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FLUOXETINE VS. ACETAMINOPHEN IN DEVELOPING ANIMAL AUTISM MODEL: A COMPARATIVE ANALYSIS

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Abstract

Autism Spectrum Disorder (ASD) includes difficulties in social interaction, communication, and the presence of repetitive behaviours and interests. While the mechanism of ASD remains unclear, a combination of genetic and environmental factors, alongside disruptions in neurotransmission systems, might play significant role in its pathophysiology. Researchers striving to comprehend the mechanics of ASD, utilize animal models through prenatal exposure of drugs like fluoxetine, valproic acid and acetaminophen to induce ASD-like behaviours in resultant offspring.

This study for the first time compares the effectiveness of fluoxetine and acetaminophen in developing animal models of autism, providing insights into which drug better elicits ASD-like behaviours. Three groups of Wistar Rats were used for the study i.e. Saline group, fluoxetine (FLX) and Acetaminophen (ACET) in which changes in the developmental parameters were assessed by comparing body weight, length and eye opening according to standardized protocol. While behavioural assessment tests include negative geotaxis, surface righting, nest seeking response, forelimb grip strength and swimming performance.

In the current study, highly significant changes were observed in motor function of FLX group in negative geotaxis test at PND 6 and 10. However, in the surface righting reflex ACET group displayed significant improvement in the re-orientation time at PND 9. In the other battery tests like nest seeking, swimming and forelimb grip strength test FLX group take more time to compete their task as compare to control, while ACET exhibited significant increase in the time to perform the forelimb grip strength task.

In conclusion, while fluoxetine may exhibit greater efficacy in inducing autism-like behaviours in animal models however it's chronic and high-dose usage raises concerns regarding elevated morbidity and mortality rates. Conversely, acetaminophen may offer a safer alternative in terms of its

potential for chronic administration, although its efficacy in modelling ASD-related phenotypes may be comparatively lower.

Key words: Autism spectrum disorders, Negative geotaxis, Surface righting reflex, Nest seeking response, swimming performance.

Introduction

Autism spectrum disorder (ASD) stands out as a prevalent condition marked by challenges in communication and social interactions, as well as the presence of repetitive behaviours and interests. ASD is an abbreviation derived from "autism," which originally comes from the Greek word "autos," meaning self-isolation (Campisi *et al*, 2018). As far as disease burden concerns, most recent data reports autistic occurrence rate greater than 2% and the overall estimated cost spent on autistic patients was $268.3 billion in 2015 only in USA (Leigh, and Du, 2015). To better understand the unknown brain mechanism behind ASD, various animal models are widely used.

While assessing the effectiveness of fluoxetine and acetaminophen in establishing preclinical autism models, several factors are under consideration. Both drugs have demonstrated the ability to elicit ASD-like behaviours in animal studies, through well-defined mechanisms. Fluoxetine primarily acts on serotoninergic pathways (Sghendo and Mifsud, 2012). Whereas acetaminophen may impact neurodevelopment via modulation of oxidative stress and inflammation (Parker *et al*, 2017). However, the potency of each drug in inducing ASD-like phenotypes may vary based on factors such as dosage, timing of exposure, and species-specific variations. Furthermore, it is crucial to evaluate the relevance of each model to human ASD pathology, as ensuring translational validity is essential for extrapolating findings to clinical applications (VeenstraVanderWeele *et al*, 2023). Fluoxetine, is a selective serotonin re-uptake inhibitor (SSRI) primarily used to treat depression and anxiety disorders. Its role in the development of animal models of autism emerged from its ability to modulate serotonin levels in the brain, which are implicated in the pathophysiology of ASD (Glover and Clinton, 2016). Several studies have demonstrated the utility of fluoxetine in inducing ASD-like behaviours in rodents, including repetitive behaviours, social deficits, and altered sensory processing. Additionally, fluoxetine has been shown to affect neurodevelopmental processes, contributing to its relevance in modelling ASD (Liu *et al*, 2021).

Acetaminophen, a widely used analgesic and antipyretic medication, has also been investigated for its potential role in the development of animal models of autism. Although its mechanism of action differs from fluoxetine, studies have suggested a link between prenatal or early-life exposure to acetaminophen and an increased risk of ASD in humans (Alemany *et al*, 2021; (Rigobello *et al*, 2021). Animal studies exploring the effects of acetaminophen exposure have reported alterations in neurodevelopment, behavioural abnormalities, and deficits in social interactions, mirroring key features of ASD. The ability of acetaminophen to modulate oxidative stress and neuroinflammation further supports its candidacy as an ASD-inducing agent in animal models (Bührer *et al*, 2021).

