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Research article

Resveratrol Ameliorates Male Reproductive Dysfunction Induced by Pirimiphos-methyl In Rats

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ABSTRACT

Exposure to Pirimiphos-methyl (POM) has been reported to adversely affect sperm functions, leading to male infertility. This study seeks to investigate the effect of resveratrol on POM induced male reproductive dysfunction. Twenty-four male Sprague-Dawley rats weighing between 165g – 200g were randomly divided into four groups of control, the POM group (received 62.5mg/kg POM/ b.w), POM + RES (received 62.5mg/kg POM and 20mg/kg body weight of resveratrol, and the RES group (received 20mg/kg body weight resveratrol) for 65 days. The results showed a significant decrease in body weight gain in the POM group, POM +RES group compared to the control. The relative liver weight of the POM group was also significantly increased compared with the control ($p<0.05$). RES + POM significantly increased the epididymal sperm count, motility, morphology, and testosterone level compared to the POM group. POM increased MDA and reduced SOD, GSH, and catalase activities, while POM + RES only reversed this trend in the MDA, catalase, and SOD levels. There was a significant increase in the cholesterol concentration of the POM group compared to the control while there were no significant differences in the HDL, LDL, and triglyceride levels across all the groups. There was distortion in the POM group's cytoarchitecture of the liver and testes compared to the control and RES groups. In conclusion, the results showed that POM disrupts sperm functions and caused hepatotoxicity in rats' livers. This study also shows that RES improves sperm parameters impaired by POM.

Keywords: *Pirimiphos-methyl, resveratrol, testosterone, organophosphate, sperm*

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INTRODUCTION

Since the beginning of the industrial age, man has continuously been exposed to various toxic chemicals. Our environments are contaminated with toxic xenobiotics that affect the food we eat, the water we drink and the air we breathe (Sengupta and Banerjee 2014). Organophosphates are chemical substances originally made to kill insects, rodents or even herbs with the aim of increasing crop yield and crop preservation. They contain powerful neurotoxic substances that can obstruct the acetylcholinesterase enzyme activity which in turn can excite cholinergic receptors producing both nicotinic and muscarinic effects in the organism such as muscle contraction and secretion in many glands (Ghafouri-Khosrowshahi *et al*., 2019, Ngoula *et al*., 2007). They could be carcinogenic, immunotoxic, and cause endocrine disruption in the release and synthesis of hormones, thereby affecting man's reproductive system (Ghafouri-Khosrowshahi *et al*., 2019). Its incessant use for agricultural purposes poses a public health concern for man and the environment (Lasram *et al*., 2009). There is an increased use of organophosphates in developing countries due to poor storage facilities, and an increase in the world population with the consequent need for food supply to the population.

Pirimiphos-methyl (POM) is a post-harvest organophosphate pesticide widely used in developing countries for the storage and preservation of grains such as corn and sorghum grain and seeds. It has also been used for the control of ants, cockroaches, bed bugs, fleas, flies, spiders, and mosquito larvae in and around domestic, public service areas, agricultural buildings, commercial and industrial areas and refuse tips (Okunola *et al*., 2014, Tamura *et al*., 2001). The reproductive organs have been shown to be among the most vulnerable organs to organophosphorus insecticides. Infertility is one of the health concerns that is associated with organophosphate exposure. Infertility has been defined as the inability of a couple to achieve pregnancy, usually after one year or more of regular and unprotected sexual intercourse (Moreira *et al*., 2021, Zegers-Hochschild *et al*., 2009). Studies have shown that exposure to POM can cause male and female reproductive dysfunctions.

