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EFFECTS OF EXPOSURE TO THE FUNGICIDE MANCOZEB ON REPRODUCTIVE AND SOMATIC PARAMETERS OF MALE MICE

EFEITOS DA EXPOSIÇÃO AO FUNGICIDA MANCOZEB NOS PARÂMETROS REPRODUTIVOS E SOMÁTICOS EM CAMUNDONGOS MACHOS

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Abstract

Mancozeb (MZB) is a pesticide that is used widely against a range of fungal diseases in plants. However, its effects on the male reproductive system are still unclear. Thus, this study analyzed the effects of MZB on the reproductive system of mice over different exposure periods. In addition, somatic parameters were evaluated. For this purpose, adult male *Swiss* mice were distributed into 6 groups (n = 8 animals/group): 3 groups orally treated with 0.37 mg.kg-1 of MZB for 15, 30, or 60 days, and 3 control groups receiving only the vehicle (water) for the same period. The results showed an increase in the glycemic index ($p < 0.05$), while the gastrocnemius muscle mass was lower in mice exposed to MZB ($p < 0.05$). The seminal vesicle of treated mice was increased ($p < 0.05$). MZB also promoted changes in the frequency of spermatogenic stages and in the number of Sertoli cells ($p < 0.05$). Moreover, epididymal sperm count was increased in the cauda segment of mice exposed to MZB ($p < 0.05$). These findings may be the result of a direct action of MZB on the thyroid pathway, which has been recognized as a target of MZB metabolites, besides being an important pathway involved in the male reproduction. However, further research is warranted to confirm this hypothesis.

Keywords: Pesticide, sperm quality, daily sperm production, glycemia.

Resumo

Mancozeb (MZB) é um pesticida amplamente utilizado contra uma série de doenças fúngicas em plantas. No entanto, seus efeitos no sistema reprodutor masculino ainda não estão claros. Assim, este estudo analisou os efeitos do MZB no sistema reprodutor de camundongos em diferentes períodos de exposição. Além disso, parâmetros somáticos foram avaliados. Para isso, camundongos *Swiss* machos adultos foram distribuídos em 6 grupos (n = 8 animais/grupo): 3 grupos tratados com 0,37 mg.kg-1 de MZB por via oral, durante 15, 30 ou 60 dias, e 3 grupos controle recebendo apenas o veículo (água) pelo mesmo período. Os resultados mostraram um aumento no índice glicêmico ($p < 0,05$), enquanto a massa do músculo gastrocnêmio foi menor em camundongos expostos ao MZB ($p < 0,05$). A vesícula seminal foi aumentada nos camundongos tratados ($p < 0,05$). O MZB também promoveu alterações na frequência dos estágios espermatogênicos ($p < 0,05$) e no número de células de Sertoli ($p < 0,05$). Além disso, a contagem de espermatozoides na cauda do epidídimo foi aumentada nos camundongos expostos ao MZB ($p < 0,05$). Esses efeitos podem ser o resultado de uma ação direta do MZB na via tireoidiana, que é reconhecida como um alvo dos metabólitos do MZB, além de ser uma via importante envolvida na reprodução masculina. No entanto, estudos adicionais são necessários para validar essa hipótese.

Palavras-Chave: Pesticida, qualidade espermática, produção espermática diária, glicemia.

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Introduction

Mancozeb (MZB) is a contact fungicide belonging to the class of dithiocarbamate pesticides, which are among the most frequently applied fungicides worldwide (FANJUL-BOLADO et al., 2020). MZB has been widely used against a variety of fungal diseases that affect different crops and ornamental plants (MELLO; CALDAS, 2024). In Brazil, there are numerous commercial products that contain Mancozeb, such as Dithane® (used in the present study), Acrobat®, Mancozeb Nortox®, Curatis®, among others.

MZB is a polymeric complex of manganese ethylene-bis-dithiocarbamate with zinc salt and exerts its biological effects through metabolites such as ethylene thiourea (ETU) and carbon disulfide (CS_2), a volatile organic compound (MELLO; CALDAS, 2024). MZB is stable in the presence of humidity and oxygen, being rapidly degraded by hydrolysis, and presents low persistence in the ground (CALVIELLO et al., 2006). However, its degradation product ETU persists in the ground for at least 10 days and, due to its high solubility and mobility, can be transported to groundwater and surface water (KOMÁREK et al., 2010).

Some dithiocarbamate pesticides, such as MZB, have been classified as hazardous compounds with toxicity to mammals and other non-target organisms (CAMPANALE et al., 2023). The role of MZB as an endocrine disruptor has already been observed (PANDEY; MOHANTY, 2015). Furthermore, increased blood pressure and the mass of organs such as the heart, kidney and liver have been reported (MORVAI et al., 2005). Higher ETU levels have also been associated with neurodevelopmental damage (STADLER et al., 2022).

