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Original Research Article

Rosmarinic Acid Restored Testicular Function and Steroidogenic Hormone Capacity in Wistar Rats Exposed to Perfluorooctanoic Acid Toxicity

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ABSTRACT

Perfluorooctanoic acid (PFOA) is an environmental toxicant which cause adverse health effects including male infertility and DNA damage in the germ line via altered oxidative stress activity. Rosmarinic acid (RA), one of the most important polyphenols, is a natural dietary nutraceutical with antioxidant property on adverse effects of toxicants. This study seeks to assess the restorative effect of RA on PFOA-induced toxicity in male Wistar rats. Twenty adult male rats (150-250g) were categorized into four groups (n =5). Animals in group 1 (control) took distilled water. Animals in categories 2 - 4 which were induced toxicity by PFOA (20 mg/kg body weight) were treated with distilled water, RA (40 mg/kg body weight) and Vitamin E (180 mg/kg body weight) respectively. Treatment was for 14 days. The epididymis was used for sperm analysis while blood sample was used for hormonal marker assay. Induction of PFOA substantively *(p<0.05)* lowered body weight, testes weight, sperm count, sperm motility, sperm morphology, testosterone, LH and FSH when compared with the distilled treated water treated control animals. Treatment of PFOA-induced toxic rats with RA significantly *(p<0.05)* restored epididymal sperm count, motility and morphology as well as body and testicular weight and those of serum hormone biomarkers in relation to Vitamin E. Results indicated that rosmarinic acid restored epididymal, testicular and hormonal PFOA-induced toxicity. This suggests that RA could be a potential therapeutic agent against PFOA- induced testicular damage.

***Keywords*:** *Rosmarinic acid,* Perfluorooctanoic acid, Male infertility, Oxidative stress, Sperm motility, Environmental toxicant.

Introduction

Male infertility is defined as the inability of a male partner to accomplish a clinical conception and/or pregnancy in a sexually matured female partner after a period of one year or more of frequent sexual activity without usage of protection. Male infertilty which could be of different types ranging from primary to secondary could be caused by a decrease in the number of spermatozoa (oligozoospermia), a decrease in sperm movement (asthenozoospermia), a decrease in sperm resilience (necrozoospermia), poor sperm structure (teratozoospermia), or a combination of two or more of these factors. An intrinsic testicular disease is responsible for the majority of cases of infertility.[1] Testes failure, maldescended testes, testicular tubule obstruction, reduced semen volume, sperm clumping, unknown infertility, scrotal constriction, erectile or ejaculatory impairment, abnormal viscosity, endocrine disorder, high sperm thickness, congenital deformity and ecological causes were identified as the most common causes and risk factors of male infertility.[2] The global predicted number of men with fertility issue range from 30,625,864 to 30,641,262.

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The highest percentage of men with fertility issue was reported in Europe.[3, 4] In Africa, the occurrence of male infertility is relatively high with a range of 20 - 35%.[5] In Nigeria, the burden of infertility with male factor being responsible is 40–50% of all infertility.[6]

Perfluorooctanoic acid (PFOA), a potent environment toxicant, is a member of the perfluoroalkyl acid (PFAA) family, which also contains perfluorooctane sulfonate (PFOS). PFOA and PFOS are sometimes known as C8 PFAAs or simply C8s because they have an 8-carbon backbone and a sulfonate (PFOS) or functional carboxylate (PFOA) group. PFOA damages male reproductive organs, causes endocrine disruption and changes testicular physiology. In animals, PFOA is a model that induces sustentacular cell dysfunction, apoptosis in the testis, and a disparity in testosterone and estradiol amount in the blood, inhibits testosterone production and produces oxidative tension in the testis, resulting in changes in semen quality, spermatogenesis, and reproductive potential.[7]

