International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2015): 6.391

Sudan III Azo Dye: Oxidative Stress with Possible Geno and Hepatotoxic Effects in Male Rats

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Running title: Sudan III dye triggered the hepatocellular and DNA damage in male rats

Abstract: ***Azo compounds were widely used in industry. Although they are not permitted in food, they had been found contaminating different foodstuffs. In the present study, the geno and hepatotoxic effects of Sudan III were estimated with relation to oxidative stress. Rats (n=18) were randomly divided into three groups each of 6. First group were left as control, the remaining two groups, were orally administrated either 125 mg/kg body weight of Sudan III (1/40 LD_{50}), or 250 mg/kg body weight of Sudan III (1/20 LD_{50}) for consecutive 45 days. Rats then sacrificed, blood and tissue samples were harvested. DNA was extracted then fragments were analyzed in the automatic multicapillary electrophoresis, the serum activity of GOT and GPT were measured. Moreover, serum Glu, Alb and TP were evaluated. Lipid profiles were assessed through measuring of Chol, Trig, HDL and LDL. Liver GSH, CAT, SOD and MDA concentrations were evaluated. Our results revealed that, oral administration of Sudan III for 45 days caused serum Glu, GOT and GPT activities to increase significantly (P<0.05) in low and high doses. The TP level was markedly decreased (P<0.05) in treated rats with low and high doses of Sudan III, while there was no significant differences in Alb for both doses between groups. Serum level of Chol, Trig, and LDL were significantly increased (P<0.05) in rats received low and high doses of Sudan III compared to that in control untreated rats, while HDL showed significant decreased (P<0.05) in both doses of Sudan III compared to that in control untreated rats. GSH, SOD and CAT were significantly (P<0.05) decreased in liver tissues of rats received low and high doses of Sudan III compared to that in control untreated rats, While MDA level was increased significantly (P<0.05) in liver tissue of rats received high Sudan III dose, beside oxidative DNA damage with appearance of new bands in both low and high doses of Sudan III. In conclusion, low and high doses of Sudan III dye triggered the hepatocellular damage and adversely altered the lipid and oxidative stress biomarkers in male rats after 45 days of oral administration with induction of genotoxicity.***

Keywords: Sudan III, Genotoxicity, Oxidative stress, Hepatotoxicity

1. Introduction

Food additives have extremely important role to meet the needs of growing population during production and presentation of plentiful, tasty and nutritious food [1]. Whether synthetic or natural food additives may be used as flavoring or coloring stuffs [2]. Colorants provide an aesthetic appearance to food stuffs for preferred by consumers [3].

Azo dyes are a class of synthetic organic colorants that are typically used in many industrial applications including solvents, oils, fats, waxes, plastics, printing inks, and floor polishes [4]. The main reason for the widespread usage is their colorfastness and low price. Azo compounds may reach the intestine directly after oral ingestion. It is exposed to digestive enzymes and microflora [5]. Azo dyes then catalyzed by azoreductases and peroxidases leading to the liberation of semiquinone radicals and aromatic amines. Later, semiquinone radicals produce superoxide, hydroxyl radicals and H_2O_2. This might lead to the reduction of the defense, thereby generating a variety of oxidative stress-related conditions [6].

Sudan dyes are azo compounds commonly used in various materials [7], [8], [9]. Due to their intense red color, these compounds have been used unlawfully as food dye, mainly in curry and paprika powders, in order to deepen the color [10]. These compounds are still a public health concern considering the studies showing the presence of these dyes in food all over the world [11]. Despite the controversial level of risk, Sudan dyes are banned as food additives for humans. The recent contamination of hot chili and derived products demands the evaluation of their effect on animal health.

There is evidence that some Sudan dyes have genotoxic effects and that ingestion of food products contaminated with the dyes triggered oxidative DNA damage [12].

The present study was conducted to evaluate the toxic effect of Sudan III on DNA, blood glucose, hepatic functions, protein profile, lipid profiles and biomarkers of oxidative stress in liver tissue of male rats after 45 days of oral administration.

