



Research Article

# Neurodevelopmental Toxic Effects of Food Additives Used in Energy Drinks on Developing Rats

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## Abstract

**Background:** Food additives are widely used in energy drinks and when taken above acceptable daily intake leads to various neurodevelopmental toxic effects. The present study aimed to evaluate the neurodevelopmental toxic effects in rat pups after pre and postnatal administration of selected food additives in pregnant animals.

**Methods:** Pregnant rats aged 160-180 days were divided into six groups with four animals per group. Group 1 treated with vehicle, group 2 standard (caffeine 25 mg/kg p.o.), groups 3-6 were treated with glucuronolactone (5 mg/kg p.o.), taurine (8 mg/kg p.o.), gluconolactone (84 mg/kg p.o.), and combination of food additives respectively till postnatal day (PND) 15. After PND 21, pups were evaluated for neurobehavioural parameters using the behavioral alteration test, Morris water maze test, locomotor activity test, Y-maze test, hot plate latency and neurobehavioural scoring. Neurotransmitters were estimated in brain tissue extract on PND 30, 45 and 60 and histological observations were examined in the brain cortex region.

**Results:** Food additive treated groups showed an increase in behavioral activity, escape latency, immobility, percentage of alterations, hot plate latency and neurobehavioural scoring at selected dose and combination compared to control ( $p < 0.001$ ). The decrease in neurotransmitter levels in the brain and marked degeneration of neurons in the cortex were observed significantly in group 6 pups.

**Conclusion:** The present results corroborate that food additives in combination induced neurodevelopmental toxic effects further mechanistic studies are suggested to understand the synergistic effect.

## Introduction

Substances of little or no nutritional value are used in the production for the storage of food or animal feed are referred to as food additives. These include antioxidants, food preservatives, food coloring agents, flavoring agents, anti-infective agents, vehicles, excipients and other correspondingly used substances. Nowadays, food and color additives are more tightly regulated than ever before.<sup>1</sup> Food additives safety study has recently raised questions about potential neurotoxic effects in the brain. The vulnerability to neurotoxins has risen significantly over the last few decades. A lot of neurotoxins are present in the food we consume, the water we drink and the air we breathe (basic requirements of everyday life is contaminated).<sup>2</sup> Neurotoxicity is the ability to cause side effects in the central nervous system, peripheral nerves or sensory organs. A chemical is neurotoxic if it may induce a persistent pattern of neuronal dysfunction or a change

in the composition or structure of the nervous system. With increasing dose, neurological changes may show up, and eventually, irreversible morphological changes are generated result in toxic effects.<sup>3</sup>

Taurine, glucuronolactone and gluconolactone are food additives present in energy drinks and they often exist as natural ingredients in food at much lower levels and are also regular human metabolites. The high chronic consumption of energy drinks results in high daily exposure to taurine, gluconolactone and glucuronolactone than the predictable mean daily exposure cause unwanted effects on the heart, kidney and brain. In adults, due to chronic habitual intake of food additives in energy drinks, soft drinks lead to several neurological disorders include migraine, seizures, endocrine disorders and neuropsychiatric disorders.<sup>4</sup> Scientific Committee on Food (SCF COMMITTEE) 1999 was uncertain of settling safe upper levels for daily intake

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of taurine and glucuronolactone due to insufficient data available. In 2003, SCF reported a new 13-week study in rats on taurine showed behavioural effects and impaired motor performance indicated neurological effects at high doses and a 13-week study in rats on glucuronolactone showed an effect on the kidneys (enlarged kidney) and gastric disturbance (diarrhea) while in hamsters, a 13-week study with glucuronolactone revealed an effect on body weight.<sup>5</sup>

EFSA, 2009 reported the daily mean chronic consumption data of humans as 0.5 cans per person and high chronic consumption as 1.4 cans per regular consumers. The amount of taurine and glucuronolactone in high chronic consumption was 23.3 and 14 mg/kg/bw/day for a 60 kg person.<sup>4</sup> Finally, EFSA 2009 concluded that exposure to taurine and glucuronolactone at the low levels does not have safety concerns at mean daily consumption. Thus, the safety concerns about the harmful effects of high chronic energy drink consumption increased. Recent publications have highlighted the overall risk and adverse effects of the overconsumption of energy drinks. But, the individual ingredients effect and their interaction was still under investigation and lacks scientific evidence. Based on the above background, the present study has been designed to systematically evaluate the neurodevelopmental toxic effects of selected food additives used in energy drinks in rats.

## Materials and Methods

### Chemicals

Glucuronolactone (GUL), gluconolactone (GL), caffeine was procured from Srineelima labs, Hyderabad and taurine (TAU) were obtained from Nutrija Lifesciences. All other chemicals were from Himedia Pvt Ltd., India.

### Animals

Sprague Dawley (SD) albino pregnant rats aged 160-180 days were obtained from the animal house of MLR Institute of Pharmacy, Hyderabad. They were housed in six groups (n=4) under standard laboratory conditions (temperature  $25 \pm 10^\circ\text{C}$ , relative humidity  $55 \pm 5\%$  and 12.00: 12.00 h dark: light cycle) with a standard pellet diet and water ad libitum. The experimental procedures were approved by Institutional Animals Ethics Committee (IAEC) as provisions of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), MLR Institute of pharmacy, Hyderabad (CPCSEA/IAEC/PR3/2019).

