Original Article

Association of Vascular Endothelial Growth Factor and Oxidative Stress in Type 2 Diabetes in Population of West Bengal

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The work was done between the periods of 2015-2019.

Abstract :

Type 2 Diabetes Mellitus (T2DM), the most frequent subtype of diabetes, comprises a complex heterogeneous group of metabolic diseases characterized by high levels of blood glucose (hyperglycaemia) and impaired insulin action and/or insulin secretion due to β cell dysfunction. Increasing evidence in both experimental and clinical studies suggest oxidative stress (OS) plays a major role in the pathogenesis of T2DM and its complications. In a physiological condition appropriate levels of Reactive Oxygen Species (ROS), generated either in restricted amounts or in a transient fashion, are essentially required to promote physiological angiogenesis and homeostatic maintenance of healthy vasculature. Uncontrolled continuous ROS production ultimately contribute to pathology and causes tissue damage. One of the most important proangiogenic factors is Vascular Endothelial Growth Factor (VEGF) which plays a key role in diabetic endothelial dysfunction, which ultimately leads to pathogenesis of vascular complications. Therefore, we aimed to investigate the association between antioxidant status and VEGF levels in plasma that may lead to vascular complications. A case-control study of one hundred and fourteen patients with T2DM (n=114) and thirty three control subjects (n=33) were screened from different areas of West

which gives rise to a risk o
damage (retinopathy, nephropathy and neuropathy) [Orasanu et al., 2009]. It is associated with reduced life expectancy, significant morbidity due to specific diabetes related microvascular complications, increased risk of macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life [WHO, 2016].

According to the Diabetes Atlas (7th edition), the global prevalence of diabetes is estimated at 415 million (8.8%), which is expected to rise to 642 million in next 25 years [IDF, 2015]. India is being referred to as the 'diabetic capital of the world' [Joshi et al., 2012]. About 31% of the population of West Bengal is suffering from T2DM [Shashank et al., 2012]. Factors associated with the pathogenesis of long-term diabetic complications are multifactorial, although persistent hyperglycemia (glucotoxicity) seems to be a key mediator. At least three distinct metabolic pathways seem to be involved in the pathogenesis of long-term complications; it is likely that all of them play a role in a tissue-specific manner [Kumar et al., 2018]. The main consequences of these pathways lead to the formation of oxidative stress (OS) which appears to be the major underlying cause of diabetic complications.

OS-dependent angiogenesis is an important contributor to the progression of cancers and chronic diseases. One of the most important proangiogenic factors is Vascular Endothelial Growth Factor (VEGF). OS induces the over expression of the VEGF gene in T2DM and leads to the development of late diabetic complications.

In this study, the relationship between the VEGF level in plasma and stress level [level of malondialdehyde (lipid peroxidation) and activity of superoxide dismutase, catalase and glutathione peroxidase] were examined. To the best of our knowledge, no previous study was found in the literature which sought to investigate the relationship between VEGF expression and antioxidant status in the context of T2DM and its complications in the population of West Bengal. Therefore, we aimed to investigate the association between antioxidant status and VEGF level in plasma that may lead to vascular complications.

Materials and Methods

Study Setting and Subjects :

A case control study was conducted, between 2015 and 2019, on all cases with Type 2 Diabetes Mellitus (T2DM) who were referred to the Department of Endocrinology and Department of Surgery of Ramakrishna Mission Seva Pratishthan, Kolkata, India. A total of one hundred and fourteen patients with T2DM (n=114) and thirty three control subjects (n=33) were included in the study. The participants were age and sex matched. The patients were confirmed T2DM by impaired fasting glucose test (> =126 mg/dl) and oral glucose tolerance test (> =200 mg/dl) [WHO, 2005]. The controls had a history of having normal glucose metabolism, and were without hypertension, hyperlipidemia, and other metabolic syndromes. We excluded patients who had fever, acute and chronic infections, malignancy, acute and chronic nephritis, cirrhosis, and congestive heart failure. All the patients were in stable conditions during assessment. Detailed personal histories were collected from the participants with the help of a questionnaire.

This study was approved by the Institutional Ethical Committee on 10th May, 2016 (Reg No. ECR/62/Inst/WB/2013 issued under Rule 122DD of the Drugs & Cosmetics Rules 1945) and was conducted in accordance with the principles of the Declaration of Helsinki. All procedures were done with the informed consent of participants. The methods in this study were performed in accordance with the relevant guidelines and regulations.

Collection of peripheral blood :

Peripheral blood samples were collected by

venepuncture both from T2DM patients and control subjects. Blood samples were collected in EDTA vacutainer and stored at 4ºC for experimental work.

