WITHDRAWN:
Atropine Reverts the Neurobehavioral Toxicity Elicited by Acute Exposure to Buprofezin in Sprague Dowley Rats

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Research Article

Keywords: Buprofezin, Atropine, Thiadiazine, Acetylcholinesterase, Moulting, CA1, CA3

Posted Date: June 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-470500/v1

EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Buprofezin (BPFN) is a thiadiazine insecticide that inhibits chitin synthesis and the moulting in case of white flies, mealybugs and leaf hoppers. The exposed insects are unable to shed their cuticle and ultimately die as moulting ensue. Neurobehavioral toxic effects elicited by buprofezin remained unclear. Furthermore, the reversal of buprofezin induced neurobehavioral toxicity by atropine was not elaborated. Thus, we explored the neurobehavioral toxic consequences of acute buprofezin exposure in adult male rats and effective reversal of these changes by pretreatment with atropine as an antidote. Acute administration of commercial buprofezin (87.9mg/kg/day through oral gavage with corn oil as vehicle) induce a wide range of neurobehavioral toxicity including damage to pyramidal cells of hippocampal CA1, and CA3,region and behavioral impairments as demonstrated through, loss of motor coordination, locomotor activity, fear loss, hearing, sensorimotor, cognitive and spatial navigation impairment following the exposure .These neurobehavioral toxic effect of acute buprofezin exposure were significantly reversed by the 15 min pre-treatment of atropine antidote before the buprofezin administration. Pre-treated atropine (20mg/kg/day;i.p) attenuates the neurobehavioral toxicity induced by buprofezin in male rats. It was suggested that acute buprofezin exposure elevated the acetylcholine level, by inhibiting the synthesis and release of acetylcholine esterase (AChE) in synapse. But the complete mechanisms are remained to be elucidated

Introduction

Pesticides are extensively used in commercial and agriculture fields to control growth of pest. (EFSA, 2013; EPA, 2011a; Eurostat, 2003). The abundance of toxic pesticides has been banned in most countries, because they are classified as organic pollutant (Stockholm Convention, 2014), yet their component and metabolic residues are still found in environment and human. (Bedi et al., 2013; Dalvie et al., 2014; Mage et al., 2004; Toan et al.,2013; Weber et al., 2010). Several pesticides are constructed in order to attack on nervous system of pests, so due to the similar neurochemical transmission system these insecticides are neurotoxic to human at different doses.

The ubiquitous application of pesticides in distinct areas has elevated the risk of environmental pollution by various xenobiotics that can be highly toxic for non-target organism containing human. Potential hazards of these pesticides, involving their neurotoxic effects on development has been reported in previous finding (Eaton et al., 2008).

The buprofezin ((Z)-2-*tert*-butylimino-3-isopropyle-5-phenyle-1,3,5-thiadiazin-4-one) belongs to thiadiazine class of pesticides that inhibit the molting process of different pests including white flies, mealybugs and leaf hoppers. (Liu and chen, 2000). In insects it inhibits the chitin synthesis and showed its action as the molting process proceeded. The exposed insects are unable to shed their cuticle and ultimately die during this molting process, developed by Nihon Nohyaku in 1981(Chen et al., 2011). BPFN render incorporation of 3H-glucosamin into chitin (Izawa et al., 1985). Because of chitin deficiency, the elasticity of procuticle is lost in whitefly nymphs and the insect was incapable to accomplish the molting. (De Cock et al., 1988).

Soil residing buprofezin was decayed into various metabolites by soil living microorganisms. (Chen et al., 2011). Delineate transformation pathway of buprofezin via pseudomonas sp. DFS35-4 a strain that metabolize buprofezin present in polluted China. Rice field soil residing Rhodococcus sp. Strain YL-1, has capability of biodegrading buprofezin into four metabolites: 2 isothiocyanato-2-methyl-propane, 2 tert-butylimino-3-isopropyl-1, 3,5-thiadiazinan-4-one N- tert-butyl thioformimidic acid formylaminomethyl ester, and 2-isothiocyanato-propane, (Li et al.,2012).

The buprofezin acute toxicity was non-significant in earthworm and fishes, mammals and eating birds, but was highly toxic to aquatic ecosystem. BPFN get accumulated in aquatic milieu thus buprofezin exposed aquatic ecosystem is at hazard. Buprofezin chronic toxicity for aquatic organism is absent, though it is extensively used for regulation of insect growth. Acute exposure to buprofezin (dermal, oral or through respiration) induce low toxicity in mammals oral and dermal LD50 > 2000mg/kg body weight.LC50 4.57mg/L air /4th and do not produce itching in skin and eyes. (EFSA, 2010).

Administration of 5000 ppm buprofezin to Sprague dawley rats enlarges liver and thyroid and turn them dark brownish color (Jürg Zarn1 et el., 2008). Alteration in serum concentration of SGPT, SGOT, urea and creatinine, thiobarbituric acid reactive oxygen species tissues of liver and kidney. Associated substantial reduction of total protein, antioxidants enzymes, the SOD, CAT, POD, and GSH non-enzymatic reduced glutathione. Damage was showed in hitomorphology of liver and kidney. Damage of liver hepatocytes, shrinkage of kidney glomerulus rupture capillaries, necrosis of tubular epithelial cell and increased Bowman's space were observed after acute exposure of buprofezin in Balb/c mice (Bibi and Qureshi, 2019).

Liver and thyroid are adversely affected by buprofezin and histopathological changes not occur in affected organism but it changes clinical chemistry. Oral administration of buprofezin for two days induce micronuclei in bone marrow erythrocyte of mouse (Inagaki, 2006).BPFN devoid carcinogenicity and reproductive toxicity in rats or mice and not induce neurotoxicity in mammals (EFSA, 2007.Buprofezin acts on liver the main site of its action. Various researches suggested that BPFN accumulated in liver and consequently induces oxidative stress. Cytochrome C oxidase activity is retarded by buprofezin, which is most important cause of energy production and thus produce (ROS) (Ji et al., 2016). Buprofezin induced chronic toxicity and carcinogenicity has suggested that liver is chief toxicity target in mice and rats. A two-year study in mice revealed that body weight of female and male mice decreased from week 6 (male) and week 9(female) onward at 5000 ppm dose, but a little decreased at 2000 ppm dose. (Jürg Zarn et al., 2008).

In earthworm the activity of AChE was intensely repressed by lufenuron subsequently by buprofezin, and then triflumuron in descendant. (Mohamed et al., 2013). The study on *B. tabaci* found all over the word horticulture, agriculture and ornamental plants have revealed that buprofezin is being used as AChE blocker to kill insects that had developed resistant to organophosphate and carbamates. (Cottage et al.,2006). It is clearly established that (CNS) is essential or production o behavior.it is also clearly recognized that human and animal behavior is altered by a several of chemical's entities. The behavior

measure has been recognized as essential in screening the potential toxicity of these agents on central nervous system. This recognition has opened a new area of research within toxicology which has been referred by Weiss and Laties (1969) as" behavioral Toxicology" and by Zbinden (1983) as "neurobehavioral toxicology".

In this study acute intoxication of buprofezin induce a wide range of neurobehavioral toxicity including damage of pyramidal cells of hippocampal neuron and behavioral impairments for example, loss of motor coordination, locomotor activity, fear loss, hearing, sensorimotor, cognitive and spatial navigation impairment following the acute exposure in rats due inhibition in synthesis and release of AChE as AChE also described as key player in activation of glial cells, brain blood flow, amyloid pathway, phosphorylation of tau protein, also function as adhesion protein for maintenance and development of synapse (Ballard et al., 2005).

We have elaborated that intoxication of buprofezin was potentially reversed by pre-administered atropine. With the advancing incidents of neurobehavioral and cognitive defects the objective of this study was to investigate the neurobehavioral toxicity, underlying mechanism, and possible therapeutic approach to overcome its neurotoxicity following acute exposure of buprofezin.

Materials And Methods

2.1 Animals

For this study Sprague dawley adult male rats weighing 200–240 gm were obtained from National Institute of health (NIH) and were accommodated in Primate Facility department of Animal Sciences Quaid -I- Azam university Islamabad. Rats were placed in steel cages in a standard temperature of 25◦c and 10/14 h light and dark period, with free availability of rodent diet and tap water.

2.2 Experimental design

The rats were separated randomly and divided into groups. First group was taken as control second group was treated with buprofezin 87.9mg/kg/day through oral gavage with corn oil for seven days. Third group was pre-treated with atropine 20mg/kg/day given intraperiotoneal (i.p) and after fifteen minutes followed by administration of buprofezin 87.9mg/kg/day for seven days. All of rats underwent behavioral test at 7th day of treatment.

