Research Article

Chlorpyrifos Impairs the Male Reproductive Functions through Inhibition of Sperm Count and Motility in Descended Testis Rats

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Received: 14-06-2024; **Revised:** 26-09-2024; **Accepted:** 05-10-2024; **Published on:** 15-10-2024.

ABSTRACT

Chlorpyrifos, a widely used organophosphate pesticide, is known for its adverse environmental and health impacts, including potential reproductive toxicity. This study examines the impact of chlorpyrifos exposure on testicular function in descended testes rats. Two graded doses (5.4 mg/k.g.bw/d and 8.1 mg /k.g.bw/d) of CPF were orally administered to descended (DT) male charles foster rats for 30 days to examine toxic effect in body weight, testis weight, histopathology of testis, biochemical alterations, sperm dynamics and reproductive hormone levels (LH, FSH and testosterone). A significant reduction of body weight and testicular weight were observed dose dependently in CPF exposed groups of rats. Sperm count, viability, reproductive hormone levels (LH, FSH and testosterone) were significantly reduced in CPF treated groups of rats compared to control rats. A significant reduction of sperm motility was also observed in CPF exposed groups. Histopathological observations showed mild to severe degenerative changes in the seminiferous tubule in the all-tests groups. Moreover, a significant reduction of sialic acid and testicular glycogen were observed, whereas the cholesterol and protein levels were increased at significant levels. These findings suggest that chlorpyrifos exposure significantly impairs testicular functions by decreasing the sperm count and reducing the motility and viability of sperms in descended testis rat.

Keywords: Chlorpyrifos, Male reproductive toxicity, Sperm count, Sperm motility, Descended testis.

INTRODUCTION

India is an agriculture-based country in south east Asia. In modern agriculture, agrochemicals like pesticide plays an important role in food grain production. In India the use of organochlorine pesticide was restricted in 1970. After that organophosphorous pesticide such as chlorpyrifos (CPF, [O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate]) have been widely used more than 50 years in agricultural field to provide sufficient food for 1.4 billion people of India. By interfering with the synaptic neural transmission of target insects, CPF, an acetylcholinesterase (AChE) inhibitor, effectively controls pests in several kinds of crop systems to reduce pest induced crop loss. CPF induce neurotoxic effect are not just confined to pests; non-target organism, including as humans and animals, are also at high risk.

Chlorpyrifos can easily enter the human body through food intake, inhalation and skin penetration.[1] CPF has been detected considerable amount in fruits, vegetables, grains, dairy products, meat, fishes, soft drinks and also detected in cervical fluid, cord blood, breast milk and meconium.[2.] Within the human body, CPF is converted into chlorpyrifos oxon (CPO) in liver by cytochrome P^{450} mediated desulfuration and subsequently hydrolyzed to diethyl phosphate and 3, 5,6-trichloro-2-pyridinol (TCPY).[3] It has been reported that CPF induces oxidative stress, inhibits the activity of AChE, promotes neurotoxicity and degeneration of dopaminergic and cholinergic neurons.[4] CPF also exerts reproductive and developmental neurotoxicity in animals due to its endocrine disrupting function.[5] It has also been reported that CPF causes disruption of the of breast cell cycle through production of reactive oxygen species (oxidative stress).[6]

In male reproductive system testis is the main organ where sperm is produced by the process of spermatogenesis under the regulation of L.H, F.S.H (Pituitary) and GnRH from hypothalamus. Testis is particularly vulnerable to CPF which can interfere with sperm production, motility, hormonal balance, and overall fertility.

CPF is a known for endocrine disrupter substance, may interfere with the hypothalamic-pituitary- testicular (HPT) axis, which regulates male reproductive function. Any disruption of the HPT axis can lead to reduced testosterone levels, spermatogenesis alterations, and impaired sperm parameters such as count, motility, and viability. Healthy and motile sperm is extremely essential to fertilize an ovum that determinants of male fertility. Although several studies have documented CPF's toxic effects on general health, relatively few have thoroughly examined its direct impact on male reproductive parameters in animal models, particularly those with descended testes—where the exposure effects can be more analogous to certain human reproductive conditions.