Pesticides can act as endocrine-disrupting chemicals, which are able to interfere with the synthesis, secretion, transport, binding, and actions of hormones responsible for reproduction (Moreira *et al*., 2021). Multiple animals- and human-based studies on exposure to environmental toxins suggest a negative impact on semen quality, in terms of sperm

concentration, motility, and/or morphology (Mima *et al*., 2019). These toxins may exert estrogenic and/or anti-androgenic effects, which in turn alter the hypothalamic-pituitary-gonadal axis, induce sperm DNA damage or cause sperm epigenetic changes (Mima *et al*., 2019, Okunola *et al*., 2014; Oyesola *et al*., 2019). Ngoula *et al*., (2007) reported that the administration of POM (62.5 and 125 mg/kg body weight) is detrimental to the reproductive potentials of male rats. Asides from the decrease in sperm density and motility, histological observations showed an enlargement of the interstitial space, inhibition of spermatogenesis, rarefaction of Leydig cells and oedema in the testes. In addition, the administration of POM has been reported to cause decreased implantation rate in rats due to decreased uterine receptivity caused by an imbalance in the level of estradiol and progesterone.

Resveratrol (RES) is a stilbenoid. It is a natural phenol, and a phytoalexin produced by several plants to protect the plants from parasitic attacks and environmental stress (Illiano *et al*., 2020). Its major sources are the skin of grapes, mulberries, raspberries, blueberries, and peanuts. As a natural phenol, it possesses a strong antioxidant function by transferring hydrogen atoms from their hydroxyl groups to the chain carrying ROO* radicals (proton-coupled electron transfer mechanism) (Foti *et al*., 2007). The valuable effects of RES have been connected to its ability to function as a potent antioxidant agent (Cai *et al*., 2003). Other actions of RES include its anti-inflammatory potential, cardioprotective function, anticancer, antimicrobial, and antiaging effects (Illiano *et al*., 2020, Price *et al*., 2012). Animal model studies have shown that RES has a positive effect on the hypothalamic-pituitary-gonad axis, sperm production and motility (Juan *et al*., 2005; Shin *et al*., 2008, Eleawa *et al*., 2014). RES was also found to decrease germ cell apoptosis, and increased mitochondrial activity, sperm motility and DNA integrity (Kasai *et al*., 2002; Zhao *et al*., 2007). We postulate that RES a potent antioxidant could alleviate the peroxidative effects and endocrine disruptions brought about by organophosphates. This study therefore investigated the effect of RES in male rats administered POM.

MATERIALS AND METHODS

Animals: Twenty-four male Sprague-Dawley rats that weighed between 165 – 200g were used for this study. The rats were randomly assigned into four groups, were kept in well-ventilated rat cages, and were allowed to feed and drank water ad libitum. They were maintained under uniform husbandry conditions of light (28±2°C, humidity at 70%, 12 hour:12hour day-night ratio) in the animal facility. Good sanitation of the cages and animal houses was ensured throughout the experimental period. All guidelines regarding the use and care of laboratory animals were strictly adhered to in accordance with the U.S. National Institute of Health (2011).

Drugs and chemicals: Pirimiphos-methyl with the trade name Guardiono-emulsifiable concentrate 50 was produced by Nantong Baoye Chemical Company Limited, China, and resveratrol (RES) was obtained from Cayman chemicals, USA (CAS 501-36-0).

Experimental group: Animals were randomly divided into four groups. The Control group received 10ml/kg body weight of distilled water (vehicle for treatment). The second group (POM group) received 62.5mg/kg body weight of POM. (Ngoula *et al.*, 2007). The third group (POM and RES group) received only 62.5mg/kg body weight of POM and 20mg/kg body weight of RES (Shati, 2019) while the last group (RES group) received 20mg/kg body weight of RES.

Sample collection: Animals were sacrificed by cervical dislocation and the reproductive organs were exposed. The testes, epididymis, and liver were carefully removed. The organs removed from each group were weighed and prepared for sperm analysis, oxidative stress, and histology. Blood was withdrawn by cardiac puncture into plain sample bottles and the serum was used for lipid profile and testosterone assays.

Sperm function analysis: Sperm motility, sperm morphology, sperm count, and viability tests were carried out on the animals as described by Akunna *et al.*, (2013) and Ali & Adel, (2013).