Oxidative stress which occurs by PFOA lead to the disparity in levels of reactive oxygen species (ROS) and enzymic and non-enzymatic antioxidants in the testes. Oxidative stress (OS) leads to lipid peroxidation and spermatozoa damage, thus, lowering the activity of testicular secretory biomarkers. In addition, the disparity in antioxidants and reactive oxygen species (ROS) caused by intrinsic and extrinsic variables attributed to diet, lifestyle, heredity and oxidative stress also contribute to male infertility. High levels of ROS result in lowered sperm content, decreased testicular parameters, heightened lipid peroxidation and elevated protein oxidation. Other notably causes of ROS include alteration in sex hormonal level, sperm DNA injury arising from mutation and compromised male sex chromosome.[8]

Rosmarinic acid (RA) lowers male infertility by reducing oxidative stress damage on reproductive organs. It is a non-artificial nutrient's antioxidant occurring that perform an important function in deactivating

injurious oxidants formed during usual intracellular exercise or disease conditions.[9] Rosmarinic acid is a polyphenolic substance containing esters of caffeic acid and 3, 4-dihydroxyphenyllactic acid. It was discovered in Rosmarinus officinalis for the first time.[10] Polyphenolic compounds have been seen to guard against a variety of illness and health hazard, including male infertility as well as acting as powerful antioxidants.[11] One of the most valuable polyphenols is rosmarinic acid. Rosmarinic acid can be found in medicinal places, herbs and spices. Rosmarinic acid is a red-orange powder with a high lipophilic and relatively hydrophilic molecular structure. It is a natural antioxidant that can be found in a variety of plants. Rosmarinic acid, a polyphenolic molecule, is an important scavenging free-radical that strengthens antioxidant defense activities on oxidative reactive oxygen species.[12] The effectiveness of rosmarinic acid as naturally occurring antioxidant in hampering and lowering lipid membrane alterations due to oxidative stress-induction. The use of rosmarinic acid as a natural feed supplement has been shown to have a variety of health benefits. Reports on impact of rosmarinic acid on toxic materials include protection against mobile phone and ultra-high frequency drift induced damage,[13] effect on testicular torsion/detorsion,[14] effect on rat liver, lung and kidney.[15] Protection against cyclophosphamide-induced gonadal toxicity.[16] Protective effect against doxorubicin-induced testicular damage.[17] Influence on male sex modulator and testes death.[18] Compromise on human sperm function subcellular Ca^{2+} level-related mode of action.[19] Action on metronidazole-induced fertility issue in male rat models.[20] Effect on sexual behaviour in in diabetic rats.[21] Effect on controlling inflammation, cell injury, and angiogenesis in rat ovary damage and detorsion models.[22] Effect on serum testosterone level after exposing with electromagnetic fields.[23] To the best of our knowledge, there has not been a study that assessed the adverse effect of synthetic fertility drugs (Vit. E) and environmental toxicants (PFOA) on male fertility. The study investigated the effects of rosmarinic acid on perfluorooctanoic acid-induced male infertility in Wistar rats.

Materials and Methods

Chemicals and reagents
Perfluorooctanoic acid, 97% purity, was procured from Ibra Hadad Nigeria and rosmarinic acid (96% purity) from Molychem, Mumbai, India. Other chemicals and reagents were of analytical grade.

Equipment
The instrument used are: Spectrophotometer (Model: Spectrumlab 752s UV visible), Incubator (Model No: DNP-9022A), Microscope, ELISA machine (Model: RT-2100C).

Preparation of PFOA and rosmarinic acid
PFOA was prepared in distilled water and administered orally to rats in the induced but not control group and in the induced and treated group. The chosen doses were based on Yuan *et al*.[24] Rosmarinic acid was dissolved in normal saline and administered orally to rats of treated group. The chosen doses were based on Zina *et al*.[25]

Experimental animals
Experimental animals were obtained from the Animal Facility Unit of the Faculty of Veterinary Medicine, University of Abuja (UniAbuja), Abuja, Nigeria. A total of 20 male Wistar rats weighing between 150 g and 250 g were randomly selected. There was 12-hrs light/12-hrs darkness. The rats were fed with water ad libitum and standard feed. The rats were allowed to familiarize with the environment for duration of 7 days before the rats were categorized into four (4) groups. The National Health Research Council's recommendations for the care and use of laboratory animals were followed. For the use of animals, an ethical approval with the number (UAECAU/2024/007) was granted by the Institutional Animal Care and Ethics (ACE) Committee of UniAbuja.