### Experimental protocol

All the pregnant rats aged 160-180 days were divided into six different groups and each group consisted of four animals (n=4). All the animals were treated with freshly prepared doses dissolved in water and administered through the oral gavage. All the doses were calculated based on human dose available in the literature and were converted to animal dose.<sup>4</sup> Treatment started from gestational day 3 (GD 3) and continued till postnatal day 15 (PND 15). Group 1 was

maintained with a vehicle, group 2 received caffeine (25 mg/kg p.o.) and groups 3 to 6 were given glucuronolactone (5 mg/kg p.o.), taurine (8 mg/kg p.o.), gluconolactone (84 mg/kg p.o.) and combination of food additives (5 mg +8 mg+84 mg/kg p.o. of three additives) respectively. On the day of parturition, the average litter size was 4-5 per animal. On PND 1, from each group, 12 pups were selected and subjected for observation as follows.

### Physical developmental in postnatal pups

Physical developmental parameters body weights were measured at weekly intervals whereas eye-opening and hair appearance was monitored daily until occurrence in pups from PND1 to PND 21.<sup>6</sup>

### Neuromotor maturation (PND 1 to PND 21)

Neuromotor maturation was accessed on PND 1, 7, 14 and 21 using three parameters righting reflex, cliff avoidance and rotating reflex.

#### Righting reflex

Pups were placed on the back and allowed to turn and the time taken to put all four paws on the base material was observed in 2 minutes.

#### Cliff avoidance

Pups were placed with forepaws and face over the edge of a tabletop. The time required to turn back from the cliff was recorded. The upper limit of 2 min was selected. A latency of 2 min was recorded, when the animal fell from the side of the table.

#### Rotating reflex

Animal pups were placed on an inclined surface with an angle of  $30^\circ$ , facing the surface with their heads pointing downward. The time taken by the pups to geonegatively rotate its body through  $180^\circ$  until the head turn upward, was reported as the time of rotation. The test's upper limit was also set at 2 min.

### Behavioral studies: Irwin protocol/functional observed battery test

The pups with significant physical and neuromotor maturation were selected (n=8) from each group and evaluated for behavioural studies.

#### Behavioral alterations

Grooming, Hind paw licking, and rearing were observed on PND 22 for 5 mins.<sup>7</sup>

#### Morris water maze test

Visual-spatial memory was tested using a water maze.<sup>2</sup> Rat pups (PND 30) were allowed to swim in the pool for the 60s on the first day without the platform. From day 2-5, offspring (PND 32-35) respectively were subjected to train for 24 trails (6 trails per day with an interval of 30s). The latency time to escape onto the hidden platform (maximum

trail of 120s) was noted. The number of such unsuccessful trials was calculated and stated as a percentage of failures on individually testing day. 120s probe trial test was performed by removing the platform on day 6. The time recorder was used to note the time spent in each quadrant.

#### Locomotor activity test

On PND 32, animals were subjected to a locomotor activity test. This locomotor activity test consists of an experimental wooden arena with square-shaped measuring 80×80×30 cm and the floor was divided into 64 squares of equal size. The parameters observed were the number of squares crossed, wall rears, rears, durations of locomotion and immobility. The visual observations in the arena were for 300s for each animal.<sup>6</sup>

#### Y-maze

It was used as an alternative spatial learning method using a continuous model of a simultaneous alternation. On PND 38 the animals were placed in the middle joint of a black, plexiglass Y-maze with three equal arms (56 × 10 × 25 cm) and left to be explored for 7 minutes. Arm entries were examined and consecutive re-entries into an arm were omitted and percentage alternation was calculated as the number of triads (set of three letters) containing entries into all three arms/maximum probable alterations. ANOVA tests were used to analyze the number of entries and percentage alternation.<sup>8,9</sup> Percent alternation = [(Number of alternations/ Total number of triads) × 100]<sup>10</sup>

#### Hot plate latency

On PND 32, the latency to respond after animal pups being placed on a 55 degrees centigrade hot platform was observed. Jump or licking of the hind paw was measured with a cut-off period of 2 mins.<sup>9</sup>

#### Katz protocol

On PND 31 Neurobehavioural scores were calculated for animals after treatment with a high dose of food additives and evaluated for neurobehavioural toxic effects.<sup>11</sup> Various parameters were considered and scores were given as follows in Table 1.

#### Neurotransmitter estimation

##### Preparation of brain tissue extracts

Four animals from each group (n=4) were sacrificed on PND 30, 45 and 60 by placing in the CO<sub>2</sub> chamber. Brains were isolated, weighed and placed in the ice-cooled temperature. Tissues were homogenized in 5ml HCl-butanol (0.85 ml of 37% hydrochloric acid in one liter) for about one min. The sample was further centrifuged at 2000 rpm for 10 min. 1ml of the supernatant layer was removed and added to the 2.5 ml heptane and 0.3 ml of 0.1 M HCl. After 10 minutes of vigorous shaking, the tube was centrifuged in the same conditions as above. Two layers were separated, the supernatant layer (organic layer) was discarded and the remaining aqueous layer is used for

