than would be predicted from the serum estradiol levels (44), however high androgen doses have also been shown to induce vitellogenin in some fish species (45, 46). Limited conversion of 8354 to estrogens and androgens could explain why this compound is considerably less effective at enhancing AFB_1 carcinogenesis in trout than DHEA. ß-estradiol has been shown to be a potent tumor promoter in this model (47). If the carcinogenic and tumor enhancing effects of DHEA are due to its steroid precursor properties, then this mechanism could be relevant to humans.

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Chapter 6

MODULATION OF AFLATOXIN-B_1 HEPATOCARCINOGENESIS IN TROUT BY DEHYDROEPIANDROSTERONE; INITIATION/ POSTINITIATION AND LATENCY EFFECTS

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ABSTRACT

Dehydroepiandrosterone (DHEA) is a strong enhancer of aflatoxin B1 (AFB_1) hepatocarcinogenesis in trout, producing increases in tumor incidence, tumor multiplicity, and tumor size when administered after carcinogen exposure. This paper describes studies examining the effects of DHEA on the time to tumor development, and the modulation of carcinogenesis when DHEA is given prior to and during AFB_1 exposure. In the first experiment, trout were initiated by a 30 min water bath exposure to 10 ppb AFB_1. Three months post-initiation, animals were started on either control diet or diet containing 444 ppm DHEA. Fifty trout per treatment were sampled prior to the start of experimental diets, and then at monthly intervals for the next seven months and examined for the presence of tumors. Tumors were not detected in initiated controls until seven months after initiation. In initiated trout fed DHEA, the first tumor was detected five months after initiation (after just two months of dietary DHEA). Six months post-initiation, twenty percent of the AFB_1-initiated trout fed DHEA had tumors, while no tumors were visible in either AFB_1-initiated controls, or noninitiated trout fed DHEA. This experiment shows that, in addition to increasing tumor incidence, tumor size, and tumor multiplicity DHEA also decreases tumor latency. The second experiment was designed to determine if the enhancing effect of DHEA on AFB_1 carcinogenesis is dependent on the time of DHEA administration relative to the time of AFB_1 exposure and if DHEA could be chemopreventive if administered prior to and concurrent with AFB_1. Trout were fed one of two levels of DHEA (888 or 1776 ppm) either prior to and during a four week initiation period of dietary AFB_1 administration, or for eight weeks following initiation with AFB_1. Nine months after initiation livers were examined for tumors. Neither exposure protocol provided protection towards AFB_1 hepatocarcingenesis. The strongest enhancement occurred when DHEA was fed during the postinitiation period. Levels of p53 and p34 cdc2 were decreased by DHEA treatment, indicating that DHEA may act through alterations in cell cycle control.

INTRODUCTION

Dehydroepiandrosterone (DHEA) is an adrenal steroid that in animal models has been demonstrated to have chemoprotective properties towards a wide variety of conditions including atherosclerosis, obesity, diabetes, immune disorders, and cancer (1, 2). DHEA is protective towards both the initiation and promotion stages of cancer, possibly through its action as an inhibitor of glucose-6-phosphate dehydrogenase (3). DHEA, however, also causes hepatomegaly, proliferation of peroxisomes, and increases in peroxisomal ß-oxidation (4-9); characteristics of a group of nongenotoxic rodent carcinogens known as peroxisome proliferators (PPs) (10). Like other PPs, DHEA is a carcinogen in rats (11-13), a species highly susceptible to both the peroxisome proliferating and carcinogenic effects of these compounds (14, 15).

We have found that DHEA is also a hepatocarcingen in rainbow trout (16), a species which responds only weakly to PPs (17, 18). In addition, DHEA strongly enhanced aflatoxin B_1 (AFB_1) hepatocarcinogenesis in trout, causing increases in tumor incidence, tumor multiplicity, and tumor size when administered following AFB_1 treatment. These effects were largely independent of peroxisome proliferation with less than a two-fold increase in peroxisomal ß-oxidation after 26 weeks on experimental diets (16).

The studies described in this paper were undertaken to further characterize the enhancement of AFB_1 carcinogenesis by DHEA. The first study examines the potential of DHEA to decrease the time to tumor appearance in AFB_1-initiated trout. We have previously shown that DHEA enhances AFB_1 carcinogenesis, causing increases in tumor incidence, tumor multiplicity, and tumor size (16). A fourth measure of enhancement of carcingenesis is a decrease in tumor latency. The second experiment was designed to determine if DHEA enhancement of AFB_1-initiated hepatocarcingenesis is dependent on the sequence of DHEA and carcinogen administration. Chemoprevention in one experimental

protocol and enhanced carcinogenesis in another has been observed with other compounds. For example, the tumor modulator indole-3-carbinol inhibits AFB_1 carcinogenesis when fed prior to and during carcinogen exposure, but promotes it when fed subsequently (19). Dietary DHEA has been shown to inhibit DNA binding of AFB_1 to hepatic DNA of rats when fed for two weeks prior to AFB_1 treatment (20), therefore we felt it was possible that pretreatment of trout with DHEA could provide protection towards AFB_1 carcinogenesis.

In this paper we report that, in addition to causing increases in tumor incidence, multiplicity, and size, postinitiation treatment with DHEA also decreases tumor latency in AFB_1-initiated trout. Treatment of trout prior to and during carcinogen administration increased AFB_1 hepatocarcingenesis, however the strongest enhancement occurred when DHEA was fed during the postinitiation period. Administration of DHEA for 8 or 12 weeks produced alterations in the levels of p53 and p34 cdc2, proteins involved in the regulation of the cell cycle. The tumor enhancing and carcinogenic effects of DHEA may be related to its effects on cell proliferation and cell cycle control.

MATERIALS AND METHODS

Materials

DHEA (5-androsten-3b-ol-17-one) and AFB_1 were purchased from Sigma Chemical Co. (St. Louis, MO). Western blotting reagents were purchased from Biorad (Hercules, CO) and Amersham (Arlington Heights, IL). Antibodies to proliferating cell nuclear antigen (PCNA) and p34 cdc2 were obtained from Paracelsian (Ithaca, NY). Antibody to p53 was purchased from Oncogene Science, Inc. (Uniondale, NY). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were spawned and raised at the Food Toxicology and Nutrition Laboratory, Oregon State University. Animals were maintained at 12-14° in circular tanks with continuously flowing well water and alternating 12-hr light and dark cycles. After spawning, trout were fed Oregon test diet (OTD), (21) until the start of the experiments.

Experimental Designs

Experiment 1. This experiment was designed to determine if treatment with DHEA decreased the time to tumor development in AFB_1-initiated trout. Ten weeks after spawning, fry were given a 30-min water bath exposure to either 10 ppb AFB_1 or a vehicle control (0.02% ethanol). Trout were fed modified OTD (100 mg/kg vitamin E) for three additional months, then were placed into treatment groups (400 fish/tank, one tank/treatment) and fed either modifed OTD or the same diet containing 444 ppm DHEA (w/w). Twenty-five fish per treatment were sampled prior to the start of experimental diets (3 months post-initiation) and then fifty fish per treatment were sampled at monthly intervals for the next seven months (until 10 months post-initiation) (Fig. 6.1).

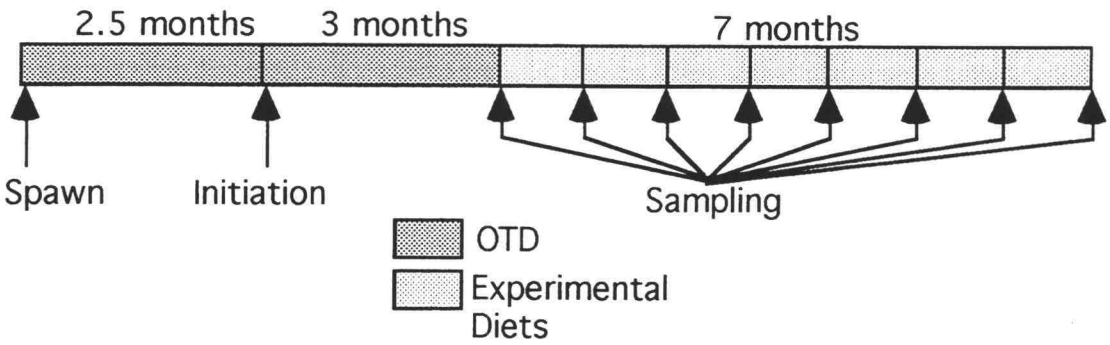

Figure 6.1. Timecourse of DHEA enhancement of AFB_1-initiated hepatocarcinogenesis (Experiment 1).

Experiment 2. This experiment was designed to determine if pre- and co-treatment of trout with DHEA relative to AFB_1 exposure would enhance or inhibit AFB_1-initiated carcinogenesis.

Figure 6.2. Time dependency of DHEA promotion of AFB_1-initiated hepato-carcinogenesis (Experiment 2).

Five months after spawning, fry were placed into treatment groups (120 fish/tank, 1 or 2 tanks/treatment) and started on a three phase dietary exposure protocol consisting of an eight week preinitiation phase, a four week initiation phase, and an eight week postinitiation phase (Fig. 6.2). During the preinitiation phase, animals were fed modified OTD (100 mg/kg vitamin E, rockfish oil base) containing 0, 888 or 1776 ppm DHEA (w/w). For the initiation phase, trout were fed diets containing the same levels of DHEA plus 0, 9, or 44 ppb AFB_1. During the postinitiation phase, animals were given 0, 888, or

Following the postinitiation phase, all animals were fed modified OTD for 30 weeks until the conclusion of the experiment.

Electrophoresis and Immunoblotting

Four fish per treatment were sampled at the end of each phase of experiment 2 in order to examine the effects of DHEA on cell proliferation and cell cycle control. Livers were removed, frozen in liquid nitrogen, and stored at -80° C until used. They were homogenized in ice cold sample buffer (10 mM potassium phosphate, pH 7.5, 20% glycerol, 0.1 mM EDTA, 0.2 mM PMSF) and protein concentrations determined according to Lowry et al. (22), using bovine serum albumin as standards. Proteins (20 µg/lane) were separated using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (23) and electrophoretically transferred to nitrocellulose (Biorad Trans-Blot®). Blots were probed with antibodies to p53 (Ab-3, mouse monoclonal), p34 cdc2 (anti-mouse, rabbit polyclonal), and PCNA (anti-rat, mouse monoclonal). Blots were then probed with horseperoxidase-linked secondary antibodies and proteins detected by chemiluminescence (Amersham Corp., Arlington Heights, IL).

Tumor Detection

Trout were killed by an overdose of 3-aminobenzoic acid ethyl ester (MS-222) and livers removed and weighed. Livers were examined for tumors under a dissecting microscope, then were fixed in Bouin's solution for future histological examination. After 48 hours livers were hand sliced to verify the presence of grossly observed tumors and to detect internal tumors.