2. Materials and Methods

2.1. Chemicals

Sudan III ($C_{22}H_{16}N_{40}$) was purchased from OXFORD lab chem. All chemicals were of the highest analytical grade.

2.2. Experimental Animals and Design

Male healthy rats (n=18) weighting 100-120 g in average obtained from the laboratory animals resource center, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt were used in this experiment, kept to acclimatize for one week before the beginning of the

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experiment. Rats were fed a standard diet and had free access to water. Animals are randomly divided into three groups. First group (n=6) were left as control and administered normal saline orally. The remaining two groups (each consists of 6 rats), were orally administrated either Sudan III 125 mg/kg body weight (1/40 LD_{50}) in plant oil vehicle as a low dose group, or Sudan III 250 mg/kg body weight (1/20 LD_{50}) in plant oil vehicle as a high dose group for consecutive 45 days. On day 45 of the experiment, rats were sacrificed under anesthesia and blood collected from heart in plain polystyrene microtubes for biochemical analysis, while liver tissue samples were rapidly collected, washed with normal saline solution (0.9% NaCl in distilled water) and placed in Eppendorf tubes at –80 °C for evaluation of antioxidants biomarkers and genotoxicity assessment by capillary electrophoresis.

All the protocols regarding the study were conducted according to the ethical guidelines for the use of animals in laboratory experiments of the Faculty of Veterinary Medicine, Suez Canal University, Egypt.

2.3. Serum and Tissue Preparations

Blood samples were left to clot at room temperature, centrifuged at 1,000 x*g* for 10 min. at 4 °C then serum was harvested and stored at -20 °C until analysis. The liver tissues were homogenized in tenfold volume of physiological saline solution by using a homogenizer (Ultra-Turrax T25, IKA; Werke; Germany). The homogenates were centrifuged at 10,000 x*g* for 1 h to remove debris. Clear upper supernatant was taken for oxidative stress biomarkers analysis and DNA capillary electrophoresis.

2.4. Biochemical Analysis of Glucose, Liver Enzymes and Proteins

Glucose was measured by enzymatic, colorimetric method (GOD/PAP) which is intended for the in vitro quantitative determination of glucose in serum (Vitro Scient assay kit, www. vitroscient.com, Egypt). Reagent is intended for the in vitro quantitative determination of Aspartate Aminotransferase (GOT) in serum (Fisher Diagnostics, Fisher Scientific Company, Middletown, VA, USA). Fisher Diagnostics kit is intended for the in vitro quantitative determination of GPT in serum. Albumin is quantitatively the major single contributor to the plasma total protein. The albumin was measured by BCG method which is intended for the in vitro quantitative determination of Albumin in serum (Fisher Diagnostics, Fisher Scientific Company, Middletown, VA, USA). Total Protein Reagent (Biuret method) is intended for the in vitro quantitative determination of Total Protein (Fisher Diagnostics, Fisher Scientific Company, Middletown, VA, USA).

2.5. Analysis of Lipid Profile

Serum levels of high-density lipoprotein (HDL), total cholesterol (TC) and triglycerides (Trig) were measured using Vitro Scient assay kit (www. vitroscient.com, Egypt).

2.6. Biochemical Analysis of Liver Oxidative Stress

SOD was done according to Cayman's super dismutase assay kit (Cayman chemical, Michigan, USA), that utilize tetrazolium salt for detection of superoxide radicals generated by xanthine and hypoxanthine. Cayman's catalase assay kit utilizes the peroxidatic function of CAT for determination of enzyme activity (Cayman chemical, Michigan, USA). Malondialdehyde (MDA) is one of many low molecular weight end-products of lipid hydoperoxide decomposition and is the most often measured as an index of lipid Peroxidation. The MDA content in liver homogenate was assayed with The NWK-MDA01 assay which is based on the reaction of MDA with thiobarbituric acid (TBA); forming an MDA-TBA2 adduct that absorbs strongly at 532 nm (Northwest Life Science Specialties, Vancouver, WA 98662). Cayman's GSH assay utilizes a carefully optimized enzymatic recycling method using glutathione reductase for the quantitation of GSH (Cayman chemical, Michigan, USA).