**Table 1.** Neurobehavioral scores by Katz protocol to evaluate neurobehavioral effects.<sup>10</sup>

	Neurobehavioral effect	Scores
<b>General behavioral deficit</b>		
Consciousness	Present	0
	No attempt (coma)	20
Respiration	Normal	0
	Abnormal	20
<b>Cranial nerve reflexes</b>		
Olfactory (sniffing food)	++	0
	--	4
Vision (follow hand)	++	0
	--	4
Corneal reflex	++	0
	--	4
Whisker (movement)	++	0
	--	4
Hearing (turning to clapped hands)	++	0
	--	4
<b>Motor deficit</b>		
Leg /tail movement	Normal	0
	Stiff	5
	Paralyzed	10
<b>Sensory deficit</b>		
Leg /tail (on pinching)	++	0
	--	10
<b>Co-ordination</b>		
Beam walking (1.5 cm)	++	0
	--	5
Placing test	++	0
	--	5
Righting reflex	++	0
	--	5
Stopping at the edge of the table	++	0
	--	5
	<b>Total</b>	<b>100</b>

neurotransmitter estimation. Ice cool temperature (0 °C) was maintained throughout the experimentation.<sup>12</sup>

##### Estimation of noradrenaline and dopamine<sup>13</sup>

Two hundred microliters of the aqueous layer was taken from tissue extract to that 0.05 ml of 0.4 M Hydrochloric acid and 0.1 ml of EDTA (pH 6-9) were mixed, accompanied by 0.1 ml of iodine solution for oxidation. After 2 minutes the reaction ceased by adding 0.1 ml sodium sulfite solution. The solution was heated to 100 °C for 6 minutes. The sample was therefore cooled to room temperature and the spectra of excitation and emission were interpreted from the spectrofluorometer. These observations were recorded at 395-485 nm for noradrenaline and 330-375 nm for dopamine.<sup>13</sup>

### Estimation of serotonin

Two hundred microliters of aqueous tissue extract was added with 0.25 ml of OPT (o-phthaldialdehyde) reagent. Then, heated up to 100°C for 10 mins. After that the sample was excited to 100°C, the readings were recorded at 360-470 nm in the spectrofluorometer.<sup>13</sup>

### Histopathological studies

On PND 90, rats were sacrificed and their brains were dissected. Brain tissues were stored in 10% neutral buffered formalin and were dehydrated with graded ethanol series, then used for histopathology study. Paraffin-embedded tissue blocks were used for fixing and a thin transverse section of 5 µm was sliced using the microtome of the cortex region. Brain sections were stained with hematoxylin and eosin stain. The observations of the brain section were carried out in 40X.<sup>14,15</sup>

### Statistical analysis

All the results were given as Mean ± SEM. The statistical analysis of all the results was carried out using two-way ANOVA followed by Dunnett's multiple comparison test. P < 0.05 was considered statistically significant. Graph pad prism, version 7.04, 2019 was used for analysis.

## Results

### Physical assessment in pups during the weaning period

Body weights of food additive treated group pups on PND 1 were insignificant compared with control. A decrease in body weights was observed in pups during PND 7 to 21 (Table 2). Opening of eyes and body hair appearance (Figure 1) was significantly delayed in group 4 and group 6 compared to control (p<0.001).

### Neuromotor maturation assessment during the weaning period

Neuromotor maturation of developing rat pups throughout the weaning period was evaluated from PND 1 to PND 21. The time taken for the righting reflexes, rotating reflexes and cliff avoidance in the food additives treated group pups was significantly declined compared with the control group (Figure 2).