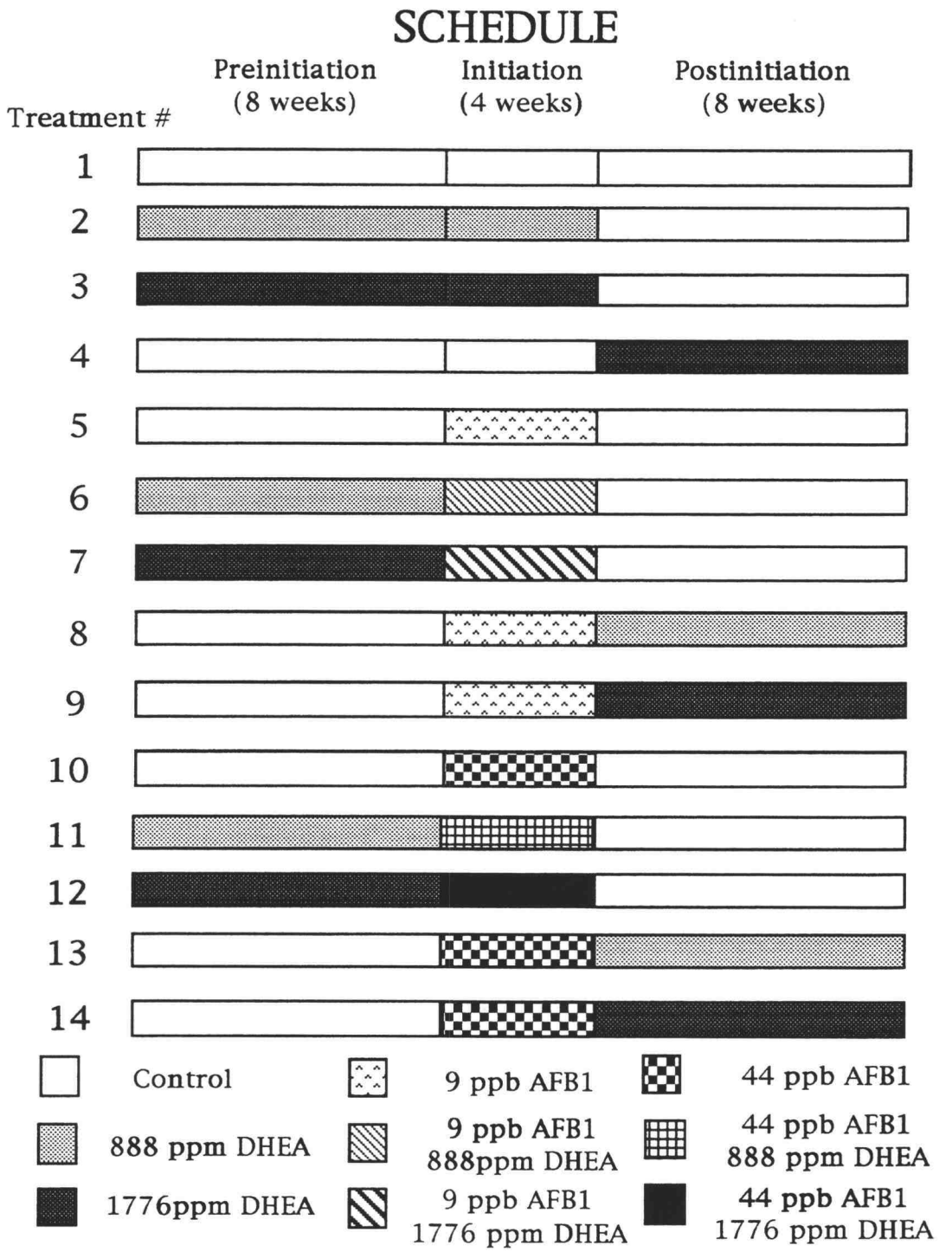

Figure 6.3. Experimental groups of time dependency study (Experiment 2).

RESULTS

Experiment 1

Treatment with DHEA decreased the growth rate of trout. Body weights were significantly lower in DHEA-fed animals beginning three months after the start of experimental diets and at each subsequent time point (Fig. 6.4). On the sixth month of experimental diets, the AFB_1-treated trout fed DHEA had significantly lower weights than sham-initiated trout fed DHEA, otherwise there were no significant differences in body weights between sham and AFB_1-initiated animals.

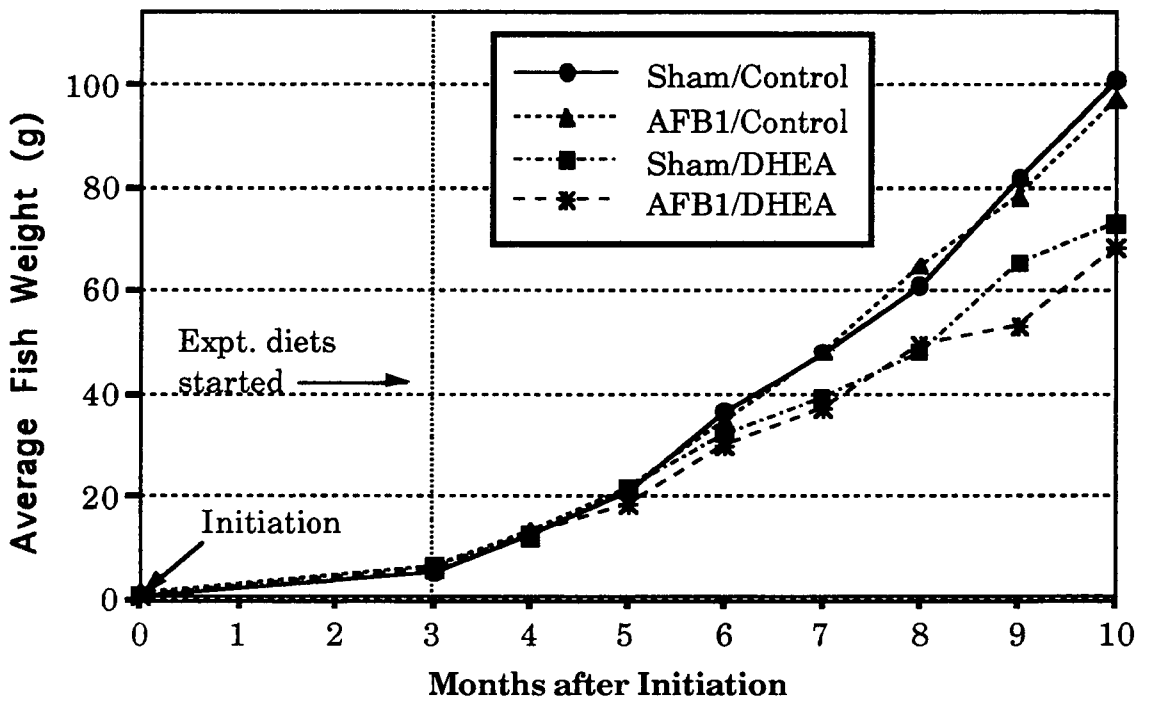

Figure 6.4. The effect of dietary DHEA on body weights. Values are means of 50 measurements per treatment group at each timepoint except 3 months post-initiation when only 25 animals were sampled.

Post-initiation treatment with DHEA caused hepatomegaly. Both absolute (Fig. 6.5) and relative liver weights (not shown) were significantly higher in the DHEA-treated compared to control-fed animals at each timepoint after the start of experimental diets. There were no significant differences in liver weights between sham and AFB_1-initiated trout.

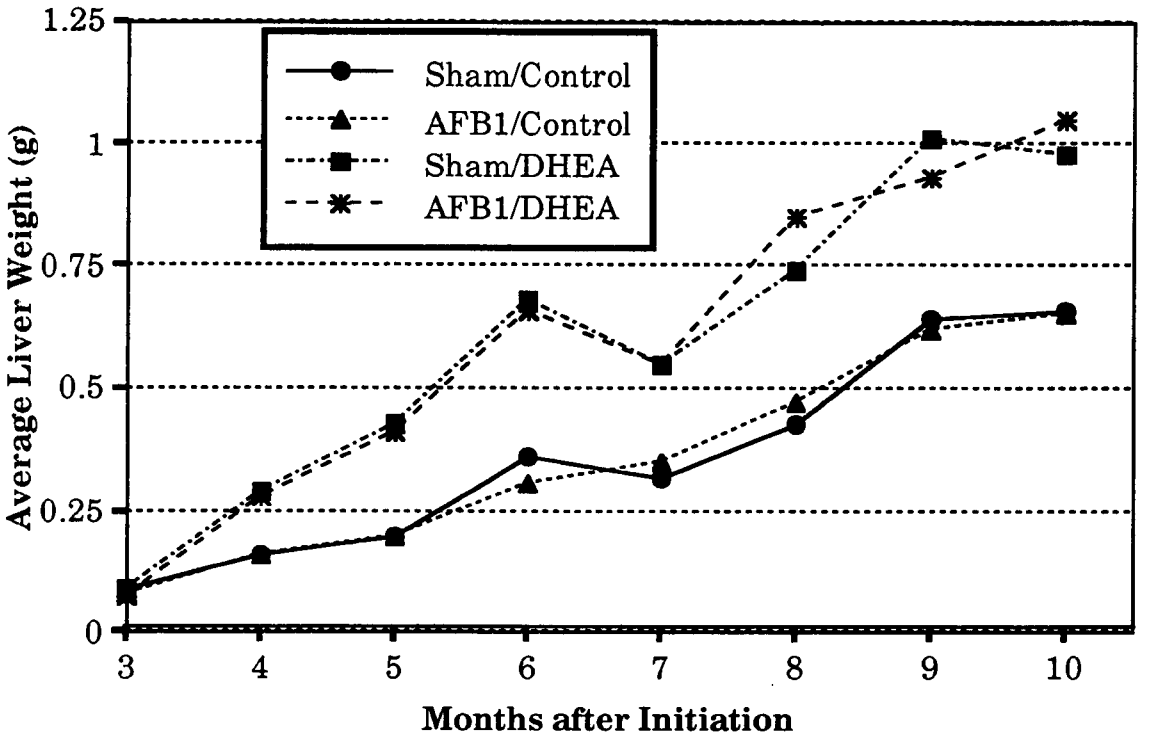

Figure 6.5. The effect of dietary DHEA on absolute liver weights. Values are means of 50 measurements per treatment group at each timepoint except 3 months post-initiation when only 25 animals were sampled.

Tumor incidence was enhanced by DHEA treatment (Fig. 6.6). At each timepoint beginning six months post-initiation, tumor incidence was significantly higher in AFB_1-initiated animals fed 444 ppm DHEA, than in initiated animals given control diet. In addition, tumors appeared sooner in the DHEA-fed trout. The first tumors appeared in

AFB_1-initiated/DHEA promoted animals five months after initiation (after two months of dietary DHEA). No tumors were detected in initiated control animals until seven months post-initiation. DHEA was also a complete carcinogen in this experiment, producing 2 and 8 percent tumor incidences in sham-initiated trout receiving 444 ppm dietary DHEA for six and seven months, respectively. As expected, the spontaneous liver tumor incidence was quite low, with only one liver tumor observed in the 375 sham-initiated animals fed control diet.

