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Toxicity and Carcinogenicity of Androstenedione in F344/N Rats and B6C3F1 Mice

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Abstract

Androstenedione was marketed as a dietary supplement to increase muscle mass during training. Due to concern over long-term use, the NTP evaluated the subchronic and chronic toxicity and carcinogenicity of androstenedione in male and female F344/N rats and B6C3F1 mice. In subchronic studies, dose limiting effects were not observed. A chronic (two-year) exposure by gavage at 10, 20, or 50 mg/kg in rats and male mice, and 2, 10, or 50 mg/kg in female mice (50 mg/kg, maximum feasible dose) was conducted. Increased incidences of lung alveolar/bronchiolar adenoma and carcinoma occurred in the 20 mg/kg male rats and increases in mononuclear cell leukemia occurred in the 20 and 50 mg/kg female rats, which may have been related to androstenedione administration. In male and female mice, androstenedione was carcinogenic based upon a significant increase in hepatocellular tumors. A marginal increase in pancreatic islet cell adenomas in male (50 mg/kg) and female (2, 10, 50 mg/kg) mice was considered to be related to androstenedione administration. Interestingly, incidences of male rat Leydig cell adenomas and female rat mammary gland fibroadenomas decreased. In conclusion, androstenedione was determined to be carcinogenic in male and female mice, and may have been carcinogenic in rats.

Keywords

Androstenedione; Rat; Mice; Cancer; Two-Year Bioassay; Toxicity

1. Introduction

Androstenedione is a weak androgen steroid hormone normally produced in both males and females to be converted to a more potent androgen or estrogen. It was marketed as a dietary supplement to aid in increasing muscle mass during training. In 2004 androstenedione was banned for sale by the Anabolic Steroid Control Act, although illegal use may continue today. Androstenedione exposure levels are not available, but they likely varied among users

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and may have followed a cyclic pattern of prolonged use with intermittent recovery periods. Oral doses of androstenedione ranged from 100 to as high as 1,200 mg/day, which equates on per weight basis to 1.4 to 17.1 mg/kg per day for a 70 kg person (Bahrke and Yesalis, 2004).

The effect of androstenedione supplements on circulating hormones and increasing muscle mass is variable and is likely affected by age, sex, exposure time, and dose. Administration of 300 mg androstenedione/day for up seven days (Leder et al., 2000) or 300 mg androstenedione/day for 28 days (Brown et al., 2004b) increased serum concentrations and excretion of androgens in men. However, administration of 200 mg androstenedione/day for 28 days (Beckham and Earnest, 2003) or 300 mg androstenedione/day for 8 weeks (King et al., 1999) did not increase serum testosterone levels in men. Prolonged androstenedione exposure to men (200 mg/day for 12 weeks) elevated serum concentrations of androstenedione, estradiol, and estrone, but not testosterone (Broeder et al., 2000). Serum androstenedione and testosterone concentrations rise considerably in women compared to men after administration of androstenedione, likely due to the low concentrations of androgens normally circulating in women (Brown et al., 2004a; Leder et al., 2002). Significant effects on muscle mass gain have not been found in men after androstenedione administration, except following a large dose (1,500 mg/day for 12 weeks) of androstenedione given to hypogonadal men, which increased circulating levels of androstenedione and testosterone at the end of the exposure period (Jasuja et al., 2005).

Only a few studies have examined the carcinogenic or tumor promoter activity of androstenedione. Male Marsh-Buffalo mice, injected subcutaneously one to two times per 2-month period, had increased numbers of fibrosarcomas at 18 months (Bischoff, 1957). Following the induction of mammary gland carcinoma in ovariectomized rats with the known carcinogen 7,12-dimethylbenz[a]anthracene, administration of androstenedione led to increased tumor size compared to the size of tumors induced in ovariectomized controls (Dauvois and Labrie, 1989). This effect was blocked by an aromatase inhibitor, indicating that conversion of androstenedione to estradiol was required for tumor enhancement. The androstenedione precursor DHEA produces hepatocellular carcinomas in rats and rainbow trout (Metzger et al., 1995; Orner et al., 1995; Rao et al., 1992). Androstenedione metabolites that are more potent androgens or estrogens, such as testosterone, estrone, or estradiol, are known carcinogens (Beamer et al., 1988; IARC, 1987; NTP, 2002).

Androgens are generally not considered to be mutagenic, clastogenic, or aneugenic. Mutagenicity was not seen with androstenedione (500 µg/disc) in a spot test using *S. typhimurium* strain TA1538, with and without induced rat liver S9 enzymes (McKillop et al., 1983). A more potent androgen, testosterone (500 µg/plate), was negative in *S. typhimurium* strains TA100, TA1535, and TA1538, with and without rat liver S9 (Ingerowski et al., 1981). Testosterone (100 µM) was tested in cultured Chinese hamster Don cells for induction of mitotic disruption, and no mitotic disturbances were noted following a 7-hour treatment period (Wheeler et al., 1986). Tsutsui *et al.* (1995) tested testosterone and testosterone propionate in Syrian hamster embryo (SHE) cells for induction of chromosomal aberrations, aneuploidy, and gene mutations; all test results were negative (Tsutsui et al., 1995).

Previously, the NTP studied the synthetic anabolic steroid oxymetholone in F344/N rats after chronic exposure, and found it to be carcinogenic in female rats based upon increased neoplasms in the liver, lung, and skin, but no clear neoplastic effects were seen in male rats (NTP, 1999). Due to the concern for adverse effects following prolonged use of androstenedione and the lack of chronic toxicity and carcinogenicity data, subchronic

toxicity studies with androstenedione were conducted by the NTP, followed by an evaluation of its chronic toxicity and carcinogenicity in F344/N rats and B6C3F1 mice.