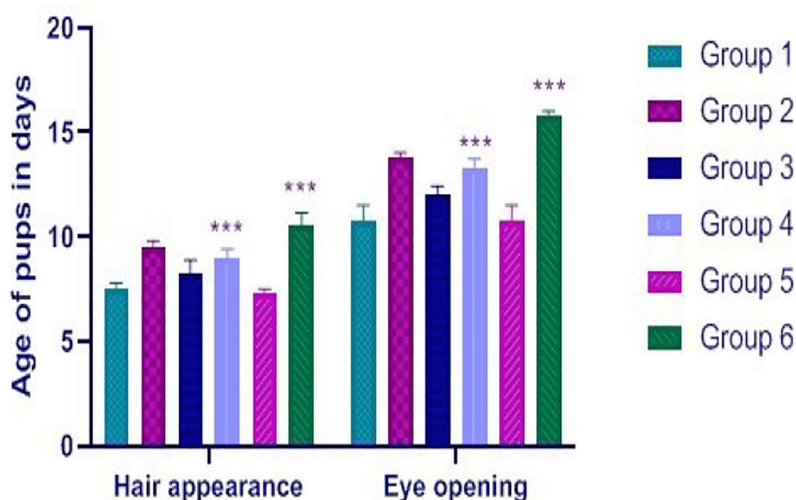
### Behavioral studies

#### Behavioral alterations

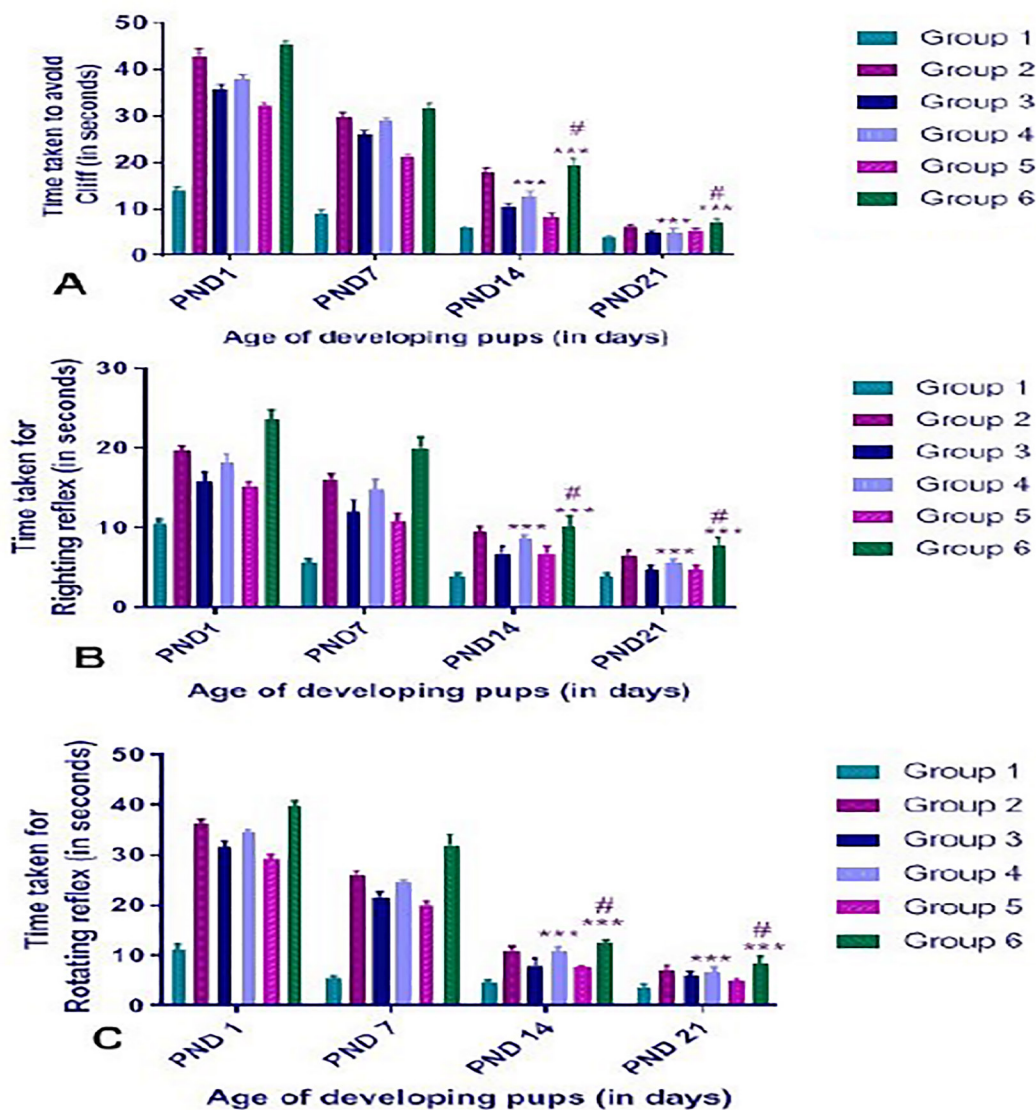
Hind paw licking and rearing behaviors were increased significantly in food additive treated group pups compared to control and they lagged in showing grooming behaviors significantly (Figure 3).

**Table 2.** Assessment of body weight in rat pups during weaning period on exposure to food additives.

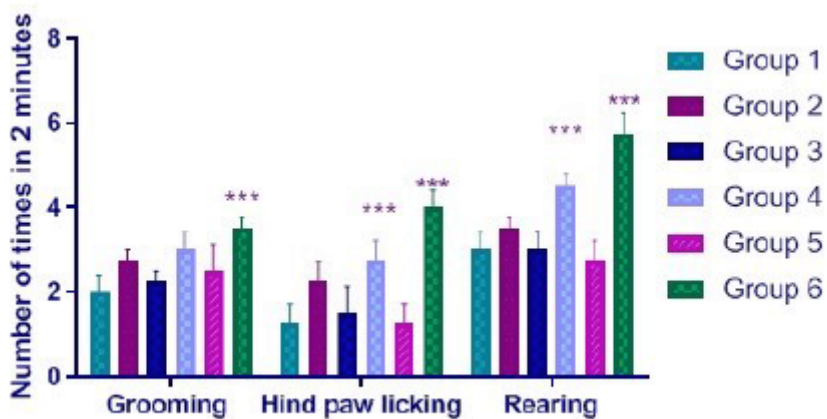
Group (n=12)	Treatment /dose	Body weight of pups in grams			
		PND 1	PND 7	PND 14	PND 21
Group I	Vehicle	5.50±0.28	17.50±0.86	23.25±0.47	30.00±0.40
Group II	Caffeine (25mg/kg p.o)	4.50±0.28	16.50±0.28	21.00±0.40	24±0.28
Group III	GUL (5mg/kg p.o)	5.00±0.40	16.75±0.47	23.00±0.40	26±0.40
Group IV	TAU (8 mg/kg p.o)	4.75±0.25	17.00±0.40	22.25±0.47	25.00±0.64***
Group V	GL (84mg/kg p.o)	5.50±0.28	17.50±0.64	23.50±0.28	25.50±0.28
Group VI	GUL+TAU+GL (5, 8, 84 mg/kg p.o)	5.25±0.47	15.25±0.64	21.00±0.40	22.50±0.64***



**Figure 1.** Effect of food additives on the body hair appearance and eye opening in the rat pups. \*\*\* represents statistical significance (p<0.001) experimental group compared with control group. Values expressed in mean±SEM (n=12).



**Figure 2.** (A-C) Effect of food additives on righting reflex, rotating reflex and cliff avoidance in the rat pups. Values expressed in mean±SEM (n=12). \*\*\* represents p<0.001 statistical significance experimental group compared with control group and # indicates the statistical significance (p<0.05) of food additive treated group compared with caffeine.