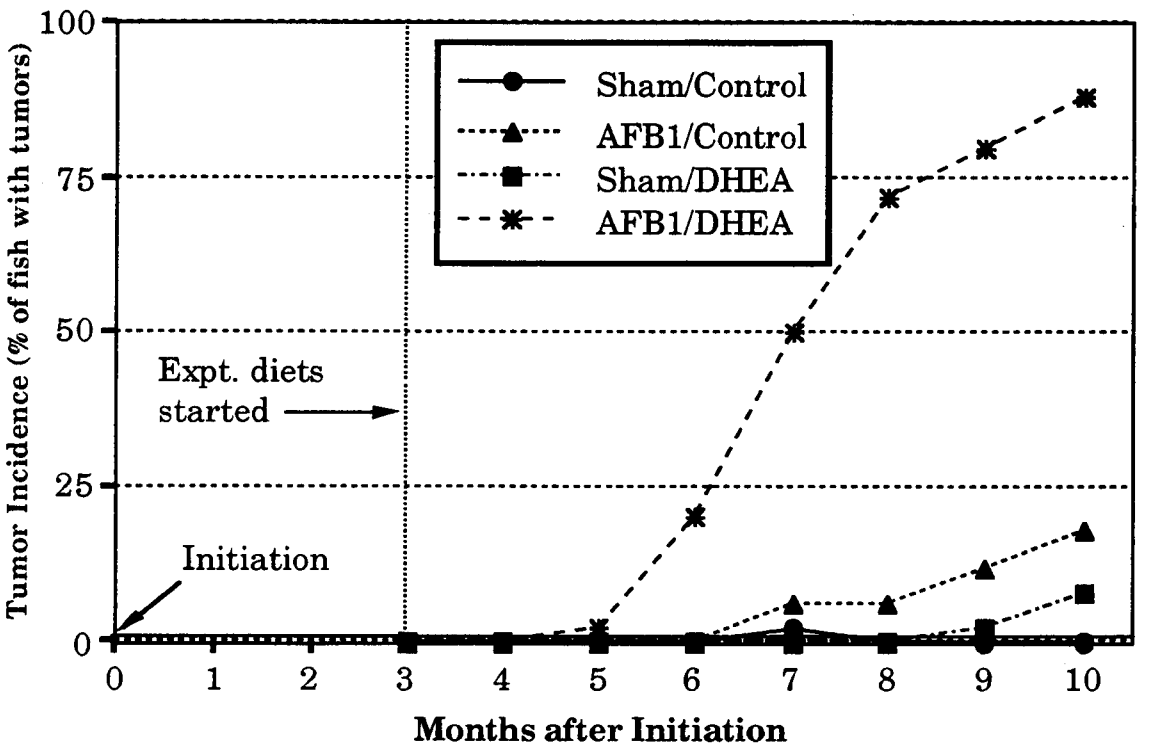

Figure 6.6. The effect of dietary DHEA on liver tumor incidence (% of animals with liver tumors). Fifty animal were sampled at each timepoint except 3 months post-initiation when only 25 animals were sampled.

In addition to increasing tumor incidence, DHEA treatment also increased tumor multiplicity (Table 6.1). There were significantly more tumors per liver in initiated trout fed DHEA than in tumor bearing animals which were fed control diet. Ten months after

initiation, the average number of tumors in initiated control trout was 1.1 ± 0.1 (± SEM). In initiated animals treated with DHEA the average number of tumors per liver was 4.0 ± 0.5. The average tumor size was also larger in DHEA-treated animals at every timepoint, however, the differences in tumor size were not statistically significant in this experiment (Table 6.2).

Table 6.1. The effect of dietary DHEA on tumor multiplicity (expressed as average number of tumors per liver in tumor bearing animals). * indicates significantly higher tumor multiplicity than initiated controls ($p \leq 0.05$).

Treatment	Months after Initiation					
	5	6	7	8	9	10
Sham/Control	0	0	1.0	0	0	0
AFB_1/Control	0	0	1.3	1.0	1.2	1.1
Sham/DHEA	0	0	0	0	1.0	1.3
AFB_1/DHEA	2.0	1.1	2.0	2.5	2.2	4.0*

Table 6.2. The effect of dietary DHEA on average tumor size (mm diameter).

Treatment	Months after Initiation					
	5	6	7	8	9	10
Sham/Control	0	0	1.0	0	0	0
AFB_1/Control	0	0	1.2	1.2	1.0	1.3
Sham/DHEA	0	0	0	0	2.0	1.3
AFB_1/DHEA	0.5	1.4	1.9	1.7	2.0	2.2

Experiment 2

Treatment of noninitiated trout with 888 or 1776 ppm dietary DHEA for twelve weeks (during the preinitiation and initiation phases of experiment 2) produced significant increases in tumor incidence and multiplicity compared to controls (Fig. 6.7).

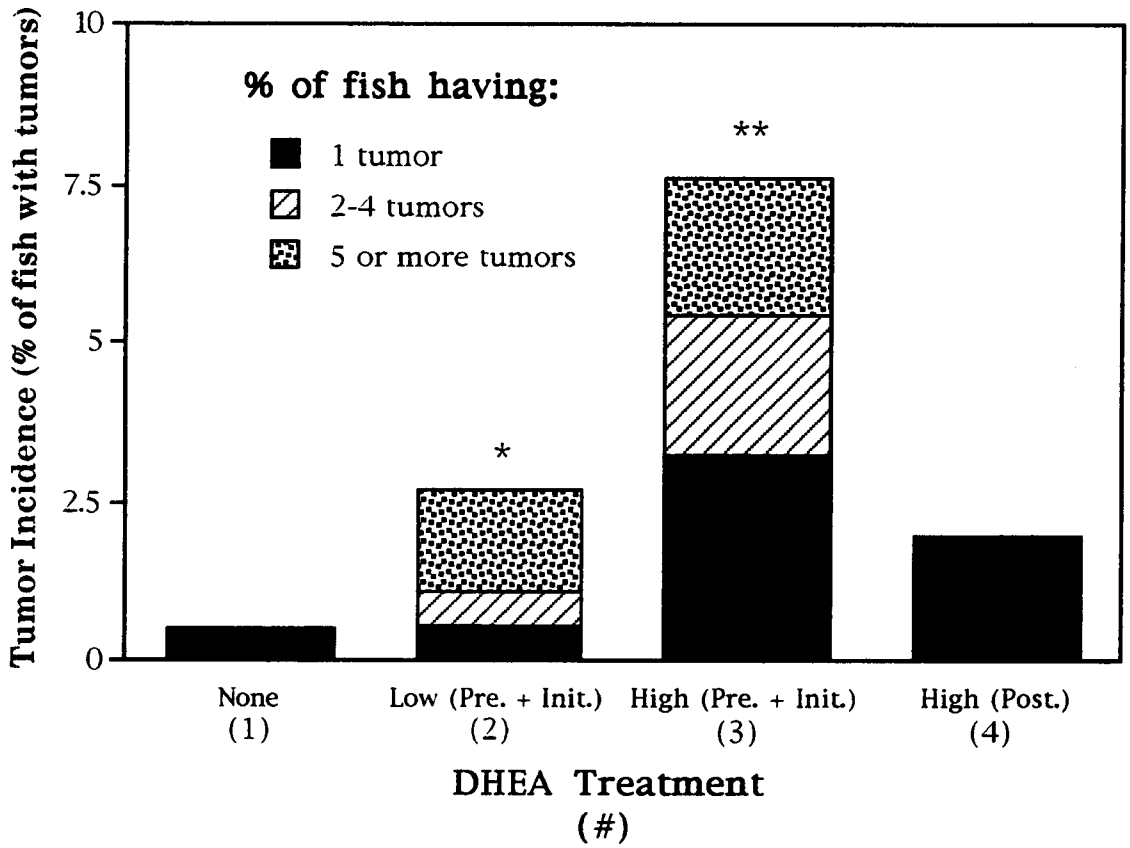

Figure 6.7. Tumor incidence and multiplicity in noninitiated trout fed low (888 ppm) or high (1776 ppm) DHEA for 12 weeks (treatment 2 and 3) or high DHEA for 8 weeks (treatment 4). Treatments marked with different symbols (*) are significantly different from one another in tumor incidence and multiplicity ($p < 0.05$). Treatment numbers are from figure 6.3.

Figure 6.8 shows tumor incidence and multiplicity in trout that were initiated with 9 ppb dietary AFB_1. Treatment with DHEA prior to, and during AFB_1 initiation increased tumor incidence and multiplicity, but only to levels approximating the additive effects of AFB_1 and DHEA carcinogenicity. Postinitiation treatment significantly enhanced tumor incidence and multiplicity.

Tumor incidences in animals initiated with 44 ppb dietary AFB_1 were only slightly higher than in those initiated with 9 ppb AFB_1 (Fig. 6.9). The pattern of enhancement by DHEA was similar to that observed with 9 ppb AFB_1 except that postinitiation treatment

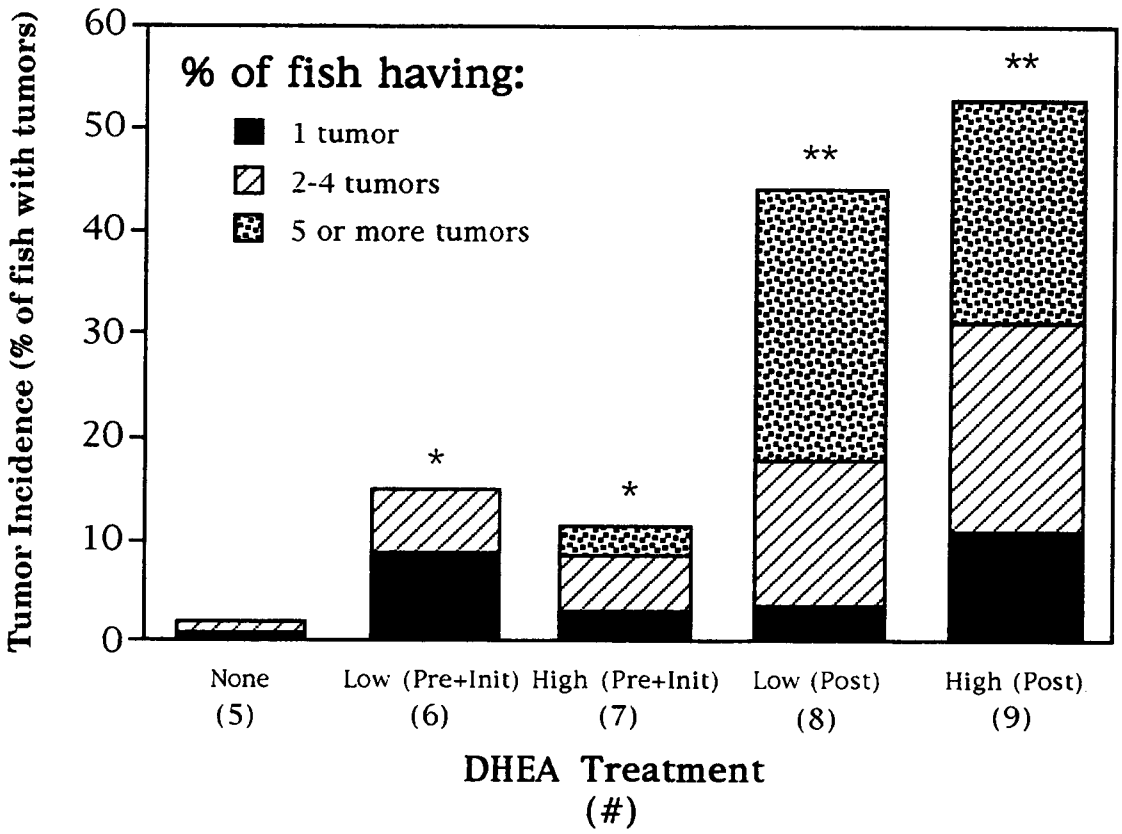

Figure 6.8. Tumor incidence and multiplicity in trout initiated with 9 ppb AFB_1. Treatments marked with different symbols (*) are significantly different in tumor incidence and multiplicity from one another ($p < 0.05$). Treatment numbers are from figure 6.3.

with 888 ppm DHEA did not produce a significantly higher tumor incidence compared with pretreatment.