2. Materials and Methods

2.1 Animals and Dosing

Androstenedione (CAS# 63-05-8, Lot H408, Steraloids, Inc., Newport, RI) was identified by infrared (IR) and proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy. The purity was determined to be > 98% by gas chromatography and high-performance liquid chromatography. Dose formulations consisted of androstenedione in 0.5% methylcellulose (Sigma Aldrich, Saint Louis, MO), which homogeneity, dosing feasibility, resuspendability, and stability were confirmed. Dose formulations were evaluated prior to the start of the subchronic and chronic study and evaluated again three times during the subchronic study and every two-three months during the chronic study. The current studies were limited to a high dose of 50 mg/kg due to dosing feasibility (i.e. ability to gavage administer the formulation) at the high dose. Dose formulations were within 10% of target.

The studies were conducted at Southern Research Institute (Birmingham, AL). Male and Female B6C3F1 mice and Fisher F344/N rats were purchased from Taconic Farms, Inc (Germantown, NY). Animals were quarantined 12-15 days and were 5-6 weeks old at the start of dosing. NTP-2000 diet (Rao, 1996) and water were provided ad libitum. Animals were randomly assigned into dose groups of approximately similar initial mean body weights. Similar sexes were grouped house (5/cage in subchronic studies; 5/cage for females and 3/cage for male rats in chronic studies), except for male mice. The gavage dose was calculated using the most recent body weight and dosing volume was 5 mL/kg for rats and 10 mL/kg for mice. Rats and mice were gavaged 5 days/week. Studies were conducted in accordance with U.S. Public Health Service animal care policy and the Guide for the Care and Use of Laboratory animals. In addition, these studies were conducted in compliance with Good Laboratory Regulations established by the Food and Drug Administration.

2.3 Subchronic study

Male and female rats and mice (n=10/sex/species) were dosed 0, 1, 5, 10, 20, 50 mg/kg/day androstenedione for 14 weeks. Animals were weighed at the beginning of the study, weekly during androstenedione administration, and then at necropsy. An additional ten rats/sex/dose were added to the study in order to collect blood, via the retro-orbital sinus, for hematology and clinical chemistry on day four and twenty four. Blood was also collected during necropsy for hematology (rats and mice) and clinical chemistry (rats only). At necropsy, the heart, right kidney, liver, lung, right testis, thymus, and uterus were weighed. Tissues for histological evaluation were fixed and preserved in 10% neutral buffered formalin, except the testis and epididymides, which were initially fixed in modified Davidson's solution prior to being transferred to formalin and the eyes, which were fixed in Davidson's solution. Tissues were trimmed, embedded, sectioned to 4-6 μ m slices, and stained with hematoxylin and eosin. A complete histopathologic evaluation was conducted on all tissues from the control and 50 mg/kg dose group. If a compound-related effect was found in the high dose, lower dose groups were examined until a no effect level was identified.

At the end of the 14 week studies, samples were collected for sperm motility, spermatid head and sperm count, and vaginal cytology evaluations on rats and mice administered 0, 10, 20, or 50 mg/kg. For 12 consecutive days prior to scheduled terminal sacrifice, vaginal smears were collected and cells were stained with toluidine blue. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus).

Homogenization-resistant spermatid nuclei and sperm count was measured using a hemacytometer and sperm motility was determined by counting motile and nonmotile sperm for five fields per slide by two observers.

2.4 Chronic study

In the chronic study, male and female rats and male mice (n=50/sex/species) were dosed with 10, 20, and 50 androstenedione mg/kg/day and female mice were dosed at 2, 10, 50 mg/kg/day for 104-106 weeks (2 years). The lower doses selected for the female mice were based upon suspected ovarian atrophy in the 14-week study (which later was not confirmed by a more detailed investigation). Animals were weighed initially, weekly for the first 13 weeks, then monthly for the rest study, and on the day of necropsy. Complete necropsies and microscopic examinations were performed on all animals. Organs were examined for visible lesions and tissues were preserved as mentioned in section 2.3. Complete histopathology was performed on all animals. More details of the pathology evaluation are available elsewhere (NTP, 2010).

Severity grades for glandular hyperplasia of clitoral gland were based on a semiquantitative evaluation of the amount of sebaceous glands in a section: grade 0 = none to only one or two small foci of glands; grade 1 = three or five small clusters of sebaceous glands; grade 2 = several moderately sized clusters or at least one larger cluster; grade 3 = several moderate or one large area of sebaceous glands. Severity of duct dilatation was graded on a semiquantitative estimate of the percentage of a 4× microscopic field that was occupied by the dilated ducts of the clitoral glands: grade 0 = neither gland occupied more than 15% of the microscopic field; grade 1 (minimal) = at least one gland occupied 16% to 25% of the microscopic field; grade 2 (mild) = at least one gland occupied 25% to 31% of the microscopic field; grade 3 (moderate) = at least one gland occupied 31% to 75% of the microscopic field; grade 4 (marked) = at least one gland occupied 75% to 100% of the microscopic field.

2.5 Genetic toxicity

The genetic toxicity of androstenedione was assessed in bacterial mutagenicity assays and rodent erythrocyte micronucleus assays that measure chromosomal damage induction. Briefly, coded samples of androstenedione were tested for induction of gene mutations (histidine reversion) in multiple strains of bacteria (*Salmonella typhimurium* TA 97, TA 98, TA 100, and TA 1535, and *Escherichia coli* WP2 uvrA/pKM101), with and without exogenous metabolic activation provided by induced male Sprague Dawley rat or Syrian hamster liver enzymes and cofactors (S9 mix) (for detailed protocol, see (Zieger et al., 1992)). In two independent experiments, androstenedione was incubated with the bacterial tester strains in buffer or S9 mix for 20 minutes at 37°C. In the first study, concentrations of androstenedione ranged from 100 to 10,000 µg/plate and both 10% and 30% rat and hamster S9 were used with *S. typhimurium* strains TA97, TA98, TA100, and TA1535. In the second study, *Salmonella* strains TA98 and TA100 were tested, along with *Escherichia coli* strain WP2 uvrA/pKM101; 10% induced rat liver S9 was used to provide metabolic activation. Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of androstenedione. In the absence of apparent cytotoxicity in the first study, a top concentration of 10,000 µg/plate was used for all tester strains. In the second study, conducted with a sample from the same lot of androstenedione used in the 2-year cancer bioassay, the highest concentrations ranged from 3500 – 10,000 µg/plate across strains.

