



## Effects of Melatonin on Behavioural Activities in Acetaminophen-Induced Autism in Rat

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### ABSTRACT

The present study aims to investigate the effect of acetaminophen in male rats after early postnatal exposure to acetaminophen on several behavioural alterations and its role to precipitate autistic-like behaviour with investigating the ameliorating role of melatonin on these parameters. Thirty healthy Albino male rat pups were used in this study. On a postnatal day 3 (PND3), the rat pups were divided into three groups (10 pups per group). Group1: As a control group, pups injected with normal saline for 7 days subcutaneously then administered with normal saline for 2 weeks orally. Group 2: pups injected acetaminophen subcutaneously in a dose (50mg/kg) once daily for 7 days, then these pups were administered with normal saline for 2 weeks. Group 3: includes pups that were injected acetaminophen subcutaneously in a dose (50 mg/kg) once daily for 7 days then administered with melatonin (10 mg/kg) for 2 weeks. Behavioural tests, such as nest seeking, were performed at PND 9; negative geotaxis test was done at "PND 15 and 17", an open field test was done at PND21. The present study demonstrated that acetaminophen exposed rat pups exhibited a significant difference in behavioural tests: nest seeking test showed that the pups of group 2 to approach the maternal bedding was (10.00±1.51) sec which was significantly higher than the time of the control group(4.50±2.19) sec. At PND15, the negative geotaxis test showed a significant increase in time spend to rotate 180° of the pups of group 2 (7.30±2.86) sec when compared with that of the control group (3.50±0.63)sec and melatonin treated group (4.56±0.63) sec. While at PND17, pups showed a significant increase in time spend to rotate 180° in negative geotaxis test in group 2 (7.40±2.90) sec compared with that of the control group (3.12 ±1.33) sec, and that of the melatonin treated group (4.18 ±1.43) sec. Data of open field test showed that pups of group 2 were traveled significantly more total distance (number of squares crossed) (31±8.34) compared to those traveled by pups of control (20±4.08) and melatonin treated groups (22±7.43). Administration of acetaminophen at dose 50 mg/kg from PND 3 to PND10 induces autistic-like behavioural manifested by several alterations in rat pups. These alterations were ameliorated by melatonin administration (10 mg/kg for 2 weeks) after exposure to acetaminophen.

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### INTRODUCTION

Autism is defined as a certain set of behaviours and is a "spectrum condition" that affects people differently and to varying degrees. While there is currently no known single cause of autism, early diagnosis helps a person receive the support and

services they need, leading to a quality life filled with opportunity (Pelsóczy, *et al.*, 2020). Some of the suspected risk factors for autism include: having an immediate family member with autism, genetic mutations, being born to older parents, low birth weight, metabolic disorder, exposure to heavy metals and environmental toxins, a history of viral infections,

and fetal exposure to the medications such as valproic acid, thalidomide or acetaminophen (Baker and Shaw, 2021).

An acetaminophen usage in newborns and young children may be considerably more strongly related to autism than its use during pregnancy. Thus, increasing acetaminophen exposure, exacerbated by inflammation and oxidative stress, is one explanation for the increased prevalence of autism (Parker, et al., 2017). Cellular stress and cytotoxicity can be produced by oxidative stress, caused by a redox state imbalance. Increased oxidative stress has been linked to various neurological diseases (Bjørklund, et al., 2020).

The use of natural products and antioxidants substances, such as melatonin, in the treatment of autistic behaviours has attracted a great deal of attention (Bittker, 2016). Melatonin is a neurohormone produced mostly by the pinealocytes in the pineal gland and is widely known for its role in controlling the circadian sleep-wake rhythm. It is formed from tryptophan, which is hydroxylated and subsequently decarboxylated to make 5-hydroxytryptamine or serotonin. Melatonin is of interest in autism spectrum disorder (ASD) because of its role in neurodevelopment abnormalities. Melatonin physiology may be involved or closely associated with the pathophysiology and behavioural expression of autistic disorder and reports of sleep-wake rhythm problems in people with autism (Lalanne, et al., 2021).

Melatonin has been demonstrated to affect basal metabolism, oxidative stress, inflammation, apoptosis, and prevent premature aging and cancer at a practical level. This strengthens interest in the research of melatonin in developmental disorders (Kubatka, et al., 2018).

