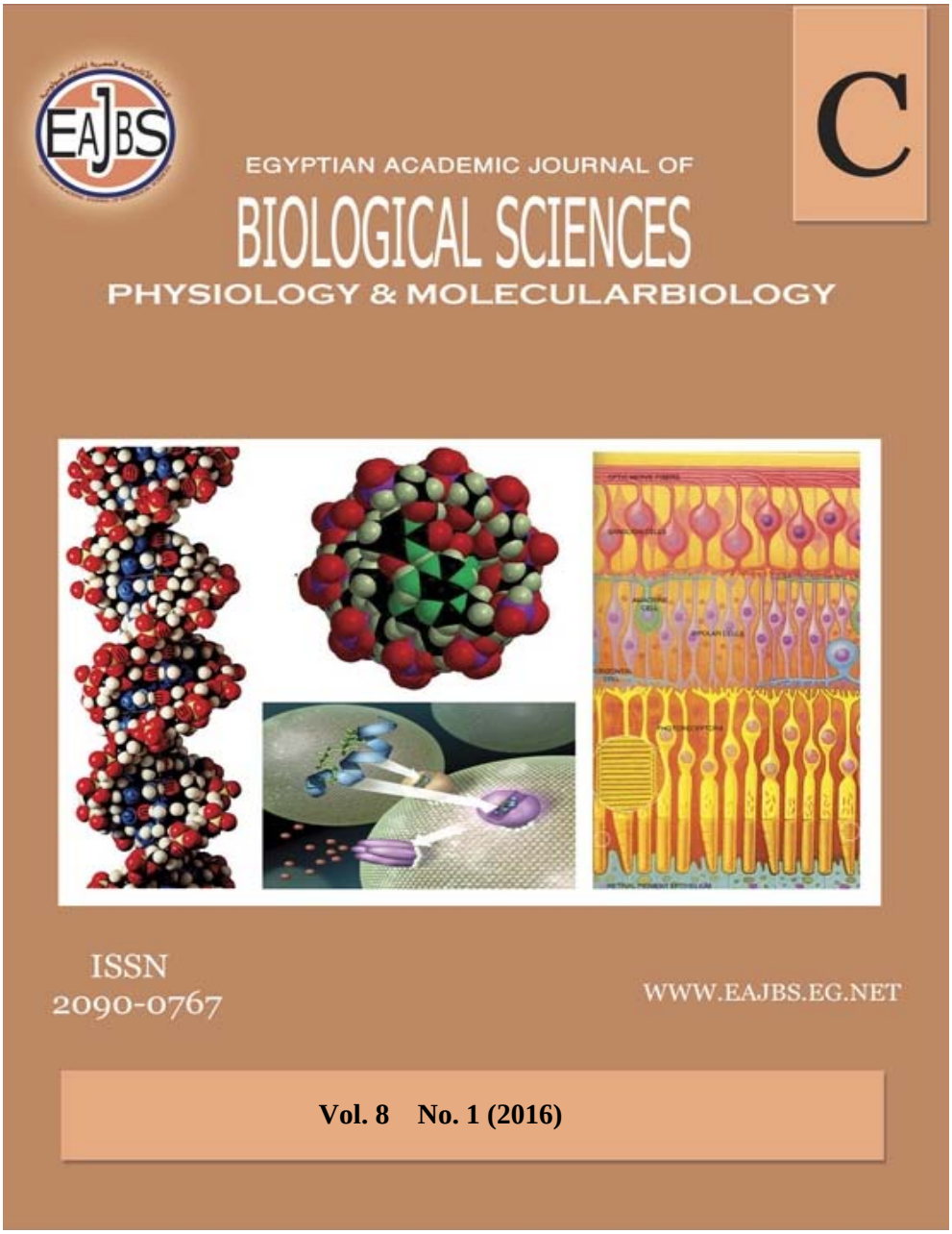

Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences, Department of Entomology, Faculty of Sciences Ain Shams University.

