AMELIORATING THE METABOLIC DISORDER IN STREPTOZOTOGIN-INDUCED DIABETIC RATS BY HAEMATOCOCUS PLUVIALIS EXTRACT

FAROUK K EL-BAZ*, HANAN F AL Y

1Department of Plant Biochemistry, National Research Centre, Giza, Egypt. 2Department of Therapeutic Chemistry, National Research Centre, Giza, Egypt. Email: elbazfk@gmail.com

Received: 15 February 2017, Revised and Accepted: 09 March 2017

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders of the endocrine system mainly divided into two types; type 1 DM (T1DM) and type 2 DM (T2DM) which characterized with hyperglycemia (high blood glucose level). T2DM represents the majority of diabetes conditions over the world, therefore; it is needed to find an economically and therapeutically effective treatment for usage in developing and under-developed countries [1]. The elevated levels of asymmetric dimethylarginine (ADMA) inhibit NO synthesis and therefore impair endothelial function and thus promote atherosclerosis and cardiovascular disorder [2] as well as type 2 diabetic patients with retinopathy [3]. Furthermore, ADMA is linked to the development of renal disease where, elevated levels of ADMA have been described [3], due to, ADMA is directly related to blood glucose levels [4].

With respect to epidermal growth factor (EGF), it is considered as an important biomarker in the restoration, mechanism, expressive on fibroblasts, epithelial cells, and promotes recovery of the damaged epithelium. In diabetic mice, these repaired cells can ameliorate flow of blood by elevating the rate of consistency of novel vessel, which also helps in the healing process [5]. It maintains the process of healing of oral and gastroesophageal ulcers through stimulation of DNA synthesis, cell migration and proliferation [6]. Moreover, EGF regulates the major function of the pancreas insulin secretion; it stimulates the secretion of insulin in a pancreatic beta-cell of mice [6]. In addition, EGF elevated the level of insulin so intercede the lowering of glucose in plasma of normal mice and diabetic one [6]. Hence, EGF is a new product that adjusts the levels of glucose in plasma and it regards as a choice for a therapeutic progress of diabetes [5].

Dynamically, the inflammatory process is a controlled procedures ranged by interfaces which perform critical adjusting function such as cytokines, chemokines, and lipid mediators; the lipoxins (LXs), resolvins, and protectins [7]. Moreover, human lipooxygenases (LOX) and their metabolites have been involved in several diseases including allergic rhinitis, cancer, asthma, allergy atherosclerosis, and diabetes [8]. Regarding to von Willebrand factor (vWF), it is endothelial cells synthesizing glycoprotein, implicated in adhesion and accumulation of platelet besides it is coagulation factor VIII carrier in plasma [9]. Further, the elevated vWF levels were connected with cardiovascular disease (CVD) risk in patients with T2DM or resistance of insulin, proposing that vWF is a critical marker unique to these disorders [9].

The rapiers used in diabetes have limited efficacy or side effects such as hypoglycemia, body weight gain, flatulence, and gastrointestinal disorders [10]. Unusual marine environments are attributed to different bioactive compounds that make marine organisms’ important source of biologically active substances using in therapeutics progress [11]. Chlorophyte alga Haematococcus pluvialis is the highest known source of astaxanthin (ASTA) in nature [12]. Where, ASTA content in H. pluvialis may exceed 4% of dry weight that is considered the highest content reported for any microorganism such as bacteria, fungi, and other microalgae [13]. Thus, H. pluvialis had a great interest for the commercial production of ASTA high value [14]. The basic and clinical research on ASTA health benefits have rapidly developed due to it has a preventive effect against oxidative stress-related diseases [15]. Moreover, ASTA has a considerable potential in the prohibition and handling of different diabetes including cancer, asthma, metabolic syndrome, rheumatoid arthritis, gastrointestinal, hepatic, and neurodegenerative diseases [16].

Keywords: Haematococcus pluvialis, Streptozotocin, Type 2 diabetes, Asymmetric dimethylarginine, Von Willebrand factor, Lipoxygenases, Histopathological examination.
The aim of this study is to investigate the potential of *H. pluvialis* extract to ameliorate the metabolic disorder in streptozotocin (STZ)-induced diabetic rats.

