Flaxseed and Vitamin E Exert Synergic Antioxidant Action on Diabetic Nephropathy in Experimental Diabetes

RALUCA ECATERINA HALIGA[1], DOINA BUTCOVAN[2], TEODOR OBOROCEANU[1], ALIN CONSTANTIN PINZARIU[1], VICTOR VLAD COSTAN[3], DRAGOS VALENTIN CRAUCIUC[4], ALLIA SINDILAR[2], RAOUL VASILE LUPUSORU[1]*, VERONICA MOCANU[1]

[1]Grigore T. Popa University of Medicine and Pharmacy, Faculty of Medicine, Department of Morpho-Functional Sciences II, 16 Universitatii Str., 700115, Iasi, Romania

[2]Grigore T. Popa University of Medicine and Pharmacy, Faculty of Medicine, Department of Morpho-Functional Sciences I, 16 Universitatii Str., 700115, Iasi, Romania

[3]Grigore T. Popa University of Medicine and Pharmacy, Department of Oral and Maxillofacial Surgery, 16 Universitatii Str., 700115, Iasi, Romania

[4]Institute of Legal Medicine, 4 Buna Vestire Str., 700455, Iasi, Romania

This study investigated the effects of flaxseed and vitamin E on diabetic nephropathy lesions in an experimental-induced model of diabetes in hamsters. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) in male Golden Syrian hamsters, and diabetic animals were fed either standard diet, or standard diet supplemented with flaxseed (150 g/kg diet), vitamin E (400 mg α-tocopherol/kg diet) or combination of flaxseed and vitamin E in the same dosages, for 20 weeks. Kidney histological evaluation of the diabetic hamsters revealed histological lesions characteristic for diabetic nephropathy, while supplementation of the diet with flaxseed and/or vitamin E improved histological aspects of diabetic nephropathy.

Key words: diabetes mellitus, flaxseed, vitamin E, oxidative stress, diabetic nephropathy

Diabetic nephropathy (DN) is a chronic complication of diabetes mellitus (DM) and one of the leading causes of end-stage renal disease. Numerous studies demonstrated that oxidative stress contributes to the development and progression of chronic diabetic complications [1,2]. It is presumed that ameliorating oxidative stress through supplementation with antioxidants might be an effective strategy to reduce these disorders. Dietary supplementation with antioxidants may be useful in maintaining a desirable pro oxidative/antioxidative balance [3], a variety of dietary sources from plants being currently of considerable interest, related to their potential health benefits in many diseases, including chronic diabetic complications [4].

Flaxseed (linseed or *Linum usitatissimum*) has been recognised as a functional food, due to its nutrient components and its demonstrated potential to prevent cardiovascular diseases [5,6]. Flaxseed is one of the richest source of n-3 polyunsaturated fatty acids (n-3 PUFA) – mainly alpha-linolenic acid (ALA; ~22% of whole flaxseed), lignans (secoisolariciresinoldiglucoside - SDG), being also an essential source of dietary fibers and proteins [7]. The lignan SDG has significant antioxidant effects by inhibiting DNA scissions, lipid peroxidation and decreasing reactive oxygen species (ROS), protecting PUFA from oxidation [8]. Despite beneficial effects of SDG and n-3 PUFA, it was shown that flaxseed consumption can reduce á and ã tocopherol concentrations in rats [9] and only vitamin E supplementation restored its plasmatic concentrations [10]. In clinical and experimental studies, vitamin E supplementation demonstrated an important role in delaying the onset and progression of diabetic complications, by increasing levels of antioxidant enzymes (superoxiddismutase – SOD, glutathionperoxidase - GSH Px) [11]. However, there are no data regarding the effects of adding vitamin E to flaxseed enriched diet on oxidative stress-induced DN.

In the present study, by hypothesizing that vitamin E supplementation could increase antioxidant protection of flaxseed, we investigated the effects of dietary flaxseed, vitamin E and their combination on DN lesions in an experimental-induced model of DM in hamsters, by examining serum and renal oxidative stress, proteinuria and kidney histopathological changes.

