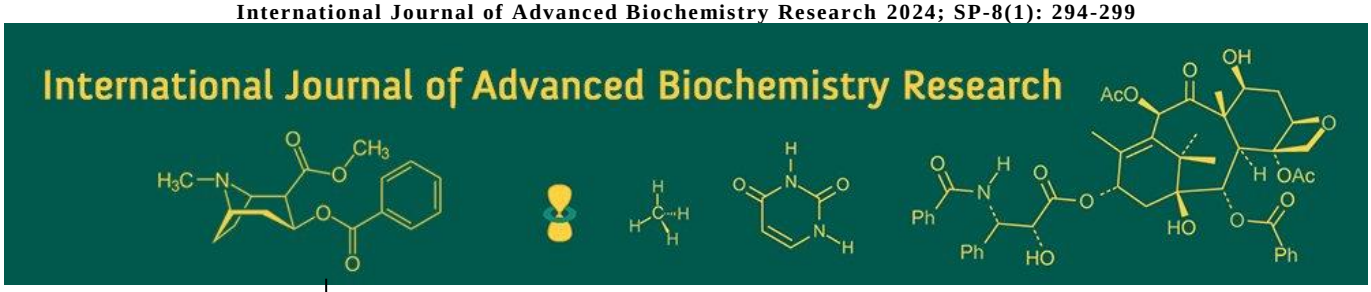

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Effect of Subacute oral Mancozeb exposure and its detrimental impact on male reproductive health in Wistar rats

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Abstract
Mancozeb is a commonly used fungicide in agriculture, and its impact on reproductive health remains a subject of concern due to its widespread use. In the pursuit of understanding the potential hazards associated with pesticide exposure, the present study was undertaken to investigate the deleterious effects of subacute oral exposure to Mancozeb on the reproductive system of male Wistar rats. To assess the effects, a comprehensive evaluation was conducted, encompassing various reproductive parameters. A total of 12 healthy Wistar rats were procured and divided into two groups: Group I (Control) and Group II (Treatment), each consisting of six animals. In Group II, Mancozeb was orally administered at a dose of 500 mg.kg^{-1} body weight for 28 consecutive days, while the control group provided with adlib feed and water without any treatment. Repeated exposure to Mancozeb resulted in alteration in various reproductive health parameters. The exposure produced mild to moderate signs of toxicity and a significant (p<0.05) increase in the absolute organ weight of the testes as well as epididymis. Reproductive parameters were notably affected, with a decrease in the count of live sperm and total sperm, along with an increase in the count of dead and abnormal sperm due to Mancozeb exposure. From the present study, it can be concluded that mancozeb fungicide produced a pronounced toxicological insult to the reproductive health of exposed animals.

Keywords: Mancozeb, fungicide, reproductive toxicity

Introduction
The world population is expected to rise from the current 7.8 billion to 8.5 billion by 2030, thereby putting immense pressure on food supplies and a growing demand for higher crop yields. A wide range of pesticide compounds including insecticides, fungicides, rodenticides, herbicides, nematicides, molluscicides, plant growth regulators and other chemicals are therefore used indiscriminately to cope up with the increased production of crops. Fungicides are awning management tools in agricultural practices, as the fungal pathogens are the number one cause of crop loss around the world (Poole and Arnaudin 2014) [26]. Fungicides have been used widely to control fungal diseases and increase crop production. Dithiocarbamates (DTCs) represent an important class of fungicides, widely used in agricultural sector. They are characterized by a broad spectrum of activity against various plant pathogens, low acute mammalian toxicity and low production costs. Mancozeb (MZ) is a fungicide belonging to the subclass of ethylene bis dithiocarbamate pesticides (Srivastava and Singh 2014) [32] having a metal atom coordinated with ethylene-bis-dithiocarbamate (EBDC) (Calviello *et al.* 2005) [6]. It is a combination of two other dithiocarbamates: maneb and zineb. The mixture controls many fungal diseases in a wide range of field crops, fruits, nuts, vegetables and ornamentals. Mancozeb interferes with enzymes containing sulphydryl groups and disrupts many core enzymatic processes of the fungal cell cytoplasm and mitochondria (Ludwig and Thorn 1960, Sijpestein 1982) [22, 30]. (Fig. 1)