**MATERIALS AND METHODS**

**Chemicals**

STZ was purchased from Sigma-Aldrich, India. All chemicals in this study were of analytical grade, products of Sigma, Merck and Aldrich. All kits were the products of Biosystems (Alcobendas, Madrid, Spain), Sigma Chemical Company (St. Louis, MO, USA), Biodiagnostic Company (Cairo, Egypt).

**H. pluvialis cultivation**

*H. pluvialis* (strain No. CCAP 34/7) was isolated by spreading 0.1 ml of water samples collected from Nile River phytoplankton using BG11 media for algal isolation [17] into petri dishes containing 1.5% agar for solidification. Then, single colonies of algae were re-cultivated in the specified liquid media as non-axenic batch cultures (50 ml) at 25±2°C and 24 hrs with continuous white fluorescent lamp intensity ≈2500 Lux. Cultivation was carried out on an open pond with a capacity of 70 L containing 55 L of growth media. After cultivation, the biomass was initially separated from the water by gravitational settling and then further concentrated by centrifugation [18], then dried at 40°C.

**Preparation of ethanol extract**

100 g of the algal powder was macerated in ethanol (80%) and shocked on shaker (Heidolph UNIMAX 2010) for 48 hrs at 150 rpm. The extract was filtered using a Buchner funnel and Whatman No. 4 filter paper, and the algal residue was re-extracted with the addition of fresh ethanol for another 2 times [19]. Combined filtrates were concentrated using Rotavapor (Heidolph-Germany) at a temperature of 40°C under vacuum to dryness. The evaporated extract so obtained was preserved at −20°C in a freeze and kept until further use.

**Animals**

About fifty male Wistar rats (180-200 g) raised in the Central Animal House, National Research Centre (NRC) were used. Animals were acclimatized to the laboratory conditions at room temperature before the experimentation. Animals were kept under standard conditions of a 12 hrs light/dark cycle with food and water in plastic cages with soft bedding. Before testing for blood glucose level or injection of STZ to induce diabetes, the rats were fasted overnight (at least 12 hrs) but had free access to water. The study was approved by the NRC Animal Ethical Committee Guidelines (approval no: 0111457) for the use and care of animals.

**Diabetes induction and animals’ treatment**

STZ was dissolved in 0.01 M citrate buffer immediately before use and induced by intraperitoneal injection of a single dose (45 mg/kg b.wt.) through the dorsal vein of the rats’ penis [20]. After STZ injection, rats had free access to food, water and were given 5% glucose solution to drink overnight to encounter hypoglycemic shock [21]. Fasted blood glucose levels were assessed 72 hrs after STZ injection as well as glycosuria to confirm the diabetic states. Rats were considered to be diabetic if glycosuria was present for three consecutive days [22]. Only rats with a fasting blood glucose level of ≥300 mg/dl and positive urine glucose were used in the experiment. The anti-diabetic glibenclamide reference drug was orally administrated at a dose of 10 mg/kg b.wt. daily for 30 days [23].

**Experimental study**

About fifty rats were randomly divided into 5 groups of 10 in each group. Group 1: Normal control. Group 2: Normal rats treated with *H. pluvialis* ethanolic extract (150 mg/Kg b.wt.) [24]. Group 3: Diabetic rats. Group 4: Diabetic rats orally treated with *H. pluvialis* extract (150 mg/Kg b.wt.). Group 5: Diabetic rats orally administered anti-diabetic glibenclamide reference drug (10 mg/kg b.wt.) daily for 30 days.

**Blood and tissue sample measurements**

Rats were fasted overnight (12-14 hrs), anesthetized by diethyl ether and blood collected by puncture of the sublingual vein in clean and dry test tube, left 10 minutes to clot and centrifuged at 3000 rpm for serum. The separated serum was used for biochemical analysis of ADMA, vWF, 15-LOX, 5-LOX, EGF, and LXA4.

**Analytical methods**

ADMA was determined in serum by ELIZA method according to Valtonen et al. [25] vWF was measured by a quantitative direct enzyme immunoassay according to Fischer et al. [26]. 15-LOX was determined in brain tissue by HPLC method according to Hoffman et al. [27]. 5-LOX was determined in brain tissue by colorimetric method according to Anthon and Barrett [28]. EGF was determined by Puchades et al. [29]. LXA4 assay was performed using a method described previously by Elsinghorst [30].

