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COMBINE COLORANTS OF TARTRAZINE AND ERYTHROSINE INDUCE EXPRESSION OF PRO-INFLAMMATORY CYTOKINES IN KIDNEY INJURY: INVOLVEMENT OF INTERLUKIN 1-A (IL-1A), INTERLUKIN 1-B (IL-1B) GENES AS KIDNEY FUNCTIONS INDICES

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ABSTRACT

Tartrazine and Erythrosine are synthetic food colorants widely used in food, pharmaceuticals and cosmetics industries with less attention on their toxic effects. This study is aimed at investigating the effect of Tartrazine and Erythrosine in the expression of Interlukin-1alpha gene (IL-1α) and Interlukin-1beta gene (IL-1β genes) in the kidneys of male wister rats. Twenty- five male wister rats divided into five groups of five rats each were administered 2.5mg/kgb.wt, 5mg/kgb.wt, 10mg/kgb.wt, and 20mg/kgb.wt of mixture of tartrazine and erythrosine respectively while Group 1 served as the control and was treated with only water and feed for 23 days. The Kidneys were harvested and preserved in an Eppendorf tube containing trizol was used to measure the expression of Interlukin 1-α gene and Interlukin 1-β gene, using modified RNA snap kit method. The result revealed an up regulation of IL-1α gene expression in groups treated with 2.5mg, 5mg, and 10mg but a down regulation in group treated with 20mg when compared with the control. Also the expression of IL-1β gene were highly up regulated at all doses when compared with the control. This implies that constant ingestion of these synthetic food colorants can increase expression of pro-inflammatory cytokines in kidney as shown by upregulation of the genes.

KEYWORD: Tartrazine, Erythrosine, Interlukin-1alpha gene, Interlukin-1beta gene, Urea.

INTRODUCTION

Interleukins (ILs) are a group of cytokines (secreted proteins and signal molecules) that were first seen to be expressed by white blood cells (leukocytes). ILs can be divided into four major groups based on distinguishing structural features. However, their amino acid sequence similarity is rather weak (typically 15–25% identity). The human genome encodes more than 50 interleukins and related proteins (Zmasek, et al 2007). IL-1α and IL-1β are the most studied members, because they were discovered first and because they possess strongly proinflammatory effect. They have a natural antagonist IL-1Ra (IL-1 receptor antagonist). All three of them include a beta trefoil fold and bind IL-1 receptor (IL-1R) and activate signaling via MyD88 adaptor, which is described in the Signaling section of this page. IL-1Ra regulates IL-1α and IL-1β proinflammatory activity by competing with them for binding sites of the receptor (Zmasek, et al 2007).

The function of the immune system depends in a large part on interleukins, and rare deficiencies of a number of them have been described, all featuring autoimmune diseases or immune deficiency. The majority of interleukins are synthesized by helper CD4 T lymphocytes, as well as through monocytes, macrophages, and endothelial cells. They promote the development and differentiation of T and B lymphocytes, and hematopoietic cells. Interleukin receptors on astrocytes in the hippocampus are also known to be involved in the development of spatial memories in mice (Dinarello 1994a).

IL-1 family is a group of 11 cytokines, which induces a complex network of proinflammatory cytokines and via expression of integrins on leukocytes and endothelial cells, regulates and initiates inflammatory responses. IL-1α and IL-1β are the most studied members, because they were discovered first and because they possess strongly proinflammatory effect. They have a natural antagonist IL-1Ra (IL-1 receptor antagonist). All three of

them include a beta trefoil fold and bind IL-1 receptor (IL-1R) and activate signaling via MyD88 adaptor, which is described in the Signaling section of this page. IL-1Ra regulates IL-1α and IL-1β proinflammatory activity by competing with them for binding sites of the receptor (Dinarello 1994b).

Nine IL-1 superfamily members occur in a single cluster on human chromosome two; sequence and chromosomal anatomy evidence suggest these formed through a series of gene duplications of a proto-IL-1β ligand. In this way, IL-1β, IL-1α, IL-36α, IL-36β, IL-36γ, IL-36RA, IL-37, IL-38, and IL-1RA are very likely ancestral family members sharing a common lineage. However, IL-18 and IL-33 are on different chromosomes and there is insufficient sequence or chromosomal anatomy evidence to suggest they share common ancestry with the other IL-1 superfamily members. IL-33 and IL-18 have been included into the IL-1 superfamily due to structural similarities, overlap in function and the receptors involved in their signalling (Dinarello 1994c). IL-1α is a "dual-function cytokine", which means it plays a role in the nucleus by affecting transcription, apart from its extracellular receptor-mediated effects as a classical cytokine. In this group also belongs IL-33.

