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Subchronic Effect Of Carbendazim On Spermatogenesis And Fertility In Male Albino Rats Before And After Accelerated Storage.

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ABSTRACT

Subchronic effect of carbendazim on male albino rats reproductive system was investigated. Carbendazim (CBZ) was orally administered daily to rats for 65 successive days at four exposed doses (1/30 and 1/10 LD_{50} before and after accelerated storage) and control group. Fertility index, testes weight, sperm counts, levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone and histopathology of testes were examined to evaluate the reproductive efficiency of treated rats. Relative to control, the ingestion of carbendazim significantly reduced the fertility index, testis weight, the sperm counts and motility. Also, levels of LH and FSH were decreased. Histopathological examination showed testicular damages and severe changes in seminiferous tubules. The results indicated that carbendazim before and after accelerated storage produced reproductive toxic effect, resulting reduced fertility in male rats, but carbendazim before accelerated storage has obvious effect on fertility in rats than post storage.

Keywords: carbendazim – testosterone - luteinizing hormone - follicle stimulating hormone - sperm

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INTRODUCTION

Fungicides are either chemicals or biological agents that inhibit the growth of fungi or fungal spores. Modern fungicides do not kill fungi, they simply inhibit growth for a period of days or weeks. Fungi can cause serious damage in agriculture, resulting critical losses of yield, quality and profit. Fungicides are used both in agriculture and to fight fungal infections in animals. Chemicals used to control oomycetes, which are not fungi, are also referred to fungicides as oomycetes use the same mechanisms as fungi to infect plants **(Latijnhouwers *et al*., 2000).**

Carbendazim is a member of the benzimidazole group of fungicides. It is a broad-spectrum systemic fungicide with protective and curative action. It is absorbed through the roots and green tissues, with translocation acropetally and acts by inhibiting development of the fungal germ tubes, the formation of appressoria and the growth of mycelia. Carbendazim products are used for the control of a wide range of fungal diseases such as mould, spot, mildew, scorch, rot and blight in a variety of crops. The target crops include cereals, fruit (pome, stone, citrus, currants, strawberries, bananas, pineapples, mangoes, avocados, etc.), vines, hops, vegetables, ornamentals, cotton, pasture, turf and mushrooms **(**APVMA, 2008).

The technical material and formulation of carbendazim may contain impurities, carbendazim has two impurities (2, 3-diaminophenazine) maximum: 0.003 g/kg of the carbendazim content found under and (2-amino-3-hydroxyphenazine) Maximum: 0.0005 g/kg of the carbendazim content found under **(FAO, 1992).**

The rapid biotransformation and excretion of carbendazim may account for its low toxicity to mammalian species. The LD50 of carbendazim in rats is greater than 10 g/kg body weight for oral ingestion (WHO, 1993). The clinical signs of toxicity after high single doses were generally non-specific. In spite of its low acute toxicity, carbendazim shows marked various deleterious effects on mammalian male reproductive systems **(Hess and Nakai, 2000)**. The aim of the present study is to investigate the effects of subchronic exposure to carbendazim, before and after accelerated storage, on fertility parameters in male albino rats.

MATERIALS AND METHODS

Carbendazim is methyl N-(1H-benzimidazol-2-yl)carbamate (C9H9N3O2). The formulation (Kemazed 50% WP) was provided from Central Agricultural Pesticides Laboratory, Agriculture Research Center (ARC), Ministry of Agriculture, Dokki, Egypt, which obtained from (Kafr El zayat pesticides & chemicals co.) company. The LD_{50} of Carbendazim for rat is 5000 mg/kg body weight for oral ingestion (Anonymous, 2009).

Accelerated storage procedure. This procedure according to CIPAC MT 46.3 **(1995).** Twenty grams of the wettable powder were put into a beaker and spread in it; a disc was placed on the surface of powder into the beaker. This disc is a loose fit in the beaker, and has produced a pressure on the surface of the sample in the beaker equal 25 g/cm^2 , then put in the oven at 72 ^oC . After 3-days, the beaker was removed and the disc was taken out and allowed to cool in the desiccators.

HPLC Determination of Carbendazim: The active ingredient percentage for carbendazim was determined before and after storage according to the method of **Shen, and Liu (2009)** with some modifications.

