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## Therapeutic effectiveness of fenugreek seeds on pancreatic toxicity in young and adult male rats exposed to water nitrate pollution

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### Abstract

Water nitrate pollution has been demonstrated to induce pancreatic toxicity, with increased risk of developing diabetes and exocrine pancreatic cancer. This study aimed to evaluate mechanisms that lead to pancreatic toxicity in nitrate exposed young and adult male rats, as well as the preventive effect of fenugreek seeds against these changes. Nitrate exposure of both ages showed almost identical changes, including significant reduction in serum insulin with elevation in blood glycosylated hemoglobin, serum glucose, acid phosphatase,  $\alpha$ -amylase and pancreatic tumor marker (carcinoembryonic antigen), indicating a tendency for developing diabetes, in association with pancreatic cancer which seemed of greatest value in the young group. An elevation in pancreas levels of nitric oxide, hydrogen peroxide and lipid peroxidation, with a reduction in the antioxidants; superoxide dismutase and catalase, as well as glutathione and its metabolizing enzymes; glucose-6-phosphate dehydrogenase and  $\gamma$ -glutamyl transferase were also demonstrated. This goes with marked changes in insulin gene profile, as indicated by appearance of new band with a molecular weight 275 bp and absence of three (1100, 825 and 750) bands as compared to the control of each age. Feeding fenugreek seeds powder to all nitrate exposed rats prevented changes in serum glucose, insulin, pancreatic tumor marker, oxidative stress biomarkers and insulin gene profile, indicating effectiveness of this plant to elicit cyto-protection against nitrate-induced pancreatic toxicity, probably through its antioxidant properties.

**Key words:** Nitrate pollution; Pancreatic cancer; Antioxidants; Fenugreek; Insulin gene profile.

## 1.Introduction

Nitrate contamination of drinking water is a major health problem for many people, particularly in the rural areas (Zhai Y *et al.*, 2016). Although nitrate occurs naturally at low levels, human activities including uncontrolled use of nitrogenous fertilizers and improper disposal of animal manure or human waste can elevate levels of nitrate in sources of drinking water (Ward *et al.*, 2005 and Stayner *et al.*,2017).At high levels, nitrates have been linked to a wide range of health hazards, including genotoxicity (Bouaziz-Ketata *et al.*, 2015), renal insufficiency and liver damage ( Anwar and Mohamed, 2015). Experimentally, it was found that increased uptake of water contaminated with nitrate may cause; hypothyroidism, methemoglobinemia (El-Wakf *et al.*,2009a,b) and hypogonadism (El-Wakf *et al.*, 2011).

An association between high nitrate levels and pancreatic toxicity was also reported. In particular, nitrate may cause chronic pancreatitis, coupled with diabetes which is more likely to occur in both young and adult population (Aschebrook- kilfoy *et al.*, 2011; El-Wakf *et al.*, 2015). Besides, increased nitrate exposure was suggested to increase risk of pancreatic cancer, which is mostly originated in the exocrine tissue and is classified as ductal adenocarcinoma (Debrin *et al.*, 2008). Possible associations between onset of diabetes and pancreatic cancer have been postulated. Some studies evidenced that diabetes may be a causative factor for risk of pancreatic cancer (Ilic and Ilic, 2016). Others indicated that patients with diabetes have approximately twice the risk of developing pancreatic cancer compared with those without diabetes (Boursi *et al.*, 2017). Although these findings are alarming, studies considering such association and the

prevention strategies are limited or not available. Thus, a need has been felt for more investigations in this vital area.

Currently, numerous herbs are available as a safe and inexpensive approach for treatment and control of wide range of diseases. Fenugreek (*Trigonella foenum graecum*) is one of the oldest medicinal plants, dating back to ancient Egyptians where it was used in incense and to embalm mummies (Basch *et al.*,2003). Fenugreek seeds have also used as anti-inflammatory, antineoplastic (Shabbeer *et al.*, 2009), antioxidant, hypoglycemic and hypocholesterolemic agent (Khole *et al.*, 2014).Additionally, fenugreek seeds have shown to possess restorative and nutritive properties (Doshi *et al.*,2012) and to stimulate healing of different ulcers (Suja Pandian *et al.*,2002). Although fenugreek has been described widely for various medicinal properties, little is known about its use against toxic effects relevant to water pollution.

Regarding the above observations, the present study was designed to characterize whether increased exposure to nitrate polluted water is an etiologic factor for the association between diabetes and pancreatic cancer in both young and adult rats, with focusing on the underlying mechanisms. Also, to evaluate effectiveness of fenugreek seeds administration against these diseases.

The present work aimed to study whether nitrate polluted water had association between diabetes and pancreatic cancer in both young and adult rats. Also to focus on the underlying mechanisms and to evaluate the possible protective effect of fenugreek seeds administration against these diseases.

