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Antioxidant and Modulatory Effect of Melatonin on Hepatotoxicity and Oxidative Stress Induced by Orange Yellow S in Male Rats

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Abstract.- Many azo dye derivatives are used as food colorants which cause cytotoxic effects. In recent years, a new trend to use antioxidants to neutralise the suspected effects of food additives has evolved. The present study investigated the possible modulatory effect of melatonin in reducing the cytotoxicity induced by the food colour additive Orange Yellow S (OYS) in male rats (*Rattus norvegicus*). The levels of thiobarbituric acid-reactive substances (TBARS) in the plasma, the activities of superoxide dismutase (SOD) and catalase in the blood and liver, the lipid profile and glucose concentration in serum and the histological appearance of the liver were also evaluated. Rats weighing 200-250 g were divided into four groups of five rats each; control, OYS-treated group, melatonin-treated and melatonin-and OYS-treated group. The treated groups were repeatedly gavaged with either 2.5 mg/kg body weight (bw) of OYS, 10 mg/kg bw of melatonin or both for three weeks. The results revealed no significant changes in liver catalase activity and serum HDL. OYS produced cytotoxic effects, while oral melatonin administration was significantly reduced the cytotoxic effect induced by OYS. The results support the use of supplemental melatonin as a modulating antioxidant agent.

Keywords: Melatonin, Orange Yellow S, antioxidant enzymes, male Wistar albino rat.

INTRODUCTION

Many people and industrial factories add food additives that are natural or synthetic compounds for coloring, as preservatives or to improve food structure. Many studies examined the heath effect of food colorants on experimental animals (Aboel-Zahab *et al.,* 1997; Sasaki *et al.,* 2002; Tanaka, 2006; Amin *et al.,* 2010; Feng *et al.,* 2012).

Food additives have attracted the attention of the public and the research community as potential causes of various human diseases. Some have genotoxic or mutagenic effects on experimental animals (Demir *et al.,* 2007; Tanaka, 2007; Turkoglu, 2007). Food additives may be among the factors responsible for hepatic cancer and nephritic failure (Nakayama *et al*., 1983; Collier *et al*., 1983; Seesuriyachan *et al*., 2007). The addition of some food additives produce significant alterations in antioxidant enzyme activity (Bansal, 2005; Amin *et al.,* 2010; Gao *et al.,* 2011) and have mutagenic effects (Oliveira *et al.,* 2010). The toxicity and carcinogenicity of food additives may result from interactions between intact molecules and cytosolic receptors (Collier *et al.,* 1983; Lubet *et al.,* 1983; Seesuriyachan *et al.,* 2007; Oliveira *et al.,* 2010), from the formation of free radicals that alter the activity of antioxidant enzymes or from arylaminazo reduction (Nony *et al.,* 1980; Pearce *et al.,* 2003).

Orange yellow S (OYS) is an azo dye produced by coupling diazotized sulfanilic acid with 2- napthol-6-sulfonic acid and isolated as sodium salt, it may be found in many sweets and soft drinks. Azo compounds are formed from arenediazonium ions conjugated through an azo linkage to highly reactive aromatic hydrocarbon compounds containing two aromatic rings, which are responsible for their intense colours (Solomon, 1996). Synthetic food additives include colouring or curing agents and/or sweeteners. For example, tartrazine and carmoisine are nitrous derivatives of azo compounds that can be metabolised to highly

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Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein;; OYS, Orange Yellow S; TBARS, thiobarbituric acid-reactive substances; SOD, superoxide dismutase.

sensitizing aromatic amines such as sulphanilic acid (Maekawa *et al.,* 1987; Amin *et al.,* 2010; Feng *et al.,* 2012), and usually absorbed in small quantities (Nihon-shokuhin-tenkabutu-kyokai, 1999).

