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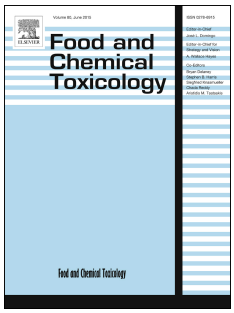

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Tartrazine Induced Neurobiochemical Alterations in Rat Brain Sub-Regions

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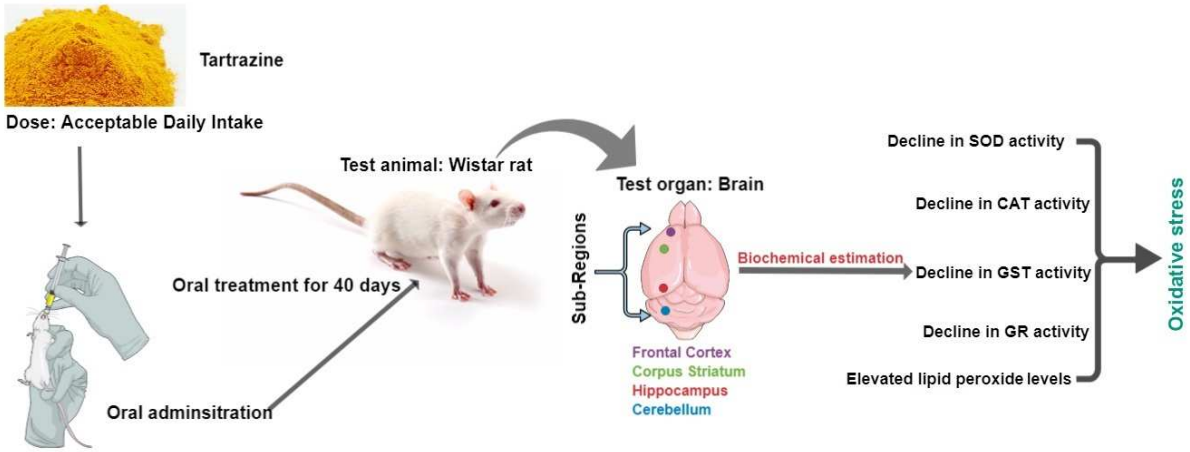


1. Introduction

Tartrazine is a synthetic azo dye primarily used as a food colorant that provides a yellow to orange color on application. Known by other names such as E102 (EFSA-European Food Safety Authority), FD&C Yellow 5(FDA- US Food and Drug Administration) or C.I. 19140(Color Index International), it is certified as a colorant by FDA for its use in food, drugs and cosmetics.

In spite of the fact that stringent guidelines on the use of these chemicals in food products have been formulated by many organizations such as JECFA (Joint FAO/WHO Expert Committee on Food Additives-JECFA 82nd Meeting Summary and Conclusion, 2016), US FDA-CGMP (Current Good Manufacturing Practice-21 CFR 74.705) and EU (European Union-EFSA, 2009), the safety limit of these chemicals being liberally used in food is still under debate. Concern regarding their human health effects, especially in children is a serious issue, when consumption of visibly alluring food products such as cotton candy, soft drinks, flavored chips, cereals (corn flakes, muesli, etc.), cake mixes, soups, sauces, some rice, ice cream, chewing gum, jam and jelly is higher in the younger population.

In 1984, the International Clinical Nutrition Review published an article summarizing several small studies conducted among school children in United States during 1970s and 1980s propounding a causal link between artificial food colorants and behavioral disorders (Schauss, 1984). In 2004, Bateman et al., reported general adverse behavioral and psychological effects with artificial food colorants in preschoolers in a population based study. Likewise, there are reports about tartrazine-related hyperactivity in children (Schab and Trinh, 2004; McCann et al., 2007).

Noticeable effects of tartrazine on the behavior of young mice have also been reported (Tanaka, 2006; Tanaka et al., 2008). Amin et al. (2010) stated that tartrazine altered biochemical markers (for example, glutathione peroxidase (GPx), superoxide dismutase

(SOD), and catalase (CAT)) in vital organs i.e., liver and kidney in young male albino rats. Tartrazine is also reported to inflame the stomach lining (increased number of lymphocytes and eosinophils) of rats when given in the diet for a prolonged period of time (Moutinho et al., 2007).