At the end of each phase of the experiment the levels of PCNA, p53, and p34 cdc2 were measured in order to determine the effects of DHEA on cell proliferation and cell cycle control. Figure 6.10 is a Western blot of trout liver homogenates from animals sampled at the end of the preinitiation period probed with an antibody to rat PCNA. After eight weeks of dietary DHEA, there are no treatment related differences in the levels of this antigen.

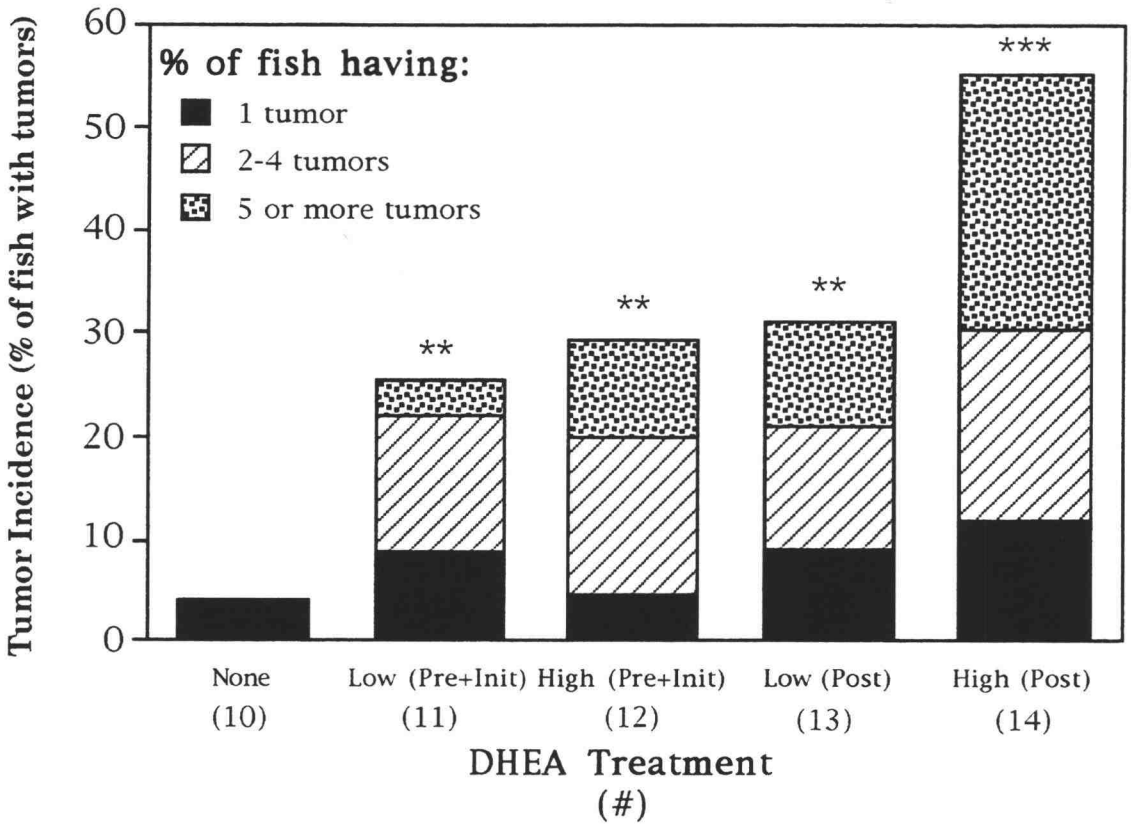

Figure 6.9. Tumor incidence and multiplicity in trout initiated with 44 ppb AFB_1. Treatments marked with different symbols (*) are significantly different in tumor incidence and multiplicity from one another ($p < 0.05$). Treatment numbers are from figure 6.3.

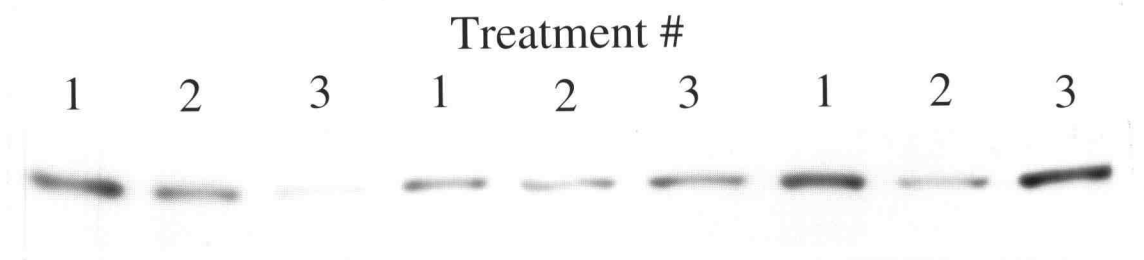

Figure 6.10. Western blot probed with antibody to PCNA. Lanes marked with #1 are from animals which were fed control diets, those marked with #2 were from trout fed 888 ppm DHEA for eight weeks, and those marked with #3 were from trout fed 1776 ppm DHEA for eight weeks.

Figure 6.11 shows Western blots which were probed with an antibody to p53 (Ab-3). This antibody (under denaturing conditions) recognizes both mutant and wild-type p53 in mammals. In control trout a double band of approximately 50 kDa reacts with this antibody. Treatment with DHEA for eight weeks (blot A) or twelve weeks (blots B and C) causes a dramatic decrease in these proteins in most, but not all trout.

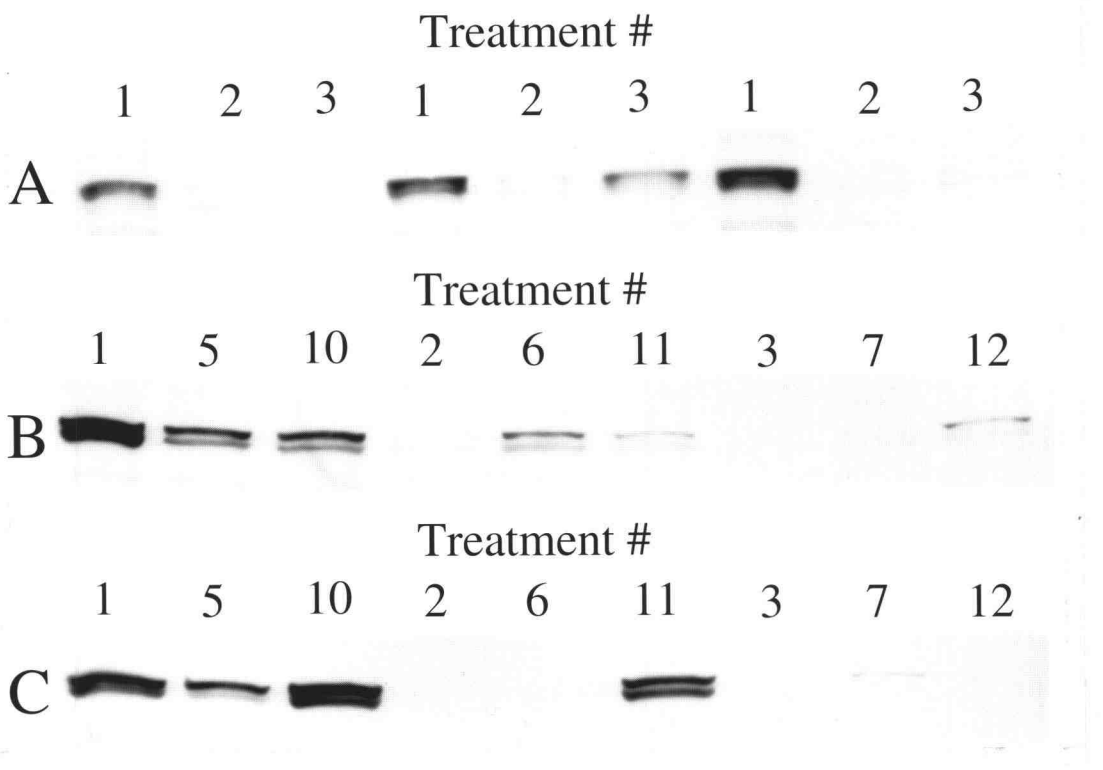

Figure 6.11. Western blots probed with antibody to p53. Blot A is from animals sampled at the end of the preinitiation phase. Blots B and C are from animals sampled at the end of the initiation period. Treatment numbers correspond to those in figure 6.3.

Figure 6.12 is a Western blot probed with an antibody to p34 cdc2. Several proteins cross react with this antibody. One protein of approximately 34 kDa is decreased dramatically by 12 weeks of DHEA treatment. Another, of approximately 65 kDa appears to undergo a shift in molecular weight.

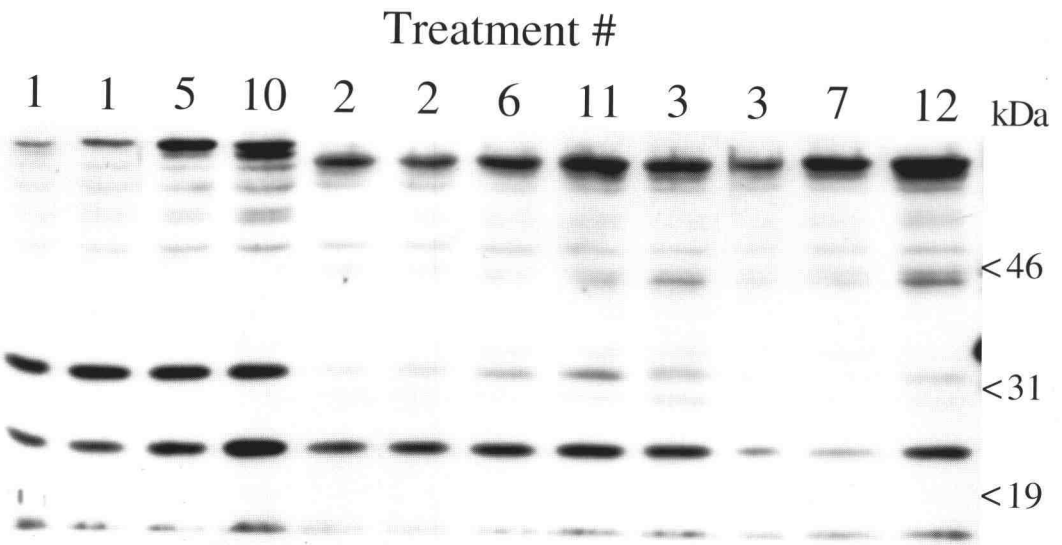

Figure 6.12. Western blot probed with antibody to p34 cdc2. The blot contains proteins from liver homogenates from trout sampled at the end of the initiation phase. Treatment numbers correspond to those in figure 6.3.