As shown by Kubrak et al. (2012) in a study conducted on goldfish (Carassius auratus), exposure for 96 hours to Tattoo®, a commercial fungicide containing MZB as the main constituent, at doses of 3, 5 or 10 $mg.L^{-1}$, equivalent to 0.9, 1.5 or 3 $mg.L^{-1}$ of MZB, respectively, promoted lymphopenia and neutrophilia and a slight induction of oxidative stress, with an increase in the antioxidant enzymes related to the gills. In rainbow trout (Oncorhynchus mykiss) exposed to a sublethal concentration of MZB (1/2 LC50=1.1 $mg.L^{-1}$) at 24-hour intervals for 3 weeks, there were some hematological effects, such as a significant reduction in hemoglobin and mean corpuscular hemoglobin and an increase in the number of red blood cells (ATAMANALP; YANIK, 2003). Moreover, the harmful effects of MZB have been observed in avian species. In a study carried out by Pandey and Mohanty (2015) with adult male red munia (Amandava amandava) birds exposed to a concentration of 0.5% of LD50 (4.3 $mg.kg^{-1}$) for 30 days, the authors reported changes in the pituitary-thyroid axis, as well as disruption of the reproductive cycle of the animals exposed to MZB.

Overexposure to dithiocarbamate pesticides and their metabolites has been documented as a major threat to mammalian health, affecting both reproductive and somatic functions (CAMPANALE et al., 2023; CHEN et al., 2023). Elevated levels of carbon disulfide (CS_2), a decomposition product of MZB, has been shown to produce degenerative changes in testicular tissue, affecting spermatogenesis and epididymal function, impairing the secretion of testicular hormones (HUANG et al., 2012), and inducing a reduction in fertility and an increase in spontaneous abortions (SHARMA et al., 2022). Additionally, somatic alterations such as hepatotoxicity, oxidative stress, and changes in body mass have been consistently reported (CHEN et al., 2023).

Despite the adverse outcomes associated with exposure to dithiocarbamate pesticides, the hazards to mammalian reproductive health are still unclear. In this context, experimental studies on the reproductive effects of MZB are important in the field of toxicology. Thus, the aim of the present exploratory study was to evaluate the effect of short-term and long-term exposure to MZB on the reproductive system of male mice. The somatic parameters of the animals were also evaluated. Is important to note that this study assessed exposition periods using doses calculated based on IDA from MZB, obtained from Brazilian regulatory agencies, and this approach is scarce in recent literature.

Material and Methods

Agrochemical compound

The fungicide Dithane® (CAS 8018-01-7), consisting of 800 $g.kg^{-1}$ (80% $m.m^{-1}$) MZB ($C_8H_{12}MnN_4S_8Zn$), was purchased from

agricultural supply stores and subsequently diluted in water prior to use. The molecular weight of MZB corresponds to 541.1 $g.mol^{-1.}$

Animals and experimental groups

Male *Swiss* mice (50 days old), weighing approximately 40 g, were obtained from the Animal Breeding Center of the Federal University of Goiás (UFG), and maintained under controlled conditions (23ºC, 12/12-h light/dark cycle) at the Maintenance Animal Center of the Laboratory of Physiology and Toxicologic Biochemistry of the Estadual University of Goiás - Campus Ceres (UEG - CERES/GO), with water and food available ad libitum. All adopted procedures were approved by the Ethics Committee of Animal Use of the university under protocol number 047/2016-CEUA/UFG.

The animals were randomly allocated into 6 experimental groups (n = 8 each) and treated intragastrically with 0.37 $mg.kg^{-1}$ of MZB diluted in water (MZB group) or with water (vehicle) in the control group, for either 15, 30 or 60 consecutive days. The dosage of MZB was based on the acceptable daily intake (ADI) for humans in Brazil, which is estimated to be 0.03 $mg.kg^{-1}$ of body mass (CALDAS et al., 2004) and was corrected according to the animal´s body surface area (BSA), as recommended by Reagan-Shaw et al. (2008).

Organ and tissue mass

After euthanasia, the reproductive and non-reproductive organs and tissues were harvested through a mid-abdominal incision. The testes, epididymis (caput/corpus and cauda units), prostate, seminal vesicle, retroperitoneal and epididymis fat tissues, soleus and gastrocnemius muscles, and the right adrenal gland were weighed for quantification of the absolute and relative mass from each animal.

Dynamics of the germ epithelium and Sertoli cell counts

Left testes were used for histological analyses for evaluation of spermatogenesis and Sertoli cell counts. For this analysis, only biological material from 6 animals per experimental groups were used. The tissues were sectioned, mounted on glass slides, and stained with hematoxylin and eosin (HE). The analysis of spermatogenesis was determined by counting 105 transversal sections of the seminiferous tubules per animal, in which the stages were classified into I–IV, V–VI, VII–VIII, IX, X–XI and XII (CARVALHO et al., 2020). For Sertoli cell counts, 10 transversal sections of the seminiferous tubules per animal at stages V–VI e X–XI were examined.