Biochemical Study :

Estimation of Plasma Malondialdehyde (MDA)**:** Plasma was separated from blood samples by centrifugation at 2500 rpm for 20 minutes. Plasma was then further analysed for malondialdehyde (MDA) level estimation. Plasma MDA was measured spectrophotometrically by the modified method of Okhawa et al. (1979).

Estimation of Superoxide Dismutase (SOD): The activity of SOD was assayed by the method of Kakkar et al. (1984).The assay of SOD is based on the inhibition of formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560nm. SOD activities are expressed as units/ml [Magnani et al., 2000].

Estimation of Catalase : The activity of catalase was assayed by the method of Sinha (1972) using dichromate-acetic acid reagent. The chromic acetate formed was measured at 620 nm. Catalase activities are expressed as units/min/ml. Estimation of Glutathione Peroxidase (GPX): For analysis of GPX activities, hemolysate of washed erythrocytes was used. The glutathione peroxidase activity was measured by the modified method of Paglia and Valentine (1967) using tert-butyl hydroperoxide as a substrate. Activity of GPX1 was calculated using the molar extinction coefficient of NADPH 6220 M^{-1} cm^{-1}. One unit of GPX1 (U) is defined as 1 µmol of NADPH oxidized to NADP per min. GPX activities are expressed as units/ml.

Measurement of plasma VEGF : Peripheral blood samples were collected from the patients and healthy volunteers to determine the plasma VEGF level. Plasma was separated from peripheral blood samples by centrifugation at 2500 rpm for 20 minutes. The plasma VEGF concentration was determined using Human VEGF (Vascular Endothelial Growth Factor) enzyme-linked immune sorbent assay kit (Fine Test, China) in Bioradi Mark^{TM} Microplate Reader. Intra - assay precision was <8% and Inter – assay precision was <10%. The detection range of the assay was 0-2000 pg/ml.

Statistical analysis :

All variables were expressed as mean ± SE (standard error). Statistical analyses were done using Independent 't' test and Chi Square test. Statistical significance was defined as P=0.05. All statistical analyses were performed by using statistical software Statistical Package for Social Sciences (SPSS).

Results :

Comparisons between control and T2DM cases in terms of Glucose concentration following Impaired Fasting Glucose and Oral Glucose Tolerance Test have been graphically presented in Figure 1a and 1b respectively.

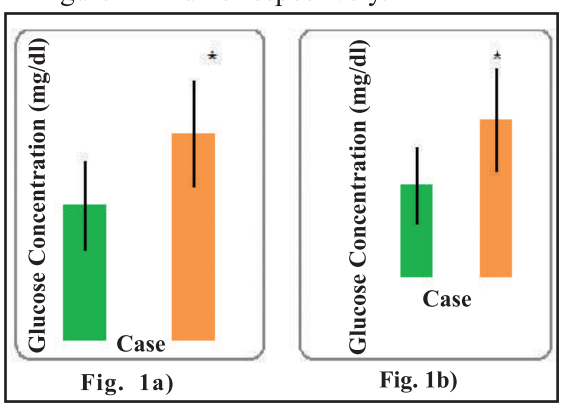


Figure 1 Comparison between Control and T2DM cases in terms of Glucose concentration following Impaired Fasting Glucose and Oral Glucose Tolerance Test

Comparison between Control and T2DM cases in terms of HbAIC (%) has been presented graphically in Figure 2.

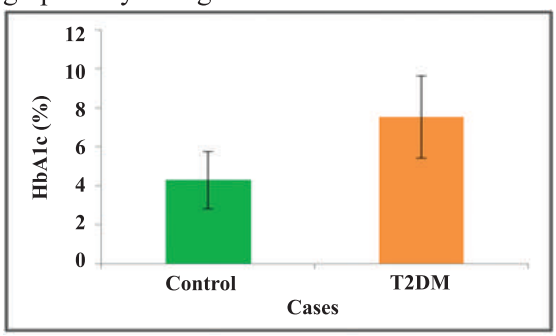


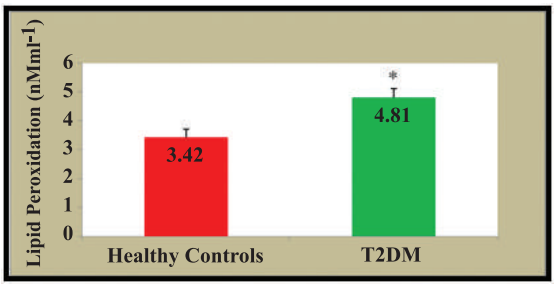
Figure 2 Comparison between Control and T2DM in terms of HbAIC (%)

