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Protective effects of melatonin against 2,2-dichlorovinyl dimethyl phosphate-induced reproductive toxicity in adult male Wistar rats

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Abstract: Organophosphorus compounds, particularly 2,2-dichlorovinyl dimethyl phosphate (DDVP), are widely used pesticides that can cause reproductive toxicity in animals. While various antioxidants have been studied to mitigate DDVP toxicity, the potential of melatonin (MLT) in preventing DDVP-induced reproductive toxicity remains underexplored. This study evaluated the fertility of melatonin-protected adult Wistar rats exposed to DDVP. Sixty adult male Wistar rats were divided into four groups: control (0.2ml corn oil orally), MLT-treated (10 mg/kg intraperitoneally), DDVP-treated (1.6 mg/kg orally), and DDVP+MLT-treated (1.6 mg/kg DDVP orally + 10 mg/kg MLT intraperitoneally). The 45-day treatment regimen was followed by assessments at 24 h, and 14 and 45 days post-treatment. Blood samples were analyzed for hormone levels using ELISA. Sperm characteristics were evaluated using standard techniques, including Wells and Awa stains for morphology and Eosin-Nigrosin stain for viability. Results showed that chronic DDVP exposure significantly decreased testosterone and LH levels ($p < 0.05$), reduced sperm motility and viability ($P < 0.05$), and increased morphologically abnormal spermatozoa ($p < 0.05$) at 45 days post-treatment. Sperm abnormalities were primarily observed in the tails. Co-administration of MLT with DDVP significantly improved these parameters ($p < 0.05$), demonstrating its protective effects against DDVP-induced spermatotoxicity. No significant changes were observed in sperm morphometry or FSH and estradiol levels across all groups ($p>0.05$). Melatonin protects against DDVP-induced male reproductive toxicity in rats by improving hormonal profiles, sperm parameters, and morphology. The data suggest the potential of MLT as a protective agent against organophosphate-induced dysfunction. Further research is recommended to investigate melatonin's protective mechanisms and long-term effects.
Keywords: Organophosphorus compounds, reproductive toxicity, spermatotoxicity, antioxidant protection

1. Introduction

Organophosphorus compounds (OP) are among the most commonly used pesticides in protecting crops and stored products from insect infestation and preventing invasion of houses by pests/vectors that can alter Public Health (Choudhary and Gill, 2001). The wide availability of most OP compounds makes toxicity common (Karaboga et al., 2024). The irreversible binding to and subsequent inactivation of acetylcholinesterase, an enzyme that normally catalyzes the hydrolysis of acetylcholine at neuromuscular junctions and other cholinergic synapses, is generally believed to be the major mechanism of OP toxicity (Shenuoda et al., 2009). Most OP are highly lipid-soluble agents and are well absorbed from the skin, oral mucous membranes, conjunctiva, and gastrointestinal and respiratory tracts (Yurumez et al., 2007). OP insecticides have relatively short biological half-lives and are fairly rapidly metabolized and excreted (Britt et al., 2000).

Dichlorvos (2, 2-dichlorovinyl dimethyl phosphate, DDVP) is a common OP pesticide used worldwide. The toxicity can occur due to exposure through water, air, or food that is contaminated by this compound (Bustos et al., 2019). It is a synthetic organic chemical that does not occur naturally in the environment but is manufactured by industry. Pure DDVP is a dense colorless liquid that evaporates easily into the air and dissolves slightly in water. DDVP has a sweetish smell and readily reacts with water. The DDVP used in pest control is diluted with other liquids and used as a spray (Bustos et al., 2019).

Exposure to DDVP causes negative effects on the function of various organs such as the heart, liver, and kidney. Also, some systems of the body are affected when exposed to high concentrations. Respiratory (Atis et al., 2002) and reproductive systems (Okamura et al., 2005) are commonly affected. In the male reproductive system, there is a disruption in spermatogenesis and there is an increase in abnormal sperm occurrence and reduction in sperm motility due to alteration in the mitochondrial enzyme activity of the spermatozoa (Uzunhisarcikli et al., 2007). There can also be damage to testicular DNA (Sarabia et al., 2009). Repeated or prolonged exposure to DDVP may result in the same effects as acute exposure including delayed symptoms. Other effects may include impaired memory and concentration, disorientation, severe depression, irritability, confusion, headache, speech difficulties, delayed reaction times, and drowsiness or insomnia (Eddleston et al., 2008). An influenza-like condition with headache, nausea, weakness, loss of appetite, and malaise has also been related to DDVP toxicity (Clark, 2002; Eddleston et al., 2008).