Experimental part

Animals and diets

In this study were used six-months old male Golden Syrian hamsters, purchased from the Cantacuzino National Institute for Research and Development in Microbiology and Immunology (Bucharest, Romania). The experiment respected the instructions of the general guidelines for the care and use of laboratory animals, recommended by the Council of European Communities [12]. All experimental procedures were approved by the Laboratory Animal Care Committee of the University of Medicine and Pharmacy Gr. T. Popa Iasi, Romania.

Throughout the experiment, the animals were housed in standard laboratory conditions, in a room with controlled temperature (21 ± 2°C), with 12 h light/12 h dark cycle. Hamsters were kept singly into mesh cages made of stainless steel, with an appropriate size, provided with continuous manure cleaning with water and proper ventilation.

Diabetes was induced in overnight fasted hamsters using streptozotocin (STZ; catalog no. S0130; Sigma-Aldrich, St. Louis, MO, USA), freshly solved in citrate buffer (50 mM, *p*H 4.5) and administered intraperitoneally, in a single dose of 50 mg/kg body weight. Citrate buffer (50 mM) was prepared from sodium citrate and citric acid (C.S. Chemical Company, Romania) solved in distilled water, with adjusted *p*H to 4.5. The day after STZ injection, hamsters with fasting blood glucose over 126 mg/dL (7 mmol/L) were considered diabetics. The control hamsters (C) received equivalent amounts of citrate buffer 50 mM, intraperitoneally too.

*email: rvlupusoru@yahoo.com

The control (C) and diabetic (DM) hamsters received the diets for a 20-weeks period, as follows: 1) groups C (n=6) and DM (n=10) received standard diet; 2) DM + Linum group (n=10) received standard diet supplemented with ground flaxseed (Linum usitatissimum; 15g /100 g of food); 3) DM + E group (n=11) received standard diet supplemented with vitamin E (40 mg á-tocopherol/100g of food; Sigma-Aldrich); 4) DM + Linum + E (n=11) group received standard diet supplemented with flaxseed (15 g%) and vitamin E (40 mg α-tocopherol/100g food). Flaxseed of the Olin variety was provided by the Department of Phytotechny, Faculty of Agronomy (Iasi, Romania).

All four diets had similar carbohydrate, total fiber, protein, and fat contents (table 1).

Animal necropsy and processing of samples

At the end of the experiment, animals were placed in metabolic cages and timed-urine collections (24 h) were made with the hamsters in the fasting state. After collections, the hamsters were anaesthetized with an intraperitoneal injection of a mixture of Ketamine, dosage of 15 mg/kg bodyweight (Romvac, Romania) and Xylazine, dosage of 10 mg/kg bodyweight (Hipervet, Romania), followed by opening the chest and collecting the blood by cardiac puncture. Blood samples were collected and serum was separated by centrifugation at 3000 rot/min, for 20 minutes at 4°C. Aliquots of serum were frozen and kept at -80ÚC for subsequent analysis.

The kidneys were dissected free of fat, immediately removed and rinsed with ice-cold phosphate buffer saline (*p*H 7.2). Fragments of kidneys were placed in a sealed container, homogenized for oxidative stress parameters analysis and stored at -20°C until analyzed. Also, fragments of kidneys were used for histopathological examination.

Metabolic and biochemical parameters

The level of serum glucose was measured by enzymatic colorimetric methods, on Tecan microplate reader (Tecan SunriseTM Touchscreen, Männedorf, Switzerland), using the AD1A716 commercially available kits (Audit Diagnostics, Cork, Ireland). Urinary protein excretion was measured by Bradford method [13].

Serum and renal thiobarbituric acid reactive substances (TBARS), as indexes of oxidative stress, were determined using the method adapted after Phelps and Harris [14]. TBARS levels were measured using TECAN microplate reader (Tecan SunriseTM Touchscreen, Männedorf, Switzerland), at a wavelength of 540 nm.

