

PROTECTIVE EFFECT OF GRAPE SEEDS EXTRACT AGAINST SODIUM NITRITE-INDUCED TOXICITY AND OXIDATIVE STRESS IN ALBINO RATS

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ABSTRACT

Sodium nitrite (NaNO_2) is important antimicrobial, flavoring, coloring and preservative agent in meat and fish product. However, nitrite may cause methemoglobinemia and other illness, and may react with certain amines to form carcinogenic nitrosamines. The study aimed to evaluate the efficacy of grape seeds extract (GSE) as a potential novel and useful strategy for the modulation of oxidative stress and toxicity induced by NaNO_2 in male rats. Sodium nitrite was used at two dose levels 1 and 2g/L and orally administrated to rats in drinking water for 4 and 8 weeks. Ingestion of NaNO_2 , resulted in significant time and dose-dependent reduction in RBCs count, WBCs count, hemoglobin (Hb) content, serum albumin and total protein (TP), plasma reduced glutathione (GSH), glutathione -S- transferase (GST), superoxide dismutase (SOD) contents in the treated rats. whereas, blood methemoglobin (MetHb), serum alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), creatinine, urea, triglycerides, total cholesterol and lipid peroxidation product (malondialdehyde, MDA) were significantly increased in dose and time dependant manner after implication of this preservative. Hyperglycemia was observed in NaNO_2 ingested rats. Moreover, histopathological examination of NaNO_2 treated rats at the end of experiment revealed marked alterations in liver, kidneys, brain, heart, lung and testes. The degree of severity of noted lesions increased with increase of administrated dose. Fortunately, synergistic administration of ethanolic GSE (150 mg/kg body weight (B.W)/day) and NaNO_2 resulted in significant amelioration of negative effect of NaNO_2 on the investigated biochemical parameters and improvement of investigated antioxidant especially with low NaNO_2 dose and longer time. Moreover, GSE administration was able to protect most of tested organs against low NaNO_2 dose and minimize the damage induced by high NaNO_2 dose. It was concluded that GSE supplementation could be considered as a promising antioxidant in reducing oxidative stress and toxicity of NaNO_2 .

Key words: Sodium nitrite, Grape seeds extract, Oxidative stress, Biochemical parameters, and Pathological examination.

INTRODUCTION

Sodium nitrite (NaNO_2) is an inorganic salt used in the manufacture of dyes and considered as one of the important food additives for fishes and meat that has been used in vivo and in vitro experiments for decades to prevent growth of *Clostridium botulinum*, the bacteria which causes botulism (Luca *et al.*, 1987). Also, it is used in drug industries and in medicine as antidote for cyanide poisoning (Filvo *et al.*, 1993). Nitrites occupy a unique position in human toxicology. They ubiquitous in the environment and can be formed from nitrogenous compound by microorganisms present in the soil, water, saliva and gastrointestinal tract (Chow and Hong, 2002). Moreover nitrite (NO_2^-) is a contaminant of

some water supplies, extensive use of nitrogenous fertilizer, decomposition of plants and sewage wastes, followed by leaching of nitrates into ground water (**Wolff and Wasserman, 1972**). Nitrite is widely consumed from the diet by animals and humans. However the largest contribution to exposure results from the in vivo conversion of exogenously derived nitrate to nitrite. Because of its potential to cause methaemoglobin (MetHb) formation at excessive levels of intake, nitrite is regulated in feed and water as an undesirable substance. Forages and contaminated water have been shown to contain high levels of nitrate and represent the largest contributor to nitrite exposure for food-producing animals (**Cockburn et al., 2013**). A human health risk assessment was also carried out taking into account all direct and indirect sources of nitrite from the human diet, including carry-over of nitrite in animal-based products such as milk, eggs and meat products. The FAO/WHO, Joint Expert Committee on Food Additive (JECFA) established acceptable daily intakes (ADIs) of 0-0.07 mg kg⁻¹ body weight for NaNO₂ (expressed as nitrite ion) (**WHO, 2003**). In order to protect animal and human health, the European Union, directive, 2002/32/EC on undesirable substances in animal feed, restricts the maximum content of nitrite in complete feeding stuff (with a moisture content of 12%) for livestock excluding birds and aquarium fish to 15 mg/kg, and the maximum content of fish meal to 60 mg/kg (**EC, 2002**).

The hazardous effect of NaNO₂ is derived from the reaction of nitrite with amines and amides to produce nitrosamines and nitrosamides, respectively. The toxic effects of nitrates and nitrites are well documented in mammalians, including impairment of reproductive function, hepatotoxicity and methaemoglobinemia, dysregulation of inflammatory responses and tissue injury, growth retardation and endocrine disturbance (**Jahries et al., 1986**). Nitrite is, however, also a natural constituent in the body, it is a potential nitric oxide (NO) donor and recent research has suggested that nitrite has important biological functions at low concentrations (**Jensen, 2007**). Nitric oxide free radical (NO[•]) is generated from the nitrite by non-enzymatic method and O^{•-} formation is found in acidic environments such as the stomach and oral cavity (**Lundberg et al., 1994 and McKnight et al., 1997**). Peroxy nitrite (ONOO⁻), the reaction product formed between NO[•] and superoxide plays a critical role in the induction of inflammatory reaction and apoptosis. O^{•-} is also associated with tumor promotion and/or progression (**Radi et al., 1991**). Anyway, nitrite when present at high concentration in blood, it can react with iron (III) of the hemoglobin, forming MetHb which has no oxygen-carrying ability. This fatal disease is called methemoglobinemia (**Zatar et al., 1999 and Sanchez-Echaniz et al., 2001**). The use of permitted colors has raised concern that they might be used in excess of the saturatory limit (100 mg kg⁻¹) or in foods in which they were not permitted (**Rao and Bhat, 2003**).

An interest in natural antioxidants, especially of vegetal origin, has greatly increased in recent years. Natural antioxidants can protect the human body from free radicals that may lead to the aging process and cause some chronic diseases including cancer, cardiovascular diseases and cataract as well as retard lipid oxidative rancidity in foods (**Craig and Beck, 1999 and Lai et al., 2001**).

Grape, one of the world's largest fruit crops, with more than 60 million tons is cultivated mainly as *Vitis vinifera* for wine production (**Amico et al., 2004**). It is estimated that around 13% of the total weight of grapes used for the wine and juice making results in grape pomace, which is a byproduct in this process (**Torres et al., 2002**). One way of using the potential of the grape pomace is the isolation of seeds and extraction of the polyphenols, since the total extractable phenolics in grape are a maximum 10% in pulp, 60 to 70 % in the seeds and 28 to 35% in the skin (**Shi et al., 2003**). Phenolic compounds from grape seeds, as other phenolic compounds have pharmacological and nutraceutical benefits both antiviral and antimutagenic (**Saito et al., 1998**).

Grape seed extract (GSE) is a natural extract from the seeds of *Vitis vinifera*. A multitude of flavonoids are contained in GSE. The most abundant of these are the proanthocyanidins, which are oligomers of monomeric flavan-3-ol units linked by carbon-carbon bonds. The major flavan-3-ols identified in GSE are (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-O-gallate (**Santos-Buelga *et al.*, 1995 and Waterhouse and Walzem, 1998**).

It has proved that, free radical scavenging capacity of GSE is 20 times more effective than vitamin E and 50 times more effective than vitamin C. GSE is beneficial in many areas of health because of its antioxidant effect to bond with collagen, promoting youthful skin, cell health, elasticity, and flexibility. Moreover, proanthocyanidins help to protect the body from sun damage, to improve vision, to improve flexibility in joints, arteries, and body tissues such as the heart, and to improve blood circulation by strengthening capillaries, arteries, and veins. The most abundant phenolic compounds isolated from grape seed are catechins, epicatechin, procyanidin, and some dimers and trimers (**Shi *et al.*, 2003**).

Here, we investigated the role of GSE in ameliorating NaNO₂-induced abnormalities in the hematological and biochemical parameters and injury of body organs associated with oxidative stress in male albino rats.

MATERIALS AND METHODS

Chemicals

NaNO₂ were obtained from Sigma Chemical Co. (St Louis, MO, USA) and all other chemicals and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany), unless stated otherwise. Kits used for the estimation of GSH, GST, SOD and MDA were purchased from Biodiagnostic, France.

Plant materials

Dried grape seeds were bought from Harrase Company Giza, Egypt. The dried seeds were ground to a fine powder with electric blender, and kept at 4 °C until further use.

Hydroalcoholic extract of dried grape seeds

About 500 g of the powder was mixed with 1000 ml of 70% ethanol in distilled water and kept for 3 days at room temperature. The extract was then filtered through a Buchner funnel. The filtrate was evaporated at 45 °C in a rotary evaporator to concentrate the solution, then lyophilized in order to obtain the dry extract and stored at 4 °C until use (**Hemmati *et al.*, 2008**).

Enough amounts of the dried extract were suspended in water and administered orally by gavages to rats for 8 weeks (150 mg/kg/day).

Animal and treatment

Thirty-six male albino rats weighing about 130-140g (provided by the Laboratory Animal Center, Faculty of Veterinary Medicine, Cairo University) were housed in stainless steel cages in animal house in Regional Center for Food and Feed, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt under controlled light and temperature conditions (12-h light/dark cycle, 22±2^oC). During the acclimation period (1 week) and experimental period (8 weeks), the normal basal diet was supplied *ad libitum*. The basal diet consisted of casein 20%, corn oil 10%, cellulose (wooden fibers) 5%, salt mixture 4%, vitamin mixture 1% and starch 60% (**Lane – Peter and Pearson, 1971**). Rats were divided

into six groups and treated for 8 week as follow: G1(normal control): Rats received purified tap water; G2: Rats received purified tap water + GSE (150 mg/kg B.W/day, orally) (**Hemmati et al., 2008**); G3 (1g/L NaNO₂): Rats provided with purified drinking water containing 1g/L NaNO₂ (**Mirvish et al., 1972**); G4 (2g/L NaNO₂): Rats provided with purified drinking water containing 2g/L NaNO₂ (**Subramaniyan et al., 2011**); G5 (GSE+1g/L NaNO₂): Rats provided with purified drinking water containing 1g/L NaNO₂+ GSE (150 mg/kg B.W/day, orally) and G6 (GSE+2g/L NaNO₂): Rats provided with purified drinking water containing 2g/L NaNO₂+ GSE (150 mg/kg B.W/day, orally).

At the 4th week and 8th week of the treatment period, blood samples were collected from the retro-orbital venous plexus under carbon dioxide anesthesia into three tubes. Two contain heparin, one used as fresh as possible for hematological parameters and other to obtain plasma. The third tube contains no anticoagulant to obtain serum. Plasma and serum separated and stored at -20°C until analysis. Rats were scarified at the end of experiment and livers, kidneys, brains, hearts, lungs and testes were collected and kept for histological examination.

Hematological analysis

Whole blood was analyzed for: Hb, RBCs and WBCs using an animal blood veterinary counter (ABC vet user Manual, RABOSIA Inc. A, France).

Estimation of methemoglobin (MetHb)

Aliquots of fresh blood were hemolyzed in water (1:5.5, v/v). After 3 min, phosphate buffer was added, and the samples were centrifuged at 14,000 rpm for 15 min. Measurements of MetHb in hemolysates was based on the absorbance of MetHb spectrophotometrically at 630 nm. Addition of cyanide eliminates the contribution of methemoglobin to the absorbance at 630 nm. The absorbance of the hemolysate at 630 nm was first determined in the absence of cyanide. To 100 µl of the hemolysate was added 50 µl of a neutralized cyanide solution containing 5.3% w/v sodium cyanide and 5.6% v/v acetic acid, and the absorbance of the cyanide-treated sample was reread at 630 nm. The absorbance in the absence of cyanide minus that in the presence of cyanide is a measure of the conversion of MetHb in the sample to cyanomethemoglobin which is proportional to concentration of MetHb (**Kohn et al., 2002**).

Biochemical assays

The ACE Alera Clinical Chemistry System Automatic Analyzer (Alfa Wasserman Corporation) was employed to measure the following parameters: ALT, AST, ALP, GGT, TP, albumin, creatinine, urea, total cholesterol, triglycerides and glucose in serum.

Estimation of antioxidant profile

The extent of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) which react with malondialdehyde (MDA) in acidic medium was measured in serum calorimetrically at 534 nm according to the method of **Onkawa et al. (1979)**. Plasma GSH was estimated calorimetrically at 405 nm based on the reduction of 5, 5 dithiobis (2-nitrobenzoic acid) (DTNB) with GSH to produce yellow compound which directly proportional to GSH concentration (**Beutler et al., 1963**). Plasma GST was determined spectrophotometrically by measuring the conjugation of 1-chloro-2,4- dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase absorbance at 340 nm. The rate of increase is directly proportional to the GST activity (**Habig et al., 1974**). Plasma SOD activity was estimated spectrophotometrically at 560 nm based on the method described by **Nishikimi et al. (1972)** which relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye.

Histopathological studies:

Livers, kidneys, brains, hearts, lungs and testes from each group were fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Sections of 4-6 μm thickness were prepared and stained with Haematoxylin and Eosin. Sections were then examined for histopathological findings (Banchroft *et al.*, 1996).