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is a ubiquitous molecule that has been studied for its antioxidant properties. The main source of melatonin in mammals is pineal glands but it also occurs in pill form (Reiter et al., 2004). It is a powerful free radical scavenger because it is involved in both direct and indirect antioxidant functions and can remove hydrogen peroxide, hydroxyl ion, peroxynitrite anion, singlet Oxygen, and peroxyl radicals (Reiter et al., 2004). MLT can interact with free radicals through consecutive reactions giving rise to many stable compounds that can be excreted by the body. It has been reported that the ability to recruit exogenous MLT varies from tissue to tissue. Moreover, tissues that accumulate more MLT present higher levels of antioxidant defense (El-Missiry, 2000). Melatonin is known to prevent the damage caused by OP pesticides (Vibhuti et al., 2015).

Several antioxidants have been used to ameliorate DDVP toxicity but the use of melatonin has not been fully studied and the potential of melatonin in the prevention of dichlorvos-induced reproductive toxicity is not well known. Therefore, this study was designed to evaluate the fertility of MLT-protected adult male Wistar rats exposed to DDVT.

2. Materials and Methods

2.1. Ethical Clearance

This study protocol was carried out according to the regulations and principles that govern both care and use of experimental animals for research purposes by the Animal Care and Use Ethics Committee at the University of Ibadan (UI-ACUREC/17/0069), with strict adherence to guidelines regarding the well-being of the animals, such as proper housing, standard feeding, humane handling and disease prevention and control.

2.2. Chemicals

Dichlorvos (DDVP) (purity 98%) and Melatonin (MLT) were purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). All other chemicals used in this study were of the highest available grades.

2.3. Experimental Animals

Sixty (60) adult male albino Wistar rats weighing 160.0 g (± 10.0 g) were used in this study. The animals were obtained from the Experimental Animal House of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria. Animals were kept in cages (60 × 60 × 50 cm) with wood shavings as bedding. All animals were kept under controlled conditions of temperature (25 ± 2.0°C), relative humidity (50 ± 15%), and normal photoperiod (12-h light and 12-h dark). The animals were fed on a standard rat diet (commercial pellet) and water was provided *ad libitum.*

2.4. Experimental Protocol

The animals were assigned into 4 groups (A-D, n = 15). Group A (control) received 0.2 ml of corn oil orally; Group B received MLT at 10 mg/kg, intra-peritoneally; Group C received DDVP (1.6 mg/kg) only by oral gavage while Group D rats were co-treated with DDVP orally (1.6 mg/kg) and MLT intra-peritoneally (10 mg/kg). The treatment which was given daily, lasted for 45 days. At 24 h, and 14 and 45 days post-treatment, 5 rats were randomly selected from each group and were sacrificed. Samples (blood, testes, epididymis, and semen) were collected for hormone assay, morphological studies, and semen characteristics, which include sperm motility, liveability, sperm morphology, and morphometry.

2.5. Blood sampling and steroid hormone analysis

Blood samples were collected via peri-orbital venous bleeding (venipuncture) into sterile non-heparinized sample tubes and kept on ice. The blood samples were subsequently transferred into microfuge tubes and centrifuged (Remi Lab World, Mumbai, India) at 3000 rpm for 15 min at 25 °C. The plasma fraction (supernatant) was collected and stored in 1.5 mL Eppendorf tubes at -20 °C.

