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Comparative Study on the Effect of Sunset Yellow Dye and Diethylnitrosamine (DEN) on Testes of Male Sprague Dawley Rats

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ABSTRACT

Sunset Yellow (SY) (E110) is a synthetic yellow azo dye commonly found in various food products such as orange sodas, powdered marinades, snack chips, ice creams, apricot jam, gelatin desserts, and cake decorations. On the other hand, Diethylnitrosamine (DEN) belongs to the nitrosamine family and is used in various applications such as a gasoline additive, lubricant additive, stabilizer in plastics, solvent in the fiber industry, and is even present in tobacco smoke. The objective of this study was to investigate the effects of SY and DEN on the testes of rats. Twenty-four male Sprague Dawley (SD) rats, aged eight weeks, were divided into four groups. The first group served as the normal control and received no treatment. The second group was solely treated with SY at a dosage of 160 mg/kg/b.wt./day via intragastric gavage (ig). The third group received an intraperitoneal (IP) injection of DEN at a dosage of 200 mg/kg. The fourth group underwent the same DEN injection as the second group, followed by treatment with SY at a dosage of 160 mg/kg/b.wt./day administered via intragastric gavage (ig). The results of the study revealed that the testes weight increased in the treatment groups (3.3±0.63; 2.95±0.63 and 3.32±0.64, for SY, DEN, and the combination of SY and DEN, respectively) compared to the control group (2.9±0.31). In terms of the lipid profile, the DEN group exhibited increased levels of serum cholesterol, triglycerides, and low-density lipoprotein (LDL), accompanied by a decrease in high-density lipoprotein (HDL). Conversely, the group treated with SY demonstrated an increase in cholesterol, triglycerides, and LDL levels, but a decrease in HDL levels. Additionally, the combination of SY and DEN showed a reduction in Glutathione reductase and Glutathione peroxidase, which are antioxidant enzymes. Furthermore, the DEN group displayed significant microscopic shrinkage of seminiferous tubules, desquamation of germinal epithelium, and increased interstitial tissue with haemorrhage and haemolysis. In contrast, the group treated with SY showed mild degenerative changes in seminiferous tubules, highly congested blood vessels, and interstitial haemorrhage.

Keywords: Antioxidant enzymes; DEN; Sunset Yellow dye; Seminiferous tubules; Testes.

INTRODUCTION

Sunset Yellow dye (SY) and Diethylnitrosamine (DEN) are chemicals that are utilized in a variety of industries and food products. Determining their safety and potential hazards to male reproductive health requires an examination of their effects on the testes. Sunset Yellow dye is a synthetic food coloring additive that is frequently used in food manufacturing to improve the appearance of various goods. On the other hand, Diethyl nitrosamine is used as a lubricant additive, antioxidant, and stabilizer in plastic products (Lawal et al., 2021).

Food additives encompass a wide range of substances added to food, whether derived from natural sources or synthetically produced. These additives play a crucial role in processed foods, contributing to their preservation, visual appeal, flavor enhancement, taste modification, and color enhancement (Alves *et al.*, 2008). Among these additives, Azo dyes, such as Sunset Yellow, stand out as widely used food coloring agents due to their stability and cost-effectiveness. In Saudi Arabia, SY was extensively consumed (30.1%) and significantly detected (34.2%) in most food consumed by school children, as determined by high performance liquid chromatography (HPLC) (Warner, 2024; Mohammed *et al*., 2023).

SY a synthetic azo dye, has a well-established history as a food colorant in beverages and various food products, including confectionery, desserts, soups, cheeses, savory snacks, sauces, drink mixes, packet soups, gelatin desserts, orange sodas, powdered marinades, snack chips, ice creams, apricot and citrus jams, lemon curd, sweets, breadcrumbs, prescription medications (particularly children's medications), preserved fruits and pharmaceutical pills (Wood et al., 2004; Hashem et al., 2010). It is soluble in water and only sporadically soluble in ethanol. SY is made from aromatic hydrocarbons derived from petroleum and is an orange-red powder or granules form (Wood *et al*., 2004; Ha *et al*., 2013).

