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RESEARCH ARTICLE

Mir-140 and Mir-34a as Molecular Markers for Apoptotic Brain in Sunset Yellow and Carmoisine Intoxicated Mice

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Abstract

Brain is the central organ in human body, that is working 24hr/7days even before we leave our mother`s womb. None surprising that care should be paid for the food consumed that consequently affecting brain environment, neurotransmitters as well as oxidative state. Carmoisine (Car) and sunset yellow (SY) are synthetic food additives extensively utilized during food processing and subsequently affecting brain health. The apoptotic effect underling the behavioral changes after oral consumption of either Car or SY remains not fully understudied. Respective biochemical and molecular biological parameters by means of one fifty adult male mice were conducted. The study extended for 3 months on 5 different groups with ten mice each; Group 1 was utilized as the control group, group 2 was treated with the acceptable daily intake (ADI) of SY (30 mg/kg BW), group 3 was treated with 10x ADI of SY, group 4 was treated with ADI of Car (4 mg/kg BW) and group 5 was administered 10x ADI of Car; all doses were given orally via gastric lavage. Exposure to higher doses of either SY or Car significantly altered the biochemical parameters; decreased both serotonin and dopamine levels and total antioxidant capacity as well. However, increased the lipid peroxidation marker malonaldehyde (MDA), upregulated the mRNA expression of the pro-apoptotic genes (Fas, Fas-L, Bax and casp3) and down regulated the transcriptional level of the anti-apoptotic gene *Bcl2*.Moreover upregulated both *miR-140 and miR-34a* expression levels and consequently down-regulated their target genes *NRF-2 and SIRT-1*, respectively. So, we can conclude that excessive oral administration of either Car or SY has apoptotic effect and care should be paid with their usage.

Keywords: MicroRNA, Apoptosis, Carmoisine, Sunset yellow, and neurotransmitters.

Introduction

Food additives have been extensively utilized in food processing, manufacturing, and packaging. Either natural or synthetic additive; it usually consumed for improvement of food quality, taste, and appearance [1]. Food additives serve as colourants, emulsifiers, stabilisers, and taste modifiers. Colouring dyes are extensively applied among all food additives[2, 3]. Among these dyes azo compounds named for their azo group (-N=N-), are the most utilized ones; for imparting a new colour to the products to be more attractive for consumers especially children [4]. Carmoisine/Azorubine(E122) (Car) is a legalised azo dye by Joint FAO/WHO Expert Committee on Food Additives (JECFA) Scientific Committee for Food (SCF) as a food additive with established ADI of 0-4 mg/kg BW/day [5, 6]. Sunset Yellow FCF (E110) is a food colorant with ADI of 30 mg/kg BW/day [7].

Sunset yellow (SY) has been listed by National Institute for Occupational Safety and Health (NIOSH) as having behavioural consequences involving coma with impact on seizure threshold only in acute high doses (LD50) of SY [8]. SY was reported to have mutagenic and carcinogenic [9], immunological [10-12] effects in mammals. Car could alter the different bio-elements levels in liver, kidney and brain [13], trigger renal failure, hepatotoxicity [14] combined with genotoxicity [15] .

Oxidative stress is a discrepancy among reactive oxygen species (ROS) production and accumulation in the living tissues [16]. Many food additives could affect normal functions of various organs with different extent, inducing excessive alteration concerning brain health and environment causing short-term memory loss [17]. Oxidative stress may incline to cellular death across diverse mechanisms. The overproduction of ROS above the normal tissue`s antioxidant capacity and their detoxification power may cause chromosomal aberration, carcinogenesis and degenerative diseases and cellular apoptosis [18-20]. Apoptosis (programmed cell death), is a cornerstone process essential for homeostasis either in initial development or in pathophysiological statuses [21].

