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Original Article *Biochemistry*

The protective effect of sulforaphane in rats fed on high cholesterol high fructose diets

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ABSTRACT

***Objective*:** To evaluate the protective role of sulforaphane in rats exposed to high cholesterol and high fructose diet.
***Design*:** Randomized experimental study.
***Animals*:** Twenty-four male Sprague Dawley rats.
***Procedures*:** Rats were allocated in groups of six animals to one of four groups. The first group was kept as a control group in which rats were fed on a basal diet for 15 weeks (Control), while in the second group (Control + SFN) rats were fed on the basal diet for 11 weeks then a sulforaphane (SFN) was given (0.5 mg/kg/day) orally for additional 4 weeks. The third group was the high cholesterol high fructose (HCF) where rats were fed on the basal diet mixed with a solution of cholesterol (1 %) and fructose (10 %) for 15 weeks, while in the fourth group (HCF + SFN) high cholesterol high fructose diet and sulforaphane rats were fed on the basal diet mixed with a solution of cholesterol (1 %) and fructose (10 %) for 11 weeks then a SFN was given orally (0.5 mg/kg/day) for another 4 weeks. Serum and plasma samples were collected to determine the glycemic status, lipid profile, antioxidant status, oxidative and nitrosative stress markers, and apoptotic marker, alongside liver tissue samples for histopathological examination.
***Results*:** Results revealed that sulforaphane alleviated the oxidative damage (decreasing MDA and NO) and improved the antioxidant status (reducing glutathione), and enhanced glycemic status through decreasing plasma glucose concentration and decreasing caspase 9 concentration.
***Conclusion and clinical relevance*:** It can be suggested that sulforaphane (SFN) can improve insulin resistance (I.R) and improve serum lipid profile.

Keywords: Sulforaphane, Caspase 9, IR.

1. INTRODUCTION

Type 2 diabetes accounts for more than 90 % of all cases of diabetes and is characterized by insulin resistance and a defect in insulin secretion from pancreatic □□cells. The relative importance of alterations in insulin sensitivity versus secretion is debatable. It is accepted that hyperglycemia, hence diabetes, does not develop without □□cell dysfunction. Alteration of pancreatic □□cell function leading to an impaired insulin secretory response to glucose is a hallmark of the transition from the pre-diabetic to the diabetic state [1]. The elevated levels of cholesterol in hepatic tissues might lead to mitochondrial damage and eventually destruction in hepatocytes [2]. Broccoli is one among the few vegetables that is claimed to possess antidiabetic potency and is commonly consumed in India. It has a beneficial hypoglycemic influence in both experimental animals and humans through improving insulin resistance in type 2 diabetic patients [3]. Moreover, one of the hallmarks of type 2 diabetes is the exaggerated hepatic glucose production due to insulin resistance, other counteracting hormones will be increased in its activity stimulation gluconeogenesis. Sulforaphane (SFN), as an active principle of broccoli, has been suggested as a new potential anti-diabetic compound as it has a promising effect on hepatic glucose production [4]. However, the molecular principle of how SFN acts in different cell types is yet only partly understood. Therefore, the current study was conducted to evaluate the potential protective effect of SFN on glycemic status, lipid profile, antioxidant status, oxidative stress markers, apoptotic factor in rats received high cholesterol high fructose diets for 15 weeks.

2. MATERIALS AND METHODS

2.1. Animals

Twenty-four male Sprague Dawley rats were used in this study and were allocated into four groups (experimental treatments). Animals were housed in metal cages, where each cage contained six rats. They were supplied with a clean drinking water that was renewed every day, and a clean

bedding material (sawdust) that was changed twice/week. Animals were left for accommodation for fourteen days prior to the induction of the experiment. The experimental protocol was approved by Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt.

2.2. Chemicals

Cholesterol powder used in the experiment was bought from Techo Pharmchem (Techo Pharmchem, Bahadurgarh, India). Fructose was obtained from El Gomhorya Company, (Egypt). Sulforaphane was obtained from MilliporeSigma (USA).