Case-control research found that taking acetaminophen early in life is linked to an elevated risk of ASD. When all children were evaluated, the investigators found that children who used acetaminophen at the age of 12 to 18 months were eight times more likely to develop ASD than those who did not. However, current research is insufficient to explain how acetaminophen causes alterations in the brain during development that led to developmental disorders (Rigobello, et al., 2021).

The present study aims to investigate the effect of melatonin on autistic-like behavior induction by acetaminophen and study the ameliorating effect of melatonin on behavioural alterations induced by acetaminophen.

## MATERIALS AND METHODS

### Modeling Experiment:

Ten pregnant Albino rats were obtained from Animal House at the College of Veterinary Medicine, University of Mosul, Iraq. The animals were kept in plastic rodent cages (28x22x18) cm with wire mesh covers.

Homogenized wood shavings were employed as bedding at ambient temperature ( $22^{\circ}\text{C}\pm 2^{\circ}\text{C}$ ) and humidity ( $55\%\pm 5\%$ ). Food and water *ad libitum* were supplied in standard light condition (12-hour light/12-hour dark cycle) (Sztainberg and Zoghbi, 2016; Dawood et al., 2020). Pregnant rats were checked every morning by a veterinarian for their general health and delivery of animals. When the newborn pups were seen, that day was considered as PND 0 (Fig. 1). At PND0, male rat pups were selected for experimental study and not the females to avoid hormonal effects in females (Jeon et al., 2018). All investigations were carried out in conformity with the institutional animal research ethics committee's requirements.



Fig. 1: A photograph of pups that were used in this work.

### Medications:

Acetaminophen ampoules (London medical, UK). Each ampoule contains 120mg /ml of acetaminophen. Melatonin tablets (Win pharmaceuticals, Canada). Each tablet contains 5 mg of melatonin.

### Experimental Groups and Procedures:

At PND 0, male rat pups were divided randomly into three (3) experimental groups and housed as ten animals per group (Philippot, et al., 2017) as following:

**The Control group** (n=10) includes pups injected subcutaneously with normal saline in the dorsal neck region once daily from PND3 to PND10. They received normal saline orally for 2 weeks to encounter the same conditions as other groups.

**The Acetaminophen group** (n=10) includes pups injected subcutaneously with a similar approach with

(50mg/kg) of acetaminophen once daily from PND3 to PND10 to precipitate autistic-like behavior. Then they received with normal saline orally for 2 weeks to encounter the same conditions of other groups (Philippot, *et al.*, 2017).

**The melatonin-treated group** (n=10) includes pups who were injected subcutaneously with a similar approach with (50 mg/kg) of acetaminophen once daily from PND3 to PND10 to precipitate autistic like behaviour (Philippot *et al.*, 2017). They were treated with melatonin (10mg/kg) orally for 2 weeks from PND11 to PND25 (El-Ansary, *et al.*, 2016).

The dose of acetaminophen injected to pups depend on this equation (Nair and Jacob, 2016) :

$$\text{Animal dose (mg/kg)} = \text{Infant dose (mg/kg)} \times \frac{\text{Infant (Km)}}{\text{Animal (Km)}}$$

The correction factor (Km) is a stable value equal to 20 in infants and 6.0 in rats. The acetaminophen dose of the infant was chosen as applied in the clinical treatment protocols (15mg/kg) (Temple, *et al.*, 2017; Asymida, *et al.*, 2020). The gavage needle was properly attached to the insulin syringe. It was introduced out of the side of the mouth with the observation of the mouth roof (Figs. 2A,B). Then melatonin was administered into the esophagus toward the stomach once the needle passed to the right length (Al-Allaf and Al-Ashoo, 2021).

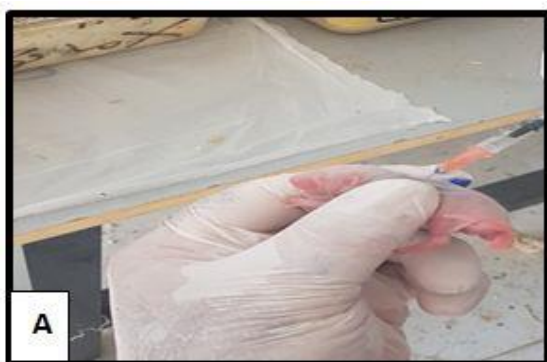


Fig. 2A: A photograph showed the procedure of acetaminophen administration.



Fig. 2B: A photograph showed the procedure of melatonin administration.