Melatonin, the pineal gland's hormone, is able to limit oxidative stress and damage. Under physiological conditions, it acts as a direct free radical scavenger via nuclear receptor or non-receptor pathways, leading to the inhibition of oxidative stress by modulating the detoxification of reactive hydroxyl radicals (OH) and neutralising singlet oxygen, peroxynitrite anion, nitric oxide and hydrogen peroxide (H_2O_2) (Tan *et al.,* 2002; Reiter *et al.,* 2004; Kharwar and Haldar, 2012; Reiter *et al.,* 2012). Reactive oxygen species (ROS) are produced during normal metabolism or as a consequence of the response to abnormal stress (Ahmed, 2012; Gunalan *et al.,* 2012). It has been implicated in the pathogenesis of ageing and diseases, including cancer (Liang and Kitts, 2014). Mammalian cells are equipped with both enzymatic and non-enzymatic antioxidant mechanisms to minimise the cellular damage that results from interactions between cellular constituents and ROS (El-Habit *et al.,* 2000; Shirazi *et al.,* 2012). Melatonin also acts as an indirect antioxidant via its stimulatory action on the gene expression and activities of antioxidative enzymes (Mayo *et al.,* 2002; Rodriguez *et al.,* 2004; El-Missiry *et al.,* 2007; Sharma *et al.,* 2008; Fischer *et al.,* 2013). Moreover, it controls reproductive functions and stimulates immune system activity and tumourigenesis (Reiter *et al.,* 2000; Singh and Halder, 2007).

The enzymatic antioxidant mechanism involves a number of enzymes, such as SOD, catalase, and glutathione peroxidase, as well as enzymes involved in recycling oxidised glutathione, such as glutathione reductase (Halliwell, 1992). Many natural antioxidant products, such as β-carotene, melatonin and vitamins A, C and E play roles in the protection against oxidative stress induced in different experimental models (El-Habit *et al.,* 2000; Canter *et al.,* 2007; Kharwar and Haldar, 2012). Hence, this work aims to study the possible modulatory effect of melatonin in reducing the cytotoxicity induced by the food colour additive OYS in male Wistar albino rats.

MATERIALS AND METHODS

Experimental animal, chemicals and treatment

Male Wistar albino rats weighing 200-250 g were obtained from the animal farm at King Saud University, Saudi Arabia, Riyadh. Handling of animals was in compliance with guidelines for the care and use of animals for scientific purposes. The animals were provided with water and standard basal diet *ad libitum* and were maintained under controlled conditions of temperature, humidity and light (12:12 h light: dark cycle). All chemicals were purchased from Sigma (St. Louis, MO, USA). OYS was purchased from a local market (96%, Kamena Industries, Canada).

Twenty adult rats were divided into the following four groups of five rats each. Group 1 (Control) did not receive any treatment. Group 2 (OYS) was orally treated with 2.5 mg/kg bw of OYS. Group 3 (Melatonin group) was administered orally with 10 mg/kg bw of melatonin according to Bhatti *et al.* (2011). Group 4 (OYS and melatonin) received both OYS and melatonin as mentioned before. Both OYS and melatonin were dissolved in distilled water and administered orally once per day for three weeks. The blood samples were collected from the rats using a fine microhaematocrit tube inserted in the inner corner of the ophthalmic venous plexus and divided into three sets: whole blood, plasma and serum. The first two sets were collected in heparinised tubes.

Oxidative stress markers, enzymatic antioxidants status and lipid profile

The TBARS levels were measured in plasma and liver as described by Yoshioka *et al*. (1979). The spectrophotometric determination of the SOD and catalase activities was carried out in both blood and liver extracts according to the methods of Winterbourn *et al*. (1975) and Bergmeyer *et al.* (1987), respectively.

The lipid profile indices were determined according to standard methods. The tests included LDL, HDL, cholesterol, triglycerides. All the indices and glucose concentration were estimated in serum using a Humalyzer model 3000 by standard enzymatic methods using Human kits (Bartham and Trinder, 1972; Richmond, 1973; Schettler and

Nussel, 1975; Izawa *et al.,* 1997; Okada *et al.,* 1998), respectively.

Histological examination

A microscopical examination of liver sections was performed according to standard methods (Yenilmez *et al.,* 2010). Pieces of liver were fixed in 10% formaldehyde, embedded in paraffin, cut into sections 5 µm in thickness (microtome, Leica Rm 2145) and stained with haematoxylin and eosin. The microscopic examination was performed under an Olympus PM 10 ADS microscope (Olympus America Inc. Melville, NY, USA).

Statistical analysis

Student's *t*-test was applied for the statistical analysis of all the experiments as mentioned by Ronald *et al*. (1983). The data are presented as the mean±standard deviation (SD) and were statistically analysed using SPSS (Statistical Package for the Social Sciences) version 20. Values were considered significant at $P<0.05$ and highly significant at $P<0.01$.