Biochemical Study :

Lipid Peroxidation In Studied Cases

Quantitative in vitro lipid peroxidation level in whole blood was estimated. Plasma malondialdehyde (MDA) level was measured for assessing lipid peroxidation level. The data were statistically analyzed by Independent 't' test to compare healthy controls and patients. The study showed significantly elevated plasma MDA level ($p<0.05$) was found in T2DM patients (4.81 ± 0.31 nM ml^{-1}) compared to control subjects (3.42 ± 0.29 nM ml^{-1}). peroxidation can be a useful biomarker for OS for the risk of T2DM progression and its complications.

Fig. 3 Lipid Peroxidation level in the Studied Cases (a) Between healthy controls and T2DM (b) Between healthy controls and different groups of T2DM based on complications.

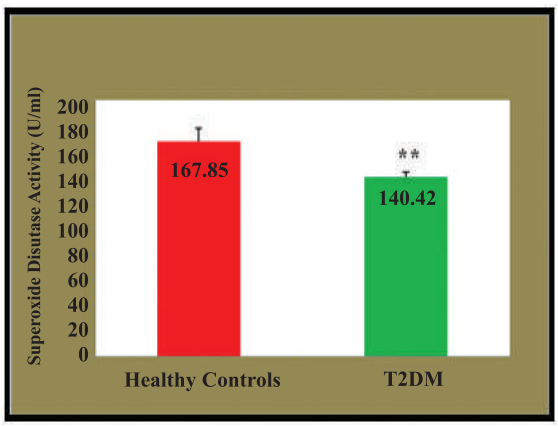


*** Statistically significant at p<0.05 (Independent *'t'* test)**
**** Statistically significant at p<0.001 (Independent *'t'* test)**

Superoxide Dismutase (Sod) Activity In Studied Cases

Quantitative in vitro Superoxide Dismutase activities in whole blood were estimated. The data were statistically analyzed by Independent 't' test to compare healthy controls and patients. In the present study, significant decrease in SOD activity ($P=0.001$) was found in T2DM patients (140.42 ± 3.72 U ml^{-1}) compared to control subjects (167.85 ± 10.56 U ml^{-1}).

Fig. 4 Superoxide dismutase activities in the Studied CasesBetween healthy controls and T2DM

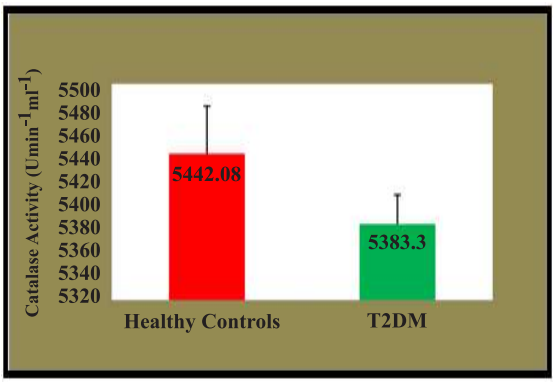


*** Statistically significant at p<0.05 (Independent *'t'* test)**
**** Statistically significant at p<0.001 (Independent *'t'* test)**

Catalase Activity in Studied Cases

Catalase is an enzyme in protecting the cell from oxidative damage by ROS. Quantitative in *vitro* Catalase activities in whole blood were estimated. The data were statistically analyzed by Independent *'t'* test to compare healthy controls and patients. Our study revealed that catalase activity had diminished in T2DM patients (5383.30 ± 24.59 U $min^{-1}ml^{-1}$) compared to control subjects (5442.08 ± 39.38 U $min^{-1}ml^{-1}$).

Fig. 5 Catalase activities in the Studied Cases Between healthy controls and T2DM

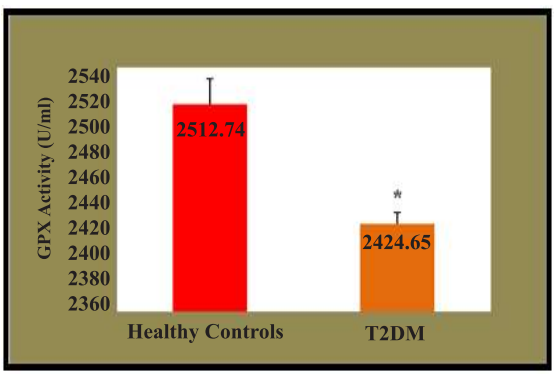


Statistically insignificant at p>0.05 (Independent *'t'* test)

Glutathione Peroxidase (Gpx) Activity In Studied Cases

Quantitative in vitro GPX activity in whole blood was estimated. A comparison between healthy control subjects and T2DM patients in respect to GPX activity was done. The data were statistically analyzed by Independent 't' test to compare healthy controls and patients. Present study found that GPX activity was significantly lower (P< 0.05) in T2DM patients (2424.65±8.53 U ml^{-1}) than control subjects (2512.74±18.96 U ml^{-1}).