MicroRNAs (miRNAs) exemplify a unique category of single-stranded, non-coding RNA of 18–25 nucleotides implicated in gene expression regulation and modification ruling a variety of human concerning diseases including apoptosis, oxidative stress, and oncogenesis [22-24]. miR-140-5p expressed in both glia and nerve cell and implicated in brain ischemia [25] and even neurodegenerative disorders [26]. Rising evidence implied that miR-140-5p play important roles in brain health and cognition [27, 28]. miR-34a is known to trigger apoptosis via the translational repression of either Sirtuin-1 (SIRT-1), a deacetylase that inhibits several pro-apoptotic proteins including p53, or the antiapoptotic protein Bcell lymphoma-2 (Bcl-2) [29, 30].

Though the precise mechanisms of MicroRNAs and antioxidant defence elements modulation are uncertain, an evidence implies that the nuclear factor erythroid 2-related factor-2 (Nrf-2) is an important transcription factor of the antioxidant stress pathway signalling pathway is the golden way for those mechanisms [31, 32]. SIRT-1 is one of the SIRT family that is a conserved protein of histone deacetylase III and consists of seven members (SIRT-1 to SIRT-7) [33]. SIRT-1 is recently reported as a target of miR-140 during apoptosis [34].

Consequently, we postulated that, the miRNA expression could be utilized as indicator for the progression of oxidative stress in addition to the apoptotic changes affecting the neuronal cells in the brain tissues of SY and Car-treated mice. In this study, the involvement of miR-140-5 & miR-34a and their targets in relation to apoptotic alterations in brain owing to SY and Car consumption was investigated.

Material and methods

Chemicals

Car (E122) with chemical formula of ($C_{20}H_{12}N_2Na_2O_7S_2$) and SY (E110) with chemical formula ($C_{16}H_{10}N_2Na_2O_7S_2$), were purchased in pure form from the local market, MDA and TAC estimation kits` were purchased from Bio diagnostic Co., Egypt, and Serotonin (CAT#: NK1120FY250) & dopamine (CAT#: NK1120FY209) ELISA kits` were purchased from Creative Biolabs neuroS.

Animals

Fifty of adult male mice weighting 20-25gm obtained from laboratory animals research center, Faculty of Veterinary Medicine, Zagazig University. The animals were kept under hygienic condition, housed in cages, well ventilated; fresh drinking water and basic laboratory diet was supplied with 15 days of acclimatization

before being experimented. All animals handled according to the procedures reviewed and approved by Zagazig University Research Center Institutional Animal Care and Use Committee (IACUC) under number ZU-IACUC/2/F/40/2021.

Experimental design

The experimental animals were randomly divided into 5 groups ten mice each; **Group1:** Mice received the basal diet and utilized as Control group, **Group 2:** Mice received 30 mg/ kg BW/day of SY that named Sunset Yellow-Low Dose (SY-LD) [7], **Group 3:** Mice received 300 mg/ kg BW/day of SY named Sunset Yellow-High Dose (SY-HD) at dose equivalent to 10 ADI, **Group 4:** Mice received 4mg/ kg BW /day of Car named Carmoisine-Low Dose (Car-LD) [14], **Group 5:** Mice received 40mg/ kg BW/day of Car named Carmoisine-High Dose (Car-HD). Both food colorants were dissolved in bi-distilled water and administered via gastric tube throughout the period of our experiment that extended to 90 days.

Sampling

Blood sampling

At the end of experiment, blood samples were collected from the eye plexuses utilizing capillary glass tubes and stored instantly on ice. Blood serum samples were collected into dry clean centrifuge tubes; the serum was collected after spinning for 15 min at 1500 xg and kept at –20℃.

Tissue sampling

Immediately after euthanizing, brain tissues divided into 2 parts one was homogenized and utilized for neurotransmitters estimation and the second part were wrapped in aluminium foil and snap frozen immediately in liquid nitrogen to minimize the action of endogenous RNases; for real time-PCR analysis for the gene expression of apoptotic genes and miRNA & their targets (miR-140, miR-34a, Nrf-2 and SIRT-1).

Biochemical analysis

The Malondialdehyde (MDA) marker of lipid peroxidation was measured in serum of mice and total antioxidant capacity (TAC) were estimated by the methods of Ohkawa *et al*. [35] and Koracevic *et al*. [36] respectively. They estimated by test reagent kits (Biodiagnostics, Egypt) according to the manufacturer's instructions. Serotonin and dopamine were measured in 10 mg of tissue homogenate from brain with ELISA kits (Creative Biolabs neuroS. London) as described by Toyokuni *et al*.[37]. Data were expressed as ng/mg of brain tissue.

