Evaluation of Neurobehavioural Toxic Effects of Taurine, Glucuronolactone and gluconolactone used in Energy drinks in young rats

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ABSTRACT

INTRODUCTION: Neurotoxic effects of food additives used in energy drinks were under investigation since 1900's but the safety concern is raising and reassurance of safety testing in animals is on demand by the public. Rigorous safety testing is aimed at dose optimization, duration of treatment and to detect the methods to accesses changes in mood and behaviour. Hence, we studied the neurobehavioural toxic effects of selected food additives used in energy drinks and their combination in rats when consumed at high dose.

METHODS: Young Sprague dawley rats were divided into six groups. Group I treated with vehicle, group II treated with 25mg/kg p.o of caffeine, group III treated with 5mg/kg p.o of glucuronolactone, group IV treated with 8mg/kg p.o of taurine, group V treated with 84 mg/kg p.o of gluconolactone and group VI treated with combination of three food additives.

Neurobehavioural changes were evaluated on day 7, 14 and 21 by behavioural parameters.

Neurobehavioural scoring and neurotransmitter estimation in rat brain tissue were estimated on day 21.

RESULTS: Significant ($p < 0.001$) changes were observed in neurobehavioural parameters and neurobehavioural scoring compared with control. Significant decrease in neurotransmitter levels in rat brain evidence the neurotoxic effects of food additives.

DISCUSSION AND CONCLUSION: This study elaborated neurobehavioural toxic effects of selected food additives glucuronolactone, taurine and gluconolactone when administered orally for duration of 21 days in young rats. High significant toxic effects were observed in animals treated with combination of food additives at high doses further justified with alteration in neurotransmitter levels in brain tissue of rats.

Keywords: Energy drinks, Food additives, Glucuronolactone, Gluconolactone, Neurobehavioural toxicity, Taurine

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1. Introduction

Dramatically we are exposed to neurotoxin naively through the food products. Today evaluation of food additives on behaviour and mood in adults is of high concern. Various regulatory bodies are encouraging to scrutinize the use of the food additives rigorously for their safety and reassurance. FDA and European Food Safety Authority has been evaluating and supporting the risk assessment and safety in use of appropriate dose of acceptable daily intake (ADI). The food additives used in many products like baby foods, cool drinks, energy drinks, soft drinks are approved by FDA after safety evaluation. But, various food additives like antioxidants, stabilizers, sweeteners, thickeners, preservatives and flavouring agents have effect on behaviour when taken in high doses which are listed under safety margin. As per 2006, FDA guidelines food additives are classified based on level of concern and safety margin into Low concern level I (12-50 ppb), Intermediate concern level II (50-250 ppb), High concern level III (250-1000 ppb) based on primary toxicological data.¹ The maximum level of additive that has no demonstrable toxic effect is called "No observed –adverse effect level" (NOAEL) and Acceptable Daily Intake (ADI) are the check parameters for each food additive. Chronic consumption per day more than ADI leads to toxicity. Risk to human health varies depending upon the type and time of exposure. Specific studies such as neurotoxicity, immunotoxicity and allergenicity are rigorously tested repeatedly to reassure safety of food additives.⁴

Common food additives used in energy drinks like taurine, glucuronolactone and gluconolactone are at elevated risk analysis. The daily exposure to taurine, glucuronolactone and gluconolactone from energy drinks in young generations was higher than the mean daily exposure (1420 ml/day of energy drink or 2.6 cans/day). In adults, chronic habitual intake of energy drinks was reported to cause several neurological disorders include migraine, seizures, endocrine disorders and neuropsychiatric disorders.² Hence, the excessive consumption of energy drinks has toxic effects on nervous system.

The safety of these food additives used in energy drinks was not documented by Scientific Committee on Food (SCF). According to EFSA 2009 data, the stimulatory effect of taurine on the central nervous system (CNS) was not clearly document.³ The major constituents of energy drinks are taurine, glucuronolactone and gluconolactone.³ Based on this background, research protocol was elaborated to assess systematically possible neurobehavioural toxic effects of individual food additives and the combination of food additives taurine, glucuronolactone and gluconolactone used in energy drinks at high doses in animals. The study includes evaluation of neurobehavioural effects, neurobehavioural scoring and neurotransmitter estimation in brain tissue of young rats to evidence possible neurobehavioural effects and reassures the safety level of food additives used in energy drinks which are listed under safety margin.