Oxidative stress parameters: The testes of the animals were washed in an ice-cold 1.15% KCL solution, blotted, and weighed. They were then homogenized with 0.1M phosphate buffer (pH 7.2). The resulting homogenate was centrifuged at 2500rmp speed for 15mins then it was removed from the centrifuge and the supernatant was decanted and stored at -20^{o}C until analysis. Estimation of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) contents of the testes was carried out as previously described by Iranloye *et al.*, (2013) and Sharma, (2014).

Lipid profile analysis: The lipid profile of the serum samples was analyzed using Mindray BS-120 automated Chemistry Analyzer (Shenzen, China). The Cholesterol, High-Density Lipoprotein, Low-Density Lipoprotein and Triglyceride concentration of the animals were determined.

Testosterone assay: An enzyme-based immunoassay system was employed to determine testosterone levels in the serum samples collected. Accu bind ELISA kit for Testosterone test system with product code 3725-300T was used. The procedure used for the analysis was as described by the kit producers (Monobind inc. USA).

Tissue preparation and histology: The left testis and the liver were fixed in Bouin's fluid for 1 week, embedded in paraffin, cut, and stained with Harris haematoxylin and eosin. The tissue sections were observed under a light microscope (Olympus, X 40) for seminiferous tubule morphology and cellular harmony. Slides were prepared as described earlier by (Survana *et al.*, 2013 Avwioro., 2014). All histologic slides are reviewed using a microscope (Leica MD-500, Leica, Microsystems. Germany) with comments made on findings.

Statistical analysis: The data collected from the analysis were presented as mean and standard error of the mean. It was analyzed using one-way analysis of variance (ANOVA) and the Turkey's post hoc test was used to show the significant differences among groups. The value of $P < 0.05$ was considered statistically significant. Analysis was performed with the GraphPad Instat Version 9.00 (GraphPad Prism Software, San Diego California, USA).

RESULTS

Body weights gain and organ weights: Fig. 1 shows the body weight changes in the rats administered with POM and RES. The result showed that there was a significant decrease in the growth of the animals administered POM and POM + RES when compared with control ($p < 0.05$). No significant change was found in the percentage body change in the RES group compared with control. Relative liver weight in the POM was significantly increased when compared with control (Table 1), while no significant difference was found in the relative weight of the testes when compared with the control.

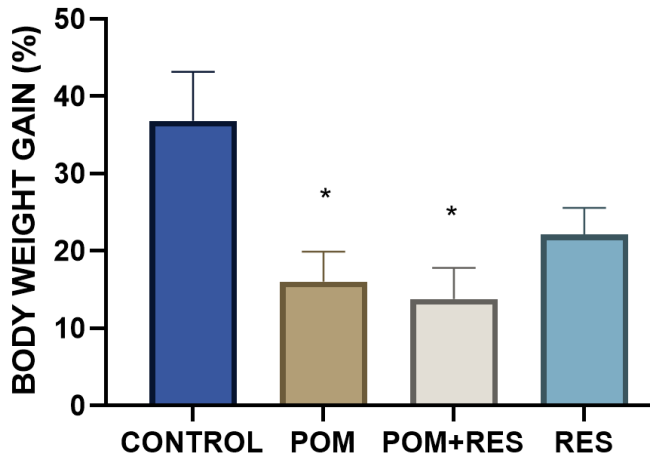

Figure 1
Body weight gain of rats treated with POM and RES.
** Connotes significant difference from control (p<0.05)*

Table 1
Relative Organ weights of rats administered POM and RES

	Control	POM	POM + RES	RES
Relative weight of liver	3.02 ± 0.09	3.75 ± 0.11*	3.34 ± 0.26	2.90 ± 0.21#
Relative weight of testes	1.23 ± 0.03	1.2 ± 0.05	1.31 ± 0.08	1.36 ± 0.76

**Signifies significant difference from control (p < 0.05), # signifies significant difference from POM*

Sperm analysis and Testosterone level: Table 2 shows the sperm parameters of rats administered POM and RES. Sperm function analysis showed significantly lower sperm motility in the POM group compared to the Control, POM +RES and RES groups ($P < 0.05$). Similar results were found in the sperm count, sperm morphology and sperm viability in which there was a significant decrease in the POM groups when compared with the control ($P < 0.05$). Fig. 2 shows the testosterone levels of rats administered POM and RES. The result showed that POM significantly decreased the levels of testosterone when compared with the control.