## Behavioral Tests:

Thirty male pups were used in our behavioural studies. Behavioural tests were performed during the daytime. Before the behavioural studies, animals were habituated to the place of the experiment for a minimum of one hour before the start of the behaviour tests, as follows:

### 1. Nest-Seeking Response:

On PND9, the latency to approach maternal bedding was assessed (n=10/group). Bedding from the maternal home cage and clean bedding were placed on paper at either corner of the testing cage (31.7x17.2x14.2cm) (Figs. 3A,B), and the observer recorded the latency to approach bedding using a mobile camera in the most time of observation is 60sec (Barrett, *et al.*, 2017).



Fig. 3A: A photograph showed nest seeking test.



Fig. 3B: A photograph showed the pup during test.

### 2. Test for Negative Geotaxis (NG):

The negative geotaxis test is an automatic vestibular response test used in rodents to detect the geogravitational stimuli and assess the sensorimotor ability (Figs. 4A,B). During this test, a wood platform (12.5 × 40 cm w × h) was used and is covered with rough paper. Pups of all groups were tested in this test at different PNDs (15 and 17). In this test, rat pups were maintained on a 45° inclined plane. The mean time to rotate to 180° was documented using a mobile camera (Ruhela, *et al.*, 2019).



Fig. 4A: A photograph showed negative geotaxis equipment.



Fig. 4B: A photograph showed a pup during the NG test.

### 3. The Open Field Locomotors Test:

Rats of all three groups underwent open field testing at PND 21. This test is performed to assess the degree of anxiety and locomotors activity in rodents (Taqa, 2013). The open-field test arena is a square box (50×50 cm) divided into 25 squares of identical size (10×10 cm). The box is virtually demarcated into a central zone and peripheral zones (Fig. 5).

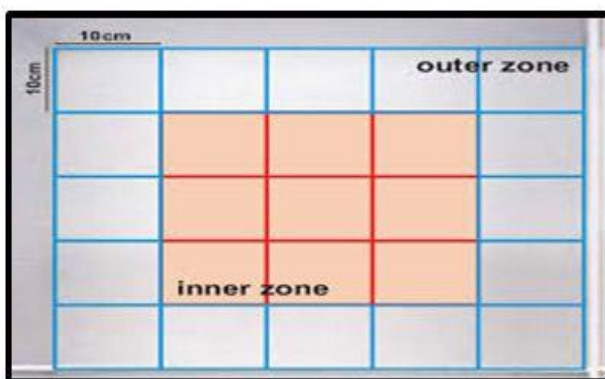


Fig.5: A photograph showed open field locomotors test.

The experimental pup was placed into the center area of the arena and allowed to explore the arena for three minutes. The video camera of the mobile measured the activity of a tested pup in the box.

The total distance that moved (total bar crossing in units) in the whole arena (outer+inner) and the total distance moved in the inner zone (centrally of bar crossing in units) were measured. The overall distance that each pup moved is considered an indicator for locomotors activity, while total distance moved in the inner zone is considered an indicator for anxiety level (Mony, et al., 2016; Wang, et al., 2020).

### Statistical Analysis:

Statistical Package Social Statistics (SPSS) version 19 was used for statistical analysis. The data were expressed as mean± SD. A one-way analysis of variance (ANOVA) with a *post-hoc* Duncan's test was used to examine the differences between groups. An independent sample *t-test* was used to examine the differences between groups in this test. Statistical analysis comparisons were considered significant at ( $p \leq 0.05$ ) (Ali and Bhaskar, 2016).

## RESULTS

### Nest-Seeking Response Results:

At PND 9 statistical analysis by independent sample *t-test* at  $p \leq 0.05$ , the time of pups of acetaminophen group to approach maternal was (10.00±1.51)sec, which was significantly higher than the time of control group (4.50±2.19)sec. (Fig. 6).

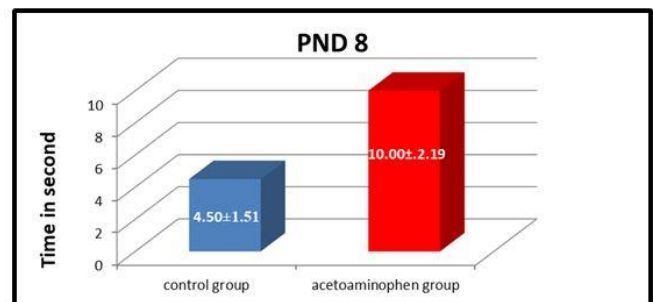


Fig.6: A histogram of the nest-seeking response data of all pups at PND9.