**Calculations:**

\[
\% \text{ Change to control} = \frac{\text{Mean of control} - \text{Mean of treated}}{\text{Mean of control}} \times 100
\]

**Statistical analysis**

All the values were expressed as mean ± standard deviation. Statistical differences between the means of various groups were evaluated by one-way analysis of variance using the Statistical Package for the Social Sciences (SPSS) program, version 11 followed by Co-state program to compare significance between groups. p-values of 0.05 or less were considered to be significant.

**RESULTS**

**Effect of *H. pluvialis* extract on ADMA, vWF, 15-LOX, 5-LOX, EGF, and LXA4 levels in different groups under investigation**

The results in Table 1 showed the effect of *H. pluvialis* extract on ADMA, vWF, 15-LOX, 5-LOX, EGF, and LXA4 levels in different groups where insignificant change in their levels was recorded in normal rats treated with *H. pluvialis* extract. While increase in ADMA, vWF, 15-LOX, and 5-LOX levels was detected in diabetic rats (430.30, 77.54, 61.05, and 81.09%, respectively), compared to normal control one. However, the EGF and LXA4 levels decreased in diabetic rats with percentages 44.17 and 51.94%, respectively. On the other hand, treatment of diabetic rats with *H. pluvialis* improved ADMA, vWF, 15-LOX, 5-LOX, EGF, and LXA4 levels with amelioration percentages 269.69, 54.77, 55.78, 72.68, 53.39, and 56.58%, respectively.

**Histopathological examination of cardiac tissue**

Microscopic examination of normal control heart revealed normal cardiac myocytes (Fig. 1). Meanwhile, the heart of diabetic rats showed vacuolation of the sarcoplasm of cardiac myocytes (Fig. 2a), congestion of myocardial blood vessels and focal necrosis of myocytes associated with inflammatory cells infiltration (Fig. 2b). Some examined sections from diabetic rats treated with *H. pluvialis* extract revealed...
Table 1: Influence of *H. pluvialis* extract on ADMA, vWF, 15-LOX, 5-LOX, EGF, and LXA4 levels

| Groups parameters | Control | Control+*H. pluvialis* | Diabetes | Diabetes+*H. pluvialis* | Diabetes+drug |
|-------------------|---------|------------------------|----------|--------------------------|---------------|
| ADMA (µmol/l)     | 1.32±0.34<sup>a</sup> | 1.27±0.22<sup>a</sup> | 7.00±0.39<sup>a</sup> | 3.44±0.21<sup>c</sup> | 4.00±0.26<sup>c</sup> |
| % Change          | -       | 3.78                   | 430.30   | -                        | 203.03        |
| % of improvement  | -       | -                      | -        | -                        | 227.27        |
| vWF (ng/l)        | 50.50±10.70<sup>b</sup> | 50.00±7.59<sup>b</sup> | 89.6±4.78<sup>b</sup> | 62.00±3.88<sup>b</sup> | 69.9±7.16<sup>b</sup> |
| % Change          | -       | 0.99                   | 77.54    | -                        | 38.41         |
| % of improvement  | -       | -                      | -        | -                        | 39.12         |
| 15-LOX (U/l)      | 9.50±0.99<sup>a</sup> | 8.11±0.38<sup>a</sup> | 15.30±1.69<sup>a</sup> | 10.00±0.56<sup>a</sup> | 9.11±0.89<sup>a</sup> |
| % Change          | -       | 14.63                  | 61.05    | -                        | 4.10          |
| % of improvement  | -       | -                      | -        | -                        | 65.15         |
| 5-LOX (U/l)       | 11.90±1.34<sup>a</sup> | 11.37±0.34<sup>a</sup> | 21.55±2.90<sup>a</sup> | 12.90±1.65<sup>a</sup> | 13.30±0.89<sup>a</sup> |
| % Change          | -       | 4.45                   | 81.09    | -                        | 11.76         |
| % of improvement  | -       | -                      | -        | -                        | 69.32         |
| EGF (pg/ml)       | 53.09±2.21<sup>a</sup> | 49.00±3.10<sup>a</sup> | 29.64±3.21<sup>a</sup> | 57.99±3.90<sup>a</sup> | 59.8±2.00<sup>a</sup> |
| % Change          | -       | 7.70                   | 44.17    | -                        | 12.63         |
| % of improvement  | -       | -                      | -        | -                        | 56.80         |
| LXA4 (Pg/ml)      | 1290.14±102.50<sup>b</sup> | 1240.3±105.00<sup>b</sup> | 620.00±67.50<sup>b</sup> | 135.00±49.70<sup>b</sup> | 129.5±90.17<sup>b</sup> |
| % Change          | -       | 3.86                   | 51.94    | -                        | 6.25          |
| % of improvement  | -       | -                      | -        | -                        | 45.69         |