Table 2.
Effect of POM and RES on sperm parameters

	Control	POM	POM + RES	RES
Sperm Motility (%)	73.4 ± 4.92	25.0 ± 6.12*	80.0 ± 8.80	77.0 ± 12.81
Sperm Count (sperm/million×10^6)	63.8 ± 4.72	19.2 ± 8.03*	70.1 ± 6.21	77.4 ± 3.55
Sperm Normal Morphology (%)	80.2 ± 1.36	54.4 ± 1.17 *	78.0 ± 4.80	89.4 ± 3.57
Sperm Viability (%)	80.4 ± 4.81	47.0 ± 5.23*	86.0 ± 6.22	85.6 ± 4.71

**Connotes significant difference from control (p < 0.05)*

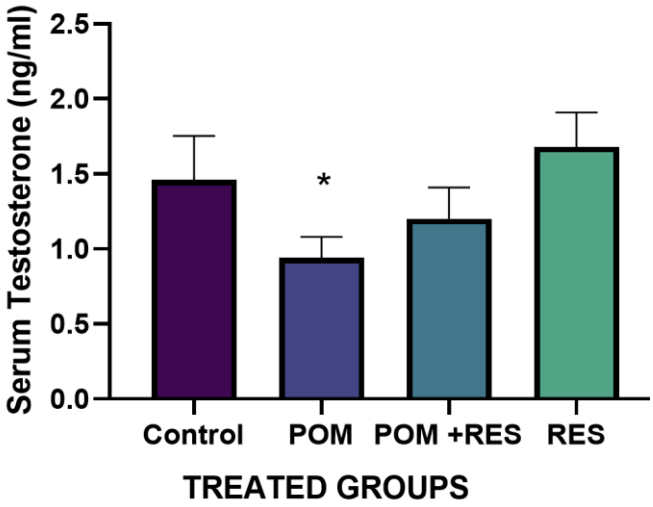

Figure 2
Serum testosterone level in animals treated with POM and RES.
** Connotes significant difference from control (p < 0.05)*

Table 3:
Malondialdehyde and antioxidant enzymes level of rats administered POM and RES

	Control	POM	POM + RES	RES
Malondialdehyde (µmol/ml)	1.83 ± 0.06	3.68 ± 0.59*	2.02 ± 0.31#	2.03 ± 0.08#
Superoxide dismutase (µmol/ml/min/mg pro)	26.19 ± 3.16	7.74 ± 0.55*	17.02 ± 2.80*#	21.78 ± 0.74#
Catalase (µmol/ml/min/mg pro)	27.31 ± 071	17.61 ± 0.42*	28.62 ± 0.89#	33.51 ± 4.73#
Reduced glutathione (µmol/ml)	17.49 ± 0.54	13.77 ± 0.78*	14.74 ± 0.38	17.83 ± 0.35#$

**Connotes significant difference from control, # connotes significant difference from POM group and $ connotes significant difference from POM + RES group (p< 0.05).*

Lipid peroxidation and antioxidant status: Table 3. shows the testicular MDA levels and antioxidant status of the rats

administered POM and RES. The result showed that POM significantly increased POM levels and reduced the activities of SOD, CAT, and reduced glutathione levels. The MDA levels was significantly reduced in the POM + RES group when compared with POM group and the activities of SOC and catalase were increased in this group when compared POM. The RES group showed a significant decrease in MDA level and a significant increase in SOD, CAT and reduced glutathione when compared with the POM group. Reduced glutathione levels were also increased in this group when compared with the POM and RES groups.

Serum lipid profile: Table 4 shows the serum lipid profile of the rats administered with POM and RES. The result showed that POM significantly increases serum cholesterol concentration in the POM group when compared with the control ($p<0.05$). There were significant differences in the HDL-cholesterol, LDL-cholesterol, and triglyceride concentrations across all the groups.

