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THIOBENCARB DECREASES TESTICULAR GERM CELL PROLIFERATION, INHIBITS SPERMATOGENESIS AND INCREASES APOPTOSIS: HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ASSESSMENTS

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ABSTRACT

Background: Herbicides were found to induce harmful health effects as oxidative stress, reproductive disorders and histopathological alterations in vital organs. Thiobencarb is heavily used in rice cultivation in Egypt. There weren't enough studies on the effect of thiobencarb on human or animal health especially on the reproductive functions. **Purpose:** Investigation of thiobencarb induced histopathological effects and alterations in the indices of spermatogenesis, germ cell proliferation and apoptosis via histological and immunohistochemical studies in albino rats. **Methods :** Twenty-five healthy adult male rats were divided into two groups : Group I : This group contained 10 rats , and was kept as control group ; Group II : This group included 15 rats and was administrated with thiobencarb at dose level (44.78 mg/kg/orally / 3 days a week (equivalent to 1/20 of LD_{50} , for 6 weeks) . At the end of sixth week, testes were processed for histological examination, immunohistochemical detection of Caspase-3 and PCNA, and histochemical demonstration of total proteins. Concerning biochemical investigations, testosterone was investigated in serum, while Malondialdhyde (MDA) and total antioxidant capacity (TAC) were estimated in testicular tissue**. Results:** Thiobencarb induced histopathological alterations as pyknosis, vaculations, and congestion of blood vessels, tubular degeneration and hemorrhage. It also significantly decreased the Johnson score of spermatogenesis, significantly increased the apoptotic index (AI), and decreased the germ cell proliferation index (PI). Moreover, thiobencarb significantly decrease the epithelial height and total protein content. Thiobencarb decreased serum testosterone and testicular TAC, and significantly increased testicular MDA.

KEYWORDS: Apoptosis, Caspase-3, Herbicides, Oxidative stress, Spermatogenesis, Thiobencarb, PCNA.

INTRODUCTION

The use of herbicides is increasing in worldwide crop production. Herbicides have contributed by dramatic increase in crop yields and in the quantity and variety of the diet. Also, they have helped to limit the spread of certain plant diseases. Selective herbicides kill certain targets while leaving the desired crop relatively unharmed.[1,2]

Herbicides can cause deleterious effects on organisms and human health, especially in agricultural workers, workers in the pesticide industry and retailers of the products from improper handling.[3,4] Herbicides have been known to cause harmful health effects ranging from skin rashes to death. The common adverse effects of human and animal exposures to herbicides include neurotoxicity, lung damage, birth defects, cancer, immunomodulation, disruption of reproductive functions and histopathological alterations in vital organs, as well as various biological effects through oxidative stress injury and mitochondrial dysfunction,[2,4,6]

Thiobencarb (*S*-(4-chlorobenzyl)-*N*, *N*-diethylthiocarbamate) is one of thiocarbamates herbicides group. Thiobencarb is used as a weed killer which is widely used in rice cultivation in Egypt and other countries. It kills weeds by inhibiting cellular division of the seedling plants. Thiobencarb is stable to hydrolysis and to anaerobic aquatic metabolism. Thiocarbamate herbicides inhibit the biosynthesis of fatty acids, proteins, isoprenoids, flavonoids, and gibberellins in higher plants.[2,6,7]

There weren't enough studies on the effect of thiobencarb on human or animal health especially the reproductive functions. In this respect, the present study aimed to investigate the effect of thiobencarb in testes of albino rats , as well as the role of thiobencarb in spermatogenesis and in germ cell apoptosis and proliferation via histological and immunohistochemical assessments.

MATERIAL AND METHODS

Chemicals used

Thiobencarb

Kafrosaturn 50% EC *(thiobencarb, thiocarbamate herbicide): IUPAC Chemical name: S*-4-chlorobenzyl diethylthiocarbamate. The commercial formulation of thiobencarb herbicide, Kafrosaturn 50% EC was obtained from Kafr El-Zayat Pesticides & Chemical Company; Egypt.