2.3 Chemical

Commercial formulation of buprofezin (Robon 250g/kg 25%w/w) linear formula C16H23N2OS and molecular weight 305.44g/mol CAS NO. 69327-76-0 were purchased from Jaffer group of companies. buprofezin is a 2-(tert-butylimino)-5-phenyl-3-(propan-2-yl)-1, 3, 5-thiadiazinan-4-one in which the C = N double bond contain Z conformation. It is a member of homopteran inhibitor of chitin biosynthesis works as an insecticide.

2.4 Histological analysis of brain

At the 7th day of treatment rats were anesthetized by (ip) administration of ketamix (0.1ml/kg body weight) and sacrificed to obtained body organs. The Brains were dissected out, weighed and subsequently immersed in freshly prepared 4% paraformaldehyde for 16 hours at 4◦C and processed for paraffin embedding. Tissue blocks containing hippocampus (-3.4 to -3.8mm posterior to bregma) were further processed for paraffin embedding and 8µ thick serial section were cut in coronal plane under microtome. The sections were stained with hematoxylin -eosin (H and E) conventional method and mounted. Hematoxylin- eosin was performed to investigate the structural changes following the buprofezin exposure and its protection with pre-treated atropine.

2.5 Motor coordination

Motor coordination was evaluated by using rotarod test, which is performed by placing rats on rotating rod that rotates around its axis; horizontal and parallel bars. Rats were placed 25–30 min in testing room prior to testing.

2.6 Rotarod Test

A rod of adjustable diameter (10.64cm) was rotated at about 4rpm. The rotation velocity was gradually increased until the rat fell off the rotating rod. When the increasing speed is measured, the time from start until the rat's fall off consider as measure of motor ability (Spyker and Avery, 1976; Rodier, 1978; Vorhees et al., 1979b). The motor coordination of control, buprofezin treated and pre-treated atropine plus buprofezin rat was quantitatively evaluated using accelerating rota-rod for rats. With laser rpm calculator. The rotarod was 10.64 cm in diameter constituted from steel rod with knurled surface for treading. Two circular plastic glass disks were fixed at the ends of the rod with diameter of 40.64cm. The disks prohibit escape and worked as a barrier between rats. The rotarod was affixed 71.12cm above the floor. Rats were mounted on the rotarod perpendicular to the long axis of the rod, with their heads facing away from the observer. As rat was mounted on the rod, the run experiment menu allows the experimenter runs experiments. When the experiment is running the current speed of rotarod is displayed on LASER rpm calculator, as well as the amount of elapsed time as the experiment has started was measured with stop watch. As the rat falls from the rotarod, the rotational velocity of the rotarod at the time of the fall is showed by LASER rpm along with the amount of time the rat elapsed on rod was calculated manually by stop watch.

2.7 Horizontal bars test

The "string test"1, "coat hanger test" or horizontal bar test was used to measure forelimb strength and coordination. We have noticed that performance depends on tightness of string therefore most experiments use a metal bar. It is noted that rat's capability to grasp the bar is inversely proportionate to bar diameter and standardly used bar has 2 mm in diameter. The bar length was 91.4 cm and were lifted 73.6cm above the floor by wooden supporting columns. We held the rats by the tail, gently put it on the

table in front of the apparatus, rapidly drags it backwards almost 20 cm so that rats align perpendicular to the bar, swiftly pick up and allowed it to grip the horizontal bar at its middle only with its forepaws, and fully relax the tail, consecutively starting the stop watch. The criterion was to measure either a time of fall from the bar prior the rat reached one end of wooden column, or measurement of time until its one forepaw touches a supporting pillar. The optimum test time was 30 sec.

2.8 Scoring procedure.

If rat failed to grip bar before first 5 sec this was attributed to poor placing and this fall was not counted. If rat fall between 1–5 sec score = 1, If rat fall 6–10 second score = 2, If rat fall 11–20 second score = 3, If rat fall 21–30 second score = 4 and If rat fall > 30 second score = 5

2.9 Parallel bars test

Two parallel steel bars 91.4cm in length and 4mm diameter were fixed 2.5 cm apart by wooden supporting columns at their ends elevated 73.6 cm above the floor. Two Parameters were measured. Maximum test time was 120 sec. Rats were held by the tail, gently put it on the bench in front of the apparatus, rapidly drags it backwards about 20 cm so that rats align perpendicular to the bar, swiftly pick up and allowed it to grip the horizontal bar at its middle only with its forepaws, and fully relax the tail, subsequently starting the stop watch. First criteria point was to measure time the rat takes to orient 90◦ from start. Second criteria point is to count the time taken by rat to reach at end of bar.

2.10 Fear conditioning

Fear conditioning tests were performed in fear conditioning test box comprised of light and dark compartments. Dark compartment was containing electric bell for cued and steel wire floor connected with electric supply for contextual fear conditioning. Length of each chamber was 73cm each compartment was 36.8cm long and wide. Both chambers contained window of 10cm in diameter. Before experiment rats were permitted to acclimatize in testing room for 30 minutes.

For contextual fear conditioning test rats were placed in testing chamber and electric foot shock of 0.14mA was imposed for 2 sec. Rats suddenly steps from shock compartment to other and freezing was examined for 5 min. For cued fear conditioning test rats were placed in same testing compartment and electric bell was turned on and electric current in steel wire was turned off, which delivered white noise tone of 90b dB for 30 sec. Rats suddenly steps in from bell containing chamber into other compartment and freezing was examined for 5 min.

For both cued and contextual fear conditioning rats were retained in conditioning test box and allowed to acclimatize for 2 minutes. Every animal then received a white noise tone of 90dB for 30 seconds and after that an electric foot shock of 0.14mA for 2 sec was subsequently delivered. The time duration between tone and shock was 2 min. After the exposure of last shock rats were left in chamber for 20 sec before removing. Freezing behavior was calculated for 5 min.

2.11 Evaluation of shock threshold for jumping, flinching and vocalization

Shock threshold for flinching, jumping and vocalization was measured of control, buprofezin and pretreated atropine plus buprofezin in passive avoidance test box provided with variable current in mA.

2.12 Step through passive avoidance

Rats were placed into passive avoidance test box containing a dark and light chamber. On the first day rats were placed into lighted chamber, and latency for the rat to step into dark chamber was recorded. Subsequently 0.2mA shock of electricity was provided for 2 sec and rat was removed. On second day rat were placed into lighted compartment, and latency for rat to step into the dark chamber was measured again with maximum time of 5 min.

2.13 Time space navigation, rearing, total locomotion activity, working memory, reference memory and memory errors measurements

Time space navigation, rearing, total locomotion activity, working memory and reference memory was measured by using “TSD MAZE, CSS OR CLOSE MAZE”. The activity of head direction cells (HD) hippocampal Grid cells, place cells, speed cells and TSD cells was assessed by preference of path selection by rats when food stimulus was baited in the both ends of path. Rearing behavior consist of rats standing upright position on its both hind paws and is index of anxiety in TSD OR CSS MAZE and the elevated plus maze. Number of rearing were assessed in TSD maze for 15 minutes. The total locomotion activity of control, buprofezin treated and pre-treated atropine plus buprofezin was analyzed manually after release of rats in 705cm long TSD maze. Rats were freely allowed to move in both long and short pathways. The total activity was measured in time scale of 15 min.

2.14 Evaluation of working and reference memory and memory errors in TSD or CSS Maze

Working memory can be demarcated as a memory for an object, recognition, or location that is used within a testing period, but not usually between the periods. It is variant from reference memory which is demarcated a memory that would typically be attained with rehearsal training and would sustain from days to months. The reference memory is mostly the memory for the ‘rules’ of a given chore. For instance, when testing object press a bar receive a food object or a water maze established with a hidden platform or entrances into the food containing pathway of the TSD or CSS Maze. Moreover, working memory enable the testing object to remember which pathway it had visited in a testing period.

2.15 Testing animal adaptation period

The rats were presented two periods of adaptation on two succeeding days before the learning process commences. The testing rats were allowed to walk around the food baited pathway of the maze for 15 min during the testing time. The testing rats were explored the TSD Maze baited with food stimulus first in long pathway, then food baited in short pathway and at the end food baited at both pathways and path

acquisition in each case was recorded. Following the adaptation period, the acquisition process was ongoing.