Histology of the Liver and Testis: The liver of the control rats showed a normal cytoarchitecture of a healthy liver, while that of the POM group revealed portal necrosis which implies inflammation of the hepatocytes. The central vein was also atrophied and abnormal (indicated in plate 1). The POM +RES group showed some inflammation and atrophy of the hepatocyte but with improvement in the blood channels flowing towards the central vein. The liver of the resveratrol group had normal cytoarchitecture. The testes of the POM group in contrast to the control and RES groups showed irregular seminiferous tubules (L), with little or no spermatozoa in the lumen as indicated in plate 2. A little abnormality is seen in the POM +RES group when compared with the POM group.

Table 4.
Effect of POM and RES treatment on serum lipid profile

	Control	POM	POM + RES	RES
Cholesterol (mmol/L)	1.8 ± 0.10	2.5 ± 0.10*	2.0 ± 0.18	1.9 ±. 0.20
HDL (mmol/L)	0.7 ± 0.95	0.9 ± 0.11	1.00 ± 0.03	0.7 ± 0.08
LDL (mmol/L)	1.1 ± 0.16	1.1 ± 0.05	1.6 ± 0.02	0.9 ± 0.14
Triglyceride (mmol/L)	0.8 ± 0.00	1.0 ± 0.10	0.8 ± 0.45	0.7 ± 0.08

**Connotes significant difference from control*

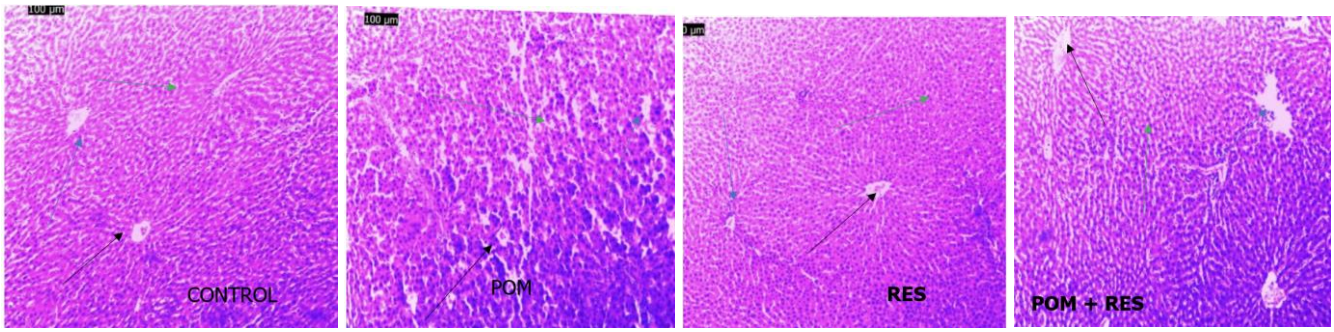

Plate 1
The photomicrograph of the Liver of rats administered POM and RES. The histology section of liver tissue (H/E) from the control and RES group shows a healthy and normal architecture of the portal triad; hepatic artery, hepatic vein, and bile duct (blue arrow). A normal parallel well-arranged plate of hepatocytes radiating from the central vein (black arrow). The endothelial lining of the hepatic sinusoid layer containing blood (green arrow) is healthy and directed towards the central vein. In the POM group, portal necrosis in the blue arrow due to inflammation of hepatocytes, the central vein (black arrow) is atrophied and abnormal, and the blood channel, the sinusoid together with its endothelial lining is partially distorted (green arrow). The POM +RES group showed some inflammation and atrophy of the hepatocyte through the plates of hepatocyte (blue arrow) radiating from the central vein.