In the present study, different functional battery tests were performed to evaluate the deviations of rodents from the typical ontogeny. Negative geotaxis, is a reflexive response used frequently for evaluating the behaviour of young rodents. This response is valuable for studying sensory and proprioceptive functions, offering insights into motor development, reflexes, activity, and vestibular function during the early stages of behavioural assessments (Harper *et al*, 2022). Surface righting reflex is another means of evaluating the behaviour response and motor ability of rodents, this reflex test basically shows the development of labyrinthine and fighting mechanisms of body (Anshu *et al*, 2022). The nest seeking test was employed to evaluate the developmental stage, attachment behaviours, and reactions of pups to environmental stimulus (Kroeze *et al*, 2016). Forelimb grip test is used to assess motor function and muscle strength in animal models, typically rodents. This test provides valuable insights to understand neuromuscular integrity, coordination, and the development of motor skills (Kroeze *et al*, 2016). Swimming performance test was used to monitor motor development and the integration of a coordinated sequence of reflex responses (Sunand *et al*, 2020).

Different developmental parameters were also analyzed in the study including body weight, body length and eye opening to observe the maturation development in rat's pups after prenatal exposure with the neurotoxicants.

This study for the first time compares the effectiveness of fluoxetine and acetaminophen in developing animal models of autism, providing insights into which drug better elicits ASD-like behaviours in rat's pups by evaluating the body weight, body length, eye opening, negative geotaxis, surface righting, nest seeking response, forelimb grip strength and swimming performance at different postnatal days.

Materials and methods

Modelling Experiment

Adult Wistar rats of both sexes (weight150-180 gm), bred and raised at Animal house, Faculty of Pharmacy, Karachi University were used for the study. The rats were maintained under standard conditions and had free access to standard food and water under standard humidity and light conditions (12 hr light and dark cycle). The study protocol received approval from the Institutional Animal Ethics Committee at Faculty of Pharmacy, Karachi University as indicated by Ethics Code: IBC KU-388/2023.

Pregnancy Determination

For the determination of pregnancy, female rats were paired overnight with males in a ratio of 1:3. Pregnancy was identified through the observation of a vaginal plug and confirmed by the presence of sperm in the vaginal smear. The detection of spermatozoa in the vaginal smear was considered as the equivalent of 0.5 days of gestation (GD 0.5). Since the vaginal plug does not persist for an extended period in females, the detection of sperm in the vaginal smear was considered an excellent predictor of pregnancy. After pregnancy confirmation, females were individually housed in separate cages with ad libitum access to food and water (Ruhela *et al*, 2019).

Acetaminophen and Fluoxetine model

24 Pregnant female rats were divided into three equal groups of 8 animals each. The FLX group received subcutaneous injections of Fluoxetine at a dose of 10 mg/kg (Svirsky *et al*, 2019) while ACET group was administered acetaminophen 15 mg/kg orally (Blecharz-Klin*et al*, 2015) starting from gestational day 1 and continuing throughout the entire pregnancy to induce autism. In contrast, the control group received saline in the same volume daily for oral route, commencing from gestational day 1 and extending throughout the entire pregnancy.

Fig. 1A: A photograph showed the procedure of Acetaminophen administration

Fig. 1B: A photograph showed the procedure of Fluoxetine administration

Grouping of pups

Females treated with Fluoxetine (FLX), Acetaminophen (ACET) and saline were permitted to rear their litters. Daily inspections for the presence of new litters were conducted twice a day, and we designated postnatal day (PND) 0 for the litter when it was first observed. The mother and the new litters were maintained together in the home cage. The rat's pups were classified into three groups:

Control group (n=12): includes rat's pups of control female that received normal saline orally during pregnancy

Fluoxetine group (n=12) includes Autistic rats pups of disease female that received Fluoxetine subcutaneously during pregnancy

Acetaminophen group (n=12) includes Autistic rats pups of disease female that received acetaminophen orally during pregnancy.

Developmental monitoring

Postnatal developmental parameters which were monitored included body weight, body length, and eye opening individually scores for each animal included in the experiment. Body weight data were recorded on PNDs 1, 5, and 10 for each pup. Body length measurements were taken from nose-to-rump on PNDs 4, 5, 6, 8, and 10 (Ruhela *et al*, 2019). For eye opening, a specific scoring system was followed, where 0 represented both eyes closed, 1 indicated one eye open, and 2 denoted both eyes open. Eye opening was assessed on PNDs 12 to 16 (Schneider and Przewłocki, 2005).

Fig 2A: A photograph the body weight

Fig 2B: A photograph showed the body length of rats pups

Fig 2C: A photograph showed the showed eye opening of rat pups of rat's pups.