2.16 Testing animal acquisition career.

During the testing animal acquisition career or (learning session), the rats were assumed three trials of acquisition per day until the rats achieved the learning criteria. The learning criteria were confronted as follows. The trial was sustained for 15 min and the training was continuous until the rats achieved the criteria of 80% correct choice; i.e., at least four correct entries out of five. The duration of this session varied depending upon condition of research procedure the maze was washed with ethanol (70%) at start of trial session and thereafter one path was baited with food stimulus. For first trial the rat was kept in central box and was permitted to choose any pathway. When the rat reached the end of pathway and ate the bait reward, the path choice was noted. Only the first approach to the baited pathway was documented as a correct choice and the maze pathway. For second trial the pathway was rebaited and entries of rat in baited pathway was recorded. Entrances into the path containing no food stimulus were recorded as Reference Memory Errors (RME). For third trial the both pathways were baited with food stimulus and path entries of rat was recorded reentries into baited pathway when both pathways are baited referred as WME. For fourth trial the both pathways were baited and choice of short pathway was recorded as correctness of TSN. Each rat was assumed four trials per day and obtained data from the four trials were averaged and included in analysis of final data. The performance pattern of rats was recorded by the percentage of the correct choices, RME and TSN (time space navigation) in TSD Maze, COSE Maze or CSS Maze.

2.21 Statistical analysis.

The data was statistically analyzed with ONE WAY ANOVA followed by Tukey$_S$ post hoc multiple comparison test. Data was demonstrated as Mean ± SEM. $P < 0.05$ represented the significant difference.

Results

3.1 Buprofezin induced Histopathological alterations of hippocampus were attenuated by pre-treated atropine

MWM test and TSD maze finding demonstrated that acute buprofezin exposure impair spatial, working and reference memory as well as time space navigation. We concentrated on histopathological alteration of hippocampus provoked by buprofezin treatment. Hippocampus can be classified into several regions on the basis of pyramidal neuron morphology as CA1-CA4.We observe the changes in hippocampus with staining. Results demonstrate that buprofezin exposure induce alteration in various regions of hippocampus. It is reported that the function of pyramidal cells in CA1 region were attributed with long term memory and degeneration of CA1 pyramidal cells may cause significant memory loss. Thus, to analyze histopathological alteration and ultrastructure variation in response to buprofezin exposure CA1 and CA3 regions of hippocampus was analyzed by hematoxylin and eosin staining.

H and E staining showed that in control group pyramidal cells were arranged orderly with intact nuclei stained clear, dark blue. However, in acute buprofezin treated rat's hippocampus H and E staining revealed more degenerative (apoptotic) neuron and pyramidal cells showed granular vacular changes and nuclear pyknosis. The cell junction and basement membrane were degenerated. In pre-treated atropine group pyramidal cell show better cell morphology compared to buprofezin treated. Cell's junctions were intact and cells were in compact form. Only few cells underwent degenerative changes. These finding clearly demonstrate the ameliorative effect of pre-treated atropine against buprofezin neurotoxicity.

3.2 Atropine reverse the impaired motor coordination induced by acute buprofezin exposure.

Motor coordination of rats was analyzed by using rotarod. the results indicated that latency of fall from rotating rotarod was significantly reduced ($p < 0.001$) in acute buprofezin exposed groups compared to control and pre-treated atropine counteract the toxic effect of buprofezin as there is less significant difference compared to control. Figure 2.A

The forelimb grip strength and coordination were assessed by using horizontal and parallel bars. Results have shown statistically significant ($p < 0.001$) impairment in fore limb grip strength in horizontal bars and was no significantly counteracted by atropine. Figure 2B, C, D, E.

In case of parallel bar time taken by rat to orient 90º and traverse time was significantly increased in buprofezin treated rats compared to control. Total distance on bars in given time duration was reduced in buprofezin exposed group. Pre-treated atropine has reversed the buprofezin effect as the there is no significant difference between control and pre-treated atropine plus buprofezin groups.

3.3 Buprofezin induced long term deficit of contextual and cued memory was revered by Atropine.

In behavior testing group of rats were trained by pairing of two sets of stimuli, mild foot shocks with an associated context and an auditory cue. After training session, we found that buprofezin treated rats' exhibit significantly less freezing compared to control and pre-treated atropine plus buprofezin exhibited no significant change in freezing compared to control. These results suggested that pre-treated atropine counteracted the loss of fear conditioning caused by acute exposure of buprofezin. Initially we tested the cued then contextual and the lastly, we paired cued and context to examine the fear conditioning. Figure 3A, B, C.

3.4 Atropine slightly counteract the impaired passive avoidance induced by acute exposure of buprofezin.

In passive avoidance test the rat learned to decrease their natural tendency to avoid from light compartment to darker compartment of training chamber. We trained the rats by placing them into a lighted compartment and measured the latency after which the rat entered into dark chamber where they receive a mild electric foot shock. Next day we assessed the latency of rat to step into dark compartment. Our results suggested that first day rat spend significant greater time in lighted compartment compared to control. While on second day in spite of receiving foot shock buprofezin treated rat latency in lighted compartment was significantly reduced compared to control and was remarkably reversed by pre-

atropine administration. These finding indicate the loss of sensory receptor by acute buprofezin exposure. Figure 4 A

3.5 Quantification of shock threshold for flinching, jumping and vocalization.

Our results showed the remarkable increase in shock threshold for flinching, jumping and vocalization in buprofezin treated rats compared to control indicating loss of general sensory perception. Pre- treated atropine rats showed no significant increase in shock threshold compared to control. Figure 4B

3.6. Effect of buprofezin on spontaneous behavior and impact of atropine

Spontaneous behavior tests were performed in TSD maze for 15 min on adult male rats. Total locomotion activity and rearing mean was measured in all testing rats. Results have shown the rats receiving acute exposure of buprofezin exhibited significant decrease in locomotion and rearing activity and pre-treated atropine rats represent no significant difference compared to control reveals reversal of buprofezin toxicity by atropine. Decrease number of rearing represent the anxiety and depression behavior in buprofezin treated rats. Figure 5 A, B

3.7. Buprofezin induced impairment in working memory, reference memory and spatial navigation was counteracted by atropine

3.7.1 Correct choice of path during acquisition career

Results were obtained by using ONE WAY ANOVA followed by Tukey post hoc multiple comparison test. Our finding has revealed a significant loss of correct path choice during acquisition session in buprofezin exposed rats compared to control. Rats were subjected to five trial for each and percentage of correct choice was calculated. At first day the control group was unable to reach 80% correct choice of path. Second day after continues trial rats ultimately obtained 80% correct choice of path. Impairment of correct path choice was counteracted by pre-treated atropine. Figure 6 A

3.7.2. Correct choice of path during navigation session

In time space navigation session, the rats were trained to obtained food stimulus from shorter pathway to reach maximum 10% of correct choice although food is baited in both pathways. We have found less than 10% of correct choice of control rats on first day and buprofezin treated rats showed significantly less correctness compared to control. Moreover, pre-treated atropine has reversed the effect. On second day control rats achieved the criteria of 10% correctness after five trials of training. buprofezin again decreased the choice correctness. These finding suggest the deterioration and apoptosis of TSD, grid cells, speed cells and hippocampal place cells following the acute exposure of buprofezin. Figure 6 B

3.8. Working memory and reference memory error during acquisition are attenuated by pre-treated atropine

3.8.1. Working memory error

Working memory is memory of object stimulus or recognition of location used in testing session. So if rat enter in food baited path it is working memory correctness and if it reenters into baited pathway when both pathways are baited it is referred as working memory error. On first day of training the working memory errors (WME) were greater in buprofezin treated rats compared to second day. Results suggested that continuous training alleviated the incidence of working memory error and pre-treated atropine significantly attenuated the working memory errors. Figure 7 A

3.8.2. Reference memory error

Reference memory is memory for rule of given condition. For example, acquisition of baited path provide food to rats. Entries of rat into pathway with no food stimulus is referred as reference memory error. Results suggested that reference memory error (RME) during acquisition session changed days after training. Buprofezin treated group exhibit more reference memory error compared to control group. On second day (RME) were comparatively more from day 2 to onward the memory errors reduced continuously. However, there was no substantial difference found in control and pre-treated atropine group. This demonstrates the reversal effect of pre-treated atropine on reference memory errors. Figure 7 B

3.13. Mechanism of buprofezin Neurotoxicity

Buprofezin intoxicated rat died soon after the administration of neostigmine (30µl/kg/day i.p) a blocker of AChE. It suggested that a small concentration of AChE in synapse was dominantly occupied by a small concentration of neostigmine consequently tremendously elevated the ACh level in synapse leading to tremor and death of rat. However pre-treated atropine delayed convulsion, tremor and death indicated a protective role of pre-treated atropine against buprofezin induced neurotoxicity. Pre-treated neostigmine plus atropine also delayed death due to sufficient availability of AChE in synapse that metabolizes the Ach in non buprofezin intoxicated rats. Pre-treated atropine plus neostigmine treatments in non buprofezin intoxicated rats also cause sudden death due to plenty of ACh in synapse as AChE and cholinergic receptors have been blocked.so these results suggested that buprofezin increases the concentration of ACh in synapse by inhibiting the synthesis and release of AChE due insufficient supply of ATP.