For mitigating any possible adverse effect of the dyes, ADI (Acceptable Daily Intake) has been used in food products with colorants. However, there are reports in which administering the ADI values of artificial food colorings as a mixture (Erithrosine, Allura Red AC, Ponceau 4R, Sunset Yellow FCF, Tartrazine, Brilliant Blue, Amaranth, Azorubine and Indigotine) to female rats before and during gestation resulted in increased locomoter activity, as well as increased exploration and anxiety in their offspring (Doguc et al., 2013). Although, the results of research carried on animals do not overlap completely with humans, but the evaluation of effects of these food dyes at permissible levels in experimental studies provides an indication to strongly consider whether or not to eliminate these colorants from human diet.

The available literature reports that intake of tartrazine by children in developing countries like UAE and India exceeds their ADIs (Rao et al., 2004; Husain et al., 2006; Dixit et al., 2010; Dixit et al., 2011) because children are the major consumers of colored food. Moreover, the prescribed limit of tartrazine in food product samples was also exceeded (Dixit et al., 2011). Thus, the exposure of the vulnerable people in the population to excessive amounts of colorants may pose a health risk.

Therefore the aim of this work was to study the effects of tartrazine, a coloring agent widely used in food products at ADI levels on some biochemical parameters associated with oxidative stress in brain sub-regions of young male Wistar rats.

2. Materials and methods

2.1 Chemicals

Tartrazine ($C_{16}H_9N_4Na_3O_9S_2$; Product No. GRM431; CAS No. 1934-21-0) was procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All other laboratory chemicals were purchased from Sigma-Aldrich (USA), Merck (Germany), Rankem (Thane, India) and HiMedia (Mumbai, India).

For biochemical assays, ultrapure water prepared from Direct-Q® Water Purification System (Merck Millipore) was used throughout experiments for the preparation of reagents and buffers.

2.2 Animals and treatment

The animal care and handling was done according to the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. The Departmental Animal Ethical Committee (DAEC) approved the experimental protocols for this study.

A total of 16 male weanling Wistar rats weighing about 20-30 g were used in the present study. They were housed in an air conditioned room at 27 ± 2^0C with a 12h light-12h dark photoperiod under standard hygienic conditions in polypropylene cages with clean dust free wood shavings (procured locally) as bedding throughout the experiment. Rats were given standard rodent pellet diet (Aashirwaad Industries, Chandigarh, India) and water *ad libitum* throughout the experiment.

2.3 Treatment schedule

Sixteen male rat weanlings (21 days old) were divided into two groups of 8 rats each and treated for a period of 40 days till they became sexually mature as indicated below:

Group I: Control- received tap water by oral gavage

Group II: Received Tartrazine- 7.5 mg/kg body weight dissolved in tap water

The dose for the treatment group was selected based on the reports of United States Environmental Protection Agency (U.S. EPA, 2011) that suggest ADI value in accordance with the test plan of the dye. For group II, ADI value of 7.5 mg/kg body weight established by JECFA (0-10 mg/g body weight) was formulated in water and administered orally once daily by oral gavage whereas group I animals received tap water and served as control group. 50% of the animals from each group were used for studying changes in the levels of total protein and TBARS while the remaining rats from each group were used for studying other biochemical variables.

2.4 Autopsy

After 40 days of treatment, rats were sacrificed by cervical dislocation. Brains were taken out, rinsed in cold saline, blotted and weighed. They were then quickly placed on a chilled glass plate resting over ice for the separation of four brain sub-regions viz., frontal cortex, hippocampus, corpus striatum and cerebellum for biochemical estimation (Scheuhammer and Cherian, 1982).