Commercial kits were used to quantify serum hormones in triplicates to avoid errors due to inter-assay. Testosterone (T), LH, FSH, and estradiol (E) concentrations in serum were determined using ELISA kits (MP Biomedicals, Ohio, USA) according to instructions by the manufacturer (Yin et al., 2012). The Beckman Coulter ACCESS 2 immunoassay system (Beckman Coulter, Fullerton, USA) kit was used to quantify E. FSH levels in serum were assayed using an FSH ELISA kit according to the manufacturer's instructions (Rapid Labs. Ltd, Colchester, UK) (Yin et al., 2012). Data for T and DHEAS were expressed in nmol/L and µmol/l, respectively, while those for E and FSH were expressed in Pmol/L and Iµ/L, respectively.

2.6. Necropsy

The animals were humanely sacrificed using ethyl ether for sedation followed by cervical dislocation. A mid-caudoventral abdominal incision was made with sterilized scissors, permitting instant access to the testis once pushed upward from the scrotum. The testes were then separated from the epididymis as described by (Oyeyemi and Fayomi, 2011).

2.7. Epididymal sperm motility and liveability

Semen samples were collected from the caudal epididymis through an incision made with a scalpel blade as described by (Oyeyemi and Fayomi, 2011). The semen was dropped on a warm glass slide and stained using warm Wells and Awa stains for morphological studies and staining for sperm liveability was carried out using Eosin-Nigrosin stain. Also, the percentage of sperm motility was carried out using 2 to 3 drops of 2.9% warm buffered sodium citrate kept at body temperature, as described by Zemjanis (1970).

2.8. Statistical Analysis

The data generated was analyzed using the Test of Homogeneity of variance, multiple comparisons, and Analysis of variance (One-Way ANOVA). SPSS Version 15 for Windows (SPSS Inc, 2006) and Microsoft Excel Professional Plus (Microsoft Corporation, 2010) were used to carry out all procedures.

3. Results

There were no significant changes in the mean values of Estradiol (E2), T, LH, and FSH in rats at 24 h and 14 days post-treatment. (Table 1).

However, at 45 days post-treatment DDVP caused a significant decrease ($P < 0.05$) in the mean serum T and LH levels of DDVP-treated rats compared to the control (Table 4). Treatment of DDVP+MLT caused a slight increase ($P > 0.05$) in the serum T and LH levels. (Table 1).

PARAMETER	CONTROL	MLT	DDVP	MLT+DDVP
		24 HOURS POST-TREATMENT		
E_2 (pg/ml)	12.14±0.45 [a]	12.37±0.38 [a]	9.70±1.62 [a]	10.20±1.62 [a]
TESTOS (ng/ml)	2.42±0.77 [a]	2.80±1.65 [a]	0.94±0.01 [a]	1.24±0.01 [a]
LH (mlU/ml)	0.32±0.01 [a]	0.34±0.01 [a]	0.14±0.02 [a]	0.29±0.02 [a]
FSH (mlU/ml)	0.15±0.01 [a]	0.13±0.02 [a]	0.11±0.01 [a]	0.12±0.01 [a]
		14 DAYS POST-TREATMENT		
E_2 (pg/ml)	10.43±1.67 [a]	11.43±1.67 [a]	8.80±0.03 [a]	9.80±0.03 [a]
TESTOS (ng/ml)	0.85±0.26 [a]	0.95±0.26 [a]	0.32±0.26 [a]	0.53±0.26 [a]
LH (mlU/ml)	0.37±0.01 [a]	0.39±0.01 [a]	0.13±0.00 [a]	0.35±0.00 [a]
FSH (mlU/ml)	0.16±0.01 [a]	0.14±0.01 [a]	0.13±0.03 [a]	0.13±0.03 [a]
		45 DAYS POST-TREATMENT		
E_2 (pg/ml)	10.67±0.45[a]	12.54±0.50 [a]	8.16±0.70[a]	9.32±0.70 [a]
TESTOS (ng/ml)	2.17±1.99[a]	2.72±1.51 [a]	0.74±0.43[c]	1.62±1.51[b]
LH (mlU/ml)	0.53±0.27[a]	0.82±0.00[b]	0.19±0.0 [a]	0.31±0.00[a]
FSH (mlU/ml)	0.38±0.07[a]	0.16±0.00[a]	0.12±0.01 [a]	0.16±0.00[a]

Values are reported as mean±SEM. [abc]: Means in the same row with different superscripts differ significantly ($P < 0.05$). E_2 = Estradiol, TESTOS = Testosterone, LH = Luteinizing Hormone, FSH- Follicle Stimulating Hormone.