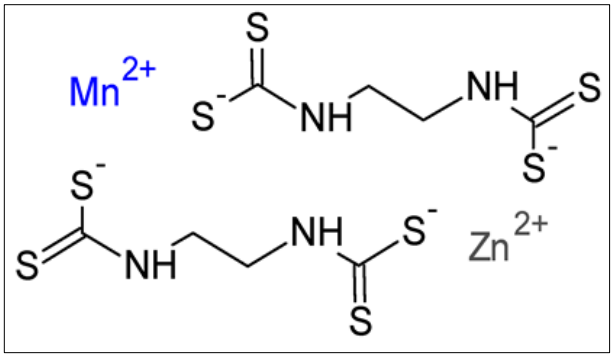


Fig 1: Structure of mancozeb (Zamora *et al*. 2019) [34]

The adverse effects of mancozeb in humans and other living organisms have not been widely studied and there are only a few reported studies evaluating the neurotoxic action of mancozeb in experimental models (Domico *et al.* 2006) [11]. This compound has been reported to have carcinogenic, teratogenic and goitrogenic effects (Chhabra *et al.* 1992) [8] as well as as structural and functional alterations of gonads (Baligar and Kaliwal 2001) [4] that leads to reproductive toxicity.

Materials and Methods

This research involved a controlled experiment, utilizing a cohort of healthy Wistar rats, with the aim of shedding light on the consequences of Mancozeb exposure and designed to evaluate Mancozeb-induced sub-acute oral Reproductive toxicity in male Wistar rats and was conducted in the Division of veterinary Pharmacology and Toxicology, GADVASU, Ludhiana, Punjab (India).

Experimental animals

A total of 12 male Wistar rats weighing about 120-150 gm were procured from National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab (India).The protocol used in the study was approved by Institutional Animal Ethics Committee (IAEC). All experiments have been carried out in accordance with the guidelines laid down by the Committee for Control and Supervision of Experiments on Animals (CCSEA), New Delhi, India.

Chemicals and reagents

Mancozeb was commercially obtained from the local market from Ludhiana as 75% WP form with brand name Manco-75. All other chemicals/reagents used for the study were of analytical grade, purchased from reputed companies.

Treatments: (Table 1)

Table 1: Experimental protocol

Specifications	Control	Treatment
No. of animals	6	6
Mancozeb	-	500 mg/kg BW
Duration	28 days	28 days
Route	-	Oral (Gavage)

Methodology and observations

Based on the EPA datasets, the oral acute toxicity dose i.e. LD_{50} value of mancozeb is >5000 mg/kg. Considering the fact and to investigate the induced toxicity in our study, a $1/10^{th}$ level of the LD_{50} i.e., 500 mg/kg/day was selected for group II. Another group *viz*: group I was kept as healthy control in which no drug was administered.

Body weight and organ weight

Body weight of all rats were recorded one day before the start of experiment for dose calculation and at weekly interval till the completion of study. At the end of experimentation percent body weight gain, organ weight and relative organ weight was evaluated.

Male Reproductive Parameters

Male mice under anaesthesia were sacrificed at end of the exposure period and the scrotal area was filled with 70 percent ethanol and dissected to reveal the full epididymis.

- **Epididymal sperm isolation:** Cauda and caput epididdymis were aseptically minced in sterile petri dishes containing 1 ml of Dulbecco's PBS (HiMedia) pre-warmed (35 °C) and screened, filtrate was used to determine the parameters of the epididymal sperm.
- **Epididymal total sperm count:** The spermatozoa were counted by hemocytometer with improved Neubaur's (Deep 1/10 mm. LABART, Germany) chamber as described by Pant and Srivastava (2003) [24].
- Total viable cells/ml = (Average number of sperm per chamber) × 10^3 × (Dilution Factor)
- **Viable Sperm Count:** Sperm smear slides were stained with Eosin-Nigrosine stain and observed under light microscope (Nikon DSRi1, Japan) for live and dead sperm count.
- **Sperm Morphology:** Spermatozoa per rat were evaluated for head and/or flagellar/tail defects by microscopy.

Statistical analysis

All the values were expressed as mean ± SE (n=6). All the values were analysed by using two way analysis of variance (ANOVA) using SPSS 20.0 version software.