Data are expressed as mean±SD of 10 rats in each group. Statistical analysis is performed using SPSS computer program (one way ANOVA) coupled with Co-state computer program, where different letters [a,b,c] are significant at p≤0.05. While, identical letters (a,b,c) are considered insignificant at p≤0.05.

DISCUSSION

The current results revealed elevation in ADMA in STZ-induced diabetic rats. The increased levels of ADMA have been also detected in diabetic patients [32]. High blood glucose level was demonstrated to enhance ADMA levels by decreased its metabolism [32]. Clinical investigations in patients also indicated that ADMA is directly related to blood glucose levels [32]. Recently, intense regulation of blood glucose levels may extend antiatherogenic influences by lowering the levels of ADMA in T2DM patients [33]. It was suggested that, the high concentrations of ADMA are distinctly connected with the cases of coronary artery disease [34], T2DM [35], type 1 diabetes [36], and chronic heart failure [37]. ADMA is related also to the progression of diabetic complication such as renal disease incidence in patients with diabetic nephropathy [3] and T2DM patients with retinopathy [38].

Further, type 2 diabetes in these results is associated with the significant elevation in vWF. High vWF levels have been correlated well with the increased levels of ADMA in type 2 diabetic patients [32]. Beside, vWF has been linked with the insulin resistance in diabetic or nondiabetic cases [40]. In this context, Frankel et al. [9] revealed that vWF is inconsistently related to CVD and this may be attributed to the link of vWF with type 2 diabetes and insulin resistance. Dysfunction of endothelial cells is a systemic disease leading to atherosclerosis and CVD. Insulin resistance and diabetic status alleviated the connection between the high vWF and the increase in the CVD risk, pointing to vWF acts as a powerful moderator [9]. In addition, intense dysfunction of endothelial cells may be key atherogenic agents that responsible for the excess of 2-4 fold increase CVD risk observed in diabetes of type 2 [9].

The present results are also declared significant increase in 5 and 15-LOX levels in diabetic rats. 5-LOX has been implicated in cancer, asthma, allergic rhinitis, osteoarthritis, and atherosclerosis, whereas 15-LOX has been engaged in diabetic status, coagulation of blood, cancer

El-Baz and Aly

Asian J Pharm Clin Res, Vol 10, Issue 6, 2017, 72-77

**Fig. 1:** Heart of normal control rats showing normal cardiac myocytes (H and E ×400)

**Fig. 2:** (a) Heart of diabetic rats showing congestion of myocardial blood vessel and vacuolation of the sarcoplasm of cardiac myocytes (H and E ×400), (b) Heart of diabetic rats showing congestion of myocardial blood vessel and focal necrosis of myocytes associated with inflammatory cells infiltration (H and E ×400)

no histopathological changes with normal cardiac myocytes (Fig. 3). Moreover, marked improvement in the histopathological picture of diabetic rats heart-treated with glibenclamide drug (Fig. 4).