2. Materials and methods

2.1. Chemicals and reagents

2.1.1 Chemicals: Glucuronolactone, gluconolactone, caffeine (food grade 99.5 %) were procured from Srineelima labs, Hyderabad, India. Taurine (food grade 99.6%) was obtained from Nutrija Lifesciences, Nagda, Madhya Pradesh, India. All other chemicals (analytical grade) are from Himedia Pvt Ltd., India.

2.1.2. Reagents

Hydrochloric acid (HCl)- Butanol solution (0.85 ml of 37% hydrochloric acid in one litre), 0.4 M HCl (3.4 ml concentrated HCl and made up to 100ml with water), 0.1 M HCl (0.85 ml concentrated HCl made up to 100 ml water), 5 M NaOH (20 gm of sodium hydroxide pellets

dissolved in distilled water and volume made up to 100 ml with distilled water), 10 M Acetic acid (57 ml of glacial acetic acid and made up to 100 ml with distilled water. Reagents and buffers like Sodium acetate buffer (EDTA pH 6.9), Heptane, sodium sulphite solution, O-phthaldialdehyde (OPT) reagent are obtained from Sigma Aldrich, Hyderabad, India.

2.1.3. Equipment

Morris water maze model MWM, Version 5.0, Orchid Scientifics, Wooden arena with 64 squares was prepared by Wood works, Hyderabad, Tissue Homozinser 160W, Refrigerated Centrifuge -Gravity labs, Spectrofluorometer model ALT 2380 (wave length range 200 to 700)

2.2. Animals

Sprague dawley albino rats of both male and female of equal ratio weighing 150-200 g were obtained from animal house of MLR Institute of Pharmacy, Hyderabad. Animals were divided into four groups and housed 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 standard pellet diet and water *ad libitum*. Experimental procedure was approved by Institutional Animals Ethics Committee (IAEC) as requirements of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), MLR Institute of pharmacy, Hyderabad (CPCSEA/IAEC/PR3/2019).

2.3. Experimental protocol

All the animals were divided into six separate groups and each group consist of six animals with equal ratio of male and female 3:3 (n=6, 3 Males + 3 females). All the doses were calculated based on human dose available in literature and are converted to animal dose.² High dose of food additives were administered and observed for the neurotoxic effects. Group I animals served as control treated with water administered through oral route, group II animals act as working standard treated with caffeine 25 mg/kg p.o. Group III animals treated with glucuronolactone 5mg/kg p.o, group IV animals treated with taurine 8 mg/kg p.o, group V animals treated with gluconolactone 84 mg/kg p.o and group VI animals were treated with combination of three food additives (glucuronolactone 5mg/kg p.o, taurine 8 mg/kg p.o and gluconolactone 84mg/kg p.o). All the animals were treated with freshly prepared doses dissolved in water and administered through oral gauge every day till 21 day.

2.4. Assessment of neurobehavioural effects

Neurobehavioural changes were observed in animals treated with respective doses for 21 days. On 7, 14 and 21 day the animals were subjected for screening of neurobehavioural effects by FOB (functional observational battery) and Irwin protocol.⁵ These include studies of behavioural alterations, Morris water maze test, locomotor activity test and Katz protocol as described below.

2.4.1. Behavioural alterations

Behavioural changes were evaluated by measuring rearing and paw licking behaviour for 5 minutes.⁶ The observations were noted by three blind observers.

2.4.2. Morris water maze test

Cognitive changes such as learning, conditioning, memory and attention was evaluated by Morris water maze test in rats.⁷ The maze was an active grey round tank (0.45 m radius, 0.5 m tall) occupied with water (22°C) to a depth of 0.15 m. An adjustable platform of size $0.06\text{ m} \times 0.06\text{ m}$ made of steel was arranged at 0.01 m under the water level and 0.13 m from the edge. Milk (1 ml) was added to made water cloudy thus the platform was hidden. On the edge of the tank the four letters nominated as north (N), south (S), east (E) and west (W), divided the tank into four portions (N-W, N-E, S-E and S-W). On day one, rats allowed to swim in the tank for one minute in the tank without hidden platform. Thus, rats were trained for swimming in tank.