Discussion

The recent study described the neurobehavioral toxicity of acute buprofezin exposure however it is previously well established that buprofezin (BPFN) frequently used as a moulting inhibitor insecticide all over the world to eradicate pests like leafhoppers, mealy bugs and whitefly (Chang et al., 2015), infiltrating the fruit crops, leafy crops and citrus crops. Its metabolic compounds are potentially hazardous to the neighboring milieu (EFSA, 2007). It has been proved extremely toxic to aquatic environment (EFSA, 2010: Ku et al., 2015) as in embryo of zebra fish reactive oxygen species (ROS) have been detected following the exposure to buprofezin and nickel. Administration of embryos and larvae of African catfish (Clarias gariepinus) to various doses of buprofezin consequences into death of embryos

when its amount of dose rises to 5–100 mg/L. In African catfish dose < 5 mg/L also carry out numerous hazardous effects during embryogenesis and development of larva. These effects comprise asymmetrical head, bleeding from pericardium, inward curvature of the lumbar and cervical regions, arcuate in body, ulcerates and accumulation of fluid in yolk sac (Marimuthu et al., 2013).

In our study we have explored that the acute oral dose of buprofezin 87.9 mg/kg/day induced wide range of neurobehavioral toxic effects. Acute intoxication of buprofezin induce a wide range of neurobehavioral toxicity including damage of pyramidal cells of hippocampal CA1 and CA3, region neurons and behavioral impairments for example, loss of motor coordination, locomotor activity, fear loss, hearing, heat shock, sensorimotor, cognitive and spatial navigation impairment following the acute exposure in adult Sprague dowley male rats. We have also found that acute intoxication of Buprofezin is potentially reversed by pre-administration of Atropine. The complete molecular and biochemical mechanism of Buprofezin neurobehavioral toxicity is not elucidated so for however we suggested that it inhibit the synthesis and release of AChE in synapse as in our experiment Buprofezin intoxicated rat was suddenly dead after the administration of neostigmine (30µl/kg/day i.p) a blocker of AChE. It put forward that a small concentration of AChE in synapse was dominantly occupied by a small concentration of neostigmine consequently tremendously elevated the ACh level in synapse leading to tremor and death of rats. This hypothesis was also supported by previous studies as activity of cytochrome and TCA cycle enzyme was rendered by buprofezin that interfere the energy metabolism and inhibit production of ATP. (Ji et al., 2016; Binukumar et al., 2010; Shan et al., 2013). Atropine function as a physiological antagonist and competitively block the acetylcholine action of muscarinic receptors and acts as antidote for excessive parasympathetic activation arised as consequence of inhibition of AChE (Johnson et al., 2000). Acetylcholinesterase (AChE) being serine hydrolase enzyme that cleaves rapidly terminate the cholinergic transmission in synapse by breakdown of Ach into choline and acetate (Soreq and Seidman, 2001). AChE also described as key player in activation of glial cells, brain blood flow, amyloid pathway, phosphorylation of tau protein, also function as adhesion protein for maintenance and development of synapse (Ballard et al., 2005). Arsenic exposure in animal model induces behavioral alteration, abnormality in nervous system shaping and development, inflammation and neuron death, (Yen et al., 2011; Flora et al., 2012). In addition, arsenic could induce toxicity in HAPI microglia (Mao et al., 2016), granular neurons of cerebellum (Liu et al., 2013) and snail neurons (Lu et al., 2009).

Further our study demonstrated the impairment of motor coordination and fore limb and hind limb grip strength as Occupational exposure to acrylamide leads to cumulative but reversible neurotoxicity described by axanopathy of peripheral nerves, ataxia, muscle weakness, tingling of hands and feet and cognitive deficiency. Because it inhibits kinesin transport, decrease the neurotransmitters and inhibition of transmission (Exon, 2006). Crofton and colleagues stated decreased grip strength in a 30-day acrylamide administration study at 15 and 20 mg/kg/days because it causes peripheral axonopathy (Crofton et al., 1996) after exposure to high oral doses of buprofezin clinical signs appear as reduced locomotor activity, tremble, runny nose, abnormal movement and urinary discontinuous urination. (*EFSA*, 2007). In present study impairment in the grip strength and motor coordination was assessed by using rotarod, horizontal and parallel bars. Similar impairments in motor activity, abnormal gait, and cognitive deficiency were

detected following exposure to bifenthrin due to oxidative stress (Farah et al., 2017) A study on earthworm described that reduction in growth rate by combined exposure of buprofezin, Lufenuron, and Triflumuron pesticide-exposed worms was observed by dose-dependent over the 28-day treated duration, which was accompanied by a decline in activity of AChE and GST. The lowest activity of AChE was noted at the highest dose of buprofezin (300 mg/kg soil) following two weeks of exposure as compared to control. The activity of AChE was intensely repressed by lufenuron subsequently by buprofezin, and then triflumuron in descendant. (Badawy et al., 2013). Similarly, our study showed that acute exposure of buprofezin inhibit the synthesis and release of AChE in synapse due to limited supply of ATP described by other study that buprofezin efficiently repressed the cytochrome c oxidase activity by binding to SCO1 active pockets and COX17, which increased the concentration of reactive oxygen species. Additionally, administration with an ROS inhibitor (N-acetyl-L-cysteine) (NAC) counteract the decreased level of ATP and cytochrome c oxidase activity, which also showed that ROS contributed in buprofezin-induced conversion of energy metabolism. After sub lethal treatment of buprofezin, the levels of the end product metabolism (ATP), end product of glycolysis (lactate) and (pyruvate) a component in initial stage of the TCA cycle were evaluated. The higher concentration of these factors after buprofezin exposure reveals the BPFN induced inhibition of TCA cycle. Pesticides can decrease the ATP concentration in HepG2 cells revealed by in vivo and in vitro studies. (Binukumar et al., 2010; Shan et al., 2013).

Pre- treated atropine counteracts the poisoning by blocking muscarinic receptors and it counteracted over parasympathetic activity. At single oral dose (24 h) of chlorpyrifos reduced the activity of plasma butyrylcholinesterase (BChE) and rats (AChE) activity in hippocampus, striatum and prefrontal cortex. The acute chlorpyrifos toxicity can be counteracted by the atropine antidote (10 mg/kg i.p.) and/or pralidoxime (40 mg/kg; i.p.) treated one hour following toxicity (Alciene Almeida Siqueira et al., 2019). In pre-clinical and clinical experiments muscarinic antagonists exhibit antidepressant effects (Drevets et al., 2013; Mancinelliet al., 1988; Witkin et al., 2014). Acute buprofezin decreases the synthesis and release of AChE in synapse. Furthermore (ip) neostigmine injection in buprofezin intoxicated rats cause tremor, and sudden death of rat also proposed that minor amount of AChE is predominately blocked by neostigmine extremely elevated the ACh level in synapse subsequent acute exposure and is counteracted by pre-treated atropine.

Buprofezin exposure impair the passive avoidance as MSK1 knockout affects numerous various forms of hippocampus-dependent memory, as evaluated by fear conditioning, Morris water maze and passive avoidance (Wilson et al.,2007). Pre-treated atropine showed counteract effect not reported in any previous study.