2.3. Experimental design

After accommodation, rats were allocated in groups of six animals to one of the four following groups: the first group was kept as a control group (Group 1) in which animals were fed on a basal diet according to NRC, (1995) (Control). The second group (Group 2), incorporated rats fed on a basal diet for 11 weeks, in addition to an oral administration of a SFN (0.5 mg/kg/day) [5] for an additional 4 weeks (Control + SNF). The third group (Group 3) included rats fed on a basal diet mixed with a solution of cholesterol 1% [6], and fructose 10 % (HCF) [7] for 15 weeks. The fourth group (Group 4) included rats that fed on a basal diet mixed with a solution of cholesterol 1% and fructose 10 % (HCF) for 11 weeks then a SFN was given (0.5 mg/kg/day) orally for another 4 weeks (Table 1).

2.4. Collection of blood samples

After 30 days of experimental induction, anesthesia of rats was induced and maintained with sodium thiopental (Pharco, Co, Egypt) (20 mg/kg) [8]. After rats were completely sedated, blood was drawn from heart through cardiac puncture in dry, clean, sterile and capped tubes that was left to clot, where clear serum sample was aspirated and transferred to clean Eppendorf for estimating serum leptin concentration [9], serum insulin concentration [10], serum total cholesterol (TC) concentration [11], serum HDL-cholesterol (HDL-c) concentration [12], serum LDL-cholesterol (LDL-c) concentration [13] and serum triacylglycerol (TAG) concentration [14]. Other portion of the collected blood was transferred to clean test tubes containing sodium fluoride for determination of plasma glucose concentration [15].

2.5. Tissue sampling

After blood collection, animals were euthanized by cervical dislocation, then were dissected and liver was removed, washed by normal saline and divided into three parts. The first part was immersed in 10 % neutral buffered formalin for histopathological examination according to Woods et al. [16]. The second part was homogenized in phosphate buffer saline (pH 7.4) for determination of liver reduced glutathione concentration (GSH) [17], hepatic malondialdehyde (MDA) concentration [18], and hepatic nitric oxide (NO) concentration [19]. The third part of hepatic tissues was used to determine caspase 9 (cas 9) concentration using flow cytometry technique [20].

2.6. Statistical analysis

Data was analyzed using SPSS v.17. One-way ANOVA was used to test for the effect of experimental treatment (diet) with LSD as a post-hoc test at a significant level of 0.05. Data was expressed as means ± standard errors [21].

Table 1. Formulated basal and high cholesterol diet used for rats throughout the study.

For each 100 kg		
Food ingredients	**Number of kilograms (Basal diet)**	**HC (high cholesterol)**
Yellow corn	70.6 kg	70.2 kg
Soya bean meal 40 %	23.3 kg	23 kg
Gluten 60 %	1.7 kg	1.5 kg
Corn oil	975 ml	975 ml
Lime stone	1.6 kg	1.5 kg
Sodium chloride	300 gm	300 gm
Dibasic phosphate	950 gm	950 gm
Premix	350 gm	350 gm
Sodium bicarbonate	300 gm	300 gm
Anti-mycotoxin	300 gm	300 gm
Cholesterol	0 kg	1 kg

* Per 1 kg vitamin-mineral premix contains: 12,000 IU vitamin A, 2,400 IU vitamin D_3, 20 mg vitamin E, 4 mg vitamin K_3, 3 mg vitamin B_1, 7 mg vitamin B_2, 25 mg niacin (vit. B_3), 10 mg pantothenic acid (vit. B_5), 5 mg vitamin B_6, 15 µg vitamin B_{12}, 50 µg biotin, 1 mg folic acid, 50 mg vitamin C, 100 mg Mn, 60 mg Fe, 60 mg Zn, 5 mg Cu, 2 mg I, 500 µg Co, 150 µg Se.

3. RESULTS

Regarding the effect of Sulforaphane on the glycemic status of rats, serum levels of glucose and leptin increased in rats of HCF group, but decreased after treatment with SFN when compared to those of other groups. Moreover, serum insulin levels increased in rats of Control + HCF group compared to those of rats in the remaining groups, and decreased in rats of HCF group after treatment with SFN when compared to other groups (Table 2).