On second day rats were trained to identify and move onto the submerged platform for 6 trails per day till fifth day. In each trail rats were released into the tank with face pointing towards water for confirm immersion. The escape latency from immersion into the tank onto the hidden platform (maximum duration of trial 2 minutes) was noted. In 2 minutes, if animal could not identify the platform it was physically directed to climb by using glass rod. Then the score of 2 minutes was noted for these trails. The number of such unsuccessful trials was calculated. For learning and memorizing the spatial cues each animal was given interval of 0.5 minute on climbing the platform.

2.4.3. Locomotory activity test

Locomotor changes such as coordination and equilibrium were assessed by locomotor activity test. This test consists of a wooden field of square designed measuring $0.8 \times 0.8 \times 0.3$ m and the flooring were divided into 64 squares of equal dimensions. Duration of immobility and locomotion in 5 minutes for each animal was recorded.⁷

2.4.4. Katz protocol (Neurobehavioural scoring)⁸

Neurobehavioural scores were calculated for animals after 21 days treatment with high dose of food additives and evaluated for neurobehavioural toxic effects (Table 1). The observations were noted by three blind observers.

2.5. Estimation of neurotransmitters

2.5.1 Preparation of tissue extract⁹

On day 21 rats were sacrificed, whole brain was dissected out and the sub-cortical region was separated and weighed. Weighed tissue was homogenised in homogenizer with 5ml HCl butanol for about one min. The homogenised tissue was then centrifuged for 10 min at 2000 rpm. The supernatant layer (1 ml) was separated and added to centrifuge tube holding 2.5 ml heptane and 0.3 ml of 0.1M HCl. After 10 mins of shaking vigorously the tube was centrifuged under identical settings. Two layers were separated, supernatant layer (organic layer) was discarded and the remaining aqueous extract was used to estimate noradrenaline, dopamine, and serotonin. All the steps were carried out at 0°C. The brain extracts were stored at - 20°C until further experimentation.

2.5.2. Estimation of noradrenaline¹⁰

0.2 ml of aqueous layer was taken from tissue extract stored at ice cool temperature after preparation extract. 0.05 ml of 0.4M HCl and 0.1 ml of EDTA (pH 6-9) were added to the aqueous extract accompanied by 0.1 ml of iodine solution for oxidation. The reaction was stopped after 2 mins by adding 0.1 ml Na₂SO₃ solution. Then, 0.1 ml of acetic acid was added after 1.5 mins. The solution was heated to 100°C for 6 mins. The sample allowed to cool, excitation and emission spectra were noted from the spectrofluorometer. These interpretations were measured at 395-485 nm for noradrenaline.

2.5.3. Estimation of dopamine¹⁰

To the 0.2 ml of aqueous phase extract 0.05 ml of HCl and 1 ml of EDTA (pH 6.9) were added were added accompanied by 0.1 ml of iodine solution for oxidation. The reaction was stopped after 2 mins by adding 0.1 ml Na₂SO₃ solution. Then, 0.1 ml of acetic acid was added after 1.5 mins. The solution was heated to 100°C for 6 mins. The sample allowed to cool, excitation and emission spectra were noted from the spectrofluorometer. These interpretations were measured at 330-375nm for dopamine

2.5. 4. Estimation of serotonin¹⁰

0.2 ml of aqueous tissue extract was added with the 0.25ml of OPT (o- phthaldialdehyde) reagent. Then, it was heated for 100°C for 10 mins. After the sample reached to ambient temperature, the readings were taken at 360-470 nm in the spectrofluorometer for the estimation of serotonin. Tissue blanks for dopamine and noradrenaline were prepared by adding the reagents of the oxidation step in reversed order (sodium sulphite before iodine). For serotonin tissue blank, 0.25 ml concentrated HCl without OPT was added. Internal standard was prepared by taking 500 µg/ml each of noradrenaline, dopamine and serotonin are prepared in distilled water: HCl butanol in 1:2 ratio. Concentration of the neurotransmitters expressed in µgm per gram wet weight of tissue was calculated by using formula.¹¹

$$\text{Concentration of unknown (Cu)} = \frac{\text{Sample O.D} - \text{Blank O.D}}{\text{Standard O.D} - \text{Blank O.D}} \times Cs$$

Cs= Concentration of standard (500µgm/ml)

O.D=optical density

2.6. Statistical Analysis

Altogether the results were studied using ANOVA followed by Dunnett's multiple comparisons. Graph pad prism, version 7.0, 2019 was the software used for analysis.