The behavioral analysis revealed that SA (sodium arsenide) treatment cause loss in learning and memory in passive avoidance as well as motor activity and balance. Additionally, acute or chronic administration to SA as revealed by other experiments induce abnormalities of CNS containing slowing of cognitive development, decreased psychomotor speed, loss of learning due to decreased number and apoptosis of pyramidal cells and was mitigated by ellagic acid. (Franzblau et al., 1989; Mathew et al., 2010; Tsai et al., 2003). Our finding also agreed with similar behavior abnormalities and loss of learning and memory due

to pyramidal neuron damage in hippocampus was attenuated by pre –treated atropine. Buprofezin induced decreased in the step- through latency was reversed by pre- treated atropine as the SA exposure has showed significant reduction in the step-through latency comparison to the control due to oxidative stress (Mehdi et al., 2018). The study reported that MSK1 knock-out animals can process the sensory information of foot shock and memorize it in association with contextual and the auditory stimulus, but loss this memory in 24h. (Wilson et al 2007). Our study reported the impairment in long tern contextual fear memory and rapid loss of sensory stimulus of foot shock in acute buprofezin exposure and was slightly counteracted by pre-treated atropine.

Various concentration of orally administered imidacloprid to female rats caused a substantial change in different features of locomotor activity and decreased in ambulation at 90 days of treatment as it inhibits AChE activity (Shipra et al., 2010). Substantial reduction in locomotion in the rats exposed with the acute dose of imidacloprid has suggested that imidacloprid or its metabolic residues has accumulated in brain. Administration of imidacloprid directly in to intraperitonium has shown to accumulated in mouse brain (Chao et al., 1997).

Although the mechanism of action of buprofezin is distinct to some extant results described the similar decline in spontaneous locomotion activity in acute buprofezin administered rats compared to control and pre-treated atropine counteract the toxicity. Similar to high dose of imadacloprid decreases the spontaneous locomotor activity. Another study also supported our results that during the peak of the BGS (brain growth spurt) (PND10) administration of single dose of endosulfan or cypermethrin, cause long lasting spontaneous behavior abnormality in adults due to alteration of protein involved in brain development, variation in locomotion, rearing and total activity without affecting body weight as compared to control mice (Lee et al., 2015).

It has also been stated that the number of hippocampal neurons declined in rats after treatment with sulfite (Akdogan et al., 2011). Additionally, it was revealed that after sulfite exposure the excitability of the spinal reflexes was increased (Küçükatay et al., 2005; Küçükatay et al., 2008). The toxic consequences of sulfite on mesencephalic cell lines have been described, as well (Reist et al., 1998).

Our study in line with these findings as pre-treated atropine has neuroprotective effect against buprofezin toxicity. Pre-treated atropine prevents hippocampal neuron degeneration, spatial memory impairment, working and reference memory loss by preventing over excitability induced neuron exhaustion and preventing the apoptosis of hippocampal neurons. It also prevents the oxidative stress of hippocampal neurons and protect against buprofezin induced cognitive impairment as curcumin inhibit lead-induced loss of memory in rats (Dairam et al., 2007). Curcumin can alleviate the cognitive deficit in diabetic rats (Kuhad et al 2007). Therefore, curcumin may preclude the oxidative stress in CA neurons and, as result, may enhance the synaptic plasticity (Noorafshan et al., 2013; Kuhad et al 2007). It is also stated that curcumin protect the neurodegeneration in Parkinson and Alzheimer disease. (Yadav et al., 2009). Additionally, it is also suggested that curcumin prevent the apoptosis of neuron. (Lin et al., 2011).

A wide range of effects produced by Trichlorethane (TCE), and ether are similar to ethanol and depressant like properties of volatile solvent described in earlier literature (Evan et al.,1996) these involved decreases in activity of CNS (alterations in posture. reduced arousal and rearing), decreases in emotionality of CNS (increased ease of removal), impair of muscle tone (disturbances in gait reduction in forelimb grip strength, increased landing foot splay and loss of psychomotor coordination on the inverted screen test), and decreased sensorimotor activity (reduced response to sensory stimuli). Though TCE and ether at concentrations of 13.300 and 30.000 ppm substantially increased landing foot splay. Flurothyl exposure did not affect increase in landing foot splay at any concentration. TCE and ether also decrease forelimb grip strength but not flurothyl. Additionally, flurothyl produccd handling-induced tremors after after animals was removed from cage, effect which was not caused by TCE, ether or ethanol. Flurothyl cause postictal depression (e.g. an increased inversion latency on the inverted screen test) Characterized by convulsion in testing animal and exhibit not any ethanol like properties (Scott e et al., 1996). The results of our study also agreed with the with these findings as the acute exposure of buprofezin in rat results in decreased brain activity, posture abnormality, decreased activity level, increased lacrimation, salivation, piloerection, abnormal muscle tone, loss of limb grip, ambulation and gait abnormalities, decreased rearing and arousal, loss of time space navigation ability, loss of sensorimotor responses, Redness of nostrils, muscle hypoplasia, decrease in body weight and body temperature. However pre-treated atropine attenuated these variations and significantly reversed these effects.

The farmer studies have various shortcoming as they devoid acute buprofezin neurotoxicity in rat model. Additionally, none of previous study has reported the mechanism underlying its neurotoxicity in rats. Further no therapeutic strategy against buprofezin toxicity was carried out. In contrary our finding has proved that acute exposure of buprofezin induce wide range of neurobehavioral toxicity in adult Sprague dowely male rats and potential antidote against its neurotoxicity

Conclusion

Conclusively our findings have explored that acute exposure of buprofezin in rats induces a profile of neurobehavioral toxicities potentially attenuated by pre-treated atropine antidote. Moreover, its neurobehavioral toxicity was induced by impairment of synthesis and release of acetylcholinesterase (AChE) in synapse. Further studies are necessary to validate molecular and biochemical mechanism involved in decreased concentration of AChE in synapse.

Declarations

Ethical Approval

This study was approved from ethical committee, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad Pakistan. All of animal procedure were carried out under the standard rules established by Bioethical Committee of Biological sciences.

Consent to participation

I have read the information for participants in this study and have had details of study explained to me. My question about the study have been answered to my satisfaction, and I understand that I may ask further question at any time.

Consent to publish

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Author contributions

I hereby declare that the work presented in the article is my own effort, and that the article is my own composition. No part of this article has been previously published in any journal.

Funding

The author is thankful to Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad Pakistan and NIH for providing Rats for experimental purpose.

Availability of data and material

Data used in this study were retrieved from “google scholar” relevant articles

Conflict of interest

The authors declare no conflict of interest.