Histopathological examination of renal tissue

Microscopically, kidneys of rat from normal control group revealed the normal histological structure of renal parenchyma (Fig. 5). Meanwhile, diabetic rat kidneys showed vacuolation of epithelial lining renal tubules and endothelial lining glomerular tuft as well as cystic dilatation of renal tubules (Figs. 6a and b). Moreover, examined sections from the diabetic group treated with *H. pluvialis* extract revealed vacuolation of epithelial lining renal tubules, endothelial lining glomerular tuft, congestion of glomerular tuft, and intertubular renal blood vessels (Figs. 7a and b). However, improved picture was observed in kidneys of diabetic rats treated with glibenclamide drug, as the examined sections revealed renal tubules with vacuolation of the epithelial lining as well as glomerular tufts with slight congestion (Figs. 8a and b).
as well as psoriasis [8]. Human reticulocyte; 15-LOX-1 is regarded as an important curative goal, especially for its function in different disorders such as atherogenic and diabetic conditions, Alzheimer’s disease, breast cancer, and stroke [8].

Concerning EGF, the present results demonstrated significant decrease in EGF in diabetic rats. Al-Ankily et al. [5] explained that STZ-induced diabetes affects the binding of 125-labeled EGF to hepatic membranes negatively. Scatchard statistics demonstrated that the reduction in the binding of EGF was related to a lower in the receptors number. These findings evidenced that, insulin deficiency is a principle cause for the reduction in hepatic EGF receptors [41]. Further, in diabetic condition, plasma, submandibular gland EGF levels as well as the granular convoluted tubules size, which manufacture EGF, were noticed to be significantly reduced [41].

Considering LXA4, the current results showed significant reduction in LXA4 level in STZ-induced type 2 diabetes. It was found that IL-4 cytokine promoted anti-inflammatory action by enhancing LXA4 formation. The general purpose of LXs are to inhibit the pro-inflammatory prostaglandins, thromboxanes, isoprostanes and leukotrienes output, beside it, stimulates wound healing and dissolve inflammatory process hence, it repair tissues as well as the function of organs to normal. Hence, when the LXs synthesis is disturbed as in this findings, it could drive to a continual inflammation and hence tissue injury [24]. It was evidenced that the synthesis of LXs are
reduced on β cells of the pancreas and subjected to toxic agents such as IL-6, TNF-α, and migration inhibitory factor (MIF), which could lead to β-cell dysfunction or destruction and the onset of diabetes [42]. Thus, polyunsaturated fatty acids (PUFAs) and their anti-inflammatory molecules as LXX, suppress the output of different pro-inflammatory mediators included MIF and high-mobility group box 1 protein. Hence, they inhibit inflammation in various disorders as diabetes [42].

Accumulating evidence suggested that ASTA of _H. pluvialis_ could do cardiac-preventative effects by ameliorating oxidative damage, inflammatory process, lipid, and glucose metabolism, hence improved architectures of cardiac and renal tissues as indicated in the present results. ASTA may also prevent diabetic nephropathy development by decrease renal oxidative stress and renal cell damage in db/db mice [43]. ASTA is commonly in isomers mixture synthesized by _H. pluvialis_ microalga and is used as a supplement in nutrition, as an antioxidant, it captures free radicals, prevents peroxidation of the lipid membrane bilayer [11]. Further, its property as antioxidant is 10-fold higher than other carotenoids, as lutein, canthaxanthin, and β-carotene [11]. In parallel, a recent meta-analysis showed that ASTA supplementation can lower glucose levels [15]. ASTA was found to be more efficient than vitamin E, it preserves rat liver mitochondria against lipid peroxidation [44]. It can induce xenobiotic metabolizing enzymes in the lung and kidney [45].

**CONCLUSION**

It could be concluded that diabetic complications are associated with metabolic disorders in the different organs including cardiac and renal tissues. However, normalization in this metabolic dysfunction is documented, on using _H. pluvialis_ extract related to its ASTA content. Further, clinical investigations are needed to use _H. pluvialis_ as a promising candidate nutraceutical for ameliorating diabetic complications.

**ACKNOWLEDGMENT**

This work was supported and funded by the project entitled "Biodiesel production from algae as a renewable energy source“. Funding organization: Research Development and Innovation program (RDI). Funding Program: EU-Egypt Innovation Fund, 2014-2017.