Physiology & molecular biology journal is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers that elucidateimportant biological, chemical, or physical mechanisms of broad physiological significance.

www.eajbs.eg.net

Citation :*Egypt. Acad. J. Biolog. Sci. (C. physiology and Molecular biology) Vol.8(1)pp11-22(2016)*

Egypt. Acad. J. Biolog. Sci., 8(1): 11 -22(2016)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbs.eg.net

Allura Red, Carmoisine and Indigo Carmine Inhibit Reactive Oxygen Species Production by Human Polymorphonuclear Leukocytes*in Vitro*

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ARTICLE INFO
Article History
Received: 22/12/2015
Accepted: 20/1/2016

***Keywords*:**
Artificial Food Colours
Neutrophils
Reactive Oxygen Species
Chemilumiscence

ABSTRACT

Evidence for Artificial Food colours (AFCs) association with allergies/intolerance/hypersensitivities and hyperactivity rea-ctions, remain controversial and inconsistent although AFCs are suspected to interact with immune system in yet to be discovered unknown mechanisms. In the present study, the effects of Allura Red, Carmoisine and Indigo Carmine on Reactive Oxygen Species (ROS) production by human Polymorph nuclear Leukocytes (PMNs) were investigated. Chemiluminescence (CL) method enhanced by luminol (5-amino 2,3-dihydro-1,4-phthalazine-dione) was employed to study the ROS generation by PMNs stimulated in vitro by Phorbol 12-myristate 13-acetate (PMA). Allura red, carmoisine and indigo carmine could produce an effectively marked and dose-dependent inhibition of ROS production by PMNs. The highest CL inhibition of 94 %, 95 % and36 % was observed respectively for allura red and carmoisine and indigo carmine at their respective highest concentration. Future studies are required to understand complete implications of the present study results.

INTRODUCTION

Food Technology has dominated and transfigured our traditional dietary patterns. Today markets are flooded with innumerable processed food products which are affordable, convenient, nutritious, and aesthetically appealing which led to their increased consumption (Shim *et al.*, 2011). Food additives, the key components which revolutionized processed food technology, serve a pivotal technological purpose in the complex foods. Various international organizations monitoring food safety issues develop list of permitted food additives for usage by food industry from time to time, based on thorough safety evaluation assessments derived from extensive toxicology data from animal studies and/or human clinical studies and from re-evaluation in case of new significant research findings.

Artificial food colour agents (AFCs) are prominent in usage among the food additives due to their ability to provide critical stability and improve product quality attributes during processing and storage conditions thereby increasing the appealing power of final processed food products to the consumers. Recent research, however, raised concerns related to AFC consumption leading to either ban of their usage in foods or exhibiting compulsory warnings on the foods using them (McCann *et al.,* 2007;European Food Safety Authority (EFSA), 2008). Also growing number of studies indicate increasing concern and negative perception in consumers about food additives in general and in particular about food colours (Aoki *et al*., 2010; European Commission, 2010; Varela and Fiszman, 2013).

Allura Red (FD and C R40; E 129), Carmoisine (E 122) and Indigo carmine (FD and C B2; E 132) are widely used AFCs for foods in many countries. Apart from being known to be possible allergens, Allura red and Carmoisine were caught in controversy recently for their possible role in etiology of hyperactivity in children (Bateman et al., 2004, McCann *et al.,* 2007). Indigo carmine, which was once considered to be extremely safe, is also being identified of late for a wide range of side effects, including a possible role as an allergen (Magner and Gerber, 1994; Miller *et al.,*1996; Naitoh and Fox, 1994; Satoh *et al.,*2001). Clinical evidence of a causal link between food colours and allergies/intolerance/hypersensitivities and hyperactivity reactions is however limited, contributing to ongoing debates in scientific circles, although they are suspected to interact with immune system in yet to be discovered unknown mechanisms (Ortolani and Pastorello, 2006; Stevens *et al.,* 2013).

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), play a vital role in immune defence against infectious agents either alone or in combination with immunoglobulins or their complements (Quinn and Guass, 2004). In response to a variety of stimuli, PMNs are a major source of reactive oxygen species (ROS), through their ability to mediate a strong oxidative burst (Nauseef, 2007). ROS is a double edged sword since low production of ROS can hamper host defences while excessive production can result in oxidative stress in tissue environment (Freitas *et al.,* 2009).