Evaluation of the ability of androstenedione to induce chromosomal damage *in vivo* was conducted in two independent rodent micronucleus assays. In the first test (protocol details in (Shelby et al., 1993)), male Fisher 344 rats received 312 or 625 mg/kg androstenedione

dissolved in corn oil by gavage three times at 24 hr intervals; vehicle control rats received corn oil only. Two cyclophosphamide positive control groups were included (15 or 25 mg/kg). Twenty-four hours after the final dosing, smears of the bone marrow cells obtained from the femurs were prepared. Air-dried slides were fixed, stained with acridine orange, and coded; 2000 polychromatic erythrocytes (PCE, or reticulocytes) were scored per animal for frequency of micronucleated cells. A second evaluation of micronucleus frequencies was conducted in mice (protocol details in (Witt et al., 2000)). At the termination of the 14-week toxicity study with androstenedione (1 – 50 mg/kg/day by gavage), blood was obtained from male and female B6C3F1 mice, and smears were immediately prepared and fixed in absolute methanol. The frequency of micronucleated cells was determined in 2,000 normochromatic (mature) erythrocytes (NCEs) per animal. In addition to assessment of micronucleus frequencies, the percentage of polychromatic erythrocytes (PCEs) among erythrocytes was determined in both the rat and the mouse studies as a measure of bone marrow toxicity.

2.5 Statistics

Survival was calculated using the methods of (Kaplan and Meier, 1958) and dose related effects were analyzed using Cox's method (Cox, 1972) for testing two groups for equality and Tarone's (Tarone, 1975) life table test for trends. Animals that died from non-natural causes (e.g. dosing accident) were not used in the analysis. The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989) was used to analyze neoplasm and non-neoplasm lesion incidence, which used a survival-adjusted quantal response procedure. Organ and body weight data were analyzed by parametric methods (Dunnett, 1955; Williams, 1971, , 1972). Spermatid and spermatozoa data were analyzed using non-parametric methods (Shirley, 1977). Trends were analyzed using Jonckheere's test and if a trend was not evident, Dunnett test was used instead of Shirley. Outliers were identified by the Dixon and Massey test. Vaginal cyclicity data were arcsine transformed and analyzed by multivariate analysis of variance (Morrison, 1976). Proportions of regularly cycling females in each dose were compared to the control group using a Fisher's exact test. For the micronucleus assays, data are presented as the mean of all animals within a dose group ($\pm$ standard error). The frequency of micronucleated cells was analyzed for increasing trend over exposure groups using a one-tailed Cochran-Armitage trend test ($\alpha = 0.05$), followed by pairwise comparisons between each exposure group and the control group. To correct for multiplicity, a test was considered positive if 1) the trend test p value was ≤ 0.025 and 2) the p value for any single exposure group was $\leq 0.025/k$ where k = the number of exposure groups. For more information relating to statistical analysis, refer to (NTP, 2010).

3. Results

3.1 Subchronic Study

3.1.1 F344/N Rats—Survival, hematology, and clinical chemistry were considered to be unaffected and no gross or histopathologic lesions were attributed to androstenedione administration. Androstenedione increased female body weight gain (Table 1), but did not increase male body weight or body weight gain. There was no effect on estrous cycle length or time of estrous stages by androstenedione administration. Although spermatid concentrations were unaffected by androstenedione administration, there was a significant decrease in cauda epididymal sperm concentrations (Table 2).

3.1.2 B6C3F1 Mice—Survival, body weight and body weight gain, and hematology were unaffected by androstenedione administration. There was a dose related increase in adrenal X-zone atrophy and decrease in X-zone cytoplasmic vacuolization in female mice while body weight or weight gain was unaffected (Table 1). Sperm motility was decreased in the

50 mg/kg dose group (Table 2). Bone marrow hyperplasia was significantly increased in the 5 and 50 mg/kg male mouse groups, which was characterized by an increase of immature cells in the marrow cavity of the femur. This effect on the bone marrow was not associated with any hematology results.

3.2 Chronic Study

3.2.1 F344/N Rats—Survival was increased in the 10 mg/kg male group ($p = 0.018$) and there were no effects on survival in the other male dose groups or in the female rats. Body weights of treated males were similar to the control. Female body weights in the 20 and 50 mg/kg dose groups were generally greater than controls after week 17 and week 9, respectively, and on average were greater than controls over selected time periods; percent increase over control for 10, 20, 50 mg/kg/day female groups: weeks 1-13: 0%, 1%, 5%; weeks 14-52: 3%, 9%, 16%; weeks 53-101: 1%, 6%, 9% respectively.

In female rats there was a positive trend ($p = 0.029$) in mononuclear cell leukemia (MCL) with the 20 and 50 mg/kg dose groups increased over control (Table 3). There was a nearly significant ($p = 0.052$) negative trend in incidence of MCL in male rats with the 20 and 50 mg/kg doses groups significantly decreased from controls. Mononuclear cell leukemia was characterized by a proliferation of neoplastic large granular lymphocytes in the spleen and liver and in the blood vessels of many other tissues. The incidence alveolar/bronchiolar adenoma increased in the male rat 20 mg/kg dose group and one carcinoma was observed in the 50 mg/kg dose group (Table 3).

In the testis, the incidences of interstitial cell adenoma (including bilateral) occurred with a negative trend ($p < 0.001$), and the incidence was significantly decreased in 50 mg/kg males (Table 3). Interstitial cell adenomas were characterized by small to large nodules of interstitial cells that compressed the surrounding seminiferous tubules; larger neoplasms effaced much of the testicular architecture. Neoplastic cells had an abundant, pale, eosinophilic to amphophilic, finely vacuolated cytoplasm, fairly well-defined cell borders, and a central nucleus. In some adenomas, large areas were composed of these basophilic cells, having a smaller round to oval nucleus with stippled chromatin and a sparse amount of eosinophilic cytoplasm with indistinct borders.