Sperm counts, daily sperm production (DSP), and sperm transit time through the epididymis

Right testes and epididymis, frozen at -20°C after collection, were thawed to estimate the quantity of mature spermatids, daily sperm production (DSP), and sperm transit through the epididymis as described previously (CARVALHO et al., 2020), with some adaptations. Briefly, the testis was decapsulated and homogenized in a solution containing NaCl (0.9%) and Triton X-100 (0.05%), followed by sonication. After dilution, the sample was transferred to Neubauer chambers, and the spermatids resistant to homogenization were counted. For determination of DSP, the number of sperm was divided by 4.84 (the number of days for which the mature spermatids are present in the seminiferous epithelium of mice). The caput/corpus and cauda epididymidis were homogenized, and sperm counted as described for the testis. Sperm transit time into the epididymis duct was calculated by dividing the number of sperm in each unit (caput/corpus and cauda) by DSP (CARVALHO et al., 2020).

Recording of body mass, food and water consumption

Body mass and the food and water consumption of the experimental animals were recorded 3 times per week throughout the treatment until euthanasia.

Blood glucose evaluation

After euthanasia, glycemia was determined by the enzymatic method of Bergmeyer and Bernt (1974), which is based on glucose enzymatic oxidation and the production of antipyrilquinonimine. The produced compound has a reddish coloration, and its intensity is measured by a spectrophotometer, with maximum absorption at the wavelength of 510 nm. The levels of glucose are given by the color intensity of the antipyrilquinonimine which is proportional to the glucose concentration, and this was expressed in

mg.dL^{-1}. For this evaluation, we used five animals per experimental group.

Statistical analysis

Statistical analyses were performed using the Prism GraphPad 8.0 software. A two-way variance analysis (ANOVA Two Way) was utilized to evaluate the statistical differences between the groups (control and MZB) and between the periods of treatment, followed by Tukey's post-hoc test. The level of significance considered was $p < 0.05$.

Results and Discussion

Reproductive Parameters

The fungicide mancozeb (MZB), which is widely used throughout the world, promoted significant changes in the protocol studied that deserve attention. In terms of reproductive effects, as depicted in Table 1, exposure of male mice to MZB for 30 days showed an increase in absolute ($F_{(1,42)} = 7.556$, $p = 0.009$) and relative ($F_{(1,42)} = 5.621$, $p = 0.023$) masses of full seminal vesicle compared to the control group during the same period. There was also an increase in the absolute ($F_{(2,42)} = 6.664$, $p = 0.003$) and relative ($F_{(2,42)} = 4.781$, $p = 0.014$) masses of this organ in the group treated with MZB for 60 days compared to the group treated for 15 days. No other parameters showed statistically significant differences.

Table 1. Absolute and relative masses of reproductive organs of adult male *Swis*s mice (n = 8/group), orally treated for 15, 30 or 60 days with 0.37 mg.kg^{-1} of Mancozeb or with water (control groups).

Parameters	Frequency					
	Control			Mancozeb		
	15 days	30 days	60 days	15 days	30 days	60 days
Absolute (g)						
Final body mass	50.0 ± 3.7	44.9 ± 4.5	49.6 ± 3.4	52.8 ± 6.3	47.4 ± 5.0	52.0 ± 4.4
Testis	0.12 ± 0.02	0.12 ± 0.01	0.13 ± 0.03	0.13 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
Caput/corpus epididymis	0.03 ± 0.007	0.03 ± 0.004	0.03 ± 0.005	0.03 ± 0.012	0.04 ± 0.008	0.03 ± 0.006
Cauda epididymis	0.02 ± 0.003	0.01 ± 0.004	0.02 ± 0.003	0.02 ± 0.008	0.02 ± 0.004	0.01 ± 0.005
Prostate	0.19 ± 0.05	0.15 ± 0.05	0.20 ± 0.05	0.19 ± 0.04	0.20 ± 0.07	0.21 ± 0.06
Full seminal vesicle	0.13 ± 0.04	0.09 ± 0.03	0.14 ± 0.04	0.12 ± 0.01$^{\#}$	0.14 ± 0.02*	0.18 ± 0.05$^{\#}$
Relative (mg.100g^{-1})						
Testis	0.25 ± 0.04	0.26 ± 0.04	0.26 ± 0.07	0.24 ± 0.03	0.28 ± 0.04	0.26 ± 0.03
Caput/corpus epididymis	0.07 ± 0.015	0.06 ± 0.012	0.06 ± 0.008	0.06 ± 0.019	0.07 ± 0.011	0.06 ± 0.008
Cauda epididymis	0.03 ± 0.007	0.03 ± 0.008	0.04 ± 0.007	0.03 ± 0.015	0.03 ± 0.007	0.03 ± 0.010
Prostate	0.37 ± 0.09	0.32 ± 0.10	0.40 ± 0.10	0.36 ± 0.06	0.42 ± 0.12	0.40 ± 0.12
Full seminal vesicle	0.26 ± 0.07	0.19 ± 0.05	0.28 ± 0.09	0.23 ± 0.04$^{\#}$	0.30 ± 0.04*	0.34 ± 0.09$^{\#}$