2.7. DNA Extraction and Capillary Electrophoresis QIAxcel

DNA extraction was done at Biotechnology Center, Faculty of Agriculture, Suez Canal University using Genomic DNA preparation kit (Jena Bioscience), DNA fragments were analyzed in the automatic multicapillary electrophoresis QIAxcel system. Isolated DNA was placed in the instrument sample tray, 10 ml of the DNA samples were automatically injected into the capillary channel and subjected to electrophoresis according to the protocol AH420 (method separation time: 420 s, method injection time: 20 s, method separation voltage: 5.0 kV and method injection voltage: 2.0 kV) of the QIAxcel DNA Screening Kit.

2.8. Statistical Analysis

Data of the present study were statistically analyzed using One-way Analysis of Variance (ANOVA), followed by means separation for multiple means comparison using Duncan's multiple range test (SPSS, version 20). Results were considered significant at probability level ≤ 0.05.

3. Results

3.1 Glucose, Liver Enzymes and Protein

Serum glucose, GOT and GPT activities were increased significantly ($P<0.05$) after the administration of low and high doses of Sudan III. Minor changes in the levels of Alb concentrations was observed after administration of low and high doses of Sudan III compared to that in control untreated rats. There was no any significant difference in albumin level between treated and control groups. The total protein level was markedly ($P<0.05$) decreased in treated rats with low and high doses of Sudan III **(Table 1).**

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Table 1: Effect of Oral Administration of Sudan III on Serum Glucose, Liver Transaminases, Albumin and Total Protein (Means ± SE)

Rats	Glu (mg/dl)	GOT (µ/l)	GPT (µ/l)	Alb (gm/dl)	TP (gm/dl)
Control	84.2 ^{c} ±1.7	37.3 ^{b} ± 1.2	62.3 ^{c} ± 1.1	4.9 ^{a} ±0.2	8.0 ^{a} ±0.2
Low Dose	90.8 ^{b} ± 2.2	60.2 ^{a} ± 1.8	66.5 ^{b} ± 0.7	4.6 ^{a} ± 0.2	6.3 ^{c} ± 0.2
High Dose	104.6 ^{a} ± 5.5	59.5 ^{a} ± 3.5	76.8 ^{a} ± 1.4	4.8 ^{a} ± 0.3	7.1 ^{b} ± 0.1

Glu: Glucose; GOT: Glutamic Oxaloacetic Transaminases; GPT: Glutamate-Pyruvate Transaminases; Alb: Albumin; Tp: Total Protein. Values are expressed as Mean ± SE, values in each column with different superscripts (a, b, c) are significantly different at $P \leq 0.05$ and highly significant different at $P \leq 0.01$. n = 6 rats/group.

3.2. Lipid Profile

Serum levels of Chol, Trig and LDL were significantly ($P<0.05$) increased in rats received low and high doses of Sudan III compared to that in control untreated rats. Serum levels of HDL were significantly ($P<0.05$) decreased in rats received low and high doses of Sudan III compared to that in control untreated rats **(Table 2).**

Table 2: Effect of Oral Administration of Sudan III on Serum Lipid Profile (Means ± SE)

Rats	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	112.5 ^{c} ±3.7	78.2 ^{c} ±1.0	32.8 ^{a} ±0.7	67.5 ^{c} ±1.0
Low Dose	120.5 ^{b} ±4.2	85.2 ^{b} ±2.3	31.7 ^{b} ±0.4	77.7 ^{b} ±1.7
High Dose	154.1 ^{a} ±7.7	154.5 ^{a} ±1.1	25.7 ^{c} ±0.6	87.3 ^{a} ±4.3

Chol: Cholesterol; Trig: Triglycerides; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein. Values are expressed as Mean ± SE, values in each column with different superscripts (a, b, c) are significantly different at $P \leq 0.05$ and highly significant different at $P \leq 0.01$. n = 6 rats/group.