## 2. Materials and methods

### 2.1. Experimental animals

This study was performed on healthy male Wistar rats of two ages; young (3 weeks old,  $35\pm 5$  gm) and adult (12 weeks old,  $175\pm 5$  gm) obtained from the Institute of Ophthalmic Disease Research, Cairo, Egypt. Rats were housed in ventilated cages in an automatically illuminated and thermally controlled room ( $25\pm 2$  °C) with 12-h light/dark cycle. Rats were fed a standard diet purchased from Meladco Feed Company (Aubor City, Cairo, Egypt) with free access to water. Experimental procedures were in accordance with the Ethical Principles adopted by Mansoura University for Care and Use of Laboratory Animals and the protocol prepared by the Institute of Laboratory Animal Resources, National Research Council "NRC" (NRC, 1995).

### 2.2. Experimental diet

Fenugreek (*Trigonella foenum graecum*) seeds were obtained from a store in Mansoura city. Fenugreek supplemented diet (5% w/w) was prepared according to El-Wakf *et al.* (2015), where seeds were washed and dried, then crushed in an electric grinder. Fenugreek powder (50gm) was added to (950gm) standard diet together with some distilled water to form small pellets that left to dry in open air, then kept in clean plastic containers. The pellets were prepared once a week and preserved below 5 °C (Raju *et al.*, 2001).

### 2.3. Experimental design

Experiments were started after one week of acclimation. Animals of each age were randomly di-

vided into four groups (six animals each) as follows: **Group I, normal control (NC)** group; received normal diet with no additives. **Group II (FG)**, animals were fed the experimental diet with fenugreek seeds' powder (5% w/w) as seen above. **Group III (NT)**, animals were fed normal diet and allowed to uptake sodium nitrate ( $\text{NaNO}_3$ ) dissolved in normal drinking water (550 mg/L) according to National Toxicology Program, (2001), where each rat approximately received 47.7 mg/kg b.wt./day. **Group IV (NT+FG)**, rats were received  $\text{NaNO}_3$  in water plus fenugreek supplemented diet in accordance with doses mentioned above. Animals were supplied with their respective treatments daily for period of four months.

### 2.4. Blood and tissue sampling

At the end of the study period, animals were fasted overnight then anesthetized using ether and sacrificed by cervical dislocation. From each of the studied rats, two blood samples were obtained; one on EDTA for glycosylated hemoglobin (Hb A1c)% determination, while the second was left to coagulate, and centrifuged at 860 xg for 15 min at 4°C, then non hemolysed sera were collected and kept at -80°C until hormonal and biochemical analysis. Pancreas was immediately removed and rinsed with ice-cold saline. Specimens of pancreas homogenate were taken, weighed and homogenized for biochemical analysis, while other specimens were removed and preserved at -80°C for later molecular analysis.

## 2.5. Biochemical analysis

In the tissue homogenates, **reduced glutathione (GSH)** was assessed according to the method achieved by Prins and Losse (1969), in which tungstate-sulfuric acid solution precipitated the protein content, then after the reaction with 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) yellow color is formed. The absorbance was determined within 30-60 sec at 412 nm against the blank. **Superoxide dismutase (SOD)** activity was assayed as described by Nishikimi *et al.* (1972). The assay relies on the ability of the enzyme to inhibit phenazine methosulphate mediated reaction of nitroblue tetrazolium dye and the increase in absorbance after 5 min was measured at 560 nm. **Catalase (CAT)** activity was determined according to the method of Bock *et al.* (1980), based on the ability of CAT to decompose  $H_2O_2$ . The decomposition of  $H_2O_2$  can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of CAT activity. The level of **malondialdehyde (MDA)**, the end product of lipid peroxidation (LPO) was determined as thiobarbituric acid reactive substance (TBARS) according to the modified method of Ohkawa *et al.* (1979). **Serum carcinoembryonic antigen (CEA)** was estimated according to the electrochemiluminescence immunoassay "ECLIA" intended for use on Roche Elecsys1010 immunoassay analyzer (Guder *et al.*, 1996). **Serum insulin** was estimated using INS-EASIA kit according to the immune-enzymatic assay (Temple *et al.*, 1992), while HbA1c % was determined using Teco diagnostics kit obtained from United States (Gonen and Rubenstein, 1978).

## Other biochemical parameters

Biochemical parameters encompassing, glucose, acid phosphatase (ACP), glucose -6- phosphate dehydrogenase (G-6-PD),  $\gamma$ -glutamyl transferase ( $\gamma$ -GT),  $\alpha$ - amylase, nitric oxide (NO) and hydrogen peroxide ( $H_2O_2$ ) were assessed using kits supplied by Bio Diagnostic Company for diagnostic reagents (Dokki, Giza, Egypt).