2.5 Biochemical Assays

2.5.1 Tissue protein levels

The total protein of the brain tissue was estimated by "Lowry Assay: Protein by Folin Reaction" (*Lowry et al., 1951*). Rat brain homogenate was prepared by homogenizing tissue in 0.1M phosphate buffer solution (pH 7.4). To 1 ml of Lowry solution, 100 µL tissue sample was added. This reaction mixture was vortexed briefly and incubated for 20 minutes at room temperature in the dark. 100 µL of Folin reagent was then added to the reaction mixture and

incubated for 30 minutes in the dark at room temperature. The absorbance values were recorded at 750 nm and the protein content was expressed in mg/mL.

2.5.2 Tissue (Thiobarbituric acid reactive substances) TBARS levels

Measurement of product of lipid peroxidation i.e., TBARS was done according to the procedure given by Ohkawa et al. (1979). The end product of lipid peroxidation is MDA (malondialdehyde) which is quantified by reacting it with TBA (Thio-Barbituric-Acid). A 10% w/v tissue homogenate was prepared in chilled 1.15% KCl solution. The reaction mixture contained 0.1 mL of sample, 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid solution of pH 3.5 and 1.5 mL of 0.8% aqueous solution of TBA. The mixture was finally made up to 4.0 mL with distilled water and heated at 95^0C for 60 minutes. After cooling with tap water, 1.0mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, v/v) were added, and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the absorbance of the organic layer (upper layer) was measured at 532 nm and the amount expressed as nMoles TBARS formed per hour per milligram of protein using molar extinction coefficient of 1.56×10^5/M per cm.

2.5.3 Tissue Glutathione reductase (GR) activity

This was determined according to the method given by Beutler et al., (1963). Accordingly, the tissue GR activity was assayed using a 1 mL cuvette containing 350 µL of phosphate buffer solution (0.3 M; pH 7.0) to which, the following solutions were added: 250 µL of oxidized glutathione (0.012 M), 250 µL Na_2EDTA (0.25 M), 100 µL of β-NADPH (3 mM) and 50 µL aliquots of supernatant obtained after centrifugation at 14000×g for 25 minutes. The decrease in absorbance was read at 340 nm at 30 seconds interval for 2 minutes for the

conversion of NADPH to NADP. The enzyme activity is expressed as nmol NADPH oxidized/min/mg protein using 6.22 mM^{-1} cm^{-1} as molar extinction coefficient.

2.5.4 Tissue Glutathione-S-transferase (GST) activity

The activity of GST was determined according to the method given by Habig et al. (1974). The tissue supernatant was obtained by centrifuging 10 % (w/v) tissue homogenate at 1500×g (for removing cellular debris) for 10 minutes followed by 10000×g (to obtain cytosolic fraction for determination of enzyme activity) for 30 minutes at 4^0C, Briefly, the reaction mixture contained 50 µL of tissue supernatant, 50 µL of CDNB (30mM) in 750 µL of phosphate buffer (0.3 M; pH 6.5). The above reaction mixture was incubated at 37^0C for 5 minutes and the reaction was initiated by the addition of GSH (30 mM). The increase in absorbance was read at 340 nm at 30 seconds interval for 6 minutes and the enzyme activity expressed as µmol G-SDNB formed/min/mg protein using 9.6 mM^{-1} cm^{-1} as molar extinction coefficient.

2.5.5 *Tissue Glutathione peroxidase(GPx) activity*

GPx activity was determined according to the method given by Wood (1970). It was assayed using a 1 mL cuvette containing 700 µL of phosphate buffer solution (75 mM; pH 7.0) to which, the following solutions were added: 25 µL of glutathione reductase solution (100U/mL), 25 µL of sodium azide (0.12 M), 50 µL Na2EDTA (0.15 mM), 50 µL of β-NADPH (3 mM) and 50 µL aliquots of supernatant obtained after centrifugation at 14000×g for 25 minutes. The above reaction mixture was equilibrated at 25^0C after mixing thoroughly and the reaction was initiated by the addition of H_2O_2 (7.5 mM). The decrease in absorbance was read at 340 nm at 1 minute interval for 5 minutes for the conversion of NADPH to

NADP. The enzyme activity is expressed as nmol NADPH oxidized/min/mg protein using 6.22 mM^{-1} cm^{-1} as molar extinction coefficient.