Molecular analysis

RNA Extraction

Four milligrams of fresh brain tissues from all treated mice were thoroughly homogenized before RNA extraction. Total RNA extraction from the brain tissue was performed using TRIzol™ reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, U.S.A.) (Catalog Numbers: 15596026 and 15596018).

For RNA quality determination, the A260/A280 ratio was analysed using the NanoDrop® ND–1000 Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, United States). RNA concentration was determined spectrophotometrically at 260 nm.

Real–time quantitative PCR (qPCR) Analysis

The real-time qPCR was performed in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) Catalog Number (#RT500S–RT500M) supplied by applied enzynomics in Life Technologies (India) Pvt. Ltd. Where the isolated cDNA was amplified following the manufacturer's instructions.

The designed primers for the target genes were listed in Table (1) [38, 39]. Primers were synthesized by Sangon

Biotech (Beijing, China). The expression levels of the miR-140-5p and miR-34a and their target genes SIRT-1 and Nrf-2 respectively as well as other genes related to apoptosis (Bcl-2, Fas, Fas-L, Bax and Caspase3) were normalized using the mRNA and miRNA expression of a known housekeeping genes; *B-actin* and *U6* respectively. Results are expressed as fold-changes compared to the control group following the $2^{-\Delta\Delta CT}$ method [40].

Table1: Specific real time PCR primers for the examined genes.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession No or Reference
Nrf-2	GCGGTTTTGTCTCGTCCTGT	CAGACGATTGCCAGGACTGT	NM_023502.2
Sirt-1	CGGCTACCGAGGTCCATATAC	ACAATCTGCCACAGCGTCAT	NM_001159589.2
Bax	CTGGATCCAAGACCAGGGTG	CCTTTCCCCTTCCCCCATTC	NM_007527.3
Bcl-2	CTGAGTACCTGAACCGGCAT	GGTATGCACCCAGAGTGATG	NM_009741.5
Fas	GTCCTGCCTCTGGTGCTTG	AGCAAAATGGGCCTCCTTGA	NM_007987.2
Fas-L	CTCCGTGAGTTCACCAACCA	ACTCCAGAGATCAGAGCGGT	NM_010177.4
Caspase-3	GGGGAGCTTGGAACGCTAAG	CCGTACCAGAGCGAGATGAC	NM_009810.3
GAPDH	GCATCTTCTTGTGCAGTGCC	TACGGCCAAATCCGTTCACA	NM_008084.3
miR-140	CGCATGGCAGTGGTTTTACCCTA	ATCCAGTGCAGGGTCCGAGG	[38]
miR-34a	ACACTCCAGCTGGGTGGCAGTGTCTTAGCT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAACCAG	[39]
U6	CGCTTCGGCAGCACATATACTAAAATTGGAAC	GCTTCACGAATTTGCGTGTCATCCTTGC	[38]

Statistical analysis

Data on blood analyses and molecular alteration of our miRNA and their targets as well as apoptotic genes were statistically analysed using the one-way analysis of variance ANOVA followed by Duncan's test using SPSS software version 20 at a significance level of 5%. All results are expressed as mean±SEM.

Results

Antioxidant capacity

Figure 1 shows that treatment with both SY-HD and Car-HD resulted in a significant ($p < 0.05$) increase in the oxidative stress that indicated by increase of serum MDA levels well as decrease of TAC compared to control as illustrated in Figures 1A and 1B respectively. Also, our results revealed a slight increase in lipid peroxidation in Car-LD with non-significant ($p \geq 0.05$) change in SY-LD as compared with control group.

Brain neurotransmitters

Our results in figure 2 revealed a significant ($p < 0.05$) decrease in Dopamine (umol/L) (Figure 2A) levels in both SY-HD and Car-HD groups. Furthermore, mice exhibited a highly significant reduction in the mean value of serotonin (ng/ml) (fig 2B) activity in both SY-HD and Car-HD groups when compared with other tested groups ($P<0.001$). Moreover, the SY-LD and Car-LD treated groups showed a significant ($p < 0.05$) decrease of both Dopamine and serotonin when compared to control group.