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Figures

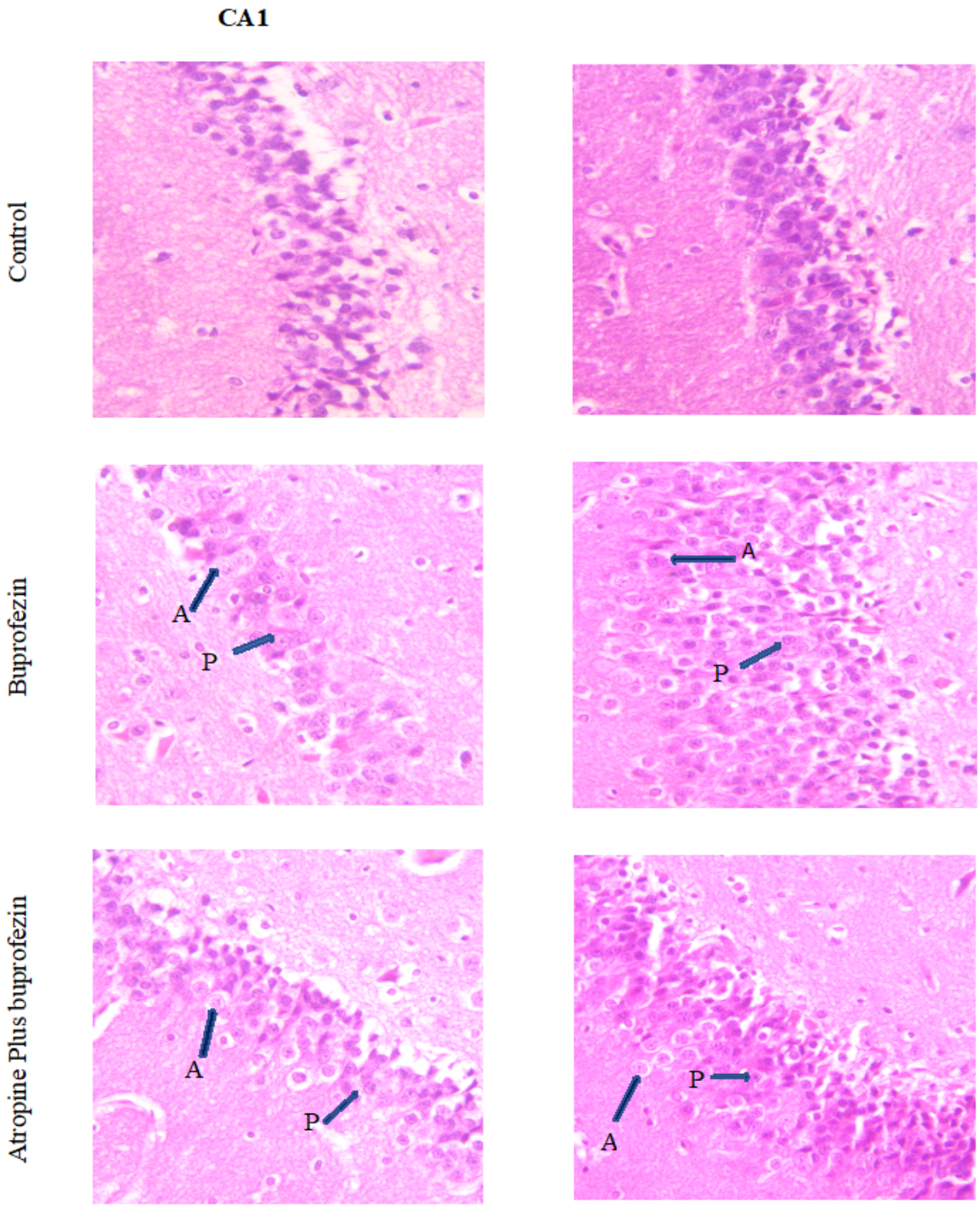

Figure 1

Photomicrograph of 8µm thick coronal section of rat hippocampus (-3.8 mm behind bregma) stained with hematoxylin-eosin (HE). The panel of each column showed control, buprofezin treated and pre-treatred atropine plus buprofezin of CA1 and CA3 region of hippocampus. (A) showed the apoptotic pyramidal cells. (P) showed the pyknotic cells. Normal cells are darkly stained. Disrupted and

misalignment of layers is shown in experimental group. However pre-treated atropine group showed less pyramidal neuron degeneration compared to buprofezin treated group

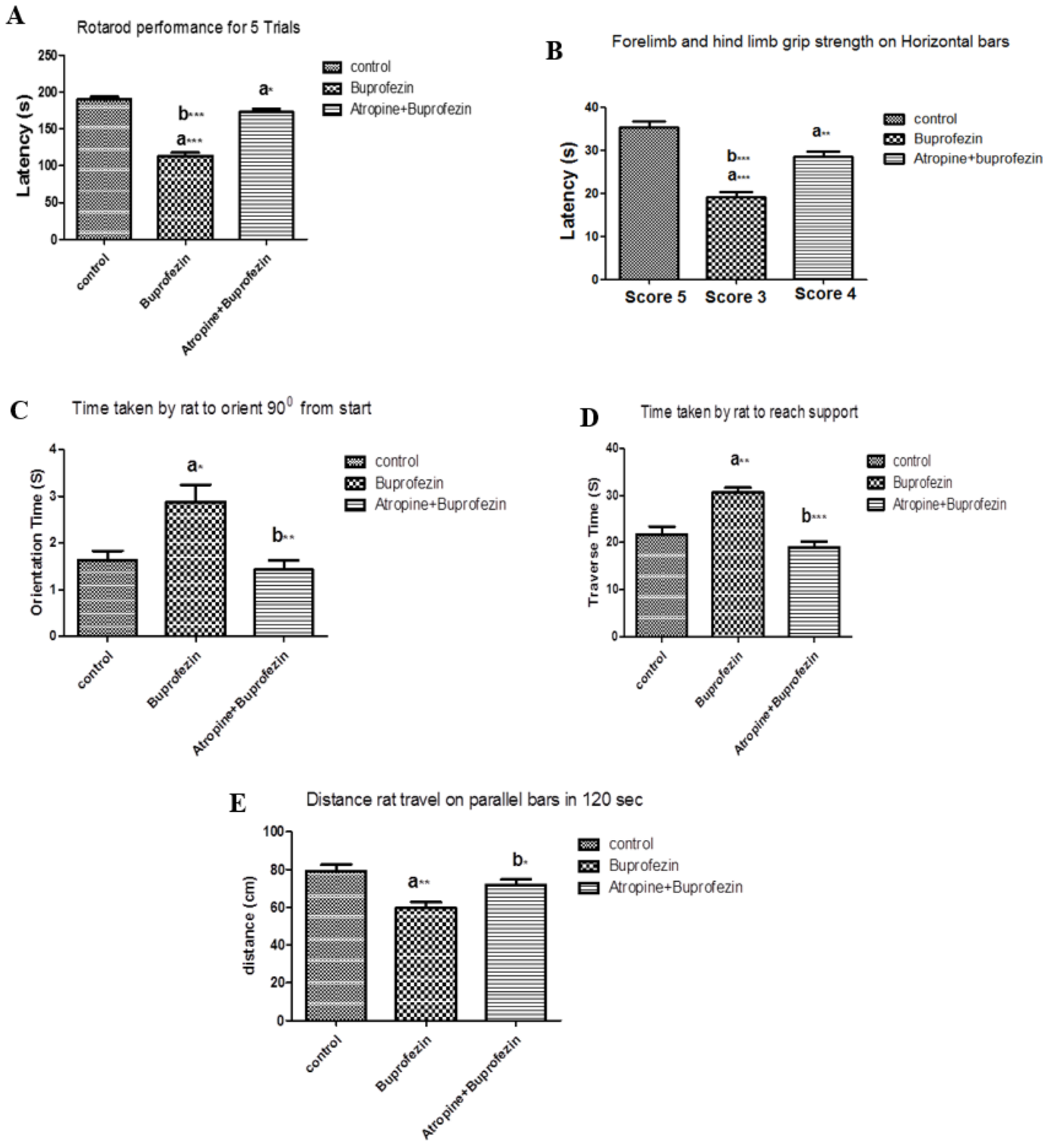

Figure 2

Showed the pre-treated atropine attenuated the buprofezin induced impairments in motor coordination. A. latency on rotarod was significantly decreased by acute buprofezin administration and substantial regain occur with pre-treated atropine. P value *p<0.05, ***P<0.001. B. Limb grip strength was significantly

reduced in buprofezin treated group and sufficiently reversed with pre-treated atropine. p value **p<0.01, ***P<0.001. C. Buprofezin treated rat's takes more time of orientation compared to control and pre-treated atropine showed mild effect. P value *p<0.05, **P<0.01. D. Exhibit the buprofezin treated rats takes more traverse time to reach support atropine showed a little effect. p value *p<0.05, **P<0.01. E. Pre-treated atropine significantly counteract the buprofezin induced decreased distance traveled on parallel bar in 120 sec