**Figure 3.** Effect of food additives on grooming, hind paw licking and rearing behaviors during on PND 22. Values expressed in mean±SEM (n=8). \*\*\* indicates statistical significance P<0.001 experimental group compared with control group.

**Morris water maze test**

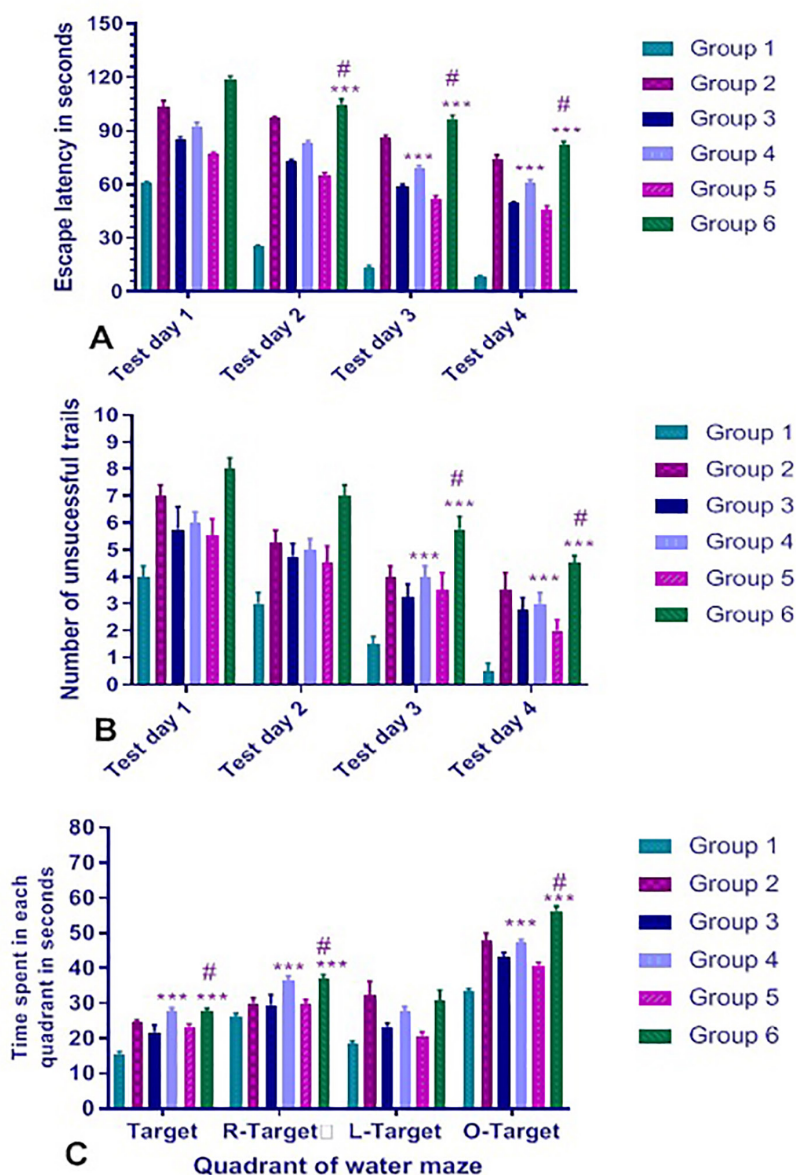
Glucuronolactone, taurine, gluconolactone and a combination of food additives treated rat pups showed longer escape latency (Figure 4A) and a higher number of unsuccessful trials (figure 4B) to reach the platform on test day 4 when compared with the control group significantly. In a probe test, the time spent by food additive treated rat pups was less compared with the control group in the target quadrant (Figure 4C). The combination of food additives treated group pups have shown significant results compared to the caffeine treated group pups indicating the decrease in cognition and spatial performance ( $p < 0.05$ ).

**Locomotor activity test**

The locomotor activity test showed a significant suppressive effect on the number of squares crossed, wall rears, rears and durations of locomotion in the rat pups (PND 32). On the other hand, an increase in the duration of immobility was significant compared to the control group. (Table 3)

**Y-Maze**

To extend the assessment of rat treated with high doses of food additives Y-maze performance was included to examine the learning and memory. A significant difference was observed in G6 and G4 groups in percentage alterations (Figure 5).

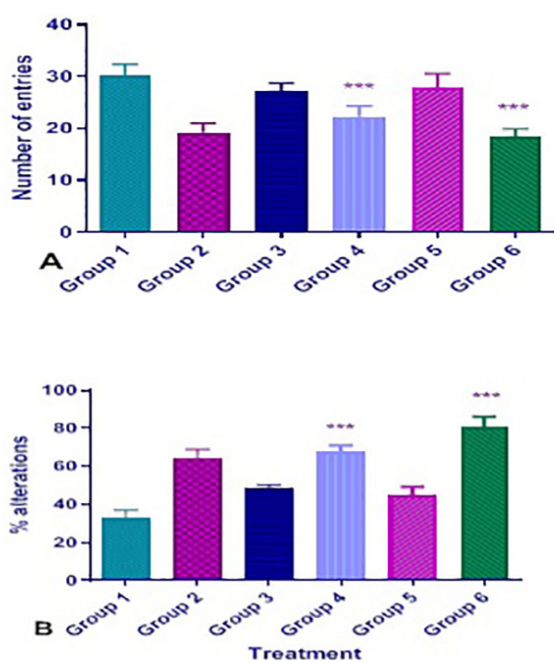


**Figure 4.** Effect of food additives in the rat pups on PND 32-35 on Morris water maze performance. Values expressed in mean±SEM (n=8). A) Escape latency on test day 1-4. B) Number of unsuccessful trails on test day 1-4. C) Time spent in target, R- target (Right side of target), L-target (left side of target), O-target (opposite of target). (\*\*\*) represents  $p < 0.001$  indicates statistical significance of the experimental group compared with the control group and # represents  $p < 0.05$  indicates the statistical significance of food additive treated group compared with caffeine treated group.