Induction of experimental animal
Toxicity was induced in the male Wistar rats by oral administration of PFOA (20 mg/kg body weight), according to Yuan *et al*.[24] Rosmarinic acid was liquefied in normal saline (0.9% NaCl) and given to animals by oral gavage (40 mg/kg body weight), according to Zina *et al*.[25]

Animal grouping and treatment
Group 1: Control group (Distilled water [40 mg/kg body weight])
Group 2: Induced by PFOA (20 mg/kg b.w) but not treated.
Group 3: Toxicity induced with PFOA (20 mg/kg b.w) + treatment with Rosmarinic acid (40 mg/kg b.w)
Group 4: Toxicity induced with PFOA (20 mg/kg b.w) + treatment with Vitamin E (180 mg/kg b.w)
Treatment of animals was once daily for 14 days.

Animal sacrifice and collection of blood
At the end of 14 days of induction and treatment, the rats were sacrificed by cervical dislocation and blood samples were taken into plain tubes. After collecting blood samples, testicular tissues of rats and epididymis were immediately dissected and collected for analysis. Testicular tissues and epididymis were divided into two equal parts. One part was homogenized using a homogenizer and the samples were used for biochemical analysis.

Sperm analysis
Principle
The sperm viscosity and translucence of the sperm solution were used to perform the analysis. Gross (volume, color, turbidity, viscosity, and pH 7.2-7.4) and microscopic (motility and spermatozoa count) examinations were performed on sperm.[26]

Assay procedure
The Spermatozoa squeezed from caudal epididymis on a microscope slide had normal saline added to it (about 2 drops), then with cover-slip concealed. Under microscope with an x40 objective and low light, the slide was viewed and scored. Immediately after the sperm sample was taken, it was analyzed for sperm motility. Eosin staining was used to study sperm motility. Microscope slide with Epididymis sperm squeezed onto it also had two drops of stain applied on it. Motile (live) sperms will not absorb the stain while non-motile (dead) sperms absorbed the stain. Using x40 microscope objectives, both unstained and stained number of sperm cells were counted and mean (average) was recorded for every slide out of which determination of % viability was achieved.
With the help of the improved Neubauer hemocytometer, sperm count was done under a microscope. The testis was carefully detached from the caudal epididymis, which was then homogenized to obtain suspension using normal saline of 2 ml. Suspension of Sodium bicarbonate-formalin was diluted in a 1:20 ratio. The sperm was counted in 2 square mm of the improved Neubauer hemocytometer chamber, which was then filled with sperm that is well diluted. By multiplying the number of sperms counted by 1000,000, sperm counts in 1 ml of fluid were calculated.
Two drops of Wall and Eosine stain was used to stain the air-dried sperm smears on microscope slides in order to assess its morphology. Under a microscope (oil immersed) X100, the slides were examined.[26]

Determination of follicle stimulating hormones (FSH) concentration
Principle
A competitive enzyme immunoassay method was used in this assay. Pre-coated on microtiter plate, the kit was goat anti-rabbit antibody.[27]

Assay procedure
All reagents were kept at room temperature for at least an hour before being used in the assay, and they were gently mixed before use. The holder was filled with the required number of coated strips.
To selected wells, 50 µl of FSH standards, control, and serum sample were introduced. All wells were filled with 100 µL of enzyme conjugates.
The plate was covered and incubated (20 – 25^{0}C) for one hour at room temperature.

All of the wells had liquid removed. 300 µl of 1X wash buffer was washed three times. Paper towels were used to blot the blood.
To all wells, TMB substrate was added in the amount of 100 µl.
At room temperature, incubated for 15 minutes.
All wells were filled 50 l of stop solution. The plate was gently shaken. Within 15 minutes of administering the stopping solution, using an ELISA Reader the absorbance was measured at 450 nm.