Agilent serious 1200 quarterly pump with ultraviolet (U.V) detector and a C18 stainless steel column (250 mm x 4.6 mm × 5µm) was used. Methanol - Acetonitril – Water (60:30:10) was used as mobile phase, at the rate of 1 ml/min, and wave length 235 nm. Under this condition the retention time (RT) of carbendazim was 1.291

Animals and Treatments: A total of 25 (3-4 months old) male albino rats of body weight ranging from 150–200 g (*Rattus Norvegicus Sprague Dawley strain*) were obtained from the animal house of Nutrition Institute, Cairo. These animals were housed in the laboratory animal center of the Faculty of Agricultural, Cairo University, Giza, Egypt. The animals were divided into five groups each (five rats) and kept under normal health laboratory conditions and adapted for 2 weeks through which they were allowed free access to tap water and fed on the standard basal diet consisting of a mixture of casein 20%, cotton seed oil 10%, cellulose 5%, salt mixture 4%, vitamin mixture 1%, and starch 60%. The first group represented the untreated check control animals, while the second and third groups were received sublethal doses of carbendazim at 1/10 (500 mg/kg) and 1/30 (166

mg/kg)of oral LD50 before accelerated storage and the fourth and fifth groups were received the same sublethal doses of carbendazim after accelerated storage for 65 days, respectively. Food and water were supplied *ad libitum* for all the groups during the period of experiment.

Animals were weighed weekly, and the body weight gain was determined. At the end of the experimental period (65 days), animals were sacrificed by decapitation, and blood samples were collected, and centrifuged at 3,000 rpm to obtain the serum which was kept frozen at −20°C until used for analysis. Testes were excised and weighed. Relative testes weight was calculated by the formula: (testes weight / body weight) × 100.

Fertility-related Parameters

a. Assessment of Sperm counts and Motility. For sperm counts and motility, immediately after sacrifice, the right cauda epididymis of each rat was minced and thoroughly mixed in 10 mL of warm (36 °C) 0.9% NaCl solution for motility evaluation. 20 µL of this mixture was observed under a light microscope (ZEISS, 40 X). Motile and nonmotile sperms were counted in 8 fields and the percentage of motile spermatozoa determined using the following formula:

Percentage of mobile spermatozoa =

(Number of mobile spermatozoa / Total number of counted spermatozoa) X **100**

For sperm density, a twenty-fold dilution was made by mixing the sperm suspension with Marcano solution and the mixture was shacked gently. Sperm were counted on a Bürker cell (Ngoula, *et al*., 2007).

b. Assessment of Sperm Viability. This technique is used to differentiate between alive and dead sperms. A drop of the eosin stain (1%) was added to 10 µl stock sperm suspension on a microscopic slide that was left to stand for 5 min at 37°C, then examined under microscope. The head of dead spermatozoa was stained with red color, while the alive spermatozoa were unstained with the eosin stain. Sperm viability was expressed as a percentage of alive sperm to the total sperm counted **(Krzanowska *et al*., 1995).**

Hormone Levels Assay. serum luteinizing hormone (LH), serum follicale stimulating hormone (FSH) and serum testosterone (T) levels were analyzed by the competitive enzyme linked immunosorbent assay (ELISA) according to the method of using a rat kit purchased from kamiya biomedical company by Chemiluminescence Immunoassay (CLIA) system **(Koivunen and Richard , 2015).**

Histopathological Examination. In histopathological examination, the right testis was fixed in 10% neutral buffered formalin overnight and rehydrated with 70% ethanol. The tissue was embedded in paraffin and 5-µm sections were cut and mounted onto slides. The slide sections were stained with hematoxylin and eosin (H&E) for light microscopic examination **(Dare *et al*., 2012).**

Statistical analysis. The data presented in this study were statistically evaluated as mean values for each group and its corresponding standard deviation (SD). Statistical analysis of differences between means was carried out using one- way analysis of variance (ANOVA). In case of a significant F-ratio, posthoc least significant difference (LSD) test for multiple comparisons was used to evaluate the statistical significant between groups at $p < 0.05$ level of significance.

RESULTS AND DISCUSSION

Accelerated storage procedure. After storage the carbendazim at 72 ±2 °C for 3 days, determination of the percentage of carbendazim by HPLC. Showed that the carbendazim active ingredient decreased to 36.80%.

Clinical Signs and Body Weight. During the experimental period, no deaths occurred and no appreciable signs of toxicity were observed in male rats when treated orally with carbendazim before and after accelerated storage for 65 days. It is well known that the changes in body and organs weight are sensitive indicators of potential toxic chemicals **(Bailey *et al*.,2004)**. The effect of carbendazim on body and testes weight is shown in

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Table (1), the results indicated that there were no significant differences in body weight between carbendazim exposure groups and the control group.