Results and Discussion

In the present investigation, evaluation of oral sub-acute mancozeb toxicity was undertaken in male wistar rats and following parameters were analysed for toxic manifestations induced by mancozeb.

- **Toxic Signs:** Daily oral administration of mancozeb at the dose rate of 500mg/kg body weight to rats for 28 consecutive days produced mild signs of toxicity like moderate degree of anorexia, listlessness, lacrimation, nasal bleeding, sluggish movement and mild degree of hind limb paralysis when compared with control group animals but no lethality. Our findings were in agreement with those of (Shukla *et al.* 1990) [29].
- **Body weight and organ weight:** All animals were weighed after the adaptation period for dose calculation at the start of experiment and from than onwards on weekly basis. There was increase in body weight and percent body weight gain in animals after 28 days of mancozeb exposure in treatment group. Kackar *et al.* (1997) [18] reported similar increase in body weight after mancozeb exposure. One study showed no significant difference in weight gain in mancozeb treated animals when compared to controls. In contrast to our study (Kackar *et al.* 1999) [19] observed a decrease in body weight gain on mancozeb exposure.

At the end of experimentation i.e., 29th day, all animals were sacrificed humanely and testes and epididymis were removed by transverse abdominal incision and weighted for absolute as well as for relative weight measurement. Significant elevation in absolute organ weight of testes and epididymis were observed. (Table 2, Fig. 2).

Table 2: Body weight and organ weight variations after exposure to Mancozeb *per os* in male wistar rats (n=6)

Parameters	Control Group	Treatment Group	P value
Initial bwt.	165.33^{a}±4.52	185.5^{b}±3.17	0.001
Terminal bwt.	188.17^{a}±6.90	208.5^{b}±5.97	0.001
% bwt. Gain	11.93±1.46	10.82±1.85	0.649
Testes + epididymis	2.67^{a}±0.08	3.23^{b}±0.11	0.002
Testes + Epididymis %	1.42±0.06	1.55±0.07	0.213

Means bearing superscripts [a, b] differ significantly ($p \leq 0.05$) in a row.

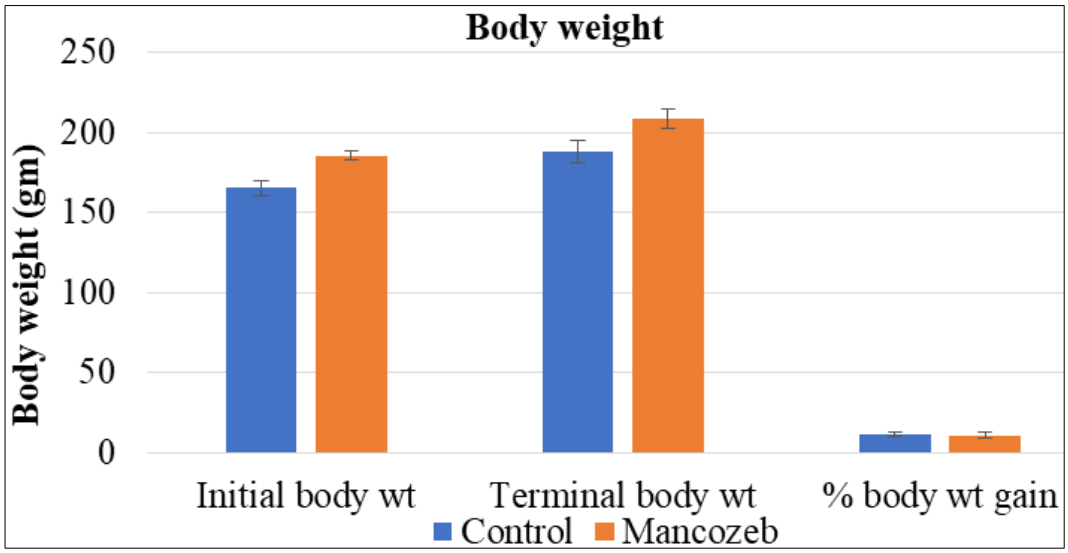


Fig 2: Effect of sub-acute exposure of mancozeb *per os* on body weight (gm) and percent body weight gain of male rats at the end of experimentation (n=6)

The analysis of relative organ weight in toxicological studies is an important criterion for identification of potentially harmful effects of chemicals (Bailey *et al*. 2004) [3]. Ahmed and Gamila (2017) [1], also reported significant increase in absolute weight of liver, kidneys, brain and testes due to mancozeb exposure. Creasy and Foster (2002) [10] suggested that changes in testes weights may reflect changes in seminiferous tubules or interstitial oedema and therefore usually contribute little further understanding of toxicity.