Results analyzed by ANOVA Two Way followed by Tukey's post-hoc test and expressed as mean ± standard deviation. *Indicates significant difference between the groups exposed to Mancozeb and control groups ($p < 0.05$). $^{\#}$Indicates significant difference between the groups exposed to Mancozeb ($p < 0.05$).

These results indicate that MZB can induce structural changes in the reproductive organs of male mice. Our hypothesis for these results is based on a previous study showing that MZB can affect the hypothalamic-pituitary-thyroid (HPT) axis, inhibiting thyroid peroxidase (TPO), and thereby reducing the synthesis of thyroxine (T4) and triiodothyronine (T3). In addition, MZB can decrease the release of thyroid stimulating hormone (TSH), which can also result in reduced levels of T4 and T3 (PANDEY; MOHANTY, 2015).

Thyroid hormones are known to directly affect the function of the male reproductive organs, including accessory glands such as the seminal vesicle. Studies in infertile men have reported that hypothyroid patients have a larger seminal vesicle volume, both before and after ejaculation, compared to hyperthyroid patients (LOTTI et al., 2016). This difference, which is related to the emptying of the seminal vesicle during ejaculation – characterized by contraction and release of secretions – was positively correlated with serum free T3 levels. In animal models, experimentally induced hyperthyroidism has been associated with a significant reduction in fluid in the loculi of the seminal vesicle in teleost (JACOB et al., 2005), suggesting massive emptying of these accessory

glands and indicating a regulatory role for thyroid hormones in the ejaculatory process.

Based on these findings, it is reasonable to postulate that the hypothyroid effect of MZB may have directly interfered with the ejaculatory process, resulting in reduced seminal vesicle emptying in the treated animals. However, further biochemical and histological analyses of the seminal vesicles and the adjacent segments of the male reproductive tract are required to more fully evaluate these changes and clarify the underlying mechanisms.

In addition to the effects observed in the seminal vesicles, short- and long-term exposure to MZB significantly modified the spermatogenic dynamics of the mice, as illustrated in Table 2. The frequency of V–VI stages of groups exposed to MZB at 15, 30 or 60 days decreased when compared to its respective controls ($F_{(1,30)} = 34.030$, $p < 0.001$). On the other hand, the spermatogenic stages X–XI were significantly increased only in the group exposed to MZB for 30 days, when compared to the control group ($F_{(1,30)} = 18.320$, $p < 0.001$). No statistically significant differences were observed for the other stages.

Table 2. Frequency of spermatogenic stages from seminiferous tubules of adult male Swiss mice (n = 6/group), orally treated for 15, 30 or 60 days with 0.37 mg.kg^{-1} of Mancozeb or with water (control groups).

Parameters	Frequency					
	Control			Mancozeb		
	15 days	30 days	60 days	15 days	30 days	60 days
Spermatogenic stages						
I–IV	25.0 ± 4.0	29.0 ± 5.0	28.0 ± 3.0	26.0 ± 6.0	32.0 ± 6.0	32.0 ± 6.0
V–VI	25.0 ± 5.0	23.0 ± 5.0	25.0 ± 2.0	18.0 ± 4.0*	16.0 ± 3.0*	16.0 ± 3.0*
VII–VIII	27.0 ± 5.0	26.0 ± 3.0	27.0 ± 4.0	30.0 ± 5.0	26.0 ± 6.0	26.0 ± 6.0
IX	21.0 ± 3.0	22.0 ± 6.0	20.0 ± 3.0	20.0 ± 5.0	19.0 ± 5.0	19.0 ± 5.0
X–XI	4.0 ± 2.0	2.0 ± 1.0	3.0 ± 1.0	6.0 ± 4.0	7.0 ± 2.0*	7.0 ± 2.0
XII	3.0 ± 1.0	3.0 ± 2.0	2.0 ± 1.0	5.0 ± 3.0	5.0 ± 3.0	5.0 ± 3.0

Results analyzed by ANOVA Two Way followed by Tukey's post-hoc test and expressed as mean ± standard deviation. *Indicates significant difference between the groups exposed to Mancozeb and control groups ($p < 0.05$).