Expression of different genes in relation to apoptosis

A significant ($p < 0.05$) upregulated gene expression of Fas, Fas-L, BAX and Caspase3 was detected both in SY-HD and Car-HD treated mice in isolated brain tissues homogenate, while they were significantly ($p < 0.05$) down-regulated in both SY-LD and Car-LD treated group but

still higher than control one (Figures; 3A, 3B, 3C, and 3D). In contrast to Bcl-2 gene that was significantly ($p < 0.05$) down-regulated in both SY-HD and Car-HD treated mice, while they were significantly upregulated in both SY-LD and Car-LD treated group but still lower than control one (Figure 3E).

MiR-140-5p and miR-34a expression level in brain tissues of treated mice

As shown in figure 4 there is A significant ($p < 0.05$) increase in gene expression of miR-140-5p and miR-34a in both SY-HD and Car-HD treated mice in isolated brain tissues homogenate, whereas they were significantly ($p < 0.05$) down-regulated in both SY-LD and Car-LD treated group but still higher than control one (Figure 4A, 4C). In contrast to Sirt-1 and Nrf-2 gene that were significantly ($p < 0.05$) down-regulated in both SY-HD and Car-HD treated mice, whilst they were significantly upregulated in both SY-LD and Car-LD treated group but still lower than control one (Figure 4B and 4D).

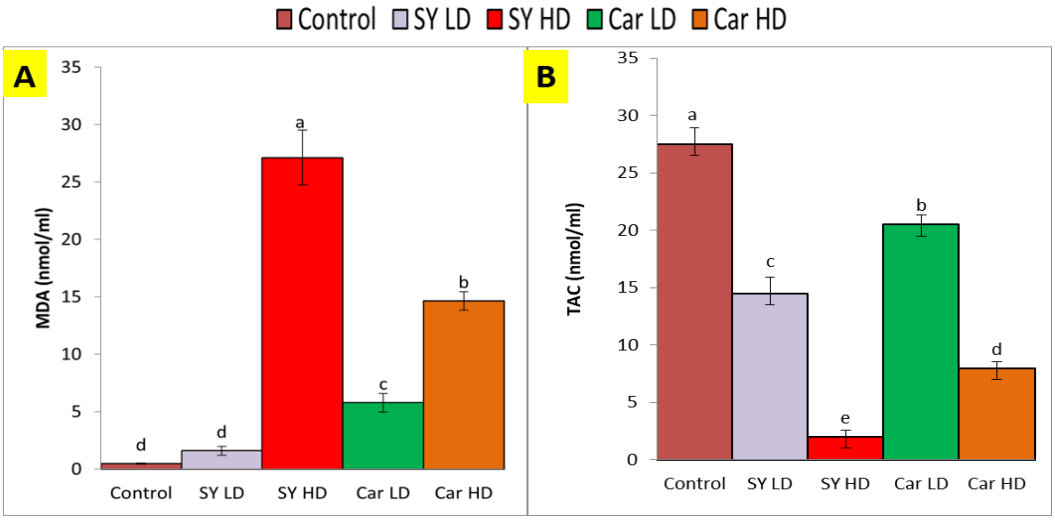

Figure (1) Effect of sunset yellow or carmoisine intoxication on serum MDA (A) and TAC (B). Values presented are mean ± SEM. Means along with different superscripts were statistically significant ($P < 0.05$).