Table 1 – Reproductive hormonal profile of male albino rats (Wistar strain) treated with 2,2-dichlorovinyl dimethyl phosphate (DDVP) and melatonin (MLT).

3.1. Sperm motility and liveability of male albino rats treated with DDVP and MLT

The sperm motility and liveability of the male Albino rats (Wistar strain) in different treatment groups at different periods are shown in Table 2. There was a significant decrease ($P < 0.05$) in the sperm motility of rats treated with DDVP at 24 h, 14, and 45 days post-treatment, compared to the control. The sperm motility also decreased consistently across the treatment periods. Concurrent administration of DDVP + MLT caused a significant increase ($P < 0.05$) when compared to the DDVP group across the same periods. There were no significant changes in the percentage of sperm liveability ($P < 0.05$) across the groups except for the rats treated with DDVP at 45 Days post-treatment. Generally, DDVP treatment caused a decrease in sperm liveability ($P < 0.05$) across the periods when compared to the control.

3.2. Sperm Morphological Abnormalities of Male Albino Rats Treated with DDVP and MLT

The total number of abnormal sperm cells in rats treated with DDVP increased significantly ($P < 0.05$) at 14 and 45-day groups post-treatment compared to the control group. There were no significant changes ($P < 0.05$) in the total number of abnormal sperm cells at 24 h post-treatment across the groups. The total number of abnormal spermatozoa increased consistently across the 3 treatment periods.

Concurrent treatment of rats with DDVP + MLT caused a significant decrease in the number of abnormal sperm cells ($P < 0.05$) at 14- and 45-days post-treatment when compared to the DDVP-treated rats. (Table 3). The most significant morphological abnormalities of spermatozoa observed in DDVP-treated rats across the 3 treatment periods were mostly abnormalities of the spermatozoa tails.

Parameters	Period	Control	MLT	DDVP	MLT + DDVP
SPERM MOTILITY (%)	24 Hours Post – Treatment	93.00±1.22[a]	87.50±2.50[b]	65.00±2.88[d]	80.00±0.00[c]
	14 Days Post-treatment	91.00±2.92[a]	75.00±2.89[c]	68.00±2.00[d]	82.50±2.50[b]
	45 Days Post-treatment	92.00±1.22[a]	82.00±2.00[b]	54.00±2.45[d]	74.00±2.44[c]
SPERM LIVABILITY (%)	24 Hours Post – Treatment	96.20±0.73[a]	96.50±0.87[a]	82.50±2.50[a]	96.80±0.73[a]
	14 Days Post-treatment	96.80±0.73[a]	96.50±0.87[a]	76.80±0.73[a]	90.50±0.87[a]
	45 Days Post-treatment	96.80±0.73[a]	94.20±2.40[a]	76.80±0.73[b]	86.20±0.73[a]

Values are reported as mean±SEM.
[abc]: Means in the same row with different superscripts differ significantly (P < 0.05)

Table 2 – Sperm motility and liveability of male Wistar rats in different treatment groups at different periods.

Parameter (%)	Period	Control	MLT	DDVP	MLT + DDVP
TOTAL ABNORMAL CELL COUNT	24 Hours Post –Treatment	13.00±0.43[a]	12.50±0.81[a]	13.66±0.81[a]	12.24±0.28[a]
	14 Days Post-treatment	11.66±0.52[a]	12.40±0.50[a]	13.75±0.23[b]	12.58±0.38[a]
	45 Days Post-treatment	12.81±0.59[a]	12.26±0.16[a]	13.84±0.45[b]	13.00±0.36[a]

Values are reported as mean±SEM
[abc]: Means in the same row with different superscripts differ significantly (P < 0.05)

Table 3 – Percentage of sperm morphological abnormalities of male Wistar rats in different treatment groups at different periods.

3.3. Spermatozoa morphometry of male albino rats treated with DDVP and MLT

There were no significant changes (P < 0.05) in the morphometric value of spermatozoa heads across the groups at different lengths of periods (Table 4). This same trend was observed for the morphometric values of the spermatozoa mid-piece and tail across the groups at the different lengths of treatment periods.