3. Results

3.1. Neurobehavioural changes

3.1.1. Behavioural alterations

Alterations in behavioural effects were observed in animals treated with high dose of individual food additives, successive increase in the behavioural effects with increase in treatment duration on 7, 14 and 21day, respectively. Taurine treated animals and combination of food additives showed increase in rearing and hind paw licking significantly ($p < 0.001$) compared with experimental group. G6, the combination of food additives has shown high significant difference ($p < 0.05$) in behavioural activity compared with caffeine as shown in figure. 1.

3.1.2. Morris water maze test

Taurine treated animals showed longer escape latency on to submerged platform in water maze compared with control. With increase in duration of treatment increase in escape latency was significant ($p < 0.001$). Combination of food additives treated animals showed high ($p < 0.05$) longer escape latency on day 21 indicating altered cognitive effects compared with caffeine treated animals. (figure.2.)

3.1.3. Locomotor activity test

Significant increase in immobility duration was seen in animals treated with individual food additives and combination of food additives ($p < 0.001$) and with increase in duration of treatment than control group when placed in wooded arena. Combination of food additives showed marked increase in immobility duration significantly ($p < 0.05$) indicating decrease in locomotion compared to caffeine treated animals. (figure. 3.)

3. 2. Katz protocol of neurobehavioural scoring

In Katz protocol animals treated with high doses of individual food additives showed high neurobehavioural scores on day 21. All the experimental groups showed significant ($p < 0.001$) scores than control animals (figure. 4.). The combination group exhibited highest neurobehavioural scoring ($p < 0.05$) indicated the increase in neurobehavioural toxic effects compared with caffeine treated animals.

3. 3. Estimation of neurotransmitters

On day 21, tissue extract was prepared, and neurotransmitters were estimated. The noradrenaline, serotonin levels were pointedly ($p < 0.001$) declined in taurine and combination of food additives treated animals than control. The combination group animals showed high significance compared with caffeine treated group. ($p < 0.05$). (See figure. 5, 6.)

The decrease in the dopamine levels were observed in taurine and combination group animals than control ($p < 0.001$). The combination animals showed high significant results when compared with caffeine treated animals indicating the altered neurotransmission in brain. ($p < 0.05$). (See figure. 7.)

4. Discussion

Food additives used in energy drinks, when consumed above the acceptable level were reported to produce toxic effects, as stated by EFSA. But, the exact ingredients and the dose responsible for toxic effects were not evaluated and documented clearly.³ This research provides evidence for neurobehavioural toxic effects for the selected FDA approved food additives used in energy drinks when consumed above acceptable daily intake. The neurobehavioural toxic effect of food additives when administered orally at doses, glucuronolactone 5mg/kg p.o, taurine 8 mg/kg p.o, gluconolactone 84 mg/kg p.o and combination of three food additives was evaluated and documented for 21 days of treatment in young rats.

Earlier studies suggested that Irwin protocol (functional observational battery test) explains many parameters provides a multidimensional method for the explanation of neurobehavioural effect.⁵ Based on Irwin protocol, the Sprague Dawley rats were treated with food additives and neurobehavioural changes were evaluated by using behavioural alterations test, Morris water maze test and locomotory test for clarification of neurobehavioural toxic effects.