### Negative Geotaxis Measurements (NG):

Negative geotaxis measurements were taken in the different time courses (PNDs 15 and 17) during this work. At PND15, the ANOVA test revealed a significant difference at ( $p < 0.05$ ) in time to rotate 180° in NG test of the pups of acetaminophen group (7.30±2.86) sec when compared with that the control group (3.50±0.63)sec and melatonin group (4.56±0.63)sec, while no significant difference in time to rotate 180° in NG test between pups of both control and melatonin treated groups.

Finally, in the NG test, there is a decrease in the time taken to rotate 180° for pups of control and melatonin-treated groups at PND17. At PND17, the ANOVA test revealed a significant difference at ( $p$

0.05) in time to rotate 180° in NG test of pups of the acetaminophen group (7.40±2.90)sec when compared with that of the control group (3.12 ±1.33) sec, and that of the melatonin treated group (4.18 ±1.43) sec, while no significant difference in time to rotate 180° in NG test between pups of the control group and melatonin groups. On the contrary, the time taken to rotate 180° was increased in pups exposed to acetaminophen alone at PND17 (Fig. 7).

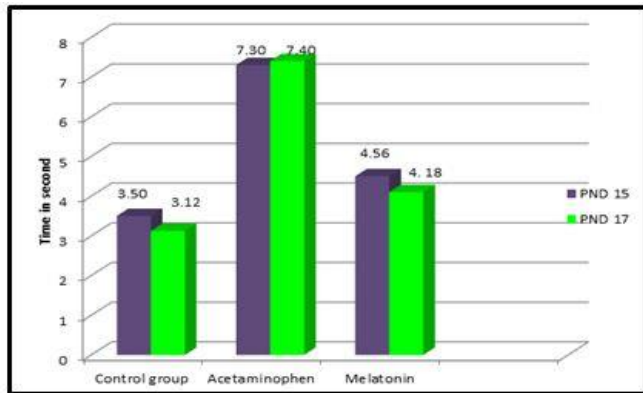


Fig. 7: Negative geotaxis data on PND 15 and 17.

### Open Field Measurements :

The result of the open-field test showed that the pups of the acetaminophen group traveled significantly more total distance (number of squares crossed) (31±8.34) compared to those traveled by pups of control (20±4.08) and melatonin treated groups (22±7.43). However, there is no significant difference between the traveled total distance by control and melatonin-treated groups. On the other hand, in the central zone, pups of the control (9.25±1.25) and melatonin treated groups (8.25±1.41) traveled a far greater distance significantly (number of squares crossed centrally) than those of the acetaminophen group (5.00±0.81). However, pups of both control and melatonin-treated groups traveled similar distances. These results demonstrate that the acetaminophen groups' locomotor activity and anxiety levels are significantly higher than control and melatonin groups (Fig. 8).

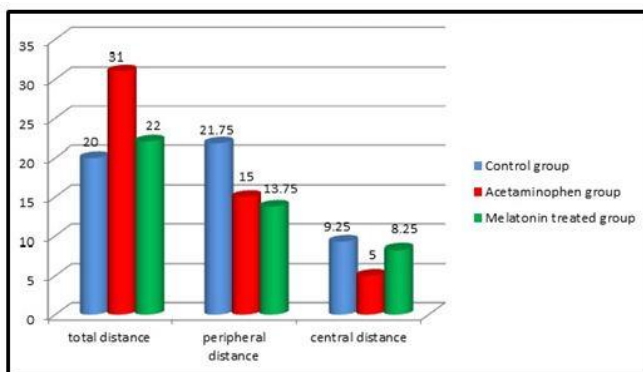


Fig. 8: A histogram of squares crossed in open field test by pups in all study groups.

## DISCUSSION

Rats are by far the most used animals in neurodevelopmental disorder studies, especially when assessing behavioral endpoints (Ellenbroek and Yoon, 2016). Animal experimentation has been one of the building blocks of biological and biomedical research, particularly in the fields of clinical medicine, pharmacology, neurodevelopmental, and toxicology (Pires, et al., 2016 and Al-Jureisy et al., 2020).

The acetaminophen model is a well-validated model of ASD (Parker, et al., 2017). Viberg et al., (2014) suggested that male rats that were exposed to acetaminophen on PND 10 altered spontaneous behavior and habituation patterns in male rats. Some studies showed that PND10 corresponds to the time around birth in humans in key developmental processes. Many studies showed comorbidities associated with postnatal acetaminophen were estimated to be about 40% of the risk of ASD among male children in the US (Philippot, et al., 2021).