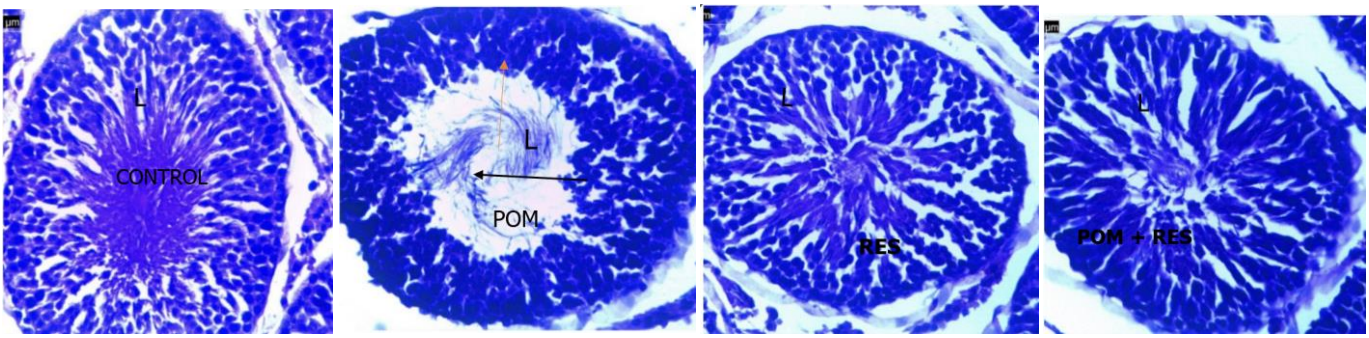

Plate 2
Photomicrograph of the testis (H&E stain) of rats administered POM and RES. The POM group contrasted with the control and RES groups. It shows irregular seminiferous tubules (L), with little or no spermatozoa in the lumen (black arrow), spermatogenesis arrest at the spermatid level (orange arrow) and the spermatogenic cell series is indistinguishable. A little abnormality is seen in the POM + RES group.

DISCUSSION

Findings on body weight following sixty-five days of POM administration and RES showed a decrease in body weight gain in POM and POM + RES groups. The liver weight of the POM group was also increased. Reports have it that, an increase or decrease in either the absolute or relative weight of an organ after administering a chemical or drug is an indicator of the toxic effects of that chemical (Iranloye and Oludare 2011). Thus, we confirm the toxic effect of POM on the rat's tissue. The increased liver weight points to the overactivity of the liver to eliminate the toxic pesticide from the body. This result supports the findings of Ngoula *et al*., 2017 who reported an increase in the relative weight of the liver in rats treated with 62.5mg/kg and 125mg/kg POM.

Pesticides such as POM are endocrine-disrupting chemicals that can interfere with hormone synthesis and processes of transport, secretion, and elimination. Thus, they could interfere with the process of spermatogenesis (Sarkar *et al*., 2000, Zoeller *et al*., 2012, Moreira *et al*., 2021). As expected, our findings showed that POM decreased testosterone levels, decreased epididymal sperm count, sperm motility, sperm normal morphology, and sperm viability. These results agree with the previous studies (Ngoula *et al*., 2017, Oyeyipo *et al*., 2011). Intervention with RES reversed the altered sperm parameters induced by POM. This could be related to the improved testosterone levels of this group. bringing about the restoration of the sperm parameters to normal values. Thus, we report that POM may affect fertility potentials in men and RES promotes the restoration of the distorted reproductive function induced by POM.

This outcome is like what was obtained by Eleawa *et al*., (2014). It has been suggested that RES has a stimulatory effect on the hypothalamic-pituitary-gonadal (HPG) axis (Shati, 2019) due to its ability to bind estrogen receptors as a mixed weak agonist/antagonist. (Juan *et al*., 2005). Testosterone is needed for the continued production of different generations of germ cells in the seminiferous tubules. Therefore, a reduction in testosterone level may lead to the separation of germ cells from the epithelium of the seminiferous tubules as observed in the histological studies of the testis in this study (Shin *et al*., 2008). RES has been found to promote mitochondrial activity thus responsible for good DNA integrity and sperm motility (Zhao *et al*., 2007, Takeo *et al*., 2014). A recent clinical trial using a resveratrol-based nutraceutical on sperm cells of patients with idiopathic infertility showed that the sperm concentration and motility of the patients increased probably due to the beneficial effects of RES on the metabolic and energetic mechanisms involved in spermatogenesis (Illiano *et al*., 2020). All these actions of resveratrol taking together might be responsible for the improved sperm count, motility, testosterone level reported in this study.