Statistical analysis:

Statistical analysis of the obtained data was done using the least significant difference test (LSD) at the 5% level of probability as outlined by **Snedecor and Cochran (1980)**. Using the Duncan test institute program used a computer in the statistical analysis.

RESULTS

In this study, the data as shown in tables (1-7) recorded that, the administration of GSE alone (G2) did not induce any obvious changes in all examined parameters along experimental periods (comparing to control (G1) ($P < 0.05$)).

Hematological analysis:

Results presented in table (1) demonstrated that NaNO_2 administration for period of 4 and 8 weeks at both 2 dose levels (rats of G3 and G4) resulted in a significant dose-dependent reduction ($P < 0.05$) of blood WBCs, RBCs count and Hb concentration. However, supplementation of NaNO_2 -intoxicated rats with GSE (G5 and G6) ameliorated the adverse effect of both NaNO_2 doses on hematological parameters especially 1 g/L NaNO_2 and at 8th week of experiment. Whereas, GSE administration restore RBCs count to normal in blood of rats treated with 1g/L NaNO_2 at 8th week of treatment (comparing with control ($P < 0.05$)).

Methemoglobin concentration:

MetHb concentration was significantly elevated (corresponding to control group ($P < 0.05$)) in time and dose dependant manner in blood of NaNO_2 -treated rats of G3 and G4 as shown in table (1). Meanwhile, co-administration of GSE with 1g and 2 g/L of NaNO_2 (G5 and G6 respectively) resulted in attenuation of MetHb formation (compared to corresponding control ($P < 0.05$)) in all experimental periods. Moreover, GSE was able to block MetHb formation in blood of 1g NaNO_2 - treated rats and restore it to normal level comparing with control group (G1).

Liver function parameters

Table (2) show the effect of GSE administration on liver function in serum of rats treated with NaNO_2 . Analysis of variance indicated that there was a significant dose dependant-increase in ALT, AST, ALP and GGT activities, whereas, a significant dose dependant-decrease in TP and albumin concentrations was detected in rats treated with both NaNO_2 doses for 4 and 8 weeks. However, GSE administration in NaNO_2 -treated rats ameliorated the NaNO_2 adverse effect as evidenced by a significant decrease of ALT, AST, ALP and GGT activities, and increase in TP and albumin concentration especially at low NaNO_2 dose and at 8th week. The activity of GGT in serum of rats co-administrated with GSE and NaNO_2 was return to normal at 8th week of treatment (compared with control ($P < 0.05$)).

Table (1): Hematological parameters and methemoglobin concentration in blood of control and treated rats during experimental periods (means \pm SE).

Parameters Groups	WBCs ($10^3/\mu\text{L}$)		RBCs ($10^6/\mu\text{L}$)		Hb (g/dL)		MetHb (g%)	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
G1 Control	20.33 $\pm 0.33^a$	21.33 $\pm 0.33^a$	11.33 $\pm 0.33^a$	11.67 $\pm 0.88^a$	16.51 $\pm 0.29^a$	17.36 $\pm 0.19^a$	0.34 $\pm 0.01^e$	0.34 $\pm 0.01^{de}$
G2 GSE	21.00 $\pm 0.58^a$	21.67 $\pm 0.67^a$	10.97 $\pm 0.63^a$	11.64 $\pm 0.20^a$	16.35 $\pm 0.26^a$	17.25 $\pm 0.15^a$	0.34 $\pm 0.01^e$	0.33 $\pm 0.01^e$
G3 1g/L NaNO ₂	16.33 $\pm 0.33^c$	16.33 $\pm 0.33^c$	8.39 $\pm 0.16^c$	7.87 $\pm 0.18^c$	14.37 $\pm 0.27^c$	13.29 $\pm 0.14^d$	0.96 $\pm 0.01^c$	1.62 $\pm 0.02^b$
G4 2g/L NaNO ₂	14.33 $\pm 0.33^d$	13.00 $\pm 0.58^d$	6.88 $\pm 0.20^d$	6.00 $\pm 0.07^d$	13.23 $\pm 0.15^d$	11.14 $\pm 0.25^e$	1.52 $\pm 0.01^a$	2.43 $\pm 0.04^a$
G5 GSE+ 1g/L NaNO ₂	18.67 $\pm 0.33^b$	19.67 $\pm 0.33^b$	9.59 $\pm 0.10^b$	10.90 $\pm 0.40^a$	15.24 $\pm 0.12^b$	16.61 $\pm 0.12^b$	0.78 $\pm 0.02^d$	0.39 $\pm 0.01^d$
G6 GSE+ 2g/L NaNO ₂	16.67 $\pm 0.33^c$	17.67 $\pm 0.33^c$	8.36 $\pm 0.09^c$	9.26 $\pm 0.13^b$	14.08 $\pm 0.08^c$	15.67 $\pm 0.19^c$	1.19 $\pm 0.01^b$	0.99 $\pm 0.01^c$
LSD _{0.05}	1.186	1.390	0.716	1.048	0.646	0.549	0.039	0.054

Within the same column, various superscript letters indicate significant differences (Duncan, $P < 0.05$).

Kidney function parameters:

The kidney function parameters results summarized in table (3) revealed that there is a significant dose-dependent increase ($P < 0.05$) in creatinine and urea concentrations in serum of NaNO₂-treated rats (G3 and G4) while GSE intake caused desperation of this increase, in addition GSE administration restore creatinine in serum of 1g NaNO₂-treated rats (G5) to normal and restore urea level in serum of both groups of NaNO₂-treated rats (G5 and G6) to normal at 8th week of treatment (compared to corresponding control ($P < 0.05$)).

Lipid profile parameters:

The data in table (4) shows that NaNO₂ induced significant dose-dependent increase in serum total cholesterol and triglycerides concentrations comparing with control ($P < 0.05$) at all experimental periods. Simultaneous administrations of GSE and NaNO₂ in G5 and G6 rats resulted in significant reduction of increased total cholesterol and triglycerides concentrations and restore cholesterol level at 4th week of treatment and triglycerides levels along experimental period to normal in serum of 1g NaNO₂-treated rats comparing with control ($P < 0.05$).

Table (2): Liver functions parameters in serum of control and treated rats during experimental periods (means \pm SE).

Parameters Groups	ALT (U/L)		AST (U/L)		ALP (U/L)		GGT (U/L)		TP (g/dL)		Albumin (g/dL)	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
G1 Control	40.00 $\pm 0.58^d$	42.00 $\pm 0.58^e$	93.33 $\pm 0.88^e$	95.00 $\pm 0.24^e$	359.00 $\pm 14.57^e$	412.00 $\pm 4.36^e$	2.33 $\pm 0.33^d$	2.67 $\pm 0.33^d$	6.76 $\pm 0.07^a$	6.86 $\pm 0.04^a$	4.14 $\pm 0.06^a$	4.19 $\pm 0.02^a$
G2 GSE	41.00 $\pm 1.53^d$	41.67 $\pm 1.20^c$	92.90 $\pm 0.61^e$	94.55 $\pm 0.55^e$	367.00 $\pm 14.73^e$	411.00 $\pm 6.66^e$	2.33 $\pm 0.33^d$	2.67 $\pm 0.33^d$	6.74 $\pm 0.05^a$	6.88 $\pm 0.06^a$	4.11 $\pm 0.05^a$	4.22 $\pm 0.02^a$
G3 1g/L NaNO ₂	55.33 $\pm 0.88^b$	62.67 $\pm 0.88^b$	109.00 $\pm 0.31^c$	128.00 $\pm 0.58^b$	590.33 $\pm 3.84^b$	627.67 $\pm 9.06^b$	5.33 $\pm 0.33^b$	6.33 $\pm 0.33^b$	5.69 $\pm 0.14^c$	4.93 $\pm 0.08^d$	3.32 $\pm 0.10^c$	3.07 $\pm 0.03^d$
G4 2g/L NaNO ₂	67.00 $\pm 1.53^a$	81.00 $\pm 1.15^a$	121.67 $\pm 1.20^a$	140.67 $\pm 1.20^a$	623.33 $\pm 5.70^a$	706.33 $\pm 3.84^a$	6.67 $\pm 0.33^a$	8.33 $\pm 0.33^a$	4.71 $\pm 0.10^d$	4.07 $\pm 0.09^e$	3.10 $\pm 0.03^d$	2.84 $\pm 0.03^e$
G5 GSE+ 1g/L NaNO ₂	50.00 $\pm 0.58^c$	45.00 $\pm 0.58^d$	105.00 $\pm 0.57^d$	99.19 $\pm 0.33^d$	489.67 $\pm 2.03^d$	436.33 $\pm 3.28^d$	3.67 $\pm 0.33^c$	3.33 $\pm 0.33^d$	6.06 $\pm 0.07^b$	6.45 $\pm 0.04^b$	3.71 $\pm 0.03^b$	4.04 $\pm 0.06^b$
G6 GSE+ 2g/L NaNO ₂	57.67 $\pm 1.45^b$	51.00 $\pm 0.58^c$	112.66 $\pm 0.67^b$	105.33 $\pm 0.34^c$	555.33 $\pm 12.20^c$	510.67 $\pm 7.88^c$	5.67 $\pm 0.33^b$	4.67 $\pm 0.33^c$	5.42 $\pm 0.08^c$	6.02 $\pm 0.13^c$	3.48 $\pm 0.01^c$	3.93 $\pm 0.04^c$
LSD _{0.05}	3.607	2.685	2.340	1.856	31.560	19.206	0.938	1.027	0.279	0.239	0.168	0.108

Within the same column, various superscript letters indicate significant differences (Duncan, $P < 0.05$).

Table (3): Kidney functions parameters in serum of control and treated rats during experimental periods (means \pm SE).

Parameters Groups	Creatinine (mg/dL)		Urea (mg/dL)	
	4 weeks	8 weeks	4 weeks	8 weeks
G1 Control	0.57 \pm 0.03 ^c	0.58 \pm 0.01 ^b	36.33 \pm 0.88 ^d	37.67 \pm 0.88 ^c
G2 GSE	0.58 \pm 0.02 ^c	0.58 \pm 0.01 ^b	36.00 \pm 1.15 ^d	37.00 \pm 1.53 ^c
G3 1g/L NaNO ₂	0.73 \pm 0.03 ^b	0.80 \pm 0.06 ^a	45.00 \pm 0.58 ^b	59.00 \pm 1.15 ^b
G4 2g/L NaNO ₂	0.90 \pm 0.06 ^a	0.93 \pm 0.09 ^a	55.33 \pm 1.76 ^a	69.00 \pm 0.58 ^a
G5 GSE+ 1g/L NaNO ₂	0.73 \pm 0.03 ^b	0.63 \pm 0.02 ^b	40.00 \pm 0.58 ^c	38.00 \pm 0.58 ^c
G6 GSE+ 2g/L NaNO ₂	0.87 \pm 0.02 ^a	0.80 \pm 0.05 ^a	42.67 \pm 1.20 ^c	40.00 \pm 1.15 ^c
LSD _{0.05}	0.107	0.149	3.406	3.193

Within the same column, various superscript letters indicate significant differences (Duncan, $P < 0.05$).

Table (4): Total cholesterol and triglycerides concentrations in serum of control and treated rats during experimental periods (means \pm SE).

Parameters Groups	Cholesterol (mg/dL)		Triglycerides (mg/dL)	
	4 weeks	8 weeks	4 weeks	8 weeks
G1 Control	37.67 \pm 1.20 ^d	38.33 \pm 1.20 ^d	106.00 \pm 1.15 ^c	107.00 \pm 1.15 ^d
G2 GSE	37.00 \pm 1.15 ^d	37.67 \pm 0.88 ^d	105.00 \pm 1.73 ^c	105.33 \pm 1.76 ^d
G3 1g/L NaNO ₂	50.33 \pm 0.88 ^b	70.33 \pm 0.88 ^b	127.00 \pm 1.73 ^b	138.33 \pm 1.45 ^b
G4 2g/L NaNO ₂	61.67 \pm 1.45 ^a	90.00 \pm 1.15 ^a	136.00 \pm 1.53 ^a	146.67 \pm 1.86 ^a
G5 GSE+ 1g/L NaNO ₂	45.33 \pm 1.20 ^c	40.00 \pm 0.58 ^d	108.67 \pm 1.20 ^c	107.67 \pm 0.88 ^d
G6 GSE+ 2g/L NaNO ₂	52.00 \pm 1.15 ^b	47.33 \pm 0.88 ^c	124.00 \pm 2.31 ^b	122.33 \pm 1.20 ^c
LSD _{0.05}	3.655	2.935	5.10	4.397

Within the same column, various superscript letters indicate significant differences (Duncan, $P < 0.05$).

Glucose concentration

As shown in table (5) NaNO₂ treatment induced significant dose-dependent increase in serum glucose level in respect to control group ($P < 0.05$). The administration of GSE significantly alleviated the hyperglycemic effect of NaNO₂. Such effect was more pronounced with 1g NaNO₂ and seemed to be time dependent as glucose level was return to normal in rats co-administrated with 1g NaNO₂ and GSE at 8th week of treatment ($P < 0.05$).