RESULTS AND DISCUSSION

In this study, biochemical analyses and histological examinations were examined to study the physiological effects of a synthetic food colourant (OYS). The modulatory role of melatonin as cytoprotective against OYS was also evaluated. The purposes of this study were to establish diagnostically normal profiles of biochemical parameters as antioxidant enzymes, lipid peroxidation in blood and liver, lipid profile in serum, glucose concentration and histological examination of liver in the all groups to evaluate the possible modulating role of melatonin.

The data in Figures 1 and 2 indicated a significant increase in SOD activity and the TBARS concentration ($p<0.05$, $p<0.001$, respectively) in the blood and liver of OYS group as compared to control group. There was no significant change in the catalase activity in the group treated with OYS in liver, whereas a significant increase of catalase activity in blood ($p<0.05$) was noted versus control group. Melatonin administration reversed the effect of OYS, as evidenced by the decrease in catalase

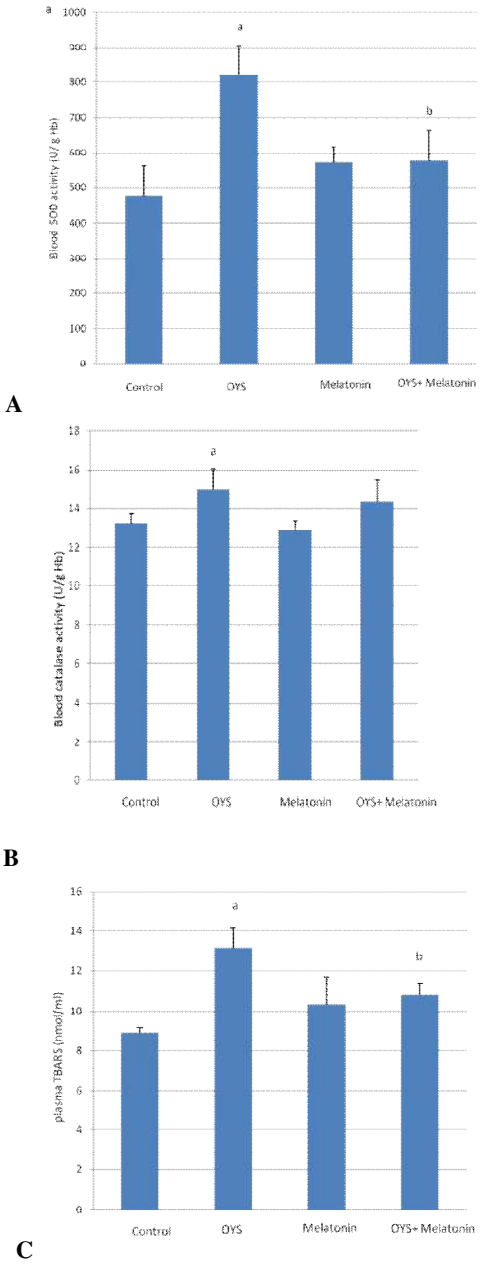

Fig. 1. Blood SOD activity (U/g Hb), catalase activity (U/g Hb), and Plasma TBARS (nmol/ml) in different animal groups. Each value represents the mean of 5 records ± S.D. $^{a}p < 0.05$, significant change with respect to Control group; $^{b}p < 0.05$, significant change with respect to OYS group.

activity in liver compared with the group treated with OYS alone (14.34±1.12 vs. 15.01±1.04). Also, the modulating effect of melatonin was noticed in