**REFERENCES**

1. Priyanka K, Singh R. A systematic review on Indian floral biodiversity as eminent reserves for alternative treatment strategy of diabetes mellitus. Int J Pharm Sci 2016;8(4):10-9.
2. Saleh DO, El-Awdan SA, Nofel SM, El-Eraky WI, El-Khatib AS, El-Baz and Aly. Circulation 2008;118(24):2533-9.
3. Lee SH, Jeon YJ. Anti-diabetic effects of brown algae derived phlorotannins, marine polyphenols through diverse mechanisms. Fitoterapia 2013;86:129-36.
4. Gammone MA, Riccioni G, D’Orazio N. Marine carotenoids against oxidative stress: Effects on human health. Mar Drugs 2015;13(10):6226-46.
5. Yuan JP, Peng J, Yin K, Wang JH. Potential health-promoting effects of astaxanthin: A high-value carotenoid mostly from microalga. Mol Nutr Food Res 2011;55(1):101-65.
6. Boussiba S. Carotenogenesis in the green alga _Haematococcus pluvialis_: Cellular physiology and stress response. Physiol Plantarum 2000;108:111-7.
7. Serhan CN. Systems approach to inflammation resolution 6. Wei F, Lin CC, Joon A, Feng Z, Troche G, Lira ME, et al. Potential and selective inhibitors of human reticulocyte 12/15-lipoxygenase as anti-stroke therapies. J Med Chem 2014;57(10):4035-48.
8. Frankel DS, Meigs JB, Massaro JM, Wilson PW, O’Donnell CJ, D’Agostino RB, et al. Von willebrand factor, Type 2 diabetes mellitus, and risk of cardiovascular disease: The Framingham offspring study. Circulation 2008;118(24):2533-9.
cardiovascular events and death in patients with coronary artery disease: Results from the AtheroGene Study. Circ Res 2005;97(5):e53-9.
35. Krzyzanowska K, Mittermayer F, Wolzt M, Schernthaner G. Asymmetric dimethylarginine predicts cardiovascular events in patients with Type 2 diabetes. Diabetes Care 2007;30:1834-9.
36. Lajer M, Tarnow L, Jorsal A, Teerlink T, Parving HH, Rossing P. Plasma concentration of asymmetric dimethylarginine (ADMA) predicts cardiovascular morbidity and mortality in Type 1 diabetic patients with diabetic nephropathy. Diabetes Care 2008;31(7):747-52.
37. Dückelmann C, Mittermayer F, Haider DG, Altenberger J, Eichinger J, Wolzt M. Asymmetric dimethylarginine enhances cardiovascular risk prediction in patients with chronic heart failure. Arterioscler Thromb Vasc Biol 2007;27(9):2037-42.
38. Malecki MT, Undas A, Cyganek K, Mirkiewicz-Sieradzka B, Wolkow P, Osmenda G, et al. Plasma asymmetric dimethyl arginine (ADMA) is associated with retinopathy in Type 2 diabetes. Diabetes Care 2007;30:2899-901.
39. Ostergård T, Nyholm B, Hansen TK, Rasmussen LM, Ingerslev J, Sørensen KE, et al. Endothelial function and biochemical vascular markers in first-degree relatives of Type 2 diabetic patients: The effect of exercise training. Metabolism 2006;55(11):1508-15.
40. Natali A, Toschi E, Baldeweg S, Ciocciaro D, Favilla S, Saccà L, et al. Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes. Diabetes 2006;55:1133-40.
41. Singla S, Singla S, Kumar A, Singla M. Role of epidermal growth factor in healing of diabetic foot ulcers. Indian J Surg 2012;74(6):451-5.
42. Das UN. Arachidonic acid and lipoxin A4 as possible endogenous anti-diabetic molecules. Prostaglandins Leukot Essent Fatty Acids 2013;88(3):201-10.
43. Naito Y, Uchiyama K, Aoi W, Hasegawa G, Nakamura N, Yoshida N, et al. Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice. Biofactors 2004;20(1):49-59.
44. Kurashige M, Okimasu E, Inoue M, Utsumi K. Inhibition of oxidative injury of biological membranes by astaxanthin. Physiol Chem Phys Med NMR 1990;22(1):27-38.
45. Jewell C, O’Brien NM. Effect of dietary supplementation with carotenoids on xenobiotic metabolizing enzymes in the liver, lung, kidney and small intestine of the rat. Br J Nutr 1999;81(3):235-42.