Animals and treatments

Twenty-five healthy adult male *Sprague Dawley* rats weighing 170±10 g were used in this study. All rats were caged in plastic cages under adequate temperature and ventilation free access to water. Rats were divided into two groups

Group I: This group contained 10 rats, and was kept as control group

Group II: This group included 15 rats and was administrated with thiobencarb at dose level (44.78 mg/kg/ orally / 3 days a week (equivalent to 1/20 of LD_{50}, for 6 weeks).[3]

Before experimentation, animals were kept under observation for one week in order to adapt and exclude any infection. The study and all procedures were approved by the Animal Care and use Committee, Menoufia University, Egypt.

Methods

1- Histological Assessments

a. Histopathological examinations

At the end of the experiment, after 6 weeks, rats were sacrificed by cervical dislocation then dissected and their testes were removed, fixed in 10% neutral formalin, dehydrated, cleared and embedded in paraffin wax. Paraffin sections of 5 microns thickness were prepared and stained with routine haematoxylin and eosin stain.[8]

b. Spermatogenesis grading by Johnsen Score

Spermatogenesis were graded according to Johnsen score.[9] All tubular sections in each observed area of testicular tissue are evaluated systematically and each is given a score from 1 to 10 as the following criteria.

- 10 complete spermatogenesis and perfect tubules.
- 9 many spermatozoa present but disorganized spermatogenesis.
- 8 only a few spermatozoa present.
- 7 no spermatozoa but many spermatids present.
- 6 only a few spermatids present.
- 5 no spermatozoa or spermatids present but many spermatocytes present.
- 4 only a few spermatocytes present.
- 3 only spermatogonia present.
- 2 no germ cells present.
- 1 neither germ cells nor Sertoli cells present.

c. Morphometric evaluation of germinal epithelial height

Images of histological slides of testes were captured from the 10x objective lens, then analyzed with ImageJ 1.45 image measurement software (NIH). The germinal epithelial height of seminiferous tubules was calculated for analysis from 10 randomly selected more circular cross-sections of the seminiferous tubules in each testis and recorded their average values. The height of seminiferous tubules epithelium was measured from the spermatogonia on the inner surface of the basal membrane through the most advanced cell types lining the lumen of the tubules. In each cross-section, two measurements were recorded and the mean was calculated.

2- Immunohistochemical Examination

a- Assessment of proliferation in Testes by PCNA

Proliferating cell nuclear antigen (PCNA) is involved in DNA replication, excision and repair. PCNA expression is an indicator to cell proliferation and spermatogenesis, as it is a complex cell cycle of rapidly proliferating cells resulting in production of sperms. In this study, PCNA was used in quantitative analysis of spermatogenesis. The tissue sections were deparaffinized and incubated with the primary monoclonal antibody to PCNA overnight at + 4°C (Dako Corp, Carpenteria, CA). Then sections were incubated with biotin-conjugated secondary antibody (Vactastain ABC peroxidase kit, Vector Laboratories). Then the slides were stained with chromagen 3, 3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.).[10]

PCNA-Labeling Index (PCNA-LI)

The staining sections of proliferating cell nuclear antigen (PCNA) immunohistochemistry were quantitatively analyzed under the light microscope with a magnification X 200. Randomly, five microscopic fields per slide were evaluated. The PCNA-LI for each seminiferous tubule was estimated as a percentage of immuno-labeled cells to all basal cells. For each section, the mean ±SD was calculated.[11]

b- Assessment of Apoptosis in Testes by Caspase-3

Immunohistochemical detection of caspase-3 was performed using 5-µm, formalin-fixed, paraffin-embedded sections using caspase 3 antibodies at 1:50 dilution (DAKO, Carpinteria, CA). The avidin–biotin complex technique was used. Antigen retrieval was performed in all cases by steam heating the slides in a 1-mmol/L solution of EDTA (pH 8.0) for 30 minutes. After blocking of endogenous biotin, staining was performed using an automated immunostainer (DAKO) followed by detection by using a streptavidin-biotin detection system (DAKO). Positive reaction for Caspase-3 was visualized as brown coloration of the cytoplasm of the in testicular cells, Negative controls were done using the same steps except that phosphate buffered saline was applied instead of the primary antibodies.[12]

Calculation of Apoptotic index (AI) (%)
Apoptotic index (AI) was calculated as the following
AI = (number of apoptotic cells/the total number of counted cells) × 100%.

