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ORIGINAL ARTICLE

Effect of melatonin on reproductive indices in untreated females following mating with F1 male Wistar rats exposed *in utero* and *via* lactation to mixture of chlorpyrifos and cypermethrin

Muftau SHITTU [1], Suleiman Folorunsho AMBALI [3], Joseph Olusegun AYO [2], Mohammed Umaru KAWU [2]

[1] Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria
[2] Department of Veterinary Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria
[3] Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Ilorin, Ilorin, Nigeria

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ABSTRACT

The study investigated effect of melatonin (MEL) on reproductive indices in untreated females mated with F1 male rats exposed *in utero* and *via* lactation to mixture of chlorpyrifos (CPF) and cypermethrin (CYP), and in the resulting F2 male rats. Sixty pregnant dams obtained *via* overnight mating of 72 nulliparous females with sexually mature males in a 1:1 mating scheme were divided into 6 groups of 10 animals each. Groups I (DW), II (S/oil) and III (MEL) were given distilled water (2 ml/kg), Soya oil (2 ml/kg) and melatonin (0.5 mg/kg) only, respectively, while group IV (CC) was co-administered with CPF (1.9 mg/kg) and CYP (7.5 mg/kg). Group V (MCC) was pretreated with MEL (0.5 mg/kg) and then co-exposed to CPF (1.9 mg/kg) and CYP (7.5 mg/kg), 30 minutes later. Group VI (CCM) was co-exposed to CPF (1.9 mg/kg) and CYP (7.5 mg/kg) and then treated with MEL (0.5 mg/kg) 30 minutes later. The regimens were administered by gavage once daily from gestation day 1 to postnatal day (PND) 21. The dams were allowed to deliver normally and the males F1 generation obtained from them was allowed to mate nulliparous females at PND 80 overnight in a ratio of 1:1 and some reproductive indices were thereafter evaluated. The mated females were evaluated for mating index, fertility index, gestation index and gestational length, while the resulting F2 male rats were evaluated for live birth index, viability index and sex ratio. The results revealed decreased mean gestational length, viability index and sex ratio in the CC group ($p<0.05$) relative to that of the other groups. Although not significant ($p>0.05$), the mating, gestation (pregnancy), fertility and live birth indices in the CC group were relatively lower when compared to that of other treated groups. In conclusion, alterations of reproductive indices from mating of nulliparous females with F1 male rats exposed *in utero* and *via* lactation to mixture of CPF and CYP were mitigated by melatonin.

KEY WORDS: chlorpyrifos; cypermethrin; reproductive indices; Wistar rats

Introduction

Insecticides are deliberately designed to be toxic and to effectively kill target insect species. Unfortunately, they also have off-target toxic effects that can harm other species, including humans and animals (Abreu-Villaca & Levin, 2017). Increase in resistance to chemical pesticides by pests has necessitated the production of insecticide mixture with different mechanism of action with resultant increase in potency and/or efficacy (Zhou *et al.*, 2011; Zhang *et al.*, 2017). However, this has come with its attendant effect on the target and non-target organisms and the environment. The adverse effect posed by pesticide mixture is still emerging.

Pesticides are one of the chemicals that have been demonstrated to interfere with sexual development, reproduction, and fertility, especially when exposure occurs during vulnerable life stages (Colborn & Caroll, 2007). Women constitute about 21% of farmworkers

Correspondence address:
Dr. Muftau Shittu
Department of Veterinary Pharmacology and Toxicology,
Faculty of Veterinary Medicine,
Ahmadu Bello University,
Zaria, Nigeria
TEL.: +2348036944273 • E-MAIL: shittumuftau4125@gmail.com

(Carroll *et al.*, 2005) with some of them entering recently treated farms, sometimes while pregnant (Ambali *et al.*, 2009). Pesticides exposure during pregnancy is a major issue since this pesticide readily crosses the placenta (Abdel-Rahman *et al.*, 2002) and, as such, has the potential to cause adverse health consequences on the developing organism. Similarly, the ubiquitous nature of pesticides makes its exposure plausible to the general populations, including nursing mothers, who may also expose the neonates to the pesticides *via* milk. This poses threat to the health and wellbeing of the developing fetus, which may eventually cause some impairment later in adult life. Pesticides penetrate maternal and paternal reproductive tissues and organs, which provides pathway for initiating harm to their offspring even before fertilization and up to gestation and lactation (Colborn & Carrol, 2007). The spermatozoa are highly vulnerable to the adverse effects of pesticides (Perry *et al.*, 2011; Marteniesa & Perry, 2013)