Determination of testosterone concentration
Principle
The competitive inhibition enzyme immunoassay method was used in this assay. Pre-coated on micro-titer plate in the kit was goat anti-rabbit antibody.[28]

Assay procedure
Before commencement the test, all reagents were warmed to room temperature (20 - 25^{0}C). The standards, control, or specimen were pipetted 25 µl into the designated well. All wells were filled with 100 µl of functioning Testosterone-enzyme conjugate reagent. To evenly distribute the chemicals, the micro-plate was gently swirled for 20-30 seconds. The plate was covered and incubated at room temperature for about one hour. All wells were drained of liquid, which was then washed three times with 300 µl of 1X wash buffer and wiped with absorbent paper towels. TMB substrate reagent (100 µl) was added to each well. For fifteen (15) minutes, the plate was covered and kept at room temperature. Each well was filled with 50 µl of stop solution and carefully stirred for 15-20 seconds. The absorbance was measured at 450nm using an ELISA Reader, 15 minutes after administration of the stop solution.

Determination of luteinizing hormone (LH) concentration
Principle
Competitive inhibition enzyme immunoassay technique is being used by this assay. Pre-coated on micro-titer plate in the kit is goat anti-rabbit antibody.[29]

Assay procedure
Before commencement of the assay, all reagents were kept at room temperature. The reagents before use were gently mixed. Required number of coated strips were placed into the holder 25 µL of LH control, standards and serum sample were pipetted. Conjugate Reagent (100 µL) was added to all wells. Plate mixed by placing on a plate shaker for 30 seconds at 600 rpm, and then incubated for 60 minutes at room temperature (20-25^{0}C). After incubation, the liquid was removed from all the wells. 1X wash buffer (300 µL) was used to wash all the wells, then blotted on paper towels. To all the wells, 100 µL of TMB substrate was added, incubated for 15 minutes at room temperature. After incubation, to all the wells, 50 µL of stop solution was added. To blend solution the plate was shake gently. After adding the stopping solution, the absorbance was read at 450 nm within 15 minutes using an ELISA Reader.

Statistical analysis
The one-way (ANOVA) and Duncan multiple range test were used to examine data from the experiments statistically (n=5). SPSS version 16 determined that differences at $p<0.05$ were significant. Graph Pad Prism 5 software was also used to present findings.

Results and Discussion

The effect of perfluorooctanoic acid and rosmarinic acid on the body and testes weight of the rat after 14 days
The rats showed significant *(p<0.05)* increase in body wright after 14 days of PFOA administration. Rosmarinic acid and vitamin E treatment led to a substantive ($p>0.05$) drop in body weight after 14 days. Mean testes weight of the left and right testis in all treated groups were significantly *(p<0.05)* lowered in comparison with the distilled water treated control rats while the administration of rosmarinic acid elevated it *(p<0.05)* (Table 1).

Table 1: Effect of rosmarinic acid on body weight and testes in PFOA-induced toxicity in rats

Body weight (g)			Testes weight (g)	
Group	**Initial Weight**	**Weight after 14 days**	**Left**	**Right**
NR + DW	245.8±5.9^a	178.6±5.2^a	1.252±0.15^a	0.928±0.20^a
PFOA + DW	168.7±3.6^b	251.5±3.3^b	0.802±0.09^b	0.765±0.08^b
PFOA + RA	196.0±3.8^c	198.0±6.2^c	0.856±0.08^c	0.824±0.06^c
PFOA + Vit. E	210.8±3.1^d	182.8±2.8^d	0.896±0.100^d	0.846±0.11^d

Mean ± SEM (n=5). Figures having superscripts varying from the reference value for each parameter down the column are essentially dissimilar *(p<0.05)*
NR = Normal rats; DW = Distilled water; PFOA = Perfluorooctanoic acid; Vit. E = Vitamin E
RA = Rosmarinic acid