IL-1α is synthesized as a precursor protein and it is constitutively stored in the cytoplasm of cells with mesenchymal origin and in epithelial cells. On the contrary, monocytes and macrophages do not contain preformed IL-1α precursors, but instead require de novo synthesis. IL-1α precursor is processed to a mature 17-kDa protein by a Ca2+-activated protease, calpain, by liberating the 16-kDa N-terminal propiece cleavage product (ppIL-1α), which contains a nuclear localization sequence (NLS), translocates to the nucleus and functions as a transcription factor. The precursor form of IL-1α, which has both the N-terminal and C-terminal receptor interacting domains, acts as a damage-associated molecular pattern (DAMP) molecule. DAMPs, also known as alarmins, are recognized by innate immunity cells by pattern recognition receptors (PRRs) and functions as danger signals for the immune system. In short, DAMPs are released from stressed cells, which undergo necrosis or pyroptosis and their intracellular components are released into extracellular space. Because of misfolding and other oxidative changes of these molecules in the context of altered pH, they are recognized by innate immunity as molecules that should not be in extracellular space. The reasons why the cell could be stressed are infection, injury, ischemia, hypoxia, acidosis and complement lysis. The IL-33 precursor molecule acts in a similar way as a DAMP molecule (Sahoo et al 2011).

The inflammatory responses in the absence of infection (such as ischemia) are only dependent on IL-1α signaling via the Interleukin-1 receptor (IL-1R), rather than TLRs signaling. IL-1α also stimulates transcription and secretion of IL-1β from monocytes, so the initiator of immune responses is likely IL-1α precursor by induction of neutrophil infiltration. IL-1β seems to be an amplifier of inflammation by recruiting of macrophages in the context of sterile inflammation.

IL-1β is synthesized as a precursor form protein only after stimulation, in contrast to IL-1α. Its expression is induced by transcription factor NF-κB after exposure of innate immune cells to alarmins. This occurs, for instance, after exposure of macrophages and dendritic cells to lipopolysaccharide (LPS), which binds to transmembrane protein such as toll like receptor 4 (TLR4) and acts as pathogen-associated molecular pattern, which is another group of alarmins (Liu et al 2010).

The synthesis of IL-1β precursor (and IL-18) is induced by stimulation of innate immune cells by Toll-like receptors (TLRs) or RIG-like receptors (RLRs), but to gain the ability to bind to IL-1 receptor, the IL-1β precursor has to be cleaved by a cysteine protease called caspase-1. Caspase-1 needs to be activated by a formation called the inflammasome which is mediated by cytoplasmic pattern recognition receptor signaling. So, the secretion of IL-1β needs these two steps and activation of different receptors to be activated. Under special circumstances IL-1β can be processed also by other proteases, like during high neutrophilic inflammation. IL-18 is also synthesized as a precursor which is cleaved by caspase-1(Liu et al 2010).

Erythrosine, also known as Red No. 3, is an organoiodine compound, specifically a derivative of fluorone. It is a red dye which is primarily used for food coloring. It is the disodium salt of 2,4,5,7-tetraiodofluorescein. Its maximum absorbance is at 530 nm in an aqueous solution, and it is subject to photodegradation (Food Product Design 2006). The IUPAC name is 2-(6-Hydroxy-2,4,5,7-tetraiodo-3-oxo-xanthen-9-yl) benzoic acid (Food Product Design 2006) while the Chemical formula $C_{20}H_6I_4Na_2O_5$ and physical properties are Molar mass 879.86 g/mol and Melting point 142 to 144 °C (288°F to 291 °F; 415 K to 417 K).It is used as food coloring, printing ink, biological stain, dental plaque disclosing agent, radiopaque medium, sensitizer for orthochromatic photographic films and Erythrosine is commonly used in sweets such as some candies and popsicles, and even more widely used in cake-decorating gels. It is also used to color pistachio shells. As a food additive, it has the E number E127 (Jennings, et al 2007).

As a result of efforts begun in the 1970s, in 1990 the U.S. FDA had instituted a partial ban on erythrosine, citing research that high doses have been found to cause cancer in rats. In June 2008, the Center for Science in the Public Interest (CSPI) petitioned the FDA for a complete ban on erythrosine in the United States (FDA 2013). A 1990 study concluded that "chronic erythrosine ingestion may promote thyroid tumor formation in rats via chronic

stimulation of the thyroid by TSH." with 4% of total daily dietary intake consisting of Erythrosine B (FDA 2013). Erythrosine can be used in colored food and ingested drugs in the USA without any restriction, however, its use is banned in cosmetics and external drugs. The lake variant is also banned from use in the United States (Jennings, et al 2007).