The safety of food additives undergoes rigorous evaluation by regulatory bodies in different countries, each holding distinct perspectives on the safety of these substances (Madhava & Sowbhagya, 2012; Soltan & Shehata, 2012). SY may cause allergic reactions, gastrointestinal distress, diarrhea, vomiting, urticaria, angioedema, migraines, and immunomodulatory effects (Yadav *et al*., 2013). The Codex General Standard for Food Additives (GFSA) outlines regulatory provisions for Sunset Yellow across a broad spectrum of food and beverage categories, with specified maximum levels ranging from 50 to 400 mg kg^{-1} (FAO, 2023). In the European region, Maximum Permitted Levels (MPLs)

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for Sunset Yellow FCF in food and beverages fall within the range of 50 to 500 mg kg^{-1}, a range consistent with that of Australia and New Zealand. Notably, in Canada and the United States, there are no specified maximum limits for the addition of Sunset Yellow FCF to foods (FAO, 2023). However, in Saudi Arabia, there are no specified maximum permitted limits for the addition of Sunset Yellow. The long-term effects of SY and other additives are not fully studied and become a topic of public concern.

Diethylnitrosamine (DEN), similar to many other food additives, belongs to the nitrosamine family and is widely used in various industries and products. This compound has multiple applications, such as a gasoline and lubricant additive, a stabilizer in plastics, a solvent in the fiber industry, and a component of cigarette smoke. Furthermore, DEN can be found in the consumption of fried food, alcoholic drinks, occupational settings, cosmetics, agricultural chemicals, and even as a byproduct of certain therapeutic drugs (Sivaramkrishnan *et al*., 2008; Gupta *et al*., 2010). The versatility of DEN's applications raises concerns regarding its potential impact on human health and the environment. Therefore, this comparative study aims to investigate, evaluate and compare the potential histopathological changes, oxidative stress levels, in the testicular tissue caused by Sunset Yellow dye and Diethylnitrosamine. The focus will be on parameters such as testicular weight, histopathology, and oxidant stress, providing valuable insights into their reproductive toxicity and potential mechanisms of toxicity. Understanding the differential effects of these compounds on testicular morphology, function, and integrity is essential for assessing their impact on testicular health and reproductive function. By comparing the effects of these two substances, this study will contribute to a better understanding of their potential impact on male reproductive health.

MATERIALS AND METHODS

Experimental animal

In this experiment, twenty four male 8-week-old Sprague Dawely (SD) rats were purchased from King Fahd Center for Medical Research (King Abdulaziz University, Jeddah, Saudi Arabia). The rats were housed with ad libitum access to food and water at constant temperature (22°C ± 2°C), and under a regular 12 h light/dark cycle (light on at 08:00) with humidity level of 55 %, without any disturbance for almost one week before starting the experiment. All phases of the experiment were carried out in compliance with the guidelines provided by the National Committee Bioethics with No. (HAO-02-T-105) Taif University, Application No. 45-297, Ministry of Education, Saudi Arabia.

Experimental design

For this study, four groups were carefully designed to investigate the effects of Sunset Yellow dye and Diethylnitrosamine (DEN) on the testes of male Sprague Dawley rats. Control Group: This group served as the baseline and received no treatment. They were used for comparison purposes to determine the natural state of the testes. Sunset Yellow Dye Group (G2): rats in this group were administered Sunset Yellow dye orally (ig) in which a dose of 160 mg/kg b.wt. was used following the method of (manal, 2024). Rats in this group were treated with the same dose of SY twice a week The third group (G3), is Diethylnitrosamine (DEN) Group: rats in this group were injected with DEN via inter-protenial (IP) one time at the beginning of the experiment using a dose of 200 mg/kg b.wt. Combined Group (G4): rats in this group were subjected to both Sunset Yellow dye and Diethylnitrosamine (DEN) treatments. This group aimed to investigate potential synergistic effects or interactions between the two substances on the testes.