## 2.6. Molecular analysis

### DNA extraction

For DNA extraction, pancreatic specimens were homogenized in 5ml sterile distilled water. The homogenate (350  $\mu$ l) was mixed with 0.6 ml of digestion buffer and 0.1 ml proteinase K (50mg/ml), vortexed and incubated overnight in water bath at 55 °C till clearing. Samples were mixed with equal volumes of phenol-chloroform isoamyl alcohol, centrifuged at 18626 xg for 3 min and the supernatant was transferred to a new eppendorf tube. To get rid of RNA, 0.003 ml RNase solution was added, mixed thoroughly and incubated in a water bath at 37°C for 30 min. DNA was precipitated by adding sodium chloride solution (1M) and iso-propanol. Mixture was inverted several times till fine fibers of DNA appeared, then centrifuged at 18626 xg for 3 min and the supernatant was removed. For washing, 1ml of 70% ethyl alcohol was added and centrifuged for 3 min at 18626 xg. DNA was collected, dried and reconstituted in a suitable volume of sterile deionized water and stored at 4 °C until use (Ausubel *et al.*, 1999).

### **Polymerase chain reaction (PCR)**

Equal volumes (1  $\mu$ l) of genomic DNA and each of insulin gene primers {forward (ACCACCA G CCCTAA- GTGATCCGCTA) and {reverse (AAGGTTTTATTTCATT-GCAGAGGGGT)} were mixed with one PCR bead. Mixture was completed to 20  $\mu$ l with sterile deionized water, inverted many times and collected again by spin at maximum speed, followed by centrifugation for 5 sec at 18626 xg.

For insulin gene amplification, PCR mixture was transferred to thermal cycler programmed for 40 cycles as follows:

1 min at 94 °C for DNA denaturing, 1 min at 57 °C for annealing and 2 min at 72 °C for new DNA extension, followed by final DNA extension at 72 °C for 7 min. PCR product and 75-20000 bp DNA ladder (Gene Ruler™) were mixed with bromophenol blue loading dye (6x) then analyzed by electrophoresis on 1% agarose gel mixed with ethidium bromide and the amplified DNA bands were visualized under UV light (Sambrook *et al.*,1998).

### **Statistical analysis**

Data analysis was done using SPSS statistical package software (version 17) for multiple variable

comparisons by one way analysis of variance (One-way ANOVA) followed by post hoc least significant difference (LSD) test. Data obtained from each group were expressed as means  $\pm$ SE. The level of significance was set as  $p \leq 0.05$  (Snedecor and Cochran,1980).

## **3.Results**

### **3.1.Serum biochemical parameters**

Feeding fenugreek supplemented diet to normal animals of both ages showed significantly reduced serum carcinoembryonic antigen (CEA),  $\alpha$ -amylase and acid phosphatase (ACP) activities only in the young group, while serum glucose, insulin and Hb A1c% showed non-significant changes compared to normal control (NC) animals. However, consumption of drinking water contaminated by NaNO<sub>3</sub> (550 mg/L) exhibited significant elevation in serum glucose, Hb A1c%, ACP and CEA, while a significant reduction in insulin level was recorded in the two studied age groups, particularly, the young compared to their control groups. The above alterations tended to be restored near to normal values on feeding fenugreek diet with nitrate contaminated water uptake in both ages relative to their nitrate exposed groups (Table 1).

### 3.2. Pancreas biochemical parameters

Obtained results revealed significantly elevated pancreatic (SOD, CAT,  $\gamma$ -GT and GSH) and significantly reduced LPO, NO and  $H_2O_2$ , with non significantly changed G-6-PD activity in fenugreek fed normal rats of both ages compared to corresponding control groups. On contrast, pancreatic (SOD, CAT, GSH, G-6-PD and  $\gamma$ -GT) were significantly reduced, but LPO, NO and  $H_2O_2$  were significantly increased in all nitrate exposed animals, especially in the young ones compared to the control animals. It should also be noted that fenugreek administration with nitrate was found to alleviate such nitrate-induced biochemical changes on comparing to nitrate exposed groups (Table 2).

### 3.3. Insulin gene changes

As illustrated in Figure 1 and 2, the extracted pancreatic DNA gave positive PCR reaction when amplified by insulin gene primers. Generally, the two age groups were identical to each other regard-

ing the PCR profiles; where the control of each age group (lane 1) produced four main bands with molecular weights 1100, 825, 750 and 600 bp. Similarly, fenugreek groups of both young and adult animals (lanes 2, 3) produced the same profile and bands like the control. In nitrate group, the first sample of each age (lanes 4) produced the same four main bands 1100, 825, 750 and 600 bp like the control; beside a new band with a molecular weight 275 bp was appeared. While the second sample (lane 5) was highly different from the control, in which three bands (1100, 825 and 750) of the four main bands were completely disappeared. Thus, nitrate exposure can be considered mutagenic for insulin gene in both young and adult animals. On contrast, PCR product of nitrate + fenugreek group of both ages (lanes 6, 7) showed a pattern near to the control groups in having the same four main bands 1100, 825, 750 and 600 bp, indicating the ability of fenugreek to protect against nitrate-induced insulin gene mutation