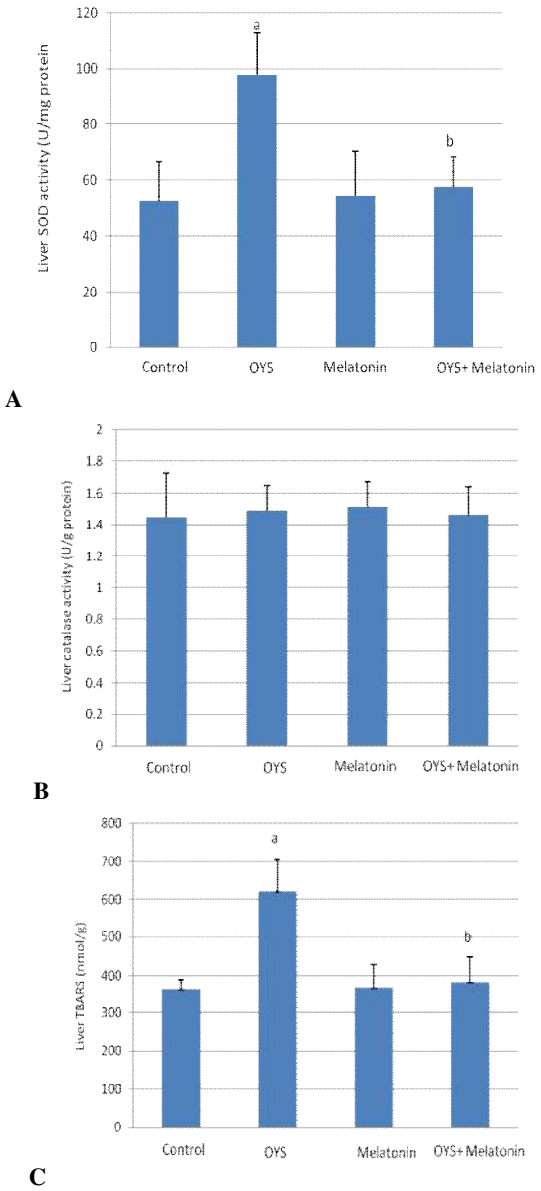

Fig. 2. Liver SOD activity (U/mg protein), catalase activity (U/g protein), and TBARS (nmol/g fresh tissue) in different animal groups. Each value represents the mean of 5 records ± S.D. [a]$p<0.05$, significant change with respect to Control group; [b]$p<0.05$, significant change with respect to OYS group.

SOD activity and the TBARS concentration (578.03±83.38 vs. 822.19±82.63 and 10.78±0.59 vs. 13.16±0.97, respectively) in the blood and (57.64±10.75 vs. 97.88±14.75 and 380.93±66.41 vs. 620.23±83.92, respectively) in the liver.

SOD and catalase provides major protection in oxidative injury by participating in the cellular system of defense against oxidative damage. The levels of ROS are controlled by antioxidant enzymes and non-enzymatic scavengers.

The TBARS are produced by the peroxidation of unsaturated fatty acids in biological membrane lipids caused by ROS and are considered as a late biomarker of oxidative stress and cellular damage. The result is a dramatic decrease in cellular membrane fluidity and the disruption of membrane integrity and function, which produce critical pathological changes (Halliwell, 1987).

Food colour additives, especially those containing azo dyes and aromatic amine structures, are cytotoxic and genotoxic compounds because they are metabolised by intestinal bacteria that produce ROS which increase oxidative stress (Sweeney *et al.,* 1994; Bansal, 2005). It should be noted that the levels of ROS are controlled by antioxidant enzymes and non-enzymatic scavengers. SOD and catalase provide a major cellular defence mechanism against oxidative damage by participating in the clearance of O_2^- and peroxide anions, respectively (Curtis *et al*., 2007). In this study, the rats treated with OYS showed a significant increase in blood and liver SOD activity; plasma and liver TBARS concentration and blood catalase activity; whereas there was no noticeable change in catalase activity in liver. Among the non-enzymatic antioxidants, melatonin can act as a direct free radical scavenger (Rodriguez *et al.,* 2004; Shirazi *et al.,* 2007; Galano *et al.,* 2011, 2013) and inhibitor of pro-oxidative enzymes (Karbownik and Reiter, 2000). Several studies have indicated that melatonin pretreatment play a modulatory role on antioxidant enzyme levels and ROS scavenging in different organs to protect against the oxidative stress induced by different treatments (El-Missiry *et al.,* 2007; Bharti and Srivastava, 2009; Bhatti *et al.,* 2011; Bharti *et al.,* 2012; Reiter *et al.,* 2012; Tamura *et al.,* 2012; Fischer *et al.,* 2013).