Effect of rosmarinic acid on epididymal sperm count, sperm morphology and sperm motility in PFOA-induced toxic rat
The PFOA-induced reproductive toxic animals showed substantive *(p<0.05)* drop in sperm count when matched with the control categories of rats. However, treatment of the PFOA-induced reproductive toxic rats with RA revealed *(p<0.05)* increased sperm count. (Figure 1).
The administration of PFOA in rats caused a significantly *(p<0.05)* high amount of abnormally shaped sperm cells when liken with control animal category (Figure 2). Treatment of RA to PFOA-induced toxic rats resulted in notably reduced *(p<0.05)* number of abnormally shaped sperm cells which was comparative with the animals treated with vitamin E (Figure 2). The morphology of the normal shaped sperm had an opposite pattern to those of the abnormally shaped one (Figure 2).
PFOA resulted in significantly high *(p<0.05)* number of non-motile sperm cells when juxtaposed with those of the control category. Treatment of the PFOA toxicity induced rats with RA resulted in

reduction *(p<0.05)* in the number of motile sperm cells which was comparable as observed with animals treated with vitamin E (Figure 3).
The number of non-motile sperm cells had an opposite pattern when liken with those of the motile ones (Figure 3).

Effect of PFOA and rosmarinic acid on serum concentration of testosterone, follicle stimulating hormone, luteinizing hormone
There was a statistically significant *(p<0.05)* reduction in the level of serum testosterone and LH in rats treated with PFOA induction when compared with the control animals. Treatment of PFOA-induced toxic rats with 40 mg/kg body weight of RA and Vit. E significantly *(p<0.05)* restored the concentration of serum testosterone and LH. Administration of RA did not significantly *(p>0.05)* affect the level of FSH in the serum of the animals when compared with the PFOA-induced toxic animals (Figure 4).
In vivo investigation of the impact of exposure to PFOA on biomarkers like sperm count, sperm morphology, sperm motility, steroidogenic hormones as well as body and testes weight can give vital clue on the reproductive performance of animal models. Exposure to environmental toxic agent is one of the main causes for male fertility challenge. As a stable substance largely located in the environment, PFOA has been reported to induce male sexual dysfunctionality in

mammal and animal models.[30] Discovering new nutraceuticals that can ameliorate the negative effects of toxicants like PFOA on male reproductive system is essential. The presence of redox properties of phenolic compounds in rosmarinic acid is responsible for its antioxidative and free radical removing property especially caffeic acid and ferulic acid which are metabolites of RA, can both positively modulate male fertility.[31]

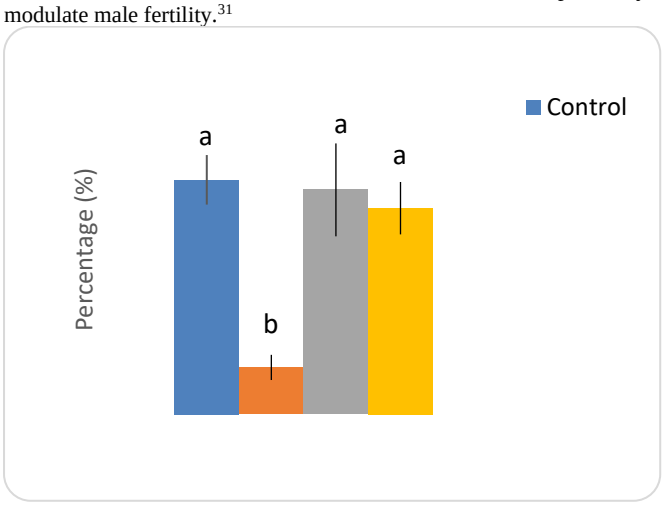

Figure 1: Effect of rosmarinic acid on sperm count in PFOA-induced toxicity in rats.
Results are expressed as Mean ± SEM (n=5)