DISCUSSION

Post-initiation treatment of AFB_1-initiated trout with DHEA results in dose-dependent increases in tumor incidence, multiplicity, and size (16). In this paper, we report that DHEA also decreases the latency period between initiation and the appearance of tumors. The first tumors were detected two months sooner in AFB_1-initiated trout treated with 444 ppm DHEA than in initiated trout fed control diet. Six months post-initiation, twenty percent of animals initiated with AFB_1 and fed DHEA for three months had tumors. No tumors were present in AFB_1-initiated control trout or in sham-initiated trout fed DHEA for three months. At this timepoint, therefore, DHEA can be considered a promoter of AFB_1-initiated hepatocarcingenesis. After seven months of dietary DHEA tumors occurred in animals treated with DHEA alone, as well as in AFB_1-initiated controls. Combined, the two carcinogens have a synergistic effect. In this experiment, as previously reported, DHEA decreases the growth rate in trout (16). This compound has been extensively studied for its antiobesity properties (24, 25), therefore decreased weight gain may be an inappropriate measure of toxicity. This dose of DHEA also caused some additional effects which are probably related to the role of this compound as a precursor of androgens and estrogens (26). Most of the males in the DHEA-treated groups showed some signs of sexual maturation (secondary sex characteristics and developing testes). In addition, many of the DHEA-treated animals had a ragged appearance to their tails and fins. This may be the result of DHEA induction of vitellogenin. Previously we have observed that treatment of trout with DHEA causes dramatic induction of this calcium-containing yolk protein precursor (27). Vitellogenesis causes mobilization of calcium from scales and other calcified tissues (28) and may be responsible for the damaged fins observed in this experiment.

In the second experiment, DHEA failed to provide any protection towards AFB_1-initiated carcinogenesis when administered prior to and during carcinogen administration.

It is still possible that treatment with DHEA before (but not during) carcinogen administration could have a protective effect, however this protocol was not examined in the current experiment. Treatment of animals with DHEA alone for twelve weeks was sufficient to produce tumors in four and eight percent of the animals fed 888 and 1776 ppm DHEA, respectively. This is of interest because nongenotoxic carcinogens do not generally produce tumors unless administered to an animal for most of its lifetime. There is some evidence that DHEA could have some genotoxic effects. In a previous study we found that about one-third of tumors examined from trout treated with DHEA had Ki-ras mutations (16). Mutations of this gene have not been detected in spontaneous liver tumors in trout, however due to the low spontaneous liver tumor incidence in trout of this age (0.1%), only a few tumors have been analyzed for ras mutations.

The most dramatic enhancement of AFB_1-initiated hepatocarcingenesis occurred when DHEA was fed during the postinitiation period. This may indicate that DHEA enhances carcinogenesis through stimulation of cell proliferation, or by providing a selective growth advantage to initiated cells. Antibodies to several proteins involved in cell proliferation and cell cycle control were utilized in this experiment. PCNA is an endogenous cell marker of DNA replication that is widely used for the measurement of cell replication. Animals treated with DHEA for eight weeks did not have higher levels of PCNA than control animals. Previously, we have found that PCNA levels increase during the first two weeks of DHEA treatment (29). After eight weeks of DHEA treatment, levels of this antigen are not elevated in liver homogenates however, this does not exclude the possiblity that certain cell populations are still rapidly dividing.

The tumor suppressor p53 is involved in regulation of the cell cycle, control of DNA repair and initiation of apoptosis (30-32). Mutations in the p53 gene are the most frequently observed genetic alteration in human cancers (33). Animals deficient in p53 are highly susceptible to spontaneous tumors (34). The p53 gene in trout has been sequenced and found to be highly homologous with mammalian p53 (35). In this experiment we used

an antibody which (under denaturing conditions) recognizes both mutant and normal mammalian p53. In blots of liver homogenates from control trout, this antibody reacts quite strongly with a double band of the approximate molecular weight of p53. Prolonged treatment with DHEA dramatically reduces levels of these proteins.

The p34 cdc2 protein is one of a family of cyclin dependent kinases which are involved in the regulation of the cell cycle (36). High levels of this kinase have been demonstrated in animals treated with peroxisome proliferators (37). In contrast to these results, however, prolonged DHEA treatment of trout downregulates a protein of approximately 34 kDa which cross reacts to this antibody. Numerous proteins from trout liver homogenates cross react to this antibody, however, so it is quite possible that the protein decreased by treatment with DHEA is not cdc2.

Although our data are limited, DHEA appears to have some effects on the expression of proteins involved in the control of the cell cycle. These changes could be involved in the modulation of AFB_1-inititated hepatocarcingenesis by DHEA. In addition, the epigenetic modulation of p53 and other tumor suppressor or protooncogenes could be involved in the carcinogenesis of many nongenotoxic carcinogens.

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Chapter 7

MODULATION OF N-METHYL-N'-NITRO-NITROSOGUANIDINE (MNNG)-INITIATED CARCINOGENESIS BY DEHYDROEPIANDROSTERONE (DHEA) IN RAINBOW TROUT

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ABSTRACT

Dehydroepiandrosterone (DHEA) and its sulfate conjugate are the major circulating steroids in human plasma. Low levels of these adrenal androgens are associated with a number of human diseases including certain cancers. In animal studies, DHEA has been shown to be chemopreventive towards both spontaneous and chemically induced cancer. In contrast to these protective properties, however DHEA is a also a peroxisome proliferator. Like other peroxisome proliferators, DHEA is a hepatocarcinogen in rats, a species highly sensitive to peroxisome proliferation. DHEA, however, is also carcinogenic in rainbow trout; a species which is relatively insensitive to peroxisome proliferation. This study examines the effects of DHEA on N-methyl-N'-nitro-nitrosoguanidine (MNNG)-initiated carcinogenesis in trout. Trout fry were given a 30 min water bath exposure to 35 ppm MNNG, a level producing primarily stomach, kidney, and swim bladder tumors. Fish were then fed diets containing 0, 55, 111, 222, 444, or 888 ppm DHEA for 7 months. Post-initiation treatment with DHEA increased liver tumor incidence, multiplicity, and size in a dose dependent manner. Liver tumor incidence ranged from 1% in MNNG-initiated controls to 99% in initiated trout treated with 888 ppm DHEA (tumor incidence in sham-initiated trout fed this level of DHEA was 37%). Kidney tumor incidence was also enhanced 2- and 4-fold over initiated controls by 111 and 888 ppm DHEA, respectively. In contrast, the number of stomach and swim bladder tumors were reduced by DHEA treatment. This study demonstrates differential effects of DHEA on MNNG-initiated carcinogenesis in liver, kidney, stomach, and swim bladder.

INTRODUCTION

The hormones dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are secreted in large quantities from the human adrenal gland. The highest serum levels of DHEAS occur in early adulthood, then decline with age to about 10-20% of maximum (1), leading to interest in the possibility that the loss of DHEA could be associated with some of the adverse changes occurring as people age (2). In humans, low levels of DHEA or DHEAS have been associated with cardiovascular disease in men, Alzheimer's disease, HIV, breast cancer, stomach cancer, bladder cancer, and lung cancer (3-8). In animal models, DHEA is chemoprotective towards a wide variety of disease conditions including atherosclerosis, diabetes, obesity, autoimmune disorders, and cancer (2, 9-13). Human clinical trials have been conducted, or are underway for the use of DHEA in the treatment of obesity, aging, cancer, diabetes, Alzeheimer's disease, multiple sclerosis, HIV, and lupus (9, 14, 15).

DHEA, however, has a number of adverse effects. DHEA is a precursor for androgens and estrogens and, in human clinical trials has been shown to increase androgen levels in female subjects (16, 17). DHEA is also a carcinogen in laboratory animals (18, 19). The hepatocarcinogenicity of DHEA in rats has been attributed to its properties as a peroxisome proliferator (20, 21), however we have also demonstrated that DHEA is both a complete carcinogen and a potent enhancer of AFB_1 hepatocarcinogenesis in rainbow trout (19), a species which resembles humans in being relatively insensitive to peroxisome proliferation (22, 23). In trout, the hepatocarcinogenicity of DHEA appears to be due to its role as a steroid precursor rather than as a peroxisome proliferator (24). Conversion of DHEA into androgens and estrogens is probably also involved in other tumor enhancing effects of this compound including stimulation of growth of the R3327 Dunning implanted prostate carcinoma in rats (25), enhancement of pancreatic carcinogenesis in azaserine

initiated rats (26); and induction of ovarian granulosa cell tumors in the genetically susceptible SWXJ-9 mice (27).

In view of DHEA's dual role as a tumor modulator, we felt it would be of interest to compare the effects of this compound on carcinogenesis of multiple organs initiated simultaneously in a single species.

MATERIALS AND METHODS

Chemicals

AFB_1, MNNG, and other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Animals and treatment

Mt. Shasta strain rainbow trout (*Oncorhynchus mykiss*) were raised at the Oregon State University Food Toxicology and Nutrition Laboratory. Animals were fed Oregon Test Diet (OTD), a casein based semipurified diet (28) and housed in circular tanks with continuously flowing 12-14° water and a 12 hour light-dark cycle. Three months after spawning 720 fry were given a 30 min bath exposure to 35 ppm MNNG. An equal number of trout were given a 30 min sham exposure to vehicle control (0.025% DMSO). Animals were fed control diet for an additional three months, then were assigned to experimental groups and given a modified OTD (100 mg Vit. E/kg dry diet) containing 0, 55, 111, 222, 444, or 888 ppm DHEA. Each treatment group consisted of a single tank containing 120 trout. Diets were prepared by dissolving DHEA in the lipid component (salmon oil) and were stored at -20° C for up to three weeks. Once thawed, diets were refrigerated for no longer than 4 days prior to feeding. Animals were fed once per day (approximately 3-5% w/w of their body weight) and diet consumption recorded. Nine months after initiation, while still sexually immature, trout were killed by an overdose of 3-aminobenzoic acid ethyl ester (MS-222). Livers, kidneys, stomachs, and swim bladders were examined for tumors, then were fixed in Bouin's solution. After fixation, livers were hand-sliced to look for internal tumors and verify previously identified tumors. Livers, stomachs, kidneys, and swim bladders were embedded in paraffin and saved for future histological examination.