Behavioral tests

Thirty rat's pups were used in our behavioural studies. The suitable time for performing the behavioural test was daytime. At least one hour before the tests started, animals were allowed to habituate to the pace of experiment. The following behavioural test was performed in our studies:

1. Negative geotaxis

Negative geotaxis (NG) is an automatic response used for evaluating sensory motor proficiency in pups. The offspring of the Fluoxetine (FLX), acetaminophen (ACET) and the control group involved in the test at different postnatal days (PNDs 6, 10, 15, and 17). During the test, rat pups were placed on a 45° inclined plane, and the meantime taken to rotate 180° was recorded. If the pups were taking more than 6o sec to rotate 180° then the test stop and restarted again from 0 sec (Ruhela *et al*, 2019).

2. Surface righting

The surface righting reflex serves to assess the motor ability of rat pups to return to an upright position on all four feet after being placed in the supine position. Each pup was gently positioned on its back, with all four limbs facing outward, and held for 5 seconds. The time taken by the pup to touch the ground with its four limbs was recorded. If a pup took more than 60 seconds, the stopwatch was stopped, and the pup was returned to its normal position. Surface righting reflex tests were conducted

on postnatal days (PNDs) 5, 6, 7, 8, and 9 to observe the developmental progression of this reflex (Anshu *et al*, 2022).

3. Nest-Seeking Response:

Nest seeking test was used to access the developmental stage of, attachment behaviours, and responses to environmental cues of pups. On postnatal day (PNDs 9-11), we evaluated the time taken by pups to approach maternal bedding. In the testing cage (31.7x17.2x14.2cm), one end of the container held a bin filled with clean bedding, while the other end contained a bin filled with bedding from the home cage. A 3 cm^2 area at the centre of the screen was demarcated. Lines were drawn above each bin on the screen. Each pup was positioned in the central demarcated region on the screen, and the time it took for them to enter the home bedding side by crossing the designated line with their front paws and head was recorded with a maximum observation time of 60 seconds (Kroeze *et al*, 2016).

4. Forelimb grip strength

This test used to evaluate the forelimb grip strength, serving as a measure of muscle strength in the pups. The Wire Suspension Test was performed on postnatal days 14, 17, and 22. It involved suspending a steel wire (20 cm long by 0.2 cm thick) between two poles positioned 25 cm from the floor. Each pup was gently held at the base of its tail and lifted above the wire until it grasped the wire with both forepaws. Subsequently, the pup was gradually lowered and released. The time it took for the pup to fall, within a maximum duration of 15 seconds was documented (Kroeze *et al*, 2016).

5. Swimming test

Swimming ability used to measure the motor development and the integration of a coordinated series of reflex responses. Swimming tests were conducted on postnatal days 22, 24, and 26 using an aquarium filled with water maintained at a temperature range of 28–29°C. Each animal was placed at the centre of the aquarium and observed for a duration of 5–10 seconds. Swimming performance was assessed based on the position of the nose and head relative to the surface of the water. The angle of swimming was categorized as follows: 0—head and nose below the surface; 1—nose below the surface; 2—nose and top of head at or above the surface, with ears still below the surface; 3—similar to category two, but with the water line at mid-ear level; and 4—similar to category three, but with the water line at the bottom of the ears.

Following the test, the pups were dried and returned to their home cages (Sunand *et al*, 2020).