Table 1 : Serum tested parameters of all studied experimental groups

Tested Parameters	Animal groups							
	Young groups				Adult groups			
	NC	FG	NT	NT+FG	NC	FG	NT	NT+FG
Glucose (mg/dl)	107.29±0.38	97.28 <sup>a</sup> ±0.36 (-9.33)	179.68 <sup>a</sup> ±1.85 (+67.47)	116.76 <sup>ac</sup> ±0.51 (+8.83)	105.40±1.25	100.13 <sup>b</sup> ±0.59 (-5.00)	161.81 <sup>b</sup> ±2.15 (+53.52)	114.71 <sup>bd</sup> ±1.74 (+8.83)
Hb A1c%	3.07±0.15	3.05±0.08 (-0.65)	4.62 <sup>a</sup> ±0.18 (+50.49)	3.27 <sup>c</sup> ±0.03 (+6.51)	3.32±0.09	3.30±0.13 (-0.60)	4.27 <sup>b</sup> ±0.13 (+28.61)	3.38 <sup>d</sup> ±0.06 (+1.81)
Insulin (μIU/ ml)	1.38±0.06	1.40±0.01 (+1.45)	1.16 <sup>a</sup> ±0.01 (-15.94)	1.30 <sup>ac</sup> ±0.01 (-5.80)	1.34±0.01	1.35±0.01 (+0.75)	1.15 <sup>b</sup> ±0.003 (-14.18)	1.28 <sup>bd</sup> ±0.01 (-4.48)
ACP (U/L)	32.89±0.93	27.91 <sup>a</sup> ±0.49 (-15.14)	61.82 <sup>a</sup> ±1.41 (+87.96)	40.16 <sup>ac</sup> ±0.35 (+22.10)	36.17±0.57	32.46 <sup>b</sup> ±0.28 (-10.26)	56.04 <sup>b</sup> ±0.57 (+54.94)	38.48 <sup>bd</sup> ±0.56 (+6.39)
α-amylase (U/L)	7.10±0.29	6.82±0.28 -3.94	10.49 <sup>a</sup> ±0.25 +47.75	7.41 <sup>c</sup> ±0.04 +4.37	8.55±0.04	8.49±0.02 -0.70	9.32 <sup>b</sup> ±0.08 +9.01	8.85±0.03 +3.51
CEA (ng/dl)	28.50±0.07	27.79 <sup>a</sup> ±0.08 (- 2.49)	29.55 <sup>a</sup> ±0.04 (+ 3.68)	28.87 <sup>ac</sup> ±0.03 (+ 1.30)	28.60±0.14	28.35±0.09 (- 0.87)	29.33 <sup>b</sup> ±0.11 (+ 2.55)	28.65 <sup>d</sup> ±0.17 (+ 0.17)

- Values are means±SE of 6 animals for each group. The values in parentheses are percentage of change from control.

- Values bearing superscript are significantly different by ANOVA test at  $p \leq 0.05$ .

NC: normal Control, FG: Fenugreek, NT: Nitrate, NT+FG: nitrate +fenugreek.

a: significant as compared to young control group ,b: significant as compared to adult control group.

c: significant as compared to young nitrate group ,d: significant as compared to adult nitrate group.