Therefore, the objective of the present study was to examine the effects of CPF on the male reproductive functions of descended testis in rat model.

MATERIALS AND METHODS

Chemicals and reagents

All the reagents used this study were of analytical grade. Chlorpyrifos (CPF) was purchased from Devidayal (sales) Limited, Mumbai, India.

Animals

Healthy adult male Charles foster rats aged 18-20 weeks and weighing 140-170 gm were used in the study. Animals were housed in polypropylene coated plastic cages containing paddy husk bedding and were maintain at an average room temperature of 25 ± 2° C with 12:12 h light and dark cycle as per recommendation of the animal ethical committee of University of Kalyani in accordance with the national guidelines.

Experimental design

After 10 days of acclimatization, the rats were distributed to three groups. The rats of the first group (Group I) were received standard laboratory diet and were designated as control group. The rats of the second and third groups were designated as Treated I and Treated II. Treated I and Treated II received 5.4mg/BW/d and 8.1 mg/BW/d chlorpyrifos for 30 days duration. After completion of treatment, rats were sacrificed by cervical dislocation and blood, testis were collected for the determination of male reproductive hormone assay, organ weight, histopathology, sperm count, motility, viability.

Table 1: Experimental Setup for the study

Groups		Rats	Dose (mg/Kg.B.Wt.)
Control	DES	**6**	Received distilled water with food for 30 days (Control group).
Treated I	DES	**6**	Received 5.4 mg CPF/Kg.B.Wt./day (i.e., 20% LD_{50}) for 30 days (T1)
Treated II	DES	**6**	Received 8.1 mg CPF /Kg.B.Wt./day (i.e., 30% LD_{50}) for 30 days (T2)

DES: Descended rats

Body weight and absolute organ weight

The body weight was calculated on the basis of the weight taken on the day of the application of first dose and it was considered as the initial body weight. The body weight taken on the 31st day was considered as the final weight. Testis was dissected out, free from adherent tissue and weighed to get absolute organ weight. Organ weight was expressed per 100gm of body weight.

Histopathological study

Bouin's fixed and paraffin impregnated testicular tissue sections were stained with normal hematoxylin-eosin stain according to the method of Bancroft et al., 2003[7] with slight modifications. Each paraffin section about 5µm thickness was kept sequentially in xylene and graded ethanol, and stained with hematoxylin for 2 minutes. After removing the excess color, the slides were counterstained with eosin and then the stained slides were dehydrated with graded ethanol, cleared with xylene and mounted with DPX. Stained sections were observed under compound microscope (Model No-Olympus OM 1) at 400X magnification. Images were obtained by SONY DSC W320 camera.

Biochemical assay

After removing the adherent tissue of the testis, the wet weight of descended testis was recorded and frozen for measurement of glycogen (Montgommery et al., 1957), cholesterol (Zlatkis et al., 1953), protein (Lowry et al., 1951) and sialic acid (Warren et al., 1959). [8-11]

Epididymal sperm count and motility

Epididymal sperm count, sperm motility and sperm viability of descended testis were calculated by modified method of Linder et al, 1986[12] Sperm count is the numbers of sperm cell/ml of semen. Total sperm count: Sperm count × Semen volume. Epididymal sperm count was measured by using hemocytometer and WBC pipette. First cover slip is placed on the top of the counting chamber (hemocytometer). Then draw liquified semen sample up to 0.5 mark of the W.B.C pipette and then again draw semen diluting solution (SDS) up to mark 11 of the W.B.C pipette. Gently wipe out the excess fluid and rotate the pipette for 1 min with palm. Discard first few drops from the pipette and then load the sperm solution into the counting chambers. Placed the loaded chamber under microscope for observation under 40 X objective.

Calculation of Sperm count: $\text{Sperm cells/ml} = \frac{\text{No of Sperm cell} \times 20}{0.02} \times 1000$

Or Sperm cells/ ml = Sperm count × 100,000

Total sperm count = Sperm count/ml × Volume (ml) of semen sample.

