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MORPHOLOGICAL CHANGES INDUCED BY COTTONSEED FLOUR IN RAT TESTES

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Gossypol, a component of the cottonseed, has anti-fertility effects. The present study was planned to determine whether diets containing whole cottonseed flour could produce morphological changes in the rat testes similar to pure gossypol. Sixteen animals were randomly divided into two groups, Control (C) and Test (T) groups. Each group comprised of 08 animals. Control group received standard diet, while test group received 70% standard diet and 30% cottonseed flour. A significant decrease in body weight, testicular weight and diet consumption was seen in the test group. Histological sections were stained in serial order by three different stains, H&E, PAS and Masson's Trichrome. Microscopic examination revealed significant increase in luminal diameter (P<0.0001) and decrease in wall thickness of seminiferous tubules (P <0.0007) of test group. Cell count per tubule was significantly decreased in test group (P <0.002). Degenerative changes in the epithelium were seen in 78.8% and degenerative cells in the lumen of the seminiferous tubules in 41.23% of tubules were observed in test group. Sertoli and leydig cell counts were not different from those of control. No disruption of basement membrane was observed. Pigment laden cells in the interstitial tissue were observed in the test group. The observed morphological changes induced by cottonseed flour suggest that cottonseed flour, by virtue of its contents of the compound gossypol is equally toxic to rat testes like pure extract of gossypol.

Gossypol, a phenolic toxin of the cotton plant is the principal toxic compound found in cottonseed.[1-2] Free gossypol in cottonseed flour and other cottonseed products is solely responsible for the toxicity.[3] During a period of 10 years, from 1930 to 1940, no child birth was reported in an area of China, due to consumption of cottonseed oil for cooking.[4] Gossypol in cottonseed is antispermatogenic. This effect is shown to be variable in different laboratory animals.[5] It was reported in a trial on 4000 males that it has 99.9% antifertility effect.[6] Gossypol may become a medical alternative to surgical vasectomy if given in low doses; high doses may cause permanent azospermia.[7]

The exact mechanism of action of gossypol is not known however but the previous experiments revealed that it affects the function of sertoli cells,[8] inhibits the T-type Ca^{++} currents in the spermatogenic cells,[9] and inhibits the effect of different enzymes.[10-13] Its cytotoxic effects were also observed on endoplasmic reticulum, mitochondria, Golgi apparatus and cell membrane.[14,15] It was also noted that gossypol produces Oxygen free radicals, which may be the underlying basis of its biological activity.[16]

The objective of this study was to find out whether a diet containing cottonseed flour produces similar damage to the rat germinal epithelium like pure gossypol.

MATERIAL AND METHODS

This experimental study was conducted in Anatomy Department, Khyber Medical College, Peshawar, during October 2002 to May 2003. Sixteen adult, male albino rats of Sprague Dawley strain, 90—110 days old, with average weight of 224 gm/animal were used. These animals were procured from the animal house of National Institute of Health (NIH) Laboratories, Islamabad. Animals were randomly divided into two groups a control group (C) and a test group (T), each comprising of 08 animals. These animals were provided optimal light and temperature. The animals were acclimatized for one week before starting the experiment.

A known variety of cottonseed, CRIS-9 was provided by Cotton Research Institute D. I. Khan. This variety of cottonseed contains approximately 1.5% gossypol. The procedure for standard diet preparation was followed, as advised by the committee report.[17]

Duration of the treatment was five weeks. Control group (C) received standard diet, Test

group (T) received 70% standard diet and 30% cottonseed flour. Each animal of both the groups was separately caged and the cages were properly labeled. The animals were fed on diets and water libitum. All the animals were weighed on an electronic scale at day zero and weekly.

On thirty-sixth day, all the animals were sacrificed. They were anaesthetized with ether and the testes dissected out by proper procedure.[18] After recording the gross parameters, the testes were washed with normal saline to remove the blood. For each testis a longitudinal cut was given to tunica albuginia, both testes were then fixed in 10% neutral buffered formalin in appropriately labeled tissue bottles.

Small pieces of testes were processed for histological sectioning. Serial sections were taken on slides. Sections were stained in a serial order, by standard procedure.[19] Three stains, Harris Hematoxylin & Eosin (H&E), Periodic Acid Schiff (PAS) and Masson's Trichrome were applied. Stained sections were studied under light microscope at magnifications of 10x, 40x and 100x. The measurements were made by means of an occulometer at magnifications of 10x and 40x.

For the study of semineferous tubules, seventeen tubules per animal were chosen. The transversely cut tubules with circular outline were selected for the study. The outer and luminal diameters were measured by two perpendicular measurements and the mean was calculated. Epithelial thickness was measured at four cross sights and the mean was worked out. Intra-luminal degenerating cells and giant cell formation were observed. Intraepithelial degenerative changes like loss of epithelium or vacuolization were noted. Sertoli cells were identified by morphology of their nuclei.

Interstitial cells of Leydig were counted at five different sites per slide per animal. Morphology of basement membrane was studied specially in PAS stained slides. Similarly, connective tissue was examined in Masson's Trichrome stained slides.

