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Acute oral coumestrol treatment induces sperm and sex steroid alterations in mice

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Abstract: Plant phytoestrogens interfere with normal estrogen-regulated functions like steroid synthesis and gonad physiology and morphology. Much evidence has been obtained by using high dose treatments or in vitro exposure to phytoestrogens but little is known about low, dietary level concentrations of these compounds, particularly coumestrol. In order to explore the possible effects on gonads and serum progesterone of oral 10, 20 or 40 µg/Kg body weight dose coumestrol were administered to three experimental groups and compared to a vehicle-only control group (n=5 animals per group) for two weeks and a similar period for treatment recovery. After treatment, testes and blood were obtained and processed for testis and sperm morphology alterations, and steroid hormone evaluation, respectively. Coumestrol treatment induces a significant dose-dependent testis volume decrease and a decrease in 17β hydroxysteroid dehydrogenase activity causing a progesterone increase in response to phytoestrogen concentration. These alterations impair the normal sperm production with an increase in abnormal head and tail shapes. These data strongly suggest a deleterious effect of oral, low concentration phytoestrogen content in adult male diets.

Keywords: Phytoestrogens, Coumestrol, Testis Alterations, Serum Progesterone, Mice

1. Introduction

Phytoestrogens are non steroid plant derived compounds that structurally resemble natural steroid hormones, mainly testosterone and progesterone in such a way that they effectively interfere for their binding to the nuclear Estrogen receptors (Björnström and Sjöberg, 2005; Kurzer and Xu, 1997). Several lines of evidence indicate that phytoestrogen consumption has either a positive or negative impact on human and animal health.

Phytoestrogen effects are quite controversial. Some reports indicate a positive influence on, for example, the control and lowering of serum lipid profile in postmenopausic women (Terzic, et al., 2012), the positive correlation between maternal phytoestrogen intake and the lower incidence of hypospadias in the human newborn male (Carmichel, et al, 2013) and the decrease in ovarian cancer risk (Bandera et al, 2011). On other side, it has been shown a 3 fold increased risk of hyperthyroidism development in subclinical patients whit a phytoestrogen rich diet (Sathyapalan, et al., 2011). There are also studies indicating that neonatal injection of 100 µg coumestrol does not alter neither the adult FSH, LH, testosterone or testis weight in rats (Awoniyi, et al., 1997).

Dietary phytoestrogen exposure is now common since soy is the main component of processed meat-based and dietary fiber-rich food complements (Cederroth, et al, 2010, 2011). One major concern is the fact that phytoestrogens are now abundant not only in adult diets but also in infant and newborns. According to the 2010 brief made by the National

Toxicology Program Center for the Evaluation of Human Reproductive Risks, more evidence of the effect of low level, dietary oral dose of phytoestrogen is needed and even though there are insufficient evidence for a developmental toxicity of phytoestrogens present in soy based baby formulas, caution should be taken in their administration since there are clear evidence for genistein to have adverse toxicity on reproductive aspects in laboratory animals (McCarver, et al., 2011).

This fact has raised the possibility of a more severe effect of soy-based foodstuff used for human nutrition and supplementation. It has been shown that perinatal exposure of female rats to coumestrol (Whitten, et al., 1995), and genistein (Jefferson, et al., 2012), induces persistent oestrus and suppresses sexual behavior once the animals reach adulthood. Also, coumestrol given orally to bats (Serrano et al, 2007) and dogs (Perez-Rivero, et al. 2009) induce morphological and sperm alterations in male gonads indicating a potential risk for reproduction of those phytoestrogens obtained directly from soy-rich diets. The goal of the present study is to evaluate the effect of acute oral coumestrol treatment at dietary concentrations on the mouse testis and to determine their effect on serum steroid content of animals treated. The results indicate a concentration dependent testis morphology alteration and disruption of the progesterone to testosterone conversion.

2. Material and Methods

Twenty adult 3 months old adult CD1male mice were distributed randomly into one of the four groups used (5 animals per group). Experimental animals were treated daily for a two week period with 10, 20 or 40 µg/kg body weight coumestrol whereas control group receive a similar amount of vehicle. Coumestrol (Sigma Chemical Co, St. Louis, MO) was dissolved at the desired concentration in 100µl Dimethyl Sulfoxide (DMSO, Sigma Chemical Co, St. Louis, MO) and given intragastrically to each animal in the corresponding group. After treatment, all animals were left untreated for two extra weeks before sacrifice. Testis were aseptically excised, blood, and epididymal sperm were obtained by direct puncture. Sperm samples were incubated in citrate-containing saline and maintained at 27°C and fixed in Carnoy (Methanol:Acetic acid) solution before Giemsa staining. All chemicals were of the highest quality available.