2.5.6 *Tissue Superoxide dismutase (SOD)* activity

Brain SOD activity was estimated by the method given by Marklund and Marklund (1974) in which the degree of inhibition of pyragallol autoxidation at an alkaline pH by SOD is used as a measure of the enzyme activity. A 10% (w/v) tissue homogenate was prepared in PBS and used for the determination of SOD and CAT activity by ethanol chloroform extraction. To 1 mL tissue homogenate, 0.25 mL absolute ethanol and 0.15 mL of chloroform was added and was shaken for 15 minutes. It was then centrifuged for 10 minutes at 2000×g. The reaction mixture for autoxidation contained 2 ml of Tris-HCl buffer (0.1 M; pH 8.2), 0.5 mL of pyrogallol and 1.5 mL of distilled water, whereas the assay mixture contained 2 ml of Tris-HCl buffer, 0.5 mL of pyrogallol and 1.5 mL of aliquot of supernatant obtained during enzyme extraction, to estimate the decrease in autoxidation by SOD. An increase in absorbance was recorded at 420 nm for 10 minutes at 30 seconds interval by spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme inhibiting the rate of autoxidation of pyrogallol by 50% as determined by change in absorbance per minute at 420 nm. The activity of SOD is expressed as Units/mg protein.

2.5.7 *Tissue Catalase* activity

Catalase activity in the brain tissue was estimated using the method given by Aebi (1974), in which, the rate of H_2O_2 decomposition through catalase is measured. The reaction mixture contained 475 µL of phosphate buffer (50 mM; pH 7.0), 25 µL of distilled water and 250 µL of H_2O_2 (30 mM) constituting uninhibited system whereas, the assay mixture contained 475 µL of phosphate buffer, 250 µL of H_2O_2 and 25 µL of brain tissue supernatant which

constitutes inhibited system. The rate of decomposition of H_2O_2 in both the systems was measured spectrophotometrically at 240 nm. The molar extinction coefficient, 43.6 M-1 cm-1 was used in determination of catalase activity, where 1 unit of activity is equal to 1 millimol H_2O_2 decomposed per minute per milligram of protein and is expressed as Units/mg protein.

2.6 Statistical Analysis

All the results were analyzed using Graphpad Prism 7 (Version 7.00) for Windows 7. Each group contained 4 animals and values were represented as mean ± SEM. Repeated measures 2-way ANOVA followed by a Sidak's multiple comparision *post-hoc* analysis was used to analyze changes in body weight. The two factors used were age (week 3 to week 9) and treatment (Tarttazine).Comparisons of mean were analyzed by Student's t-test for body weight gain, brain weight and biochemical estimation observations. P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 were considered as statistically significant, highly significant and extremely significant respectively.

3. Results

3.1 Effect on Body weight, Body weight gain and Brain weight

Body weights of treated and control animals were recorded daily throughout the 40 day experimental period. The average weekly weight of the tartrazine treated animals was highly significantly lower than that of the control animals (Fig.1). Also, highly significant decline in body weight gain in the tartrazine treated group (42.350 ± 2.906** gram) was observed compared to control group (73.400 ± 2.626 gram). On the contrary, there was no significant change in brain weight of tartrazine treated animals (1.532 ± 0.04105 gram) when compared to that of control group animals (1.479 ± 0.04120 gram).