Table (5): Glucose concentrations in serum of control and treated rats during experimental periods (means \pm SE).

Parameter Groups	Glucose (mg/dL)	
	4 weeks	8 weeks
G1 Control	109.33 \pm 2.19 ^e	113.33 \pm 2.19 ^{de}
G2 GSE	108.67 \pm 4.63 ^e	106.00 \pm 4.62 ^e
G3 1g/L NaNO ₂	138.00 \pm 1.15 ^c	144.67 \pm 1.20 ^b
G4 2g/L NaNO ₂	170.67 \pm 1.20 ^a	181.00 \pm 1.53 ^a
G5 GSE+ 1g/L NaNO ₂	121.33 \pm 1.45 ^d	114.33 \pm 2.19 ^d
G6 GSE+ 2g/L NaNO ₂	148.33 \pm 1.20 ^b	122.67 \pm 1.86 ^c
LSD _{0.05}	7.177	7.766

Within the same column, various superscript letters indicate significant differences (Duncan, $P < 0.05$).

Antioxidant profile:

Data in table (6) illustrated that NaNO₂ treatment significantly reduce ($P < 0.05$) plasma GSH, GST and SOD levels in dose-dependent manner in all experimental periods. GSE administration significantly elevate of GSH, GST and SOD levels in plasma of NaNO₂-intoxicated rats especially with 1g NaNO₂ dose (G5) and at 8th week of experiment comparing with corresponding control ($P < 0.05$). GSH and GST levels were restored to normal level at 8th week of experiment as a result of GSE intake in 1g NaNO₂-treated rats.

Serum MDA level (lipid peroxidation product) was increased parallel to dose and time of NaNO₂ intake in G3 and G4 rats comparing with control ($P < 0.05$). The administration of GSE ameliorated the NaNO₂-induced elevation in lipid peroxidation as evidenced by a significant decrease ($P < 0.05$) in MDA production in G5 and G6 rats. Such effect was more pronounced with 1g NaNO₂ and seemed to be time dependent as compared to control rats. The results were shown in table (7).

Table (6): Antioxidants parameters in plasma of control and treated rats during experimental periods (means \pm SE).

Parameters Groups	GSH (mg/dL)		GST (u/ml)		SOD (u/ml)	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
G1 Control	0.53 $\pm 0.01^a$	0.52 $\pm 0.01^a$	61.33 $\pm 0.88^a$	64.00 $\pm 0.58^a$	542.23 $\pm 7.08^a$	544.86 $\pm 8.67^a$
G2 GSE	0.54 $\pm 0.01^a$	0.53 $\pm 0.02^a$	61.32 $\pm 0.34^a$	63.69 $\pm 0.35^a$	528.99 $\pm 8.77^a$	550.78 $\pm 9.66^a$
G3 1g/L NaNO ₂	0.42 $\pm 0.01^c$	0.34 $\pm 0.01^c$	58.29 $\pm 0.36^b$	52.34 $\pm 0.18^c$	436.86 $\pm 7.48^c$	392.44 $\pm 5.23^d$
G4 2g/L NaNO ₂	0.34 $\pm 0.01^d$	0.28 $\pm 0.01^d$	55.00 $\pm 0.57^c$	48.29 $\pm 0.30^d$	336.55 $\pm 10.99^d$	263.44 $\pm 10.99^e$
G5 GSE+ 1g/L NaNO ₂	0.46 $\pm 0.01^b$	0.50 $\pm 0.01^a$	59.00 $\pm 0.58^b$	63.11 $\pm 0.12^a$	481.96 $\pm 2.23^b$	503.04 $\pm 5.26^b$
G6 GSE+ 2g/L NaNO ₂	0.40 $\pm 0.01^c$	0.45 $\pm 0.01^b$	57.65 $\pm 0.29^b$	60.27 $\pm 0.37^b$	426.69 $\pm 2.88^c$	471.35 $\pm 8.81^c$
LSD _{0.05}	0.030	0.032	1.670	1.075	22.402	25.839

Within the same column, various superscript letters indicate significant differences (Duncan, $P < 0.05$).

Table (7): Lipid peroxidation (Malondialdehyde levels) in serum of control and treated rats during experimental periods (means \pm SE).

Parameter Groups	MDA nmol/ml	
	4 weeks	8 weeks
G1 Control	1.86 \pm 0.02 ^e	1.82 \pm 0.07 ^e
G2 GSE	1.86 \pm 0.04 ^e	1.78 \pm 0.04 ^e
G3 1g/L NaNO ₂	6.35 \pm 0.10 ^b	7.32 \pm 0.14 ^b
G4 2g/L NaNO ₂	8.02 \pm 0.06 ^a	10.17 \pm 0.12 ^a
G5 GSE+ 1g/L NaNO ₂	3.85 \pm 0.44 ^d	2.78 \pm 0.10 ^d
G6 GSE+ 2g/L NaNO ₂	5.03 \pm 0.13 ^c	3.65 \pm 0.13 ^c
LSD _{0.05}	0.591	0.329

Within the same column, various superscript letters indicate significant differences (Duncan, $P < 0.05$).

Histopathological Results:**Liver:**

Microscopically, examination of liver of rats from G1 (untreated, control) revealed the normal histological structure of hepatic lobules. Also no histopathological changes were noticed in liver of rats from G2 which treated with GSE (fig. 1). Conversely, the examined sections from G3 which treated with 1g/L NaNO₂ showed focal hepatic necrosis associated with inflammatory cells infiltration (fig. 2), kupffer cells activation and sinusoidal leucocytosis (fig. 3). While the liver of rats from G4 which treated with 2g/L NaNO₂ showed dilatation and congestion of hepatic sinusoids (fig. 4), portal infiltration with mononuclear cells, focal hepatic necrosis associated with mononuclear cells infiltration (fig. 5), cytoplasmic vacuolization of hepatocytes and necrosis of sporadic hepatocytes (fig. 6). Meanwhile, some examined sections of liver of rats from G5 which treated with 1g/L NaNO₂ and GSE showed kupffer cells activation (fig. 7), while other sections from same group revealed no histological changes (fig. 8). Moreover, liver of rats from G6 which treated with 2g/L NaNO₂ and GSE showed cytoplasmic vacuolization of hepatocytes (fig. 9).

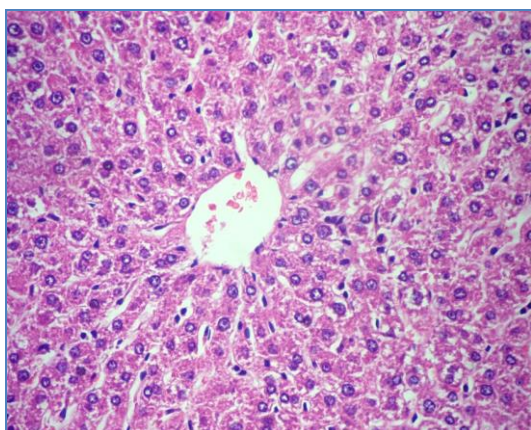


Fig. (1): Liver of rat from G2 showing the normal histological structure of hepatic lobule (H and E X 400).

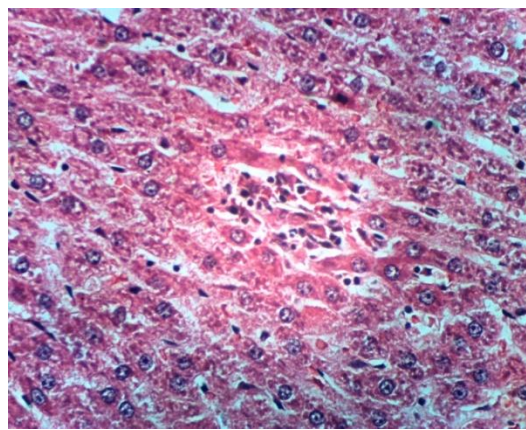


Fig. (2): Liver of rat from G3 showing focal hepatic necrosis associated with inflammatory cells infiltration (H and E X 400).

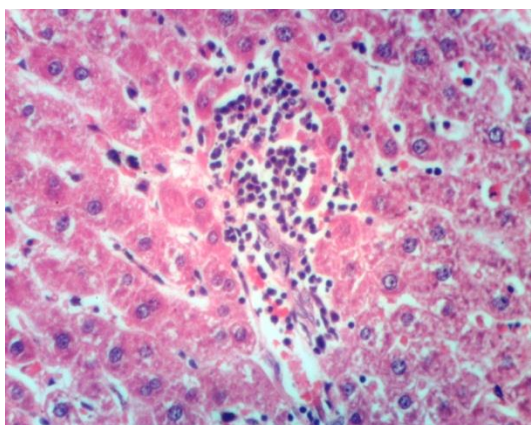


Fig. (3): Liver of rat from G3 showing kupffer cells activation and sinusoidal leucocytosis (H and E X 400).

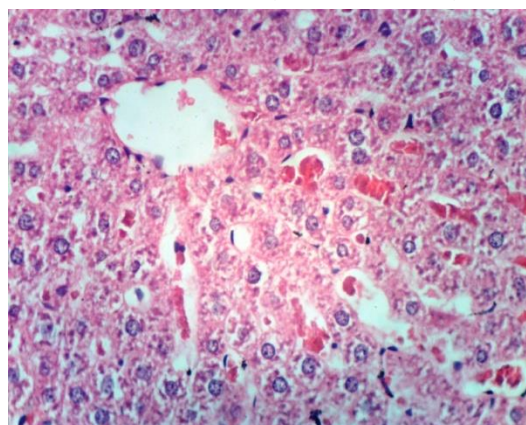


Fig. (4): Liver of rat from G4 showing dilatation and congestion of hepatic sinusoids (H and E X 400).

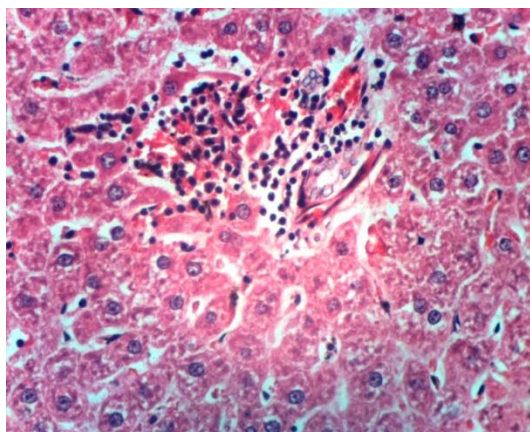


Fig. (5): Liver of rat from G4 showing portal infiltration with mononuclear cells, focal hepatic necrosis associated with mononuclear cells infiltration (H and E X 400).

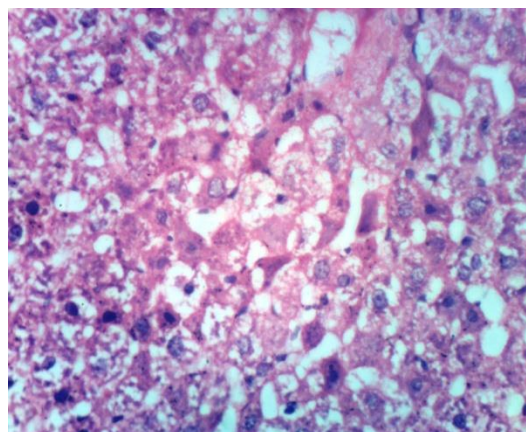


Fig. (6): Liver of rat from G4 showing cytoplasmic vacuolization of hepatocytes and necrosis of sporadic hepatocytes (H and E X 400).



Fig. (7): Liver of rat from G5 showing Kupffer cells activation (H and E X 400).

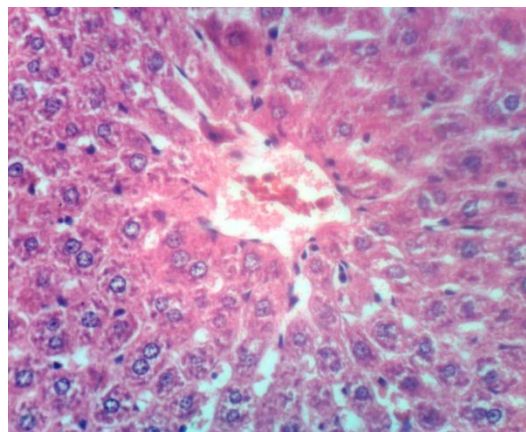


Fig. (8): Liver of rat from G5 showing apparent normal hepatocyte (H and E X 400).

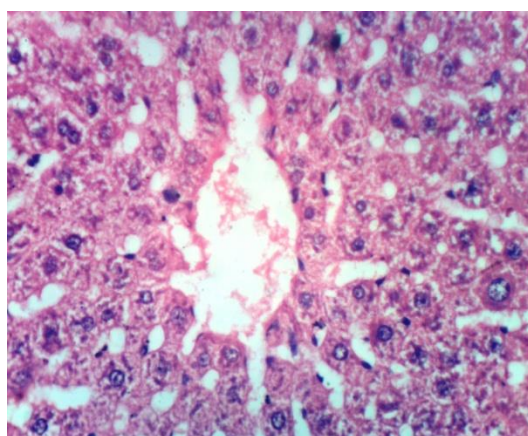


Fig. (9): Liver of rat from G6 showing cytoplasmic vacuolization of hepatocytes (H and E X 400).