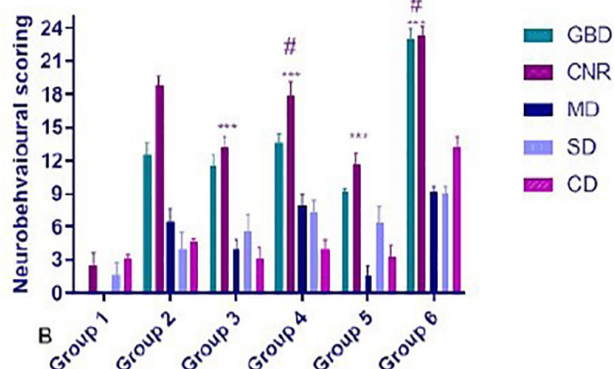
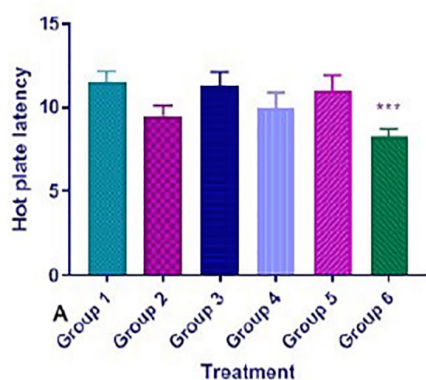
**Table 3.** Effect of food additives on locomotor activity in rat pups on PND 32

Group (n=8)	Treatment /dose	Number of squares crossed	Wall rears	Rears	Locomotion duration in seconds	Immobility duration in seconds
Group 1	Vehicle	346.5±7.8	32.2±1.5	13.5±1.7	143.7±7.4	101.2±2.9
Group 2	Caffeine (25mg/kg p.o)	101.7±7±3.4	16.5±1.8	9.5±0.6	94.5±2.5	175.2±6.7
Group 3	GUL (5mg/kg p.o)	115.2±7.8	23.2±1.2	10.0±0.9	99.2±7.4	160.7±6.7
Group 4	TAU (8 mg/kg p.o)	107.5±5.5	19.7±0.8***	10.2±1.1	89.2±4.1***	169.0±6.1***
Group 5	GL (84mg/kg p.o)	123.5±5.3	22.7±1.3	11.7±0.8	122.2±2.5	160.0±0.0
Group 6	GUL+TAU+GL (5, 8, 84 mg/kg p.o)	89.7±8.2***, #	18.5±1.0***, #	8.5±0.6***, #	85.2±5.2***, #	196.5±5.9***, #

Values expressed in mean±SEM (n=8). \*\*\* indicates statistical significance P<0.001 experimental group compared with control group. '# indicates statistical significance P<0.05 represents the experimental group compared with caffeine treated group.



**Figure 5.** Effect of food additives on Y-Maze performance in rat pups on PND 38. A) Number of entries. B) Percentage alterations. Values expressed in mean±SEM (n=8). \*\*\* indicates statistical significance P<0.001 experimental group compared with control group.



**Figure 6.** (A). Effect of food additives on hot plate test and (B). Neurobehavioural scores in rat pups on PND 32. GBD- General behavioral deficits (score 40) CNR- Cranial nerve reflexes (score 20) MD- Motor deficit (score 10) SD- Sensory deficit (score 10) CD- Co-ordination (score 20). Data is represented as mean±SEM (n=8) \*\*\* indicates p<0.0001 showed significant differences between the experimental group and vehicle group. # indicates p<0.05 showed significant differences between the experimental group and caffeine treated group.