Table 2. Pancreas tested parameters of all studied experimental groups

Tested parameters	Animal groups							
	Young group				Adult group			
	NC	FG	NT	NT+FG	NC	FG	NT	NT+FG
SOD (U/g wet tissue)	131.33±1.34	145.44 <sup>a</sup> ±1.15 (+10.74)	91.32 <sup>a</sup> ±2.47 (-30.47)	124.79 <sup>ac</sup> ±0.59 (-4.98)	138.93±1.92	145.61 <sup>b</sup> ±0.99 (+4.81)	99.52 <sup>b</sup> ±2.25 (-28.37)	133.72 <sup>bd</sup> ±0.89 (-3.75)
CAT (µmol/sec /g wet tissue)	2.51±0.04	3.19 <sup>a</sup> ±0.15 (+27.09)	0.47 <sup>a</sup> ±0.01 (-81.27)	2.48 <sup>c</sup> ±0.06 (-1.19)	2.80±0.20	3.54 <sup>b</sup> ±0.37 (+26.43)	0.69 <sup>b</sup> ±0.02 (-75.36)	2.76 <sup>d</sup> ±0.05 (-1.61)
γ-GT (U/g wet tissue)	22.09±0.34	42.17 <sup>a</sup> ±0.75 (+90.90)	10.75 <sup>a</sup> ±0.35 (-51.34)	9.54 <sup>ac</sup> ±0.02 (-11.54)	40.82±0.73	53.63 <sup>b</sup> ±1.27 (+31.38)	30.80 <sup>b</sup> ±1.11 (-24.55)	40.28 <sup>d</sup> ±0.50 (-1.32)
G-6-P-D (mU /g wet tissue)	40.26±0.40	41.04±0.82 (+1.94)	25.07 <sup>a</sup> ±0.45 (-37.73)	35.80 <sup>ac</sup> ±0.24 (-11.08)	38.08±0.06	38.65±0.26 (+1.50)	24.36 <sup>b</sup> ±0.71 (-36.03)	34.20 <sup>bd</sup> ±0.72 (-10.19)
GSH (mg/100g wet tissue)	22.50±0.34	24.85 <sup>a</sup> ±0.90 (+10.44)	15.80 <sup>a</sup> ±0.54 (-29.78)	20.40 <sup>ac</sup> ±0.40 (-9.33)	34.00±0.73	36.92 <sup>b</sup> ±0.58 (+8.59)	27.29 <sup>b</sup> ±0.44 (-19.74)	32.92 <sup>d</sup> ±0.45 (-3.18)
LPO (nmol/g wet tissue)	302.85±0.91	296.68 <sup>a</sup> ±1.73 (-2.04)	400.21 <sup>a</sup> ±2.19 (+32.15)	312.36 <sup>ac</sup> ±0.97 (+3.14)	473.56±0.70	465.56 <sup>b</sup> ±1.11 (-1.69)	595.57 <sup>b</sup> ±1.12 (+25.76)	514.44 <sup>bd</sup> ±0.53 (+8.63)
NO (µmol/g wet tissue)	11.96±0.19	10.28 <sup>a</sup> ±0.09 (-14.05)	14.76 <sup>a</sup> ±0.31 (+23.41)	12.32 <sup>c</sup> ±0.39 (+3.01)	11.87±0.04	10.33 <sup>b</sup> ±0.10 (-12.97)	14.30 <sup>b</sup> ±0.25 (+20.47)	12.19 <sup>d</sup> ±0.11 (+2.70)
H <sub>2</sub> O <sub>2</sub> (mM/g wet tissue)	1.27±0.03	0.91 <sup>a</sup> ±0.01 (-28.35)	3.79 <sup>a</sup> ±0.07 (+198.43)	1.49 <sup>ac</sup> ±0.01 (+17.32)	1.07±0.04	0.88 <sup>b</sup> ±0.02 (-17.76)	2.15 <sup>b</sup> ±0.04 (+100.93)	1.24 <sup>bd</sup> ±0.07 (+15.89)

- Values are means± SE of 6 animals for each group. The values in parentheses are percentage of change from control.

- Values bearing superscript are significantly different by ANOVA test at  $p \leq 0.05$ .

NC: normal Control, FG: Fenugreek, NT: Nitrate, NT+FG: nitrate +fenugreek.

a: significant as compared to young control group ,b: significant as compared to adult control group.

c: significant as compared to young nitrate group ,d: significant as compared to adult nitrate group.