3.3. Oxidative Stress Biomarkers

GSH, SOD and CAT were significantly ($P<0.05$) decreased in liver tissues of rats received low and high doses of Sudan III compared to that in control untreated rats. While MDA level was increased significantly ($P<0.05$) in liver tissue of rats received low and high Sudan III doses compared to control untreated rats **(Table 3).**

Table 3: Effect of Oral Administration of Sudan III on Antioxidant Enzymes (Means ±SE)

Rats	GSH (nmol/ml)	CAT (nmol/ml)	SOD (µ/ml)	MDA (nmol/ml)
Control	56.7 ^{a} ±0.7	66.1 ^{a} ±0.8	46.7^{a} ±1.0	10.7 ^{c} ±0.1
Low Dose	45.2 ^{b} ±0.4	57.1 ^{b} ±0.5	34.2^{b} ±0.4	14.4 ^{b} ±0.2
High Dose	39.5 ^{c} ±0.2	38.2 ^{c} ±0.4	28.1^{c} ± 0.5	19.0 ^{a} ±0.3

GSH: Reduced Glutathione; CAT: Catalase; SOD: Super Oxide Dismutase; MDA: Malondialdehyde. Values are expressed as Mean ± SE, values in each column with different superscripts (a, b, c) are significantly different at $P \leq 0.05$ and highly significant different at $P \leq 0.01$. n = 6 rats/group.

3.4. DNA Capillary Electrophoresis

DNA undergoes fragmentation with appearance of new bands in both doses after oral administration of Sudan III for 45 days as shown in

Table 4 and Figure 1. (Samples Key: DNA ladder, L1, L2, L3, H1, H2, H3, C).

Table 4: Comparative DNA Size (bp) Among Different Study Samples.

Samples	*DNA size (bp)*
A1	1,525,075,010,002,000,000,000,000,000,000,000,000
A2	15, 57, 83,100,143,944, 10000
A3	15,236, 10000
A4	15,516, 10000
A5	15, 166, 207, 10000
A6	15, 229, 10000
A7	15, 53, 74, 84, 126, 10000
A8	1,510,000

A1: DNA ladder; A2:low dose1; A3:low dose2; A4:low dose3; A5:high dose1; A6:high dose2; A7:high dose3;A8: control

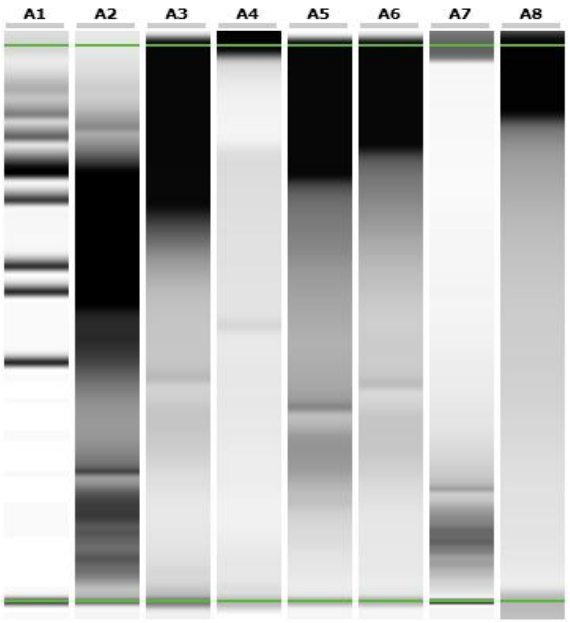

Figure 1: Showed appearance of new bands of DNA in both Sudan III doses compared to control sample.