In female rats, there was a decreasing trend ($p < 0.001$) in mammary gland fibroadenoma incidences and the incidence was significantly decreased in 20 and 50 mg/kg females compared to controls (Table 3). Furthermore, the incidences of multiple fibroadenomas were decreased. The incidences of fibroadenoma, adenoma, or carcinoma (combined) were significantly decreased in 20 and 50 mg/kg females, mainly due to the decreased incidences of fibroadenoma, relative to control females. Fibroadenomas consisted of both ductular and/or alveolar epithelium and fibrous connective tissue. Smaller neoplasms usually contained a higher proportion of glandular tissue, and larger ones consisted almost entirely of connective tissue. In larger neoplasms, the majority of the neoplasm was composed of fibrous tissue with only a few atrophied glands remaining. The central portions of larger neoplasms were often necrotic, and only faint outlines of glands were identified.

The incidence of mammary gland hyperplasia was significantly decreased in all dosed female groups, and the incidences of mammary gland cyst were significantly decreased in 10 and 50 mg/kg females (Table 3). Mammary gland hyperplasia was characterized by increased layers of ductular and/or alveolar epithelial cells, enlarged lobules, and enlarged ducts filled with secretory product. Alveolar epithelial cells often had a vacuolated cytoplasm. Mammary gland cysts were characterized by large dilated ducts that were more prominently distended with secretory product than the majority of the enlarged ducts in the hyperplastic mammary glands.

3.2.2 B6C3F1 Mice—Female survival was unaffected by androstenedione dosing. In males, there was a marginal increase in survival ($p = 0.059$) in the male 10 mg/kg dose group and the other dose groups were not affected. Dosed male body weights were similar to controls and female body weights were decreased by approximately 6% in the 50 mg/kg dose group.

Hepatocellular neoplastic incidence increased in both dosed male and female mice (Table 4). There were positive trends ($p = 0.024$) in the incidences of hepatocellular adenoma in male and female mice, and the incidences in the 50 mg/kg groups were significantly increased. The incidences of hepatocellular carcinoma in all dosed groups of females were significantly increased. There were significantly increased incidences of multiple hepatocellular adenoma (males and females) and multiple hepatocellular carcinoma (females). The incidences of hepatoblastoma and multiple hepatoblastoma were marginally increased in dosed males. There was a positive trend in the incidences of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma (combined) in males, and the incidence in the 50 mg/kg group was significantly increased.

Hepatocellular adenomas were characterized by well-circumscribed nodular lesions larger than a liver lobule and composed of well-differentiated hepatocytes of variable size and tinctorial properties. The cytoplasm varied among eosinophilic, basophilic, vacuolated, or a mixture. Normal lobular architecture was lost, and hepatic plates at the margins impinged at sharp angles to the surrounding normal liver plates. Central veins and portal tracts were not readily apparent, although some were trapped in the expanding mass at the periphery. Some adenomas were solid and others had plates one to three cell layers thick. Cellular atypia and mitotic figures were occasionally observed. Focal areas of fatty change (lipidosis) within adenomas were occasionally noted. Hepatocellular carcinomas were not always well demarcated and often had irregular borders as cells infiltrated into the surrounding parenchyma. Cellular atypia and mitotic figures were common. Nucleoli were often enlarged and multiple. Cells had eosinophilic, basophilic, vacuolated, or mixed tinctorial appearances. Some carcinomas had a solid growth pattern, while a trabecular pattern was also common. Necrosis was noted in some tumors. Incidence of pancreatic islet adenoma increased in 50 mg/kg males and 10 and 50 mg/kg females, and a decreased incidence of hyperplasia occurred in 50 mg/kg males, but these differences were not statistically significant (Table 5). In 50 mg/kg males, first incidence of pancreatic islet adenoma decreased with dose, and one animal had multiple pancreatic islet adenomas. Pancreatic islet adenomas were characterized by an increased size of a single islet with a more uniform population of cells having a pale pink, lacy cytoplasm and central nuclei with a delicate chromatin pattern.

The incidence of malignant lymphoma (lymphocytic, histiocytic, mixed, or undifferentiated) was significantly decreased in 50 mg/kg female mice. Malignant lymphoma was characterized by a proliferation of sheets of neoplastic lymphocytes in various organs, particularly the liver and spleen. Variable types of malignant lymphoma were represented.

Several non-neoplastic changes in female mice were noted in the clitoral gland, kidney, and submandibular gland (Table 6). Within the clitoral gland, glandular hyperplasia was characterized by increased amounts of sebaceous glands located around the ducts. Individual sebaceous gland cells were of normal size and tinctorial staining. Duct dilatation was characterized by an increase in the size and number of duct profiles. The ducts were lined by a keratinizing squamous epithelium, although the lining was often attenuated and consisted of a single layer of flattened squamous epithelial cells. The dilated duct contents were composed of pale, basophilic, amorphous material and keratin debris, although the cyst contents were often lost in processing.

In the female mouse kidney, glomerular metaplasia increased in the 50 mg/kg dose group (Table 6). Glomerular metaplasia was characterized by a cuboidal appearance of the parietal epithelium of Bowman's capsule as compared to the normally flattened epithelium of vehicle control female mice. The cuboidal appearance of the epithelial cells involved more than 50% of the surface of the parietal epithelium of a glomerulus. The parietal epithelium of Bowman's capsule in control male mice is predominantly cuboidal, whereas this epithelium in control female mice has a predominantly flattened or squamoid appearance; a normal sexual dimorphism in this species (Figure 1).

Cytoplasmic alteration was increased in the submandibular salivary gland for the dosed female mice groups (Figure 2). This lesion was characterized by an increase in prominence and size of the convoluted ducts due to increased amounts of eosinophilic granular material in the cytoplasm of the duct epithelial cells. In addition, the nuclei of cells with cytoplasmic alteration tended to be more basally located within the cytoplasm. The appearance was similar to the submandibular salivary gland of male mice, as the granular ducts in male mice normally exhibit a sexual dimorphism with increased amounts of granular eosinophilic material in the cytoplasm and a more basilar location of nucleus beginning at approximately 20 days of age.