Neutrophils, which were initially thought to be passive players in response to external stimulation, are now being increasingly understood as dynamic players in a variety of mechanisms which may have functional implications and consequences within disease models (Beyrau *et al.,* 2012). There is a growing body of evidence which suggests a higher role for the neutrophils participation in allergic processes (Macdo well and Peters, 2007; Monteseirín, 2009) and inflammatory processes and diseases (Kolaczkowska and Kubes, 2013). It is therefore interesting to understand the phenomena of AFCs triggering effect on the extent of ROS production by neutrophils which probably can unveil some of the tangled knots in the allergic/intolerance/hypersensitivities and hyperactivity associations with food colours.

In view of the foregoing discussion, the present study was planned to investigate the in vitro effect of food coloursallura red, carmoisine and indigo carmine on the production of ROS by isolated human neutrophils using a luminol-dependent CL assay (Allen *et al.,* 1972).

MATERIALS AND METHODS

Experimental work was carried out at the Department of Clinical Nutrition, College of Applied Medical Science, University of Ha'il, Kingdom of Saudi Arabia.

Reagents and Chemicals

Polymorphonuclear isolation medium was obtained from Robbins Scientific Corporation (Sunnyvale, CA, USA). Lysis buffer (NH_4Cl-0.87%), Phorbol 12-myristate 13-acetate (PMA), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and phosphate buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Conical centrifuge tubes and plain vacutainer tubes were purchased from Medical Technical Trading EST (Riyadh, Saudi Arabia). Evacuated blood collection tubes (10ml; EDTA(K_3) were obtained from Terumo (Terumo Europe N.V., Leuven, Belgium).Food additive colours (allura red, carmoisine and indigo carmine) were purchased from United Flavors and Fragrances Co. Ltd. (Sahab, Jordan). All chemicals used were of reagent grade and used without further purification.

Separation of PMNs

Isolation of PMNs was done as previously described by Shalabiand Al-Tuwaijri, 1996; Mustafa and Al-Tuwaijri, 1992. PMNs were isolated from heparinized venous blood obtained from normal individuals in sterile containers containing 0.47 mol/L of anticoagulant (EDTA(K_3)). All participants gave their informed consent before participating in the study. Ten ml of blood was carefully layered over 3.5ml of PMN in a 15ml centrifuge tube and centrifuged at 1800rpm for 30min in a Jouan – B4centrifuge. The resulting neutrophil layer was extracted and washed with 10ml of PBS, pH 7.4, and centrifuged at 1500rpm for 10min. Five ml of 0.87% NH_4Cl were added to the PMN pellets and left to stand in a water bath at 37°C for 10min to lyse the erythrocytes. The cells were then washed three times with PBS, and after the last wash, the pellet was resuspended in 1ml of PBS. The PMNs were counted and adjusted to $5x10^5$ cells/ml.

PMN viability tests

The percentage of viable PMNs was estimated by the trypan blue exclusion method, which was carried out by a microscopic count of cells not stained by 0.2% trypan blue; the quantity was expressed as percentage of unstained cells to total cell numbers (Shalabiand Al-Tuwaijri, 1996). Only PMNs with viability above 80% were used in the experiments (Mustafa and Al-Tuwaijri, 1992).

Preparation of PMA (PMNs stimulant)

The PMNs were stimulated by PMA, which is a non-physiological stimulant(Mustafa and Al-Tuwaijri, 1992).

PMA (Sigma Chemical Co.) was used to enhance and stimulate the metabolic burst of phagocytic cells. It was dissolved in dimethyl-sulfoxide (DMSO) to give a stock solution of 2mg/ml. This stock solution was stored at –20°C until it was used. Working solution was freshly prepared by diluting 50µl of stock solution with 10ml PBS.

Measurement of ROS by chemiluminescence (CL) technique

CL was measured by using an Auto Lumat Plus (Berthold, Bad Wilbad, Germany) with a controller set at a constant temperature of 37ºC. The luminometer was connected to an IBM-compatible computer, which recorded the light output, in mV, continuously for 30 min.

In brief, a 100µl PMN suspension was placed in 400µl PBS and 100µl food colours. Luminol (200µl) and the stimulant PMN (200µl) were then added. For control, a 100µl PMN suspension was placed in 500µl of PBS and then Luminol (200µl) and the stimulant PMN (200µl) was added. The cuvette contents were mixed gently. The light output was

recorded in mV for 30 min, and the maximum peak response was noted.