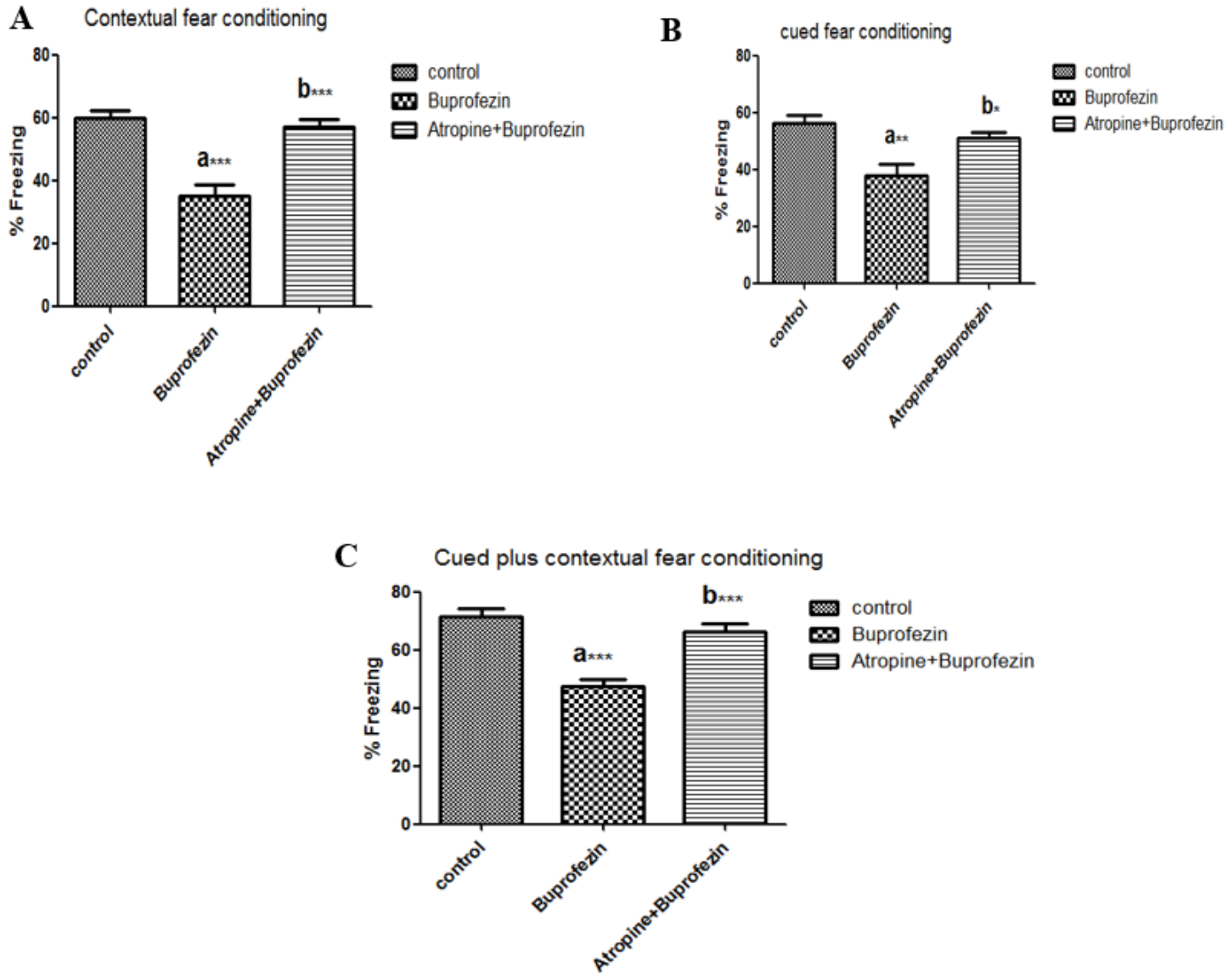

Figure 3

A. Demonstrate the loss of fear condition by acute exposure of buprofezin is significantly reversal by pre-treated atropine. P value ***p<0.001 B. substantial reversal of cued fear against buprofezin induced loss of fear condition. P value **p<0.01 C. Buprofezin induced loss of cued plus contextual fear was significantly reverted by pre-treated atropine. P value ***p<0.001

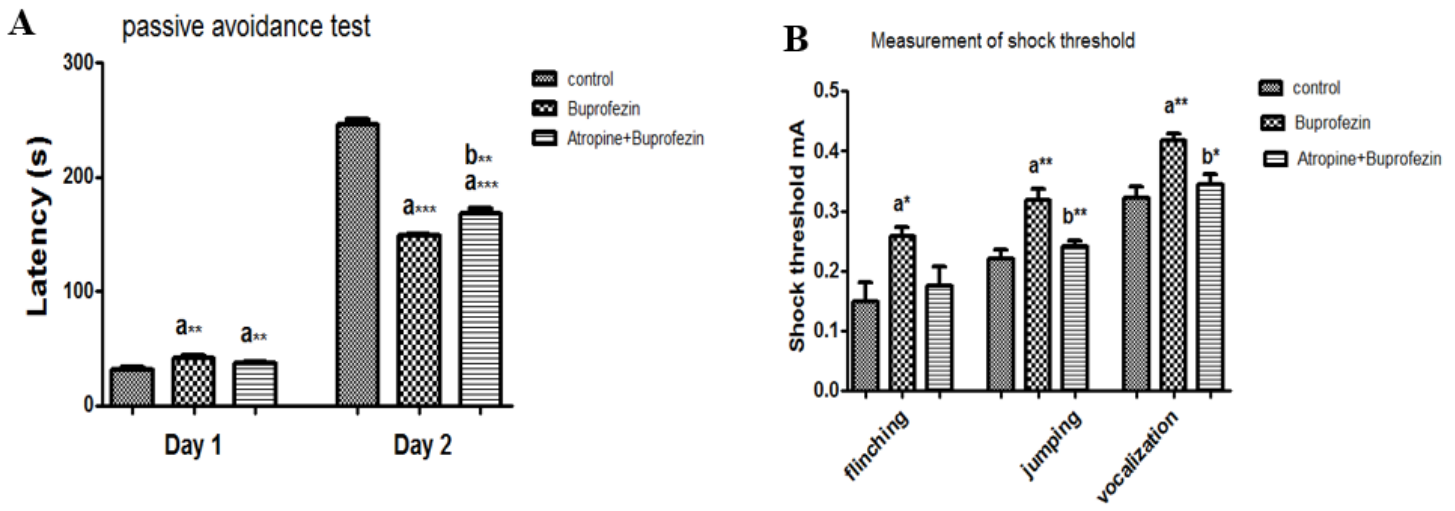

Figure 4

A. Plot showed the protective effect of pre-treated atropine against buprofezin induced loss of passive avoidance. .Buprofezin treated rat represent impaired passive avoidance after electric shock at day 2 and was slightly counteracted by pre- treated atropine. P value ***p<0.001, **p<0.01.3.5 B. Plot exhibit the quantification of shock threshold for flinching, jumping and vocalization in buprofezin treated and pre-treated atropine rats. Pre-treated atropine significantly counteracts the increased in shock threshold for flinching, jumping and vocalization. P value *p<0.05, **p<0.01.

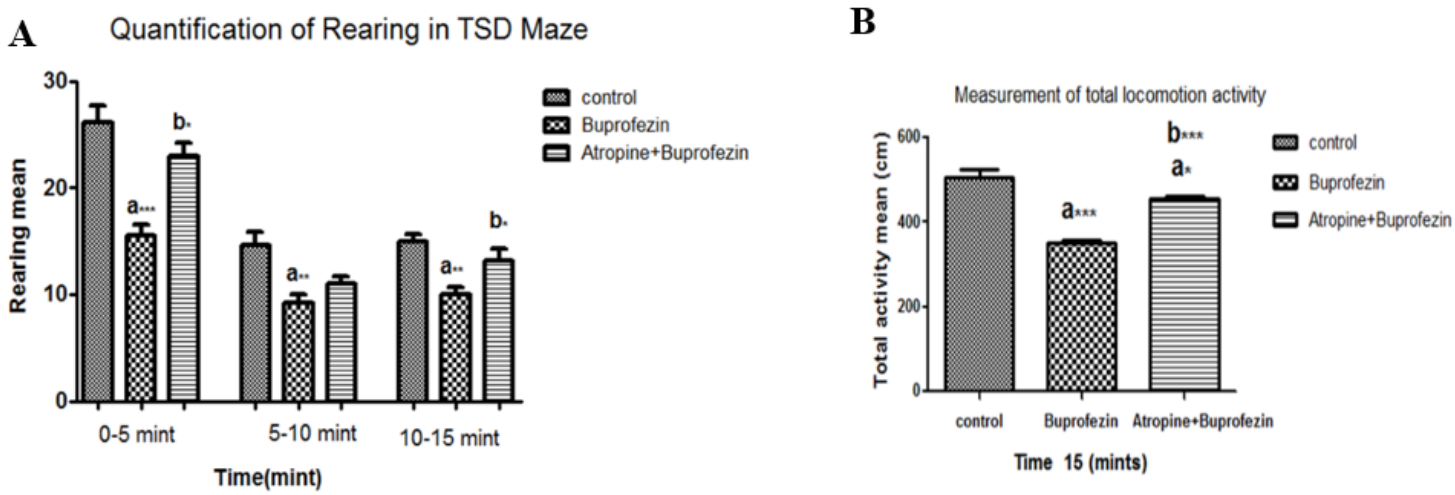

Figure 5

A. Demonstrated the significant protective effect of pre-treated atropine against buprofezin induced anxiety (less no of rearing) in TSD maze for 15 minutes duration. P value *p<0.05, **P<0.01***p<0.001 B. Represent the substantial reversal of total locomotion activity by pre-treated atropine caused by acute buprofezin exposure in 705cm long TSD maze. *p<0.05, ***p<0.001