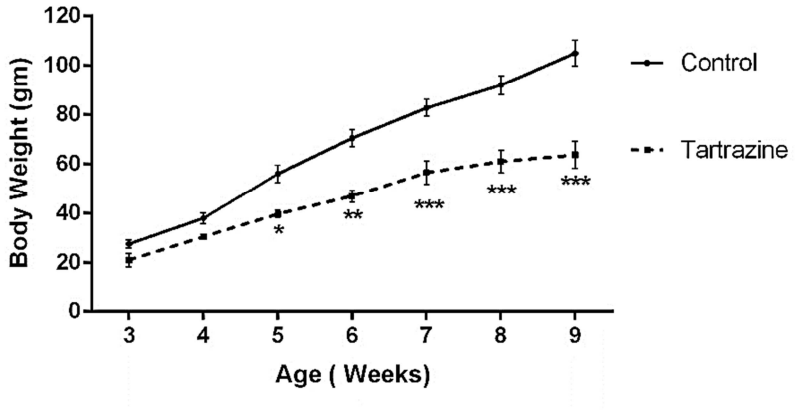
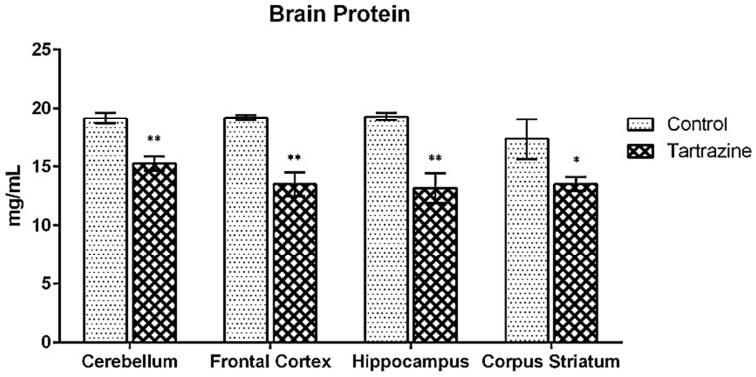


Fig.1. Effect of ADI of Food Azo dye Tartrazine on body weight in male Wistar rats. Values are mean ± SEM. ***, **, ***** indicates significant ($p \leq 0.05$), highly significant ($p \leq 0.01$) and extremely significant ($p \leq 0.001$) difference from Control group respectively.

3.2 Effect on Tissue protein levels and Tissue TBARS (Thiobarbituric acid reactive substances) level in brain sub-regions

Tissue protein levels in all the brain sub-regions decreased significantly whereas there was a significant increase in lipid peroxide levels in all brain sub-regions as shown by the marked increase in TBARS levels in Fig.2.



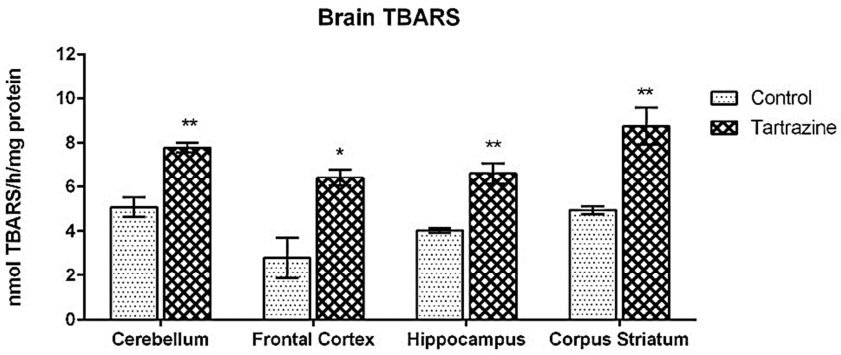
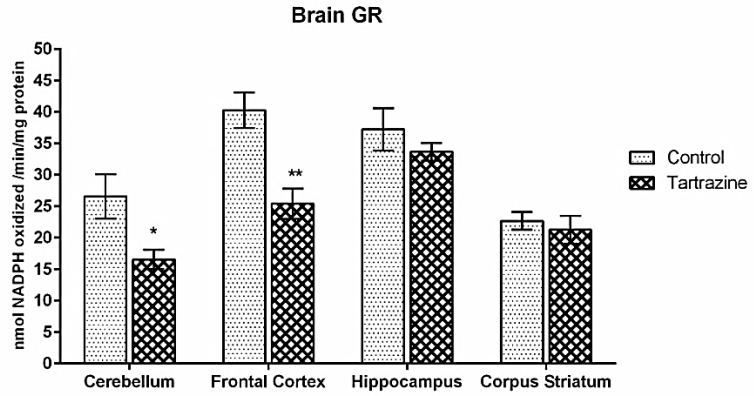


Fig.2. Effect of ADI of Food Azo dye Tartrazine on tissue protein levels and TBARS levels in Brain Sub-regions viz., Cerebellum, Frontal Cortex, Hippocampus and Corpus Striatum of rats. Values are mean ± (SEM) of 4 rats per group. *****, ****** indicates significant ($p \leq 0.05$) and highly significant ($p \leq 0.01$) difference from Control group respectively.