Preparation of luminal

Luminol (5-amino 2,3-dihydro-1,4-phthalazine-dione)was used to enhance chemiluminescence (CL); 1.77mg of luminol was dissolved in 1ml of DMSO (10M) and stored at –20°C. Then, 100µl of this stock solution was further diluted with 10ml of PBS prior to use.

Concentration of food colours used to prime PMNs

The concentrations of food colours (allura red, indigo carmine, and carmoisine,) used here is in accordance with the amounts approved by various international agencies.

Table 1: Concentrations of colour additives used in the study

Allura red (E129) and Carmoisine (E122), water soluble. Stock solution 120g/L = 120mg/ml Indigo carmine (E132), water soluble. Stock solution 150g/L = 150mg/ml			
Tube Number	**Dilution**	**Final Concentration (mg/ml)**	
		For Allura red (E129) and Carmoisine (E122)	**Indigo carmine (E132)**
1	1:10	1.200	1.500
2	1:10	0.900	1.125
3	1:10	0.600	0.750
4	1:10	0.300	0.375
5	1:10	0.120	0.150
6	1:100	0.090	0.1125
7	1:100	0.060	0.075
8	1:100	0.030	0.0375
9	1:100	0.012	0.015

Accepted daily intake for Allura red = 0–7 mg/kg/day (JECFA, 2005)
Accepted daily intake for Carmoisine = 0–4 mg/kg/day (JECFA, 2005)
Accepted daily intake for Indigo carmine = 0–5mg/kg/day (JECFA, 2005)

Statistical analysis

Independent Student's t-test was used to analyse the data for significant inter group comparisons. The values were considered significant if the P value was <0.001 or <0.05. Values are expressed as the mean ± standard error of mean (SEM) of four observations.

RESULTS

Effect of food colours on viability of PMNs

In order to rule out any adverse cytotoxic effects of food colours and PMA, the viability of incubated PMNs were tested at the lowest and highest concentrations of food colours used in the study, as described previously (Metcalf *et al.*,1986). The results showed that different types of food colours did not alter the cell viability of the neutrophils since there was no blue staining of the nucleus. The percentage of viable cells as evaluated by trypan blue (0.2% v/w) was within normal limits after incubation with various food colours. PMNs incubated with allurared, carmiosine and indigo carmine for 30 min prior to PMA stimulation also exhibited a time-dependent increase in ROS release and a maximum increase in CL peak response (mV) occurred at 13–18 min, 10–12 min and 6-12 min respectively after stimulation, followed by a gradual decline in ROS release. Thus, loss of cell viability over time or resulting from exposure to food colours were not factors in this study.

Effect of allura red (E129) on ROS production by PMNs

Significant inhibition of CL peak response (Table 2) was observed when the PMNs were incubated with various concentrations of allura red (1.2–0.03mg/ml) for 30 min and stimulated with PMA. The lowest significance of ($p<0.05$) inhibition percentage was seen at a concentration of 0.03mg/ml while at a concentration of 0.012 mg/ml, it did not induce any statistically significant change in CL response as compared with the control.

Table 2: Effect of various concentrations of allura red (E129) on chemiluminescence peak response and percentage of inhibition of isolated human PMNs stimulated with PMA

Allura red (mg/ml)	CL max. peak response (mV)	P value*	CL inhibition (%)
Control	661.40 ±21.30	-	-
1.2	39.160 ±1.640	< 0.001	94.08
0.9	55.430 ±2.280	< 0.001	91.62
0.6	83.660 ±3.350	< 0.001	87.35
0.3	179.41 ±7.000	< 0.001	72.87
0.12	380.37 ±13.85	< 0.001	42.49
0.09	452.71 ±16.23	< 0.001	31.55
0.06	529.87 ±18.33	< 0.001	19.89
0.03	606.47 ±19.99	< 0.05	8.30
0.012	688.75 ±23.14	NS	-4.14

* Student's t test. NS = not significant

Values are expressed as the mean ± SEM of four observations.

