Effects of cottonseed flour on immunohistochemical localization of androgen receptors (AR) in rat testes

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SUMMARY

The objective of the present study was to investigate the effect of the consumption of the gossypol rich cottonseed flour (CSF) on immunohistochemical localization of androgen receptors (AR) in rat testes. For that, 50 male rats were divided into 5 equal groups according to the CSF content (0%, 5%, 10%, 20% and 40%) added to the standard ration for 20 and 60 days. The corresponding gossypol doses measured by spectrophotometry were 0.641, 1.282, 2.654 and 5.128 g/kg of food respectively. The AR localization in Leydig, Sertoli and peritubular myoid cells was determined by semi-quantitative immunohistochemistry after fixation of testes in 10% neutral buffered formalin and staining with the streptavidin-biotin-peroxidase method. All dietary CSF treatments significantly decreased the AR nuclear expression in the 3 cell types except for 5% CSF in the Leydig cells. These effects were dose and time dependent and the variations coefficients varied from -1.8% to -84.9% in the Leydig cells, from -22.5% to -99.3% in the Sertoli cells and from -35.5% to -88.9% in the myoid cells. These results clearly demonstrated the adverse effects of the CSF and gossypol on the AR expression in rat testes, particularly in the Leydig and Sertoli cells which can lead to infertility.

Keywords: Cottonseed flour, rat, androgen receptor, immunohistochemistry, testes, Leydig cells, Sertoli cells, Myoid cells.

Introduction

Cottonseed and its by-products are commonly used as food supplements in livestock rations. However, cottonseed and cottonseed by-products contain gossypol, a yellow, polyphenolic glycoside known to have cardiotoxic and hepatotoxic effects [14, 15]. In addition, it has been reported that gossypol interferes with fertility of male in various animal species, including human [9, 19, 22]. The infertility effect of gossypol was discovered first in China, where cotton cake and fiber from the plant had been consumed by animals and by man. COUTINHO et al. [6] reviewed that the infertility dose of gossypol is 30 mg/kg of body weight in the rat, but in man gossypol is much more efficient, and infertility is achieved with only 0.3 mg/kg of body weight. Gossypol is known to cause testicular dysfunction and infertility, and its use has been proposed as a male contraceptive [6]. However, the mechanism by which gossypol affects the testes is still not fully understood. Available data about gossypol suggest that it prevents spermatogenesis [19].

Androgens are critical steroid hormones that determine the expression of the male phenotype as well as the initiation and maintenance of spermatogenesis [10]. Their actions are mediated by the androgen receptor (AR), a member of nuclear receptor subfamily. Androgen and AR play important roles in the male spermatogenesis and fertility [5, 16, 28]. To our knowledge, there is no report about the effects of cottonseed flour (CSF) or gossypol on immunohistochemical localization.
of AR. The objective of the present study was to investigate the effect of consumption of cottonseed flour (CSF) on immunohistochemical localization of androgen receptors in rat testes and provide additional mechanistic information on the gossypol effect on testes.

**Materials and Methods**

**ANIMALS AND PROTOCOL DESIGN**

A total of 50 male-Wistar rats, 3.5 months old, weighing approximately 350-400 g, were used. The animals were fed with a standard diet for 10 days, housed as 5 animals per cage and subjected to daily lighting regime of 12 h light followed by 12 h dark. They were divided into 5 equal groups of 10 animals each according to the cottonseed flour dietary supplementation level for 20 or 60 days: 0% (Group I, control), 5% (Group II), 10% (Group III), 20% (Group IV), and 40% (Group V). Cottonseed flour was prepared by separation of seed content from outer layer by cracking followed by sifting and the resulting flour was added to the diet before mixing and making pellets. Food and water were provided ad libitum throughout the study. The contents of cottonseed flour used in this study were analyzed according to AOAC techniques [1]. The free gossypol concentration was determined by spectrophotometry in the Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Ankara, Turkey. After 20 and 60 days, 5 rats from each treatment group were decapitated under deep ether anaesthesia. The experimental protocol was approved by local ethic committee of Veterinary Control and Research Institute. Testicles were immediately removed and fixed in 10% neutral buffered formalin.