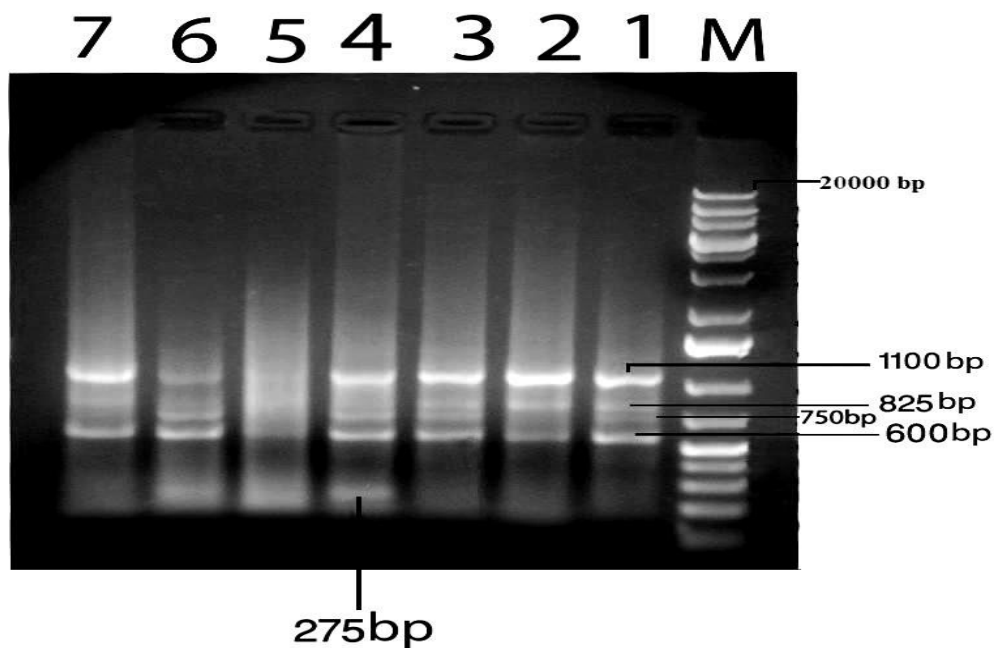


Figure 1 : Agarose gel electrophoresis of PCR product from pancreatic tissue in different young groups. Lane 1 is control (NC), lanes 2,3 is fenugreek (FG), lanes 4, 5 are nitrate (NT) and lanes 6, 7 are nitrate with fenugreek (NT+FG) respectively. Lane M is 1kb ladder "75- 20000 bp" DNA marker.

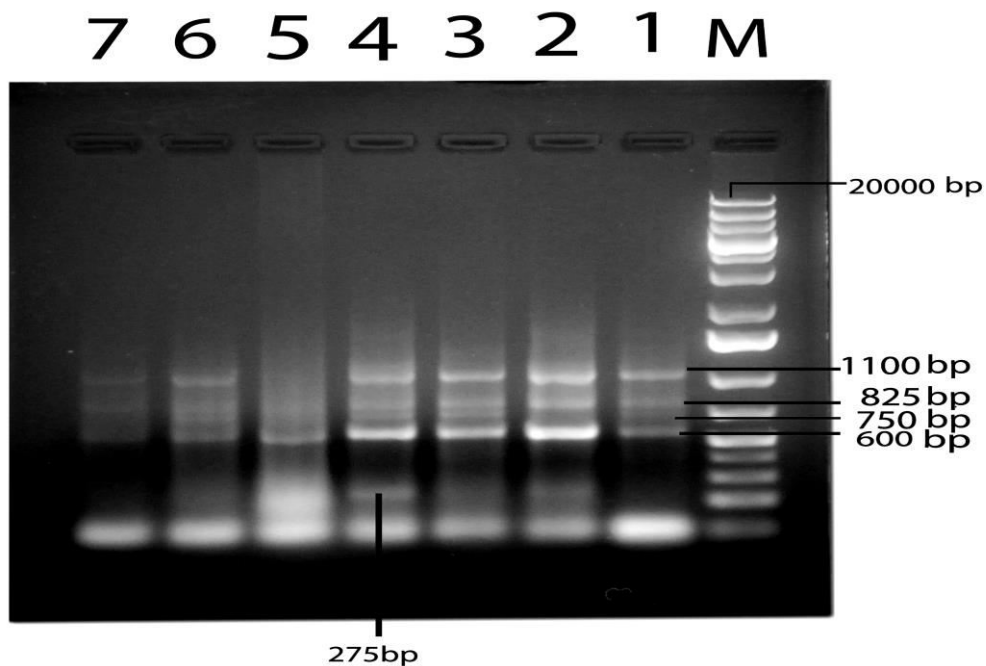


Figure 2: Agarose gel electrophoresis of PCR product from pancreatic tissue in different adult groups. Lane 1 is control (NC), lanes 2, 3 are fenugreek (FG), lanes 4, 5 are nitrate (NT) and lanes 6, 7 are nitrate with fenugreek (NT+FG) respectively. Lane M is 1kb ladder "75- 20000 bp" DNA marker.

#### 4. Discussion

Various studies have addressed a relation between incidence of diabetes and nitrate exposure (Marienfeld *et al.*, 2007; Benson *et al.*, 2010; El-Wakf *et al.*, 2015). Others extended to describe nitrate as a causative or inductive factor for pancreatic cancer that predominantly arises in the exocrine portion (Debrin *et al.*, 2008). Thus, suggesting nitrate as an environmental toxicant for both exocrine and endocrine pancreas. Subsequently, a correlation between diabetes and pancreatic cancer has been suggested by several researchers (Pannala *et al.*, 2009; Zavoral *et al.*, 2011).

Observations from clinical studies indicated that diabetic patients have a risk of evolving pancreatic cancer approximately twice the nondiabetic ones (Huxley *et al.*, 2005). Also, there is evidence that the majority of diabetics associated with pancreatic cancer is diagnosed either concomitantly with the cancer or before cancer was developed (Pannala *et al.*, 2009). Therefore, it appears appropriate to suggest that recently-developed pancreatic cancer may be a consequence of diabetes and that events associated with diabetes are involved in promoting the development of pancreatic cancer.