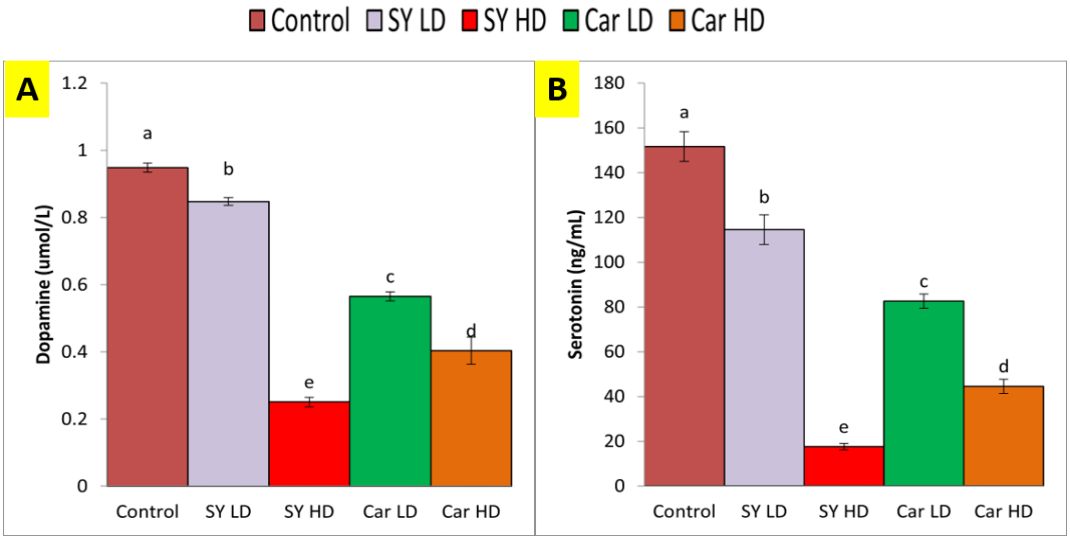

Figure (2) :Effect of sunset yellow or carmoisine intoxication on brain neurotransmitters of mice; A. Dopamine and B. Serotonin. Values presented are mean ± SEM. Means along with different superscripts were statistically significant ($P < 0.05$).

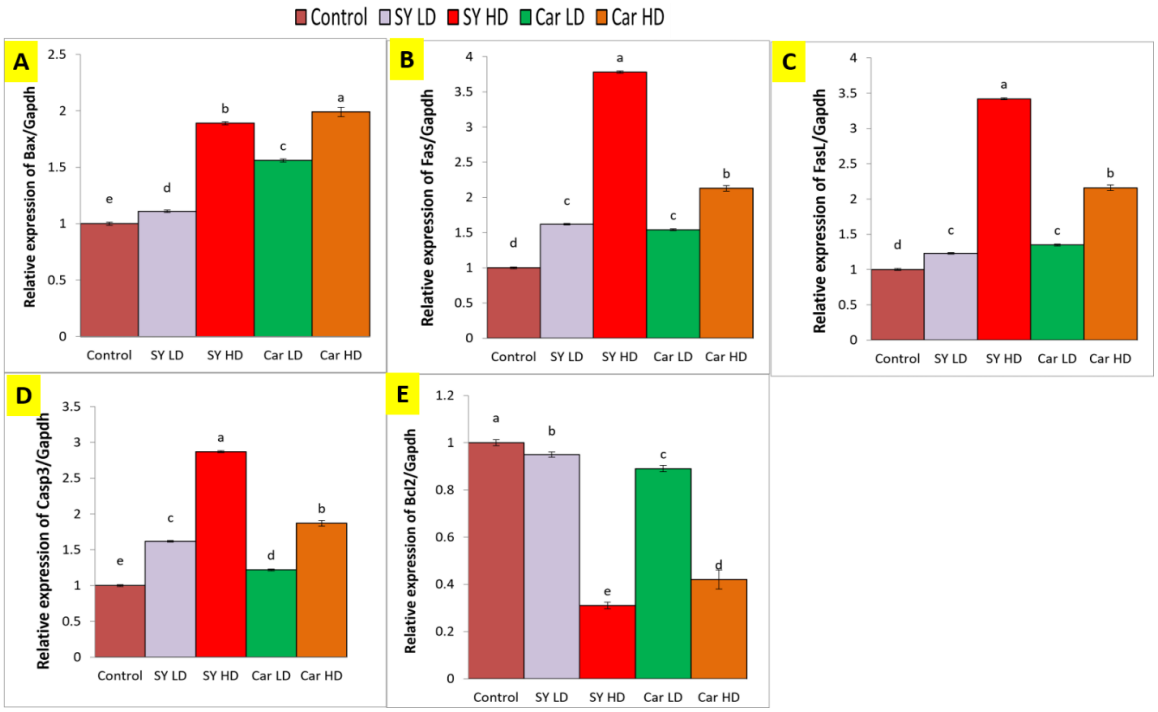

Figure (3): Effect of sunset yellow or carmoisine intoxication on gene expression of apoptotic genes in brain tissue of mice; A. Bax, B. Fas, C. FasL, D. Caspase-3 and E. Bcl2. Values presented are mean ± SEM. Means along with different superscripts were statistically significant ($P < 0.05$).