Chlorpyrifos (CPF) (*0-0*-diethyl-0-3,4,6-trichloro-2-pyridyl phosphorothionate) is one of the most extensively used organophosphate (OP) insecticide in agricultural and domestic pest control worldwide (Guardia-Escote *et al.*, 2018). It was first marketed in USA in 1965 and its use has increased rapidly, in part due to banning of chlordane for termite control in 1988 (Steenland *et al.*, 2000). Like other OP insecticides, the main mechanism of systemic toxicity is due to inhibition of acetylcholinesterase (AChE) resulting in cholinergic toxicity (Eaton *et al.*, 2008). However, toxicity has been recorded at doses that do not affect AChE activity or long after restoration of its activity (Saulbury *et al.*, 2009), prompting the search for alternative mechanisms. Oxidative stress is one of the other molecular mechanisms implicated in CPF poisoning (Ambali & Aliyu, 2012).

Cypermethrin (CYP) is a class II synthetic pyrethroid with a wide range of application in agriculture, forestry, domestic, horticulture and veterinary medicine (Yavasoglu *et al.*, 2006). Its use has increased tremendously due to high bioefficacy, enhanced stability and considerably low mammalian toxicity (Yavasoglu *et al.*, 2006) compared to the organochlorines. Apart from the inhibition of central neurotransmission through prolongation of sodium ion channels in the nerve cell membrane, the induction of oxidative stress has also been implicated in its toxicity (Wielgomas & Krechniak, 2007).

Oxidative stress, which damages biomolecules in the body through redox imbalance, has been implicated in the toxicity associated with OPs (Ambali *et al.*, 2010; Shittu *et al.*, 2012), pyrethroids (Wielgomas & Krechniak, 2007) and their mixtures (El-Demerdash, 2011a, b). However, during oxidative stress, the protective/neutralizing antioxidant systems are overwhelmed due to increased ROS generation. Therefore, to overcome this challenge of excessive oxidant production, supplementation with exogenous antioxidant molecules becomes imperative (Ambali *et al.*, 2012).

Melatonin (N-acetyl-5-methoxytriptamine) is a hormone produced from the pineal gland during the dark phase of the circadian cycle and it is also a highly conserved antioxidant molecule (Reiter *et al.*, 2010). It plays significant role in many body processes including control of reproductive functions, modulation of immune system activity, inhibition of tumorigenic processes and inhibition of oxidative stress (Hardeland *et al.*, 2009). Its role as an antioxidant and free-radical scavenger (Lekic *et al.*, 2010) and its involvement in the enhancement of body antioxidant systems have been well documented (Olukole *et al.*, 2018; Pang *et al.*, 2018).

One of the consequences of exposure of non-target organism to insecticide mixture is the adverse reproductive outcome. Indeed, exposure to pesticide mixture has been implicated in varying reproductive deficits involving sperm qualities and anomalies in male reproductive organs (Rim, 2017). Exposure to variety of insecticides, even in trace amounts, has been shown to cause adverse health consequence in both males and females including infertility, increased mortality of offspring, and behavioral changes (Russel *et al.* 1987; Berteau *et al.* 1989; Prendergast *et al.* 1989). Cypermethrin (Singh *et al.*, 2020), CPF (Zhang *et al.*, 2020) and their combination (Alaa-Eldin *et al.*, 2017) have been reported to adversely affect reproductive functions. In addition, a study has shown that mating of pesticides exposed male rats with non-exposed females have adverse effect on reproductive outcomes and fetal viability (Elbetieha *et al.*, 2001). Since oxidative stress has been shown to be central to the pathophysiology of insecticides-induced adverse health consequence, the present study was therefore aimed at evaluating the effect of melatonin on reproductive indices in nulliparous female rats mated with F1 male rats exposed *in utero* and *via* lactation to mixture of CPF and CYP and in the resulting F2 male rats. We hypothesize that melatonin will not mitigate the reproductive indices in female rats mated with F1 male rats exposed *in utero* and *via* lactation to mixture of CPF and CYP and in the resulting F2 male rats.

Materials and methods

Experimental animals

Seventy-two (72) healthy adult nulliparous female and 72 male Wistar rats weighing between 140–160 g used for this experiment were obtained from the Laboratory Animal House of the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria. The animals were housed in the Department of Veterinary Anatomy Research Laboratory, Ahmadu Bello University, Zaria, Nigeria. They were fed pellets made from grower's mash (Vital Feeds Ltd., Jos, Nigeria). They were given access to water *ad libitum* and acclimatized to the laboratory environment for two weeks prior to use.