The data in Table I showed a significant increase in serum LDL, cholesterol, triglycerides and glucose concentration ($P<0.05$, $P<0.01$, $P<0.01$, $P<0.01$., respectively) and no significant change in the HDL in the group treated with OYS versus control group. Melatonin administration to OYS

Table I.- Serum LDL, HDL, Cholesterol, triglycerides and glucose in different animal groups.[§]

Group of animals	LDL (mg/dL)	HDL (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Glucose (mg/dL)
Control	14.34±1.22	9.18±3.22	30.60±1.23	27.05±2.64	142.72±5.97
OYS	15.50±0.92	10.00±2.67	44.25±7.33	40.44±5.03	169.68±7.18
*P*1 value*	<0.05	>0.05	<0.01	<0.01	<0.01
Melatonin	13.62±1.70	9.96±1.86	32.40±1.47	31.62±3.89	141.62±7.79
*P*1 value	>0.05	>0.05	<0.01	>0.05	>0.05
*P*2 value**	>0.05	>0.05	<0.05	>0.05	<0.01
OYS + Melatonin	12.18±0.68	8.52±2.88	31.60±0.86	32.82± 4.12	149.40±8.81
*P*1 value	<0.05	>0.05	<0.05	<0.05	>0.05
*P*2 value**	>0.05	>0.05	<0.05	<0.01	<0.01

[§] Each value represents the mean of 5 records ± S.D.
* The significance of changes from control value
** The significance of changes from OYS value

reversed the effect of OYS as evidenced by the decrease in serum LDL, cholesterol, triglycerides and glucose concentration compared with the group treated with OYS alone (12.18±0.68 vs. 15.50±0.92, 31.60±0.86 vs. 44.25±7.33, 32.82±4.12 vs. 40.44±5.03 and 149.40±8.81 vs. 169.68±7.18, respectively).

These results are correlated well with these reported by Aboel-Zahab *et al.* (1997) who obtained a significant increased in serum total cholesterol and triglycerides level in rats whose diets were supplemented with food colouring which were added to chocolate like tartrazine and carmoisine.

Cholesterol is a soft, waxy substance among the lipids in the blood stream and in the body cells. It is an important part of healthy body because it is used to form cell membranes and to produce certain hormones. The total body content of cholesterol depends on the balance between the amount of cholesterol formed in the body plus that absorbed from diet. Intestinal cholesterol absorption represents another major route for the entry of cholesterol into the body, and, thus, this source can influence the plasma LDL-cholesterol concentration (Turley, 2004).

Melatonin affects the metabolic and physiological status of lipid metabolism in obese animals (Hussein *et al.,* 2007). The increase of cholesterol level in blood serum is considered as an indication of liver diseases and the disruption of membrane structure and function. This change may affect membrane fluidity and permeability. It could also affect the activity of membrane associated enzymes and transport system (Singh *et al.,* 1988). In the present study the increased cholesterol level in OYS group is accompanied by liver damage and the subsequent increase in LDL, triglycerides and glucose concentration, whereas no significant change was observed in HDL level in the same group. It is reported that the elevation in blood LDL and cholesterol concentration results in the increase of heart attack and stroke (Scirica and Cannon, 2005). In the present work, the administration of melatonin reversed the effect of OYS on cholesterol, LDL, triglycerides and glucose concentration which indicates that melatonin plays a hypocholesterolic role by two means; first through enhancing the catabolism of cholesterol to bile, second by inhibiting cholesterol synthesis and accumulation of LDL (Chan and Tang, 1995). This process is mediated by several mechanisms including a decrease in the number of LDL receptors (Muller-Wieland *et al*., 1994) and by the release of inhibitory G protein that couples the receptors causing inhibition of fatty acid transport (Dauchy *et al*., 2003). The decrease of glucose level in the melatonin treated group may occur due to the ability of melatonin to stimulate insulin secretion from the pancreas (Fabis *et al*., 2002). This hypoglycemic effect counteracts the hyperglycemic effect of the food colourant in the OYS group.