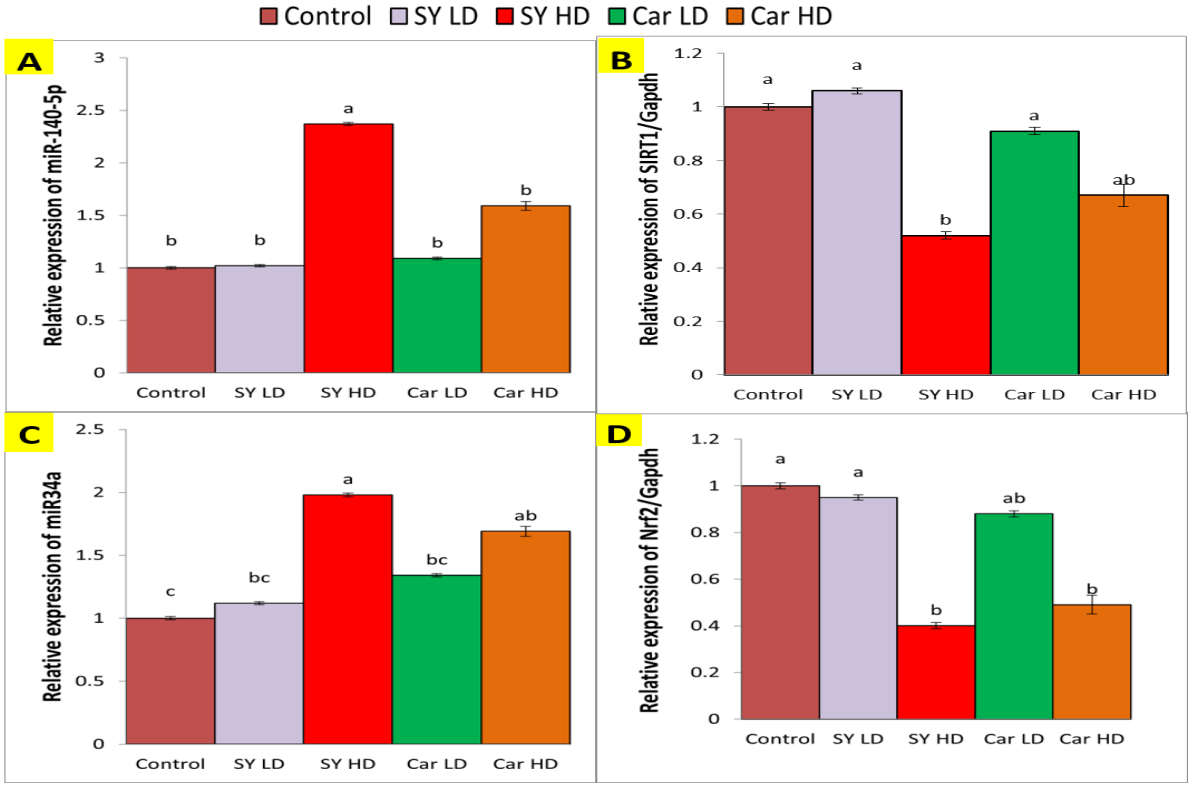

Figure (4) : Effect of sunset yellow or carmoisine intoxication on miR-140 & miR-34a and their targets` expression level in brain tissue of mice; A. miR-140, B. SIRT-1, C miR-34a and D. Nrf-2. Values presented are mean ± SEM. Means along with different superscripts were statistically significant ($P < 0.05$).