Sperm viability test is performed by eosin- nigrosine dye exclusion staining technique. First, few drops of freshly collected semen (within 60 mins) was placed in the slide and add 5 drops of eosin- nigrosine stains (or 2 drops of 1% eosin and 3 drops of 10 % nigrosine stain). Mixed the stain with semen sample for 10- 20 sec. Then prepare a smear into another slide and allow to dry. Observed under microscope with 40 X or 100 X magnification. Dead sperm cells show pink stains at the head region of the sperm and live sperm remain unstained with eosin-nigrosine stain. Percentage of the viability of the sperm was measured by counting 100 random sperm cells. Sperm motility was measured by normal counting the movement of sperm cells under microscope. Few drops of liquified semen sample

were taken in slide and covered with a coverslip and observed under microscope and counted by observing 100 or 200 random sperm cells.

Hormonal study

Blood was collected from different groups of descended and cryptorchid rats and levels of serum testosterone and luteinizing hormone (LH) were measured by enzyme linked immunosorbent assay (ELISA) method using kits of ERBA Diagnostic GmbH, Mannheim, Germany.

Statistical analysis

The data were expressed in terms of mean ± SEM. The data were analyzed by Student's 't' test, one way and two-way analysis of variance (ANOVA) and software 'R' wherever applicable. [a]P≤0.05, [b]p≤0.01, [c]p≤0.001 were considered significant. The number of each experiment is indicated by the alphabet 'n' in the results.

RESULTS AND DISCUSSION

Effect of CPF on body weight and organ weight:

The mean body weight of chlorpyrifos exposed groups of descended testes rats were decreased significantly in a dose dependent manner compared to control groups of rats. The mean testis weight of chlorpyrifos exposed groups of descended testes rats were also decreased significantly in a dose dependent manner compared to control groups of rats. **(Figure 1: A; B).** Moreover, a marked depression in food and water intake was observed in rats treated with chlorpyrifos. The results suggest that chlorpyrifos may reduce both body and testis weights, likely by altering the hypothalamic set-point controls for food and water intake towards a state of satiation. This effect may partly result from enhanced synaptic transmission within neural circuits, due to chlorpyrifos-induced irreversible inhibition of acetylcholinesterase (AChE)-an enzyme that terminate synaptic transmission by cleaving acetylcholine to acetate and choline at the post synaptic neuron of hypothalamus.

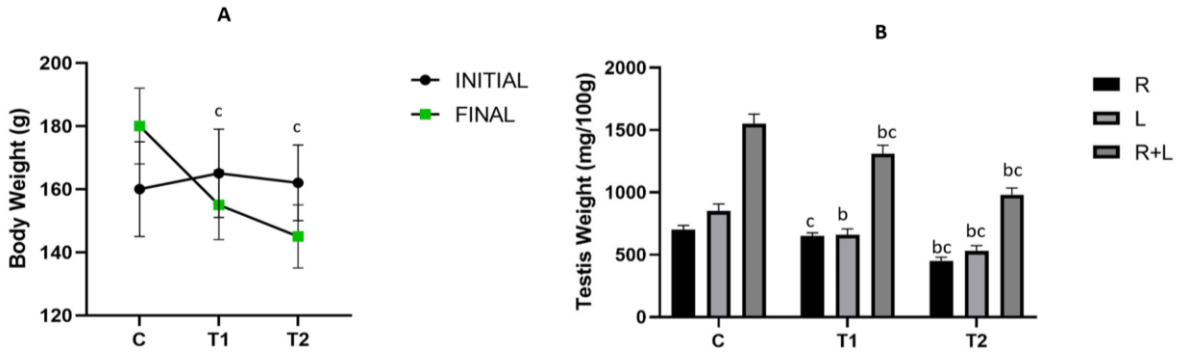

Figure 1: Bardiagrams showing the effects of CPF on the body weight (A) and testicular weight changes (B) in control and treated groups of descended rats compare to control groups of rats. C: Control, T1: Treated 1(CPF applied 5.4mg/day/kg.BW for 30 days), T2: Treated2 (CPF applied 8.1mg/day/kg.BW for 30 days). Values are represented as Mean±SEM. [a] p<0.05, [b] p<0.01, [c] p<0.001 *vs.* control groups of rats. R-Right testis; L- Left testis