Tartrazine is a synthetic lemon yellow azo dye primarily used as a food colouring dye(Amin et al 2010). It is also known as E number E102, FD & C Yellow 5, Acid Yellow 23, Food Yellow 4, and trisodium 1-(4-sulfonatophenyl)-4- (4-sulfonatophenylazo)-5-pyrazolone-3-carboxylate). Tartrazine is a commonly used color all over the world, mainly for yellow, and can also be used with Brilliant Blue FCF (FD&C Blue 1, E133) or Green S (E142) to produce various green shades. Tartrazine is water-soluble and has a maximum absorbance in an aqueous solution at 425 nm. Like many azo dyes tartrazine is manufactured using petrochemicals as starting stock, and was formerly manufactured using chemicals derived from coal tar as the starting material (Mehedi et al 2009). Tartrazine is used in various area such as Food, Beverages, Snacks, Dessert and confectionary, other processed foods and Personal care and cosmetic product.

This study is aimed at investigating the effects of Combine Colorants of Tartrazine and Erythrosine on kidney injury in albino rats using Interlukin 1-α (IL-1α), Interlukin 1-β (IL-1β) genes as indicators.

MATERIALS AND METHODS

Animals: Twenty-five (25) albino rats weighing about 116.00-171.00g were obtained from animal house Federal University of Technology, Owerri and kept in the animal farm, Madonna University, Elele, Rivers State. The rat feeds were obtained from Elele market, Rivers State.

Reagents: Commercially prepared RNA Snap Kit was used. TRI reagent (Zymo Research, USA, Cat:R2050-1-50, Lot: ZRC186885), chloroform (BDH Analytical Chemicals, Poole, England Cat: 10076-6B), centrifugation at 15,000 rpm/15 min (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States), isopropanol (Burgoyne Urbidges & Co, India, Cat: 67-63-0), 70% ethanol (70 ml absolute ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y), 30 ml of nuclease-free water (Inqaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ML), 2.0 % agarose gel (Cleaver Scientific Limited: Lot: 14170811), Tris (RGT reagent, china, Lot: 20170605), Borate (JHD chemicals, China, Lot 20141117) and EDTA buffer (pH 8.4).

Food colorant: The synthetic food colorants (tartrazine and erythrosine) used for this study was obtained from Sigma company United States of America. Equal weight of tartrazine and erythrosine were dissolved in distilled water for different concentrations of the mixture. i.e 1.25mg tartrazine and 1.25mg erythrosine for 2.5mg/kg, 2.5mg tartrazine and 2.5mg erythrosine for 5mg/kg, 5mg tartrazine and 5mg erythrosine for10mg/kg, and 10mg tartrazine and 10mg erythrosine for 20mg/kg.

Ethical approval: The research was approved by the ethical committee of the institution, the standard rules and regulations for the use of animal for research purposes was strictly adhered to as approved by the committee.

Methods

LC_{100} Study: Preliminary study was done to determine the lethal concentration (LC_{100}) and lethal concentration (LC_{50}) of erythrosine and tartrazine on the rats. Twenty-five albino rats were divided into five (5) groups of five (5) rats each and was treated with erythrosine and tartrazine synthetic food colorants thrice (3) a week for 23days. Physical changes (weakness, abnormal movement, diarrhoea), behavioural changes (aggressiveness, rational movement, bitting and hyperactivity) and death was observed.

Animal studies: The animals were acclimatized for one week and fed *ad libitum* with normal rat feed and water at 12hours of daylight and 12 hours of darkness. The twenty-five (25) albino rats were divided into five (5) groups of five (5) rats each. The animals in groups 2, 3, 4, and 5 were treated with combined administration of tartrazine and erythrosine at concentrations of 2.5mg/kg, 5mg/kg, 10mg/kg, and 20mg/kg respectively using cannula and syringe once daily and thrice (3) a week for three weeks while Group 1 served as the control and were fed *ad libitum* with water and rat diet. After 23days of administration, all groups of the rats were sacrificed by chloroform sedation. Each animal in each group was sacrificed and kidney was collected and preserved in trizol solution for gene expression and profiling.

Gene expression and profiling: Modified RNA Snap kit method was used as described by Omotuyi, *et al.*, (2018).

Principle of polymerase Chain Reaction (PCR): The PCR involves the primer mediated enzymatic amplification of deoxyribonucleic acid (DNA). It is based using the ability of DNA polymerase to synthesize new strand of DNA complimentary to the offered template strands. Primer is needed because DNA polymerase can and a nucleotide only into a preexisting 3'OH group to add the first nucleotide. DNA polymerse then elongate its3 end by adding more nucleotides to generate an extended region of double strands DNA. The PCR is used for RNA Isolation, RNA Quantification, PCR amplification, and Agarose gel electrophoresis while Amplicon image processing and semi-quantification was used for identification.