In fact, the current work tried to evaluate the effect of acetaminophen on the behavioral test responses to postnatal exposure to acetaminophen in rats which are still not well understood. Multiple human and animal studies suggested that prenatal and postnatal acetaminophen use is associated with abnormal offspring neurodevelopment. The mechanism may involve disrupted endocrine function, which has been shown in animal studies to affect fetal brain development. Another possibility is that acetaminophen disrupts brain development through dysregulation of oxidative stress (Tovo-Rodrigues, et al., 2018).

In this work Nest Seeking Test (olfactory discrimination) was performed for all pups at PND 9 (Zou, et al., 2015). Pups of the control group were approached maternal bedding significantly higher than those of the acetaminophen group (impaired in nest seeking responses). These observations are in accordance with those of Saeedan et al., in 2018, who suggested that impaired in nest seeking responses in pups that were exposed to acetaminophen postnatally may be due to acetaminophen alters neural circuits in brain regions that are implicated in ASD, such as the amygdala a collection of cells near the base of the brain (Saeedan, et al., 2018). There are two, one in each hemisphere or side of the brain. The amygdala undergoes significant functional, morphological, and physiological maturation during the first two weeks of pups' development. Insult and disruption of these circuits during this period can dramatically alter socioemotional behavior and lead to behavioral

abnormalities and impaired to recognize the nest (**Barrett, et al., 2017**).

Amygdala abnormalities in the structure and neuronal density during social tasks have been reported in patients with ASD (**Ko et al., 2015**). Similar results were reported by Klein *et al.* in 2020, who suggest that the evaluation of sensory functions indicated impaired olfactory discrimination. In general, impairment of nest-seeking behavior may be caused by: 1) motor deficit; 2) reduction of social interest by the smell of mother and siblings or 3) decreased perception and processing of any olfactory stimuli (**Klein, et al., 2020**).

The second behavioral test was NG. It is the upward migration of rats on an inclined plane. As a result, NG is acting as a major behavioral tool for evaluating neurodevelopmental illnesses, potentially useful even at a young age when other behavioral tests are unavailable. As a result, the goal of our research was to assess the role of NG as an early behavioral diagnostic of autism (**Lefter, et al., 2020**). It is an automatic unlearned response and directional movement against gravitational signals that aid in the study of sensory or proprioceptive function as well as the evaluation of motor development (reflexes) activity, visuospatial development and vestibular activity (**Liu, et al., 2015**)

In our study, there is a significant increase in the rotation time 180 degrees among the pups of the acetaminophen group compared to those of control and melatonin-treated groups. These findings were similar to Ghanem *et al.*, in 2016 who confirmed that acetaminophen has direct neurotoxic effects on rat brain neurons *in vitro* and *in vivo* through his work and also suggested this toxic effect may be due to acetaminophen metabolism by the cytochrome P-450 CYP2E1 isoform which generates a highly reactive metabolite N-Acetyl-P Benzoquinoneimine. This highly reactive compound is conjugated with GSH, producing a decrease in glutathione levels in neurons that leads to neuronal death (**Ghanem, et al., 2016**).

In the early behavioral investigation, NG is important as a controller of vestibular functions in the developmental sequence after sensory function starts. This is an innate postural response that enhances in the early age of rats. Actually, NG has been validated in many neurological and neurodevelopmental conditions such as decline recurrent seizure in neonates, infantile spasm, ASD, and others (**Ni, et al., 2015**). The geotaxis development in the acetaminophen rat model of ASD with more data collected over time. It was used in the ASD animal model to examine mesocortical

dopaminergic activity. The mesocortex structurally transitional between the neocortex and the allocortex includes the cingulate gyrus, fascicular gyrus, and isthmus. The allocortex and mesocortex incorporate the limbic lobe, an artificial construct formed from parts of other lobes and located on the medial aspect of the hemisphere, where it forms a ring around the corpus callosum and rostral brainstem (**Wang, et al., 2021**).