Chemicals and Doses

To ensure accurate administration and dosage, the following chemicals and doses were used in the experiment: Sunset Yellow FCF Dye (SY): Highly purified SY FCF dye (Sigma-Aldrich) with the chemical formula $C_{16}H_{10}N_2Na_2O_7S_2$ and CAS No. 2783-94-0 was orally administered to the animals using intragastric gavage (ig) technique. The dosage of SY was determined based on the permitted acceptable daily intake (ADI) recommended by authoritative bodies such as the EU, WHO, and FAO (EFSA, 2015). Diethylnitrosamine (DEN): DEN was obtained from Sigma-Aldrich, St. Louis, MO, USA. It was dissolved in a normal saline solution (0.9%) and injected intraperitoneally (IP) into the rats at a dosage of 200 mg/kg body weight (Solt & Farber, 1976). This dosage was selected to elicit the desired effects of DEN on the testes. Combined Group: In the fourth group, SY was administered to the animals two days following the DEN injection. Throughout the experiment, SY was then administered twice a week at a calculated dose of 160 mg/kg body weight (b.wt.).

Sample collection after treatment

At the end of experiment, all animals were fasted overnight then anesthezied by diethyl ether. Blood was taken from the abdominal aorta of each rat in EDTA and non-EDTA glass tubes to obtain plasma and serum. Complete blood pictures (CBC) for all rats were measured from the collected blood samples by automatic methods (Sysmex kx-21n automated hematology analyzer; JAPAN CARE CO., LTD). This included hemoglobin (Hb), white blood cells (WBCs), red blood cells (RBCs), Platelets, Hematocrits, PCV and others.

Centrifugation was used to separate the serum for 10 minutes at 3000 rpm. The obtained serum was kept cold until it was subjected to biochemical examination (Almatrafi, 2024). The testes quickly removed and washed by cold saline. Some sections from these organs were fixed in 10% buffered formalin for histopathological assessments.

Body weight measurements

Body weight measurements were taken at the beginning and end of the experiment for all experimental groups. The mean value ± standard error was calculated and recorded for each group. Additionally,

the weight of the testes was also measured at the end of the experiment.

Estimation of Complete blood picture (CBC)

The collected blood samples were subjected to hematological analysis to evaluate various parameters including Hemoglobin (Hb), Red Blood Cell Count (RBC), Platelet Count, Hematocrit red blood cell volume (HCT), mean Cellular Vvolume (MCV), Cellular Hemoglobin Concentration (MCHC), Lymphocytes (LYM), Monocytes (MON) and Granulocytes (GR). Hematological parameters were analyzed using automated hematology analyzers to ensure accuracy and reliability of the results.

Estimation of Lipid profile

Serum total cholesterol was measured using the method of Richmond (1973), serum triglyceride was measured using the method of Fossati and Prencipe (1982), serum HDL-C was determined according to the method of Burstein and Scholnick (1973), Serum LDL-C calculated consistent with this equation: LDL-C = TC – TG/5 –HDL-C = mg/dl was determined accordance to method of Ahmadi *et al*. (2008).

Histopathological examination

A histopathological investigation was performed using the protocol described by Yoon *et al.* (2001). In order to evaluate and define any structural alterations, anomalies, or pathological findings within the kidneys and spleens of the experimental rats, a thorough microscopic examination of tissue samples was conducted. The objective of the histopathological examination was to offer significant information regarding the morphological changes and any histological damage caused by the experimental treatments. This information was then utilized to assess the overall health of the organs and the effects of the treatments throughout the study.

Data analysis

The data obtained were replicated and recorded as means ± SE. One-way analysis of variance (ANOVA) was utilized to assess the impact of YS, DEN and combination between them in compare to control group of male Sprague Dawley rats. Additionally, the Duncan Multiple Range Test was employed to compare the various treatments.

RESULTS

Variation in body weight in different groups

The variation in body weight among the different treatment groups was analyzed using the data obtained from Table (1). It is evident that there is no significant difference in the change in body weight between most of the treatment groups, except for Group 4, where the combination of YS and DEN was administered. In this group, a highly significant synergistic effect ($p \leq 0.01$) on body weight was observed compared to the control group. Similarly, significant differences were also observed in the weight of the testes among the different treatment groups, following the same pattern.