**IMMUNOHISTOCHEMICAL ANALYSIS**

For immunohistochemical examination, the tissue sections were deparaffinised and rehydrated through a series of graded alcohols and stained with the streptavidin-biotin-peroxidase method [3]. Endogenous peroxidase activity was blocked by 10 minutes incubation in 3% H2O2 in methanol and blocking of nonspecific binding was achieved with incubation in PBS containing 10% normal goat serum for 30 minutes at room temperature. Sections were then incubated in a moist chamber at 4°C overnight with an affinity purified polyclonal antibody targeted against rat AR [18], named PG 21, diluted at 1:50 in PBS. Thereafter, sections were washed with PBS three times (10 minutes each) and treated with biotinylated goat anti-rabbit antibody for 30 minutes followed by streptavidin-peroxidase for 30 minutes. Antibody binding was visualized using glucose oxidase - diamino benzidine (DAB), nickel ammonium sulphate (GDN) substrate [20] for 10 minutes. After washing with distilled water, sections were counterstained with eosin. The localization of AR immunostaining was examined by a light microscope [18, 21]. The AR positive cells (i.e. Sertoli, Leydig and myoid cells) were counted in 5 randomly selected microscopic fields under a 40 X magnification, and arithmetic means were determined.

**STATISTICAL ANALYSIS**

Statistical analysis of the data was performed using SPSS software [23]. Kruskall Wallis and Wilcoxon’s signed rank tests were used for the significance of differences among the groups and days, respectively. Differences were considered as significant when P value was less than 0.05.

**Results**

**DETERMINATION OF DIETARY GOSSYPOL CONTENTS**

The CSF (dry matter: 95.6%) was rich in crude fat (38.9%), in crude proteins (31.8%) and in nitrogen free extract (NEA) (12.8%) but weakly provided crude fibre (9.2%). The free gossypol concentration in CSF used was determined as 12.820 g/kg of CSF, corresponding to dietary supplies of 0.641, 1.282, 2.654 and 5.128 g/kg of food in the groups II (5% CSF), III (10% CSF), IV (20% CSF) and V (40% CSF) respectively.

**AR IMMUNOLABELLING**

The AR expression was specifically determined in nuclei of the Leydig cells, peritubular myoid cells (figure 1) and in the Sertoli cells (figure 2). However, no AR immunostaining was found in any type of the germinal cells. The semi-quantitative evaluation of the somatic cells in the testes revealed that the frequencies of AR positive cells in the CSF treated groups were significantly lower than in the control group (figure 3 and Table I).

The variations of the numbers of AR positive cells counted in 5 randomly selected microscopic fields under a 40 X magnification according to the dietary CSF contents and to the duration of CSF supplementation were reported in Table I. In controls (0% CSF), the AR expression was maximal in the Leydig cells and minimal in the peritubular myoid cells whereas the number of AR positive Sertoli cells was intermediate. After a 20 days long CSF dietary supplementation, the frequency of AR positive cells in testes from supplemented rats significantly decreased compared to controls (P < 0.01) except for positive Leydig cells from rats treated with 5% CSF (group II) which the number did not significantly differ with value recorded in controls. Moreover, the depletion in AR positive Sertoli and myoid cells significantly increased according to the dietary CSF content (group II vs. groups III, IV and V: P < 0.01 for both cell types, group III vs. groups IV and V: P < 0.01 for the Sertoli cells and groups III or IV vs. group V: P < 0.01 for the myoid cells). The variation coefficients of the frequency of the AR positive Sertoli and myoid cells according to the CSF amount in the ration ranged from -22.5% (for 5% CSF) to -94.9% (for 40% CSF) and from -35.5% (for 5% CSF) to -80.6% (for 40% CSF), respectively. The frequency of AR positive Leydig cells was also significantly depressed in rats supplemented with 20% and more CSF compared to controls (P < 0.01) but, as the parameter weakly decreased according to the CSF dietary

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FIGURE 1: AR immunostaining of Leydig cells (thick arrow) and peritubular myoid cells (thin arrows) in the testis of a rat fed with 5% CFS for 20 days. ABC method, Eosin counterstain, X 400.

FIGURE 2: AR immunostaining of Sertoli cells (arrows) in the testis of a rat fed with 10% CFS for 20 days. ABC method, Eosin counterstain, X 400.