Renal antioxidative capacity in DM was investigated by the levels of superoxiddismutase (SOD) and reduced glutathione (GSH) in kidney homogenate. Renal levels of GSH were determined by an enzymatic method, based on the oxidation of GSH by 5,5' dithiobis(2 nitrobenzoic acid) (DTNB), in the presence of GSH reductase and $NADPH_2$, measured at a wavelength of 405 nm [15]. Renal SOD levels were measured with the method adapted by Minami and Yoshikawa [16]. Renal concentrations of proteins were determined by Bradford assay, using Bradford reagent (Sigma, code B6916) and bovine serum albumin (Fluka, code 05481) (adapted after 13).

Morphological study of kidneys

Kidneys were processed for histological examination (formalin-fixed renal tissue was processed for paraffin embedding and the samples were cut into 4-6 μm) and stained with hematoxylin-eosin (HE) and periodic acid Schiff's (PAS) stain (Bioptica Milano SpA, Milan, Italy). Hematoxylin stains the cell nuclei in blue, whereas eosin stains eosinophilic structures in various shades of red. PAS stains the nuclei in blue and glycogen deposits in magenta.

Table 1
COMPOSITION OF THE DIETS

Ingredients	**Standard diet**	**Standard diet supplemented with flaxseed**	**Standard diet supplemented with vitamin E**	**Standard diet supplemented with flaxseed + E**
	g/kg diet	g/kg diet	g/kg diet	g/kg diet
Carbohydrates	330	330	330	330
Fibers	110	110	110	110
Proteins	240	240	240	240
Lipids	160	160	160	160
Saturated	96	96	96	96
Monounsaturated	7	7	7	7
Polyunsaturated	57	57	57	57
n-6 PUFA (sunflower oil)	57	23	57	23
n-3 PUFA (flaxseed*)		**34**		**34**
Colin	3	3	3	3
Vitamin A	200,000 IU	200,000 IU	200,000 IU	200,000 IU
Vitamin C	1	1	1	1
Vitamin D	10,000 IU	10,000 IU	10,000 IU	10,000 IU
Vitamin E	0.05	0.05	**0.4 (400 mg)**	**0.4 (400 mg)**
Calcium	6.2	6.2	6.2	6.2
Phosphate	3.9	3.9	3.9	3.9
Potasium bicarbonate	20	20	20	20

* Flaxseed are represented by OLIN variety, provided by the Department of Phytotechny from the Agronomy Faculty Iasi, Romania and were chromatographyc analyzed. The composition of flaxseed was: 40,2% oil (55,6% linolenic acid) and 19,5% proteins.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Student's t test and Bonferroni's multiple comparison test (Statistical Software Package SPSS®, version 13, SPSS Inc., Chicago, IL, USA). Unpaired Student's t-tests were performed to determine whether there were significant differences between groups ($p<0.05$).

Results and discussions

Serum glucose levels

Supplementation of the diet with flaxseed and combination flaxseed+vitamin E significantly decreased glycemia in DM groups compared to unsupplemented diabetic group (table 2).

Proteinuria levels

DM was associated with significant increase in urinary concentrations of proteins compared to control group, representing a marker of DN. Supplementation of the diet with flaxseed and/or vitamin E had beneficial effects, significantly decreasing proteinuria (table 2).

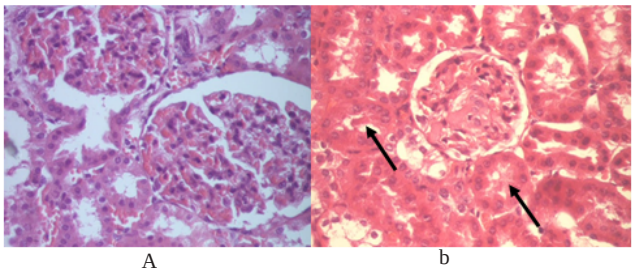
Parameters of oxidative stress

Our main results regarding oxidative stress parameters are summarized in table 3.In DM groups, serum and renal TBARS were increased compared to C group, as markers of oxidative stress. The levels of these markers significantly decreased by the addition of flaxseed or vitamin E, while combined flaxseed + vitamin E diet induced a more significant decrease of these parameters. The renal SOD and GSH significantly increased by the addition of combined flaxseed + vitamin E diet, with the highest values compared to single flaxseed or vitamin E supplementation.