In the current study, nitrate exposed rats exhibited marked diabetic state, characterized by elevation in glucose concentration and Hb A1c%, accompanied with reduction in insulin level. The study also revealed higher tendency for pancreatic cancer, through elevated serum level of carci-

noembryonic antigen (CEA) that often accompany conditions of pancreatic tumor (Yegneswaran and Pitchumoni, 2010). This goes concomitantly with shift towards elevation in serum  $\alpha$ -amylase activity. Although  $\alpha$ -amylase is mostly used to detect pancreatitis; increased pancreatic output of amylase with elevation of its serum level is indicative to exocrine pancreatic cancer in some studies (Rückert *et al.*, 2010). For all, the present study tends to suggest that high nitrate intake from drinking water is potentially toxic for both endocrine and exocrine pancreas, with a link between diabetes and pancreatic cancer may exist in the two tested age groups, in particular the young one. A result that may be explained by the fact that young individuals usually have low gastric acidity, where nitrate favors conversion to other toxic nitrogen compounds that increased susceptibility at the young age (Gupta *et al.*, 2008; El-Wakf *et al.*, 2009a,b). As support, it was reported that gastric acidity of the young children is not as strong as adults that favors the growth of nitrate-reducing bacteria, leading to conversion of nitrates into high levels of the potentially toxic nitrites ( $\text{NO}_2$ ) (Kusaka *et al.*, 2016).

Previously, it was indicated that nitrate toxicity relies mainly on its ability to generate oxygen free radicals and to induce oxidative stress (El-Wakf *et al.*, 2011). Therefore, it appears appropriate to suggest oxidative stress as a general pathophysiological pathway by which nitrate exposure can lead to diabetes in association with

pancreatic cancer. Related to this, it was evidenced that diabetes and pancreatic cancer may share a number of underlying mechanisms; however oxidative stress may play a key role (Malati 2007; Abdal Dayem *et al.*, 2010).

Findings from the present study demonstrated increased pancreatic  $H_2O_2$  and NO following nitrate exposure which in turn may elicit oxidative stress as contributing factor in developing diabetes. In support, it was reported that nitrate can cause oxidative damage to pancreas through elevation of  $H_2O_2$  and NO (Brindicci *et al.*, 2010). Moreover, it was explained that the major target of nitrate damaging species ( $H_2O_2$  and NO) is pancreatic  $\beta$ -cells, which are particularly prone to attack because  $\beta$ -cells are unique in containing low levels of free radicals- scavenging enzymes (Wierusz-Wysocka *et al.*, 1997). A relation between *in vivo* NO and onset of diabetes has been consequently evidenced. This effect is probably linked to interaction of NO with superoxide anion ( $O_2^-$ ) to form peroxynitrite ( $ONOO^-$ ) which is tissue destructive (Brindicci *et al.*, 2010). Peroxynitrite may also considered mutagenic because it can cause deamination of DNA and inactivation of DNA repair enzymes (Hou *et al.*, 2007). Besides, peroxynitrite can oxidize DNA and may potentially cause single-strand DNA breaks through attack on the sugar-phosphate backbone (Xu *et al.*, 2002). Considering this, it can suggest that increased  $H_2O_2$  and NO in response to nitrate exposure may enhance pancreatic DNA damage, causing insulin gene mutations, as seen in the current study. An effect which may directly aid in developing diabetes.

In parallel, a number of studies have suggested increased free radicals and oxidative stress to play a key role in initiation and progression of organs carcinogenesis, including pancreatic cancer (Crohns *et al.*, 2013). In particular, oxidative stress has been reported to affect cellular membrane integrity which in turn may affect structure and function of cellular organelles, as lysosomes. Increased activity of lysosomal enzymes, as acid phosphatase was linked with certain types of cancer in human (Goto *et al.*, 2001; Malati, 2007). Accordingly, the presently increased activity of acid phosphatase in serum of nitrate exposed rats may be considered as marker of oxidative stress contributing to development of pancreatic cancer.

Increased oxidative stress refers to an imbalance between intracellular production of reactive oxygen species (ROS) and the cellular defense mechanisms. Oxidative stress is known to be associated with increased lipid peroxidation and reduced antioxidant activity. Lipid peroxidation arises due to conversion of cellular polyunsaturated fatty acids to toxic products known as lipid peroxides, including malondialdehyde (MDA). Owing to MDA cytotoxicity and inhibitory action on cellular protective enzymes, it is suggested to act as a tumor promoter (Manimaran and Rajneesh, 2009). Accumulation of MDA is emphasized to increase during carcinogenesis with accompanying depletion of SOD and CAT activities and GSH content (Amin *et al.*, 2012). SOD is considered the first line of defense against deleterious effects of oxygen free radicals in the cells by catalyzing dismutation of superoxide radicals ( $O_2^-$ ) to  $H_2O_2$  and molecular oxygen. CAT is responsible for detoxification of

H<sub>2</sub>O<sub>2</sub> which is an effective inhibitor of SOD (De duve and Baudhhuin 1996); thus the reduction in the activity of CAT may reflect inability of tissues to eliminate H<sub>2</sub>O<sub>2</sub>. CAT protects SOD against inactivation by H<sub>2</sub>O<sub>2</sub>, while SOD protects CAT against inhibition by O<sub>2</sub><sup>-</sup>. Thus, the balance of this enzyme system may be essential to get rid of ROS generated in the tissues. In this concern, GSH represents an important defense mechanism in protecting cells against ROS. Decreased GSH content may be occurred by reduced activity of the enzymes, G-6-PD and  $\gamma$ -GT being required for maintaining cellular GSH level (El-Wakf 1996; Ansari and Mahmood, 2015). This effect when taken with reduced activities of both SOD and CAT may indicate enhanced oxidative stress leading to increased risk of pancreatic cancer, as seen in the current study.