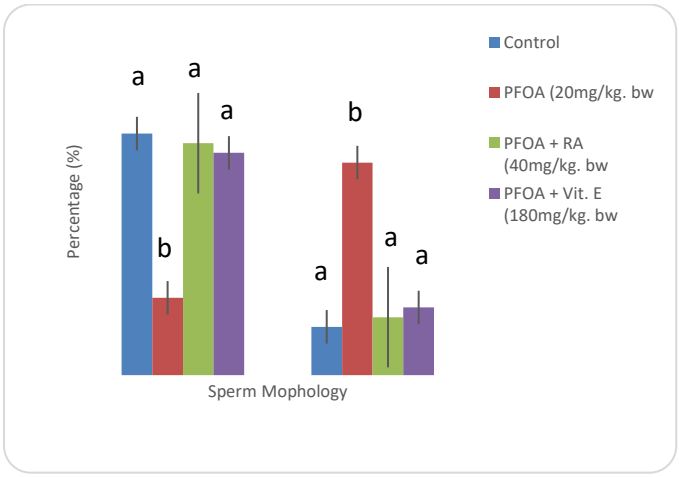

Figure 2: Effect of rosmarinic acid on sperm morphology in PFOA-induced toxicity in rat.
Results are expressed in Mean ± SEM (n=5)

Body weight is considered as the mass or quantity of heaviness of an organism. The rise in body weight observed after 14 days of PFOA administration in the present study may present with heightened leptin concentration which reduces sperm count, sperm motility and sperm biosynthesis (spermatogenesis) as well as high level of sperm DNA fragmentation. The lowered weight by noticed after 14 days of treating PFOA-toxic rats with rosmarinic acid is indicative of restoration in the altered motility, synthesis and DNA fragmentation of sperm cell.[32]
Testes weight is a biomarker for ascertaining swelling, atrophy or hypertrophy. The reduction in weight of testes by PFOA may be tied to testicular distortion which manifests as testes degeneration and damage of component testicular cells. The rise in testes weight by

rosmarinic acid in the present study could be related to hypertrophy which presents as its androgenic and synthetic action by its elevated testicular secretory activity as well as increased size of the seminiferous tubules, which is made up of closely 85% of the testicular space.[33]

Sperm count is the number of active sperm produced in the body of a man. Sperm are male reproductive cells present in the ejaculate**.** The substantive decrease in the sperm count in the PFOA treated animals may be adduced to alteration in epididymal-sperm producing organ by PFOA toxicity. The increase in sperm count by rosmarinic acid is indicative of its restorative and enhanced testicle capacity, as well as the improved fluid productive action of seminal vesicle and prostate gland which combines with sperm to form semen.[34]
Sperm morphology is the size, shape, appearance of individual sperm when view under the microscope and a contributing biomarker to male fertility. The high amount of abnormally shaped sperm cells following PFOA administration may result in infertility as higher numbers of abnormally shaped sperm are associated with other irregularities of the semen such as low sperm count or motility. The reduction in number of abnormally shaped sperm cells in the PFOA treated group treated with rosmarinic acid may indicate amelioration and an increase in its ability to fertilize an ovum successfully.[35]
Sperm motility is the capacity of sperm to swim effectively through the female reproductive part to bring about fertilization of an ovum. The lowered sperm motility observed in this study following PFOA administration can be a cause of male factor infertility which presents with many factors including abnormal sperm synthesis, obstruction of normal sperm delivery or reduced sperm generation. The increase in sperm motility by RA is an indication that RA may have acted by enhanced sperm synthesis/steroidogenesis and delivery to desired female reproductive package.[36]
Testosterone formation in the interstitial cells of the testicles is chiefly regulated by the hypothalamic-pituitary-testicular region of the brain. The male sex hormone, testosterone enhances sexual differentiation in men, androgen formation at puberty and general male fertility. Lowered testosterone level facilitates the release of gonadotropin-releasing hormone by hypothalamic brain region, which then acts on the pituitary gland to release LH and FSH and conversely. A compound capable of sustaining or facilitating the hypothalamic-pituitary gonadal loop and/or the negative feedback effect on gonadotropins and testosterone can exhibit a robust action on the androgen-based productive and releasing function of the testes, including spermatozoa production and male sex fecundity. Consequently, the lowered serum testosterone level by PFOA in the present investigation may indicate inadequate secretion of gonadotropin-releasing hormone by hypothalamus which may hamper male sexual segregation, pubertal androgen synthesis and fecundity. The increase in testosterone level by RA may be adduced to improved function activity of the interstitial cells of the testes which would facilitate sperm production.[37]
Luteinizing hormone (LH), a glycogen-protein rich hormone, is released together with follicle-stimulating hormone by the gonadotrophin cells in the adenohypophysis (anterior pituitary). LH is essential for male normal sex action and triggers the interstitial cells of the testes to generate testosterone. Lowered level of LH in the present by PFOA administration may be adduced to interference with Interstitial function resulting in male sexual dysfunction and infertility. The increased serum LH concentration by following administration of RA in this study may be indicative of enhanced functional action of the interstitial cells of the testes to biosynthesize testosterone and enhance normal male fertility.[38]
FSH signals the testes thereby stimulating sperm production. FSH stimulates the Sustentacular cells to extrude inhibin which stimulates the formation of Sustentacular- Sustentacular compacted regions (zonula occludens) and thus supporting the semen-generating cells. The decrease in the level of serum testosterone and FSH may be adduced to the toxic effect of PFOA on the spermatogenic function of testes which may impair fertility. The non-significant effect in FSH level by RA administration imply maintenance of the synthetic function of the testes.[39]