3- Histochemical Demonstration of Total Protein
For histochemical purposes, sections of 5 microns thickness were cut. Total proteins demonstrated by Mercury Bromophenol Blue method.[13]

4- Image analysis of Total Protein
Digital images were analyzed by a semi-quantitative scoring system (Image J software, Java based application for analyzing images). The histochemical-stained sections were analyzed in 10 microscopic fields under high-power field (×400) microscope. In each field, the degree of reaction of total protein (blue) area was recorded. Percentage of positive stained area (%) was calculated as mean of 10 fields / slide.

5- Biochemical Investigations
Investigation of serum Testosterone
Before sacrificing animals, blood samples were collected from the retro-orbital venous plexus by using clean capillary tubes inserted in the medial canthus. Blood samples were left to clot at room temperature, and then centrifuged at 4000 rpm for 15 minutes to separate serum. Sera were obtained by centrifugation of the blood samples and stored at - 20°C until used to determine testosterone hormone. Testosterone concentration in serum was measured by enzyme-linked immunosorbent assay (ELISA) according to Sizonenko[14] using immunoassay kit produced by Monobind Inc, USA.

Determination of Malondialdhyde and total antioxidant capacity in testicular tissues
Estimation of both Malondialdehyde (MDA) and total antioxidant capacity (TAC) were carried out according to Sedlak and Lindsay.[15] Kits were purchased from Bio diagnostic Co. Cairo, Egypt.

6- Statistical analysis
Data were analyzed using student's *t-test* by a computer statistical package (SPSS program version 16; SPSS Inc., Chicago, USA). Results were presented as mean ± SD. A P value (P <0.05) considered significant.

RESULTS
1- Histological Results
Histopathological Observations
Histological examination of testicular sections of control group showed typical structure of testis with normal seminiferous tubules with normal appearance of spermatogonia, spermatocytes, spermatids, spermatozoa, Sertoli cells, and normal intertubular connective tissue (Fig 1a). In comparison with control animals, Thiobencarb treated rats showed advanced histopathological alterations, such as vaculations, sever interstitial hemorrhage, pyknosis, tubular degeneration and atrophy (Fig 1b). Moreover, blood vessels were dilated and congested with blood (Fig 1c). An inhibition of spermatogenesis was recorded in large number of tubules (Fig.1d). Data shown in table (1) summarized the recorded histological alterations in thiobencarb treated groups with a comparison with the control group.

Histological scoring of spermatogenesis by Johnsen system
Histological evaluation of spermatogenesis via Johnsen score of control group was in normal values (9.73 ± 0.45) as all seminiferous tubules were uniformed and contained the normal sequence of spermatogenic cells (spermatogonia, spermatocytes, spermatids and sperms) (Fig 1a). On the other hand, compared to testis sections of animals of control group, rats treated with thiobencarb showed significant decrease (P <0.01) in Johnsen score (4.13 ± 1.92) as testicular tubules configuration, cellularity, and spermatic density were significantly decreased (Table 2, fig 1d). Johnson scoring for this group exhibited various grades; such as score (1) where neither germ cells nor Sertoli cells were present , score(2) as there were Sertoli cells only but no germ cells present , score (3) as only spermatogonia were present , score (4) as only a few spermatocytes were present , score (5) as there were no spermatozoa or spermatids but many spermatocytes were present , score (6) where only a few spermatids present and score (7) as no spermatozoa but many spermatids present.

Morphometric results
A significant reduction in the germinal epithelia height of seminiferous tubules was recorded in testes of animals treated with thiobencarb (46.20 ± 3.96) when compared with the control group (124.80 ± 3.83) (Table 3).

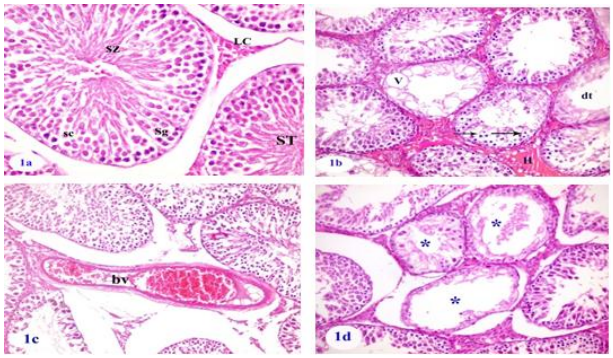

Figure 1(a): Section in testis of control rat showing normal seminiferous tubules (st), with normal spermatogonia (sg), spermatocytes (sc), spermatozoa (sz) and leydig cells (LC),(H&E,X400). (b) Thiobencarb treated rat showing degenerated tubules (dt), vaculations (v), pyknosis (arrows) and hemorrhage (H)., (X200). (c). Thiobencarb treated rat showing congested blood vessel, (H&E X200). (d). Thiobencarb treated rat showing inhibition of spermatogenesis in large number of tubules (*), (H&E, X200).