Parameters	Period	Control	MLT	DDVP	MLT + DDVP
HEAD (µm)	24 Hours Post – treatment	1423.95±226.85[a]	2272.55±142.55[b]	2138.05±306.55[a]	2140.00±129.70[a]
	14 Days Post-treatment	2380.40±255.50[a]	2698.30±308.60[a]	2557.70±112.50[a]	2295.50±174.10[a]
	45 Days Post-treatment	2380.40±255.50[a]	2698.30±308.60[a]	2557.70±112.50[a]	2295.50±174.10[a]
MID-PIECE (µm)	24 Hours Post – treatment	7411.25±178.85[a]	8425.90±1078.70[a]	7530.60±111.10[a]	7933.25±306.45[a]
	14 Days Post-treatment	8264.75±714.95[a]	8048.40±91.00[a]	799.65±414.75[a]	7689.35±197.05[a]
	45 Days Post-treatment	8264.75±714.95[a]	8048.40±91.00[a]	799.65±414.75[a]	7689.35±197.05[a]
TAIL (µm)	24 Hours Post – treatment	11550.45±592.55[a]	10471.30±1700.40[a]	10932.55±1305.75[a]	12208.50±128.20[a]
	14 Days Post-treatment	11108.20±1280.30[a]	9385.60±892.50[a]	11439.65±834.65[a]	12038.80±62.10[a]
	45 Days Post-treatment	11108.20±1280.30[a]	9385.60±892.50[a]	11439.65±834.65[a]	12038.80±62.10[a]

Values are reported as mean±SEM
[abc]: Means in the same row with different superscripts differ significantly (P < 0.05)

Table 4 – Spermatozoa morphometry in the male Wistar rats in different treatment groups at different periods.

4. Discussion

This study examined the protective effects of MLT against reproductive toxicity induced by DDVP in adult male Wistar rats. Our findings demonstrate that chronic exposure to DDVP negatively impacts several reproductive parameters, while co-administration of MLT mitigates many of these effects.

Our results showed no significant changes in serum levels of E2, T, LH, and FSH at twenty-four hours and fourteen days post-treatment across all groups. This suggests that acute and subacute exposure to DDVP does not immediately impact the hypothalamic-pituitary-gonadal (HPG) axis in male rats. However, chronic exposure (forty-five days post-treatment) to DDVP caused a significant decrease in serum T and LH levels compared to the control group. This finding aligns with previous studies by Dirican and Kalender (2012) and Ezeji et al. (2016), who reported similar decreases in T levels following DDVP administration. The reduction in T and LH levels is concerning, as these hormones play crucial roles in spermatogenesis (Ramaswamy and Weinbauer, 2014). The mechanism by which DDVP affects T and LH levels likely involves disruption of the hypothalamic-pituitary axis, possibly through oxidative stress or direct effects on hormone-producing cells. DDVP has been shown to cross the blood-brain barrier (Karaboga et al., 2024), which could explain its effects on the hypothalamus and pituitary gland.

Interestingly, FSH and E2 levels remained unchanged even after chronic exposure. This differential impact on hormones suggests that DDVP may have a selective effect on the HPG axis, primarily affecting the LH-T pathway while sparing the FSH and E2 pathways. The reason for this selectivity is unclear and warrants further investigation.

Co-administration of MLT with DDVP led to a slight increase in serum T and LH levels, although this increase was not statistically significant. This trend suggests a potential protective effect of MLT on the HPG axis, consistent with findings by Yu et al. (2018) who reported that MLT administration could enhance T production. The mechanism behind MLT's ability to boost

testosterone production involves its interaction with the HPG axis at multiple levels: (1) MLT stimulates GnRH release from the hypothalamus, which in turn increases LH secretion from the pituitary, (2) MLT directly enhances LH receptor expression in Leydig cells, increasing their responsiveness to LH, and (3) MLT acts as a potent antioxidant in testicular tissue, protecting Leydig cells from oxidative stress-induced damage and maintaining their steroidogenic capacity (Cagnacci et al., 1995; Shi et al., 2013; Yu et al., 2018).