Testes were measured and volume estimated according to Klomberg et al, (2002). Sperm morphology was evaluated in stained samples from at least 200 cells taken at 10 different random fields. Serum was obtained from clotted blood and frozen until processing. Progesterone and testosterone content was determined by ELISA by using hormone specific immobilized antibodies, a second HRPO-conjugated antibody was used to determine the amount of trapped hormone after ABTS-H_2O_2 addition (Calbiotech Inc, Spring Valley, CA). Oxidized ABTS absorbance was determined in a diode 509 nm plate reader. Statistical significance was determined by using the Student t test.

3. Results

One major concern with dietary phytoestrogens is related to their negative effects on reproduction. In the present study, coumestrol induces a dose-dependent testis volume decrease being the 40 µg/Kg dose the one that induces a 60% loss in volume when compared with vehicle only control group ($p<0.05$).

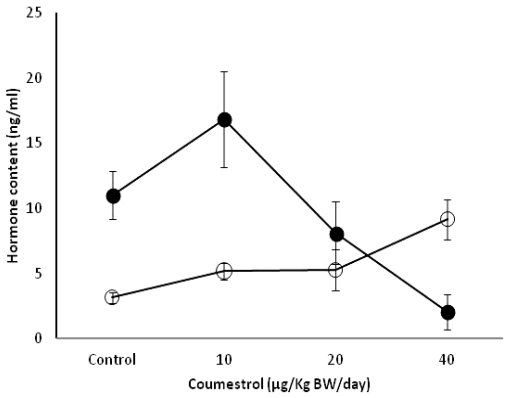

Fig 1. *Circulating steroid hormone alterations induced by acute oral coumestrol administration. Sera was obtained as described under Material and Methods. Testosterone content (solid circles) and progesterone (open circles) were determined by ELISA. Data are mean and SD from 5 independent determinations.*

It has been reported that phytoestrogen genomic action is through binding directly to either αER or βER nuclear Estrogen Receptor (Björnström and Sjöberg, 2005; Li, et al, 2013). When phytoestrogen is absent, estrogen could bind to the cytoplasmic form of the nuclear receptor. Ligand binding allows ER dimer formation and translocation into the nucleus where the complex interacts with specific response sequences (ERE) on the promoter they regulate. The molecular structure of phytoestrogens effectively interferes with these interactions modifying the otherwise estrogen-evoked response (Whitten and Patisaul, 2001). A primary response of gonads is on steroidogenesis. It has been well documented that estrogen biotransformation require the participation of testis enzymes like the aromatase (CYP19a1) and 17β dihydroxysteroid dehydrogenase (DHSD17β) expressed in Leydig interstitial or Sertoli tubular cells that participate in sperm terminal differentiation (Vitale et al, 2013). As can be seen in figure 1, acute coumestrol treatment impairs the steroidogenic process in treated animals. Progesterone accumulation could indicate the lack or at least the lowering in activity of the 17 beta hydroxysteroid dehydrogenase enzyme.

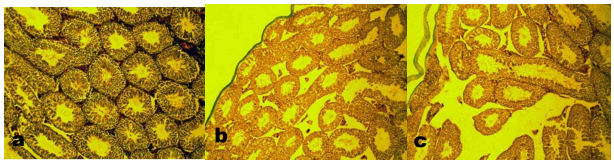

Figure 2. *Effect of coumestrol on mouse testis. The homogeneous distribution of testis tubuli (a) is disturbed after acute coumestrol treatment in either 10 µg/Kg (b) or 40 µg/Kg (c) dose (100X)*

The impairment of the estrogenic pathway inside the testis effectively interferes in sperm production rendering to alterations in tubule abnormalities. As can be seen in figure 2, one simple effect of this alteration is the increase in extratubular material rendering to a less compact architecture in response to coumestrol concentration.

Sperm production was also affected. As shown in figure 3, at least a 10% of the total epididymal spermatozoa from treated mice are abnormal. The most common alteration is in the tail that could be either the complete loss of the organelle or the presence of a characteristic bent in either the middle or the upper part of the tail, close to the implantation site. The presence of the cytoplasmic drop in less than 5% of the spermatozoa is a common finding in this kind of sample.