Table 1: Thiobencarb Induced Histopathological Alterations in Testes.

Groups / Alterations	Control	Thiobencarb
Congested Blood Vessels	Ø	++++
Pyknosis	Ø	+++
Vaculations	Ø	++++
Tubular atrophy	Ø	+++
Germ cell separation	Ø	++
Interstitial Hemorrhage	Ø	++++
Tubular degeneration	Ø	+++

Ø, absent; +, mild; +++, moderate; ++++ severe.

Table 2: The Spermatogenesis Results According to Johnsen Score System (Mean ± SD) .

Parameters / Groups	Control G.	Thiobencarb G.
Johnsen Score	9.73±0.45	4.13 ± 1.92 *
	P <0.01	

* Significant decrease when compared with control group. ; (n= 10).

Table 3: Thiobencarb Induced Alterations in Tubular Epithelial Heights (Mean ±SD).

Parameters / Groups	Control G.	Thiobencarb G.
Epithelial Heights (µm)	124.80 ± 3.83	46.20 ± 3.96 *
	P <0.01	

* Significant decrease when compared with control group. ; (n= 10).

2- Immunohistochemical Results

a. Caspase-3

Microscopic examination of control group revealed minimal expression of the caspase-3 in the testicular tissue (Figs 2a). Rats treated with thiobencarb showed significant increase in apoptotic cells as indicated by increased Caspase-3 expression (Fig 2b). Moreover, thiobencarb treated group exhibited significant increase in the apoptotic index (AI) when compared with control group (table 4).

b. Proliferative Cell Nuclear Antigen (PCNA)

Examination of sections of control rats showed a strong positive reaction for PCNA which is expressed in the nuclei of spermatogonia and early spermatocytes (Figs 3a). On the other hand, thiobencarb treated rats showed decrease in cell proliferation, as the number of PCNA-positive cells was reduced remarkably (Fig. 3b). Furthermore, Proliferative index (PI) was significantly decreased in thiobencarb treated group when compared with control group (table 4).

3- Histochemical Results

Examination of testes of control rats showed total proteins appeared as strong deeply stained granules in all spermatogenic cells' nuclei and cytoplasm, moreover the tunica albuginea, interstitial connective tissue and the tubular boundaries were stained deeply. Microscopic examination and Image analyzing showed that thiobencarb induced significant reduction in total protein content in testicular tissues , as most spermatogenic cells were degenerated and contained diffused proteins when compared with control group (Fig 4 , table 5).

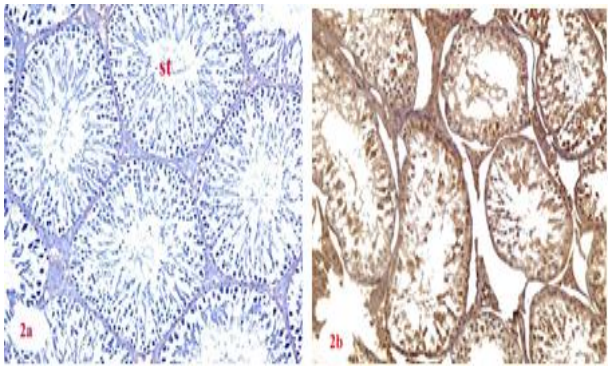

Figure 2: Immunohistochemical alterations of Caspase-3 activity (X 200). (a) Rat of control group showing minimal immunoreactions of Caspase-3. (b) Thiobencarb treated rat showing Caspase-3 strong immunoreaction in spermatocytes and leydig cells (brown color).