Chemicals

Commercial grade Chlorpyrifos (20% E.C. TERMICOT®, Sabero Organics, Gujarat, India) and Cypermethrin (10% EC, GLOBATHRIN®, Heranba Industries Limited,

Vapi, India) were obtained from a reputable agrochemical outlet in Kaduna, Nigeria. Melatonin (MEL, 3 mg/Tablet, Nature Made Nutritional Product, USA) was obtained from a reputable pharmaceutical outlet in Ilorin, Nigeria. Soya oil (Grand Pure Soya Oil, Grand Cereals Limited, Jos, Nigeria) was obtained from a sale outlet in Zaria, Nigeria.

Sample preparation

Chlorpyrifos (CPF) was dissolved in S/oil to make a 1% working solution, while cypermethrin (CYP) and melatonin (MEL) were dissolved in distilled water to make 1% and 1.5 mg/2 ml concentrations, respectively.

Animal breeding and dosing protocol

Seventy-two sexually mature female nulliparous Wistar rats weighing between 140–160 g were bred with 72 adult males to obtain 60 pregnant animals after being mated overnight in a 1:1 mating scheme, based on guidelines on reproductive toxicity study No 421 of the Organization for Economic Co-operation and Development (*OECD*) (OECD, 1995). Evidence of mating was assessed in each female animal by the presence of spermatozoa deposit in the vagina or the presence of vaginal plug. Day 1 of gestation was regarded as the day copulation plug/spermatozoa were found in the vagina. The sixty pregnant dams were thereafter weighed and assigned at random into 6 groups of 10 animals each. Group I (DW) was given distilled water (2 ml/kg), while group II (S/oil) was given Soya oil only (2 ml/kg). Group III (MEL) was dosed MEL (0.5 mg/kg [Umosen *et al.,* 2012)]) only, while group IV (CC) was co-administered with CPF (1.9 mg/kg, 1/50th of the LD_{50} (Shittu, 2018) and CYP (7.5 g/kg, 1/50th of the LD_{50} (Shittu, 2018). Group V (MCC) was pretreated with MEL (0.5 mg/kg) and then co-exposed to CPF (1.9 mg/kg) and CYP (7.5 mg/kg), respectively, 30 minutes later. Group VI (CCM) was co-exposed to CPF (1.9 mg/kg), and CYP (7.5 mg/kg) respectively, and thereafter treated with MEL (0.5 mg/kg), 30 minutes later. The regimens were administered to the dam by gavage once daily from gestation day 1 (GD 1) to postnatal day (PND) 21. The dams delivered normally. At PND 80, the F1 generation male rats from each group (n=10) were cohabited with nulliparous females overnight in a 1:1 mating scheme. The pregnant dams were evaluated for reproductive indices comprising of gestational length, mating index, fertility index and gestation (pregnancy) index while the F2 generation rats from each group (n=10) were evaluated for live birth index, sex ratio and viability index as described by United States Environmental Protection Agency (1996). The study was conducted in accordance with international guidelines for animal welfare.

Male mating index: The male mating index was calculated as follows: Number of males mated/Number of males cohabitated × 100.

Fertility index: This parameter was assessed by recording the number of dams that were apparently pregnant at gestation day 14 in order to be sure of state of pregnancy after an overnight cohabiting with males and calculated as follows: Number of co-habited females pregnant/ Number of non-pregnant couples co-habited × 100.

Gestation (pregnancy) index: This was assessed by counting the number of females that delivered live pups at parturition and the number of females with evidence of pregnancy after co-habitation. It was calculated as follows: Number of females that delivered live young/ Number of females with evidence of pregnancy co-habited × 100.

Gestational length: This was evaluated based on the number of days between establishment of pregnancy (GD 1) and the day of parturition.

Live birth index: Live birth index was determined at the end of gestation period of the co-habited females with the male animals in the F1 generation. This was done by visual counting of the number of live offspring (F2) littered by individual dam versus the total number of offspring delivered. It was calculated as follows: Number of live offspring/ Number of offspring delivered × 100.

Viability index: This index was determined by counting the number of F2 litters that were alive at PND 4 relative to those that were delivered and then calculated as follows: Number of live offspring at lactation day 4/ Number of live offspring delivered ×100.