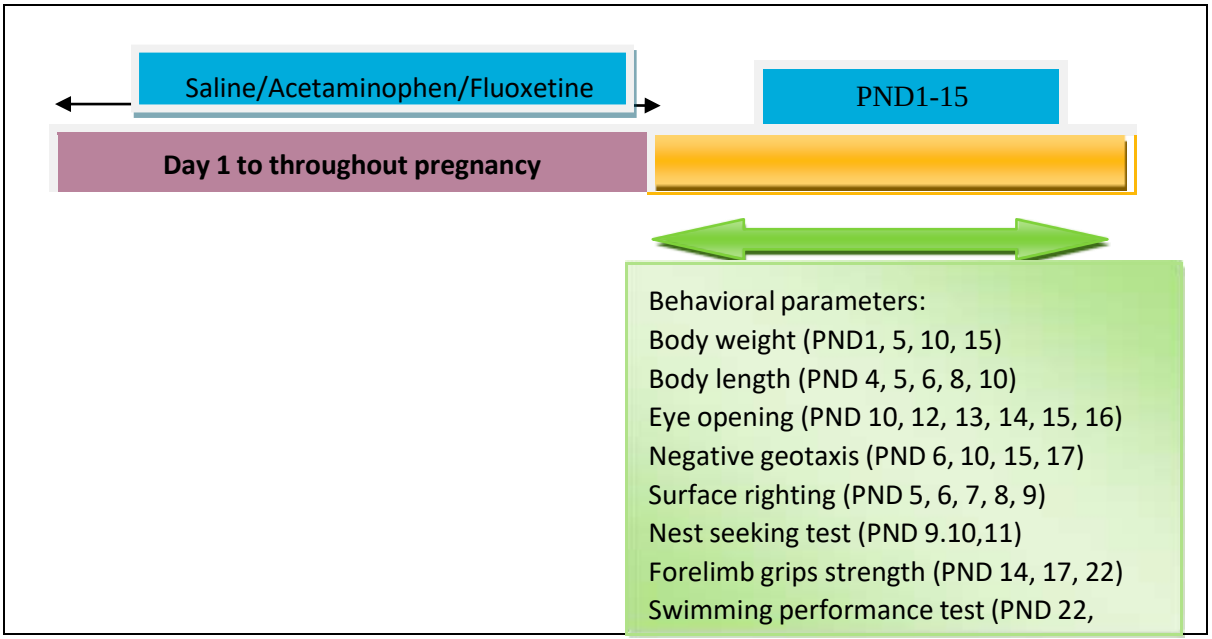

Fig 3: Experimental protocol

Statistical Analysis:

SPSS (Statistical Package for Social Sciences) version 26 was used in the study for statistical analysis. The data were expressed as mean ± SD. A one-way analysis of variance (ANOVA) with a *post-hoc* Bonferroni test was used to examine the differences between groups. Statistical analysis comparisons were considered significant at ($p \leq 0.05$) and highly significant at ($p \leq 0.001$) *(*Ali and Bhaskar, 2016). **Results**

Postnatal Growth and Maturational Development

Offspring were weighed on PNDs 1, 5, 10, 15 and 20**.** Eye opening was measured on PND 12- 16 and body length was observed on PND 4, 5, 6, 8 and 10 as shown in Table 1.

Table 1: Postnatal weight, length and eye opening of pups exposed to Saline, FLX and ACET

	Saline	FLX	ACET
	Body weight		
PND1	5.91±0.26	5.68±0.31	5.4±0.15**
PND5	6.9±0.47	6.24±1.95	6.68±0.36
PND10	9.71±0.13	9.08±0.92*	9.45±0.25
PND15	14.13±0.8	14.78±0.75	13.9±0.91
PND20	18.22±1.64	17.21±1.12	17.95±1.3
	Body length		
PND 4	4.98±0.05	4.29±0.42	5.63±0.23
PND 5	5.04±0.16	4.91±0.11	5.8±0.16
PND 6	5.41±0.25	5.3±0.15	6.04±0.11
PND 8	5.8±0.16	5.75±0.23	6.07±0.63
PND 10	6.25±0.26	6.03±0.06	6.36±0.34
	Eye opening		
PND 12	2±0	0±0	0±0
PND 13	2±0	0±0	0±0
PND 14	2±0	0±0	0±0
PND 15	2±0	0±0	0±0
PND 16	2±0	2±0	2±0

Saline: Normal rats' pups of control female rats that received Saline orally during pregnancy
ACET: Autistic rats pups of disease female rats that received Acetaminophen orally during pregnancy
FLX: Autistic rats pups of disease female rats that received Fluoxetine s.c during pregnancy Values are mean ± SEM (n=12) Significant difference by Bonferroni test.
* = Control as compared to Disease groups

Table 2: Physical data of dams and litters from pregnant rats treated with saline, ACET and FLX during gestation

	Gestational days (avg)	Total pups per litter on PND 1	Number of live pups per litter at the end of study	Pregnancy weight gain(GD20GD0/GD0)
Saline	22.3±0.32	12±0	12±0	40.88±0.9
ACET	22±0.78	11.66±0.49	11.55±0.52*	41.7±0.66*
FLX	21.18±1.24*	7.3±0.48**	5±0.42**	47.36±0.49**

Saline: Normal rats' pups of control female rats that received Saline orally during pregnancy
ACET: Autistic rats pups of disease female rats that received Acetaminophen rally during pregnancy
FLX: Autistic rats pups of disease female rats that received Fluoxetine s.c during pregnancy Values are mean ± SEM (n=12) Significant difference by Bonferroni test. * = Control as compared to Disease groups

Behavioral parameters

Effect of ACET and FLX on negative geotaxis (NG)

There was a highly significant increase in the time taken by FLX pup to re-orient 180 degree on the 45° inclined plane at PND 6 and PND 10, when compare to saline. On the contrary, the acetaminophen group has displayed no significant difference in time taken to rotate 180° as compare to saline at all PNDs.