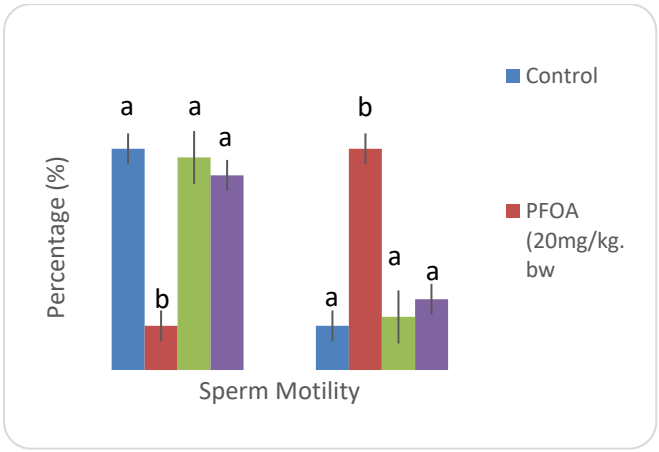

Figure 3: Effect of rosmarinic acid treatment on sperm motility in PFOA-induced toxicity in rat.
Results are expressed as Mean ±SEM (n=5)

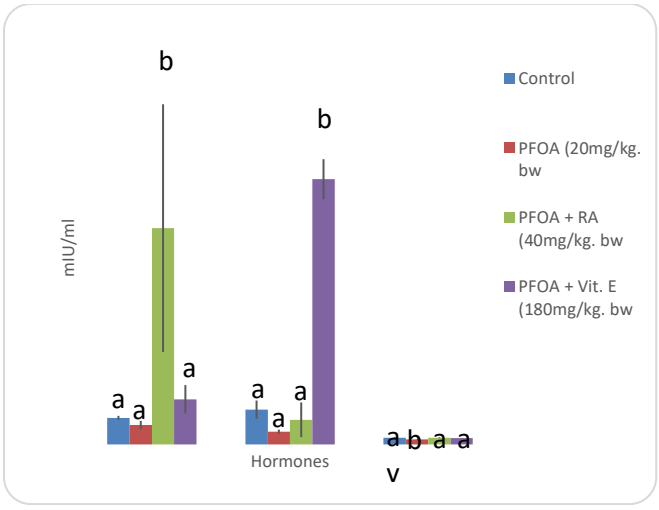

Figure 4: Effect of rosmarinic acid on testosterone, follicle stimulating hormone and luteinizing hormone concentrations in PFOA-induced toxicity rats.
Results are expressed as Mean ± SEM (n=5). Bars are significantly different from each other at *P<0.05*).

Conclusion

The investigation indicated that rosmarinic acid offered protection for PFOA-induced testicular toxicity and damage via amelioration of oxidative stress and modulation of spermatogenesis.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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