Complete blood picture

The complete blood picture provides important hematological parameters that offer valuable information about various diseases and conditions. DEN and DEN + SY group showed a significant ($p \leq 0.05$) decrease in WBC, RBC, HGB, HCT, platletes, monocytes and lymphocytes. While SY showed a significant ($p \leq 0.05$) increase in WBC, RBC, HGB, HCT, platelets, monocytes and lymphocytes (Table 2).

Lipid profile in different study groups

The lipid profile in the different study groups revealed significant findings. The groups that received DEN, DEN+SY, and SY showed a significant ($p \leq 0.05$) increase in serum cholesterol, triglyceride, and LDL levels compared to the control group. Additionally, there was a significant ($p \leq 0.05$) decrease in HDL levels in these groups, as shown in Table (3).

Evaluation of antioxidant enzymes

Glutathione reductase (GR) activity

GR levels in groups (2), (3) and (4) were significantly decreased when compared with the G1 as -ve control. However, injection with DEN (G3) alone recorded a highly significant ($p \leq 0.001$) decrease in the antioxidant enzyme levels (Table 3). Meanwhile, GR levels were significantly less in group (2) compared to control group (Table 4).

Glutathione peroxidase (GPx) activity

The GPx activity levels in treated group showed the same pattern with more influences where SY groups and DEN groups showed significant decrease in enzyme activity in compared to control group.

Table (1): Effects of SY and DEN and a combimation of both of them on body and testes weight in Sprague Dawley rats

Weight parameters (g)	Treatments			
	G1 (-ve control)	G2 (SY)	G3 (DEN)	G4 (SY+DEN)
Initial body wt.	120±23.3	118.2±31.2	118.9±21.5	121.9±11.1
Final body wt.	236±33.8	237.6±44.2	232.5±30.1	292.5±23.4
Weight gain	116±10.5	119.4±13.0	113.6±8.6	170.9±12.3
Testes wts.	2.9±0.31	3.3±0.63	2.95±0.63	3.32±0.64

Data are presented in means ±SD.

Table (2): Effect of sunset yellow dye (SY), Diethylnitrosamine (DEN) and combination of SY+DNE on hematological parameters in Sprague Dawley rats.

Hematological Parameters	Treatments			
	G1 (-ve control)	G2 (SY)	G3 (DEN)	G4 (SY+DEN)
WBC ($X10^3$ /µL)	7.9±1.13[a]	9.73±1.61*	6.13±0.55*	6.03±0.38*
RBC ($X10^6$ /µL)	7.78±0.93	8.13±0.46	6.5±0.94*	5.9±0.36*
HGB (g/dl)	13.8±1.7	14.23±0.74	12.1±0.55*	11.83±0.22*
HCT (%)	39.67±1.15	40±3.89	34.2±2.69*	33.33±2.94*
MCV (fL)	51.66±7.17	49.12±2.84	53.30±6.91	56.76±7.16
MCH (Pg)	17.89±2.07	17.53±1.19	18.88±2.43	20.09±0.97
MCHC (g/dl)	34.88±3.86	35.82±3.48	35.59±3.64	35.75±3.60
PLT ($X10^3$ /µL)	380±48.88	393.33±17.51*	320±15.17*	276.67±18.89*'**
LYM ($X10^3$ /µL)	3.42±0.30	4.23±0.78	2.53±0.26	4.57±1.39*'**
MON ($X10^3$ /µL)	0.13±0.05	0.2±0.09	0.23±0.14	0.2±0.08
GRA($X10^3$ /µL)	0.5±0.28	0.5±0.09	0.37±0.08	1.42±0.35*'**

Data are presented in means ±SD. Raw with * are significantly different at level $p \leq 0.05$; **, significant at $p \leq 0.01$

Table (3): Effect of sunset yellow dye (SY), Diethylnitrosamine (DEN) and combination of SY+DNE on Cholesterol, Triglyceride, HDL and LDL of SD male rats.