Dietary habits and nutrition are known to play important role in alleviating oxidative stress being related to development of several health problems (Prabhu and Krishnamoorthy 2010). Fenugreek is a herb whose seeds and leaves are used not only as food but also as ingredients in the traditional medicine. Recently, fenugreek seeds have been demonstrated to possess a number of phytochemicals being accepted as natural antioxidants. These include vitamins, flavonoids, terpenoids, carotenoids, coumarins, curcumins, lignin and saponin (Zheng and Wang 2001). As support, it was demonstrated that fenugreek seeds prevented *in vitro* oxidative hemolysis and lipid peroxidation induced by H<sub>2</sub>O<sub>2</sub> in human erythrocytes (Kaviarasan *et al.*, 2004; Al-Amri and Al-rasheedi, 2016). Moreover, dietary supplementation with fenugreek caused a reduction in bi-

omarkers of oxidative damage, coupled with increased antioxidant potential in the diabetic rats (Ravikumar and Anuradha 1999). Epidemiologic evidence also indicated that long term feeding of fenugreek seeds showed no signs of toxicity in diabetic patients (Goyal *et al.*, 2016). Thus; it can suggest that fenugreek can be used safely for protecting against free radicals - mediated diseases, as diabetes.

Other protective action of fenugreek seeds is through reducing initiation and progression of tissue tumors (Alizadeh *et al.*, 2009). Consumption of phenolic rich foods, as fenugreek has been proved to reduce incidence of tissue cancer (Randhir *et al.*, 2004). However, fenugreek does not act directly as an anticarcinogenic agent, but it can suppress generation of ROS through its antioxidant activity, thereby decreasing radical mediated sequence of events leading to carcinogenesis. As support, the antioxidant constituents; flavonoids of fenugreek seeds have shown to be apoptotic in human carcinoma cells (Ahmed *et al.*, 2000; Al-Amri and Al-rasheedi, 2016). Apoptosis is a type of cell death, and agents with ability to induce apoptosis in tumor cells have the potential to be used for antitumor therapy. It appears that fenugreek flavonoids can differentially induce apoptosis in cancer cells, but not in their normal counter parts, thereby inhibit the growth of cancer cells without affecting the growth of normal cells (Alizadeh *et al.*, 2009). This action differentiates fenugreek from chemical agents that kill both cancer and normal cells. Thus, fenugreek seems to act selectively via attacking cancer cells. Evidence supporting this was provided by (Alizadeh *et al.*, 2009) who reported that fenu-

greek has the ability to induce *in vitro* cytotoxic effect to a panel of cancer, but not normal cells. Treatment with fenugreek also showed growth inhibition to breast, pancreatic and prostate cancer cell lines (Shabbeer *et al.*, 2009). Moreover, a potential protective effect of fenugreek seeds was observed against 7,12 - dimethylbenz (a) anthracene (DMBA) induced breast cancer in rats (Amin *et al.*, 2005).

In the current study, use of fenugreek seeds has demonstrated marked protection against nitrate induced- pancreatic toxicity. The protective signs included, a reduction in the levels of H<sub>2</sub>O<sub>2</sub>, NO and MDA in pancreatic tissue, in association with increased values of SOD, CAT, GSH, GGT and G-6-PD. This, in all, goes with consequent improvement in values of glucose, HbA1c% and insulin, as well as pancreatic tumor marker (CEA),  $\alpha$ -amylase and acid phosphatase activities. Thus, it can suggest that fenugreek having the ability to reduce nitrate-induced endocrine and exocrine pancreatic toxicity, primarily

through inhibiting various oxidative pathways linking between diabetes and pancreatic cancer.

In conclusion, nitrate exposure can be considered as a portent agent for deterioration of both endocrine and exocrine pancreas. Accordingly, an association between high nitrate level in drinking water and incidence of diabetes and pancreatic cancer has been presently demonstrated in the two investigated ages, particularly the young one that comprise the most susceptible group to nitrate toxicity. However, feeding fenugreek seeds to all nitrate exposed rats seemed to positively affect incidence of these diseases. So, fenugreek seeds can be advised to be included in diet of population inhabiting rural areas with high nitrate levels in drinking water, in particular the more sensitive young children.

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