Effect of sub-acute oral mancozeb exposure on reproductive parameters in male wistar rats: Effect of repeated oral sub-acute mancozeb exposure on male reproductive parameters such as live and dead sperm count, sperm abnormalities and total sperm count was analyzed at end of 28 days. Exposure of mancozeb resulted into significant decrease in the live sperm count and increase in dead sperm count in mancozeb-treated rats when compared with control group. Mancozeb-treated rats showed significantly higher level of abnormal sperm count including detached head, coiled tail, damaged head etc. as compared to control group rats. In the present study, there was significant decreased in the total sperm count of mancozeb-treated male rats at end of the exposure period as compared to control group. (Table 3, Fig 3, Fig 4)

Table 3: Effect of sub-acute oral mancozeb exposure on reproductive parameters in male wistar rats (n=6)

Group/Parameters	Control	Treatment
Live sperm (%)	80.33^{b}±2.11	62^{a}±2.13
Dead sperm (%)	19.67^{a}±2.11	38^{b}±2.13
Abnormal sperm (%)	5.33^{a}±0.49	17.17^{b}±0.74
Total sperm count (*10^{7}sperm/epididymis)	46.17^{b}±0.99	33.32^{a}±0.70

Means bearing superscripts [a, b] differ significantly ($p<0.05$) in a row (values: Mean ± SE; n=6).