Statistical analysis

Tumor incidence was assessed using logistic regression and Fisher's exact test (two-tailed). The nonparametric Kruskal-Wallis test with chi-square approximation of p-values was used to compare the average number of tumors per tumored animal. In groups with differences, pairwise comparisons were made by the Wilcoxon rank sum test. Body weights, liver weights, and tumor size were log transformed and compared by ANOVA followed by the LSD multiple range test for pairwise comparisons. Calculations were performed on the statistical package SAS, version 6.04 (SAS Institute, Cary, NC).

RESULTS

Animals treated with DHEA had lower body weights at terminal sacrifice compared to controls (Table 1). Food consumption was 3% lower in tanks of trout fed 888 ppm DHEA, however, all other treatment groups had caloric intake similar to controls (not shown). There was some mortality in trout treated with the highest level of DHEA. Eighteen percent of animals initiated with MNNG and fed 888 ppm DHEA died. Six percent of sham-initiated animals fed the same dose of DHEA died during the experiment. Mortality in all other experimental groups was ≤ 1%. Liver weights and liver somatic indexes (% of body weight made up by liver) both were increased in a dose dependent manner by DHEA treatment.

Table 7.1. Effect of dietary DHEA on body weight, mortality, and liver weight

Initiation	DHEA (ppm)	Mort. (%)[a]	Final Body Wt. (g)[b,c]	Final Liver Wt. (g)[b,c]	Liver Somatic Index (%)[b,c]
Sham	0	1	92.3 ± 2.4	0.49 ± 0.01	0.53 ± 0.01
MNNG	0	1	95.4 ± 2.1	0.58 ± 0.02	0.60 ± 0.01
Sham	55	0	71.1[d] ± 1.8	0.49 ± 0.01	0.69[d] ± 0.01
MNNG	55	1	81.1 ± 1.6	0.52 ± 0.01	0.64 ± 0.02
Sham	111	0	72.7[d] ± 1.7	0.54[d] ± 0.01	0.74[d] ± 0.01
MNNG	111	0	77.0[e] ± 1.7	0.55 ± 0.02	0.72[e] ± 0.01
Sham	222	0	81.3 ± 1.8	0.64[e] ± 0.02	0.78[d] ± 0.01
MNNG	222	1	78.3[e] ± 1.6	0.65[d] ± 0.02	0.83[e] ± 0.01
Sham	444	1	79.6 ± 1.5	0.77[e] ± 0.02	0.97[d] ± 0.01
MNNG	444	1	74.1[e] ± 2.0	0.77[d] ± 0.03	1.03[e] ± 0.02
Sham	888	6	75.4[d] ± 1.9	1.01[e] ± 0.03	1.34[d] ± 0.02
MNNG	888	18	77.7[e] ± 2.0	1.51[d] ± 0.09	1.94[e] ± 0.01

a Mortality is % of animals that died while on experimental diets.
b ± SE
c n = 100 for all groups except the MNNG-initiated fed 888 ppm DHEA, where n = 94.
d Significantly different from sham control at $p < 0.05$.
e Significantly different from MNNG control at $p < 0.05$.

In this experiment, as demonstrated previously (19), DHEA was a complete liver carcinogen in rainbow trout (Fig. 7.1). Tumor incidence (% of animals with tumors) increased in a dose-dependent manner, ranging from 1% of animals fed 55 ppm DHEA, up to 37% of animals fed 888 ppm DHEA. No liver tumors were observed in sham-initiated animals fed control diet.

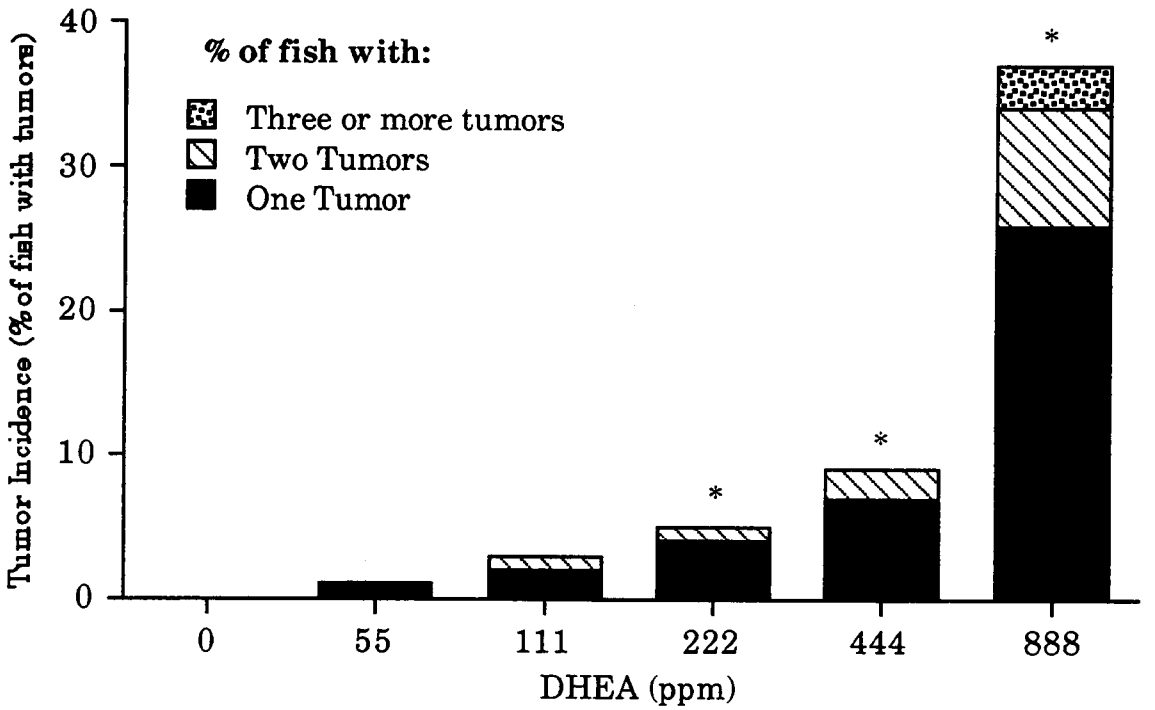

Figure 7.1. Liver tumor incidence and multiplicity in sham-initiated trout. * indicates significantly higher ($p < 0.05$) tumor incidence than sham-initiated controls.

In addition to being a complete carcinogen, DHEA also dramatically enhanced MNNG-initiated hepatocarcinogenesis (Fig. 7.2). Tumor incidence increased with increasing doses of DHEA from 1% in the MNNG-initiated controls to 99% in MNNG-initiated trout fed 888 ppm DHEA. The tumor size (in animals with tumors) ranged from 0.5 mm in diameter in the single tumor observed in the MNNG initiated controls to an

average of 3.5 ± 0.2 mm (± SE) in initiated animals fed 888 ppm DHEA. Tumor multiplicity increased in a dose-dependent manner with a single tumor per liver in the initiated controls and 55 ppm DHEA groups; and an average of 1.06 ± 0.06, 1.58 ± 0.14, 2.87 ± 0.29, and 6.03 ± 0.44 in the 111, 222, 444, and 888 ppm DHEA-treated groups, respectively.

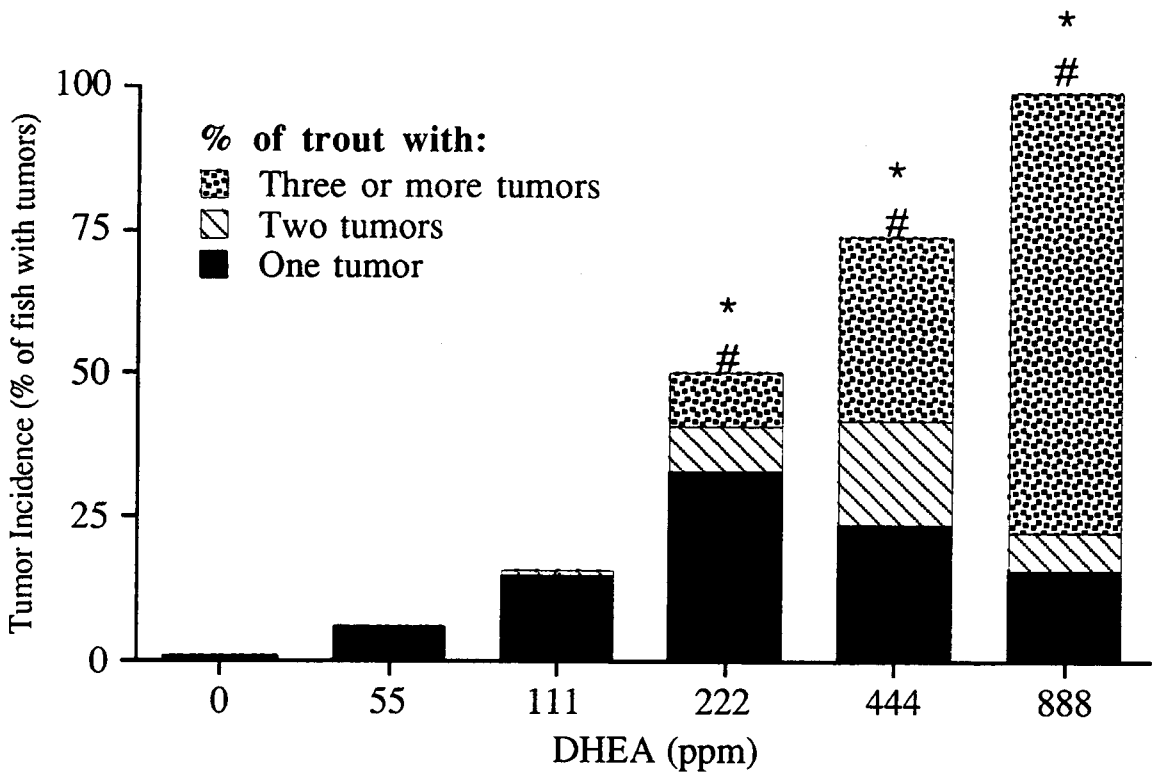

Figure 7.2. Liver tumor incidence and multiplicity in MNNG-initiated trout. * indicates significantly higher ($p < 0.05$) tumor incidence than MNNG-initiated controls. # indicates significantly higher ($p < 0.0005$) tumor multiplicity than next highest treatment.

One kidney tumor occurred in a sham-initiated trout fed 222 ppm DHEA for 30 weeks. This observation, although not statistically significant, is of interest because renal tumors are extremely rare in untreated trout of this age. In MNNG-initiated trout, the observed kidney tumor incidence was higher in each of the DHEA-fed groups than the

controls, although only the 111 and 888 ppm DHEA treatments were significantly higher at the 0.05 level of significance (Fig. 7.3).