Another study by **Blecharz-Klin et al., (2016)** demonstrated that postnatal acetaminophen exposure to rats results in changes in the level of catabolic end-products of serotonin (5-HIAA) and noradrenaline (MHPG) degradation because of acetaminophen on monoaminergic neurotransmission in the cerebellum. Since the cerebellum has an important role in motor control, any cerebellum dysfunction often presents with motor signs. It is active in coordination, precision, and timing of movements and motor learning. This may be the reason why acetaminophen increase in time taken to rotate 180 degrees. While **Saeedan et al., (2018)** suggested that the reduction of the Purkinje cell in the cerebellum was observed in rats exposed to acetaminophen may adversely affect the normal functions of the cerebellum and lead to a significant reduction in performance on NG test by rats exposed postnatally to acetaminophen. **Jaswal et al., (2019)** suggested there has been no evidence reported to date indicating that acetaminophen alters attentional processes such as those associated with visual target identification, for example, processes impacted by off-task attentional states that precede more evaluative stages of analyses in the afferent stream of visual stimulus processing.

Regarding the melatonin-treated group, there is a significant difference in values between pups exposed to acetaminophen alone and pups of the melatonin-treated group in time taken to rotate 180 degrees. This study showed improvement in values of the melatonin-treated group during NG performance. These findings are in accordance with **Bazrgar, et al., (2015)** who observed that exposed to lead (Pb) may impair the motor activities of rats and suggested that melatonin treatment can improve motor deficits and oxidative stress protecting the cerebellum against Pb toxicity. Furthermore, **Motallebzadeh, et al., (2020)** suggest that melatonin showed a capacity for neuroprotective activity in treating irradiation-induced oxidative stress and apoptosis in the brainstem of rats; however, the use of melatonin as a neuroprotective agent in humans requires further study, particularly clinical trials.

In this work, Open-Field Test was performed to assess the locomotors activity and anxiety-like behaviour in pups of control, acetaminophen and melatonin-treated groups. Our observation indicated

features of alteration in locomotors activity (hyperactivity) during the open-field test as the total distance was greater in rats exposed to acetaminophen compared to those of control and melatonin-treated rats.

These findings are consistent with those of other researchers (Mony, *et al.*, 2016). The explanation of this feature may be due to many reasons. At the beginning and according to the anatomy of central motor structures which including the motor cortex, cerebellum, basal ganglia, and spinal cord, Brain-Derived Neurotrophic Factor exerts both neurotrophic and direct electrophysiological effects via a high-affinity tyrosine receptor kinase B receptor and a common low-affinity p75 neurotrophin- receptor (Ueda, *et al.*, 2020). The loss of BDNF usually leads to neurodegeneration in these motor centers and eventually results in several severe motor diseases such as vestibular syndrome (Mandel, *et al.*, 2009).

Many studies confirmed that compounds that act on cannabinoid systems such as acetaminophen lead to alteration BDNF level in the brain region; this may explain the alteration in motor activity that occurred in the acetaminophen group. Our findings are in accordance with those of other (Stergiakouli, *et al.*, 2016). In addition, some studies observed significant alteration on various neurotransmitters (e.g., serotonergic, noradrenergic, dopaminergic and neurotrophic) following developmental exposure to acetaminophen, which leads to alteration motor activity (Essawy, *et al.*, 2017).

On the other hand, the values of the open field test showed that the rats exposed to acetaminophen alone traveled to the central zone less than those in the control and melatonin-treated groups. These results demonstrate that anxiety levels in acetaminophen groups are significantly higher than control and melatonin-treated groups. Brain regions such as the amygdala, hippocampus and cortex, directly involved in the regulation of emotional behaviour, contain high densities of CB1 receptors, which are responsible for the analgesic effect of acetaminophen and responsible for the anxiogenic effect of acetaminophen (Wang, *et al.*, 2020). Chen *et al.* (2018) suggested that acetaminophen increases anxiety-like behaviour and impairs recognition memory in mice. It was mediated by the activation of the endocannabinoid system, as a cannabinoid type-1 receptor antagonist reversed it compared to controls.

This study showed an improvement in the parameters of open field test after melatonin administration; these findings are similar to Genario, *et al.*, (2020) who reported in an animal study that

melatonin increased levels of gamma-aminobutyric acid (GABA) in certain parts of the brain. Higher GABA levels can have a calming effect and reduce anxiety symptoms (Genario, *et al.*, 2020).

## CONCLUSION

The exposure of pups to acetaminophen induces behavioral alterations such as delay to approach the maternal bedding, increase in time taken to turn 180 degrees, an increase in locomotors activity, lowering in the functional memory outcomes, have a higher percentage of side-preference rates, and disruption in the social behavior compared to control and melatonin treated groups. All these alterations indicating precipitate of autistic-like behaviour, were reduced by giving melatonin.

## Declaration of Conflicting Interests

The authors revealed that there was no potential conflicts of interest.

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