### Hot plate latency

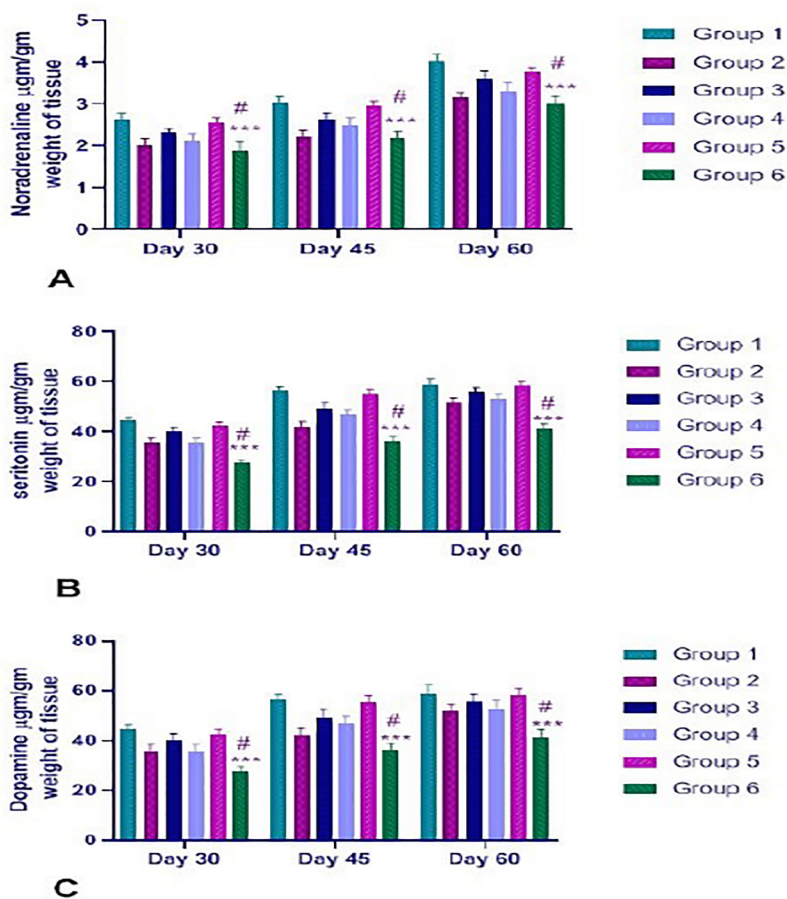
There was no significant difference in reaction latency to a 55 ° C hot plate between control, Group 3 and group 5 rat pups (Figure 6A). In rats exposed to the combination of food additives and taurine decreased in response latency (p<0.001) indicated a hyperalgesic effect.

### Neurobehavioral scoring in rat pups on PND 32

In Katz protocol, rat pups of individual food additives and combinations showed high scores compared with control group animals (Figure 6B). Group 6 showed highest-scoring indicates the increase in neurobehavioural toxic effects compared with caffeine treated animals.

### Estimation of neurotransmitter

Neurotransmitter estimation was done on PND 30, 45 and 60 using whole brain extract of rat pups. A significant decrease in neurotransmitter levels of noradrenaline (Figure 7A) and serotonin (figure 7B) was observed in taurine and combination of food additives treated animals (p<0.001). High significant decrease in dopamine (Figure 7C) levels was observed in combination group pups. With the increase in the age of pups, an increase in neurotransmitter levels was observed.

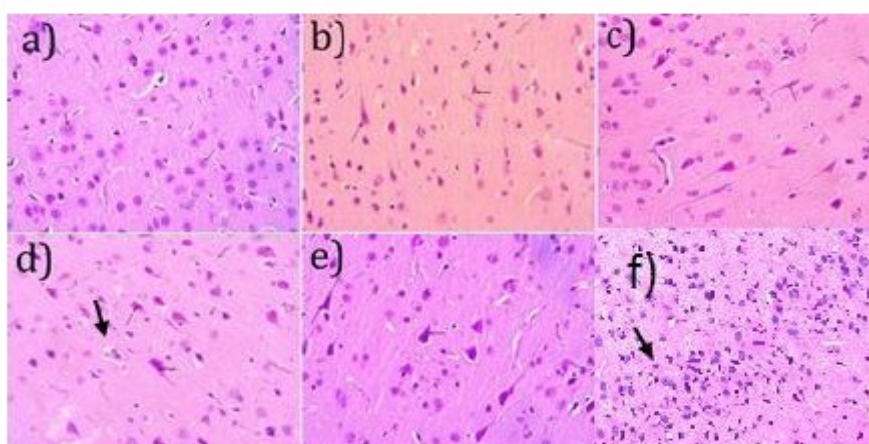


**Figure 7.** Effect of food additives on Noradrenaline, serotonin and dopamine levels in whole brain tissue of rat pups on PND 30, 45 and 60. Data were represented as mean ± S.E.M. (n=4). \*\*\* indicates statistical significance p<0.001 showed significant differences between the experimental group and control group. # indicates statistical significance p<0.05 showed significant differences between the food additives treated group and caffeine.

**Histopathology studies**

Using hematoxylin and eosin stain microscopical examination of the cerebral cortex region was examined in rat pups. All the additive treated rats showed normal

morphology of neurons. The taurine treated group showed less significant degeneration than caffeine treated animals. Combination group pups had shown marked neurodegeneration (See Figure 8).



**Figure 8.** Effect of food additives on histopathological changes in cortex region of brain in rat pups (40X). a) Group 1- control: showed normal morphology. b) Group 2-Standard Caffeine treated: Degeneration of neurons was examined. c) Group 3-Glucuronolactone: No death of neurons was observed. d) Group 4-Taurine: Less degeneration of neurons was observed. e) Group 5- gluconolactone: No degeneration of neurons was observed. f) Group 6- Combination of food additives: Marked degeneration of neurons was examined.



## Discussion

Food additives used in energy drinks undergo rigorous safety testing before they are approved for safe use. However, they are not routinely tested for their impact on mood and behaviour. The present study provides evidence for neurodevelopmental toxic effects particularly behavioural changes for selected food additives used in energy drinks at selected dose. Pregnant animals were used following OECD guidelines 426. In this protocol, pregnant animals were treated with food additives from GD 3 till PND 15.<sup>16</sup>

The decrease in physical maturation, neuromotor maturation and behaviours of pups in food additive treated rats may be due to the neurodevelopmental alteration during the formation of a fetus as suggested in the previous neurotoxic studies of aluminum exposure during pregnancy and lactating period of mice.<sup>6</sup> Earlier studies suggested that Irwin protocol (Functional assays) explained the data from many endpoints for each subject. In this study, different behavioral parameters were measured to understand neurobehavioural effects multidimensionally.<sup>17</sup> Previous studies indicated that behavior is a gross indicator of neural system integration. Neurotoxic substances can have effects on multiple cellular targets, ranging from specific ion channels or neurotransmitters causing changes in a behavioral activity such as arousal, body reflexes, etc.<sup>17</sup> Thus, change in behavioural activity indicated by a decrease in arousal, hind paw licking and rearing may be related to the alteration in neurotransmitter level.