Total RNA isolation

Total RNA was isolated from whole tissue following modified RNA snap kit method as described by Omotuyi, *et al.,* (2018). Briefly, tissues were homogenized in cold (4 °C) TRI reagent. Total RNA was partitioned in chloroform following centrifugation at 15,000 rpm/15 minutes. RNA from the clear supernatant was precipitated using equal volume of isopropanol. RNA pellet was rinsed twice in 70% ethanol in 30 ml of nuclease-free water. The pellets were air-dried for 5 min and dissolved in RNA buffer (1 mM sodium citrate, p^H 6.4).

cDNA conversion: Prior to cDNA conversion, total RNA quantity (concentration (µg/ml) = 40 * A_{260}) and quality (≥ 1.8) was assessed using the ratio of A_{260}/A_{230} (A=absorbance) read using spectrophotometer (Jen-way UV-VIS spectrophotometer model 6305, UK). DNA contamination was removed from RNA was removed following DNAse I treatment (NEB, Cat: M0303S) as specified by the manufacturer. 2 µl solution containing 100 ng DNA-free RNA was converted to cDNA using M-MuLV Reverse transcriptase Kit (NEB, Cat: M0253S) in 20 µl final volume (2 µl, N^9 random primer mix; 2 µl, 10X M-MuLV buffer; 1 µl, M-MuLV RT (200 U/µl); 2 µl, 10 mM dNTP; 0.2 µl, RNase Inhibitor (40 U/µl) and 10.8 µl nuclease-free water). The reaction proceeded at room temperature O/N. Inactivation of M-MuLV Reverse transcriptase was performed at 65°C for 20 minutes.

Polymerase Chain Reaction (PCR) amplification and agarose gel electrophoresis

PCR amplification for the determination of genes whose primers (Primer3 software) (Ye et al 2012) are listed below were done using the following protocol: PCR amplification was performed in a total of 25 µl volume reaction mixture containing 2 µl cDNA (40 ng), 2 µl primer (100 pmol) 12.5 µl Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB, Cat: M0486S) and 8.5 µl nuclease-free water. Initial denaturation at 95 °C for 5 minutes was followed by 20 cycles of amplification (denaturation at 95 °C for 30 seconds, annealing (TM values for each primer pair in appendix 2) for 30 seconds and extension at 72 °C (for 60 seconds) and ending with final extension at 72 °C for 10 minutes. In all experiments, negative controls were included where reaction mixture has no cDNA. The amplicons were resolved on 2.0% agarose gel in Tris-Borate -EDTA buffer (pH 8.4).

Amplicon image processing and semi-quantification

In-gel amplicon bands images captured on camera were processed on Keynote platform. Gel density quantification was done using Image-J software (Rueden et al 2017). Each point represent relative expression (test gene band intensity/ internal control band intensity)*100) plotted using Numbers software (Mac OSX version).

Statistical analysis

Data obtained were subjected to statistical analysis using the gene expression was read by Image-J Software. Results were presented in graphs.

RESULT AND DISCUSSION

Interlukin-1alpha (IL-1α)

As shown in figure 1 below there was an upregulation in the expression of IL-1α gene in groups treated with 2.5mg/kg and 5mg/kg of tartrazine and erythrosine when compared to the control and other group. There was also a down regulation in the expression of interlukin-1alpha gene in group treated with combined administration of 20mg/kg of tartrazine and erythrosine when compared to the control and other groups. From the result, there was upregulation of IL-1α gene in the groups treated with 2.5mg, 5mg and 10mg of combined dose of tartrazine and erythrosine with down regulation of the gene in group treated with 20mg of tartrazine and erythrosine when compared with the control. This indicates highly inflamed environment, which is a hallmark for glomerulonephritis which can also result in kidney failure (Bernstein, and Treyzon 2007). Furthermore, there was up regulation of IL-1β gene in groups treated with 2.5mg, 5mg, 10mg, and 20mg of both tartrazine and erythrosine when compared with the control.

IL-1β is a master pro-inflammatory mediator that functions to regulate the expression of various other pro-inflammatory cytokines, adipokines and chemokines through the involvement of various transcriptional mediated pathways (Akash, et al 2012). From the result this indicates that the cystolic pro-IL-1α is a principal inflammation triggering moiety in necrotic cells which includes traumatic injury and hyperthermia (Garlanda et al 2013). The up regulation of these genes can lead to impaired kidney function. This is as a result of treatments given to the animal.