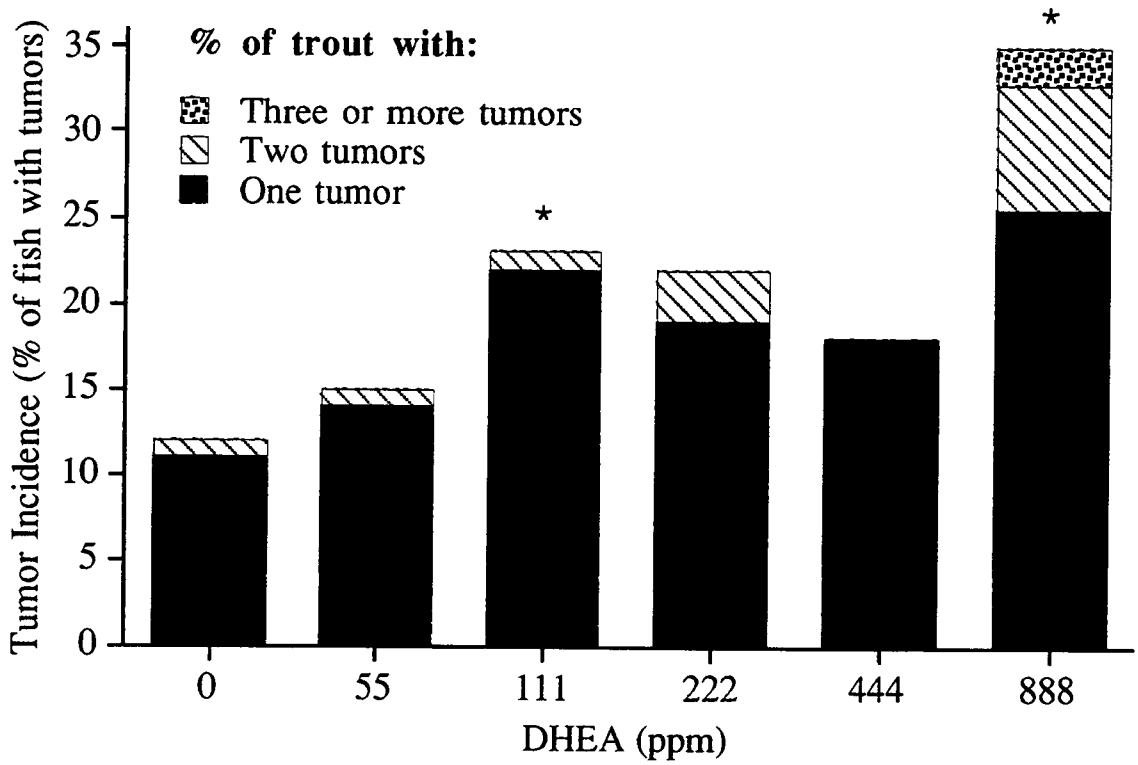

Figure 7.3. Kidney tumor incidence and multiplicity in MNNG-initiated trout. * indicates significantly higher ($p < 0.05$) tumor incidence than MNNG-initiated controls. The p-value for tumor incidence in the 222 ppm DHEA group is 0.06.

In contrast to DHEA's effects on liver and kidney carcinogenesis, DHEA appeared to offer some post-initiation protection towards stomach and swim bladder tumors. Although the stomach tumor incidence was not significantly different between DHEA-treated animals and initiated controls, the total number of stomach tumors per treatment was significantly suppressed in all DHEA-treated groups (Fig. 7.4).

DHEA treatment also reduced MNNG-initiated swim bladder carcinogenesis. Tumor incidence in DHEA-fed animals was significantly lower than initiated controls for all doses except the 444 ppm DHEA (Fig. 7.5).

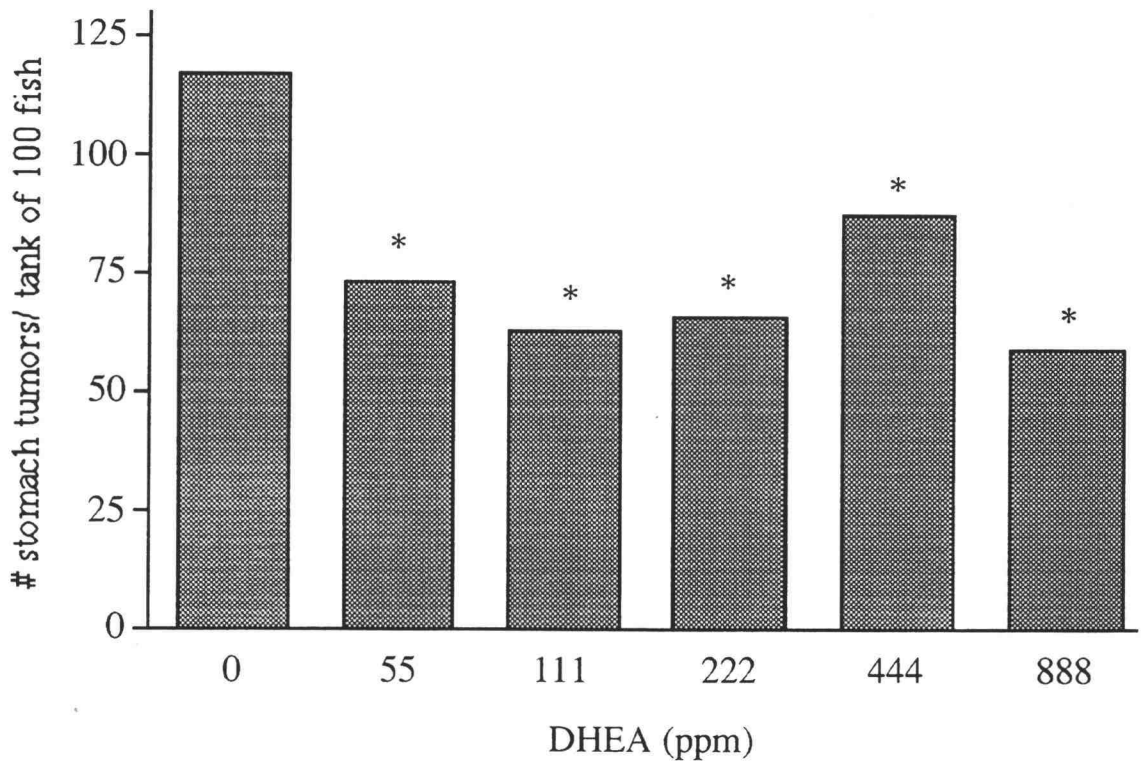

Figure 7.4. Number of stomach tumors in MNNG-initiated trout. * indicates significantly lower ($p < 0.05$) total number of tumors than MNNG-initiated controls (for statistical analysis, a maximum number of 6 tumors per stomach was assumed).

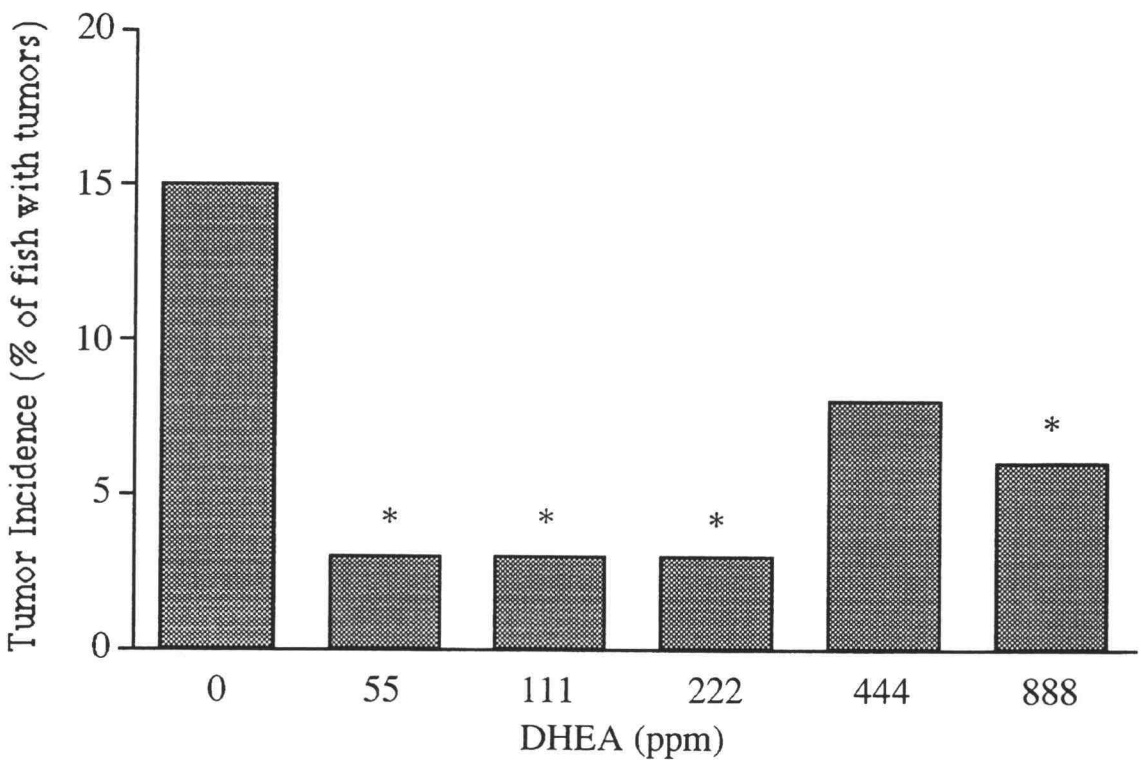

Figure 7.5. Swim bladder tumor incidence in MNNG-initiated trout. * indicates significantly lower ($p < 0.05$) tumor incidence than in MNNG-initiated controls.

DISCUSSION

Previous studies in our laboratory have found that DHEA is a potent enhancer of AFB_1-initiated hepatocarcinogenesis in trout. This study was designed to determine if DHEA enhancement of carcinogenesis was carcinogen or organ specific. Initiator specific promotion by the peroxisome proliferators clofibrate and WY 14,643 of the carcinogens N-acetylamino-fluorine and diethylnitrosamine has recently been demonstrated (29). We selected MNNG as the initiator in the current study because, unlike AFB_1, MNNG is a direct acting carcinogen and because a single exposure of trout fry to MNNG can produce tumors in four different organs. This provides a unique model for the study of modulation of carcinogenesis in more than one tissue without the need for multiple initiators or surgical procedures. In this experiment initiation conditions were selected that would produce predominantly stomach tumors, moderate numbers of kidney and swim bladder tumors, and a very low incidence of liver tumors. As predicted, the incidence of liver tumors was extremely low (1%) in the MNNG-initiated controls. Post-initiation treatment with DHEA dramatically enhanced liver tumor incidence to almost 100% in initiated animals fed 888 ppm DHEA. Since sham-initiated trout produced tumors in only 37% of the animals, the combined effects of MNNG-initiation and DHEA treatment were clearly more than additive. DHEA may be acting by enhancing proliferation of initiated cells (30).