Previous literature indicated that behaviour is a measure of the integration of neural function and alteration in behaviour was used to evaluate neurobehavioural toxic effect.⁵ In this study, alteration in behavioural activity was assessed by considering behavioural parameters like paw licking and rearing behaviours which were considered as indicators of grooming. An increased anxiety level by any stimulus was reported to change paw licking and rearing behaviour.¹² Similar alteration in paw licking and rearing behaviour was caused by taurine and combination group which clearly indicates the alteration in neuronal functioning by the selected food additives. Previous studies evaluated the cognitive effects in rats by using water maze test reported increase in duration of escape latencies indicated the decrease in cognition.¹³ In the present study the increase in duration of escape latency to submerged platform was observed with taurine and combination group significantly. The decreased cognition may be due to the decrease in cyclic GMP levels as reported with cognitive impairment and neurobehavioural deficit reported with aluminium toxicity studies.¹⁴ Similar decrease in cGMP levels was reported with taurine in cardiomyocytes.¹⁵ Our study indicated that neurotoxicity caused by food additives progressively increased with days of exposure from 7th day to 21st day. Earlier studies stated that locomotor activity indicates attentiveness.¹⁶ In the present study, the decrease in locomotor activity indicated by increase in duration of immobility in taurine and combination group which affirms decrease in attentiveness lead to altered neurological functioning.¹⁶

Previous studies reported that taurine showed dose correlated behavioural changes in rats. Chewing of limbs after treatment with taurine indicated the central pharmacological and neuromodulator effect of taurine.¹⁷ In this study, taurine treated group also showed altered behavioural activity which confirmed its potent neuromodulator effect on neurotransmitters of brain. In an sub-acute toxicity study for 14 days in rats, gluconolactone showed mortality,

abnormal clinical signs, body-weight changes (on days 1, 2, 3, 7, 10, and 14), and gross pathological changes in brain but was not focused on neurobehavioural symptoms.¹⁸ Our study for the first time have shown changes in the behavioural activity in gluconolactone treated animals and may hint neurotoxicity when consumed higher than the acceptable doses. These changes were high when given in combination with taurine.

Neurological scales/scores tested motor, sensory and reflex functions in rats, mice and dogs were used to detect effects on brain injury.⁸ In the present research work, the Katz protocol of neurobehavioural scores were calculated considering various parameters like general behavioural deficits, cranial nerve reflexes, motor deficit, sensory deficit and co-ordination were used to evaluate the neuronal damage in animals. High score of neurobehavioral deficits were observed in animals receiving combination of food additives, rather than individually. This indicates chances of increase in brain neuronal damage and can be correlated with the decrease in neurotransmitters.

Selected food additives were hypothesized to enhance neurotransmitter activity concentrated in the sub cortical regions according to the literature.¹⁹ So, subcortical regions of whole brain extracts were used to estimate neurotransmitters. The decrease in noradrenaline, serotonin and dopamine levels indicated the neurochemical alterations and neurotoxic effects on sub chronic administration of drug was suggested by previous studies.²⁰ In addition the former studies, also focused the participation of serotonin in the cognition and memory and altered serotonergic neurotransmission by toxic substances was reported.²¹ The neurotransmitter modulatory effect of these selected food additives was mentioned in previous literature.²² Corroborating the earlier studies noradrenaline, serotonin and dopamine were decreased prominently in the current study. The combination of Taurine, gluconolactone and glucuronolactone caused more noticeable changes in the neurotransmitters levels than when given alone, which indicates risk of more neuronal damage, modulation and toxicity. These changes are supporting the observed neurobehavioral deficits caused by the food additives.

This study raises the concern on the safety of mentioned food additives at the doses studied considering the aspect of simultaneous consumption of these food additives via energy drinks, though safety of these additives were established and approved, but individually and at a different exposure level. Further, histopathological studies are needed for correlation of neurobehavioural toxic effect.

5. Conclusion

In conclusion, this study elaborated neurotoxic effects of glucuronolactone, taurine and gluconolactone used in the energy drinks when consumed above acceptable daily intake. This study has shown significant neurobehavioural toxic effects accompanied with altered neurotransmitter levels in rats treated with combination of selected food additives. Further, investigation is required to understand the mechanism and interaction between food additives. Appraisal of developmental neurotoxic effects of these food additives in combination will also be noteworthy.