Histological changes in renal tissues

In control group, histological exam of the kidneys with H-E staining revealed normal glomerular morphology, permeable capillaries, thin capillary wall, free Bowman space and intact Bowman's capsule (fig.1A), while in diabetic group with standard diet, histological evaluation (HE and PAS) revealed changes characteristic for DN, consisting of increase of glomerular size, active mesangial cells and PAS positive mesangial deposits, thickening of the basal capillary membranes; the capillary lumens could not be seen, being reduced, filled with erythrocytes (fig.1B).

Supplementation of the diet with lignans and n3 PUFA from flaxseed and/or vitamin E improved the histological aspects of DN, microscopic exam revealing reduction of



A b

Fig. 1. Images of the kidney. (A) Kidney from control group fed with standard diet (H-E staining; magnification, x 20). Free Bowman space and intact Bowman's capsule, thin capillary wall. (B) Kidney from DM group fed with standard diet (H-E staining; magnification, x 20). Basal capillary membrane with mucopolysacharides deposits, mezangium with active mezangial cells and eosinophilic substances; thickening of the capillary basal membrane, reduced capillary lumens (arrows)

PAS positive neutral mucopolysacharides (MPS) deposits, glomerules with visible, permeable capillary lumens (fig.2A), less thickened glomerular capillary membrane (fig. 2B, 3).

Hyperglycemia in DM leads to increased oxidative stress and decreased antioxidant defense, oxidative stress being the main cause of DN [1]. In the present study, we investigated the antioxidative effects of dietary supplementation with flaxseed, α tocopherol and their combination on an animal model of DM. We used 15% flaxseed diet supplementation in hamsters, similar in energetic load to 40 g/day dosage used in human trials, corresponding to approximately 10% of total energy intake [17].

Flaxseed has been investigated in experimental and clinical studies for its potential role in preventing cardiovascular diseases. The use of whole grain flaxseed significantly reduced reactive oxygen species generation, mainly due to the lignan SDG and its metabolites, enterodiol

Table 2

MEAN ± SD VALUES FOR GLYCEMIA AND URINARY PROTEIN EXCRETION IN STUDIED GROUPS

MEASURES	Control	DM	DM+Linum	DM+E	DM+Linum+E
Glycemia (mg/dl)	62.5±6.9	362.4±59.9§*	163.4±46.6*	220.6±43.8	139.1±42.6*
Urinary protein excretion (mg/dl)	0.142±0.1	0.204±0.2*	0.166±0.2*	0.125±0.2*	0.102±0.2*

Values are means±SD

§ $p< 0.05$ as compared to Control group

*$p< 0.05$ as compared to group fed with standard diet

MEASURES	Control	DM	DM+Linum	DM+E	DM+Linum+E
Serum TBARS (nmol/ml)	1.5±0.3	4.3±0.3§	2.6±0.3*	2.8±0.3*	2.1±0.3*
Renal TBARS (nmol/mg protein)	0.13±0.1	0.17±0.2	0.14±0.1	0.13±0.2*	0.12±0.2*
Renal SOD (U/mg protein)	0.12±0.02	0.08±0.01*	0.13±0.03*	0.15±0.02	0.16±0.02*
Renal GSH (μmol/mg protein)	0.03±0.01	0.01±0.001	0.05±0.01	0.05±0.02	0.07±0.03§*