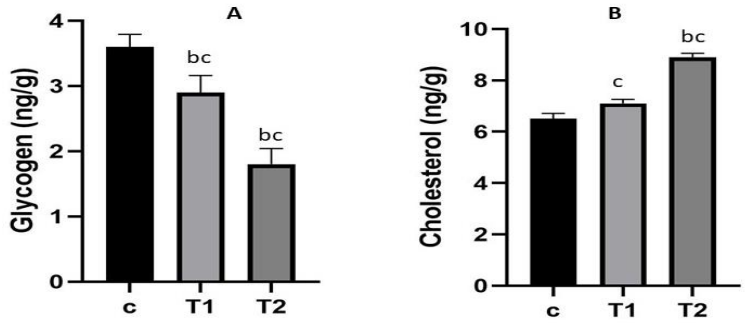

Figure 2: Bardiagrams showing the changes in biochemical contents (A. Glycogen; B. Cholesterol) due to exposure of CPF of descended testes of male charles foster rats. Values are Mean± SEM. [a] P<0.01; [b] P<0.001; [c] P<0.05 Vs corresponding control groups of rats. C: Control, T1: Treated 1(CPF applied 5.4mg/day/kg.BW for 30 days), T2: Treated2 (CPF applied 8.1mg/day/kg.BW for 30 days).

Biochemical content:

Effect of CPF on glycogen and cholesterol contents:

CPF induce biochemical alteration in the testis. The levels of testicular glycogen of descended testis were decreased significantly of treated groups of rats than the respective control groups **(Figure 2: A)** whereas testicular cholesterol level significantly increase compared to control groups of rats **(Figure 2: B)**. CPF may interfere with the enzyme involved in glycogen metabolism. Increased levels of cholesterol indicate decreased androgen level of CPF exposed groups of rats. Decreased androgen level may impair the process of spermatogenesis.[14]

Effect of CPF on Protein and sialic acid:

The levels of testicular proteins of descended testis were decreased significantly of treated groups of rats than the respective control groups **(Figure 3: A)** whereas testicular sialic acid level significantly increase compared to control groups of rats **(Figure 3: B)**. Increased protein content in the CPF exposed group of rats may be due to stimulation of the growth proteins and ribonucleic acid (RNA) synthesis. Reduced level of sialic acid content in the CPF exposed group of rat indicates the suppression of LH, FSH and testosterone. Reduced level of Sialic acid content in the CPF treated rats might be due to loss of viability and fertilizing ability of spermatozoa.[13]

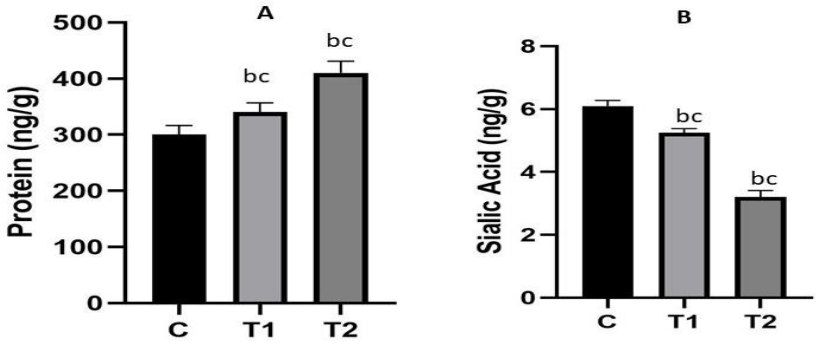

Figure 3: Bardiagrams showing the changes in biochemical contents (A. Protein; B. Sialic Acid) due to exposure of CPF of descended testes of male charles foster rats. Values are Mean± SEM. [a] P<0.01; [b] P<0.001; [c] P<0.05 Vs corresponding control groups of rats. C: Control, T1: Treated 1(CPF applied 5.4mg/day/kg.BW for 30 days), T2: Treated2 (CPF applied 8.1mg/day/kg.BW for 30 days).