3.3 Genetic Toxicity

Androstenedione was not mutagenic in either of two independent bacterial mutation assays conducted with and without induced rat or hamster liver metabolic activation enzymes. *In vivo*, no statistically significant increases in the frequencies of micronucleated PCEs (reticulocytes) were observed in bone marrow of male F344/N rats administered androstenedione (312.5 or 625 mg/kg) by gavage once daily for 3 days. Following 3 months of androstenedione administration (1 to 50 mg/kg), androstenedione administration did not affect the frequency of micronucleated NCEs in male B6C3F1 mice. In female mice, a small increase in the frequency of micronucleated NCEs was observed at the highest dose tested (50 mg/kg); although not significantly elevated above the vehicle control ($P=0.0142$; significance set at $p \leq 0.005$), this increase resulted in a significant trend ($P=0.001$), and the test in female mice was therefore judged to be equivocal (Table 7). No significant changes in the percentages of PCEs among total erythrocytes were seen in either the rats or mice, suggesting no androstenedione-associated toxicity in the bone marrow.

4. Discussion

Prior to the banning of over-the-counter sales, androstenedione was used as a dietary supplement by individuals who likely believed it would increase muscle mass during training. Due to concern for adverse health effects associated with its chronic use, the NTP conducted genetic, subchronic, and chronic toxicity studies of androstenedione in male and female rats and mice. The health effects of androstenedione will vary since androstenedione is an androgenic hormone that can be metabolized to a more potent androgen (e.g., testosterone) or to an estrogen, and different hormonal effects may occur depending on the metabolism and presence of steroid receptors within a specific tissue, which may vary with the age and sex of the animal.

In the 14-week studies, androstenedione did not demonstrate any dose-limiting toxicity effects in male and female rats and mice. Female rat body weights increased compared to the vehicle controls in both the 3-month and 2-year studies. A similar result occurred with oxymetholone, an androgenic anabolic steroid, which increased female rat body weights in subchronic and chronic studies (NTP, 1999). The increase in female rat body weights by androstenedione may be due to increased muscle mass, but neither muscle mass nor adipose mass were evaluated. The increased incidences of adrenal gland X-zone atrophy and X-zone

cytoplasmic vacuolization seen in female mice after a 14-week exposure indicate that androstenedione, or a metabolite, had an androgenic effect. Regression of the adrenal X-zone in female mice normally occurs rapidly during the first pregnancy. Regression of the X-zone in female mice can be stimulated by administration of androgens and can be delayed in males via castration (Holmes and Dickson, 1971; Tomooka and Yasui, 1978).

The reduction in sperm concentration in the rat cauda epididymis that occurred in the 3-month study, in the absence of effects on spermatid numbers, suggests androstenedione may be interfering with sperm maturation. This effect is different from previous studies which observed a decrease in spermatid and spermatozoa concentrations. Administration of testosterone or estrogen via implants decreases spermatid concentrations within the testes and results in reduced fertility (Robaire et al., 1979; Robaire et al., 1984) and the anabolic steroid oxymetholone, reduced sperm concentrations in the epididymides and spermatid numbers (NTP, 1999). Sperm motility in male mice was significantly decreased at the top dose, and similarly, oxymetholone reduced male mouse sperm motility (NTP, 1999). Since sperm motility is achieved within the epididymis, androstenedione administration may also have affected sperm maturation in male mice. These effects indicate a potential for exogenous androstenedione to produce adverse effects on fertility and reproductive performance. The genetic toxicity studies in bacteria and tests on male rodents reported here were negative, with the exception of an equivocal response in the peripheral blood micronucleus test in the high dose female mice exposed to androstenedione for 14 weeks. These results are in agreement with other published genetic toxicity test results (McKillop et al., 1983).

In the chronic study, there were statistically significant increased incidences of MCL, a common neoplasm in female F344/N rats (Haseman et al., 1998). Due to the low incidence in vehicle controls (10%) compared to the historical range for all routes (8% to 40%; mean, 22%) the relationship of this increased incidence to androstenedione administration was uncertain. Androstenedione also significantly increased the incidence of alveolar/bronchiolar adenoma or carcinoma (combined) in mid-dose male rats. Since these lung neoplasms were only marginally increased in the mid-dose (20 mg/kg) group and there was no reduction in survival or body weight in the 50 mg/kg group, it is unclear if the marginally increased incidence in the mid-dose group was compound related because of the lack of a dose response.

Interestingly, there were significant decreases in the incidences of mammary gland hyperplasia, mammary gland cysts, and mammary gland adenomas in female rats in the chronic study, which suggests that androstenedione administration ameliorated these lesions' endocrine mechanism(s). The decrease in the incidences of testicular interstitial cell adenoma in F344/N male rats, a neoplasm common to this strain (Haseman et al., 1998), also suggests that androstenedione administration had a similar effect in this 2-year study. The origination of testicular interstitial cell adenoma is thought to be through endocrine-mediated mechanisms (Cook et al., 1999), which androstenedione administration may have alleviated. Oxymetholone administration had a similar effect on testicular adenomas in rats (NTP, 1999). A previous study that administered testosterone, estradiol, or a luteinizing hormone (LH) receptor agonist to Fisher 344 rats demonstrated that these hormones reduced the high levels of circulating LH in older animals and inhibited formation of Leydig cell adenomas (Chatani et al., 1990). Androstenedione, or an androstenedione metabolite, likely had a similar effect on circulating LH levels, which is believed to be a stimulating factor for interstitial cell adenoma formation in the Fisher 344/N rat testis.

In the chronic mouse study, androstenedione was carcinogenic in male and female mouse liver. Although B6C3F1 mice have a high background rate of liver tumors, males more so than females, the increase in adenomas, multiple adenomas and carcinomas, and marginal

increase in hepatoblastomas indicates carcinogenic activity in the male mouse liver. In female mice, androstenedione increased the incidence of adenomas and carcinomas and multiple carcinomas. In contrast, male and female rats displayed no evidence of androstenedione-induced hepatocellular neoplasms, which may be due to differences in metabolism between the two species and/or genetic susceptibility. Androstenedione carcinogenicity within the liver is consistent with other androgens, which are known hepatocellular carcinogens (IARC, 1987). The incidence of these neoplasms in mice might have declined if androstenedione administration had been stopped. Stopping treatment with oxymetholone, a human liver carcinogen, results in regression of hepatic tumors (Fremond et al., 1987; Montgomery et al., 1980; Obeid et al., 1980; Velazquez and Alter, 2004). The androgen receptor also contributes to tumor promotion in the liver. Male mice lacking a functional androgen receptor in the liver have a considerably lower prevalence of hepatocellular tumors after a *N,N*-diethylnitrosamine challenge compared to wildtype mice (Kemp et al., 1989).