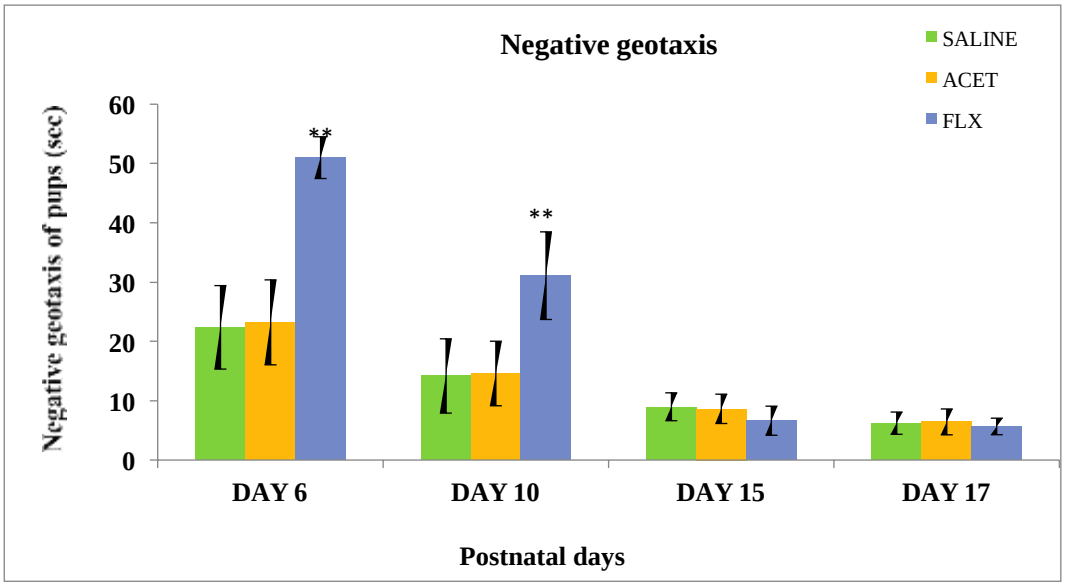

Fig 4: Negative geotaxis of Saline, Acetaminophen and Fluoxetine pups
Values are mean ± SEM (n=12) Significant difference by Bonferroni test
* = Control as compared to Disease groups $^{*}p<0.05$ were consider as significant
$^{**}P<0.001$ were consider as highly significant

Effect of ACET and FLX on surface righting reflex

In surface righting reflex there was highly significant ($p < 0.001$) increase in the re-orientation time of ACET pups at PND 9, but Fluoxetine group take less time to re-orient on their four paws, displaying highly significant ($p < 0.001$) lower surface righting reflex as compare to saline group at PND 9.

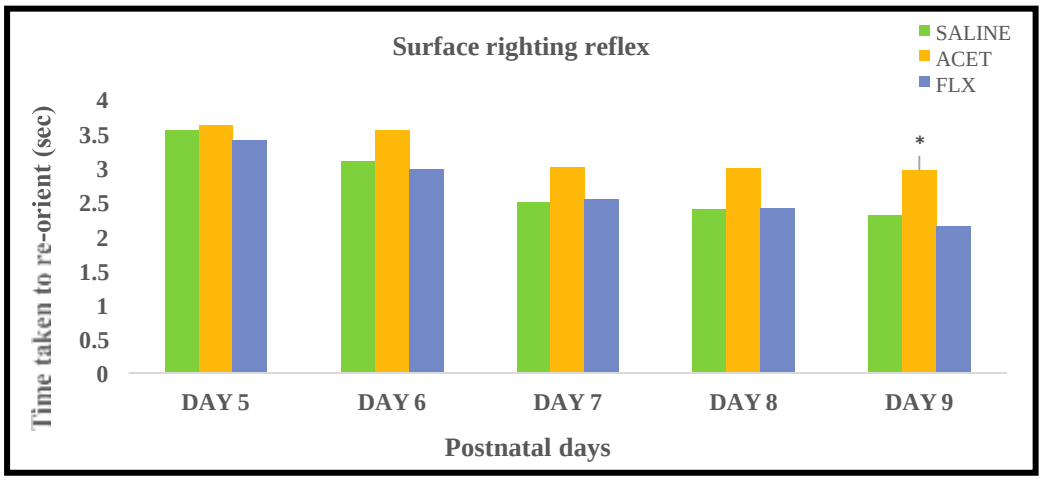

Fig 5: Surface righting reflex of Saline, Acetaminophen and Fluoxetine pups

Values are mean ± SEM (n=12) Significant difference by Bonferroni test

* = Control as compared to Disease groups * p<0.05 were consider as significant

** P<0.001 were consider as highly significant

Effect of ACET and FLX on nest seeking test:

In nest seeking response the FLX pups displayed highly significant ($p < 0.001$) decrease in the latency to reach the home bedding as compare to saline group at PND 9, 10 and 11. On the contrary, ACET group displayed no significant change in the latency to reach home bedding.