Discussion

Food colorant is any material added to food or beverage to change its colour and appearance. Over many ages, adding colour to food and drink has been a common practise. Colour was commonly used to act as a visual indicator for quality, to induce flavour recognition, and to match consumer expectations. Colour is still used in the production of food like biscuits, pastries, cakes, and processed meats [1-3].

Several adverse consequences have been documented based on various research studies, including behavioural impacts on children, and even adult cognition. or neuro-developmental effects [41,42].

To elucidate the mechanisms involved in the up regulation of miR-140-5p in response to oxidative stress, we investigated the relationship between miR-140-5p expression and ROS production. Lipid peroxidation is one of the most concerning disturbance aroused from lowered antioxidant activities in neurons leading to brain injury and neurodegenerative disorders [43]. Our findings synchronized with Khayyat *et al.* [44] and Moeen *et al.* [45] who reported visible increase in MDA level and a marked decline in total antioxidant capacity in SY and Car-treated groups respectively.

Extreme ROS accumulation in macrophages could trigger a cascade of cellular alterations, in RNA, DNA, lipids, and protein, which can lead to cell metabolic dysfunction and apoptosis [32]. Apoptosis is a crucial way of controlling cell populations; however it is therefore imperative to be under control. Homeostasis in the normal cell death is tightly delimited by restraining of the oxidative stress. Consequently, faulty signalling across these pathways be able to and cancer development as well as initiation of apoptosis [20].

Apoptosis is sparked via two key paths described as the intrinsic (mitochondrial) and extrinsic pathways (receptors-dependant). The intrinsic one is triggered by oligomerization of BAK and BAX (pro-apoptitic members of BCL-2 family) forming pores in the outer mitochondrial membrane, liberating cytochrome c into the cytosol where it binds to Apoptotic protease activating factor-1 (Apaf-1), that enlists procaspase-9, creating the apoptosome that migrates to nucleus contributing to Direct DNA degradation and apoptosis [46]. On the other hand, the extrinsic pathway is triggered by several mechanisms where death ligand (Fas-L) or TNF-α binds to the corresponding cell surface receptors; death receptors & TNF receptor 1 (TNFR1) respectively. Later, these proteins provoke signalling complexes formation of TNFR1-Fas-associated death domain protein (FADD) or associated death domain protein (TRADD) and procaspase-8 that forming death inducing signal complex (DISC) then triggering Caspase-3 & multiple caspase-substrates cleavage and subsequently cell death [21, 46].

Car, according to Basu and Kumar [47], can link with B form of DNA that resulted in various conformational changes in its structure. Similarly SY caused apoptotic induction via membrane & DNA damage as well as cell division inhibition as reported by Prajitha and Thoppil [48].

Extensive studies of the apoptotic signal transduction pinpointed caspase (cysteine-protease) being as the effectors where the apoptotic triggering stimuli exist, many activated caspase enlisted to exterminate the cells [49,50]. In the current study, Car caused progression of apoptotic environment in brain tissues that was confirmed by enhanced Fas, Fas-L, BAX and Caspase3 gene expression as a pro-apoptotic gene. Bcl-2 family have been previously recognized, that comprises both a pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl-2) [51]. Augmented gene expression of Fas, Fas-L, Bax and cleaved Caspase3 was detected both in high doses of SY &Car-treated mice in isolated brain tissues homogenate, that proved activated apoptotic process in the brain tissues of treated mice.

In contrast to Bcl-2 gene that was significantly down regulated both in high doses of SY & Car-treated mice in isolated brain tissues homogenate, that evidenced blocked apoptotic cascade in the brain tissues of treated mice. In accordance with the obtained results, significant reduction ($p<0.05$) in Bcl-2 gene as has been recorded in previous study [44] as it considered an anti-apoptotic one through blocking Bid redistribution and the downstream caspase.