Data was collected and analyzed using the two tailed Student's t Test.[20]

RESULTS

General Observations:

Animals of Control group (C) remained active throughout the experimental period. Test group animals showed gradual decrease in their activity and were irritable. Body weight and diet consumption by the test group were decreased. There was a significant decrease in the mean weight of the paired testes of test group when compared with controls (Table 1).

Table 1: *Comparison of diet consumption, body weight and testes weight.*

Group	Diet consumed per week (gm)	Initial body weight (gm	Final body weight (gm)	Body weight changes	Paired tests weight (gm)
Control (C)	851.80 ± 30.83	224.75 ± 27.61	306.87 ± 34.18	+82.12	3.23 ± 0.31
Test (T)	328.60 ± 11.71	206.00 ± 11.69	179.50 ± 19.44	-26.5	2.51 ± 0.13

Table 2: *Comparison of microscopic observations between control and test group.*

Parameters	Control group	Test group	Level of significance
Tubular diameter (Outer) (µm)	224.42 ± 18.57	328.98 ± 19.99	p > 0.1
Tubular diameter (Luminal) (µm)	97.03 ± 11.01	132.58 ± 14.16	P < 0.0001
Tubular Wall thickness (µm)	63.66 ± 4.23	54.20 ± 4.48	P < 0.0007
Cell count per tubule per section	244.62 ± 39.31	187.25 ± 16.78	P < 0.002
Degenerating cells in lumen (percent of tubules)	0	41.23%	
Degenerative changes in epithelium (percent of tubules)	0	78.08%	
Intraepithelial vacuolization (percent of tubules)	00	36.21%	
Sertoli cell count per tubule	22.55 ± 2.61	22.08 ± 1.26	P > 0.6
Leydig cell counts / HPF	6.52 ± 2.2	6.65 ± 0.84	P > 0.8

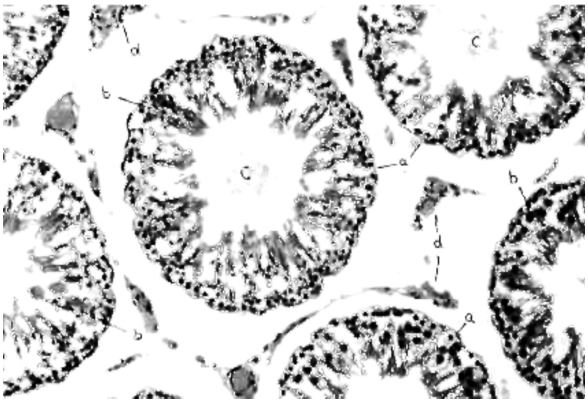

Fig. 1: A photomicrograph of the histological section of rat testis of Control group showing normal seminiferous tubules. (a) seminiferous epithelium, (b) tubular lumen, (c) and interstitial tissue, (d). H and E stain ×290.

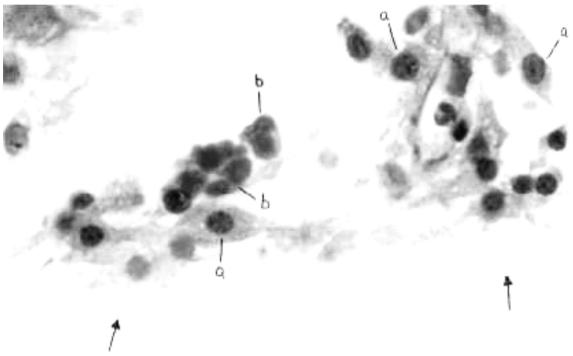

Fig. 3: A photomicrograph of 5 µm thick histological section of test group rat testis, showing interstitial tissue (arrow), Leydig cell. (a) A pigment laden cell, (b) is obvious in this sections. PAS stain × 1480.

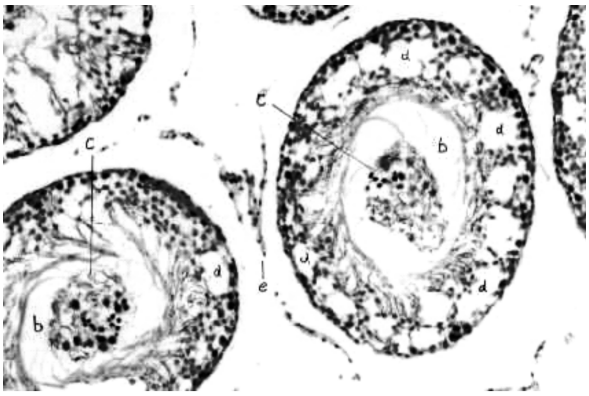

Fig. 2: A photomicrograph of 5 µm thick histological section of Test group rat testis, showing seminiferous tubule. (a) wide tubular lumen, (b) degenerating spermatogenic cells, (c) in the lumen, intraepithelial vacuoles, (d) in the epithelium and interstitial tissue, (e) surrounding the tubules. PAS stain ×290.