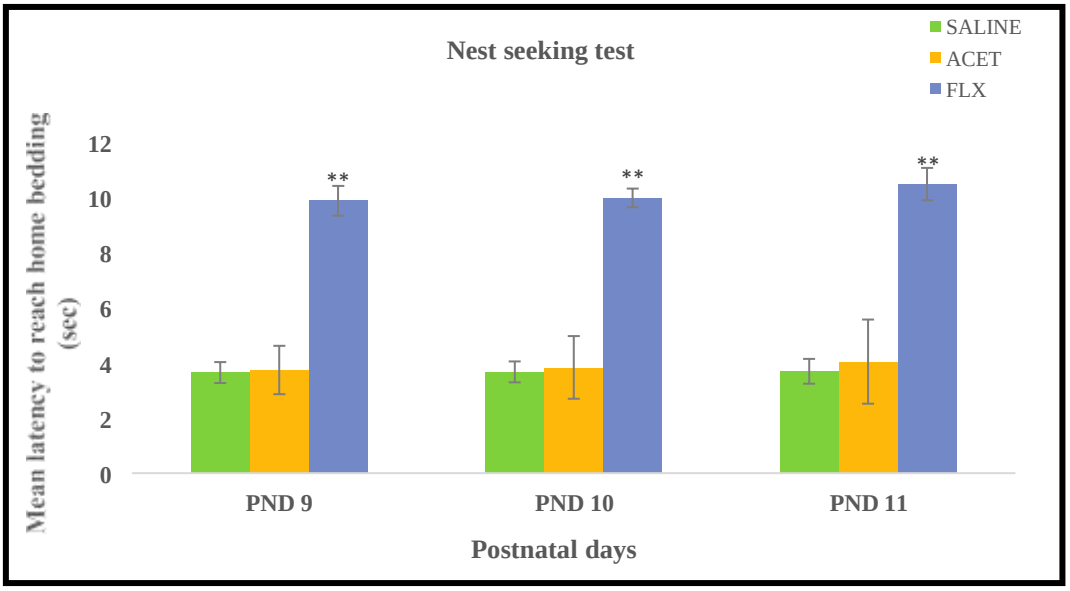

Fig 6: Nest seeking test of Saline, Acetaminophen and Fluoxetine pups

Values are mean ± SEM (n=12) Significant difference by Bonferroni test

* = Control as compared to Disease groups * p<0.05 were consider as significant

** P<0.001 were consider as highly significant

Effect of ACET and FLX on forelimb grip suspension

There was highly significant (p<0.001) decreased in the forelimb grip strength of the FLX group compared with the saline group at PND 14, 17 and 22. However, ACET group showed significant (p<0.05) decrease in the forelimb grip strength at PND 14, 17 and 22.