The results indicate a potential impact of MZB on the regulation of germinal epithelium dynamics. Spermatogenesis is a biological phenomenon mediated by an intricate network of autocrine, paracrine, and endocrine signaling that involves a coordinated sequence of cell proliferation and differentiation characterized by morphological changes for sperm formation (HOLSTEIN et al., 2003). Thus, alterations in stages V–VI and X–XI may result in structural damage to the sperm, considering that these stages are involved in the process of germ cell proliferation and differentiation throughout the spermatogenic cycles (OAKBERG, 1956).

Data from the literature depicts a positive correlation between the metabolic products of MZB, most notably carbon disulfide (CS2) and ethylene thiourea (ETU), and disorders of spermatogenesis (HUANG et al., 2012; NITTOLI et al., 2021). Multi-species studies suggest that ETU may disrupt testicular T3 signaling and alter the expression of genes that regulate testicular cell proliferation and differentiation (NITTOLI et al., 2021). In rodents, in vivo and in vitro studies have demonstrated the modulating effect of T3 on the proliferation and differentiation of somatic and germ cells (HERNANDEZ, 2018). The changes in spermatogenic stages revealed in the present study may result from the direct action of MZB or its metabolites on molecules that regulate the proliferation and differentiation of testicular cells, possibly related to interference with the HPT axis. However, the mechanisms by which MZB interferes with the spermatogenic stages of adult male mice need to be further elucidated.

Another interesting result found in our protocol was in the number of Sertoli cells with evident nucleoli observed in the groups exposed to MZB. The quantity of Sertoli cells was measured in stages V–VI (Figure 1A) and X–XI (Figure 1B) of the germinal epithelium, which showed changes after exposure to 0.37 mg.kg-1 of MZB. In stages V–VI, Sertoli cell numbers increased in mice exposed for 30 days to MZB ($F_{(2,30)} = 6.968$, $p = 0.003$), when compared to 15 days of exposition. In stages X–XI there was also an increase of Sertoli cells in mice

exposed for 60 days when compared to mice exposed for 15 days to MZB ($F_{(2,30)} = 12.410$, $p < 0.001$).

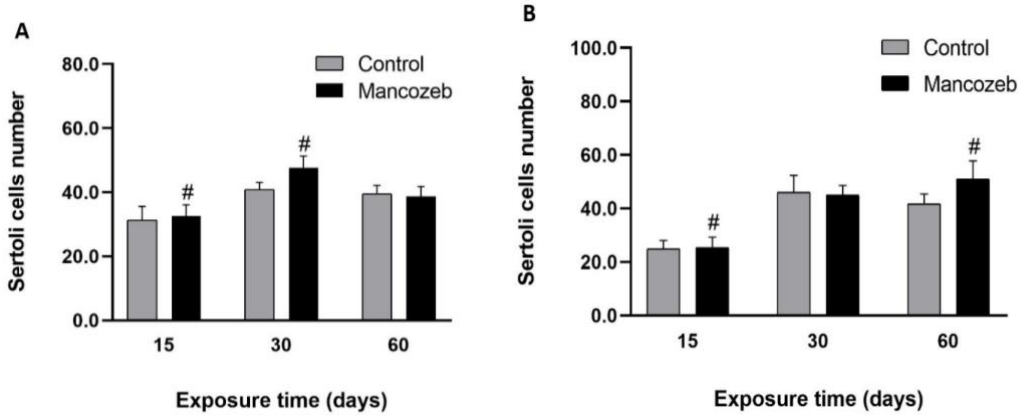

Figure 1. Number of Sertoli cells per section of seminiferous tubules at stages V–VI (A) and X–XI (B) of adult male Swiss mice (n = 6/group), orally treated for 15, 30 and 60 days with 0.37 $mg.kg^{-1}$ of Mancozeb or with water (control groups). Results analyzed by ANOVA Two Way followed by Tukey's post-hoc test and expressed as mean ± standard error. #Indicates significant difference between the groups exposed to Mancozeb ($p < 0.05$).

Sertoli cells are essential for the formation and maintenance of sperm in the seminiferous tubules. Changes in these cells can affect testicular arrangement and spermatogenesis (SETCHELL; BREED, 2006). Thus, the increase in the number of Sertoli cells associated with the duration of the exposure to MZB are suggestive of a compensatory biological response, evidenced by an increase in somatic cell activity in the seminiferous tubules in the face of alterations in the spermatogenic stages of the animals treated with this fungicide.