Previous studies evaluated the cognitive effects in rats by using the water maze test.<sup>14</sup> Longer the latency to escape and increase in unsuccessful trails indicates the decrease in cognition was reported. In the present research work, glucuronolactone, taurine and combination treated food additives groups increase in the latency time suggested the decrease in cognition correlating the previous study. In the water maze test, the four days trail to find the hidden platform provided a measure of the hippocampal-dependent spatial reference memory, whereas the probe test was a measure of spatial learning. Therefore, in this study, the decrease in time spent in the target quadrant in the probe test indicated a decrease in spatial learning in combination with treated group animals hints the altered cognitive effect. Earlier studies stated that locomotor activity specifies attention and its decrease showed sedative action. Sedation and muscle relaxation were concerned with the GABA receptor. Alteration of these receptors results in various neurological disorders.<sup>18</sup>

The decrease in locomotor activity and an increase in the duration of immobility on PND 32 in combination group pups was indicative of altered neurological function may be due to alteration of GABA receptors. Y-maze was an extra evaluation of spatial learning and memory in which an increase in percentage alteration was observed significantly in group 6 pups hint the cognitive deficit. A decrease in hot plate latency was observed in food additives treated animal pups indicated a slight thermal hyperalgesic effect.

Previous studies reported that taurine has shown dose-related behavioral changes in both sexes of rats. The activity of occasional chewing of limbs increased after dosing with taurine mentioned the central pharmacological and neuromodulator effect of taurine.<sup>19</sup> In this study, taurine treated group pups had shown altered behavioral activity, a decrease in locomotor activity and cognitive effects confirming the earlier reports. Earlier studies done on glucuronolactone in rats were focused on the kidneys and No Observed Adverse Effects Level (NOAEL) was estimated as 1000 mg/kg p.o./day.<sup>19</sup> In the present protocol, animals were exposed to a high dose of glucuronolactone, a projecting change in the neurobehavioural effects were noticed. Previous literature state that a 14-day study in rats was done on gluconolactone showed mortality, abnormal clinical signs, body-weight changes (on days 1, 2, 3, 7, 10, and 14), and gross pathological changes in the brain has not focused on neurobehavioral symptoms.<sup>5</sup> Our study for the first time indicates that gluconolactone treated animal pups have shown variable changes in the neurobehavioural activity and may hint developmental neurotoxicity when consumed higher than the acceptable doses.

Earlier research showed neurological scales/scores measured motor, sensory, and reflex functions in rats, mice and dogs used to detect effects on brain injury.<sup>20</sup> In this study, the Katz protocol showed significant increase neurobehavioural scores in food additive treated groups. Various parameters like general behavioral deficits, cranial nerve reflexes, motor deficit, sensory deficit, co-ordination were observed. Alteration in these parameters indicated neuronal damage further supported by histopathological studies.

Earlier studies proved the relation between neuroendocrine and neurobehavioural studies in neurodevelopmental research.<sup>7,21</sup> Based on earlier reports, in this study, alteration of noradrenaline levels confirms the relation with alteration of neurobehaviour in food additive treated animals. In neurotoxic studies of 3-nitropropionic acid decrease in dopamine and serotonin levels in the brain homogenate was observed indicated neurochemical alterations lead to neurobehavioural changes.<sup>22</sup> The decrease in dopamine and serotonin levels in the present study signified the same effect on developing brain linking the above studies. Previous literature states that neurodegeneration in the cortex region of the brain indicated the disease state like Alzheimer's disease emphasis neurotoxicity.<sup>15</sup> Hence, histopathological changes observed in this research enumerates the neurodegeneration of neurons in the cortex confirms the neurotoxicity of taurine and a combination of food additives.

Finally, the study elaborates on the neurobehavioural effects of individual food additives and the combined effect of the food additives for the first time at a selected dose using multiple behavioural parameters. Ethical considerations and duration of treatment limit an in-depth understanding of neurotoxicity. Further research was suggested to find the mechanism involved in the synergistic effect of additives.

## Conclusion

The present results corroborate that the food additives at the selected dose and in combination induced neurobehavioural and neurotransmitter alterations in rat pups. This indicated the neurodevelopmental toxic effects which were further supported by neurohistological alterations in the cerebral cortex of developing rat brain. This study provided evidence for the neurodevelopmental toxic effects of food additives used in energy drinks when consumed above the daily acceptable intake. Further research is obligatory to understand the mechanism in individual effect and synergistic effect of food additives.

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## Ethical Issues

The study is in compliance with ethical guidelines OECD Test number 426: Developmental neurotoxicity study. The experimental procedures were approved by Institutional Animals Ethics Committee (IAEC) as provisions of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), MLR Institute of pharmacy, Hyderabad (CPCSEA/IAEC/PR3/2019).

## Author Contributions

RB and SD: Conception, design, supervision resources, analysis, and/or, interpretation literature search, writing manuscript and critical review RB: Materials data, collection and/or processing. All authors have read and agreed to the published version of the manuscript.

## Conflict of Interest

There is no conflict of interest.

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