Effect of CPF on Sperm count, motility, TSI and Sperm Viability:

The cholinergic sign (lacrimation and tremor) of descended exposed to CPF are due to inhibition of AChE leading to accumulation of ACh in cholinergic receptors.[15]Testis, the main male reproductive organ produces sperm by the process of spermatogenesis and testosterone, a hormone that plays a key role in male sexual development. Healthy motile sperm is extremely essential to fertilize an ovum. Suppression of any stage of the complex process of spermatogenesis in the seminiferous tubule of the testis may lead to male infertility.[16] In this study, the semen quality of male rats exposed to chlorpyrifos (CPF) was examined as a holistic indicator of the toxic effects of CPF on male reproductive system function, involving the key roles of the hypothalamus, pituitary, and testis. In this study, a significant reduction in sperm count, sperm motility, and sperm viability was observed in the descended rats of the CPF-exposed group compared to the control groups of rats.

The exposure of chlorpyrifos on descended testis rats caused a significant reduction in sperm count, sperm motility and sperm viability and tissue somatic index (TSI) compared to the control groups of rats. **(Figure 4: A; B; C; D).** The low concentration of sperm count in the CPF exposed group may be resulted from reduced testosterone concentration due to low GnRH-FSH-LH output. Similarly lower FSH concentration in the CPF treated rat resulted lower sperm count since this hormone plays an important role in the process of sperm formation.[17] The lower sperm count in the CPF exposed group of rats may also be due to oxidative stress in the testis. Generation of excessive reactive oxygen species (ROS) in the seminiferous tubule by CPF may provide unconducive environment which suppresses the process of spermatogenesis.[18-19] The plasma membrane of the spermatozoa is made up of poly unsaturated fatty acid (PUFA). Reactive oxygen species (ROS), formed due to exposure of CPF in the seminiferous tubule, attack the plasma membrane of the spermatozoa which are rich in polyunsaturated fatty acid. Spermatozoa prevent the CPF induced oxidative stress by cellular antioxidant defense mechanism such as SOD, catalase and ascorbic acid. CPF induce oxidative stress in the testicular tissue by lowering the scavenging enzymes and glutathione. Thus, excessive generation of free radical in testicular tissue by CPF, resulting lower concentration of scavenging enzyme and deficient DNA damage repairmen, may be responsible for reduction of sperm count.[20] Suppression of the gonadal function and reduction of sperm count may be due to germ cell apoptosis induced by free radical (ROS).[21]The ability of sperm to fertilize an ovum is depends on sperm motility and any negative impact on sperm motility may impairs the process of fertilization.[22]

In the present study we found significant inhibition of the sperm motility of the descended rat of CPF exposed groups compared to control. Low ATP content in the spermatozoa may be responsible for the significant inhibition of the sperm motility.[23]CPF may alter the enzymatic activities of the oxidative phosphorylation, resulting low adenosine tri phosphate (ATP) content in the spermatozoa. [24-25] ATP is required for forward sperm movement and low ATP level in spermatozoa may be responsible for reduction of sperm motility which hampers the process of fertilization leading to infertility.[24]

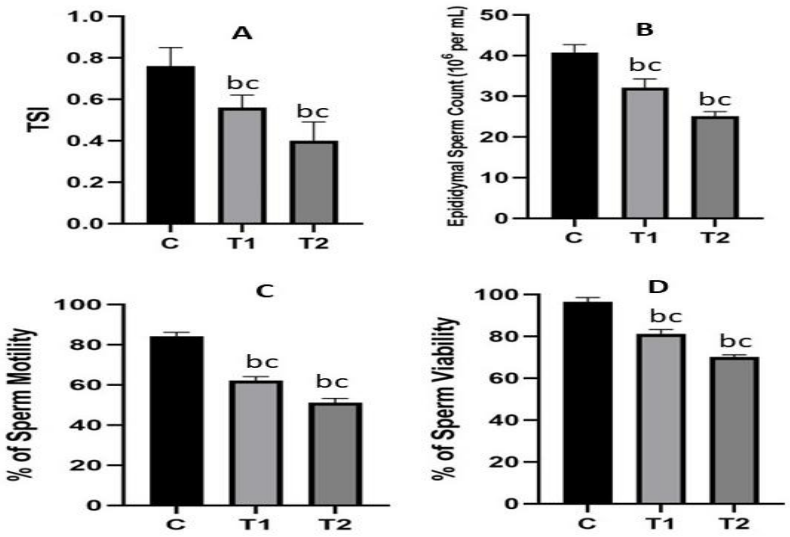

Figure 4: Bardiagrams showing the changes in TSI (A. Tissue Somatic Index); B. Epididymal Sperm Count; C-% of sperm motility; D- % of sperm viability of descended testes of male charles foster rats. Values are Mean± SEM. [a] P<0.01; [b] P<0.001; [c] P<0.05 Vs corresponding control groups of rats. C: Control, T1: Treated 1(CPF applied 5.4mg/day/kg.BW for 30 days), T2: Treated 2 (CPF applied 8.1mg/day/kg.BW for 30 days)