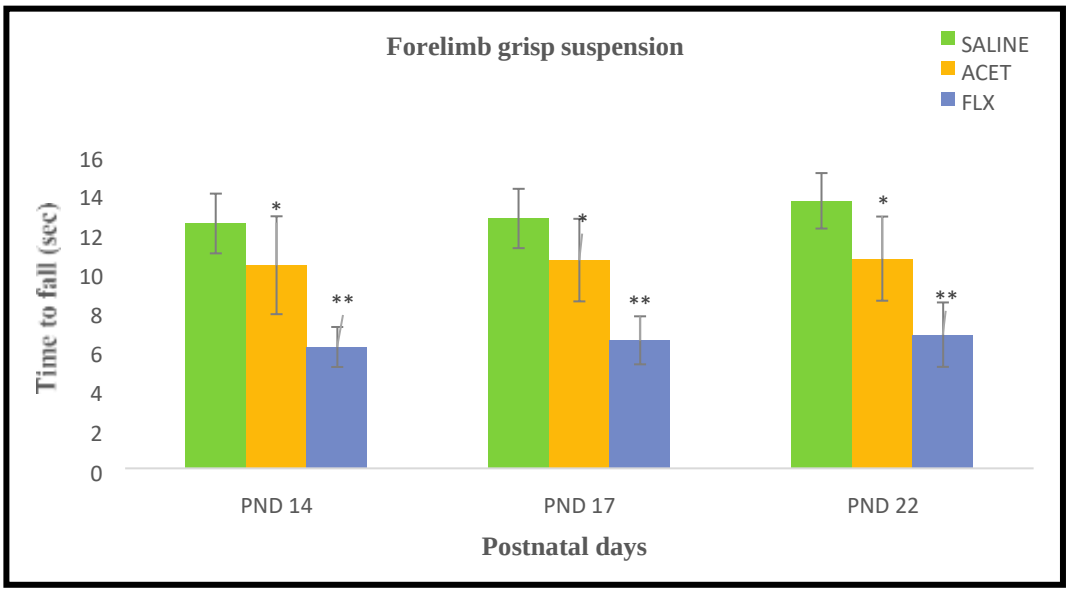

Fig 7: Forelimb grip suspension test of Saline, Acetaminophen and Fluoxetine pups

Values are mean ± SEM (n=12) Significant difference by Bonferroni test

* = Control as compared to Disease groups * p<0.05 were consider as significant

**P<0.001 were consider as highly significant

Effect of ACET and FLX on swimming performance test

There was highly significant decreased in the swimming performance of the FLX group compared with the saline group (p<0.001) at PND 22, 24 and 26. However, ACET group showed no significant change in the swimming rate at PND 22, 24 and 26.

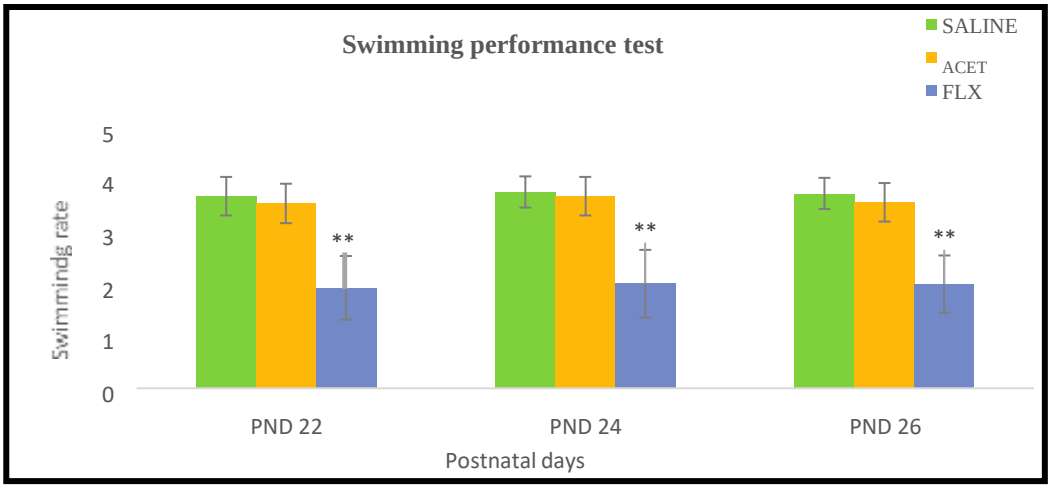

Fig 8: Swimming performance test of Saline, Acetaminophen and Fluoxetine pups

Values are mean ± SEM (n=12) Significant difference by Bonferroni test

* = Control as compared to Disease groups * p<0.05 were consider as significant **P<0.001 were consider as highly significant

DISCUSSION:

The current study conducted to analyse the effect of prenatal acetaminophen and Fluoxetine exposure in offspring's maturation development and behaviour which is still not clearly understand. Previous researches have proved that prenatal acetaminophen exposure is directly connected with the abnormalities in the offspring neurodevelopment (Taqa *et al*, 2021). As, acetaminophen is commonly used during pregnancy for managing pain and fever and increases the risk of autism spectrum disorders in the children's (Bührer *et al*, 2021). The mechanisms effecting the development of fetal brain by acetaminophen exposure may include endocrine function disruption and oxidative stress

dysregulation (Tovo-Rodrigues, *et al*., 2018). Similarly, SSRIs especially fluoxetine is the most frequently prescribed anti-depressant used in pregnancy for managing maternal depression and increases the likelihood of ASDs in offspring (Arzuaga *et al*, 2023). The mechanism responsible for this is that serotonin is very crucial neurotransmitter required for the development of fetal brain, SSRIs exposure effect the brain development through their detrimental effect on serotonin receptors, by developing hyperserotonaemia (Bhat *et al,* 2023).

The key elements of the present study were to examine the effect of acetaminophen and Fluoxetine exposure during pregnancy resulted (1) in delayed maturation of certain reflex like surface righting, negative geotaxis, nest seeking response, forelimb grip strength and swimming performance (2) change in body weight and body length and delay in eye opening.