Table 1: Body weights of male rats exposed to carbendazim before and after accelerated storage for 65 days

Treatment	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Body weight gain %
Control	190±16[a]	279±30.5[a]	89	100
CBZ 1/10 LD_{50} before storage	193±20.5[a]	282±21[a]	89	100
CBZ 1/30 LD_{50} before storage	182±22[a]	286±37[a]	104	116
CBZ 1/10 LD_{50} after srorage at 72°C for 3 days	190±12.5[a]	288±15.5[a]	98	110
CBZ 1/30 LD_{50} after storage at 72°C for 3 days	189±14[a]	285±21.5[a]	96	107

Results are presented as means ± SD. ([a]) Indicate no significant differences at $P < 0.05$ compared with the control group, respectively

In table (1), there were no statistical differences between the final body weight and body weight gain of the male rats in 1/10 and 1/30 LD50 treated groups before and after accelerated storage and the control group. It means that carbendazim does not induce effects on body weight of rats. These results are in agreement with **(Scholz & Weigand, 1972)** who noted that there was no apparent effect on body weight and no compound-related effects on weight gain when Wistar rats received diet containing carbendazim at a concentration less than 1000 mg/kg bw per day for 90 days.

Table 2: Testes weight of male rats exposed to carbendazim for 65 days

Treatment	Final body weight (g)	Testis	
		weight (g)	Ratio %
Control	279±30.5[a]	3.13±0.89[a]	1.2
1/10 LD_{50} before storage	282±21[a]	1.7±0.24[c]	0.6
1/30 LD_{50} before storage	286±37[a]	2.03±0.57[ab]	0.7
1/10 LD_{50} after storage at 72°C for 3 days	288±15.5[a]	1.99±0.5[ab]	0.69
1/30 LD_{50} after storage at 72°C for 3 days	285±21.5[a]	2.43±0.56[ab]	0.82

Results are presented as means ± SD, ([a,ab,c]) Indicate significant difference in testes weight at $P < 0.05$ compared with the control group, respectively.

From the results in table (2), it is interesting to notice that, testes weight was substantially different in rats treated with 1/10 and 1/30 of the LD50 before and after accelerated storage compared with the control group . Testes weight was reduced in rats treated with 1/10 of the LD_{50} before accelerated storage more than those of the treated after storage. Microscopically; testes were atrophied and discolored. Microscopic findings; tubular degeneration and evidence of a spermatogenesis which suggests that carbendazim had adverse effect on development of rat testes these observations are in consonance with earlier investigations **(Lu, *et al*., 2004; and Yu, *et al* ., 2009).** Also these results are in agreement with **(Nakai *et al*., 1992)** who mentioned that the mean of testes weight showed a dose-dependent decrease when male rats were given carbendazim for 70 days and that decline was significant at doses of 100 mg/kg bw and above.

Fertility-related Parameters, Data of fertility parameters; sperm count, sperm motility and viability of sperm are shown in Table (3). A statistical significant reduction in caudal epididymal sperm count was noted at dose levels of 1/10 and 1/30 of the LD_{50} before accelerated storage treatments more than the same two doses after accelerated storage this may be due to the degradation of carbendazim active ingredient after exposure to stored at72°C for 3 days. Sperm count in male rats was significantly reduced to about 23% and 35% respectively in the carbendazim-treated males with 1/10 LD_{50} and 1/30 LD_{50} before accelerated storage compared with the control group. Sperm motility was altered primarily in those males received carbendazim

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for 65 day that exhibited very low sperm count. The Sperm motility was significantly decreased parallel with the increasing of its dose, there was no motility was detected in male rats treated with 1/10 of the LD_{50} before accelerated storage. furthermore, viability of sperm was significantly decreased in the 1/10 and 1/30 of the LD_{50} before accelerated storage treated groups , till it reaches 5% in male rats treated with 1/10 of the LD_{50} before accelerated storage compared to control group.

Table 3: Sperm characteristics in male rats exposed to carbendazim for 65 days.