4. Discussion

Treatment with Sudan III orally for 45 days resulted in significant increase in GOT and GPT activities in low and high doses. This increase of liver enzymes may be attributed to the hepatocellular damage resulting from chemical toxicity, where these enzymes levels showed an intimate relationship to cell necrosis and/or increased cell membrane

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permeability that leads to discharge of the enzyme to blood stream and increase of its level in serum [13]. Our results are coincident with the study of **Salih et al., (2014)** [14] who detected a significant increase in liver enzymes (GOT and GPT) activities in a dose-dependent manner after oral or subcutaneous administration of hair dye (PPD is the main constituent in hair dye and is a derivative of paranitro-aniline). While, the marked decrease in total protein level was markedly decreased in treated rats with low and high doses of Sudan III may attributed to the cellular damage caused by Sudan III metabolites or due to oxidative stress [15]. In relation to the other studies, the present findings are in agreement with other researchers **Saito et al., (1990)** [16] who find that administration of hair dye to rats lead a significant decrease in total proteins. These alterations in hepatic enzymes and proteins might indicate hepatic toxicity due to different doses of Sudan III.

Serum levels of cholesterol, Trig and LDL were considerably increased in rats received high and low doses of Sudan III. In contrary to the present findings, **Salih et al., (2014)** [14] reported that low cholesterol level observed in his study is usually associated with hepatocellular damage, hepatic lipidosis and obstructive liver diseases [17].

In the present study, administration of low and high doses of Sudan III for 45 days caused significant decreased in antioxidant parameters (GSH, CAT and SOD) with significant increase of MDA in treated animals compared to untreated ones, and this alterations may caused by oxidative stress and formation of reactive oxygen species (ROS) in hepatic tissue [18]. GSH plays a crucial role in protecting the cells against oxidative damage that originates from possible toxic compounds in food. Inline to our present results, in vivo and in vitro studies showed that aromatic amines were possibly responsible for the toxic effects of azo dyes and significantly decreased GSH levels [19]. Moreover, **Amin et al., (2010)** [3] found that rats who received high (500 mg/kg BW) doses of tartrazine, the GSH levels was declined in liver homogenates significantly, as compared to control. **Siraki et al., (2002)** [20] found that incubation of hepatocytes with aromatic amines lead to depletion in hepatocytes GSH. Depletion of GSH puts cells at oxidative risks. The increase in oxidative stress biomarkers in this study in contrary to the previous research might be due to the different type of dye used and this may need more clarification through more detailed experiment.

The imbalance between ROS and the antioxidant capacity causes the oxidative stress which may cause severe damage to several biomolecules including DNA [21]. And this came in agreement with our results at which DNA showed fragmentation and appearance of new bands in liver tissue samples after oral administration of low and high doses of Sudan III for 45 days.
Because of the large number of azo dyes and their extensive usage, research on their adverse effect on animal health is important. In the present study, we evaluated different parameters in male rats after they exposed to different oral doses of Sudan III dyes. It is suitable for any extracted DNA of sufficient quality, al-lows rapid analysis of a large number of samples.

Hassan and Yassein, (1998) [22] showed that, changes in DNA fingerprint (i.e. band patterns) observed reflect DNA alterations in genome from single base changes (point mutations) to complex chromosomal rearrangements and that DNA fingerprinting offers a useful biomarker assay in assessment of genotoxicity. Appearance of new PCR products occurred because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations (resulting in new annealing events), and/or large deletions (bringing two pre-existing annealing sites closer), and/or homologous recombination (two sequences that match the sequence of the primer). Apparent bands may also be the results of genomic template instability related to the level of DNA damage, the efficiency of DNA re-pair and replication with a strong relation to oxidative stress.

5. Conclusion

Sudan III adversely altered the hepatic enzymes, lipid and oxidative stress biomarkers in liver of rats not only at high doses but also at low doses with apparent genotoxic effects on DNA. This is highlight the importance and risk of Azo dye present in animal and human feed.

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