FIGURE 3: AR immunostaining (arrows) in testis from one negative control rat (0% CFS) (A.) and from a rat fed with 20%CSF (B.) on day 60. ABC method, Eosin counterstain, X 200.

<table>
<thead>
<tr>
<th>Control (0% CSF)</th>
<th>Group I (5% CSF)</th>
<th>Group II (10% CSF)</th>
<th>Group III (20% CSF)</th>
<th>Group IV (40% CSF)</th>
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<tr>
<td></td>
<td>Number</td>
<td>VC (%)</td>
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<td>VC (%)</td>
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<td><strong>Leydig cells</strong></td>
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<td>On day 20</td>
<td>33.6 ± 1.6a</td>
<td>-1.8%</td>
<td>18.5 ± 1.4b</td>
<td>-44.9%</td>
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<td>On day 60</td>
<td>34.5 ± 1.8a</td>
<td>-7.0%</td>
<td>15.4 ± 1.6b</td>
<td>-55.4%</td>
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<td><strong>Sertoli cells</strong></td>
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<td>On day 20</td>
<td>13.8 ± 0.8a</td>
<td>-22.5%</td>
<td>6.7 ± 1.0c</td>
<td>-51.4%</td>
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<tr>
<td>On day 60</td>
<td>14.2 ± 1.1a</td>
<td>-38.7%</td>
<td>4.4 ± 0.5c</td>
<td>-69.0%</td>
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<td><strong>Myoid cells</strong></td>
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<tr>
<td>On day 20</td>
<td>9.3 ± 0.7a</td>
<td>-35.5%</td>
<td>4.6 ± 0.1c</td>
<td>-50.5%</td>
</tr>
<tr>
<td>On day 60</td>
<td>9.9 ± 0.9a</td>
<td>-45.5%</td>
<td>4.8 ± 0.6b</td>
<td>-51.5%</td>
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VC (%): Variation coefficient given by the following formula: 100 x (NT – NC) / NC where NT: number of AR positive cells in the treated group and NC: number of AR positive cells in the control group.

Different superscripts indicate significant differences (P < 0.01) between groups.

Different superscripts A, B in the same column indicate significant differences (P < 0.05) according to the time exposure.

TABLE I: Counts of androgen receptor (AR) positive Leydig, Sertoli and peritubular myoid cells in testes from controls (not supplemented) or rats dietary supplemented with 5%, 10%, 20% and 40% CFS for 20 days or for 60 days. Positive cells were counted in 5 randomly selected microscopic fields under a 40 X magnification. Results are expressed as means ± standard errors.
content, the relation dose-effect was less marked as far as the positive Leydig cells were concerned; however, the variation coefficients decreased from -1.8% with 5% CSF in the ration to -62.3% with 40% CSF.

After a 60 days long CSF dietary supplementation, the reduction of the numbers of AR positive cells, was aggravated whatever the cell type. All numerations of positive cells were significantly depressed in supplemented rats compared to controls (P < 0.01) except the number of Leydig cells recorded in rats treated with 5% CSF. Significant differences (P < 0.05) in frequencies of AR positive Leydig cells between the 2 supplementation duration (20 days and 60 days) were observed in rats treated with 20% and 40% CSF. Whereas the number of positive myoid cells from rats treated for 60 days compared to the value measured in rats treated for 20 days was significantly reduced only with the highest CSF content, the frequency of positive Sertoli cells was significantly lowered after 60 days in rats treated with 5% and 20% CSF (P < 0.05). In addition, the variation coefficients of the frequencies of the AR positive cells were markedly higher when treatment has lasted 60 days for the 3 cell types: they varied according to the CSF content in the ration from -7.0% to -84.9% for the Leydig cells, from -45.5% to -88.9% for the myoid cells and from -38.7% to 99.3% for the Sertoli cells (Table I).

Furthermore, the intensity of the nuclear AR labelling gradually declined according to the CSF content and to the exposure duration in the 3 cell types.