Neurotransmitters are category of biological messengers between cells synchronizing countless body concerning processes [52]. In present study, there is a significant decrease in Serotonin and dopamine that comes in accordance with several previous experiments revealed the negative effect of the synthetic food colorants on the hormone balance in the experimental animals [53, 54]

miRNA expression provides a great progression in diagnosis, monitoring as well as clarifying the underlining mechanism for numerous diseases alterations; leading to the improvement of innovative diagnostic and therapeutic prospects [55]. The intracellular process for inactivation of ROS is important for maintaining physiologic redox equilibrium by regulating the production and activity of several cellular anti-oxidative defence components, and hence plays a key role in oxidative stress development. Any disruption in the Nrf-2 signalling pathway can lead to oxidative damage-related diseases [31].

Consistent with previous findings, we observed up-regulation of miR-140-5p by way of increased oxidative stress and ROS levels that could explained by suppressing the protein expression of Nrf-2 [56]. Furthermore, the role of miR-34a in apoptosis regulation has been widely emphasized in a wide variety of cell types [57, 58]. Our results come in accordance with Guo *et al*. [57] who postulated that miR-34a triggers senescence to a certain extent through molecular embarrassment of SIRT-1 as previously postulated by Zhao *et al.* [59]

Conclusion

miR-140-5 & miR-34a and their targets were extensively contributed to oxidative process & apoptotic cascade alterations in brain owing to SY and Car consumption. Additionally, the use of synthetic colours in various foods has adverse effect on biochemical as well as molecular analysis specifically at high level for a long time. On The Other Hand, it leads to the change in brain environment and increase apoptotic & oxidative indices. So, we suggested low administration of food colouring in food products mainly during childhood.

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الحمض النووي الريبوزي الميكرو 140 و34أ كمؤشرات جزيئيه لموت خلايا الدماغ المبرمج في الفئران المسممة بالكارموزين ولون الغروب الاصفر

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الملخص العربي

المخ هو العضو المركزي في جسم الإنسان ، ويعمل 24 ساعة / 7 أيام حتى قبل أن نغادر رحم أمهاتنا. ليس من الغريب زيادة الاهتمام بجودة الأغذية المستهلكة والتي تؤثر بالتالي على بيئة الدماغ والنواقل العصبية وكذلك حالة الأكسده. الكارموزين (Car) ولون الغروب الاصفر (SY) من الإضافات الغذائية الاصطناعية والتي تستخدم على نطاق واسع أثناء معالجة الطعام وبالتالي تؤثر على صحة الدماغ. لا يزال تأثير موت الخلايا المبرمج الكامن وراء التغيرات السلوكية بعد تناول الكارموزين(Car) أو SY غير مفهوم بشكل واضح. تم إجراء معاملات بيوكيميائية وجزيئية بيولوجية عن طريق استخدام خمسين ذكر بالغ من الفئران. امتدت الدراسة لمدة 3 أشهر في 5 مجموعات مختلفة باستخدام عشرة فئران لكل منها. تم استخدام المجموعة 1: كمجموعة ضابطة ، وعولجت المجموعة 2: بالمقدار اليومي المسموح (ADI) من SY (30 مجم / كجم بالوزن) ، وعولجت المجموعة 3: بـ10 اضعاف ADI من SY ، وعولجت المجموعة 4 :باستخدام ADIمن Car (تم إعطاء 4 مجم / كجم من وزن الجسم) والمجموعة 5: بعشر أضعاف ADIمن Car باستخدام انبوب المعده عن طريق الفم. أدى التعرض لجرعات أعلى من SY أو Car إلى تغيير كبير في المؤشرات البيوكيميائية ؛ خفض مستويات كل من السيروتونين والدوبامين والقدرة الكلية المضادة للأكسدة كذلك. ومع ذلك ، زاد malonaldehyde (MDA)، وكذلك زاد التعبير الجيني لكلا من الجينات proapoptotic (Fas-L، Bax ,casp3 ,Fas) وقلت مستوى Bcl-2.علاوة على ذلك ، ارتفعت مستويات التعبير الجيني لكلا من miR-140 و miR-34a وبالتالي قللت من مستوي التعبير الجيني لجيناتها المستهدفة NRF-2 و SIRT-1 على التوالي. لذلك ، يمكننا أن نستنتج أن الإفراط في تناول الفم عن طريق الفم للسيارة أو SY له تأثير موت الخلايا المبرمج ويجب دفع الحذر عند استخدامها.