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	Reviewers comments	Author answers
1	The English is not still clear. There are several syntax errors and grammar mistakes. I have indicated this before, but the authors did not consider my suggestions.	The grammar was thoroughly checked as per the suggestions of the reviewers, which were found mainly in the Introduction section. Page No 1; 2 para, last two lines, third para 1 st line, page no. 2, first para 3 line and 6 th line .
2	Why to use English English and later American English throughout the manuscript? The authors should prefer American English.	The preferred language was set to American English and the whole manuscript was recorrected.
3	Figures are all in different styles. Some are colored; some are in black and white. They should have the same style.	All figures were converted to black and white. Fig No. 4
4	Do not start the sentences with arabic numbers or abbreviations.	Sentences starting with arabic and abbreviation were corrected and rewritten in Page No 2 , under sub heading Reagents first word; page no 3, under Animals sub heading first word.
5	Conclusion part still needs to be improved.	According to reviewer suggestions, the conclusion was improvised.
6	What are the strengths and weaknesses of your study? Please add	Strengths – 1) For the first time gluconolactone neurotoxic effects at doses given were evaluated in this study. (Mentioned in discussion page no. 10 first para second line) 2) The combination effect of selected food additives was significantly evaluated. (Mentioned in discussion part, Page no 9 ;second para,11 and 19 th Line) Weakness- Lack of histopathological data page no. 11, 1 st line.
7	The studies reported in discussion section does not even give the route, dose and period of exposures and the discussion part is still not advanced.	The route, dose and period of exposures were mentioned clearly in the methodology section, but as per suggestions of the reviewer, the details were included in discussion page no 8 first para, 6th line. Few advances in the research area, relevant were also updated in the discussion section.

Figures

Figure 1. Assessment of neurobehavioural alterations of food additives on exposure to high doses

for 7, 14 and 21 days in SD rats by (A). Number of rearing (B). Number of paw licking. Data were represented as mean $\pm$ S.E.M.(n=6). ^ap<0.001 showed significant differences between the experimental group and control group. ^bp<0.05 showed significant differences between the food additives treated group and caffeine.

Figure 2. Assessment of neurobehavioural effects of food additives on exposure to high doses for 7, 14 and 21 days in SD rats by escape latency in seconds using Morris water maze test. Data were represented as mean $\pm$ S.E.M. (n=6). ^ap<0.001 showed significant differences between the experimental group and control group. ^bp<0.05 showed significant differences between the experimental group and caffeine.

Figure 3. Assessment of neurobehavioural effects of food additives on exposure to high doses for 7, 14 and 21 days in SD rats by duration of immobility using locomotor activity test. Data were represented as mean $\pm$ S.E.M. (n=6). ^ap<0.001 showed significant differences between the experimental group and control group. ^bp<0.05 showed significant differences between the food additives treated group and caffeine.

Figure 4. Assessment of food additives on exposure to high doses for 21 days in SD rats for neurobehavioural effects by neurobehavioural scoring using Katz protocol. Data were represented as mean $\pm$ S.E.M. (n=6). ^ap<0.001 showed significant differences between the experimental group and control group. ^bp<0.05 showed significant differences between the experimental group and caffeine.

GBD- General behavioural deficits (score 40) CNR- Cranial nerve reflexes (score 20) MD- Motor deficit (score 10).SD- Sensory deficit (score 10) CD- Co-ordination (score 20).

Figure 5. Effect of food additives on Noradrenaline levels in brain tissue of rats exposed to high doses for 21 days. Data were represented as mean $\pm$ S.E.M. (n=6). ^ap<0.001 showed significant differences between the experimental group and control group. ^bp<0.05 showed significant differences between the food additives treated group and caffeine.

Figure 6. Effect of food additives on serotonin levels in brain tissue of rats exposed to high doses for 21 days. Data were represented as mean $\pm$ S.E.M. (n=6). ^ap<0.001 showed significant differences between the experimental group and control group. ^bp<0.05 showed significant differences between the food additives treated group and caffeine.

Figure 7. Effect of food additives on dopamine levels in brain tissue extract of rat exposed to high doses for 21 days. Data were represented as mean $\pm$ S.E.M. (n=6). ^ap<0.001 showed significant differences between the experimental group and control group. ^bp<0.05 showed significant differences between the food additives treated group and caffeine.

Uncorrected proof