Treatments	Lipid parameter measuerd			
	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control (G1)	53±7.79a	113.67±1.75	14.66±3.56	15.6±11.34
SY (G2)	57.4±8.25	118.03±3.05	15.67±1.21	18.13±7.34
DEN (G3)	60±7.40	120.07±3.91*	13.33±2.73	22.65±9.04
DEN+SY (G4)	67.67±4.50*	122.3±4.73*	9.67±1.86*	33.53±3.37*,**

Data represents as means±S.D. of 6 observations; *: Significant vs. G1 at $p<0.05$.; **: Significant vs. G2 at $p<0.05$;
HDL: High-density lipoprotein; LDL: Low-density lipoprotein

Table (4): Effect of sunset yellow dye (SY), Diethylnitrosamine (DEN) and combination of SY+DNE on Antioxidant enzyme levels detected in different treatment groups

Treatment groups	GR (mmole/min/g tissue)	GPx (µmol/g tissue)
-ve control (G1)	3.58±0.06	38.48±1.45
SY (G2)	2.99±0.05	23.115±3.53
DEN (G3)	1.51±0.38**	15.715±3.6**
DEN+SY (G4)	2.265±0.09**	13.55±2.57**

Data represents as means±S.D. of 6 observations; **: Significant difference at $p<0.01$

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However, highly significant difference was recorded in combination treatment and recorded the lowest enzyme value (13.55± 2.57) compared to SY (23.115±3.53) alone. For DEN group, no significant difference was detected when compared with SY+DEN (Table 4).

Histopathological analysis

Histopathological examination of testes stained with H&E showed normal architecture testicular parenchyma with seminiferous tubules, separated by interstitial tissue, intact germinal epithelium of seminiferous tubule, myoid cells surround the basement membrane and interstitial tissue (Figure 1a-b). DEN group showed shrinkage of seminiferous tubules, desquamation of germinal epithelium, increased interstitial tissue with hemorrhage and hemolysis, degenerative changes and desquamation of tubular epithelium in addition to hemorrhage and hemolysis in interstitial tissue (Figure 1c-d). Treatment with DEN and SY showed irregular outline of basement membrane of seminiferous tubules, mild degenerative changes in germinal epithelium, interstitial tissue containing congested blood vessels, mild hemorrhage, pyknosis and degenerative changes in germinal epithelium of seminiferous tubule, in addition to mild vacuolation of interstitial tissue (Figure 1e-f). Groups that received SY only showed mild degenerative changes in seminiferous tubules, highly