The increase of pancreatic islet cell adenomas, a rare neoplasm, was considered related to androstenedione administration due to the incidence in the 50 mg/kg group exceeding concurrent and historical control rates from all routes of administration. Male mice had a similar increase at the high dose, which also was considered related to androstenedione administration since it exceeded the historical control range from all routes of exposure. In addition, the day of first incidence decreased with increasing dose in male mice, which is supportive of an effect in the pancreas. It is not clear by what mechanism androstenedione administration would induce these neoplasms, but the mouse pancreatic islet β cells express the androgen receptor, which may play a role in β -cell proliferation (Li et al., 2008). CYP 17, the steroidogenic enzyme responsible for converting progestins to androgens, has been identified in rat pancreatic islets (Ogishima et al., 2008), but has yet to be identified in mouse islet cells. The relation of androgens, pancreatic islet cells, and insulin is not well understood, but it is of interest due to polycystic ovary syndrome, in which individuals have increased levels of circulating androstenedione, and decreased insulin sensitivity (Schuring et al., 2008).

The decrease in the incidence of malignant lymphoma in female mice, a common type of neoplasm for this strain (Haseman et al., 1998), may be due to endocrine action since the immune system is sensitive to steroids (Beagley and Gockel, 2003; Bouman et al., 2005). Male B6C3F1 mice have a considerably lower background rate of malignant lymphoma compared to the female mice (average: 3% versus 21% for all routes), and the androgen action of androstenedione could be responsible for reducing malignant lymphoma incidence in female mice.

Androstenedione administration resulted in histological changes, indicating masculinization, in the kidney (Figure 1), submandibular salivary gland (Figure 2), and clitoral gland of female mice. The submandibular salivary gland is a sexually dimorphic and androgen-sensitive tissue. The withdrawal of androgens in male mice results in demasculinization within this gland and administration of androgens to females induces masculinization (Chretien, 1977; Kronman and Spinale, 1965; Sawada and Noumura, 1991). In addition to the submandibular gland changes, androstenedione administration resulted in glomerulus metaplasia in female mice, an appearance similar to that of the male mouse. This effect within the female mouse glomerulus was also noted after oxymetholone administration (NTP, 1999). The hormonal responsiveness of the clitoral gland to androgens is not well understood (Traish et al., 2002), but the increased incidence of clitoral gland hyperplasia in female mice may be related to the androgenic effects of androstenedione.

There were similar findings between oxymetholone and androstenedione in the chronic exposure rat gavage studies (Table 8), but there were some noted differences. Oxymetholone is a potent anabolic steroid, but it displays poor androgen receptor binding, while androstenedione binds the androgen receptor, albeit less potently than dihydrotestosterone, and displays limited evidence of an anabolic effect (Jasuja et al., 2005; Kicman, 2008; Saartok et al., 1984). In the chronic study, oxymetholone increased neoplasm incidences in the liver and skin of female rats, while androstenedione did not. The differences in liver tumor incidence may be related to differences in metabolism. The 17' methyl group within oxymetholone decreases the rate of liver metabolism and 17 α -alkylated androgens have side effects of hepatotoxicity (Snyder, 2001). However, androstenedione, as an endogenous non-17' methylated hormone, likely has a faster metabolism. The skin neoplasms observed with oxymetholone-treated female rats may be related to oxymetholone's anabolic activity, as anabolic steroids induce skin lesions, and oxymetholone accumulates within this site (NTP, 1999). In contrast there is limited evidence of anabolic activity by androstenedione (Jasuja et al., 2005). The effects of diet, NIH-07 for oxymetholone versus NTP-2000 for androstenedione, on outcome are not known, but dietary differences may also be a contributing factor in differences between the two studies.

In summary, there was clear evidence of androstenedione carcinogenic activity in the mouse liver, which is generally consistent with other androgens. The pancreatic islet adenomas in male and female mice were considered to be related to androstenedione administration. The incidences, both increases and decreases, of neoplasia in rodents administered androstenedione were generally similar to oxymetholone, an anabolic androgen. The exceptions may be due to differences in androgen receptor binding, metabolism, and anabolic activity. In male and female rats, there was equivocal evidence of carcinogenic activity based on lung neoplasms and mononuclear cell leukemia, respectively. Androstenedione administration reduced the incidence of neoplasms in several tissues that are well known endocrine targets, suggesting an ameliorative effect within these tissues, possibly due to compensation for adverse endocrine-mediated mechanisms that arise during the aging process.

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Androstenedione manuscript highlights for Food and Chemical Toxicology

- We investigated the chronic toxicity and carcinogenicity activity of androstenedione
- Androstenedione may or may not be carcinogenic in F344/N rats
- Androstenedione was carcinogenic in B6C3F1 mice
- Androstenedione decreased tumors in endocrine sensitive reproductive organs

Figure 1.

Glomerular metaplasia in female mice. In male mice this epithelium is normally cuboidal whereas in female mice it is normally flattened. A) kidney image from a female mouse in the control group. Note the flattened parietal epithelium lining Bowman's capsule (indicated by arrows). B) kidney image from a female mouse in the high dose group. The parietal epithelium lining Bowman's capsule is cuboidal, similar to males.

Figure 2.

Submandibular gland cytoplasmic alteration in female mice. In male mice the ducts are larger and contain more intracytoplasmic granular material than females. A) salivary gland image from a female mouse in the control group. B) salivary gland image from a female mouse in the high dose group with larger ducts and increased amount of eosinophilic intraductal material (indicated by arrows).

Table 1

Summary of female rat and mouse body weight and relevant histological findings in female mice after a subchronic (14 week) exposure to androstenedione (n=10/dose; mean $\pm$ SEM).