4. Discussion

Major concerns have been recently raised about the safety of phytoestrogen-containing food (Carmichel, et al, 2011; Jefferson et al, 2012). Phytoestrogens can impair the mammalian normal reproductive function through the binding to both cytoplasmic ER or directly to certain enzymes that regulate key functions during steroidogenesis (Barnes, 2010, Ye, et al, 2011). Coumestrol has been shown to act either as an agonist or antagonist of estradiol in a dose dependent as biphasic potent ER regulator (Vitale, et al, 2013).

Most of the well documented effects on fertility of phytoestrogens have been obtained by using high concentrations and continuous chronic exposures. Little is known about the dietary, acute exposure to phytoestrogens. In any case, the dramatic effects of phytoestrogens can be measured not only in individuals treated directly with geninstein (LeeCole, et al, 2011) or coumestrol (Tinwell, et al., 2000) but also in utero exposure. In utero exposure can reduce the time to achieve sexual maturity (Zawatski and Lee, 2013). Human exposure to soy phytoestrogens as early in lifetime as in lactation is now a common practice in the West hemisphere when lactose intolerance is suspected or detected (Barnes, 2010).

Oral acute consumption of low microgram and dietary-equivalent amounts of coumestrol has effects on testis morphology. When compared to normal testes, seminiferous tubules from treated animals are fragile with high interstitial space (fig. 2) even when an equal period of possible recuperation was taken. Gonad characteristics are also altered as the loss in testis volume indicates. Finally, the high serum progesterone observed in treated animals strongly indicates the alteration in steroidogenesis.

As mentioned earlier, the effect of phytoestrogens are rather controversial. For example, rats whose pregnant mothers were treated with genistein-containing pellets and then either maintained under phytoestrogen treatment or normal diets have differential effects that indicate the innocuity of phytoestrogen diets even thought there are small decreases in testosterone serum content at d21 after birth that are normalized at adulthood (Roberts et al, 2000). Our results are in agreement with those of Tarragó-Castellanos et al. (2006) in that they also found a significant decrease in testosterone content and alterations in testis ultra-structure that indicate an spermatogenesis inhibitory effect in coumestrol-injected male rats. One major difference with other highly used phytoestrogen administration protocols is that we use the oral route that requires much less coumestrol amount when compared to the intramuscular or subcutaneous injection. Also, is the most common exposure via to these naturally occurring endocrine disruptors for mammals.

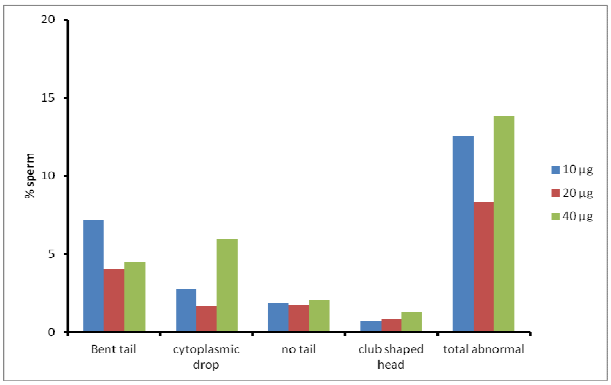

Figure 3. *Effect of coumestrol on mouse sperm morphology. Epidydimal spermatozoa from treated mice were obtained and stained as described in Material and Methods. Abnormal spermatozoa were identified according to the main alteration observed. Data are from 200 sperm in at least 5 independent preparations.*

The molecular mechanism for seminiferous tubuli induced by phytoestrogens is far from being elucidated since there are no studies focusing this subject. One possible explanation is the global alteration of the proliferation in the basal spermatogonial compartment regulated by the Wnt/beta catenin system (Kerr et al., 2014). Also, this alteration could indicate an exacerbation of the apoptotic process. In this study this question was not addressed and requires further investigation

Sperm morphology alterations found in epidydimal spermatozoa clearly indicate the impairment of the spermatogenic process since head alterations are usually generated during sperm nuclear remodeling that occurs directly in the testis (Fuentes-Mascorro, et al., 2000). As can be seen on figure 3, head shape is present in all dose used and even when this alterations has a minor contribution to altered sperm, they represent the alteration of small amount of oral phytoestrogens.

Regarding to the cytoplasmic drop of the abnormal sperm, they represent the impairment of the seminiferous tubule, particularly the Sertoli cell function, and the epididymal sperm maturation process during epidydimal transit (Yanagimachi, 1994).

Collectively, the data shown indicate the potential reproductive alterations that the use of low, dietary equivalent amounts of coumestrol can induce in an acute exposure even after a post exposure period equivalent to that of the exposure. Further research is needed in order to establish the molecular, physiological and behavioral

alterations induced by phytoestrogens, particularly coumestrol on different mammals.

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