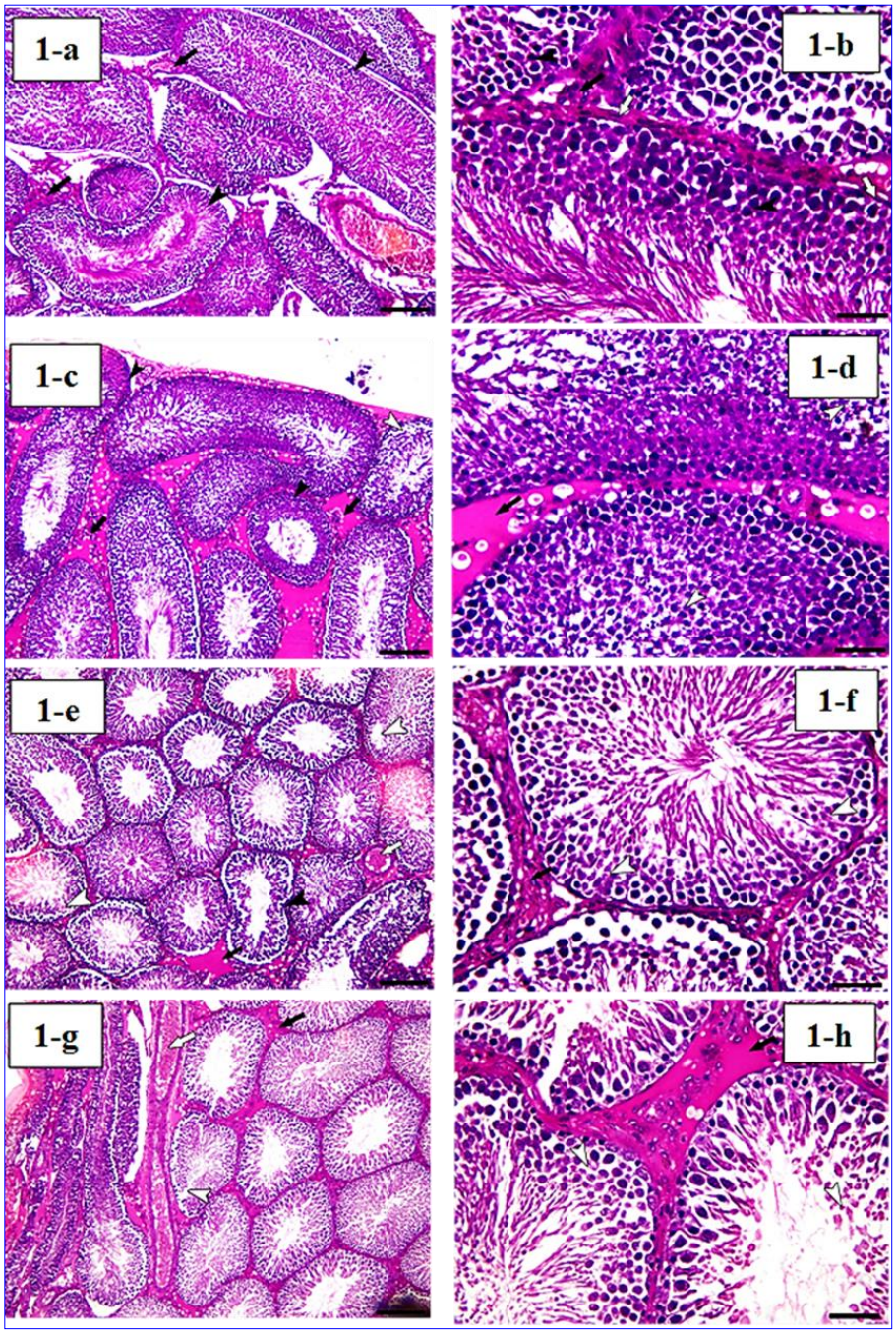

Figure (1):(a-b) Photomicrograph of testes of control group (a) showing normal architecture testicular parenchyma with seminiferous tubules (black arrow heads), separated by interstitial tissue (black arrows). Bar=200µm, (b) showing intact germinal epithelium of seminiferous tubule (black arrow heads), myoid cells surround the basement membrane (white arrows) and interstitial tissue (black arrow). Bar= 50 µm. (c-d) Photomicrograph of testes of DEN group (c) showing shrinkage of seminiferous tubules (black arrow heads), desquamation of germinal epithelium (white arrow heads) and increased interstitial tissue with hemorrhage and hemolysis (black arrows). Bar= 200 µm, (d) showing degenerative changes and desquamation of tubular epithelium (white arrow heads) in addition to hemorrhage and hemolysis in interstitial tissue (black arrow).Bar= 50 µm. (e-f) photomicrograph of testes of DEN and SY treated group (e) showing irregular outline of basement membrane of seminiferous tubules (black arrow heads), mild degenerative changes in germinal epithelium (white arrow heads), interstitial tissue containing congested blood vessels (white arrows) and mild hemorrhage (black arrow). Bar= 200 µm, (f) showing pyknosis and degenerative changes in germinal epithelium of seminiferous tubule (white arrow heads), in addition to mild vacuolation of interstitial tissue (black arrow). Bar= 50 µm. (g-h) photomicrograph of testes of SY treated group (g) showing mild degenerative changes in seminiferous tubules (whit arrow heads), highly congested blood vessel (white arrows) and hemorrhage in interstitial tissue (black arrow).Bar= 200 µm, (h) mild degenerative changes and accumulation of cellular debris in the lumen of seminiferous tubules (white arrow heads) in addition to hemorrhage in interstitial tissue (black arrow).Bar= 50 µm. Stain H&E.

congested blood vessel, hemorrhage in interstitial tissue, mild degenerative changes and accumulation of cellular debris in the lumen of seminiferous tubules in addition to hemorrhage in interstitial tissue (Figure 1g-h).