PMN count=$5x10^5$ cell/ml. Luminol=10^{-4M}. PMA=2µg/ml. CL% inhibition=a-b/a x 100, where (a) is the CL response of the control and (b) is the CL response of the experiments.

Effect of carmoisine (E122) on ROS production by PMNs

A significant inhibition in CL peak response was observed at a concentration of 1.2–0.03mg/ml of carmoisine priming on PMNs (Table 3). Carmoisine exhibited maximal inhibition at a concentration of 1.2mg/ml; however, 0.012mg/ml of carmoisine did not induce statistically significant changes in CL response when compared with the control.

Table 3: Effect of various concentrations of carmoisine (E122) on chemiluminescence peak response and percentage of inhibition of isolated human PMNs stimulated with PMA

Carmoisine (mg/ml)	CL max. peak response (mV)	P value*	CL inhibition (%)
Control	809.82 ±33.52	-	-
1.2	39.940 ±1.800	< 0.001	95.07
0.9	53.090 ±2.340	< 0.001	93.44
0.6	78.290 ±3.400	< 0.001	90.33
0.3	153.32 ±6.630	< 0.001	81.07
0.12	305.02 ±12.70	< 0.001	62.33
0.09	386.02 ±16.43	< 0.001	52.22
0.06	481.03 ±20.09	< 0.001	40.60
0.03	610.65 ±25.41	< 0.001	24.59
0.012	744.72 ±31.12	NS	8.04

* Student's t test. NS = not significant.

Values are expressed as the mean ± SEM of four observations.

PMNs count=$5x10^5$ cell/ml. Luminol=10^{-4M}. PMA=2µg/ml. CL% inhibition=a-b/a x 100, where (a) is the CL response of the control and (b) is the CL response of the experiments.

Effect of indigo carmine (E132) on ROS production by PMNs

Table 4 shows that the inhibition was minimized when the PMNs were treated with indigo carmine at a concentration of 0.375mg/ml and stimulated with PMA; however, concentrations of 0.15–0.015mg/ml did not induce statistically significant changes in CL response compared to the control.

Table 4: Effect of various concentrations of indigo carmine (E132) on chemiluminescence peak response and percentage of inhibition of isolated human PMNs stimulated with PMA

Indigo carmine (mg/ml)	CL max. peak response (mV)	P value*	CL inhibition (%)
Control	520.25 ±23.19	-	-
1.5	329.79 ±17.86	< 0.001	36.61
1.125	416.17 ±22.19	< 0.001	20.01
0.75	442.86 ±22.67	< 0.001	14.88
0.375	449.75 ±21.36	< 0.05	13.55
0.15	468.59 ±21.27	NS	9.93
0.1125	477.40 ±21.45	NS	8.24
0.075	485.09 ±21.53	NS	6.76
0.0375	504.61 ±22.33	NS	3.01
0.015	498.22 ±21.89	NS	4.23

* Student's t test. NS = not significant.

Values are expressed as the mean ± SEM of four observations. PMN count=$5x10^5$ cell/ml. Luminol=10^{-4M}. PMA=2µg/ml. CL% inhibition=a-b/a x 100, where (a) is the CL response of the control and (b) is the CL response of the experiment.

DISCUSSION

Artificial Food colours have attracted the consumer's attention in terms of both sensory perceptions and safety concerns. Beginning with Feingold assertion in 1975 (Feingold, 1975) to latest renewed attention created by Southampton study (McCann *et al.,* 2007), AFCs safety evaluation was always at the centre of scientific discussion and public attention. Southampton study prompted European Food Safety Authority to take a precautionary stance (EFSA, 2008) while U.S. Food and Drug Administration (FDA, 2011) concluded that these latest developments did not warrant further agency action leading to debates on the issue (Weiss, 2011; Arnold *et al.*,2012; Nigg *et al*., 2012). Further, in a very recent review, Nigg *et al.* (2012) pointed that nearly all studies they considered for review examined only combinations of food colours and suggested there is an urgent need for studies that quantify comparative effects of individual food colours and additives.