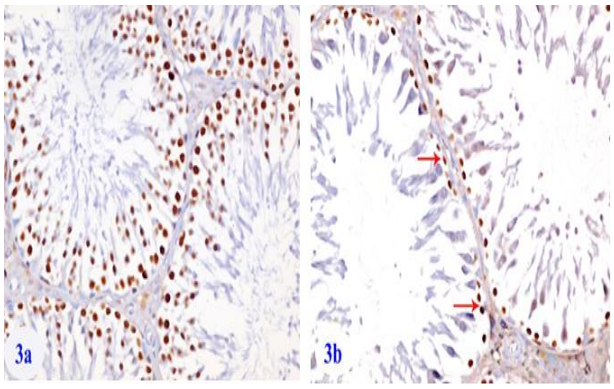

Figure 3: Immunohistochemical alterations of PCNA activity (X, 400). (a) Rat of control group showing strong expression of PCNA immunostain in the most of nuclei of spermatogonia and early spermatocytes. (b) Thiobencarb treated rat showing a decrease in the number of PCNA-positive cells (arrows).

Table 4: Apoptotic index (AI)(%) and proliferative index (PI) (%) of germ cells in the testicular tissues (Mean ± SD , n=10).

Parameters / Groups	AI (%)	PI (%)	
Control G.	0.77 ± 0.25	89.16 ± 6.30	P<0.05
Thiobencarb G.	8.12 ± 1.51*	36.83 ± 4.79 **	

(*) Significant decrease against control group,
(**) Significant increase against control group.

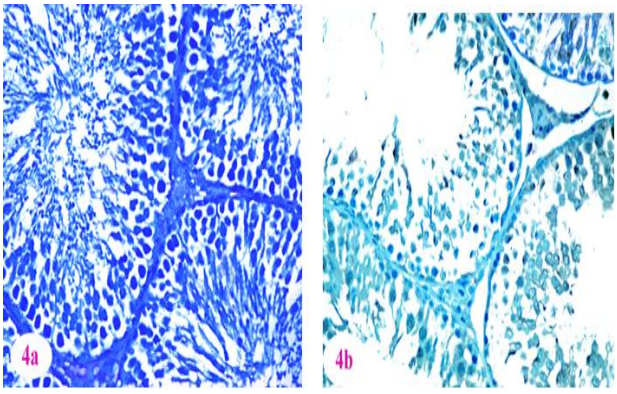

Figure 4a: Normal protein content in the testis of a control rat. b. Thiobencarb treated rat showing marked reduction of the protein content in the germ cells. (Mercury-Bromophenol Blue stain, X400).

Table 5: Alterations in proteinic content (%) induced by thiobencarb treatment (Mean ± SD).

Parameters \ Groups	Control G.	Thiobencarb G.
Total protien content (%)	51.40 ± 4.61	27.20 ± 3.43 *
	P <0.01	

* Significant decrease when compared with control group. ; *(n= 10)*

4- Biochemical Results

Data represented in table (6) showing that treating rats with thiobencarb induced significant decrease in serum testosterone and testicular TAC (total antioxidant capacity), in contrast thiobencarb induced a significant increase in testicular MDA.

Table 6: Effect of thiobencarb on serum testosterone, testicular TAC and MDA (Mean ± SD, n= 10).

Parameters / Groups	Serum Testosterone (ng/ml)	Testicular TAC (mM/g tissue)	Testicular MDA (nmol /g wet tissue)
Control G.	2.02 ± 0.20	91.71 ± 3.72	13.48 ± 3.08
Thiobencarb G.	0.72 ± 015*	20.07 ±2.97*	66.87 ± 4.58**
	P<0.05		

* Significant decrease when compared with control group.
** Significant increase when compared with control group.

DISCUSSION

The present work recorded that rice herbicide thiobencarb caused marked testicular alterations and disorders. Histopathological observation showed that thiobencarb induced hemorrhage, pyknosis, and congestion of blood vessels, tubular vaculations and degeneration. Thiobencarb induced a significant decrease in spermatogenesis according to Johnson score. Moreover, the epithelial height of seminiferous tubules was significantly decreased.