Although it is postulated that adult rodent Sertoli cells are not proliferatively active but remain quiescent due to a balance between inhibitors and inducers of the cell cycle (CHAUDHARY et al., 2005), these cells show differential expression of cell cycle regulatory genes that distinguish them from other quiescent cells (BEUMER et al., 1999). *In vivo* studies have indicated that mouse Sertoli cells cultured under optimized conditions can grow for long periods before entering senescence (AHMED et al., 2009). These cells can exhibit sustained proliferation and delayed maturation due to the loss of Conexin 43 (Cx43), a protein present in the seminiferous tubules that is essential for arresting cell proliferation and maturation (SRIDHARAN et al., 2007). Given that Cx43 can be regulated by T3 and that thyroid hormones play a critical role in Sertoli cell proliferation, it is possible that depletion of these hormones promotes delayed differentiation and induces Sertoli cell proliferation (GILLERON et al., 2006). This highlights the need for further studies on the effects of MZB on factors that regulate cell proliferation, with Cx43 being a potentially important target.

The functional activity of the germinal epithelium was analyzed by quantifying DSP and germinal cells. As demonstrated in Table 3, no significant effect of MZB, compared to the control group or time of exposition, on testicular spermatids concentration per organ ($F_{(2,42)} = 1.654$, $p = 0.204$) or per gram of testis ($F_{(2,42)} = 0.390$, $p = 0.680$), as well as in DSP ($F_{(2,42)} = 1.658$, $p = 0.203$) was observed. The epididymal sperm transit time, considering the caput/corpus ($F_{(2,42)} = 2.329$, $p = 0.110$) and cauda ($F_{(2,42)} = 1.328$, $p = 0.276$) segments, was not affected. Regarding epididymal spermatid concentration, there was an increase only in the spermatids per gram in the cauda epididymis of mice exposed to MZB for 15 days compared to its respective control ($F_{(2,42)} = 5.302$, $p = 0.009$).

Table 3. Sperm count, daily sperm production (DSP) and sperm transit time through the epididymis from adult male Swiss mice (n = 8/group), orally treated for 15, 30 or 60 days with 0.37 mg.kg^{-1} of Mancozeb or with water (control groups).

Parameters	Frequency					
	Control			Mancozeb		
	15 days	30 days	60 days	15 days	30 days	60 days
Testis						
MS (10^6 testis^{-1})	15.6 ± 6.5	17.6 ± 5.7	17.5 ± 8.0	23.1 ± 7.3	15.9 ± 7.0	19.1 ± 7.0
MS (10^6 g^{-1} testis^{-1})	126.8 ± 49.4	159.8 ± 37.4	150.2 ± 71.6	190.4 ± 73.9	122.6 ± 43.6	146.9 ± 49.2
DSP (10^6 testis^{-1} day^{-1})	3.2 ± 1.4	3.6 ± 1.2	3.6 ± 1.7	4.8 ± 1.9	3.3 ± 1.4	4.0 ± 1.4
Caput-corpus epididymis						
MS (10^6 epididymis^{-1})	5.0 ± 4.2	2.8 ± 1.4	4.1 ± 2.1	4.2 ± 1.6	3.0 ± 1.8	3.7 ± 1.7
MS (10^6 g^{-1} epididymis^{-1})	139.4 ± 93.8	103.2 ± 36.2	132.3 ± 60.4	137.6 ± 53.2	93.5 ± 46.9	108.2 ± 40.9
Sperm transit time (days)	1.4 ± 0.6	0.8 ± 0.1	1.2 ± 0.6	0.9 ± 0.4	0.9 ± 0.2	0.9 ± 0.2
Cauda epididymis						
MS (10^6 epididymis^{-1})	2.0 ± 0.4	2.69 ± 1.13	4.3 ± 2.7	3.7 ± 1.4	3.1 ± 1.9	3.2 ± 2.0
MS (10^6 g^{-1} epididymis^{-1})	122.8 ± 8.9	229.7 ± 86.7	220.8 ± 117.9	275.2 ± 106.5*	187 ± 93.8	216.4 ± 81.8
Sperm transit time (days)	0.7 ± 0.3	0.7 ± 0.3	1.2 ± 0.3	0.9 ± 0.5	0.9 ± 0.2	0.8 ± 0.3

Results analyzed by ANOVA Two Way followed by Tukey's post-hoc test and expressed as mean ± standard deviation. *Indicates significant difference between the groups exposed to Mancozeb and control groups ($p < 0.05$). MS: Mature spermatids.

It is important to note that the changes in the germinal epithelium induced by MZB treatment did not affect sperm production in the testes. However, an increase in the amount of sperm in the cauda epididymis was observed during the 15-day treatment, with no change in sperm transit time. The rodent cauda epididymis, the main storage site for spermatozoa that are progressively released during ejaculation (ROBAIRE et al., 2006), expresses the thyroid hormone receptor isoforms TRα1 and TRβ1 (DE PAUL et al., 2008). Based on literature data regarding the positive correlation between thyroid hormone levels and seminal vesicle emptying, our results suggest a hypothyroid effect of MZB in the cauda epididymis of mice during short-term exposure. In this context, the depletion of thyroid hormones may have impaired epididymal contraction, resulting in greater sperm storage, an effect that was restored in the longer treatment periods (30 and 60 days).