Kidney:

Microscopically, kidneys of control, untreated rats (G1) and rats administrated GSE (G2) revealed the normal histological structure of renal parenchyma (fig. 10). However, kidneys of rats from G3 which treated with 1g/L NaNO₂ revealed vacuolization of endothelial lining glomerular tuft with presence of protein cast in the lumen of renal tubules (fig. 11), hypertrophy of glomerular tuft (figs. 12 and 13), periglomerular fibroblasts proliferation (fig. 12) and vacuolization and congestion of glomerular tuft (fig. 13). Also, kidneys of rats from G4 which treated with 2g/L NaNO₂ revealed eosinophilic protein cast in the lumen of renal tubules (fig. 14), hypertrophy and congestion of glomerular tuft, perivascular edema (fig. 15) and atrophy of glomerular tuft (fig. 16). No histological changes were found in kidneys of rats from G5 and G6 which co-treated with 1 and 2g/L NaNO₂ (respectively) and GSE (figs. 17 and 18).

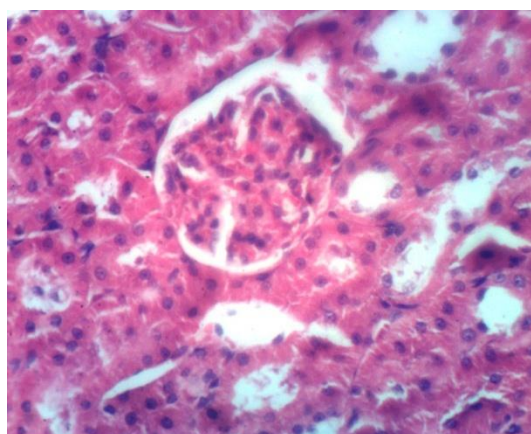


Fig. (10): Kidney of rat from G2 showing the normal histological structure of renal parenchyma (H and E X 400).

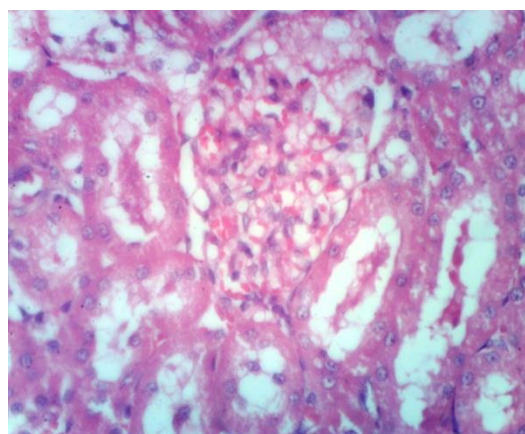


Fig. (11): Kidney of rat from G3 showing vacuolization of endothelial lining glomerular tuft with presence of protein cast in the lumen of renal tubules (H and E X 400).

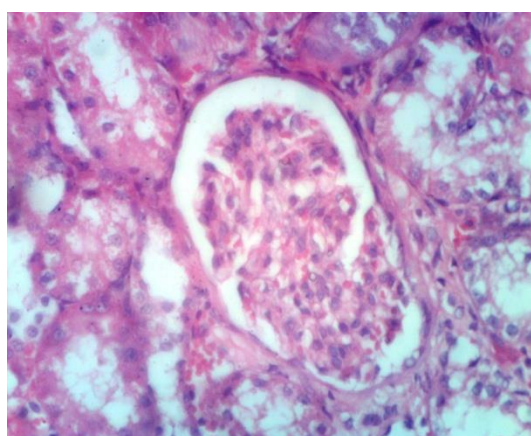


Fig. (12): Kidney of rat from G3 showing hypertrophy of glomerular tuft and periglomerular fibroblasts proliferation (H and E X 400).

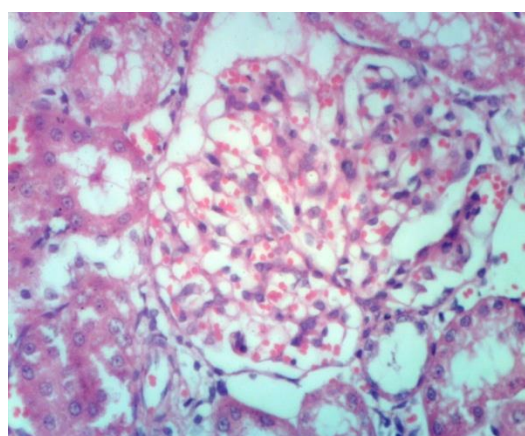


Fig. (13): Kidney of rat from G3 showing hypertrophy, vacuolization and congestion of glomerular tuft (H and E X 400).

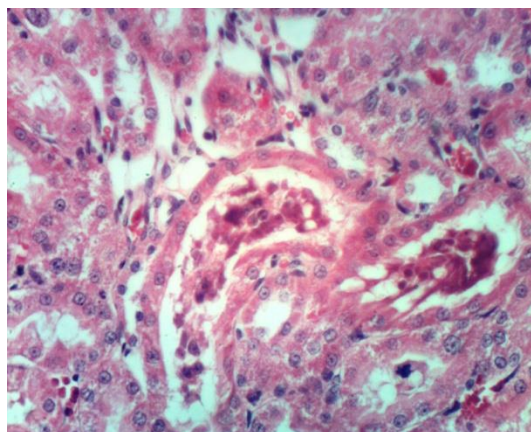


Fig. (14): Kidney of rat from G4 showing eosinophilic protein cast in the lumen of renal tubules (H and E X 400).

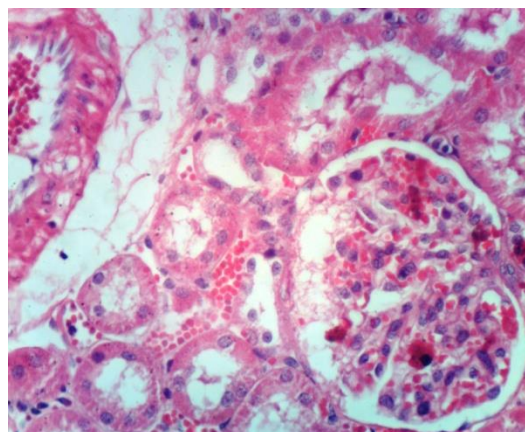


Fig. (15): Kidney of rat from G4 showing hypertrophy and congestion of glomerular tuft, perivascular edema (H and E X 400).

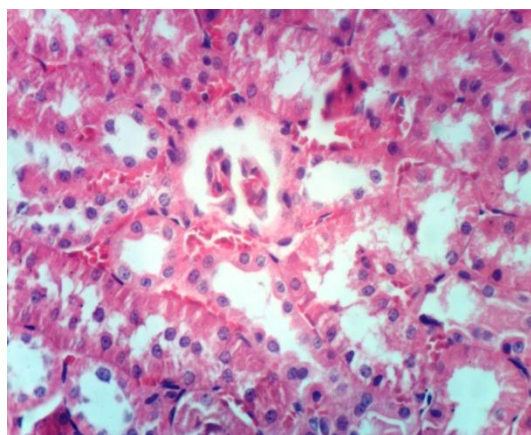


Fig. (16): Kidney of rat from G4 showing atrophy of glomerular tuft (H and E X 400).

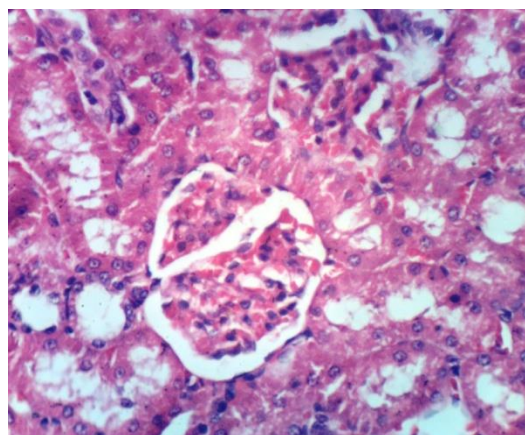


Fig. (17): kidney of rat from G5 showing apparent normal renal parenchyma (H and E X 400).

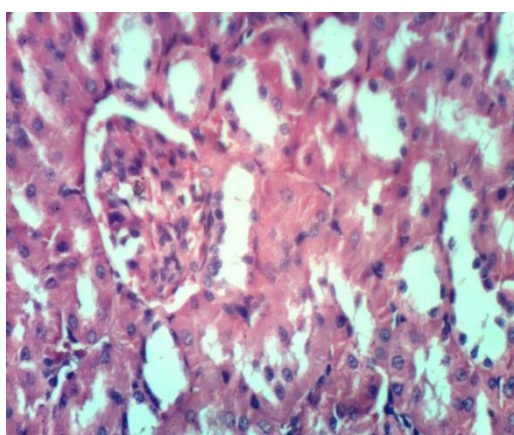


Fig. (18): Kidney of rat from G6 showing apparent normal renal parenchyma (H and E X 400).

Brain:

Examination of brain of control, untreated rats (G1) and GSE-administrated rats (G2) revealed normal histological structure (fig. 19). Conversely, brain of rats from G3 which treated with 1g/L NaNO₂ showed pyknosis of neurons, neuronophagia (fig. 20) and menengial hemorrhage associated with inflammatory cells infiltration (fig. 21). Moreover, brain of rats from G4 which treated with 2g/L NaNO₂ showed focal hemorrhage (fig. 22), hemorrhage in Virchow space (fig. 23), necrosis of neurons (fig. 24) and congestion of cerebral blood vessels (fig. 25). Meanwhile, brain of rats from G5 which administrated with 1g/L NaNO₂ and GSE showed pyknosis of neurons (fig. 26). While, brain of rats from G6 which administrated with 2g/L NaNO₂ and GSE showed neuronophagia of pyknotic neurons (fig. 27).

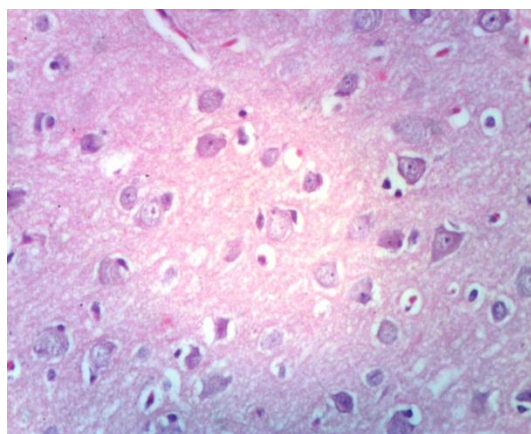


Fig. (19): Brain of rat from G2 showing the normal histological structure (H and E X 400).

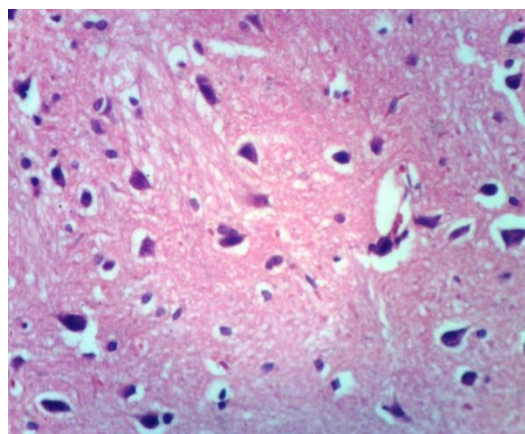


Fig. (20): Brain of rat from G3 showing pyknosis of neurons, neuronophagia (H and E X 400).

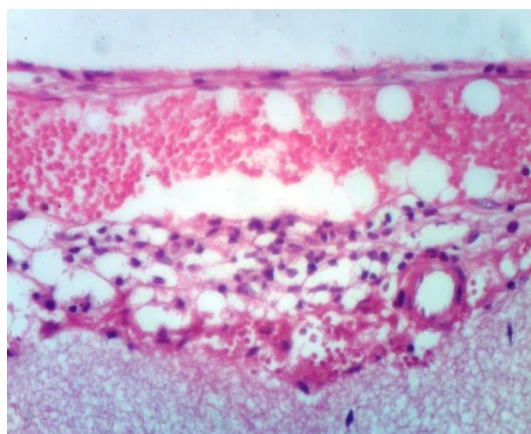


Fig. (21): Brain of rat from G3 showing menengial hemorrhage associated with inflammatory cells infiltration (H and E X 400).

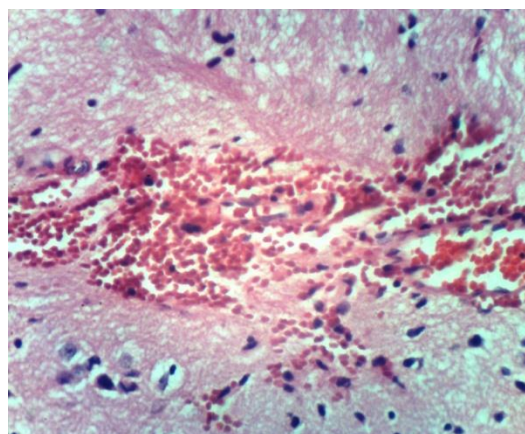


Fig. (22): Brain of rat from G4 showing focal hemorrhage (H and E X 400).