Food additives safety assessments mostly are dependent on toxicological tests because of complexities involved in obtaining causal links from epidemiological studies. Epidemiological studies dealing with food allergies / intolerance reactions to food additives, however, strongly suggest causal links between them. It is generally agreed that food additives in particular AFCs may cause non-IgE-mediated intolerance reactions in some predisposed individuals or sub populations causing asthma, rhinitis, urticaria, itchiness, and migraines in largely unidentified mechanisms (Orlatoniand Pastorello, 2006; Stevens *et al.,* 2013). Studies, nevertheless, suggest hypersentivity reactions to food antigens could possibly be associated with inflammatory cells like eosinophils, neutrophils and mast cells (van Odijk *et al*., 2006; Stevens *et al.,* 2013). Especially, neutrophils, of late are being identified, as playing critical role in allergic inflammatory processes giving rise to immense interest in these fascinating cells (Mocsai, 2013; Gunzer, 2014).

In the present study we tried to attempt to understand the in vitro effects of allura red, carmoisine and indigo carmine on isolated human PMNs ROS production as measured by luminol dependent CL. The activation of PMNs to produce NADPH oxidase-mediated respiratory burst is an important mechanism of immune protection against bacterial and parasitic infections in humans (Gray *et al.,* 2013). In vitro, a wide variety of stimuli are used to trigger these activities. In the present study, soluble phorbolmyristate acetate, was

used to trigger the ROS process since it is known to activate protein kinase C which results in NADPH oxidase activation.

Chemiluminescence is widely in use for assessment of the capacity of ROS production by PMA and has been accepted as the most sensitive methodology (Wardman, 2007). Luminol helps in further enhancing the sensitivity of native CL because luminol deoxygenation results in a high quantum yield of photons (Freitas *et al.,* 2009). The major findings of the present study show that allura red, carmoisineand indigo carmine are capable of producing a marked and dose-dependent inhibition of ROS production either by interfering at enzymatic generation stage or by suppressing different reactive species. The highest CL inhibition of 94 %, 95 % and 36 % was observed respectively for allura red and carmoisine and indigo carmine at their respective highest concentration. The results suggest that allura red and carmiosinehave potent inhibitory activity on ROS production as compared to indigo carmine. They exhibited significant inhibitory effects even at lower concentrations. A recent study (Al-Shammari *et al.,* 2014) on food preservatives and flavouring additives effects on ROS production of PMNs also reports dose dependent inhibitory effects of sodium benzoate (E211). Both allura red and carmoisine which were considered for the present study were part of colour mixes tried in Southampton study along with preservative sodium benzoate (McCann *et al.,* 2007). Further studies are however required to understand the underlying mechanisms for these inhibitory effects of AFCs on ROS production and their consequences on immune and inflammation pathways.

It is well-known that ROS generated by stimulated PMNs play a critical role in host defences against invading microorganisms. In addition, recently, an extracellular killing mechanism by neutrophils known as neutrophil extracellular trap (NET) has been identified. NET formation is dependent on NADPH oxidase-mediated respiratory burst when stimulated with PMA and is likely to follow the pathway of activation of protein kinase C and others (Hakkim *et al*., 2011;Gray *et al.*, 2013). The results of the present study, therefore, at the outset may propose that increased intake of AFCs may reduce first line of defence against infectionsby inhibiting ROS production and may perhaps even NET formation by neutrophils.

The results of the present study could be interesting from the view point of certain subpopulations of children who are increasingly athigher risk of exposure to food additives because of clustered dietary behaviours and whose immune system is still vulnerable. Factors influencing absorption of AFCs: intestinal motility, dye characteristics, pH, and intestinal integrity also advocate that certain subsections of population could be more vulnerable for effects of AFCs, although it is still uncertain whether food dyes are absorbed intact or only as metabolites especially in children (Stevens *et al.,* 2013). Further, Stevens *et al.* (2013) suggest that “leaky gut”, as one plausible reason for increased absorption of AFCs in some children, which itself could have been a result of long term usage of antibiotics and/or nonsteroidal anti-inflammatory drugs; or food allergies; excessive fructose intake etc. For example, obese children have been observed with habitual consumption of excess amounts of soft drinks and sweetened beverages as compared to normal weight children in many studies which can increase their overall consumption of AFCs and also put them at risk of developing “leaky guts” because of excessive fructose intake (Moreno *et al.,* 2010; Stevens *et al.,* 2013). Increased intestinal permeability was also noted in patients with a history

of food allergies and allergic disorders. A complete discussion on this topic is beyond the scope of this article. It is, however, very important to understand the results of this study from the perspective of the newly emerging knowledge about neutrophils and their immunomodualtory effects in various settings and its ROS contribution to immune and inflammatory diseases. Stevens *et al*. (2013) in their discussion on how AFCs might cause behavioural and physical symptoms in children points fingers towards hypersentivities and its involvement with unidentified immune factors as one probable mechanism. A very recent study further identifies neutrophils as a potential immune factor playing a major role in hypersentivities (Weber *et al.,* 2015).