Treatment	Sperm characteristics			
	Sperm count (10^6)	Sperm count (%)	Sperm motility(%)	Viability of sperms(%)
Control	37 ± 4.35^a	100	97 ± 1^a	98 ± 1^a
CBZ 1/10 LD_{50} before storage	8.6 ± 0.57^e	23.24	0 ± 0^e	5 ± 1^e
CBZ 1/30 LD_{50} before storage	13 ± 1^d	35.13	36 ± 2^d	40 ± 2^d
CBZ 1/10 LD_{50} after storage at 72°C for 3 days	14.6 ± 1.5^c	39.45	16 ± 2^c	20 ± 2^c
CBZ 1/30 LD_{50} after storage at 72°C for 3 days	21 ± 2^b	56.75	68 ± 6^b	75 ± 4^b

Results are presented as means ± SD. ($^{b, c,d,e}$) Indicate a significant difference at $P < 0.05$ compared with the control group, respectively.

Also **Yu, *et al*.,(2009)** found that administration of carbendazim into male Wistar rats, daily for 65 days, reduced fertility indices; testes weight, sperm count and sperm motility. this might be due to the atrophic of seminiferous tubules, the reduction of number of germ cells and the Carbendazim inhibition of meiotic transformation and spermatogenesis**.** In addition Pang et al. (2005) suggested that the decrease in sperm mortality in male rats following administration of ornidazole (a nitroimidazole derivative) may be due to inability of spermatozoa to obtain ATP through the glycolytic pathway or due to the inhibition of energetic transferase or non-protein substances in the epididymus. Similarly, Raji et al. (2007) reported a reduced sperm motility that results from the alteration in testicular superoxide dismutase activity after metronidazole administration to male albino rats. It is therefore reasonable to suggest that the decrease in the sperm count is the outcome of spermatogeic arrest following carbendazim intoxication as revealed by the current histological examination.

Hormone levels, Serum hormone concentration levels, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone (T) are shown in Table (4).The results showed that the concentration levels of serum LH and FSH had a decreasing tendency with increasing dose of carbendazim which was statistically significant. Where it had the highest effect in carbendazim 1/10 LD_{50} before storage. Although, there were slight decrease in serum testosterone level. These findings would suggest that carbendazim-induced testicular damage is accompanied by compensatory changes in the hypothalmic and pituitary regulation of the testes **(Lu, *et al* .,2004 and Liu, *et al*.,2006).** These results are in agreement with **(Yu, *et al* .,2009)** who reported that, the levels of serum LH,FSH and T had a dose-related effect with increasing dose of carbendazim.

Table 4: Serum hormone levels in male rats exposed to carbendazim before and after accelerated storage for 65 days

Treatment	Serum hormone levels		
	LH (mlu/ml)	FSH (mlu/ml)	T (ng/ml)
Control	0.126 ± 0.03^a	0.116 ± 0.037^a	0.233 ± 0.057^a
CBZ 1/10 LD_{50} before storage	0.056 ± 0.028^{ab}	0.056 ± 0.03^{ab}	0.116 ± 0.028^c
CBZ 1/30 LD_{50} before storage	0.086 ± 0.02^{ab}	0.07 ± 0.026^{ab}	0.160 ± 0.01^{bc}
CBZ 1/10 LD_{50} after storage at 72°C for 3 days	0.0533 ± 0.02^{ab}	0.07 ± 0.01^{ab}	0.156 ± 0.04^{bc}
CBZ 1/30 LD_{50} after storage at 72°C for 3 days	0.136 ± 0.032^a	0.073 ± 0.015^{ab}	0.196 ± 0.005^{ab}

Results are presented as means ± SD. (^{ab,bc,c}) Indicate a significant difference at $P < 0.05$ compared with the control group, respectively.