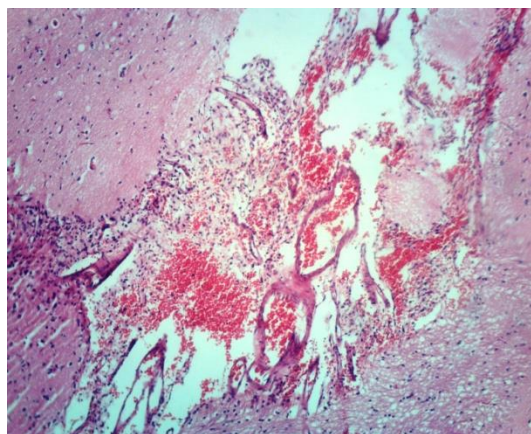


Fig. (23): Brain of rat from G4 showing hemorrhage in Virchow space (H and E X 400).

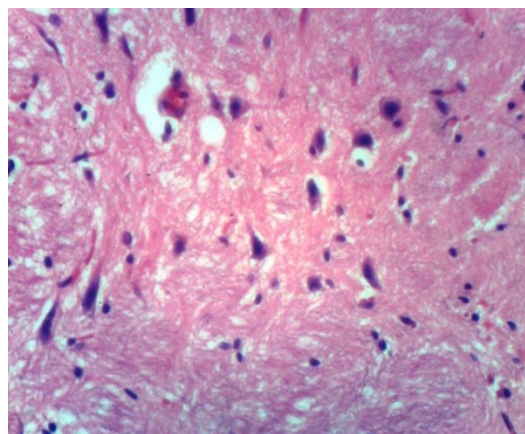


Fig. (24): Brain of rat from G4 necrosis of neurons (H and E X 400).

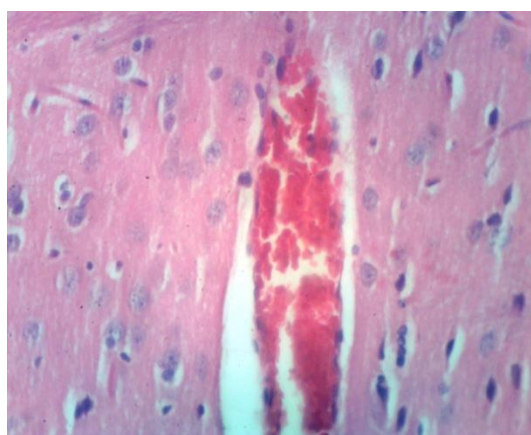


Fig. (25): Brain of rat from G4 showing congestion of cerebral blood vessels (H and E X 400).

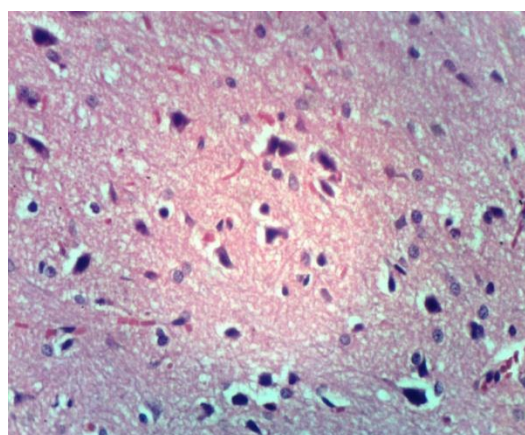


Fig. (26): Brain of rat from G5 showing pyknosis of neurons (H and E X 400).

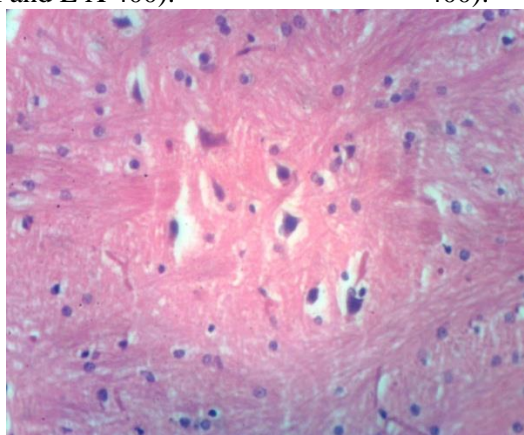


Fig. (27): Brain of rat from G6 showing neuronophagia of pyknotic neurons (H and E X 400).

Heart:

Histopathological examination of rats from G1 (control) and G2 (GSE-treated rats) revealed normal cardiac myocytes (fig. 28). However, heart of rats from G3 (treated with 1g/L NaNO_2) showed congestion of myocardial blood vessels (fig. 29) and myolysis of focal myocytes (fig. 30). Also, examined heart from G4 which treated with 2g/L NaNO_2 showed

congestion of myocardial blood vessels (fig. 31) and zenker's necrosis of sporsdic myocytes (fig. 32). No histological changes were noticed in heart of rats from G5 which treated with 1g/L NaNO₂ and GSE (fig. 33). While, heart of rats from G6 which treated with 2g/L NaNO₂ and GSE showed congestion of myocardial blood vessels (fig. 34).

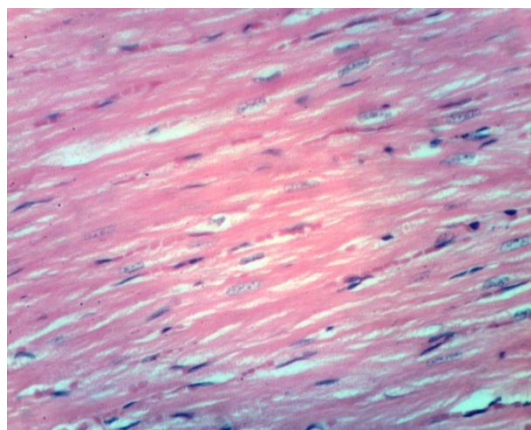


Fig. (28): Heart of rat from G2 showing the normal cardiac myocytes (H and E X 400).

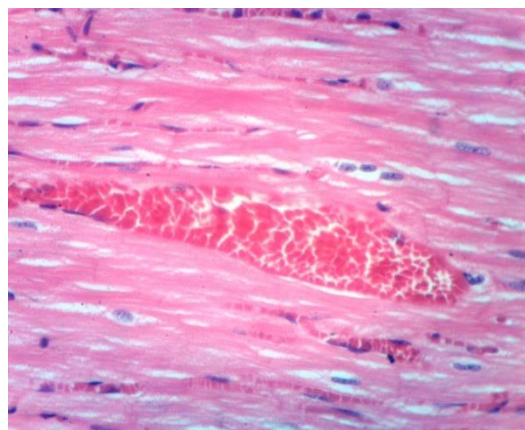


Fig. (29): Heart of rat from G3 showing congestion of myocardial blood vessels (H and E X 400).

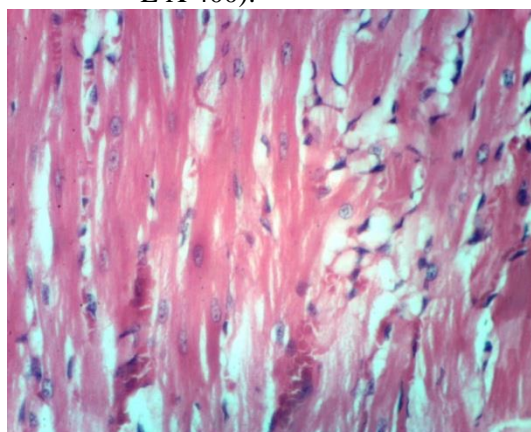


Fig. (30): Heart of rat from G3 showing myolysis of focal myocytes (H and E X 400).

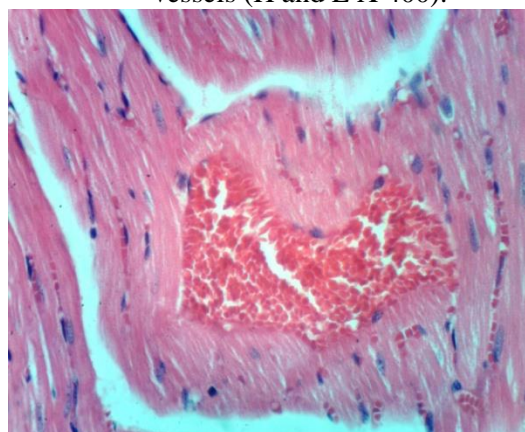


Fig. (31): Heart of rat from G4 showing congestion of myocardial blood vessels (H and E X 400).

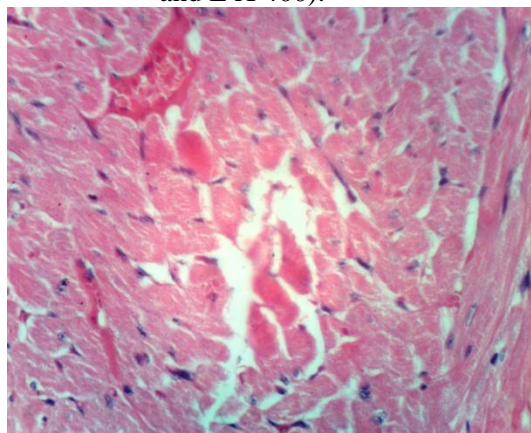


Fig. (32): Heart of rat from G4 showing zenker's necrosis of sporsdic myocytes (H and E X 400).

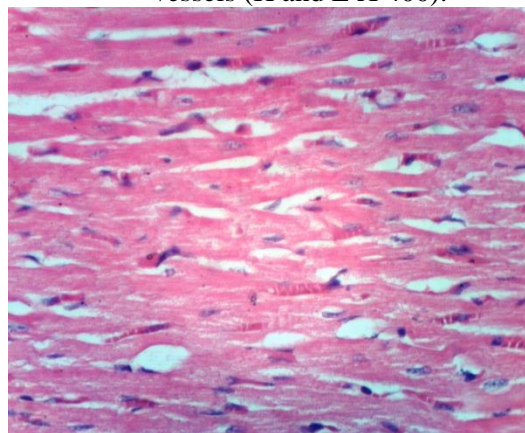


Fig. (33): Heart of rat from G5 normal cardiac myocytes (H and E X 400).

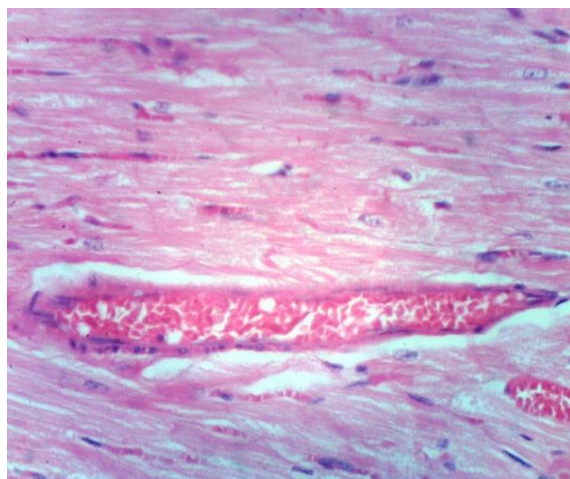


Fig. (34): Heart of rat from G6 showing congestion of myocardial blood vessels (H and E X 400).

Lungs

Microscopically, lungs of control, untreated rats (G1) and GSE-treated rats (G2) showed normal histological structure (fig. 35). Conversely, examined lungs of rats from G3 (treated with 1g/L NaNO₂) showed atelectasis (fig. 36) and bronchitis (fig. 37). The examined lungs of rats from G4 (treated with 2g/L NaNO₂) showed perivascular inflammatory cells infiltration (fig. 38), interstitial pneumonia (fig. 39) and pulmonary hemorrhage (fig. 40). While, no histological changes were noticed in lungs of rats from G5 (treated with 1g/L NaNO₂ and GSE) and in some examined sections of lungs of rats from G6 (treated with 2g/L NaNO₂ and GSE) (fig. 41), whereas, other sections showed focal interstitial pneumonia (fig. 42).

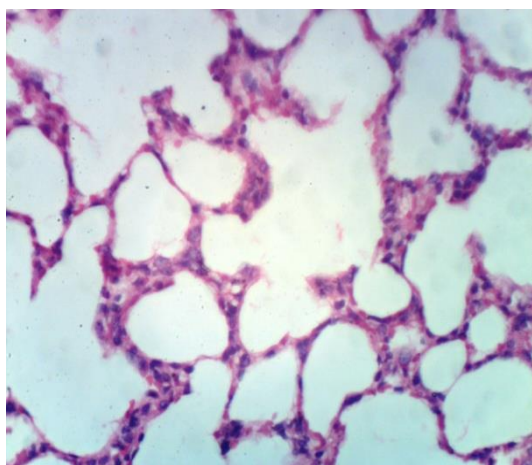


Fig. (35): Lung of rat from G2 showing the normal histological structure (H and E X 400).