Androstenedione (mg/kg)	0	1	5	10	20	50
Female Rats Final weight (g)	189.3 $\pm$ 2.4	199.9 $\pm$ 3.7	198.1 $\pm$ 2.3	198.2 $\pm$ 2.3	205.9 $\pm$ 3.4**	199.1 $\pm$ 3.6
Female Rats Weight Gain (g)	97.5 $\pm$ 3.0	111.1 $\pm$ 3.6*	106.6 $\pm$ 1.9	105.7 $\pm$ 2.2	115.8 $\pm$ 3.0**	110.6 $\pm$ 3.9*
Female Mice Final weight (g)	30.9 $\pm$ 1.0	32.0 $\pm$ 1.5	31.6 $\pm$ 0.9	30.9 $\pm$ 1.0	30.8 $\pm$ 0.8	29.3 $\pm$ 0.7
Female Mice Weight Gain (g)	11.7 $\pm$ 1.0	12.6 $\pm$ 1.3	12.4 $\pm$ 0.8	11.7 $\pm$ 0.8	11.8 $\pm$ 0.8	10.2 $\pm$ 0.6
Female Mice Adrenal Cortex X-Zone:						
Atrophy ^a	0	0	6** (1.7)	9** (1.8)	10** (2.5)	10** (2.8)
Cytoplasmic Vacolization ^a	10 (1.6)	10 (1.7)	10 (1.4)	10 (1.2)	1** (1.0)	0**

^aSeverity of lesion in parentheses: 1=minimal, 2=mild, 3=moderate, 4=marked

* p < 0.05

** p < 0.01

Table 2

Summary of andrology results after subchronic androstenedione exposure to male B6C3F1 mice and F344/N rats (n = 10/dose; mean $\pm$ SEM).

Androstenedione (mg/kg)	0	10	20	50
Rat Spermatid heads (10^3 /mg testis)	124.9 $\pm$ 3.3	127.1 $\pm$ 4.8 ^a	131.8 $\pm$ 4.5	126.50 $\pm$ 3.6
Rat Spermatid heads (10^6 / testis)	169.4 $\pm$ 5.5	172.1 $\pm$ 4.1 ^a	178.0 $\pm$ 6.9	169.4 $\pm$ 5.9
Rat Sperm (10^3 /mg cauda epi)	575 $\pm$ 27	475 $\pm$ 28 [*]	463 $\pm$ 27 [*]	407 $\pm$ 21 ^{**}
Rat Sperm (10^6 /cauda epi)	112 $\pm$ 5	98 $\pm$ 5	95 $\pm$ 6	85 $\pm$ 5 ^{**}
Rat Sperm motility (%)	75.7 $\pm$ 2.2	76.0 $\pm$ 2.1	73.8 $\pm$ 2.4	74.7 $\pm$ 1.5
Mouse Spermatid heads (10^3 /mg testis)	189.5 $\pm$ 3.3	208.7 $\pm$ 11.0	219.2 $\pm$ 11.3 [*]	209.8 $\pm$ 11.3
Mouse Spermatid heads (10^6 / testis)	19.4 $\pm$ 0.7	22.2 $\pm$ 1.0	23.3 $\pm$ 1.1 [*]	20.9 $\pm$ 0.8
Mouse Sperm (10^3 /mg cauda epi.)	780 $\pm$ 110	958 $\pm$ 54	840 $\pm$ 54	879 $\pm$ 77
Mouse Sperm (10^6 /cauda epi)	18 $\pm$ 2	19 $\pm$ 1	18 $\pm$ 1	19 $\pm$ 1
Mouse Sperm motility (%)	67.2 $\pm$ 4.4	67.2 $\pm$ 2.5	68.8 $\pm$ 1.4	56.7 $\pm$ 3.9 ^{**}

^a
n = 9

^{*}
p < 0.05

^{**}
p < 0.01

Table 3

Incidences of lung, testis, mammary gland neoplastic and non-neoplastic lesions in male and female F344/N rats after chronic exposure to androstenedione.

Tissue Lesion	Androstenedione (mg/kg)			
	0	10	20	50
Mononuclear Cell Leukemia (male) ^b	26/50 (59.0) ^a	22/50 (47.8)	18/50 (39.9) [*]	18/50 (39.2) [*]
Mononuclear Cell Leukemia (female) ^c	5/50 (10.4)	11/50 (23.3)	18/50 (38.4)	15/50 (30.3)
Alveolar bronchiolar Adenoma (male) ^d	0/50 (0)	0/50 (0)	5/50 (11.6)	2/50 (4.6)
Alveolar bronchiolar Adenoma or Carcinoma (male) ^e	0/50 (0)	0/50 (0)	5/50 (11.6)	3/50 (6.9)
Interstitial Cell Adenoma (male)	42/50 (91.1)	39/50 (83.8)	36/50 (79.2)	26/50 (58.1) ^{***}
Interstitial Cell Adenoma – Bilateral	29/50	29/50	28/50	10/50 ^{**}
First Incidence (days)	464	540	558	607
Mammary Gland (female)				
Hyperplasia ^f	48 [2.2]	40 ^{**} [2.3]	35 ^{**} [2.0]	23 ^{**} [1.8]
Cyst ^f	15 [2.1]	3 ^{**} [1.7]	9 [2.1]	3 ^{**} [2.7]
Fibroadenoma	35 (72.3)	31 (66.1)	22 (47.6) ^{**}	12 (26.9) ^{***}
Multiple Fibroadenomas	17	13	6 ^{**}	3 ^{**}
Fibroadenoma, Adenoma, or Carcinoma	37 (75.7)	32 (68.2)	22 (47.6) ^{**}	12 (26.9) ^{***}

^aPoly-3 estimated neoplasm incidence after adjustment for mortality in parentheses

^bHistorical control incidence: methylcellulose vehicle 49/100, range 46-52% (unadjusted); all routes 553/1399 range 8-58%

^cHistorical control incidence: methylcellulose vehicle 17/100, range 10-24% (unadjusted); all routes 297/1350, range 8-40%

^dHistorical control incidence: methylcellulose vehicle 1/100, range 0-2% (unadjusted); all routes 34/1399, range 0-8%

^eHistorical control incidence: methylcellulose vehicle 1/100, range 0-2% (unadjusted); all routes 47/1399, range 0-10%

^fSeverity of lesion in brackets: 1=minimal, 2=mild, 3=moderate, 4=marked

*
p < 0.05

**
p < 0.01

p < 0.001

Table 4

Incidences of hepatocellular neoplastic lesions in male and female B6C3F1 mice after a chronic exposure.