Effects of CPF on the histopathology of testis

In the present study, mild to severe degenerative changes and atrophy of the seminiferous tubule was observed in chlorpyrifos treated groups of rats in a dose-dependent manner (5.4 and 8.1 mg./Kg.b.wt./day) for the periods of the exposure (30 days) of chlorpyrifos compared to control groups of rats. Degeneration and atrophy of the spermatogonia, spermatids, sperms, sertoli cells and leydig cells were also observed in CPF exposure groups of rats compared to control groups of rats. Some of the lumen of the seminiferous tubule devoid of sperm and filled with debris. **(Table 2)**. CPF induce neurotoxic effects in the testis by inhibiting AChE, altering neuronal control of reproductive functions. Besides, disrupted neuronal signaling in the testis may impair the testicular blood flow and interfere with spermatogenesis affecting spermatogenic cells like spermatogonia, spermatids, sperms, sertoli cells and leydig cells. Moreover, in the seminiferous tubule, CPF exposure generate ROS and increase oxidative stress induced degeneration of various germ cells and supportive cells.

Table 2: Showing effects of CPF on testis in descended rats

Groups	Dose (mg/Kg.B.Wt.)		Spermatogonia	Spermatids	Sperm	Sertoli cells	Leydig cells
Group I	0	**N/DES**	+	+	++	++	+
Group II	5.4 CPF	**N/DES**	+	-/+	-	-/+	-
Group III	8.1 CPF	**N/DES**	-	-	- - -	- -	-

(-) = Absence; + = few; ++ = Moderate; +++ = Highly; N/DES: Normal descended

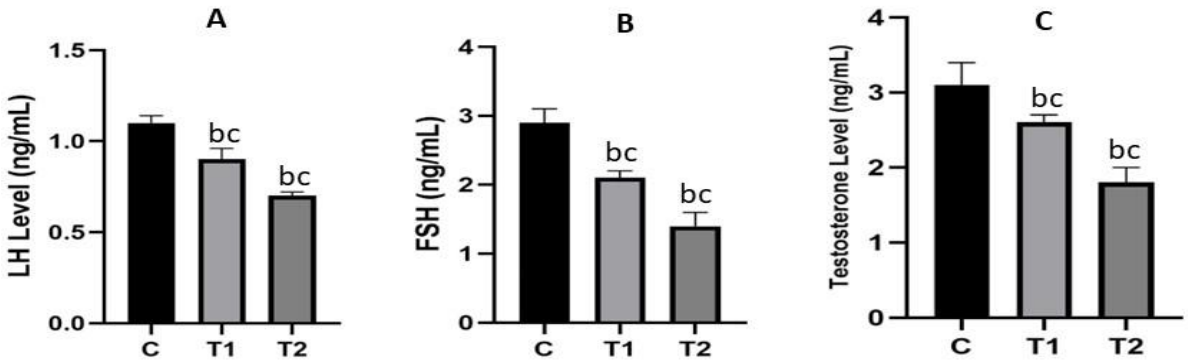

Figure 5: Bardiagrams showing the changes in male reproductive hormone levels. (A-LH; B-FSH; C-Testosterone) of descended testes of male charles foster rats. Values are Mean± SEM. [a] P<0.01; [b] P<0.001; [c] P<0.05 Vs corresponding control groups of rats. C: Control, T1: Treated 1(CPF applied 5.4mg/day/kg.BW for 30 days), T2: Treated2 (CPF applied 8.1mg/day/kg.BW for 30 days).