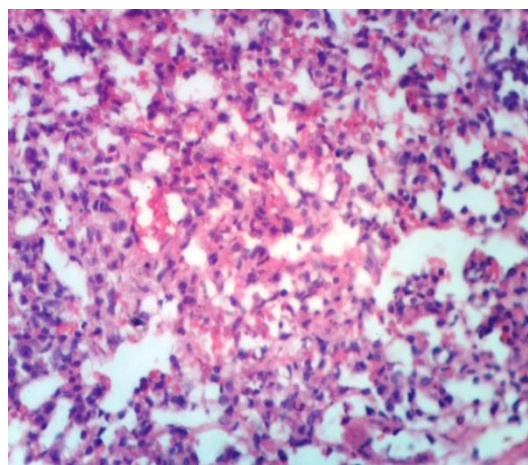


Fig. (36): Lung of rat from G3 showing atelectasis (H and E X 400).

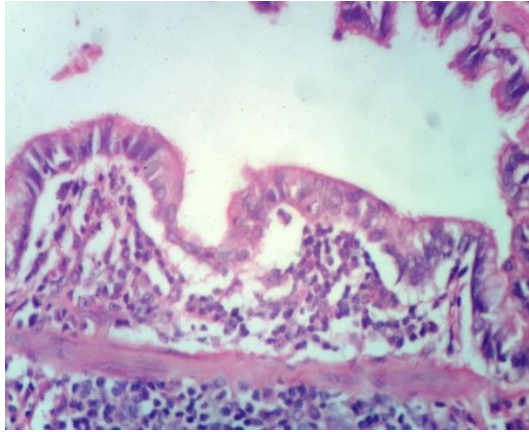


Fig. (37): Lung of rat from G3 showing bronchitis. Note mononuclear cells infiltration in bronchial mucosa (H and E X 400).

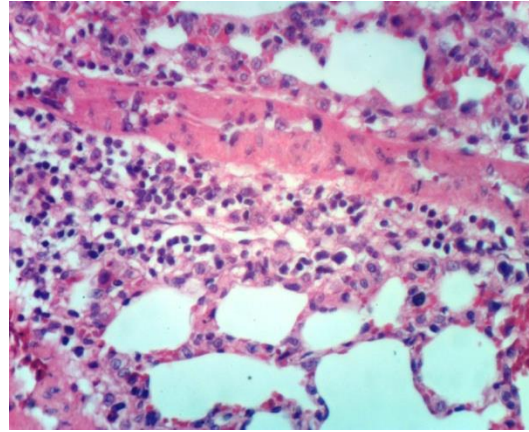


Fig. (38): Lung of rat from G4 showing perivascular inflammatory cells infiltration (H and E X 400).

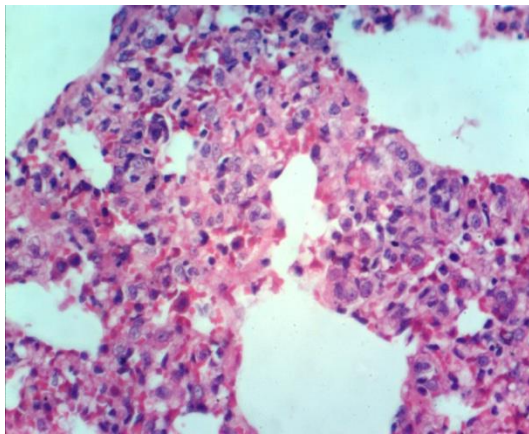


Fig. (39): Lung of rat from G4 showing interstitial pneumonia (H and E X 400).

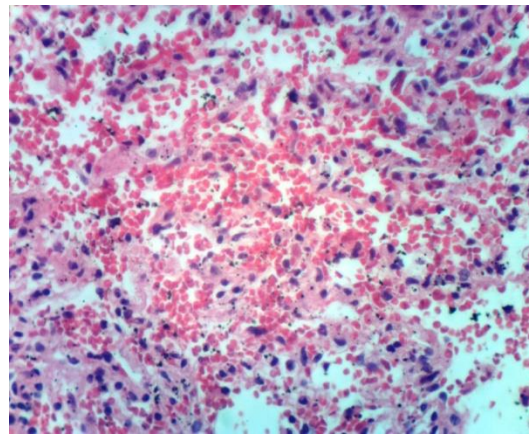


Fig. (40): Lung of rat from G4 pulmonary hemorrhage (H and E X 400).

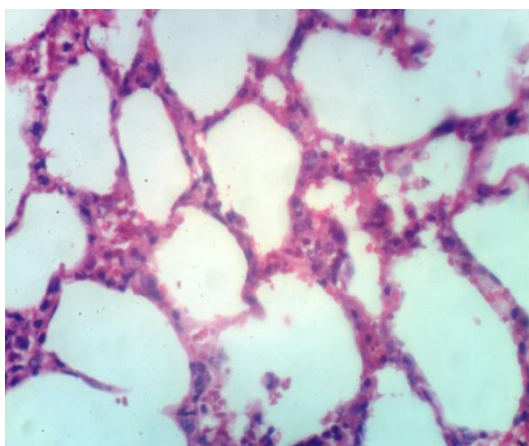


Fig. (41): Lung of rat from G5 showing no histopathological changes (H and E X 400).

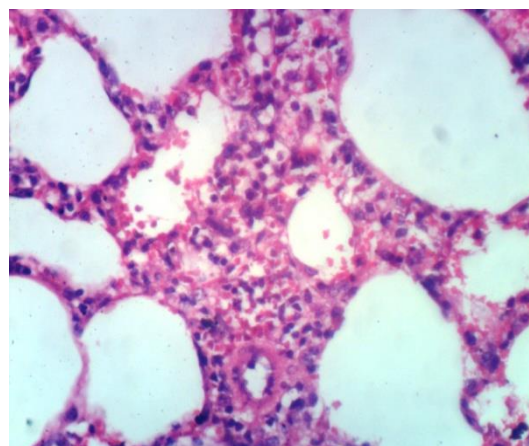


Fig. (42): Lung of rat from G6 focal interstitial pneumonia (H and E X 400).

Testes:

Microscopically, examination of testes of rats from G1 (control, untreated) and rats from G2 (GSE-treated rats) showed normal seminiferous tubules and complete spermatogenesis (fig. 43). However, testes of rats from G3 which treated with 1g/L NaNO₂ revealed degeneration of spermatogoneal cells lining seminiferous tubules and necrosis leydig cells (fig. 44). Also, examination of testes of rats from G4 which treated with 2g/L NaNO₂ revealed interstitial edema (fig. 45) and degeneration of spermatogoneal cells lining seminiferous tubules (fig. 46). Meanwhile, testes of rats from G5 and G6 which treated with 1 and 2g/L NaNO₂ respectively in combination with GSE revealed no histological changes (figs. 47 and 48).

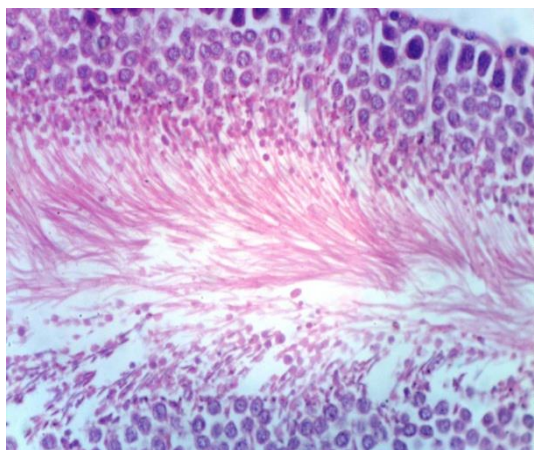


Fig. (43): Testis of rat from G2 showing the normal seminiferous tubules and complete spermatogenesis (H and E X 400).

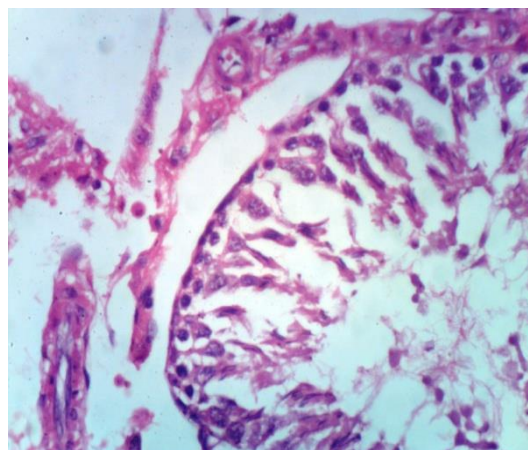


Fig. (44): Testis of rat from G3 showing degeneration of spermatogoneal cells lining seminiferous tubules and necrosis of Leydig cells (H and E X 400).

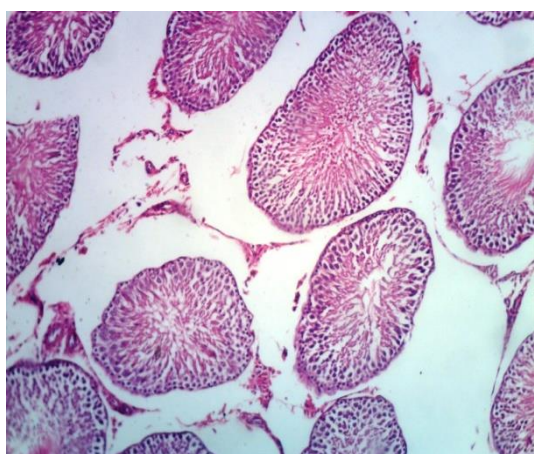


Fig. (45): Testis of rat from G4 showing interstitial edema (H and E X 200).

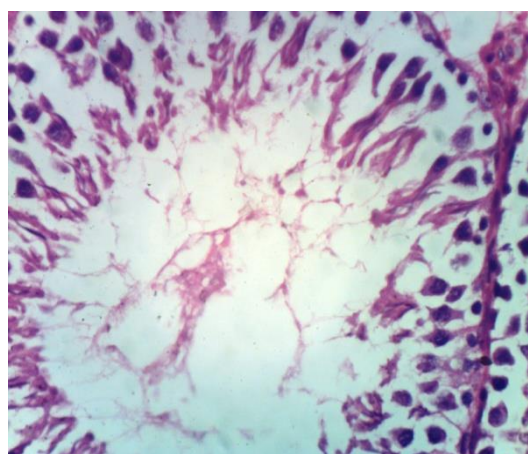


Fig. (46): Testis of rat from G4 showing degeneration of spermatogoneal cells lining seminiferous tubules (H and E X 400).

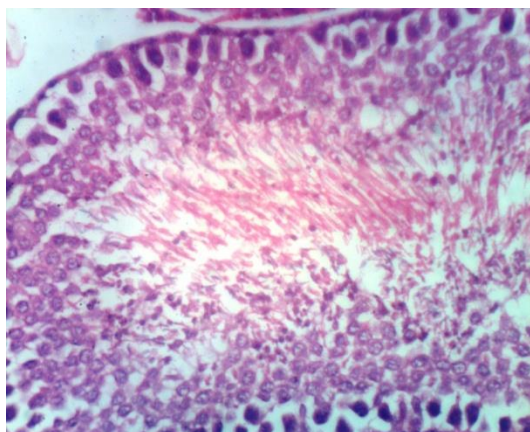


Fig. (47): Testis of rat from G5 showing normal seminiferous tubules and complete spermatogenesis (H and E X 400).

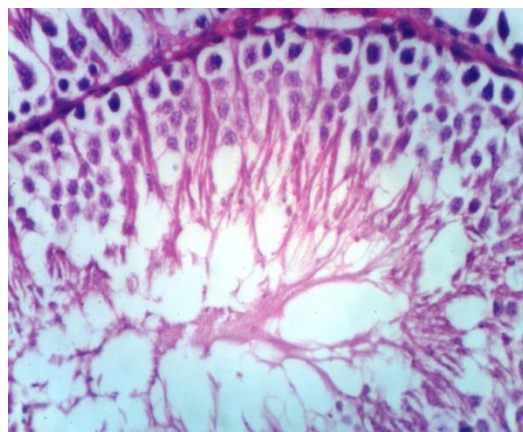


Fig. (48): Testis of rat from G6 showing no histological changes (H and E X 400).

DISCUSSION

NaNO_2 is a main preservative in cured meat products, fish and some types of cheese and occurs naturally in many foods, particularly vegetables (Dejam *et al.*, 2005). However, toxicity to humans and animals is well documented in nitrite overexposure (RCHAS, 2000 and WHO, 2007). The NaNO_2 and other additives may react with amines of the foods in the stomach and produce nitrosamines and free radicals. Such products may increase lipid peroxidation, which can be harmful to different organs including liver and kidney (Choi *et al.*, 2002). On the other hand, many plant-derived drugs used in modern medicine are developed by ethnomedical leads and subsequent ethnopharmacological studies. Plants containing flavonoids have been reported to possess strong antioxidant properties (Raj and Shalini, 1999). The antioxidant activity of GSE make it candidate for addition to food and beverages to retard deterioration; it is possible that the antioxidant activity of GSE ingested with these foods would also support physiological defenses against in vivo-generated free radicals species (Ricardo-DaSilva *et al.*, 1991 and Bentivegna and Whitney, 2002).