To the best of our knowledge, the effects of exposure to thiobencarb on mammalian testis have not been previously reported. The current findings agreed with other investigations carried out on the reproductive toxicity induced by molinate, which is a thiocarbamate herbicide widely used in rice culture. Molinate was found to induce marked histopathological alterations in testicular tissue as ; disorganization of the seminiferous epithelium , vaculations of Sertoli cells , inhibition of spermatogenesis , phagocytosis of spermatids , increase of multinucleated giant cells , and absence of spermatozoa and late spermatids. Moreover, in some cases, there was a complete absence of all germ cells in the seminiferous tubules.[16,17]

The hypothesized spermatogenesis inhibitory mechanism of molinate is inhibition of the hydrolysis of cholesterol from high-density lipoprotein in the testicles, followed by an androgen withdrawal syndrome on spermatogenesis.[17]

Immunohistochemical results revealed that intoxication with thiobencarb induced significant reduction in germ cell proliferation and proliferation index (PI), as PCNA expression was minimal, on the other hand apoptotic index (AI) and immunoreactivity of Caspase-3 were significantly increased in thiobencarb treated group when compared with control group.

Many investigations discussed the apoptotic and antiproliferative effects of pesticides on testis. Sakr and Shalaby[18] reported that carbamate pesticide carbofuran induced testicular apoptosis as indicated by Immunohistochemical examination. The examination of testes of carbofuran-intoxicated rats revealed a marked increase of caspase-3 expression in germ cells (spermatogonia and spermatocytes), moreover the number of the Bax positive staining cells in Leydig cells was increased.

Heikal et al.[19] studied the effect of Methomyl, a carbamate insecticide, on the alterations in the expression of apoptosis-related genes (CASP3, CASP9, Tp53 and Bcl2) in testicular tissues in rats, and they observed that, the expression level of CASP3, CASP9, Tp53 and Bcl2 genes was significantly increased in methomyl treated rats than in control.

Histochemical demonstration of total protein and image analyzing revealed that thiobencarb induced significant reduction in testicular proteinic content. Many pesticides were known to reduce the proteinic content of testicular tissue. Insecticide deltamethrin was found to induce a

marked decrease in the protein content in testicular spermatogenic cells when compared with normal control animals.[20] Furthermore, a marked decrease of carbohydrates and proteins in testes was observed in Carbofuran treated mice when compared to control mice.[21] These findings may suggest that the inhibition of spermatogenesis may be resulted from the reduction in protein synthesis in spermatogenic cells.

Currently, biochemical results recorded a significant decrease in serum testosterone hormone in thiobencarb treated rats when compared with control animals. In accordance with this, Molinate has been shown to reduce serum testosterone which results in a delayed release of the late spermatids and testicular toxicity.[22]

Concerning oxidative stress and antioxidant status, thiobencarb caused significant increase in testicular malondialdhyde (MDA), while testicular total antioxidant capacity (TAC) was decreased significantly.

Many herbicides were found to induce testicular oxidative stress. Atrazine and Haloxyfop-*p*-methyl Ester herbicides a significant decrease in the testicular antioxidant status. The testicular levels of non-enzymic antioxidants as glutathione and ascorbic acid, and enzymic antioxidants as GST, SOD and CAT were significantly reduced in rats administered with HPME or atrazine. HPME induced a significant increased level of testicular malondialdhyde (MDA).[23,24]

Carbamates were known to generate reactive oxygen species (ROS), results in oxidative stress and increase lipid peroxidation with a reduction in CAT, SOD and GST levels in experimental models in different organs.[25] Oxidative stress in the testis is one of the major factors that induce germ cell apoptosis and inhibits spermatogenesis. ROS induced apoptosis in the testis was observed mainly in spermatogonia and spermatocytes.[26] The antioxidants protect germ cells against oxidative DNA damage and play important roles in spermatogenesis.[27-29]

Increased lipid peroxidation results in sperm immobilization, reduced acrosomal reaction and membrane fluidity, and DNA damages and also causes high frequencies of single and double DNA strand breaks. Excessive ROS production disrupts both the inner and outer membranes of mitochondria then leading to the release of cytochrome-C protein, which activates Caspases and induces apoptosis.[30]

CONCLUSION

The present study concluded that, rice herbicide thiobencarb induces reproductive toxicity in male albino rats. Thiobencarb induced apoptosis and decreased the germ cell proliferation as indicated by the increase of caspase-3 and decrease in PCNA in germ cells. In addition, thiobencarb caused histopathological alterations, decreased significantly each of spermatogenesis, tubular epithelial height and total protein content. Thiobencarb decreased serum testosterone and induced oxidative stress in testicular tissue. Further studies are necessary in order to discover different ameliorative and protective antidotes to help farmers and workers in the pesticide industry to avoid the toxic effects of thiobencarb.

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