Somatic Parameters

With respect to the somatic effects of exposure to MZB analyzed in this study, Table 4 shows that the exposure of male mice to MZB for 15, 30 and 60 consecutive days did not result in any significant differences in body mass gain ($F_{(2,54)} = 0.325$, $p = 0.724$). Furthermore, exposure of any duration to MZB was not associated with any significant changes in food ($F_{(2,98)} = 0.748$, $p = 0.476$) or water ($F_{(2,98)} = 0.517$, $p = 0.598$) consumption. These results suggest a generally healthy state of the animals, despite the prolonged treatment.

Table 4 - Somatic parameters of adult male Swiss mice (n = 8/group), orally treated for 15, 30 and 60 days with 0.37 mg.kg^{-1} of Mancozeb or with water (control groups).

Parameters	Frequency					
	Control			Mancozeb		
	15 days	30 days	60 days	15 days	30 days	60 days
Body mass gain (g)	2.0 ± 5.0	1.5 ± 2.1	1.2 ± 4.0	2.8 ± 3.6	0.2 ± 2.8	0.4 ± 6.7
Food consumption (g)	73.2 ± 31.4	64.67 ± 21.0	70.3 ± 15.1	82.2 ± 37.2	74.4 ± 22.1	71.5 ± 14.2
Water consumption (mL)	90.4 ± 37.1	93.7 ± 13.6	99.8 ± 30.6	94.7 ± 37.4	100.1 ± 17.9	91.6 ± 30.7

Results analyzed by ANOVA Two Way followed by Tukey's post-hoc test and expressed as mean ± standard deviation ($p < 0.05$).

The results of the analysis of non-reproductive organs and tissues, as shown in Table 5, revealed only a decrease in the relative mass of the gastrocnemius muscle from mice exposed to MZB for 60 days, when compared to the group exposed for 30 days ($F_{(2,42)} = 4.318$, $p = 0.020$).

The blood glucose levels of the mice exposed to MZB (Figure 2) showed no significant differences between the control and MZB groups in this study ($F_{(2,24)} = 1.126$, $p = 0.341$). On the other hand, between MZB-treated groups, glycemia was significantly increased in the 30-day group compared to the group exposed for 15 days ($F_{(2,24)} = 3.923$, $p = 0.034$). However, it is important to point out that the blood glucose levels found in the mice treated for 30 days was in the normal range for *Swiss* mice (130–210 mg.dL^{-1}) (SANTOS et al., 2016).

Table 5. Absolute and relative masses of non-reproductive organs/tissues of adult male Swiss mice (n = 8/group), orally treated for 15, 30 and 60 days with 0.37 mg.kg^{-1} of Mancozeb or with water (control groups).

Parameters	Frequency					
	Control			Mancozeb		
	15 days	30 days	60 days	15 days	30 days	60 days
Absolute (g)						
Final body mass	50.0 ± 3.7	44.9 ± 4.5	49.6 ± 3.4	52.8 ± 6.3	47.4 ± 5.0	52.0 ± 4.4
Soleus muscle	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.007	0.009 ± 0.003	0.011 ± 0.004	0.011 ± 0.002
Gastrocnemius muscle	0.22 ± 0.027	0.22 ± 0.020	0.23 ± 0.063	0.22 ± 0.055	0.24 ± 0.054	0.20 ± 0.037
EA tissue	0.41 ± 0.252	0.51 ± 0.240	0.51 ± 0.194	0.51 ± 0.209	0.52 ± 0.196	0.50 ± 0.167
RA tissue	0.21 ± 0.185	0.22 ± 0.134	0.23 ± 0.133	0.23 ± 0.119	0.22 ± 0.100	0.31 ± 0.143
Right adrenal gland	0.006 ± 0.005	0.003 ± 0.001	0.005 ± 0.003	0.004 ± 0.001	0.004 ± 0.002	0.003 ± 0.002
Relative (mg.100g^{-1})						
Soleus muscle	0.03 ± 0.004	0.02 ± 0.005	0.03 ± 0.016	0.02 ± 0.005	0.02 ± 0.008	0.021 ± 0.003
Gastrocnemius muscle	0.45 ± 0.051	0.50 ± 0.062	0.47 ± 0.110	0.42 ± 0.099	0.50 ± 0.103$^{\#}$	0.38 ± 0.055$^{\#}$
EA tissue	0.91 ± 0.466	1.12 ± 0.487	1.01 ± 0.337	0.97 ± 0.368	1.14 ± 0.514	0.97 ± 0.298
RA tissue	0.47 ± 0.327	0.46 ± 0.263	0.56 ± 0.234	0.42 ± 0.211	0.48 ± 0.213	0.60 ± 0.247
Right adrenal gland	0.01 ± 0.011	0.007 ± 0.002	0.010 ± 0.007	0.007 ± 0.001	0.008 ± 0.004	0.007 ± 0.003

Results analyzed by ANOVA Two Way followed by Tukey's post-hoc test and expressed as mean ± standard deviation. $^{\#}$Indicates significant difference between the groups exposed to Mancozeb ($p < 0.05$). EA: Epididymal adipose tissue; RA: Retroperitoneal adipose tissue.