POM altered the oxidative status by increasing the MDA levels and reducing the antioxidant activities. Some studies have corroborated that acetylcholinesterase enzyme inhibition is significantly associated with an increase in ROS and a decrease in antioxidant capacity (Ranjibar *et al*., 2002, Prakasem *et al*., 2001, Cortés-Iza *et al*., 2018). In addition, several studies have shown that pesticides including POM can cross the blood-testis barrier to destroy the biological membranes of the testes to induce oxidative stress and lipid peroxidation (Kumar *et al*., 2008; Duzguner *et al*., 2010, Ngoula *et al*., 2014, Hussein *et al*., 2013 UriosteguiAcosta *et al*., 2020). The scavenger activity of resveratrol confers on it a protective role against sperm DNA damage caused by oxidative stress (Mongiol *et al*., 2021, Alamo *et al*., 2019, Guo and Cairns, 2019) suggesting its beneficial effect on pesticide-induced oxidative damage on sperm cells. Another study also showed that RES confers protection on frozen bull sperms which are highly sensitive to lipid peroxidation. The authors reported that RES decreases MDA and protected mitochondrial function thereby increasing their blastocyst percentage and blastocyst quality following IVF (Li *et al*., 2018). Results from this study showed that RES reduced MDA levels and increased antioxidant activities. Therefore, resveratrol treatment can enhance antioxidant capacity, and effectively improve any genital damage caused by POM induced oxidative stress.

Elevated levels of pesticide pollutants are associated with raised serum lipids which are a major risk factor for cardiovascular disease (Pothu *et al*., 2019, Goncharov *et al*., 2008). Our study showed increased cholesterol levels in POM administered rats. Cholesterol is the main precursor for steroidogenesis, and it is synthesized mostly in the liver (Johnson and Bridgham, 2001). Although cholesterol level was increased, the testosterone level was decreased in this group thus suggesting a suppressed process of steroidogenesis by POM. In another study using hepatocytes of Atlantic salmon fish, the study reported that POM is a potent toxicant affecting lipid and vitamin metabolism, as well as glutathione turn-over (Olsvick *et al*., 2017). RES is believed to reduce cholesterol levels by decreasing the activities of enzymes responsible for its production (Medes *et al*., 2016, Cheng *et al*., 2019). A recent meta-analysis showed that RES supplementation reduced total cholesterol among patients with metabolic syndrome and related disorders but did not affect triglycerides, LDL-and HDL-cholesterol concentrations (Akbari *et al*., 2020). This is in line with the results of our studies in which cholesterol levels were decreased by RES.

Histological observations from this study further buttress the effect of RES on POM-administered rats. Distortion in liver architecture was noticed in the POM and POM +RES groups. These changes were also reported by Nessiem *et al*., 2013 and Nosiri *et al*., 2017. The observed changes in the structure of the liver in exposure to POM may be due to the decrease in glycogen content of the liver tissues due to the significant inhibition of acetylcholinesterase (Nosiri *et al*., 2017). There were also histological changes in the testis of the POM and POM +RES treated groups. Spermatogenesis occurs in the testes thus this results in the testicular tissue damage buttresses the result of the reduced sperm parameters observed in the POM group. This is suggested to be because of POM blockage of the dihydrotestosterone-dependent androgen receptors reflecting the negative effect on sperm parameters (Nessiem *et al*., 2013). However, there is less testicular tissue

damage in the POM+RES groups when compared to the POM group. This may be a result of the ameliorative effect of resveratrol in improving sperm parameters.

In conclusion, POM negatively affects sperm parameters and has a degree of toxicity to the reproductive system and the liver. RES on the other hand demonstrated its protective role in male reproductive dysfunction caused by POM. Therefore, we suggest that RES has fertility-promoting effects on rats exposed to pesticides administration. This research also calls for further control on the use and exposure of farmers to organophosphates pesticides.

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