Our results revealed a significant decrease in sperm motility in DDVP-treated rats across all treatment periods. We observed a progressive decline in motility from twenty-four hours to forty-five days post-treatment compared to the control group. This decrease in motility could severely impair the fertilization capacity of spermatozoa, potentially leading to infertility. These findings corroborate those of Okamura et al. (2005) and Oya et al. (2017), who also observed decreased sperm motility following DDVP exposure. The mechanism behind this effect likely involves DDVP-induced alterations in mitochondrial enzyme activity of spermatozoa, as suggested by Uzunhisarcikli et al. (2007). DDVP, being an organophosphate, could inhibit acetylcholinesterase in sperm, leading to an accumulation of acetylcholine and subsequent disruption of sperm motility (Nelson, 1964).

Sperm viability was also adversely affected by DDVP exposure, with a significant decrease observed after forty-five days of treatment. This finding aligns with the work of Taylor et al. (2010), who reported that pesticide exposure can negatively impact male fertility. The decrease in sperm viability could be due to DDVP-induced oxidative stress in the testes and epididymis, leading to increased sperm cell death (Juárez-Rojas et al., 2022).

Importantly, concurrent treatment with MLT significantly improved both sperm motility and viability across all treatment periods, indicating a protective effect against DDVP-induced sperm toxicity. This protective role of MLT on sperm parameters has been previously reported by Rocha et al. (2015) and Sarabia et al. (2009). MLT's protective effect is likely due to its potent antioxidant properties, helping to neutralize reactive oxygen species generated by DDVP metabolism and protecting sperm cell membranes from oxidative damage (Galano, 2011).

DDVP treatment led to a significant increase in morphologically abnormal sperm cells, particularly after fourteen and forty-five days of exposure. This observation is consistent with the findings of Oya et al. (2017) and Yucra et al. (2006), who reported similar increases in sperm abnormalities following organophosphate exposure. The most prominent abnormalities were observed in the sperm tails, which could explain the reduced motility observed in this study. DDVP may interfere with the process of spermiogenesis, particularly affecting the formation of the sperm tail. This could be due to the compound's ability to disrupt microtubule assembly or affect the expression of genes involved in sperm tail formation (Clark et al., 2004).

Co-administration of MLT with DDVP resulted in a significant decrease in the number of abnormal sperm cells at fourteen and forty-five days post-treatment. This suggests that MLT helps preserve the structural integrity of spermatozoa, aligning with the findings of Rocha et al. (2015). MLT achieves this protective effect through several mechanisms: (1) Reducing oxidative stress during spermatogenesis by directly scavenging reactive oxygen species, (2) Enhancing the activity of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in testicular tissue, and (3) Modulating gene expression involved in sperm formation, particularly genes related to tail development (Minucci and Venditti, 2022; Zi et al., 2022)

Despite the observed changes in sperm morphology, our study found no significant alterations in the morphometric assessment of spermatozoa head, mid-piece, and tail across all treatment groups and periods. This finding suggests that while DDVP affects sperm morphology and function, it does not impact the overall size and shape of spermatozoa components. This is consistent with the observations of Ratcliffe et al. (1987) in their study on pesticide exposure and sperm morphometry. The stability of sperm morphometry in the face of other significant changes highlights the complex nature of DDVP's effects on male reproductive function. It suggests that DDVP may primarily affect the internal structures or biochemical processes of spermatozoa rather than their gross morphology. This underscores the need for comprehensive assessments in toxicological studies, including functional and ultrastructural analyses in addition to morphometric measurements.

5. Conclusion

Melatonin demonstrates significant protective effects against 2,2-dichlorovinyl dimethyl phosphate-induced reproductive toxicity in adult male Wistar rats, as evidenced by improved hormonal profiles, sperm parameters, and morphology. These findings suggest that melatonin has a protective action against organophosphate-induced male reproductive dysfunction. Further research is recommended to investigate melatonin's protective mechanisms, optimize dosing regimens, and evaluate its long-term effects and safety. Studies should also explore melatonin's efficacy against other organophosphates and its potential use in occupationally exposed human populations.

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