Sex ratio: This parameter was determined at the end of gestation period of the cohabited F1 male generation and sexually mature female. This index was determined by measuring the anogenital distance of each of the resulting F2 pups with a vernier caliper and then calculated as follows: Number of male offspring/Number of female offspring × 100.

Data analysis

All data obtained were expressed as mean ± standard error of mean (±SEM) and subjected to one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test to determine significance of the differences in the mean of the parameters obtained between various experimental groups. Graphpad Prism version 4.0 for windows (2003) from Graphpad Prism software, San Diego, Califonia (www.grapphpad.com) was used. Values of $p<0.05$ were considered significant.

Results

Effect of treatments on mating index

There was no significant ($p>0.05$) change in the mating index in between the groups. However, the mating index in the CC group was comparatively lower relative to that of the DW (15%), S/oil (18%), MEL (18%), MCC (17%) and CCM (18%) groups (Figure 1).

Effect of treatments on fertility index

There were no significant differences ($p>0.05$) in the fertility index in between the groups. However, the mean fertility index in the CC group decreased relative to that of the DW (17%), S/oil (18%), MEL (18%), MCC (18%) and CCM (15%) groups, respectively (Figure 2).

Effect of treatments on gestation (pregnancy) index

Although there were no significant changes ($p>0.05$) in between the groups, the gestation (pregnancy) index in the CC group was relatively lower when compared to that of DW (17%), S/oil (18%), MEL (17%), MCC (18%) and CCM (16%) groups, respectively (Figure 3).

Effect of treatments on gestation length

There was a significant ($p<0.05$) reduction in the mean gestational length in the CC treated group relative to that of the DW, S/oil, MEL, and MCC groups. In addition, there was a significant ($p<0.05$) increase in the gestation length in the S/oil group when compared to CCM

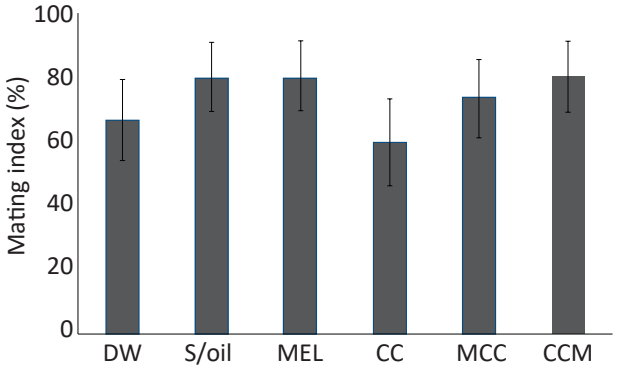

Figure 1. Effect of gestational and lactational exposure to Distilled water (DW), Soya oil (S/oil), Melatonin (MEL), Chlorpyrifos and Cypermethrin (CC), Melatonin + Chlorpyrifos + Cypermethrin (MCC) and Chlorpyrifos + Cypermethrin + Melatonin (CCM) on mating index (n=10).

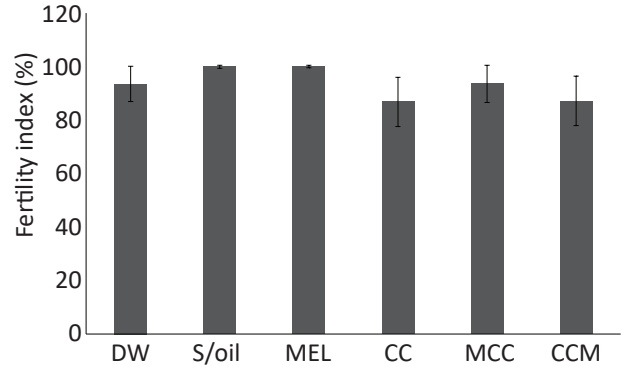

Figure 2. Effect of gestational and lactational exposure to Distilled water (DW), Soya oil (S/oil), Melatonin (MEL), Chlorpyrifos and Cypermethrin (CC), Melatonin + Chlorpyrifos + Cypermethrin (MCC) and Chlorpyrifos + Cypermethrin + Melatonin (CCM) on fertility index (n=10).