Tissue Lesion	Androstenedione ^a			
	0 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg
<i><u>Males</u></i>				
Adenoma	32 (71.2)	38 (78.6)	29 (63.9)	43 (91.8)**
Adenoma Multiples	16	27*	23	34**
Carcinoma	26	33	28	32
Carcinoma Multiples	7	12**	10	17**
Blastomas	3 (6.9)	8 (16.4)	7 (15.7)	8 (17.6)
Carcinoma Multiples	0	1	1	3
A, C, or HB ^b	41 (85.2)	47 (94.1)	43 (89.4)	48 (98.5)*
<i><u>Females</u></i>				
Adenoma	14 (31.6)	16 (34.6)	18 (39.1)	28 (61.1)**
Adenoma Multiples	4	7	7	17**
Carcinoma	5 (11.3)	13 (28.2)*	15 (32.0)*	15 (32.7)*
Carcinoma Multiples	1	2	5	4
A, C, or HB	17 (38.2)	23 (49.5)	27 (57.0)	32 (69.8)***

^aPoly-3 estimated percent incidence after adjustment for mortality in parentheses^bA=adenoma, C=carcinoma, HB=hepatoblastoma*
p < 0.05**
p < 0.01***
p < 0.001

Table 5

Incidences of non-neoplastic lesions of the pancreas and islet cell adenomas in male and female B6C3F1 mice after chronic exposure to androstenedione via gavage.

Androstenedione				
<i>Males</i>	0 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg
Hyperplasia ^a	12 [2.3]	14 [2.5]	11 [2.3]	6 [2.0]
Adenomas ^{b,c}	2/50 (4.6)	2/50 (4.1)	2/50 (4.5)	5/49 (11.1)
Multiple Adenomas	0	0	0	1
Day of First Incidence	729	729	620	493
<i>Females</i>	0 mg/kg	2 mg/kg	10 mg/kg	50 mg/kg
Adenomas ^{b,d}	0/49 (0.0)	2/50 (4.4)	4/49 (9.0)	4/48 (8.9)
Day of First Incidence	-	729	729	654

^aSeverity of lesion in brackets: 1=minimal, 2=mild, 3=moderate,4=marked

^bPoly-3 estimated percent incidence after adjustment for mortality in parentheses

^cHistorical control incidence: methylcellulose vehicle 2/100, range 0-4% (unadjusted); all routes 17/1435, range 0-6%

^dHistorical control incidence: methylcellulose vehicle 1/95, range 0-2% (unadjusted); all routes 11/1478, range 0-2%

Table 6

Incidences of non-neoplastic lesions in female B6C3F1 mice after chronic exposure to androstenedione via gavage.

Tissue Lesion ^a	Androstenedione (mg/kg)			
	0	2	10	50
Clitoral Gland Hyperplasia ^b	0/47	2/47 [1.0]	13/49 ^{**} [1.9]	41/50 ^{**} [2.0]
Clitoral Gland Duct Dilation ^c	0/47	2/47 [1.0]	17/49 ^{**} [1.4]	49/50 ^{**} [2.6]
Kidney Glomerulus Metaplasia	2/50 [1.5]	1/50 [2.0]	5/50 [1.0]	27/50 ^{**} [2.0]
Submandibular Salivary Gland Cytoplasmic Alteration	0/49	17/49 ^{**} [1.2]	40/49 ^{**} [1.4]	45/50 ^{**} [2.5]

^a Severity of lesion in brackets: 1=minimal, 2=mild, 3=moderate, 4=marked;

^b Severity grades for glandular hyperplasia and duct dilatation were based on a semiquantitative evaluation as described in the methods section.

^{**}
p < 0.01

Table 7

Frequency of MN-PCE (mean $\pm$ SEM) in B6C3F1 mice following administration of androstenedione by gavage for 14 weeks¹

Male mice			
	Dose (mg/kg)	MN-NCE/1000	% PCE
	0	2.6 ± 0.51	2.9
	1	2.9 ± 0.62	2.5
	5	2.8 ± 0.30	3.0
	10	2.7 ± 0.54	3.0
	20	2.4 ± 0.46	2.9
	50	2.1 ± 0.10	2.5
	Trend p = 0.880 ²		
Female mice			
	Dose (mg/kg)	MN-NCE/1000	% PCE
	0	1.6 ± 0.43	2.8
	1	1.3 ± 0.20	2.8
	5	1.4 ± 0.29	3.3
	10	2.0 ± 0.57	3.2
	20	1.3 ± 0.12	3.2
	50	3.1 ± 0.40 ⁴	2.6
	Trend p = 0.001		

¹ Five animals per group; NCE, normochromatic erythrocytes; PCE, polychromatic erythrocytes (reticulocytes)

² One-tailed Cochran Armitage trend test, significant at p $\leq$ 0.025

⁴ p = 0.0142; for significance, p must be $\leq$ 0.005.

Table 8

Comparison of chronic exposure results between oxymetholone (F344/N rats) and androstenedione (F344/N rats and B6C3F1 mice)

<i>Tissues with Neoplasm or Neoplasm</i>	<i>Oxymetholone^a</i>		<i>Androstenedione</i>	
	Male Rats	Female Rats	Male Rats	Female Mice
Testis	↑		↑	
Mammary Gland		↓	↑	
Lung		↑	↑ ^b	
Liver		↑		↑
Pituitary Gland		↓		
Skin		↑		
Pancreatic Islet				↑
MCL ^c Lymphoma ^d	↓	↓	↓	↑ ^b

^a NTP, 1999

^b Although a statistical increase, this finding may have been related to androstenedione administration

^c Present in rats

^d Present in mice