Regarding the effect of Sulforaphane on the lipid profile of rats, rats of HCF group showed increased levels of serum TC, serum TAG, serum LDL-c, however these levels were decreased after treatment with SFN (Table 3). Rats of the HCF

showed the lowest values for serum HDLc, and the highest values after treatment with SFN.

Rats of the HCF group displayed lower concentrations of hepatic GSH and NO compared to those in the Control and Control + SFN groups (Table 4). Treatment with SFN induced a marked increase in concentration of GSH and NO in rats of HCF group compared to those of HCF + NSF. Hepatic MDA concentrations were higher in rats of HCF group compared to those in the other groups. However, these high levels of MDA decreased after treatment with SFN (Table 4).

Table 2. Means ± SEM Plasma glucose (mmol/L), serum insulin (µIU/ml) and serum leptin (pg/ml) in rats fed on basal (Control), basal and Sulforaphane (Control + SFN), high cholesterol high fructose (HCF), and high cholesterol high fructose supplemented with Sulforaphane (HCF + SFN) diet**S**

Group	Serum glucose mmol/L	Serum insulin µIU/ml	Serum leptin pg/ml
Control	4.42 ± 0.09^{b}	2.22 ± 0.075^{bc}	1.80 ± 0.40^{c}
Control + SFN	4.29 ± 0.16^{c}	2.72 ± 0.076^{a}	1.80 ± 0.115^{c}
HCF	13.80 ± 0.46^{a}	2.43 ± 0.066^{ab}	6.40 ± 0.577^{a}
HCF + SFN	6.43 ± 0.42^{b}	1.91 ± 0.073^{c}	3.66 ± 0.176^{b}

In each column, means having different letters differ significantly

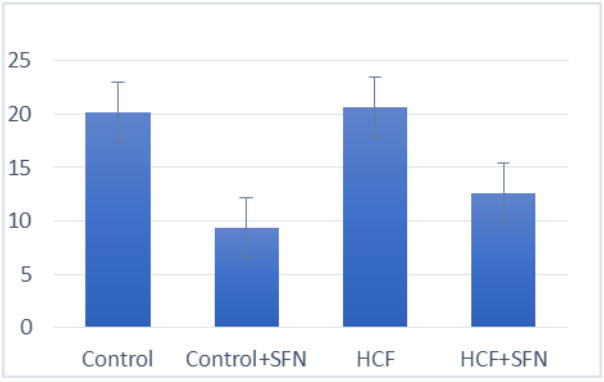

Figure 1. Flow cytometric assay of CAS 9 concentration in rats received basal (Control), basal and Sulforaphane (Control + SFN), high cholesterol high fructose (HCF), and high cholesterol high fructose supplemented with Sulforaphane (HCF + SFN) diets.

There was a significant reduction in hepatic concentrations of CAS 9 in rat's treated with SFN in both Control and HCF group (Figure 2). Results of the histopathological examination of hepatic tissue are illustrated in Figure 2. In Figure 2A liver tissue displayed normal hepatocytes (arrow) in normal histological architecture with normal blood vessels and normal hepatic sinusoids (HE, 400x). In Figure 2B, liver displayed normal hepatocytes (arrow) in normal radial arrangement around central vein (HE, 400x). In Figure 2C, liver showed a massive hemorrhage replacing hepatic parenchyma with hepatocytes with a signet ring appearance (cytoplasm contains sharp clear vacuoles with pushed flattened nuclei to the cell membrane) (arrows) (HE, 100x). In Figure 2D, liver showed focal hepatocytes with a signet ring appearance. Moreover, there were focal lymphocytic infiltrates in hepatic parenchyma (arrow). (HE, 400x).