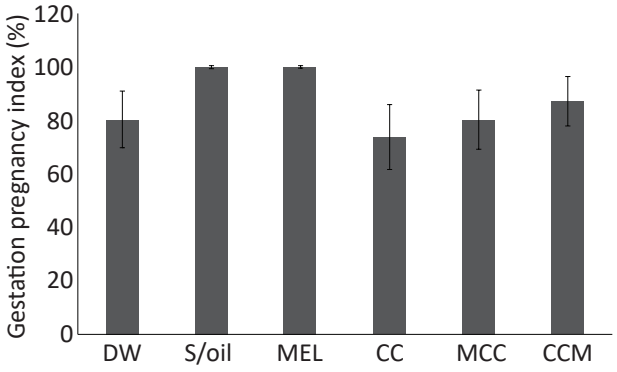

Figure 3. Effect of gestational and lactational exposure to Distilled water (DW), Soya oil (S/oil), Melatonin (MEL), Chlorpyrifos and Cypermethrin (CC), Melatonin + Chlorpyrifos + Cypermethrin (MCC) and Chlorpyrifos + Cypermethrin + Melatonin (CCM) on gestation index (n=10).

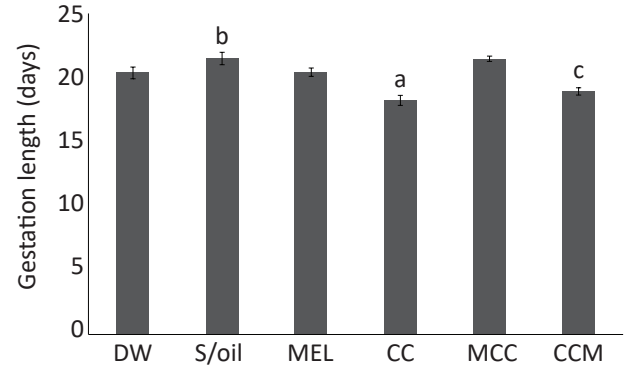

Figure 4. Effect of gestational and lactational exposure to Distilled water (DW), Soya oil (S/oil), Melatonin (MEL), Chlorpyrifos and Cypermethrin (CC), Melatonin + Chlorpyrifos + Cypermethrin (MCC) and Chlorpyrifos + Cypermethrin + Melatonin (CCM) on gestational length (n=10). [a]$p<0.05$ versus DW, S/oil, MEL and MCC groups; [b]$p<0.05$ versus CCM group; [c]$p<0.05$ versus MCC group.

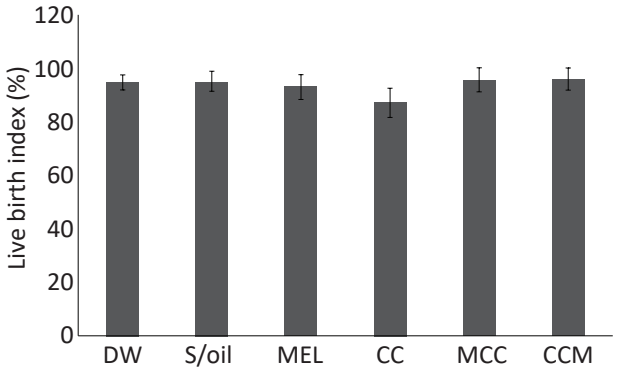

Figure 5. Effect of gestational and lactational exposure to Distilled water (DW), Soya oil (S/oil), Melatonin (MEL), Chlorpyrifos and Cypermethrin (CC), Melatonin + Chlorpyrifos + Cypermethrin (MCC) and Chlorpyrifos + Cypermethrin + Melatonin (CCM) on live birth index (n=10).

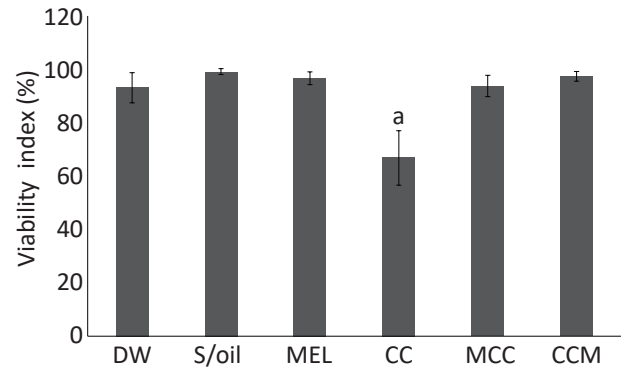

Figure 6. Effect of gestational and lactational exposure to Distilled water (DW), Soya oil (S/oil), Melatonin (MEL), Chlorpyrifos and Cypermethrin (CC), Melatonin + Chlorpyrifos + Cypermethrin (MCC) and Chlorpyrifos + Cypermethrin + Melatonin (CCM) on viability index (n=10). [a]$p<0.05$ versus DW, S/oil, MEL, MCC and CCM groups.

group. The mean gestation length in the CCM group decreased significantly (p<0.05) relative to that of the MCC group (Figure 4).