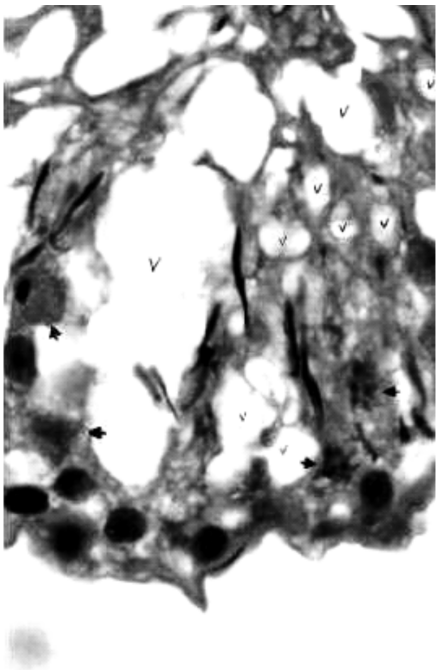

Fig. 4: A photomicrograph of a section of seminiferous tubular wall from a test group rat testis, showing small and large intraepithelial vacuoles (v) Degenerating cells (arrow heads) and a cell with pyknotic nucleus (arrow) can also be seen. Masson's Trichrome stain × 1480.

Microscopic Observations:

General morphology of the tubules appeared normal in Control as well as test group, with the exception of one animal in test group with irregular margins and kinking of some tubules (Table 2).

Outer diameter of the seminiferous tubules of control group was 224.42 ± 18.57 µm and in comparison the test group showed no significant change (Fig. 1). There was a statistically significant increase in luminal diameter of the seminiferous tubules of Test group animals. (P <0.0001) (fig. 2). Tubular wall thickness of control group was 63.66±4.23 µm, it was significantly decreased in test group (P <.0001). Cell count per tubule was significantly decreased in T group (P <.002) when compared with control. Tubules showing degenerative cells in the lumen, degenerative changes in the epithelium and intraepithelial vacuolization were observed only in T group (fig. 2, 4). No disruption of the basement membrane was observed in any slide of either control or test group. It was

observed in PAS as well as in H&E stained slides. There was no significant change in Sertoli and Leydig cell count in the test group when compared to control. Meiotic cell divisions in spermatogenic cells were commonly seen in control, while it was occasionally observed only in T group animals. Connective tissue elements of both the groups were normal in appearance, with the exception of the presence of pigment laden cells in the interstitial tissue of test group. This pigment was of yellowish color in H&E stained slides, reddish in PAS and yellowish green in trichrome stained slides (fig. 3).

DISCUSSION

The available literature is lacking in morphological effects of cottonseed flour on small animals. The present study is, therefore, an endeavour in that direction. This study provides data for evaluation of cottonseed flour induced alterations in the histology of rat testes. In this experiment body weight changes and diet consumption by the animals are in agreement with the study by Sotelo, with diet No. 4 which consisted of 24% cottonseed flour fed to rats.[21] Many workers have reported that Gossypol, in addition to infertility also affects other organs.[1,22-24] This general toxicity, probably account for weight loss and dislike for food. Decrease in testes weight of test group animals are similar to values as reported previously.[4,21]

On microscopic observations, there was no significant difference in the diameter of seminiferous tubules of Control and Test groups. This is in complete agreement with the previously reported observations.[25-27] The luminal diameter of the tubules of T group animals was significantly increased ($p < 0.0001$) when compared with the control group. This finding is similar to reported observations on larger animals.[26-28] No observations are available in small animal studies. This experiment provides evidence that changes in luminal diameter occurs in all species. Tubular wall thickness of the tubules was significantly reduced in T group animals, a finding similar to that reported previously.[26-28]

Cell count per tubule per section was significantly decreased in T group. This is in agreement with the observations by other authors,[25] although their study of tubules was selective while in our study, selection of tubules was random.

A disruption of basal lamina was not observed in any section examined. A broken membrane and the presence of spermatogenic cells in the interstitial tissue has been reported by Arshami.[26] In the present experiment we have also observed small breaches in the basement membrane of a few tubules in both groups of animals. But after application of specific stains to demonstrate basal lamina in serial sections it was proved to be due to a technical fault during cutting of sections.

In this study, we have consistently observed the presence of degenerating cells in the tubular lumen of test group. This finding is in agreement with previous experiments.[29] Degenerative changes in the epithelium, were also constantly observed in the present study. These degenerative changes consisting of decrease in cell layers and intraepithelial degenerative cells are in agreement with the observations by others.[26-28,30] The observations of intraepithelial vacuoles in the present study is similar to that of Hikim,[25] who reported it as a characteristic of gossypol treated rats.

Number of sertoli cell per tubule and leydig cell count were not different from control group. These observations are consistent with previous studies.[28,30,31] Interstitial tissue appeared normal with the exception of the presence of pigment - laden cells, that confirm previous observations.[29] These cells were found in all slides of test group. The cell type containing pigment was most probably a tissue macrophage. The exact nature of the pigment however, needs further investigations.

This study **Concludes** that the morphological changes induced by cottonseed flour suggest, by virtue of its content of toxic compound gossypol, that is equally toxic to rat testes like pure extract of gossypol.

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