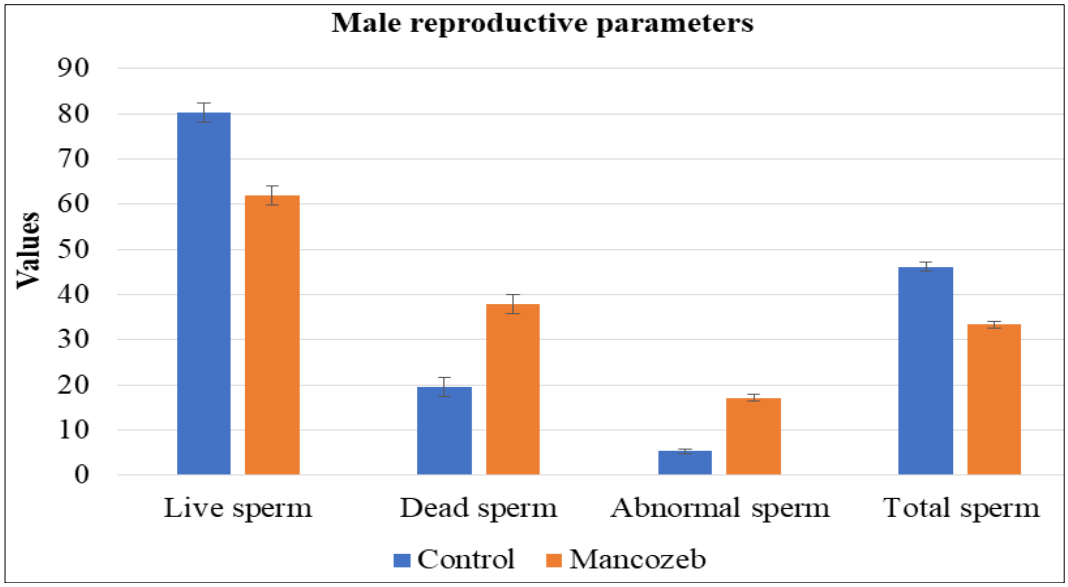
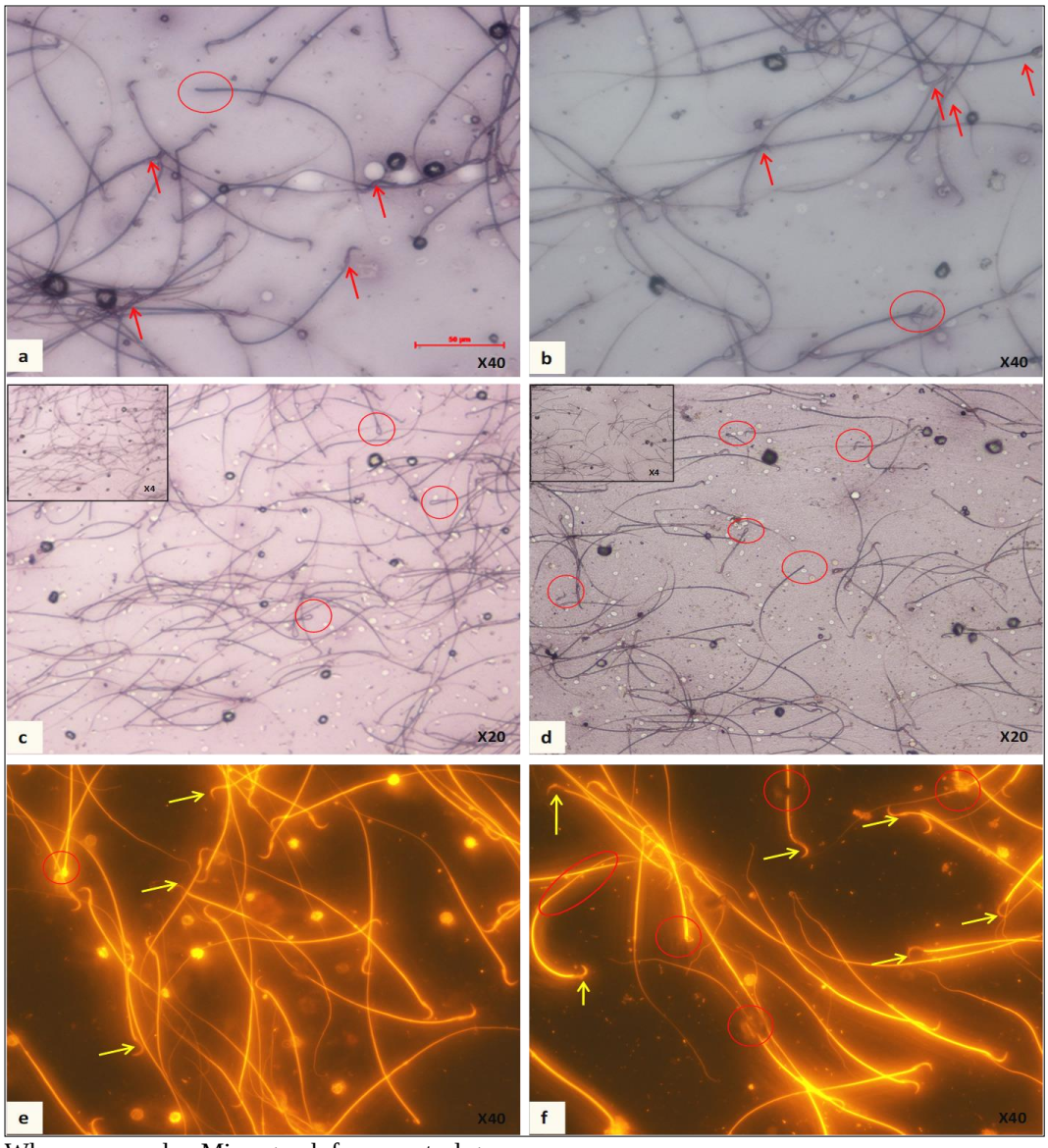


Fig 3: Effect of mancozeb exposure on male reproductive parameters in control and mancozeb group rats (n=6)



Where; a, c and e: Micrograph from control group.
b, d & f: Micrograph from treatment (mancozeb) group.
a-d: Under bright field.
e-f: Fluorescent field.

Fig 4(a-f): Representative photomicrograph of sperms from experimental male wistar rats

Arrow: dead sperm; Circle: sperm defects broken head, detached tail, damaged head
Eosin-nigrosin staining; original magnification: a, b, e and f: x40; c and d: x20.