Effect of treatments on live birth index

There were no significant (p>0.05) changes in the live birth index in between the groups. The mean live birth index in the CC group decreased marginally by 17% when compared individually to that of the DW, S/oil, MEL, MCC and CCM groups (Figure 5).

Effect of treatments on viability index

There was a significant (p<0.05) decrease in the viability index in the CC group, compared to that of the DW, S/oil, MEL, MCC and CCM groups, respectively (Figure 6).

Effect of treatments on sex ratio

There was a significant (p<0.05) decrease in the mean sex ratio between CC group relative to that of DW and MEL groups. In addition, group co-exposed to CC decrease significantly (p<0.01) in mean sex ratio when compared to the S/oil and MCC groups (Figure 7).

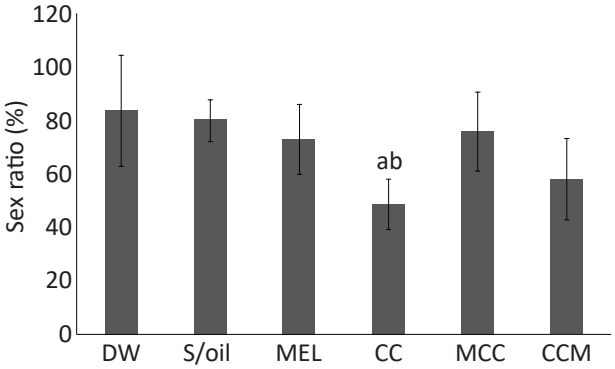

Figure 7. Effect of gestational and lactational exposure to Distilled water (DW), Soya oil (S/oil), Melatonin (MEL), Chlorpyrifos and Cypermethrin (CC), Melatonin + Chlorpyrifos + Cypermethrin (MCC) and Chlorpyrifos + Cypermethrin + Melatonin (CCM) on gestational length (n=10). [a]p<0.05 versus DW and MEL groups; [b]p<0.05 versus S/oil and MCC groups.

Discussion

Studies have shown that male factors account for 40%–50% of infertility in human (Hirsh, 2003; Brugh & Lipshultz, 2004). Erectile dysfunction, as one of the major problems contributing towards male infertility, afflicts as much as 10% of the male population (Read, 1999). It has also been reported that pesticides may cause erectile dysfunction (Soliman *et al.*, 2008). The present study showed that the group exposed to the insecticide mixture had the least mating index, which may be partly due to erectile dysfunction. This may be due to the antiandrogenic effect of the insecticide mixture. Chlorpyrifos (Shittu, 2018; Shittu *et al.*, 2014) and CYP (Umosen & Uchendu, 2014) are known to alter sex hormone concentration *via* oxidative injury to the pituitary gland (Shittu *et al.*, 2012), therefore affecting FSH and LH that regulate the number of Leydig cells and consequently testosterone secretion (Ramaswamy & Weinbauer, 2014). Testosterone, the male hormone, is the major driver of male reproductive development and function (Kaur *et al.*, 2015), and therefore any interference with its secretion will interfere with sexual potency. The improvement in mating index in group pretreated with MEL may be ascribed to apparent increase in testosterone concentration, as demonstrated by our earlier study (Shittu, 2018). Melatonin promotes and modulates testosterone concentration through increased endocrine activity in Leydig cells (Deng *et al.*, 2018; Yu *et al.*, 2018) and its antioxidant properties.