3.3 Effect on Tissue Glutathione reductase (GR), Glutathione-S-transferase (GST) and Glutathione peroxidase (GPx) activities in brain sub-regions

Tartrazine administration significantly decreased GST activity in all the brain sub regions. There was also a significant decline in GR activity in cerebellum and frontal cortex, whereas hippocampus and corpus striatum were less affected. However, Gpx activity on the other hand was seen elevated in all the brain sub regions with a significant increase in corpus striatum.



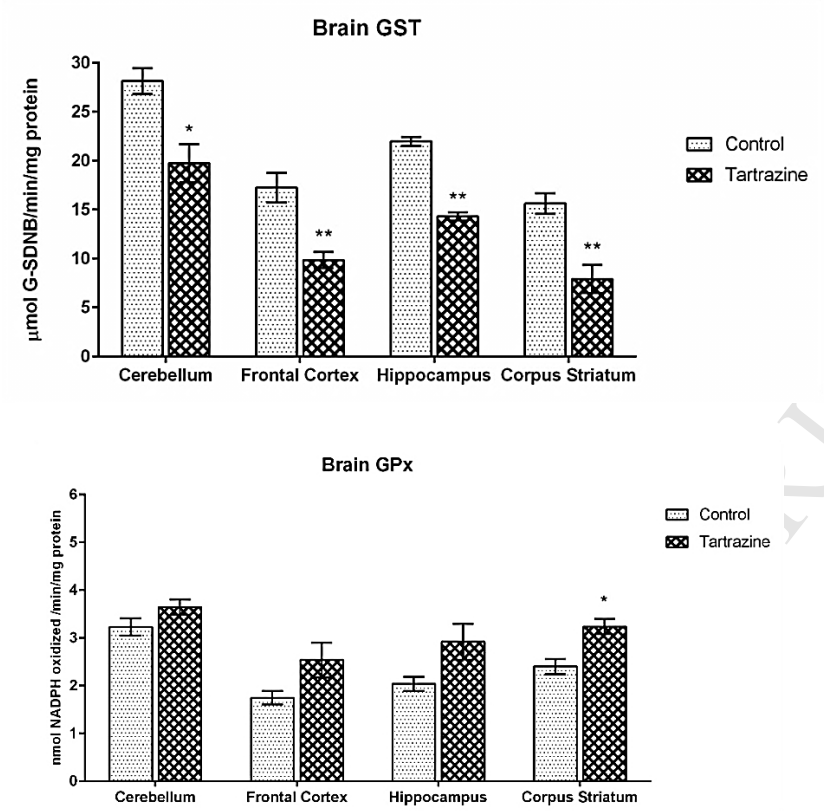


Fig.3. Effect on GR, GST and GPx activities in Cerebellum, Frontal Cortex, Hippocampus and Corpus Striatum of rats following administration of ADI of Tartrazine. Values are mean ± (SEM) of 4 rats per group. *****, ****** indicates significant ($p \leq 0.05$) and highly significant ($p \leq 0.01$) difference from Control group respectively.

3.4 Effect on Tissue Superoxide dismutase (SOD) and Catalase activities in brain sub-regions

A significant decrease in SOD activity in the cerebellum, frontal cortex and hippocampus was observed thereby leaving the corpus striatum less affected .Likewise, CAT activity showed a significant decrease in all the brain sub regions except the hippocampus as compared to controls.