The recent immunological research is changing our perceptions about neutrophils and its ROS functions and it is now widely being appreciated that they may have diverse complex roles in autoimmune and inflammatory disease development than previously thought or understood (Björkman *et al.,* 2008; Mayadas *et al.,* 2014). Excess ROS production has been long been considered as culprit of inflammatory tissue damage for surrounding host cells and tissues. However, in contrast, congenital abnormalities in human patients effecting genetic deletions in components of the NADPH oxidase and myeloperoxidase (MPO) pathways and their associations with autoimmunity have challenged this much accepted theory. A recent study by Ferguson *et al*. (2008) suggests a possibility that ROS deficient cells, specifically decreased production of ROS from intracellular compartments of PMNs actually may drive inflammatory diseases. Björkman *et al.* (2008), in their editorial, provide a supporting compelling argument to this rather "counterintuitive idea that decreased ROS may actually drive inflammatory responses" and suggests it is time for us to change our "old view of ROS merely as antimicrobials and mediators of tissue damage". Mayadas *et al.(*2014), while acknowledging the research suggesting roles for proteases and oxygen radicals in disease causation, raises an important point that "the challenge is to decipher whether neutrophils are the actual source of these products". In addition, immune system has inbuilt counter acting mechanisms to effectively protect from overt neutrophil activation (Gunzer, 2013).

It is very early to conclude that food colours may be actually driving inflammatory responses and to accept that perhaps this is the most plausible explanation for the elusive causal link between food colours and allergies/intolerance/ hypersensitivities and hyperactivity reactions. However it is tempting at this point to look at the possibility of such an association given the fact that allergic reactions to food additives can be considered as triggers or aggravating factors in sensitive individuals, rather than aetiological factors (Gultekinand Doguc, 2013).In addition there are studies which suggest that severity of autoimmune diseases increase with decreased production of ROS (Bengtsson *et al.,* 2014; Mossberg *et al.,* 2007; Mossberg *et al.*, 2009).

Future studies probing the effects of synthetic as well as food colours on both intracellular and extracellular ROS production with the help of other PMNs stimulants like N-formyl-methionyl-leucyl-phenylalanine (fMLP), opsonized zymosan and/ orother CL enhancers like iso Luminolare required. It would be interesting also for the future studies to consider the interaction of antioxidants with different synthetic food colourants. Antioxidants are added to many foods to prevent the degradation of food components and degradation or fading of food colours but their combined effects on the intracellular ROS production by

PMNs could be of research interest (Iwasaki *et al.,* 2014). Also, given the well-established ROS linked pathogenesis of diseases like cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease), rheumatoid arthritis (Valko *et al.,* 2007), future recommendations could include conducting further studies on sub groups of diseased human volunteers. Also other underpinnings like age effects, effects of combinations of food colours, mode of action physiologically for each food colour need to be further understood to arrive at definite conclusions.

CONCLUSIONS

To summarize, allura red and carmoisine have a potential inhibitory effect even at moderate concentrations on intracellular ROS production by PMNs in vitro. This study results in general could be more useful as a beginning point for further exploration of the link between food colours and ROS production and its comprehensive effects on general health of populations in view of augmented exposures to the food colours.

ACKNOWLEDGEMENTS

The authors thank Deanship of Research and staff of the Clinical Nutrition Department, University of Hail for their continuous support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Eyad Al Shammariconceived, designed and carried out the experiments; Suneetha Epurustatistically analysed the data and participated in the drafting of the manuscript; RafiaBano, Mohd Adnan and Saif Khan wereinvolved in literature collection, interpretation of the results and critical revision of the manuscript.

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