One of the challenges currently ravaging human race is infertility and subferitility, a situation that has been exacerbated by environmental contaminants including pesticides (Sengupta & Banerjee, 2014; Chiang *et al.*, 2017). Indeed, OPs (Peiris-John & Wickremesinghe, 2008; Shittu *et al.*, 2013) and pyrethroids (Jurewicz *et al.*, 2014; Umosen & Uchendu, 2014) have been shown to adversely alter reproduction. In the present study, the decrease in fertility index in the F1 male rats in the CC group may be due to testicular damage and sperm abnormalities with respect to concentration, morphology and progressive motility (Shittu, 2018), apparently due to oxidative injury to spermatozoa and testes resulting from *in utero* and lactational exposure to mixture of CPF and CYP. Furthermore, oxidative damage to the testes may have also been partly responsible for the lower fertility index of the F1 male rats. The male testes are involved in spermatogenesis and synthesis of sexual hormones. These two functions work in collaboration with hypothalamus-pituitary-gonadal axis (Bretveld *et al.*, 2007). Any alteration in the complex regulation of hormone production and spermatogenesis can result in impairment or cessation of spermatogenesis leading to infertility (Sharma & Goyal, 2014). Melatonin, in the present study, improved fertility index adversely altered by the pesticide mixture, partly due to its antioxidant and free radical scavenging effect. Furthermore, Mel has been shown to directly influence the hypothalamo-pituitary-gonadal axis, as melatonin receptors (MT1 and MT2) have been identified in hypothalamic neurons governing the release of pituitary gonadotrophs (Dubocovich & Markowska, 2005) in gonadotropins of the anterior pituitary (Balik *et al.*, 2004) and in both female and male gonads (Woo *et al.*, 2001; Frungieri *et al.*, 2005) as well as in the reproductive adnexae (Ekmekcioglu, 2006). Although MT1 and MT2 receptors have been identified in testicular cells, particularly in Leydig cells (Izzo *et al.*, 2010), it is MT2 receptor that is responsible for enhancement of sperm capacitation. Melatonin also regulates testicular growth and testosterone production in testicular cells (Frungieri *et al.*, 2005). Melatonin stimulates flagellar motility, which is required for successful penetration of the zona pellucidum, hence fertilization of the egg (Reiter *et al.*, 2009). Anti-apoptotic potentials of melatonin contribute to prolongation of sperm survival (Fernando & Rombauts, 2014), hence successful fertilization.

The present study revealed that cohabitation of F1 male rats from dams co-exposed to CPF and CYP with untreated females resulted in an apparent decrease in gestation or pregnancy index. This shows that many mated females were not able to carry the pregnancy through to parturition, partly due to morphological defect in the spermatozoa of the F1 male (Shittu, 2018). Genetic defects in male germ cells have been shown to allow fertilization but results in early embryonic death (Hjollund *et al.*, 2004). Although the present study did not evaluate the effect of the pesticide mixture on germ cell, an earlier study by Recio *et al.* (2001) demonstrated that OP insecticides significantly increased sperm chromosome nullisomy involving the sex chromosomes. Therefore, this abnormality in sperm chromosome, which may have contributed to morphological defect in the spermatozoa of F1 male rats co-exposed to CPF and CYP (Shittu, 2018), might have led to increased embryonic death, hence low gestational index in the female rats cohabited with F1 male rats. Melatonin has been shown in this study to improve the gestation index in dams mated by F1 male rats gestationally and lactationally exposed to CPF and CYP and this may be partly due to its antioxidant and anti-apoptotic effect.

The present study has shown that breeding of untreated female rats with F1 male rats that have been exposed to CPF and CYP *in utero* and *via* lactation caused shortening of the gestational length, hence pre-term delivery. This may be apparently due to male factor, since that was the sex that was exposed to the insecticide combination. This decrease in gestational length may be attributed to the fertilization of female ova by defective sperm cells, probably due to defect in the genetic composition of the sperm caused by insecticide-induced oxidative injury. In addition, contamination of the conceptus by the pesticides may have trigger prenatal stress, which is known to reduce gestation length (Paris *et al.*, 2011). *In utero* exposure to OP pesticides has been shown to shorten duration of gestation (Eskenazi *et al.*, 2004), a situation the authors attributed to depression of AChE activity, which causes increased acetylcholine concentration that stimulates contraction of the uterus. Pretreatment with MEL normalized the gestation length, hence prevented the pre-term delivery. This may be linked to the ability of MEL to protect spermatozoa from genetic damage due to its antioxidant effect. A decrease in the live birth index in the female mated by the F1 rats exposed to the pesticide mixture has been reported only in the current study. This may be related to the oxidative stress-induced increase in morphological alterations of sperm cells, thereby causing a defective and non-viable F2 generation. The outcome of the present study has been corroborated by finding from a study by Garry *et al.* (2002) in humans that showed a decrease in the number of live birth index caused by increased frequency of pregnancy loss through miscarriage, prematurity, or still-birth in the spouses of applicators exposed to mixtures of pesticides. Improvement in the live-birth index in the MEL groups may be ascribed partly to the antioxidative effect of melatonin as it stabilizes the integrity of the DNA (Ostgen *et al.,* 2019) and create favorable environment for fertilization, blastocyst formation and embryogenesis (Pang *et al.,* 2018).