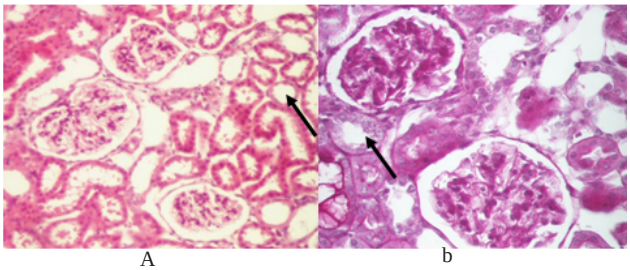
Values are means±SD

§ $p< 0.05$ as compared to Control group

*$p< 0.05$ as compared to group fed with standard diet

Table 3

MEAN ± SD VALUES FOR SERUM AND RENAL OXIDATIVE STRESS PARAMETERS IN STUDIED GROUPS



A b

Fig. 2. Images of the kidney. (A) Kidney from DM group with flaxseed supplemented diet (H-E staining; magnification, x 10). Reduced glycogen deposits; glomerules with permeable capillary lumens (arrows). (B) Kidney from DM group with vitamin E supplemented diet (PAS staining; magnification, x 20). Reduced neutral mucopolysacharides deposits, visible and permeable glomerular capillaries lumens (arrows)

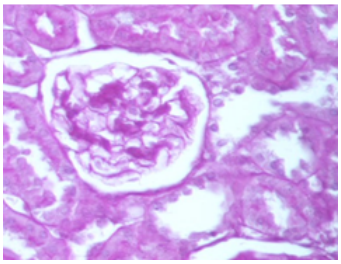


Fig. 3. Image of the kidney. Kidney from DM group with combined flaxseed-vitamin E supplemented diet (PAS staining; magnification, x 20). Weak positive PAS reaction in glomerules; intact Bowman's capsule, visible urinary space

and enterolactone [4]. Few studies have been conducted to evaluate the effects of dietary supplementation with flaxseed on DN, regarding the relationship between oxidative stress, antioxidative protection and renal histopathological changes [18]. Concerns were also raised regarding the supposition that diet supplementation with flaxseed could decrease plasma vitamin E in rats [9].

Our study confirmed previous studies upholding that DM increased oxidative stress, expressed as serum and renal TBARS (measured in kidney homogenate) [2]. The lipid peroxides formed under diabetic conditions have been counteracted by dietary inclusion of flaxseed, vitamin E or their combination, the highest decrease of serum TBARS being determined by combined flaxseed+vitamin E diet, suggesting the strong antioxidant capacity of this association. Renal lipid peroxidation was significantly dimin-ished by supplementation of the diet with flaxseed or α tocopherol alone, while combination flaxseed +vitamin E further significantly decreased renal TBARS.

Increased oxidative stress is associated with histological renal changes of DN [19,20]. Recent clinical studies investigated the relationship between oxidative stress and antioxidant system in diabetic conditions, showing significant correlations between the extent of microalbuminuria and markers of oxidative stress [21]. All three diets in our study had protective effects on DN, suggested by the improvement of histological lesions and decreased renal TBARS levels compared with unsupplemented diabetic group. Also, combined flaxseed+vitamin E diet had a better effect on antioxidant protection in diabetic conditions, as shown by the raise of SOD and GSH in renal homogenate. The combined supplementation was also associated with a more significant decrease in plasma glucose levels and urinary protein excretion in diabetic animals, indicating that the renal protective effects were in concordance with glycemic control.

The results of this experimental study suggest that higher antioxidant effects of flaxseed associated with vitamin E are determined by their synergic actions. Flaxseed has antioxidant properties through its content in lignans, mainly SDG, demonstrated by inhibition of lipid peroxidation or by direct hydroxyl radical scavenging activity [4,22]. There are few experimental studies suggesting that dietary flaxseed has protective effects on the kidneys in animal models of chronic renal disease. Thus, in a study on obese rats, dietary protein substitution with flaxseed meal reduced proteinuria, glomerular and tubulointerstitial lesions and flaxseed meal was more effective than soy protein in reducing proteinuria and renal histologic abnormalities [18].