The determination of early life body weight is a fundamental indicator of the health issues, this enables to focus on the early interventions and future disease prevention. The impact of acetaminophen exposure on the body weight and their perinatal outcomes like preterm birth and low gestational age is still under observation (de Castro *et al*, 2022). In the current study ACET treated rat's pups showed lower birth weight as compare to saline from PND1 to PND 20 but the FLX group has significantly lower body weight at PND 10 as compare to saline group. As the same results were reported in the previous researches, in which women who utilized SSRIs during pregnancy will show higher chance of giving birth to infants with low birth weight as compared to those not exposed to SSRIs. Additionally, another study reported an association between SSRIs and reduced birth weight (Hutchison *et al*, 2018). Eye opening is another developmental parameter analyzed in our study which indicates a maturational delay in eye opening of rats prenatally exposed to ACET and FLX. This delayed eye-opening time supports the notion of impaired glutamatergic synapse maturation in the superior colliculus (Zhao *et al,* 2013). The first behavioural test conducted in the present study was NG. In our study, we observed a significant delay in the time taken by pups to rotate 180-degree specifically in the offspring exposed to Fluoxetine as compared to those in the saline group especially at PND 6 and 10 showing possible damage to vestibular system. This was proven from the previous study where delay in the motor development was directly link with that long lasting prenatal SSRI exposure (Handal *et al,* 2016). However, ACET group showed no significant difference as compare to saline rats. Another test was surface righting reflex in which ACET and FLX group showed highly significant delay in the reorientation time at PND 9 as compare to saline. These outcomes confirmed the previous research which reported the in vivo and in vitro neurotoxic effect of acetaminophen on neuron of rat's brain. The neurotoxic mechanism of acetaminophen involves the formation of N-Acetyl-P Benzoquinoneimine (a highly reactive metabolite) after the acetaminophen metabolism. This reactive metabolite reduces the level of glutathione by forming conjugate with GSH, results in neuronal death (Ghanem, *et al*., 2016). Moreover, the neuroimpairment mechanism of SSRIs involved the increase in the level of serotonin and brain derived neurotrophic factor (BDNF), this results in neurogenesis especially in cortex and hippocampus region of the brain and development of serious psychological disorders like autism spectrum disorder (ASD), schizophrenia, attention deficit hyperactivity disorder (ADHD), anxiety and depression (Millard, 2019).

Swimming tests, which serve as a measure of motor coordination and activity levels, demonstrated poorer performance in individuals treated with fluoxetine, depicting the reduced swimming activity commonly observed in autistic individuals. Additionally, deficits in nest seeking behaviour, a complex social and exploratory behaviour, further reflects the social communication challenges often observed in individuals with ASD. Moreover, the diminished performance in the forelimb grip test, which assesses motor coordination and strength, reflects the motor impairments frequently encountered in autism. These results shed light on the varying effects of different pharmacological interventions on behaviours resembling those seen in autism, offering insights into potential avenues for therapeutic interventions and furthering our understanding of the disorder's underlying mechanisms (Kroeze *et al*, 2016).

In the current study, the comparative evaluation between prenatal fluoxetine and acetaminophen exposure in various behavioural tests reveals significant differences, highlighting distinct characteristics associated with autism. Fluoxetine induced autism model was well validated as

compare to acetaminophen and displayed more resemblance with autistic characteristics, as evidenced by its poorer performance in negative geotaxis, surface righting, swimming tests, nest seeking behaviour and forelimb grip tests as compared to acetaminophen.
This difference may be due to the dose of FLX and ACET administered. As, FLX administered in the highest dose of 10mg/kg while the dose of ACET was very low that is 15 mg/kg which results in more autism-like characteristic in FLX group but also reported higher mortality rate.

Limitation of the study

While animal models can provide valuable insights, they may not fully replicate the complexity of human conditions like autism. Moreover, wide range of doses was not part of our study.
Beside the behavioural test mentioned in our study more tests were also be performed.

Conclusion

The prenatal exposure of pups to acetaminophen and Fluoxetine induces alteration in behaviour parameters such as delay in eye opening, increase in time taken to turn 180 degrees, an increase in the time taken to come back on its four feet after lying in supine position, delayed performance time in nest seeking, forelimb grip strength and swimming test as compare to control group. All these alterations indicate characteristics of autistic-like behaviour in rats pups exposed to acetaminophen and Fluoxetine during pregnancy. Fluoxetine has shown a greater tendency to induce ASD-like behaviours in animal studies compared to acetaminophen, with a notable increase in mortality rates.

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