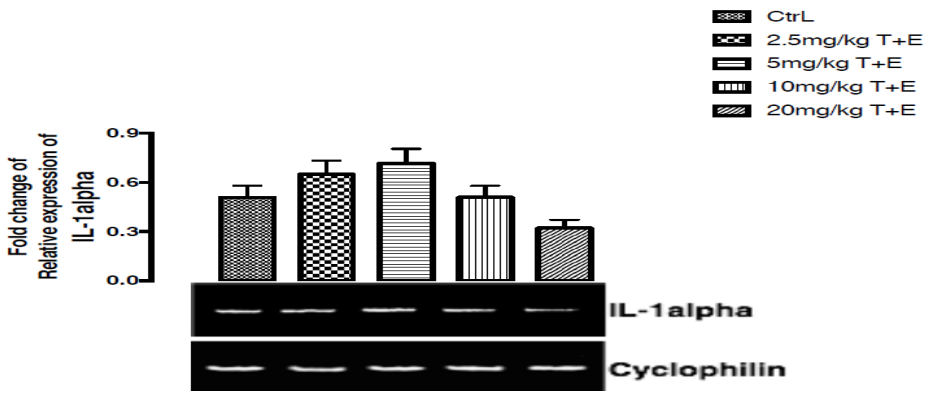

Figure 1: The effect of the combined treatment of T+E in the expression of interlukin-1alpha gene after 23 days.

interlukin -1beta(IL-1β)

As shown in figure 2 below, there was a significant upregulation in the expression of IL-1β gene in groups treated with 2.5mg/kg of tartrazine and erythrosine, 5mg/kg of tartrazine and erythrosine, 10mg/kg of tartrazine and erythrosine and 20mg/kg of tartrazine and erythrosine respectively when compared to the control. Gulati *et al* (2016) has suggested that, cytokines may be useful biomarkers for health and disease and act as diagnostic, prognostic and therapeutic agents. Cyclophilin are housekeeping genes also called constitutive genes which is needed for the maintenance of essential cellular functions and present in both normal and pathological conditions. In a similar to study by Wopara *et al* (2021) there was upregulation of Caspace 9 and TNF in groups treated with 2.5mg, 5mg. It can therefore be said that administration of these food colorants at concentrations of 2.5mg and 5mg are a potential risk factor for kidney dysfunction because the combined food colorants caused increase expression of pro-inflammatory cytokines in kidney. This is because the kidney is a delicate organ in the body whose major function is to filter and excrete waste products, keep the body in proper equilibrium and enhance the production of calcitriol and erythropoietin, therefore damage to the kidney will lead to malfunction of this processes in the body.

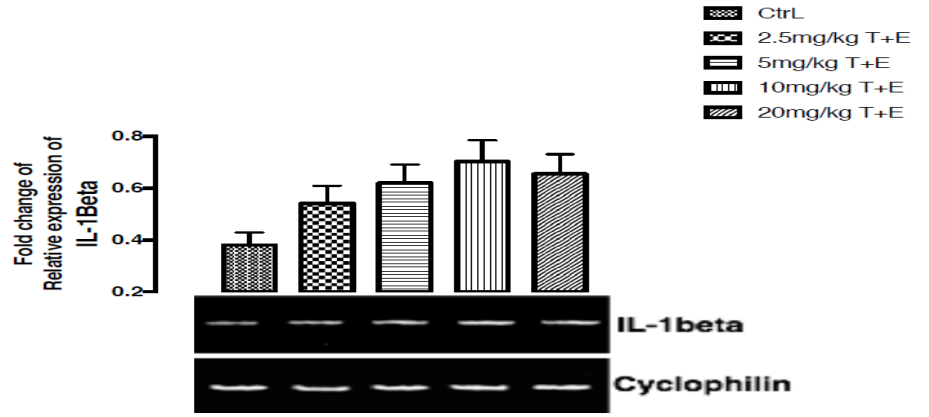

Figure 2: The effect of the combined treatment of T+E in the expression of interlukin-1beta gene after 23 days.

The Urea (Mmol/l) concentration increased from 4.62±0.46, 4.80±0.38, 6.07±0.38 in control and groups treated with 2.5mg and 5mg of tartrazine and erythrosine respectively before decreasing to 4.27±0.67 and 4.90±0.43 respectively at 10mg and 20mg of tartrazine and erythrosine respectively as shown in table 1.

CONCLUSION

The study has shown that combine administration of tartrazine and erythrosine can increase expression of pro-inflammatory cytokines in kidney.

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