**Discussion**

Results of the present study indicated that the addition of CSF to the diets of rats at 5%, 10%, 20% or 40% resulted in a decrease in the AR immunopositive cell numbers in testes depending on the dose and exposure time. It has been reported that androgens mediate a wide range of developmental and physiological responses, especially in male sexual differentiation and pubertal sexual maturation, maintenance of spermatogenesis, and male gonadotropin regulation [10, 16, 28]. The effects of androgen are mediated through the androgen receptor, a 110-kDa ligand–inducible nuclear receptor that regulates the expression of target genes through binding to a receptor, a 110-kDa ligand–inducible nuclear receptor that regulates the expression of target genes through binding to a receptor.

There are different hypotheses dealing with the effects of cottonseed or gossypol on the Leydig cells. Although several researchers have reported that cottonseed or gossypol did not affect the Leydig cells [12, 17, 19, 21], some others showed that long-term and high dose treatment with gossypol resulted in a change in the Leydig cell morphology such as nuclear shrinking and fat droplets in the cytoplasm [9, 26, 31]. Besides, it has been reported that gossypol decreased the androgen secretion [11]. Likewise, foamy vacuolisation of the Leydig cells was previously described in rats fed with a diet containing 20% and 40% CSF [24]. In the current study, the addition of 5% CSF to the diets has not significantly affected the frequency of AR positive Leydig cells in rat testes, but larger amounts (10%, 20% and 40%) have significantly induced a marked depression of the AR expression in these cells depending of the CSF content and of the exposure duration at a lesser extent. In a study conducted on the Leydig cells of AR knockout mice, TSAI et al., [25] reported decreases in the lumen formation and in the diameter of seminiferous tubules, arrest of the germ cell development and the sperm absence in the epididymis. In agreement with that, we have previously reported in rats that a dietary 10% to 40% CSF supplementation caused a significant decrease in sperm motility and induced evident pathological changes including degeneration, disorganization or necrosis of germinative epithelium, and intra-tubular giant cell formation in testicular tissue depending on the dose and exposure time whereas 5% CSF in diet failed to induce modification of the sperm motility or degenerative lesions in the testicular tissue [24]. WANG et al. [28] concluded that a functional androgen receptor in Leydig cells was essential for the maintenance of spermatogenesis.

There are similar reports on the effects of cottonseed or gossypol on Sertoli cell morphology. It is well known that the Sertoli cells are more sensitive to gossypol than the other somatic cells in testes [24, 30], and gossypol causes vacuolisation, degeneration, exfoliation and disappearance of Sertoli cells [19]. These cells serve as the principal structural element of the seminiferous epithelium, providing physical support and immunological barrier in favour of normal germ cell development and maturation in testes [8]. It was stated that the androgen receptor in the Sertoli cells plays a central role in male spermatogenesis [4, 7, 13, 25, 27]. TSAI et al., [25] reported that in Sertoli cell-specific AR knockout mice, the same anatomical anomalies than those seen in the Leydig cell-specific AR knockout mice occurred leading to infertility. In addition, mating tests have shown that Sertoli cell-specific AR knockout mice failed to impregnate the fertile female mice [25]. The results of the present study revealed that CSF decreased the AR positive Sertoli cell numbers in all rats fed with CSF and that the repression of the AR expression was considerable (near 100%) in rats treated with 40% CSF content in the diet.

The peritubular myoid cells in the testes are mesenchymal cells that surround the Sertoli cells and form the outer wall of the seminiferous tubules. These cells are responsible for contractions of the seminiferous tubules to induce peristaltic-like waves and aim the transport of spermatozoa through the tubular lumen and into the epididymis [2, 28]. ANTHONY and SKINNER [2] indicated that androgen can act on peritubular myoid cells to stimulate the production of a paracrine factor, termed P-Mod-S which modulates some functions of the Sertoli cells. ZHANG et al. [29] reported that these cells contain a large amount of androgen receptors, and that the depletion of the receptor in these cells caused a decrease in the testis size and oligosperma in mice whereas their fertility remained yet unaffected. In agreement with that, the present study demonstrates that the AR expression in myoid cells was also dramatically depressed in rats supplemented with CSF whatever the dose.

In conclusion, the dietary CSF addition markedly and gradually decreased the androgen receptor expression in the Leydig, Sertoli and peritubular myoid cells in rat testes according to the dose and to the exposure duration. Consequently, the androgen dependent effects in spermatogenesis may be dramatically attenuated causing infertility.
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References