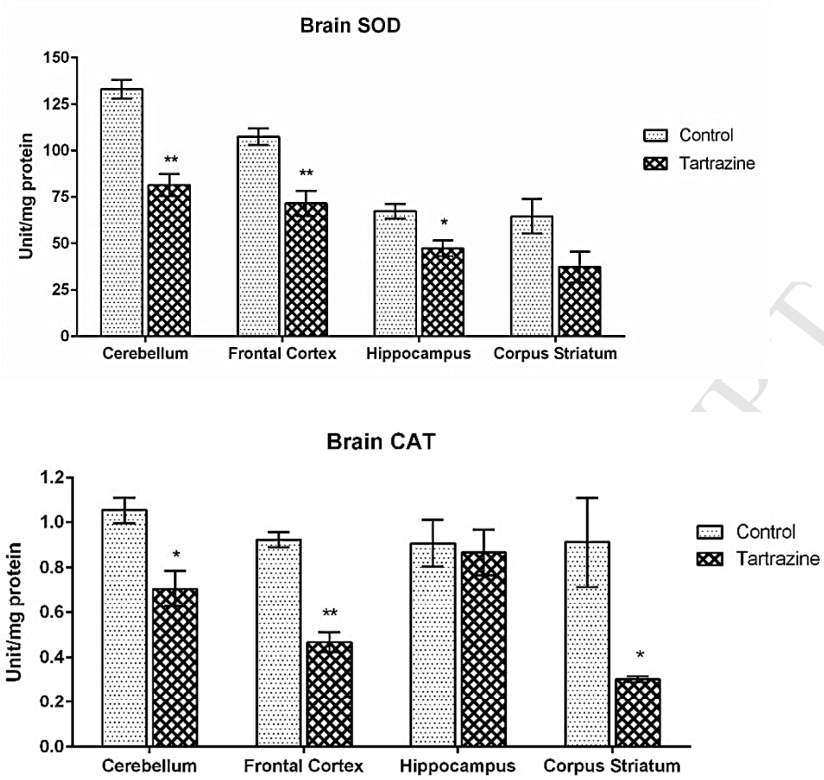


Fig.4. Effect on SOD and CAT activities in the Cerebellum, Frontal Cortex, Hippocampus and Corpus Striatum of rats following administration of ADI of Tartrazine. Abbreviations and unit used: SOD, Superoxide Dismutase; 1 Unit is equals the amount of enzyme inhibiting the rate of autoxidation of pyrogallol by 50%; CAT: Catalase; 1 Unit equals to 1 millimol H_2O_2 decomposed per minute per milligram protein. Values are mean ± (SEM) of 4 rats per group. *****, ****** indicates significant ($p \leq 0.05$) and highly significant ($p \leq 0.01$) difference from the Control group respectively.

4. Discussion

We investigated the toxic effects of ADI of the food azo dye, tartrazine on oxidative stress biomarkers. Extensive application of the dye in our food industry has led to serious concerns towards its liberal use in food stuffs. There is literature available on the toxicity of food azo dyes at high and low doses but not on ADI of these dyes.

The ADI dose of tartrazine chosen for this study was 7.5 mg/kg body weight. This was based on the ADI range of 0-10 mg/kg body weight as established by JECFA (2016).

Post tartrazine administration, there occurred a significant decrease in body weight gain which may represent the dye's adverse effect, as, loss in body weight is considered as a reliable sensitive toxicity indicator (Ezeuko et al., 2007). This is in consonance with the findings of Amin et al., (2010) who reported a loss in body weight in male rats after 30 days oral administration of tartrazine at a dose double the ADI (15 mg/kg/body weight).

In the present study, decrease in tissue protein levels in all brain sub-regions is indicative of a highly catabolic environment as a decline in protein profile suggests stress in metabolic activities of cells and tissues and an impairment of protein synthesis in animals. The catabolic process may be proteolysis, leading to a rapid decline in proteins to meet the energy demand in stressful conditions (Rajini et al., 2014).

Sweeny et al. (1994) found that various azo dye products are genotoxic only after reduction by gastrointestinal bacteria, *Enterococcus faecalis*. The mechanism may involve oxygen radicals and superoxide free radical formation. Azo compounds contain an aromatic ring linked by an azo bond to second naphthalene or benzene ring (Amin et al. 2010). When they reach the intestine after oral ingestion, azo reductases from intestinal bacteria and the liver's cytosolic and microsomal enzyme fraction reduce the azo bond to produce aromatic amines, which have been found in the urine of dyestuff workers and experimental test animals following administration of food azo dyes (Cerniglia et al., 1986).