Effects of CPF on male reproductive hormone:

A significant reduction in the level of LH, FSH and testosterone was observed of the descended testes rat in CPF exposed groups of rats compared to control rat **(Figure 5 A, B, C).** Results indicate that the CPF may inhibit the function of testis presumably by inhibiting the secretion of gonadotropins (LH and FSH) from anterior pituitary and testosterone from leydig cell of testis.

The reduction of gonadotropins (LH and FSH) in blood may be due to CPF induced suppression of gonadotropins gene (LH and FSH gene), involved in the release of LH, FSH and steroidogenesis.[26] CPF induce prolong suppression of the neural transmission of the neural circuit of hypothalamus by inhibiting the brain AChE may impairs the synthesis and/ or release of GnRH from hypothalamus and thus the synthesis and/or release of gonadotropins (LH and FSH) from pituitary.

Low concentration of LH in blood may be responsible for reduction of testosterone levels in blood because LH directly stimulate leydig cell to produce testosterone. Therefore, the reduction of reproductive hormones (LH, FSH and testosterone) may be due to disruption of the hypothalamo-pituitary-gonadal axis (HPG axis).[27]

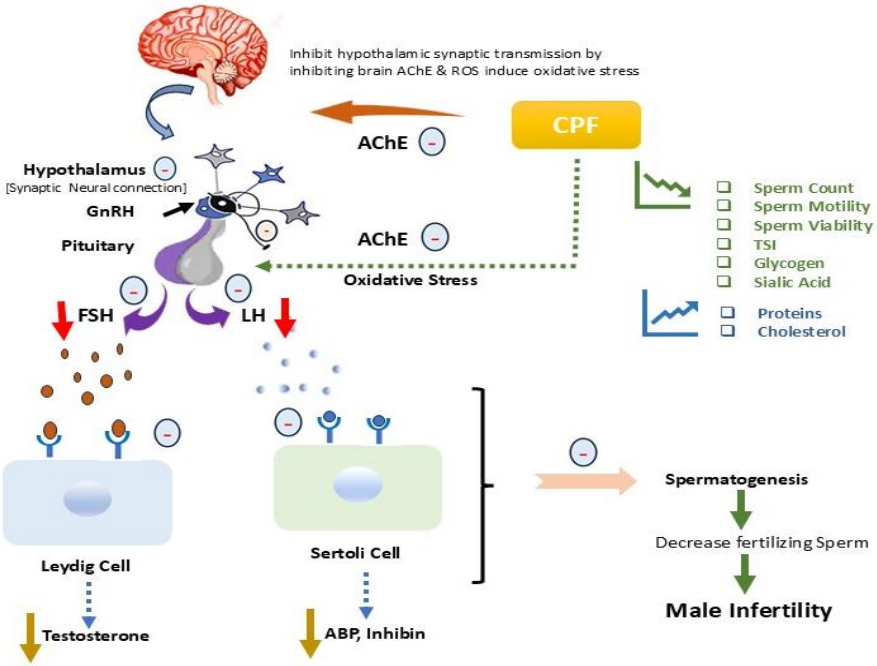

Figure 6: Diagram showing that CPF probably suppresses the synaptic transmission of the neural circuit of the hypothalamus of brain by inhibiting the AChE and ROS induce oxidative stress, resulting low GnRH-LH, FSH –Testosterone output which impairs the process of spermatogenesis leads to infertility.

Another reason of low levels of reproductive hormone is the CPF induced oxidative stress in the pituitary gland and testis. Low testosterone level in blood of treated rats may be due to oxidative stress induced direct damage of the leydig cells that secret testosterone.[28] Thus, it was predicted that CPF induced neuro-endocrine disruption of HPG axis **(Figure 6)** along with oxidative stress mediated degeneration of the pituitary gland and testis may have responsible for reduction of male reproductive hormone (LH, FSH and testosterone).

CONCLUSION

From the results it is concluded that Chlorpyrifos (CPF) suppresses the male reproductive system functions by decreasing the sperm count and reducing the motility and viability of sperm in rat. The CPF induced decrease in sperm count and other effects on spermatozoa might be due to CPF induced inhibition of the secretion of LH, FSH from anterior pituitary and testosterone from testis by shifting the set-point control of the function of hypothalamo-hypophyseal-testicular axis.

Source of support: The author received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest: The author declared that there are no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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