Conclusions

To our knowledge, the present study is the first one which demonstrates that the combined flaxseed+vitamin E diet had beneficial effects on histological lesions of DN, associated with changes in metabolic parameters, significant reduced glucose concentrations, urinary protein excretion and renal oxidative stress. Thus, concerns that flaxseed supplementation of the diet could decrease vitamin E actions were not confirmed by this study, showing that the combination of flaxseed and vitamin E could be better for renal protection in DM, by decreased oxidative stress and increased antioxidant protection in renal homogenate, these results being in concordance with improvement of glycemic control and reducing of urinary protein excretion.

References

1.PEDRAZA CHAVERRI, J., SaNCHEZ-LOZADA, L.G., OSORIO-ALONSO, H., et al., Oxid. Med. Cell. Longev., **2016,** article 6043601, 2016, p.1.
2.KUMAWAT, M., SHARMA, T.K., SINGH, I., et al., N. Am. J. Med. Sci., **5,** no. 3, 2013, p. 213.
3.JOHANSEN, J.S., HARRIS, A.K., RYCHLY, D.J., et al., Cardiovasc. Diabetol., **4,** article 5, 2005, p. 1.
4.PRASAD, K., Int. J. Angiol., **9,** no. 4, 2000, p. 220.
5.GOYAL, A., SHARMA, V., UPADHYAY, N., et al., J. Food. Sci. Technol., **51,** no. 9, 2014, p. 1633.
6.PRASAD, K., Circulation, **99,** no. 10, 1999, p. 1355.
7.SINGH, K.K., MRIDULA, D., REHAL, J., et al., Crit. Rev. Food. Sci. Nutr., **51,** no. 3, 2011, p. 210.
8.MOREE, S.S., KAVISHANKAR, G.B., RAJESHA, J., Phytomedicine, **20,** no. 3-4, 2013, p. 237.
9.FRANK, J., ELIASSON, C., LEROY NIVARD, D., et al., Br. J. Nutr., **92,** no. 1, 2004, p. 169.
10.FLIS, M., SOBOTKA, W., ANTOSZKIEWICZ, Z., et al**.,** J. Anim. Feed. Sci., **19,** no. 4, 2010, p. 539.
11.BABURAO JAIN, A., ANAND JAIN, V., J. Clin. Diagn. Res., **6**, no. 10, 2013, p. 1624.
12.GENERAL GUIDELINES FOR THE CARE AND USE OF LABORATORY ANIMALS RECOMMENDED BY THE COUNCIL OF EUROPEAN COMMUNITIES, Official Journal of European Communities, Brussels, no. 1-18, 1986.
13.BRADFORD, M.M., Anal. Biochem., **72**, 1976, p. 248.
14.PHELPS, S., HARRIS, W.S., Lipids, **28**, no. 5, 1993, p. 475.
15.TIETZE, F., Anal. Biochem., **27**, no. 3, 1969, p. 502
16.MINAMI, M., YOSHIKAWA, H., Clin. Chim. Acta., **92**, no. 3, 1979, p. 337.
17.MOCANU, V., HALIGA, R., PADURARU, O., et al., Journal of Biologically Active Products From Nature, **1**, no. 3, 2011, p. 144.
18.VELASQUEZ, M.T., BHATHENA, S.J., RANICH, T., et al., Kidney Int., **64**, no. 6, 2003, p. 2100.
19.MAKNI, M., SEFI, M., FETOUI, H., et al., Food. Chem. Toxicol., **48**, no. 8-9, 2010, p. 2407.
20.STOIAN, A., EARAR, K., BUDACU, C., et al., Rev. Chim. (Bucharest), **67**, no. 11, 2016, p. 2355.
21.SHAO, N., KUANG, H.Y., WANG, N., et al., J. Diabetes. Res., **2013,** article 232404, 2013, p.1
22.HU, C., YUAN, Y.V., KITTS, D.D., Food. Chem. Toxicol., **45**, no. 11, 2007, p. 2219

Manuscript received: 3.02.2017