In the present study, the histological examination of liver sections prepared from rats treated with OYS revealed severe cellular

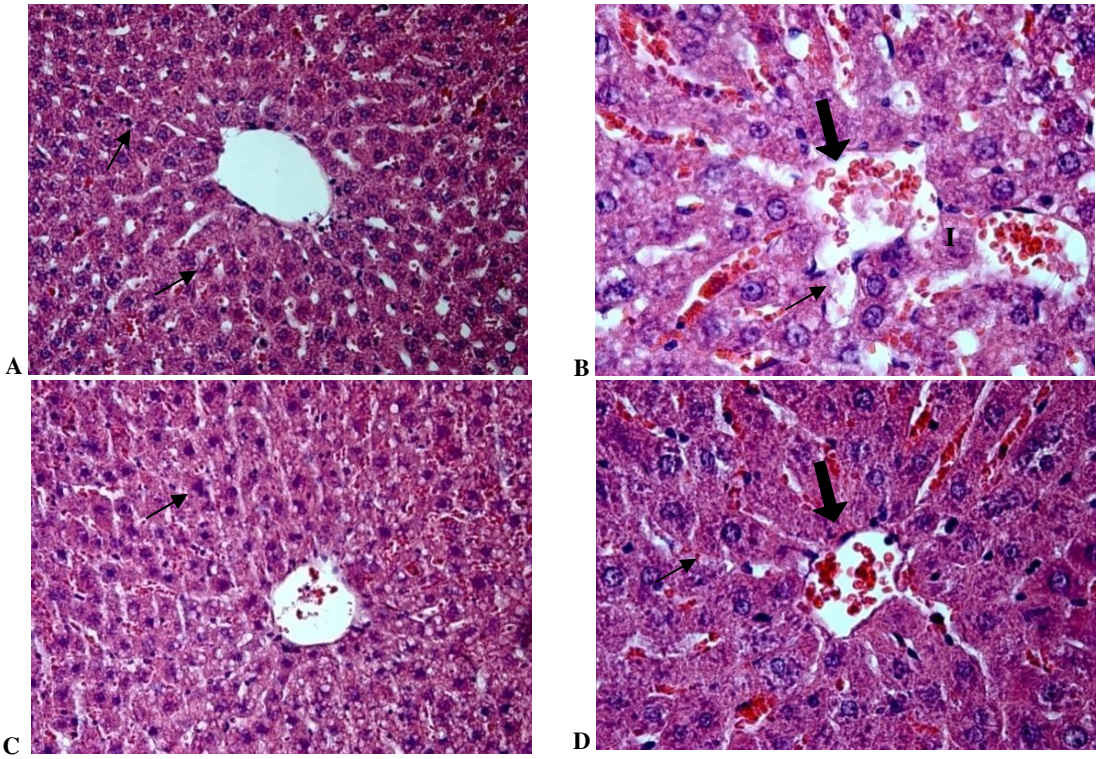

Fig. 3. Histological structure of rat liver showing the effect of melatonin on food colorant additives induced hepatic damage. A, Control group showing normal appearance of the hepatic strands radiating from the central vein (↑). Notice the hepatocytes, blood sinusoids and bile canaliculi; B, OYS group showing lymphocytic infilteration (I), congestion of the central veins (↟), obliteration of the blood sinusoids, and pyknotic nuclei (↑); C, Melatonin group showing normal hepatic structure with mitotically dividing hepatocytes in some areas (↑); D, OYS and melatonin group showing slight congestion of the central veins (↟) and normal hepatic architecture (↑).
Stain: Haematoxylin and Eosin; Magnification: A, C 20x; B, D. 40x.

destruction. This histological profile has also been reported in other works (Aboel-Zahab *et al*., 1997; Mekkawy *et al.,* 1998; Amin *et al*., 2010). Increased ROS and free radical generation can damage the hepatic tissues of rats, resulting in marked hepatic lesions (Suzuki *et al.,* 1998). Sarhan *et al.* (2014) indicated the hepatotoxicity by the increase of serum ALT and AST levels in rats treated with OYS.

The histological examination of liver sections from the OYS-treated animals revealed lymphocytic infiltration, congestion of the central veins, obliteration of the blood sinusoids, and pyknotic nuclei. There were no remarkable adverse effects observed in the group treated with melatonin. In general, there was appreciable improvement after treatment with melatonin (Fig. 3).

CONCLUSIONS

Because it is difficult to persuade consumers of food colorant, especially children, for dispensing them, a new trend is seeking a naturally occurring compound that reduces the harmful effects of food additives. The results of this study suggest that melatonin can serve as a powerful, naturally occurring antioxidant capable of neutralising the deleterious biochemical and histological effects of OYS in blood and liver and can be used in concurrent with food additives.

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