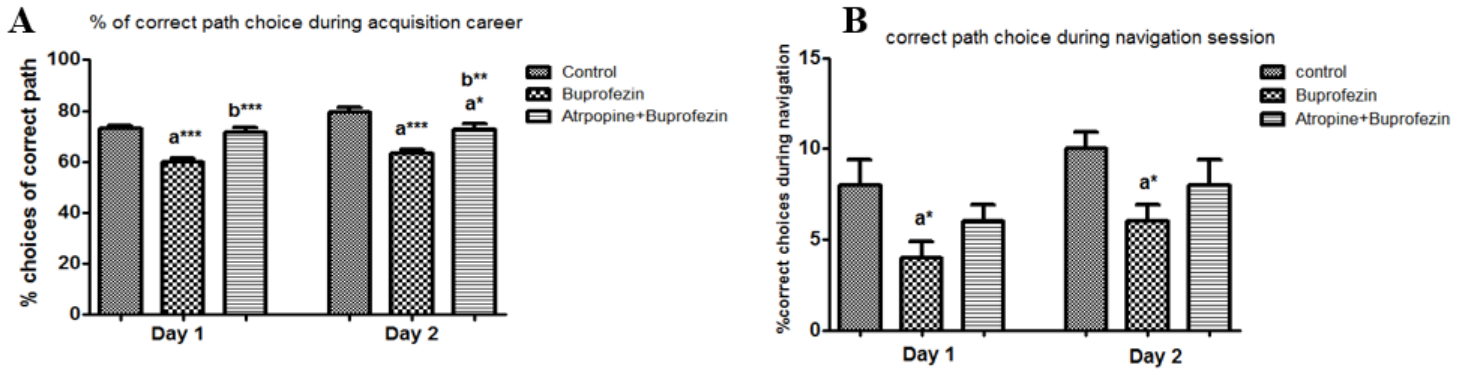

Figure 6

A. Showed the % of correct path choice during acquisition session and reversal of buprofezin induced incorrect path choices by pre-treated atropine in adult male rats. Pre-treated atropine has shown to increase % of correct path choice. P value *p<0.05, **P<0.01***p<0.001.B. Represent the reduced % of correct path choice during navigation on day 1 and its counteraction by pre-treated atropine. Correctness of path selection is increased with training on day 2. *p<0.05

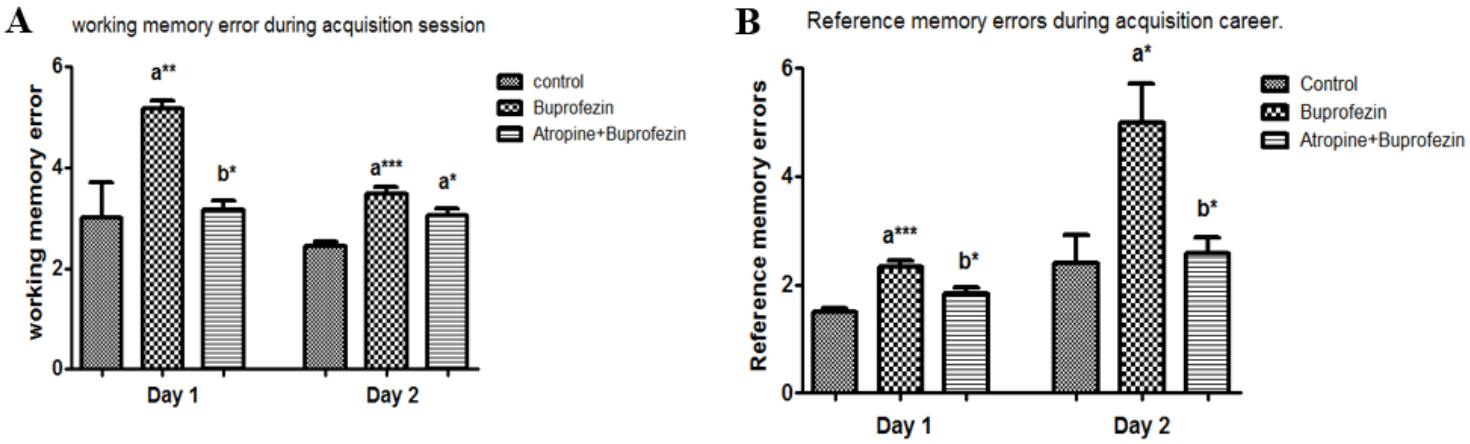

Figure 7

A. Revealed the significant reduction of working memory error (WME) during acquisition session with pre-treated atropine induced by acute exposure of buprofezin. P value *p<0.05, **P<0.01***p<0. 001.B. Describe the attenuation of reference memory errors (RME) caused by acute exposure of buprofezin with pre-administration of atropine. Memory errors reduced continuously from day two to onwards. *p<0.05, ***p<0.001

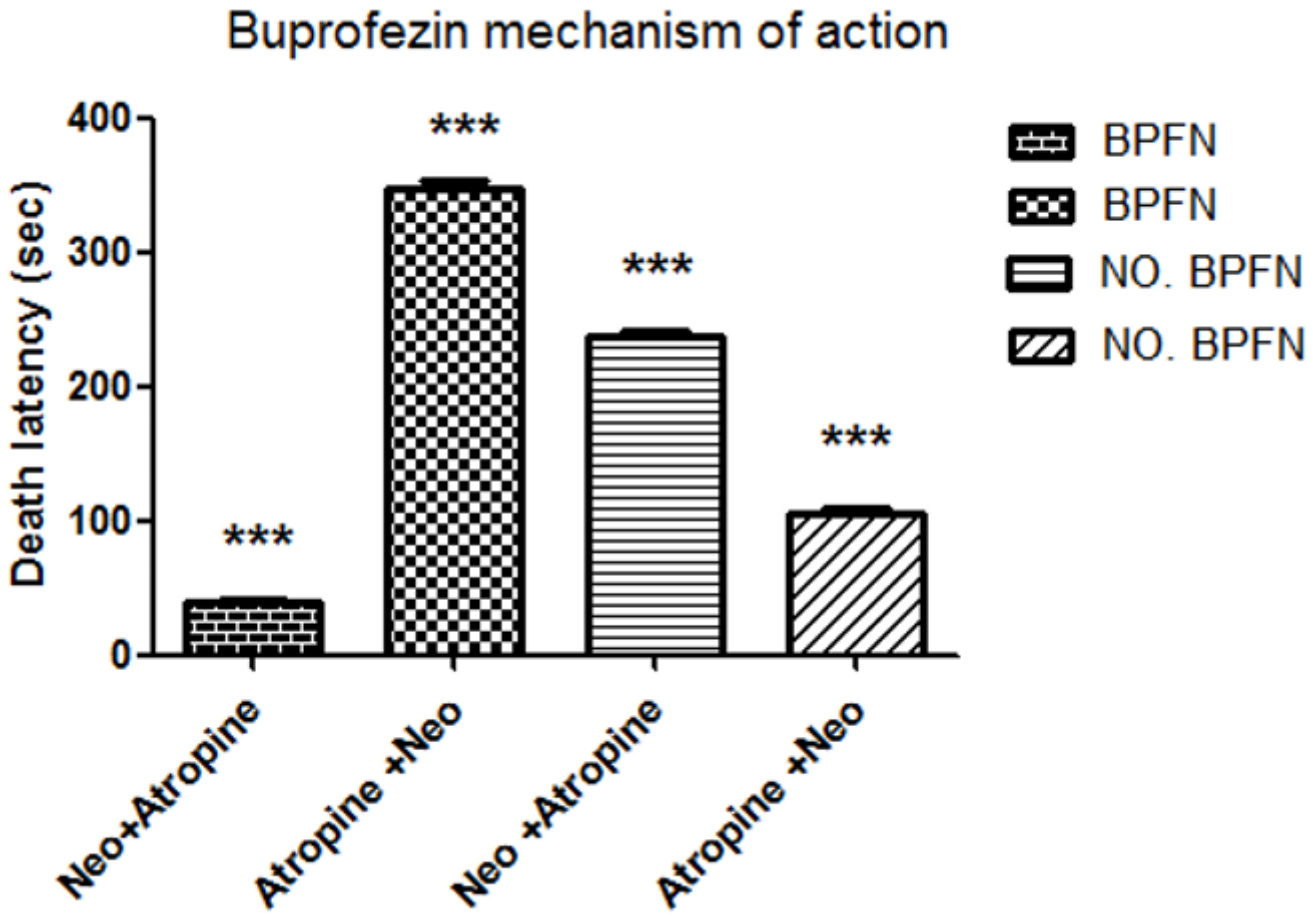

Figure 8

Showed the latency of death following the alternative exposure of neostigmine plus atropine in buprofezin intoxicated and non-intoxicated rats. P value <0. 001.significant latency difference was exhibited by each group. Data was expressed as mean ±SEM