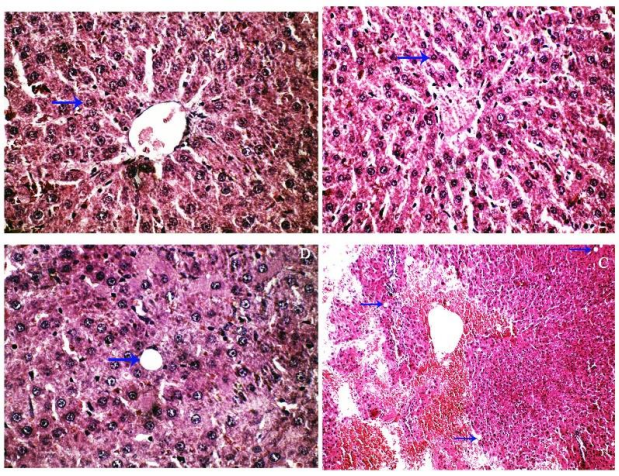

Figure 2. Histopathological examination of hepatic tissue in rats received basal (Control), basal and Sulforaphane (Control + SFN), high cholesterol high fructose (HCF), and high cholesterol high fructose supplemented with Sulforaphane (HCF + SFN) diets. (A) Hepatic tissue of rats in (Control), (B) Hepatic tissue of rats in SFN, (C) Hepatic tissue of rats in (HCF) and (D) Hepatic tissue of rats in (HCF + SFN) group.

4. DISCUSSION

Numerous studies showed that insulin resistance precedes the development of hyperglycemia in subjects that eventually developed type 2 diabetes (T2D). However, it is increasingly being realized that T2D only develops in insulin resistant subjects with the onset of β cell dysfunction [22]. Moreover, cholesterol loading on MIN6 cells derived from pancreatic β cells leads to cholesterol-induced apoptosis in a time- and dose-dependent manner. Also, treatment with methyl-β-cyclodextrin that removes cholesterol from plasma membrane prevented the cells from cholesterol- induced apoptosis [23]. Sulforaphane (SFN) is an isothiocyanate derived from cruciferous vegetables such as broccoli and cabbage, and it plays a major role in energy metabolism [24].

HCF rats showed a significant impairment in glucose tolerance to exogenously administered glucose, as shown by elevated glycemic levels at 60–120 min post glucose challenge, compared with the control group [25]. The molecular mechanism of SFN protection against diabetic nephropathy is through Nrf2 activation of antioxidant enzymatic defenses (NADPH-quinone oxireductase and c- glutamyl cysteine synthetase) suggesting that this same pathway could exert an

insulin-independent action in the reduction of blood glucose [26].

In addition, SFN has the potential to increase energy expenditure by enhancing uncoupling protein 1 (UCP1) expression in inguinal and epididymal adipose tissue depots [27]. Sulforaphane could also attenuate hyperglycemia-induced endothelial damage via inhibition of the mitochondrial ROS production and inactivation of the hexosamine and PKC pathways and protein glycation [28].

A threefold increase in insulin levels was detected in HCF rats, relative to controls [25]. In conclusion, Bahadoran et al. [3] mentioned that 4 weeks supplementation with broccoli sprout rich in SFN had favorable effects on decreasing serum insulin and improving IR in type 2 diabetes. Shawky et al. [29] revealed that fasting plasma insulin levels were higher by 2.1-fold in high fructose and cholesterol fed rats when compared to those of the control group. This may indicate a dysfunction of adipose tissue in maintaining appropriate levels of leptin to overcome the state of leptin resistance observed in obese subjects particularly where insulin resistance is developed [30].

Previous studies have shown that in high fat diet- or high fructose/cholesterol diet-fed mice with SFN or SFN precursor (glucoraphanin) a marked in body weight gain and plasma leptin concentration resulted [27]. Sulforaphane prevents cholesterol-induced lipid per-oxidation as the exposure to cholesterol doubled lipid peroxidation in Min6 cells. This was completely averted in the presence of 10 µM SFN [31].