The gastrointestinal microflora are reported to metabolize tartrazine into aromatic amine sulphanilic acid (Mountinho et al., 2007).^{38}S-labelled sulphanilic acid has also been reported to enter the brains of developing rats by diffusing across blood-brain barrier (Goldenring et al., 1982).

Therefore, it may be probable that these aromatic amines can generate reactive oxygen species such as hydroxyl radical, hydrogen peroxide and superoxide anion as a part of their metabolism leading to oxidative stress (Bansal 2005) as evident in the results of the present

study which showed declines in the levels of SOD and CAT in the brain tissue homogenate. The degree of impaired ability to detoxify reactive oxygen free radicals and hydrogen peroxides via SOD and catalase appeared to be more in the cerebellum and the frontal cortex as compared to the corpus striatum and hippocampus in our findings. Thus, the significantly low CAT and SOD activities in the brain tissue of treated rats indicates the severity of the tartrazine-induced oxidative stress condition.

Previous studies have shown that increased generation of free radicals is a result of the decreased SOD and CAT activities, causing superoxide anion radical to accumulate. This might be responsible for increased lipid peroxidation of cellular membranes, generating MDA, the degradation end product of lipid peroxidation which reflects the damage caused by reactive oxygen species (Qin et al., 2009). In the present study, tartrazine significantly increased MDA levels in all brain sub-regions indicating damage caused by reactive oxygen species.

Further our study has shown an increase in GPx activity in all brain sub-regions suggesting a counteracting mechanism towards insufficient activity of CAT, GST and GR. Under physiological conditions, the enzyme GST initiates detoxification by catalyzing the conjugation of GSH to the electrophilic foreign compounds for their elimination from the system (Dynyelle and Kenneth, 2003), GR constantly works to replenish reducing equivalents for efficient working of glutathione assisted antioxidant enzyme whereas, GPx plays an important role in reducing the oxidative stress induced by toxicants (Sachdeva et al., 2015). Moreover, this significant decrease in CAT, GST and GR activity in tartrazine-exposed rats may also be attributed to the increased levels of lipid peroxides as evident in the present study, where a more pronounced increase in TBARS levels was seen in the cerebellum, hippocampus and corpus striatum as compared to the frontal cortex. A similar trend was observed by Gao et al. (2011), when Sprague-Dawley rats were administered 250

and 500 mg/kg/body weight of tartrazine for a period of 30 days resulted in decline in activities of SOD and CAT as well as rise in the level of MDA.

Among the brain sub-regions evaluated for biochemical alterations, the cerebellum and frontal cortex were found to be more prone to oxidative stress as evident in the findings of all the biochemical tests conducted in the present study. Various researches have earlier shown that the regional distribution of the antioxidant enzymes varies distinctly in the brain (Brannan et al., 1980; Goss-Sampson et al., 1988; Ansari et al., 1989; Verma et al., 1992), and so does the metabolic rate that could be responsible for differential oxidative damage in the brain sub-regions (Shukla et al., 1988; Hussain et al., 1995). Our study also indicates that the vulnerability to oxidative stress of the brain is region-specific which is in concomitance with the report by Baek et al. (1999).

5. Conclusion

Food azo dye tartrazine can elicit adverse effects and can alter biochemical markers in brain even at prescribed ADI levels. This study provides some interesting observations for ADI level exposure to azo food dye tartrazine and the related toxic manifestations. Moreover, we may assume that tartrazine-induced oxidative stress condition might be the result of its metabolic product sulphanilc acid, as a possible mechanism of toxicity. Therefore, it is necessary to create awareness regarding the human health effects of these synthetic food dyes to consumers.

6. Conflict of Interest

The authors declare that there are no conflicts of interests.

7. Acknowledgments

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Highlights

- Acceptable daily intake of tartrazine (food azo dye) by Wistar rat induced oxidative stress in brain, when exposed from postnatal day 21 to 60.
- Activities of anti-oxidant enzymes were significantly altered.
- Lipid peroxidation was increased.
- Vulnerability to oxidative stress in brain is region specific.