The data of this study have shown that NaNO_2 administration at dose level of 1g and 2g/L in drinking water for 4 and 8 weeks resulted in significant dose- dependent decrease of blood WBCs, RBCs count and Hb concentration. These results are in agreement with the finding of Helal and Elsaid (2006) showed significantly decrease of RBCs%, WBCs%, Ht% and Hb% in rats received NaNO_2 (10 mg/kg/day) orally for 30days. Moreover, Gluhcheva *et al.* (2012) stated that acute treatment of rats with NaNO_2 induces significant hematological and rheological changes therefore monitoring of these parameters is necessary when exposed to high doses or prolonged treatment with the compound. The decrease of RBCs count and Hb concentration in our study was associated with increase of MetHb formation in blood of NaNO_2 -intoxicated rats. Such increase was noted by Imaizumi *et al.* (1980) who reported that maximum levels of MetHb (45 – 80%) were reached one hour after dosing Sprague-Dawley rats with 150 mg/kg B.W NaNO_2 . Also, In Sprague-Dawley rats receiving a single dose of 30 mg/kg B.W of NaNO_2 in aqueous solution by gavage (10 - 15% of LD_{50}), plasma nitrite and MetHb levels were increased after 2.8 minutes and maximum effects (plasma nitrite = 15%, MetHb = 12%) were observed after 22.5 minutes (Hirneith and Classen, 1984). The experimental model of Kohn *et al.* (2002) predicts that 10% of the Hb is oxidized to the ferric form after oral doses of 15.9 mg/kg NaNO_2 in male rats and 11.0 mg/kg NaNO_2 in female rats and after intravenous doses of 8.9 and 7.1 mg/kg in male and female rats, respectively. The chemical reactivity of NaNO_2 with Hb may enhance heme- or

iron-mediated toxicities. Nitrite is known to cause free radical generation, as it can stimulate oxidation of ferrous ions in oxyhemoglobin to form MetHb as well as various ROS (reactive oxygen species) (**Gladwin et al., 2004 and Baky et al., 2010**). The nitrite ion, its metabolites, and lipid peroxidation products are supposed to react with sulfhydryl groups of the lipid bilayer and protein components of erythrocyte membrane and change its structure (**Maeda et al., 1987**). Nitrite-promoted Ca^{2+} influx in blood cells activates phospholipases, which increase the proportion of phospholipids with a rigid structure in the membrane (**Kaya and Miura, 1982**). Moreover, WBCs decrease is associated with the failure of the hematopoietic tissues to produce new WBC (**Tan et al., 1992**). It is suggested that the decrease in RBCs and Hb concentration may be attributed to microcytic and/or hypochromic anemia possibly as a consequence of the toxic effect of NaNO_2 on bone marrow, spleen and liver (**Aboel-Zahab et al., 1997**).

This study has shown that GSE alone does not have any negative impact on hematological parameters and MetHb formation. In the current study, the decrease in hematological parameters and increase in MetHb formation was reversed near normalcy by the administration of GSE in all experimental periods, moreover RBCs and MetHb were return to normal at end of experiment in 1g NaNO_2 -treated rats. Similarly, no significant changes in hematological parameters in male and female Sprague-Dawley rats provided with GSE in the diet at levels of 0.5, 1.0, or 2.0%, for a period of 90 days (**Wren et al., 2002**) and in male and female rats provided with GSE in the diet at levels of 0.63, 1.25 or 2.5%, for a period of 3 months (**Bentivegna and Whitney, 2002**). While the relative improvement of these parameters may be due to suggested antioxidant activity of GSE in vivo includes stimulating enzyme production of nitric oxide, oxygen radical scavenging and inhibition of nitrositive stress (**Bagchi et al., 2000 and Roychowdhury et al., 2001**).

The current study revealed that NaNO_2 induced dose-dependent liver damage as evidenced by changes in liver function parameters and histopathological examination. The administration of NaNO_2 for 8 weeks induced dose-dependent increase in ALT, AST, ALP and GGT activities and significant dose dependant-decrease in TP and albumin. Thus increased concentrations of these enzymes in serums normally indicate hepatocellular damage and leakage of these enzymes from liver cells into the blood stream (**Carey, 2000**). Similar to our results, **Helal and Elsaid (2006)** reported increased activities of ALT, AST, ALP and GGT and decrease concentrations of TP and albumin in NaNO_2 -treated rats. Also, **Krishnamoorthy and Sangeetha (2008), Hassan et al. (2009) and Efurube et al. (2013)** reported increase of ALT, AST, GGT and ALP activities in rats as a result of treatment with NaNO_2 (300 mg/kg B.W for 30 days, 80 mg/kg B.W for 3 months and single dose of 6.20mg, respectively). **El-Sheikh and Khalil (2011)** fed rats with 0.2% NaNO_2 for 6 weeks and recorded decrease of TP and albumin. The increase in the activity of AST, ALT, GGT and ALP enzymes in the serum of NaNO_2 -treated rats could be attributed to the toxic effect of nitroso-compounds, formed in the acidic environment of the stomach, in causing severe hepatic necrosis **Hassan et al. (2009)**. However, the indicated inhibitory effect of NaNO_2 on the biosynthesis of protein could be attributed to stimulation of the thyroid and the adrenal glands by NaNO_2 which can lead to a blockage in protein synthesis, fast breakdown, and increased rate of free amino acids, and decreased protein turnover (**Eremin and Yocharina, 1981**). In addition, nitrite interactions results into nitric oxide release, which can inhibit total protein synthesis (**Kolpakov et al., 1995**). The severity of hepatic lesions in our study was found to be parallel to NaNO_2 concentration in water. The liver of 1 g/L NaNO_2 -treated rats showed focal hepatic necrosis associated with inflammatory cells infiltration, kupffer cells activation and sinusoidal leucocytosis while the liver of 2 g/L NaNO_2 -treated rats showed the same previous lesions besides dilatation and congestion of hepatic sinusoids, portal infiltration with mononuclear cells, cytoplasmic vacuolization of hepatocytes and necrosis of

sporadic hepatocytes. Such hepatic damage by NaNO₂ was previously recorded by **Gomaa and Abd Elaziz (2011)** in rats by 80 mg/kg NaNO₂ oral administration for 2 months. Also, **Abebe et al. (2013)** recorded severe haemorrhage within the sinusoid and the portal triad was infiltrated by inflammatory cells in liver of NaNO₂-treated rats (25 and 50mg/ kg). While, **Efuruibe et al. (2013)** were reported mild periportal cellular infiltration as a result of NaNO₂ administration in rats. Such liver damage could be explained by formation of free radicals (ONOO⁻) from nitric oxide. Both NO and oxygen radicals could react further to produce other oxidant and nitro compounds such as peroxy nitrite to induce liver injury (**Liaudet et al., 2000 and Chow and Hong 2002**) and it play an important role in death of liver cells (**Krishnamoorthy and Sangeetha, 2008**).

This study has shown that GSE alone does not have any negative impact on liver function parameters and histology of liver. As revealed previously in toxicological studies on GSE by **Wren et al. (2002) and Bentivegna and Whitney (2002)**. The current results revealed that co-treatment with GSE and NaNO₂ resulted in a significant improvement in all liver function parameters and liver histology as indicated by significant alleviation of toxic effect of NaNO₂ on ALT, AST, ALP, TP and albumin levels in serum. Especially with low NaNO₂ dose and at 8th week of treatment, but it failed to normalize them except GGT, which restored to normal. Moreover the hepatic structure of 1g NaNO₂ and GSE-treated rats was reversed to normal. The protective effect of GSE on liver against oxidative damage was previously recorded by **Dulundu et al. (2007)** who reported that GSE administration was significantly able to decrease the increase of AST and ALT levels as a result of biliary obstruction induced by bile duct ligation in serum of rats due to its antioxidant and antifibrotic prosperities. **Chis et al. (2009)** suggested that the GSE enhanced the antioxidant defense against reactive oxygen species produced under hyperglycaemic conditions, hence protecting the liver cells. **Yousef et al. (2009)** reported that GSE antagonized cisplatin-induced increase in the activities of AST and ALT and decrease concentrations of TP and albumin in rats. Furthermore, **El-Beshbishy et al. (2010)** observed that oral intake of GSE to tamoxifen-intoxicated rats resulted in attenuation of histopathological changes in liver and corrected all liver function parameters (ALT, AST, ALP and GGT). More interesting **Hassan (2012)** noted that the ethanol grape seed extract (EGSE) was more effective than water grape seed extract (WGSE) against hepatotoxicity of alcohol in rats. Hepatoprotective activity of the grape seed extracts could be attributed to the antioxidant effect of the constituents and enhanced antioxidant defenses.

Kidneys represent the major control system maintaining body homeostasis. The serum concentration of urea and creatinine determine renal function and are thus biomarkers for kidney disease (**Levey et al., 1999**). The present results revealed dose-dependent increase of creatinine and urea concentration in serum of NaNO₂-intoxicated rats. Such finding was previously reported in rats by **Hassan et al. (2009) and El-Sheikh and Khalil (2011)**. These effects could be attributed to the changes in the threshold of tubular re-absorption, renal blood flow and glomerular filtration rate (GFR) (**Zurovsky and Haber, 1995**). The elevation in kidney function biomarker was synchronized with observed damage in kidney. The noticed lesions in kidneys of both NaNO₂-treated groups (1g and 2g/L) showed the same degree of severity. Kidney of NaNO₂-intoxicated groups revealed vacuolization of endothelial lining glomerular tuft with presence of protein cast in the lumen of renal tubules, hypertrophy of glomerular tuft, periglomerular fibroblasts proliferation, vacuolization and congestion of glomerular tuft, perivascular edema and atrophy of glomerular tuft. Such kidney damage by NaNO₂ was previously recorded by **Gomaa and Abd Elaziz (2011) and Abebe et al. (2013)**.

This study has shown that GSE alone does not have any negative impact on kidney function parameters and histology of kidney. As revealed previously in toxicological studies

on GSE by **Wren *et al.* (2002)** and **Bentivegna and Whitney (2002)**. The current results revealed that co-treatment with GSE and NaNO₂ resulted in a significant improvement in all kidney function parameters and Kidney histology which indicated by significant alleviation of toxic effect of NaNO₂ on creatinine and urea levels in serum especially with low NaNO₂ dose and at 8th week of treatment. Both of them were restored to normalcy level at the end of experiment. Moreover the GSE administration reversed toxic effect of both NaNO₂ dose and restore kidney histology to normal. The protective effect of GSE on kidney against oxidative damage was previously recorded by **Yousef *et al.* (2009)** and **Saad *et al.* (2009)** they reported that GSE antagonized cisplatin-induced increase in concentration of creatinine and urea in serum of rats and damage in Kidney genomic DNA. The mechanism underlying GSE nephroprotection may be due to the marked radical scavenging ability of GSE proanthocyanidins (**Sato *et al.*, 2005**).

Lipids play an important role in cardiovascular disease, not only by way of hyperlipidaemia and the development of atherosclerosis, but also by modifying the composition, structure, and stability of cellular membranes. Excess lipids in the blood are considered to accelerate the development of arteriosclerosis and are the major risk factor in myocardial infarction. High levels of circulating cholesterol and its accumulation in heart tissue are well associated with cardiovascular damage (**Salter and White, 1996**). The results of the present study revealed time and dose-dependent increase in total cholesterol and triglycerides concentration in serum of NaNO₂-treated rats. Such results have been provided by **Helal and Elsaid (2006)** in NaNO₂-intoxicated rats. These biochemical changes were coincident with the histopathological changes in heart tissue induced by NaNO₂ in the present study which represented by congestion of myocardial blood vessels and myolysis of focal myocytes in 1g/L NaNO₂-treated group. And congestion of myocardial blood vessels and zenker's necrosis of sporsdic myocytes in 2g/L NaNO₂-treated group. In an early chronic toxicity study (**Gruener and Shuval, 1973**), rats were given nitrite in the drinking water at doses equivalent to 0, 10, 100, 200 and 300 mg/kg B.W/day) corresponding to 0, 6.7, 67, 124, 201 mg/kg B.W per day for nitrite). At the highest dose, focal degeneration and fibrosis of the heart muscle as well as dilatation of coronary arteries were also observed. More recently **Gomaa and Abd Elaziz (2011)** reported necrosis of myocardial muscle cells in NaNO₂-treated rats.

It worthy to note that GSE were had no negative effect on total cholesterol, triglycerides and heart tissue. The hypercholesterolemic, increased triglycerides and heart injury induced by NaNO₂ in the present study were significantly alleviated by GSE administration. GSE administration restores cholesterol level at 4th week of treatment, triglycerides levels along experimental period in serum and heart histology to normal of 1g NaNO₂-treated rats. These results are in agreement with finding of **Karthikeyan *et al.* (2007)** showed that the administration of grape seed proanthocyanidins significantly maintained the cholesterol, phospholipids, triglycerides, and free fatty acids levels in serum and heart tissue of the isoproterenol -induced myocardial injury in rats. The experiments conclude that grape seed proanthocyanidins possess cardioprotective and hypolipidemic effect on the treatment of ISO-induced myocardial injury. Also, **Yousef *et al.* (2009)** reported that GSE antagonized cisplatin-induced increase in cholesterol and triglycerides. Furthermore **Jiao *et al.* (2010)** concluded that the hypocholesterolemic activity of grape seed proanthocyanidins was most likely mediated by enhancement of bile acid excretion and up-regulation of cholesterol-7 α -hydroxylase (CYP7A1). On the other hand, potential mechanisms by which grape polyphenols may exert cardioprotective effects include a reduction in oxidative stress, modulation of the inflammatory cascade, improvement in vascular endothelial function (eg, flow-mediated dilatation [FMD]), and protection against

atherothrombotic episodes including myocardial ischemia and inhibition of platelet aggregation (Cui *et al.*, 2002 Shanmuganayagam *et al.*, 2002 and Delmas *et al.*, 2005).