In a study conducted by Hoshida et al. [32], they found that hypercholesterolemia induced microvascular dysfunction characterized by loss of endothelium-derived nitric oxide. However, the intake of dietary phase 2 protein inducers would ameliorate both hypertension and atherosclerotic changes by scavenging of superoxide anion through the activation of nitric oxide [33]. Oral ingestion of broccoli sprouts, as a dietary source of SFN, protected smooth muscle cells from oxidative injury by inducing cellular and mitochondrial antioxidants and phase-II enzymes (superoxide dismutase, catalase, reduced glutathione, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, and NADP(H) quinone oxidoreductase) [34]. In a previous study conducted by Korish & Arafah, [35], the ingestion of high dietary cholesterol resulted in a significant increase in TC, TAG, LDL-c and VLDL-c, and decreased the HDL-c levels. Additionally, the results of the current work corroborate with those of Choi et al. [36], who found that mice fed on a high-fat diet plus 0.1 % SFN for 6 weeks had low levels of total cholesterol and triglycerides. In another study by de Souza et al. [37], a significant lower levels of serum TAG were observed, however the supplementation of 0.5 mg/kg of SFN in the diet improved this alteration in serum lipid profile.

A study carried out by Pham et al. [38] illustrated the presence of two major pathways for caspase activation; the death receptor and mitochondrial pathway consistent with known amplification loop of the caspase pathways, confirming that sulforaphane acts within the death receptor pathway of caspase activation.

Conclusion

It can be concluded that SFN can produce a hypoglycemic effect on rats that suffered from insulin resistance, and could also have a hypolipidemic effect.

Table 3. Means ± SEM Serum total cholesterol (mmol/L), serum triacylglycerol (mmol/L), serum HDL (mmol/L) and serum LDL (mmol/L) in rats received basal (Control), basal and Sulforaphane (Control + SFN), high cholesterol high fructose (HCF), and high cholesterol high fructose supplemented with Sulforaphane (HCF + SFN) diets

Group	Serum cholesterol (TC) mmol/L	Serum Triacylglycerol (TAG) mmol/L	Serum HDL-c mmol/L	Serum LDL-c mmol/L
Control	2.36 ± 0.07^{c}	1.59 ± 0.03^{c}	0.89 ± 0.02^{a}	1.01 ± 0.01^{c}
Control + SFN	1.62 ± 0.08^{d}	1.05 ± 0.02^{c}	0.63 ± 0.05^{b}	0.92 ± 0.02^{c}
HCF	3.61 ± 0.05^{a}	5.94 ± 0.30^{a}	0.35 ± 0.01^{c}	2.99 ± 0.06^{a}
HCF + SFN	2.88 ± 0.06^{b}	2.79 ± 0.25^{b}	0.60 ± 0.05^{b}	1.92 ± 0.18^{b}

In each column, means having different letters differ significantly

Table 4. Means ± SEM Hepatic reduced glutathione (mg/g), malondialdehyde (nmol/g) and nitric oxide (μmol/g) in rats received basal (Control), basal and Sulforaphane (Control + SFN), high cholesterol high fructose (HCF), and high cholesterol high fructose supplemented with Sulforaphane (HCF + SFN) diets.

Group	Reduced glutathione (GSH) concentration (mg/g tissue)	Malondialdehyde (MDA) concentration (nmol/g tissue)	Nitric oxide (NO) concentration (μmol/g tissue)
Control	4.44 ± 0.587[a]	7.17 ± 0.965[b]	4.73 ± 0.962 [bc]
Control + SFN	4.31 ± 0.193[a]	3.77 ± 0.443[c]	4.63 ± 0.691 [c]
HCF	1.77 ± 0.587[b]	12.08 ± 0.775[a]	9.00 ± 1.431 [a]
HCF + SFN	5.10 ± 0.588[a]	7.52 ± 0.559[b]	13.41 ± 1.850 [ab]

In each column, means having different letters differ significantly

Conflict of interest statement

The authors declare that there is no any conflict of interest in the current research work.

Permission of Animal Ethics Committee

The current research work was approved by Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University.

Authors´ contributions

Wael El-Shahat conducted the experiment and analytical procedures, Mohamed EL-Adl performed sample collection, statistical analysis, research writing and correspondence for research, Mohamed Hamed conducted histopathological examination, and Youssef El-Saedy revised the manuscript and supervised the whole work.

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