Many exogenous factors (chemicals, drugs, etc.) that act in many different ways that can interfere with testicular steroidogenesis by the complex physiological mechanism that regulates the Leydig cell function (Cooke 1998) [9]. Consistent to our results, Joshi *et al.* (2005) [17] reported, damaged seminiferous tubules, with complete spermatogenic arrest in mancozeb-treated rats and the lumen contained cellular debris, degenerated sertoli cells and was devoid of sperms.
Male infertility may result from impaired Leydig cell activity (Payne *et al.* 1980) [25]. Spermatogenesis is a mechanism that includes LH and FSH gonadotropin production under hypophysial hormonal regulation and operates Leydig and Sertoli cells, respectively (Steinberger 1971) [33]. Interactions between Leydig cells-Sertoli cells are also needed for the normal development of intratesticular testosterone (Skinner 1991) [31]. Changes in the metabolism of testosterone may be associated with several specific physiological conditions (Dufau *et al.* 1979) [12]. The amounts of intratesticular testosterone supporting natural spermatogenesis are 100 times greater than those of blood plasma level reached by regular development of the Leydig and Sertoli cells (Dufau *et al.* 1979, Russell *et al.* 1998) [12, 27].
Many studies support the findings of present study. Imidacloprid treatment has been shown to increase cell death, as well as increased immature sperms and decrease the immotile sperm velocity with substantial decrease in serum testosterone level in rats (Najafi *et al.* 2010) [23], and responsible for induction of sperm deformities in earthworms have been recorded (Zang *et al.* 2000) [35]. In fact, lower sperm counts in the epididymis, increased teratozoospermia and sperm nuclei chromatin defects in mice following treatment with Parathion (Guillermino *et al.* 1998) [15].
Mancozeb is known to have toxic and adverse effect on reproductive organs and might have toxic effect on testicular cells which could be responsible for loss of membrane structure and testicular function leading to altered functionality of testes. Similarly, male rats received carbendazim orally resulted in reduce fertility index and severe induce seminiferous tubular atrophy (Carter *et al.* 1987, Lu *et al.* 2004) [7, 21]. Gray *et al.* (1990) [14] reported reduced reproductive potential of the rats treated with carbendazim due to inhibition on sperm production and foetal viability, altered sperm morphology, testicular and epididymal weights, sperm count and change in testicular histology. Barlas *et al.* (2002) [5] reported decrease in the total serum testosterone levels and dihydrotestosterone levels, detrimental effects in the testicular tissue such as vacuolization, disorganization, necrosis in germinal epithelium and multinucleated giant cells in the lumen of the seminiferous tubules in carbendazim-treated rats.
Carbendazim was reported to be capable of causing prolonged infertility in Japanese quails with decrease in testicular weights and seminiferous tubular diameter (Aire 2005) [2]. Sakr and shalaby (2014) [28] reported degeneration of seminiferous tubules, loss of spermatogenic cells, apoptosis and decreased height of epithelium in carbendazim treated animals. Carbendazim known to induce testicular toxicity in mammalian cells by inhibition of microtubule assembly formation and direct alteration of testicular function through germ cell depletion and altering Sertoli and Leydig cell functions leading to sloughing of immature spermatids (Gray *et al.* 1989, Hess *et al.* 1991, Lim and Miller 1997b) [13, 16, 20].

Summary and Conclusion
Dithio-carbamate fungicide mancozeb was administered orally at the dose rate of 500 mg/kg ($1/10^{th}$ of LD_{50} i.e; 5000 mg/kg) to Wistar rats. Mancozeb produced mild signs of toxicity like moderate degree of anorexia, listlessness, lacrimation, nasal bleeding, sluggish movement and mild degree of hind limb paralysis as compared to control group rats. However, no lethality was observed in treatment as well as in control group animals.
There was overall increase in body weight and per cent body weight gain in animals after 28 days of mancozeb exposure in treatment group as compared to control group. Significant elevation in absolute organ weight of testes and epididymis were observed mancozeb exposure. No significant alteration in per cent body weight gain was recorded in mancozeb-exposed animals. Exposure of mancozeb resulted into significant decrease in the live sperm and total sperm count while increase in dead and abnormal sperm count in rats.
Although, mancozeb is widely used in agricultural practices as fungicide, our study revealed that mancozeb exposure for 28 consecutive days produced certain toxicological manifestations in the exposed rats.
Consequently, the outcomes of this study suggest that Mancozeb fungicide, when administered at the tested dose, imposes a substantial toxicological burden on the reproductive health of exposed animals and may have detrimental implications for both human and animal reproductive health.

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