Our results clearly showed that there was a significant dose dependent-increase in serum glucose concentration of NaNO₂-treated rats in all experimental periods. The same results was obtained by Hassan *et al.* (2009) and Gomaa and Abd Elaziz (2011) in rats and by Abdul-Ameer and Abed (2012) in mice. The findings suggest nitrite-stimulation of gluconeogenesis (Wiechetek *et al.*, 1992), and glucose shift from tissue to blood or an impairment of glucose mobilization. Furthermore, nitroso-compounds can alter the antioxidant system causing disturbance in the metabolic processes leading to hyperglycemia (Anil *et al.*, 2005).

However, in our study serum glucose levels were ameliorated upon GSE supplementation and return to normal level in serum of 1g NaNO₂-treated rats at 8th week. The hypoglycemic effect of grape seed proanthocyanidins was previously reported by El-Alfy *et al.* (2005) and Terra *et al.* (2009). The mechanism underlying such protection is mediated via prevention and restoration of pancreatic antioxidant defense systems and stimulating insulin release by pancreatic β -cells. An additional mechanism of the antihyperglycemic action of grape seed may be through stimulating the surviving pancreatic cells to release more insulin (El-Alfy *et al.* 2005). A recent study by Pinent *et al.* (2004) has also shown that grape-seed procyanidins possess an insulinomimetic activity on insulin sensitive cell lines.

Lipid peroxidation, a type of oxidative degeneration of polyunsaturated fatty acids (PUFA), has been linked with altered membrane structure and enzyme inactivation (Karthikeyan *et al.*, 2007). Consequently, lipid peroxidation is known to induce cellular damage and is responsible for ROS induced organ damage. Lipid peroxidation of membranes is regulated by the availability of substrate in the form of PUFA, the availability of inducers, such as free radicals and excited state molecules, to initiate propagation, the antioxidant defense status of environment, and the physical status of the membrane lipids (Anandan *et al.*, 1998). GSH is one of the essential compounds for regulation of variety of cell functions. It has a direct antioxidant function by reacting with superoxide radicals, peroxy radicals and singlet oxygen followed by the formation of oxidized glutathione (GS-SG) and other disulfides. GST is GSH-dependent antioxidant enzymes (Karthikeyan *et al.*, 2007). GST catalyses the conjugation of GSH via the sulfhydryl group, to electrophilic centers on a wide variety of substrates. This activity is useful in the detoxification of endogenous compounds such as peroxidised lipids (Valavanidisa *et al.*, 2006). The enzyme SOD catalyzes dismutation of the superoxide anion (O⁻²) into hydrogen peroxide (H₂O₂), which is then detoxified to H₂O by catalase (Mukherjee *et al.*, 2003).

Results of this study showed that NaNO₂ induce oxidative stress in rats as evidenced by dose-dependent increase of serum MDA and dose-dependent decrease of plasma GSH, GST and SOD. Oxidative stress induced by NaNO₂ was previously fully described by Krishnamoorthy and Sangeetha (2008); El-Sheikh and Khalil (2011) and Gomaa and Abd Elaziz (2011).

The present data indicated that treatment with GSE alone didn't alter any of oxidative stress parameters and significantly alleviated the toxic effect of NaNO₂ on antioxidant defense system especially on lower dose and longer time as GSH and GST levels were restored to normal level at 8th week of experiment as result of GSE intake in 1g NaNO₂-treated rats. Such enhancement in antioxidant profile by GSE intake against oxidative stress induced by many chemicals were previously recorded such as isoproterenol (Karthikeyan *et al.*, 2007), cisplatin (Yousef *et al.*, 2009 and Saad *et al.*, 2009) and streptozotocin (Chis *et al.*, 2009).

Present histopathological results revealed that NaNO₂ treatment for 8 weeks induced dose-dependent neurotoxicity. Brain of 1g/L NaNO₂-treated rats showed pyknosis of neurons, neuronophagia, menengial hemorrhage associated with inflammatory cells infiltration while brain of 2g/L NaNO₂-treated rats showed focal hemorrhage, hemorrhage in Virchow space, necrosis of neurons and congestion of cerebral blood vessels. NaNO₂ induced neural toxic effects by exerting oxidative stress and retrograde the endogenous antioxidant system (**Hassan et al., 2010**). Moreover hypoxic neurotoxicity was induced by 30 mg/kg of NaNO₂ intake dissolved in normal water for 14 day and 75 mg/kg of subcutaneous injection NaNO₂ (**Premanand and Ganesh, 2010 and Zaidi, 2010**, respectively).

Meanwhile, present study revealed that GSE intake significantly reversed neurotoxic effect of both NaNO₂ dose which induced oxidative stress and restore brain tissue to normal. This suggestion run with data of **Balu et al. (2005)** demonstrated that GSE enhanced the antioxidant status and decreased the incidence of free radical induced protein oxidation in aged rats thereby protecting the central nervous system from the reactive oxygen species.

In the present investigation, treatment of rats by NaNO₂ for 8 weeks resulted in lung injury as revealed by histological study. The degree of severity of pathological lesions was correlated to concentration of NaNO₂. Microscopically, lungs of 1g/L NaNO₂-treated rats showed atelectasis and bronchitis. Whereas the examined lungs of rats treated with 2g/L NaNO₂ showed perivascular inflammatory cells infiltration, interstitial pneumonia and pulmonary hemorrhage. Histopathologic changes in lung as a result of NaNO₂ toxicity were also observed by **Gruener and Shuval (1973)** who stated that, the changes in the lungs consisted of dilatation of the bronchi with infiltration of lymphocytes and alveolar hyperinflation. **U.S. EPA (1995)** reported dilated bronchi, fibrosis and emphysema at 1000 ppm NaNO₂ or above.

Present histopathological results revealed that GSE was able to protect lung against low NaNO₂ dose and minimize damage of lung induced by high NaNO₂ dose. The beneficial effect of GSE on lung tissue was previously reported by **Hemmati et al (2008)** revealed that the use of GSE can provide a protective action against the harmful and fibrogenic effect of silica on lung tissue.

Present histological examination revealed that NaNO₂ induce dose-dependent testicular damage. Testes of rats administrated with 1g/L NaNO₂ revealed degeneration of spermatogoneal cells lining seminiferous tubules and necrosis leydig cells. Also, examination of testes of rats treated with 2g/L NaNO₂ revealed interstitial edema and degeneration of spermatogoneal cells lining seminiferous tubules. The effect of NaNO₂ on testis was previously reported by many studies. **Akintunde et al. (2013)** suggested that nitrite may change the testicular oxidative status and play a role in testicular dysfunction that causes infertility. Testes of rats received 0.04mg/kg B.W, 0.06mg/kg B.W and 0.08mg/kg B.W nitrite by oral route for 30 days showed Seminiferous tubules with focal areas of lost germ cells, hyperplasia of Leydig cells and at 0.06mg/kg dose level, there was arrest of spermatogenesis while there was no evidence of spermatogenesis at 0.08mg/kg nitrite dose. **Pavlova et al. (2013)** reported that intraperitoneal injection of male Wistar rats with NaNO₂ at dose of 50 mg.kg⁻¹ B.W and sacrificed at different time intervals (1 h, 5 h, 24 h and 48h) following the administration resulted in disorganization of seminiferous epithelium and assemblance of undifferentiated germ cells in the luminal area of the tubules.

Present histopathological results revealed that GSE was able to protect testes against both NaNO₂ dose and restore testicular tissue to normal. Such protective effect of GSE was recorded against sodium fluoride induced testicular damage (**El-Demerdash et al., 2008**).

Generally the cytotoxic and oxidative stress activity of NaNO₂ may be due to NO[•] and ONOO⁻ formed from nitrite. Such product may increase lipid peroxidation which can be

harmful to different organs (**Chow and Hong, 2002**). The high oxidative stress indicator lipid peroxidation could be attributed to the oxidative cytotoxicity of nitrite (**Patsoukis and Georgiou, 2007**). Moreover, nitrite can react with secondary and tertiary amines forming N-nitrosocompounds (nitrosamines) (**Walker, 1990**), which are associated with high risk of stomach, liver and esophagus carcinomas (**Mitacek et al., 1999 and Kim et al., 2002**). Nitrosamines have strong toxic, mutagenic, neuro- and nephrotoxic and carcinogenic effect. These may interpret the distortion in lung, testis, brain, heart, liver and kidney and reflected by the changes in the investigated parameters.

Meanwhile, GSE could reduce organ injury through its ability to balance the oxidant-antioxidant status, and to regulate the release of inflammatory mediators (**Sehirli et al., 2008**). GSE has been shown to have conjugated structures between the B-ring catechol groups and 3-OH free groups of polymeric skeleton allowing being effective free radical scavengers and metal chelators (**Yilmaz and Toledo, 2004**). As GSE scavenges free radicals, the resulting aroxyl radical formed has been shown to be more stable than that generated from other polyphenolics to prevent DNA damage (**Bradham et al., 1998**). Also, **Devi et al. (2006)** found that intake of proanthocyanidin which is a naturally occurring antioxidant from GSE in low quantity is effective in up-regulating the antioxidant defense mechanism by attenuating lipid peroxidation.

Conclusion: Sodium nitrite (preservative) has harmful effects on all tested parameters and induces oxidative damage in liver, kidney, brain, heart, lung and testis tissues in dose-dependent manner. GSE not only has antioxidant properties that can enable it to scavenge the reactive oxygen species resulted from the ingestion of the food additives, but also it can normalize the changes induced by low NaNO₂ dose in most examined organs. It has hypoglycemic and hypocholesterolemic actions.

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التأثير الوقائي لمستخلص بذور العنب ضد السمية و الجهد المؤكسد الناتج عن نترتيت الصوديوم في الجرذان

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يعتبر نترتيت الصوديوم من أهم المضادات الميكروبية وكذلك كمادة حافظة و محسنة للطعم و اللون في منتجات اللحوم و الأسماك. ومع ذلك، قد يسبب النترتيت ميتيموجلوبينية الدم و أيضاً العديد من الأمراض الأخرى ، و كذلك يتفاعل مع بعض الأمينات لينتج مركبات النتروزامين المسرطنة. هدفت هذه الدراسة إلى تقييم فعالية مستخلص بذور العنب بوصفها طريقة جديدة و مفيدة للتخلص من الاكسدة و السمية الناجمة عن نترتيت الصوديوم في ذكور الجرذان. تمت إضافة جرعتين من نترتيت الصوديوم (1و2 جم/لتر) لمياة الشرب لمدة 4 أسابيع و 8 أسابيع. أظهرت النتائج ان المعاملة بنترتيت الصوديوم قد سببت انخفاض في عدد كرات الدم الحمراء، كرات الدم البيضاء و محتوى الهيموجلوبين في الدم و الألبومين و البروتين الكلى في المصل و الجلوتاثيون، GST , SOD في البلازما بشكل متناسب مع الجرعة و الوقت. و أيضاً تسببت في ارتفاع ملحوظ في الميتيموجلوبين في الدم و ALT , AST , ALP , GGT , الكرياتينين، اليوريا، الدهون الثلاثية، الكوليسترول الكلي، السكر و MDA في المصل بشكل متناسب مع الجرعة و الوقت. أظهر الفحص الميكروسكوبي لأنسجة الجرذان المعاملة بنترتيت الصوديوم في نهاية التجربة تغيرات باثولوجية في الكبد و الكلى و المخ و القلب و الرئة و الخصيتين. كما لوحظت زيادة في شدة الأعراض متناسبة مع زيادة الجرعة. أدت المعاملة بالمستخلص الإيثانولي لبذور العنب (150 مجم / كجم من وزن الجسم / يوم) الى مغايرة كل التأثيرات السلبية لنترتيت الصوديوم على كل التحاليل البيوكيميائية المختبرة و كذلك تحسن واضح في مستوى مضادات الأكسدة. وعلاوة على ذلك كان التأثير الإيجابي لمستخلص بذور العنب أكثر وضوحاً مع الجرعة المنخفضة من نترتيت الصوديوم و الوقت الأطول. أيضاً اظهر مستخلص بذور العنب قدرة على حماية معظم الأنسجة المختبرة من التأثير الضار للجرعة المنخفضة من نترتيت الصوديوم و أيضاً تخفيف حدة الأعراض الناتجة عن الجرعة المرتفعة. خلصت الدراسة إلى أن مستخلص بذور العنب يمكن اعتباره أحد مضادات الأكسدة الواعدة الممكن إستخدامها في الحد من الاكسدة و السمية الناجمة عن نترتيت الصوديوم.

الكلمات الدالة: نترتيت الصوديوم، مستخلص بذور العنب، الجهد المؤكسد، التحاليل البيوكيميائية و الفحص النسيجي.