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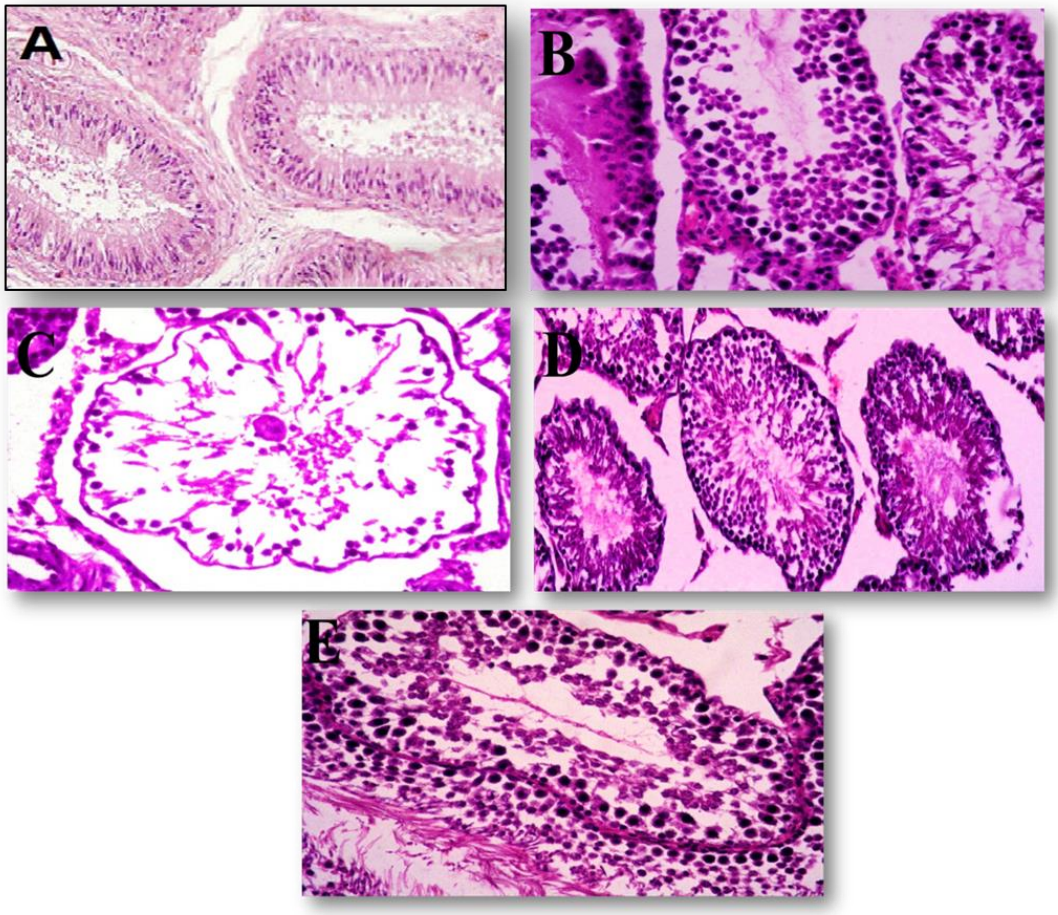
Histopathological examination. Testicular histopathology examination showed a dose-dependent effect of carbendazim on spermatogenesis. The control rats showed a normal process of spermatogenesis, a regular arrangement of spermatogenic epithelium existed in seminiferous tubules (Figure A). The rats exposed to carbendazim provoked severe alterations in the seminiferous tubules namely the loss, derangement and necrotic germ cells appeared exfoliated into the tubular lumen, multinucleated giant cells appeared in the lumen, derangement and sloughing of spermatogenic cells , the vacuolization of sertoli cell cytoplasm and the disruption of sertoli cell cytoskeleton . The highest effect was in the animals treated with the dose carbendazim at 1/10 of the LD_{50} before storage (Figure C) then carbendazim 1/30 of the LD_{50} before storage (Figure B) and carbendazim 1/10 of the LD_{50} after storage (Figure E) . The lowest effect was in the dose carbendazim 1/30 of the LD_{50} after storage (Figure D) . The above results are consistent with the previous studies **(Lu, *et al*., 2004; Naki, *et al*., 1992; Yu, *et al*., 2009).**



A= control group; B= low dose of carbendazim 1/30 LD_{50}; C= high dose of carbendazim 1/10 LD_{50} D= low dose of carbendazim 1/30 LD_{50} after storage at 72°C for 3 days; E= high dose of carbendazim 1/10 LD_{50} after storage at 72°C for 3 days

Fig 1: A photomicrograph of testis of male rat from:

Generally, in the present study, and administration of male rats with carbendazim , at the doses of 1/10 and 1/30 LD_{50} before and after storage, reduced the testes weight accompanied with significant reduction in all fertility indices . These findings were confirmed by histopathological observations, since most of the seminiferous tubules were degenerated and atrophied. Moreover, marked decline in reproductive and sex hormone levels (FSH, LH and T) was recorded, with respect to normal control.

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CONCLUSION

In view of these data, it can be concluded that an exposure of carbendazim before storage promotes reproductive toxicity and reduces the fertility in male rats more than after storage. Further studies are needed for more understanding of the mechanism of carbendazim effect, under storage condition on fertility.

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