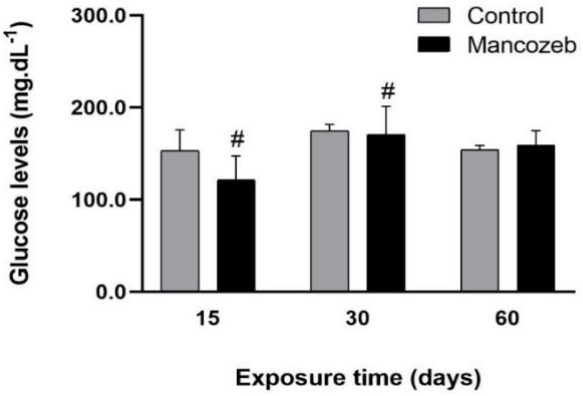

Figure 2. Blood glucose levels (mg.dL^{-1}) of adult male *Swiss* mice (n = 5/group), orally treated for 15, 30 or 60 days with 0.37 mg.kg^{-1} of Mancozeb or with water (control groups). Results analyzed by ANOVA Two Way followed by Tukey's post-hoc test and expressed as mean ± standard error. $^{\#}$Indicates significant difference between the groups exposed to Mancozeb ($p < 0.05$).

In terms of somatic parameters, one factor related to metabolism deserves attention: the increase in glycemia in mice exposed to MZB for 30 days compared to the group exposed for 15 days. These results need to be considered together with the data on the relative mass of the gastrocnemius muscle, which presented a time-dependent decrease in MZB-treated mice between 30 and 60 days of exposure. There is evidence that alterations in thyroid hormones can affect glucose uptake by tissues, as shown in hypothyroidism, which is characterized by ineffective T3 synthesis (CHIDAKEL et al., 2005). The imbalance in glucose uptake may in turn activate

counterregulatory mechanisms such as muscle proteolysis, which releases amino acids for hepatic gluconeogenesis (PAULUSMA et al., 2022).

Considering that prolonged muscle proteolysis can lead to loss of muscle mass (CASTANEDA, 2002), it is plausible that the significant reduction in gastrocnemius mass in animals exposed to MZB for 60 days (24%) is a consequence of increased muscle proteolysis due to thyroid hormone deficiency. Indeed, skeletal muscle is an excellent indicator of metabolic disturbances associated with pesticide toxicity (HE et al., 2020), as evidenced by morphological changes and variations in muscle fiber volume, diameter, and conformation (SIMÃO et al., 2011). However, the underlying mechanisms are poorly understood.

Conclusion

In this exploratory study, it was demonstrated that short- and long-term exposure (15 to 60 days) to commercial MZB at a dose of 0.37 $mg.kg^{-1}$, obtained by IDA, promoted changes in reproductive parameters in the experimental model used, when compared to the control group or between treatment periods. This fungicide, even at the equivalent dosage of the acceptable daily intake determined for humans in Brazil, increased the number of spermatozoa in the cauda epididymis, and there was also an increase in the absolute and relative mass of the seminal vesicle. In the testes, there were changes in the proliferative stages of spermatogenesis and in the number of Sertoli cells. In addition to the reproductive effects, significant changes were observed between the MZB-treated groups, including a reduction in the relative mass of the gastrocnemius muscle and an increase in blood glucose levels, but with no significant effect on body mass gain, water consumption, or food intake by the animals.

These results may be related to an imbalance in thyroid hormone levels, possibly due to MZB or its metabolites interfering with the HPT axis. This hypothesis is supported by previous studies demonstrating the potential of MZB to reduce the release of thyroid hormones, which are widely involved in various physiological systems, including male reproduction. However, further studies are needed to confirm these hypotheses, to elucidate the mechanisms underlying these changes, and to assess the implications of this hormonal interference over longer periods of treatment. It is important to evaluate the thyroid axis along with histological analyses and the sexual behavior of animals.

Despite some limitations in the present study, such as the lack of analysis on sperm viability and motility as well as sexual behavior evaluation, the findings pointed to a reproductive risk, particularly under long-term exposure to IDA from MZB. These results bring a caution for reproductive safety of this pesticide.

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