The present study recorded a decrease in viability index in group exposed to insecticide mixture. This is consistent with the findings from previous studies in pups following *in utero* CPF exposure (Morgan & El-Aty, 2008; Shalaby *et al.*, 2013). The obtained result may be due to pesticides associated development disorders that cause non viability of the offspring sired by insecticide exposed male rats.

Numerous studies have shown that prenatal and neonatal exposure to pesticides affect the survivability of the offspring (Recio *et al.*, 2001; de Castro & Chiorato, 2007). Bell *et al.* (2001) reported an increased risk of fetal death due to congenital anomalies when synthetic pyrethroids were used in the same township, range, or section during the 3rd to 8th week of pregnancy. The reduction in pups' viability recorded in the current study may be partly related to defective spermatozoa of the F1 males that were exposed to the pesticide mixture *via in utero* and lactation. The union of the defective spermatozoa and the eggs from the ova of untreated dams may have resulted in production of defective F2 conceptus, hence non-viable pups. Pretreatment by melatonin has shown, in this study, to restore the pups' viability index altered by the pesticide mixture partly due to its antioxidant and free radical scavenging effect. Antioxidants have been shown to neutralize the free radical produced by the pesticide mixture and prevent damage to DNA, lipids, proteins, and other biomolecules (Trushina & McMurray, 2007) thereby preventing free-radical induced fetotoxicity.

The sex ratio, which is the ratio of male to female offspring, may be a simple and non-invasive way to monitor the reproductive health of a population. Astolfi & Zonta (1999) noted a trend towards a declining proportion of male births in many industrialized countries, a development partly attributed to chemical contaminants including pesticides. Alteration in sex ratio is one of the adverse reproductive outcomes that have been partly attributed to increased chemical abundance and exposure (Terrell *et al.*, 2011). The present study revealed a decreased sex ratio in the CC group, which was indicative of the presence of more females than males. Huang and Li (2014) reported a decrease in male to female ratio following maternal exposure of rats to CYP. Studies have also reported a decrease in sex ratio of children born to pesticide manufacturers and applicators (Ryan *et al.*, 2002; Figa-Talamanca *et al.*, 2003). Decrease in the sex ratio in the CC group may also be attributable to effect of both pesticides on the gene responsible for sex determination or gender differentiation during embryogenic development. Mammalian offspring sex ratios can be biased *via* prenatal mechanisms, including sperm selection and sex-specific embryo loss (Beery & Zucker, 2012). Therefore, the increased number of female births in the CC group may also be associated with high number of defects in the spermatozoa that carry the sex-determining chromosomes. Indeed, our earlier study recorded poor semen quality in the spermatozoa of

F1 generation rats co-exposed *in utero* and *via* lactation to CPF and CYP (Shittu, 2018). A study by Bae *et al.* (2016) reported that sperm defect is associated with excess number of female births. Given that the Y chromosome is the sex-determining chromosome in men, a study by Eisenberg *et al.* (2011) in an infertile cohort of 185 men undergoing a semen fluorescence *in situ* hybridization (FISH) test from 2003 to 2010 in the United States showed that poor semen quality, which was reflected by semen volume, sperm concentration, and total motile sperm count, significantly decreased the odds of having a Y chromosome-bearing sperm. Garry *et al.* (2002) reported a 21% decrease in the number of male children born to male pesticide applicators. Epidemiological and reproductive analyses further revealed that more female children were born to applicators with lower testosterone levels (Bae *et al.*, 2016). Shittu (2018) reported a lower testosterone concentration in F1 rats that were exposed to mixture of CPF and CYP *via in utero* and lactation. From the foregoing, the lower testosterone concentrations in the F1 male rats exposed to CPF and CYP *in utero* and *via* lactation may have been responsible for the fewer number of male births in the F2 generation. The improvement in sex ratio in favor of the male sex in the group pretreated with melatonin may be partly due to its antioxidant and free radical scavenging properties, which protect the pituitary glands and the testes from insecticide-induced oxidative injury, which may ordinarily impair optimal testosterone secretion in the F1 male rats. Indeed, our earlier study has shown an improved testosterone concentration in the F1 male rats in the group pretreated with melatonin before exposure to mixture of CPF and CYP (Shittu, 2018).

Conclusion

In conclusion, melatonin was able to mitigate the adverse reproductive indices in female rats mated with F1 male rats exposed in *utero* and *via* lactation to mixture of CPF and CYP and in the resulting F2 male rats, apparently due to its antioxidant and free radical scavenging properties.

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