DISCUSSION

The data recorded the effects of SY and DEN on body weights and testes weights. Interestingly, our findings indicated that there was no significant effect ofSY and DEN on these parameters. These results are consistent with previous studies by Liao (2001) and Gaunt et al. (1967), which also did not observe significant changes in weights and testes weights.

Moving on to the hematological data, we observed a significant decrease in WBC, RBC, HGB, HCT, lymphocytes, monocytes, granulocytes, and platelets in comparison to the normal control rats. This finding aligns with the study conducted by Bendong et al. (2012), who also noted a significant decrease in WBC counts in rats that received DEN. Similarly, Gnanaraja and Veeru (2014) reported a decrease in RBC and HGB counts but an increase in WBC counts in mice treated with 175 µl/kg of DEN. Similar results were obtained by a study done by Almatrafi (2024) when used grape seed proanthocyanidin extract to ameliorate the toxic effect of Diethylnitrosamine DEN.

Furthermore, Arirudran *et al*. (2014) found that DEN administration led to a decrease in lymphocytes, monocytes, granulocytes, RBCs, HGB, HCT, MCV, MCH, and MCHC, but an increase in WBC counts. In our study, SY administration resulted in a significant decrease in WBCs, RBCs, HGB, HCT, and platelets compared to the negative and positive control groups. These findings are consistent with the research conducted by Mackenzie et al. (1992), who found a reduction in total WBC counts as a result of caramel treatment in rats.

Overall, our results regarding the hematological parameters are in line with previous studies, providing further support for the impact of SY and DEN on these measures. However, it is important to note that each study may have specific differences in methodology, dosage, and duration of treatment, which can contribute to variations in the observed effects.

Several studies have examined the effects of SY on testes, providing a basis for evaluating its impact as a commonly used food coloring in Saudi Arabia. SY is a synthetic azo dye commonly added to the food and pharmaceutical industries (Morrison et al., 2012). Therefore, the aim of this study was to investigate the effects of SY on the biochemical parameters related to testicular histopathology in rats. Nitrosamine compounds, such as diethylnitrosamine, have been shown to induce tumor growth in organs like the lungs, liver, kidneys, and spleen (Yamada et al., 2006).

The results of this study demonstrated an increase in serum cholesterol, triglyceride, and LDL levels, along with a decrease in HDL levels after DEN injection compared to the normal group. These findings are consistent with the research conducted by Jayaraman and Christina (2013). Additionally, Ramalingam and

Vaiyapuri (2014) reported a significant increase in cholesterol and triglyceride levels in rats treated with DEN. Similarly, treatment with SY resulted in a significant increase in these parameters, which aligns with the findings of Soltan et al. (2012), Amin et al. (2010), and AL-Shinnawy et al. (2009).

The biochemical findings obtained in this study are supported by the histopathological observations of the kidney and spleen. Microscopic analysis of the kidney revealed shrinkage of seminiferous tubules (indicated by black arrowheads), desquamation of germinal epithelium (indicated by white arrowheads), and increased interstitial tissue with hemorrhage and hemolysis in the DEN group. These findings are consistent with the research conducted by Emre Kaya et al. (2019) and Aitken & Roman (2008).

On the other hand, treatment with SY resulted in mild degenerative changes in the seminiferous tubules, highly congested blood vessels, and hemorrhage in the interstitial tissue. These observations are in agreement with the findings reported by Sarhan and Al-Sahhaf (2011) and Ismail (2016).

CONCLUSION

In conclusion, this comparative study aimed to provide a comprehensive understanding of the effects of Sunset Yellow dye and diethylnitrosamine (DEN) on the testes of male Sprague Dawley rats. Our meticulous examination and analysis aimed to contribute to the existing knowledge in the field of reproductive toxicology, emphasizing the need for further investigation and raising awareness about the potential risks associated with these substances. By conducting a comparative analysis of Sunset Yellow dye and DEN, we aimed to shed light on their relative potency, target organs, and underlying mechanisms of action. This valuable information can then be utilized to inform risk assessment and guide regulatory decision-making processes concerning the safety of these chemicals in food, consumer products, and industrial applications.

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