In addition to dramatically enhancing liver carcinogenesis, treatment with DHEA also enhanced kidney tumor incidence in MNNG-initiated trout. There is one previous report of DHEA enhancement of renal carcinogenesis. An increased incidence of renal adenocarcinomas but not adenomas was found in dimethylnitrosamine-initiated rats treated with 6000 ppm DHEA for 26 weeks (31). Histopathology has not yet been performed on the tumors from our study, however tumors in the DHEA-treated animals tended to be larger (although not significantly) than in the initiated controls. In addition, the high mortality in the MNNG-initiated trout fed 888 ppm DHEA may have been due to renal

carcinomas. Several of the animals from this treatment that died prior to sampling had large, externally visible kidney tumors. At our facility, approximately 30,000 trout are sampled annually. No spontaneous kidney tumors have been observed in at least the last three years. It is therefore of interest that in this study a single kidney tumor was found in a trout that was treated with 222 ppm DHEA. In addition, we have just completed a study in which the same levels of DHEA were fed to non initiated trout for 42 weeks. In this experiment, five out of 1000 DHEA-fed trout developed kidney tumors (Orner et al., unpublished data).

There are a number of mechanisms through which DHEA could be acting to enhance carcinogenesis in the kidney. One possible mechanism is related to the renal toxicity of DHEA. In immature trout, treatment with DHEA causes induction of the estrogen inducible yolk protein vitellogenin (24, 30). This lipoprotein is normally secreted in response to ß-estradiol in mature female trout and is rapidly taken up by the ovaries. In juvenile trout, vitellogenin secretion can be induced but the protein accumulates outside the ovaries (32). The high toxicity of ß-estradiol in young trout has been attributed, in part, to kidney function impairment resulting from the accumulation of vitellogenin in the tubules, glomeruli, and sinusoids of the anterior kidney (32). We have observed similar renal toxicity in animals treated with high ($\geq$ 444 ppm) doses of DHEA. The effects of DHEA on renal carcinogenesis could be the result of the accumulation of this protein, through cytotoxicity and proliferative cell growth. If so, this would be a mechanism specific to non-mammalian species with little relevance towards humans.

However, the fact that enhancement of renal carcinogenesis has also been observed in rats (31) would indicate that this response is not unique to fish. Previous studies in our laboratory indicate that DHEA may have some genotoxicity. Although DHEA is negative in short-term mutagenicity assays (33-35), we detected Ki-*ras* mutations in about 30% of liver tumors in trout treated with DHEA (19). No Ki-*ras* mutations have been observed in spontaneous tumors in trout, however the number of tumors examined is quite small due to

the low spontaneous incidence of liver tumors in trout. A direct effect of DHEA on DNA is, therefore, possible but requires further investigation.

There is also the possibility that the renal effects could be related to DHEA's properties as a peroxisome proliferator. Although peroxisome proliferation is typically considered a hepatic response, peroxisomes were first described in the rodent kidney (36). Trout do not appear to respond in the same manner as rats and mice to peroxisome proliferators, however, no studies have examined peroxisome proliferation in extrahepatic tissues in trout. Only slight increases in hepatic peroxisomal ß-oxidation occur when trout are treated with DHEA, however significant decreases occur in peroxisomal catalase (24, 30). This could result in the same sort of prooxidant condition that has been proposed as a possible mechanism of carcinogenicity of peroxisome proliferators in rodents (37).

In contrast to its effects on liver and kidney, treatment of MNNG-initiated trout with DHEA provided protection towards stomach and swim bladder carcinogenesis. In humans, low plasma levels of DHEA and DHEAS have been associated with an increased risk of developing gastric cancer (8), however this is the first report of DHEA chemoprotection towards stomach cancer in an animal model. DHEA provided significant protection towards swim bladder carcinogenesis at doses as low as 55 ppm; one-fourth the dose which significantly enhanced liver carcinogenesis. Therefore, it appears that the cancer chemopreventive properties of DHEA are not limited to rodents.

Despite its chemoprotective effects towards cancer, however, DHEA also has carcinogenic and tumor enhancing properties in multiple organs and multiple species (18, 19, 25-27, 31). A high priority should be placed on determining if the carcinogenic effects of this compound pose a risk to human health, particularly since DHEA continues to be used in human chemoprevention studies.

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Chapter 8

CONCLUSIONS

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SUMMARY

The studies described in this thesis demonstrate that rainbow trout are extremely sensitive to the carcinogenicity and tumor enhancing properties of DHEA. DHEA is a complete carcinogen in trout at doses 20-fold lower than doses shown to be hepatocarcinogenic in rats. There are a number of possible explanations for this. Trout are highly sensitive to genotoxic carcinogens such as AFB_1, possibly because of their low ability to repair bulky DNA adducts. In chapter 3, we show that tumors in DHEA treated trout have Ki-ras mutations. Therefore, DHEA may have some genotoxic properties. If so, this could explain the high sensitivity of trout to DHEA carcingenesis. Alternately, the tumor enhancing and carcinogenic effects of DHEA may be due to its steroid precursor effects. The trout carcingenesis model differs from rodent models in that trout tumor studies are completed prior to sexual maturation. The effects of DHEA as an estrogen or androgen may differ according to what sort of hormonal environment is present (1). In juvenile trout, the steroidal effects of DHEA may be particularly potent because they have such low endogenous hormone levels. It is also possible that a low dose of DHEA given to trout results in a greater bioavailable dose of DHEA than the same treatment in mammals because more of the compound is present in an active form. In humans, about 99% of DHEA exists as the sulfate conjugate, DHEAS. After short-term treatment of trout with dietary DHEA, serum levels of DHEA and the sulfate are approximately equal (chapter 4), possibly due to the low DHEA sulfotransferase activity in trout (2). The ratio of DHEA to DHEAS therefore, is much higher in trout than in humans. As a result, a higher proportion of DHEA is available for direct conversion into steroids. Some of the effects of DHEA, however appear to be due to the sulfate conjugate. For example, it has recently been demonstrated that DHEAS may be the active form for peroxisome proliferation (3). This could be part of the reason why trout are less susceptible to the peroxisome proliferating effects of DHEA as well, although it would not explain the low response of trout to other

peroxisome proliferators. Another explanation for the high sensitivity of trout to DHEA could be due to a lack of chemoprotective effects to balance out the carcinogenic properties. DHEA carcinogenicity clearly occurs through a mechanism which can occur in trout. It is possible, however, that properties responsible for chemoprevention by DHEA in rodents, are not present in trout. Inhibition of G6PD may be involved in DHEA's anticancer effects (4-6). We did not observe any inhibition of G6PD in red blood cells of trout treated with DHEA or 8354 for two weeks (Chapter 5). *In vivo* studies in rodents, however have also failed to demonstrate inhibition of this enzyme (7). Further studies on the ability of DHEA to inhibit trout G6PD are necessary before we can conclude that this is a mechanism which does not occur in trout. Another possible mechanism of DHEA chemoprevention is through glutathione-S-transferase induction (8). Previous studies from our laboratory suggest that chemopreventive agents which act through the induction of GST are not effective in the trout model (9). Susceptibility to the carcinogenic, but not the chemopreventive effects of DHEA may account for the high sensitivity of trout to DHEA carcinogenesis. In trout initiated with MNNG, however, there is significant protection towards carcinogenesis of the stomach and swim bladder.

SUGGESTIONS FOR FUTURE STUDY

In this thesis, we present limited evidence that DHEA could be genotoxic. Many more spontaneous tumors must be examined for ras mutations, however before we can rule out the possiblity that DHEA is simply promoting tumors bearing spontaneous or pre-existing *ras* mutations. In addition, DHEA's ability to bind to DNA should be thoroughly examined. *In vitro* incubation of rat microsomes with DHEA has been shown to produce DNA and protein-interacting species (10). We conducted one *in vivo* DNA binding experiment by injecting trout with [^{3}H] DHEA (11),. In this study there was no evidence of DNA binding, however the dose used was many orders of magnitude lower than the carcinogenic dose. It would be extremely expensive to conduct this *in vivo* assay with carcinogenic doses of [^{3}H] DHEA, however it would be relatively simple to determine if DHEA could be activated to DNA-binding intermediates by trout microsomes *in vitro*. To examine DHEA binding to DNA *in vivo*, the most appropriate method would probably be to examine DNA of DHEA-treated trout for DNA adducts using ^{32}P-postlabeling.

Studies in this thesis confirm reports that trout, like humans are relatively insensitive to peroxisome proliferation (12-16). An understanding of the mechanism of trout resistance to peroxisome proliferation is necessary, however, before we can conclude that trout are appropriate models for predicting human risk from these compounds. The difference in response between humans and rodents does not appear to be due to the lack of a functional peroxisome proliferator activated receptor (PPAR), as a human PPAR has been cloned that is able to activate the rat acyl CoA oxidase and rabbit CYP4A6 response elements (17). It should be fairly simple to determine if trout also have a functional PPAR. We have already established that trout have several proteins which cross react with a mouse PPARα antibody (unpublished data). The next step is to conduct gel mobility shift assays of trout nuclear extracts using a labeled peroxisome proliferator response element (PPRE) as a probe, followed by supershift assays using the PPARa antibody to confirm that the

mobility shift is due to binding of a PPAR. The trout PPAR(s), if present, could be cloned, sequenced, and compared to *Xenopus*, human, and mouse PPARs.

Another possible explanation for trout being weak responders to peroxisome proliferators could be related to the low levels of ω-hydroxylation of fatty acids produced by trout P450s. Induction of P4504As (which catalyze ω-hydroxylation of fatty acids) may be involved in the peroxisome proliferation response in rodents as induction of these enzymes occurs prior to the induction of ß-oxidation (18).

Another area which should be further explored is the possiblity that mature trout might respond more like rodents to peroxisome proliferation than the juveniles used for the studies contained in this thesis. Testosterone has been shown to enhance the induction of peroxisomal b-oxidation in clofibrate-treated rats (19). It may be that in immature trout, induction of peroxisome proliferation is limited due to the lack of steroid hormones. We conducted a preliminary study to address this question by gavaging mature male trout with clofibrate or corn oil. In this study, trout still appeared to be weak responders to peroxisome proliferation, however there were some increases in liver wt. and ß-oxidation. A more effective dosing strategy might give more dramatic results.

As discussed above, one explanation for the high sensitivity of trout to DHEA carcinogenesis is that trout have a limited ability to sulfate DHEA. Cloning and sequencing of the trout sulfotransferases would allow this hypothesis to be further examined by comparing trout, human, and rodent sulfotransferases.

The MNNG experiment should be repeated using lower doses (possibly ranging from 10 to 100 ppm w/w). In the MNNG-modulation experiment (chapter 7), significant protection towards swim bladder and stomach carcingenesis was provided by doses as low as 55 ppm. This dose slightly (but not significantly) enhanced liver carcinogenesis, however it is possible that lower doses could provide significant protection towards stomach and swim bladder carcinogenesis without enhancing liver or kidney carcinogenesis. In rats, DHEA has recently been found to have chemoprotective effects

towards MNU-induced breast cancer at 120 ppm, a dose which is less than 4% of the maximum tolerated dose (3000 ppm) (20). This is considerably lower than the doses which have been found to be hepatocarcinogenic in this species. It is possible that very low doses of DHEA in trout could be protective towards stomach and swim bladder carcingenesis without promoting hepatocarcinogenesis.

A comparison of G6PD inhibition *in vitro* by DHEA (and the analog) in trout, rat, and human samples would help to establish if this mechanism of protection is present in trout.

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