Fig. 6GPX activities in the Studied Cases Between healthy controls and T2DM



*** Statistically significant at p<0.05 (Independent *'t'* test)**

**** Statistically significant at p<0.001 (Independent *'t'* test)**

VEGF Estimation in Studied Cases

VEGF levels in plasma was compared between healthy controls and T2DM. Elevated VEGF level was found in T2DM patients compared to healthy controls and it was found statistically significant (P< 0.001).

Table 1. VEGF level in Plasma in Studied Cases

Group	VEGF Level in plasma (pg/ml) (mean±SE)	p-value
Healthy Controls	317.9±12.9	-
T2DM	526.8±34.3	0.000**

*** Statistically significant at p=0.05 (Independent *'t'* test)**

**** Statistically significant at p=0.001 (Independent *'t'* test)**

Discussion :

The number of diabetes cases worldwide has increased according to the estimation by the International Diabetes Federation, of which T2DM constitutes approximately 90% to 95%. Up to 80% of mortality with T2DM is directly associated with vascular complications. Many experimental and clinical studies have shown that oxidative stress is an important factor in the pathogenesis of both Type 1 and Type 2 Diabetes Mellitus. At least three distinct metabolic pathways seem to be involved in the pathogenesis of long-term complications; it is likely that all of them play a role in a tissue-specific manner [Kumar et al., 2018]. One of the factors is the process of non-enzymatic glycation of proteins, lipids and nucleic acids, with subsequent formation of advanced glycation end products (AGEs) [Brownlee, 1994]. Other factors are activation of protein kinase C and disturbances in polyol pathway. One of the main consequences of these adverse actions is the formation of oxidative stress (OS). Oxidative stress represents

an imbalance between the production and manifestation of Reactive Oxygen Species (ROS) especially free radicals and a biological systems ability to detoxify the reactive intermediates or to repair the resulting damage. The increase in the level of ROS in diabetics could be due to their increased production and/or decreased destruction by enzymatic catalase and superoxide dismutase antioxidants. DM causes oxidative degradation of lipids in cell membranes resulting in cell damage. Lipid degradation leads to increased susceptibility to lipid peroxidation.

OS-dependent angiogenesis is an important contributor to the progression of cancers and chronic diseases. VEGF is the most potent and primary endothelial specific angiogenic growth factor, both in physiological and pathological conditions. VEGF signaling is ultimately required for normal vascular development and homeostasis, but it is also actively engaged in tumor progression by promoting growth of tumor vasculature [Shibuya et al., 2008]. This signaling pathway often seems to be affected by ROS. Thus, the reciprocity between oxidative stress and angiogenesis has been centered on the VEGF signaling pathway. VEGF appears to play a central role in mediating microvascular pathology in late diabetic complications.

Antioxidants are agents that protect, prevent, or reduce the extent of oxidative damage to biomolecules (proteins, lipids, and nucleic acids) causing cellular dysfunction and alteration in lipid profile leads to increased susceptibility to lipid peroxidation. These agents may be enzymatic, non-enzymatic, or metal chelators. The enzymatic antioxidants include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). SOD, a copper, zinc and manganese-containing enzyme, reacts with superoxide radicals to form hydrogen peroxide, which is then converted to water by GPX (a glutathione-dependent selenoprotein), or catalase, a heme enzyme. The present study indicates that the diminished activity of SOD, catalase and GPX in the plasma of T2DM patients might be due to antioxidant depletion. Presence of ongoing free radical activity and breakdown of protective antioxidant species occurs due to the depletion of the antioxidant. The reduction in the activity of SOD may be due to excessive oxygen radical production from autooxidation of glucose, glycated proteins and glycation of antioxidative enzymes, which limit the capacity to detoxify oxygen radicals. Diminished catalase activity may result in increased damage to various proteins, lipids and DNA [Tiwari et al., 2013; Asmat, Abad and Ismail, 2016]. Our study found that GPX activity was significantly lower ($P< 0.05$) in T2DM patients than control subject. This suggested that the antioxidant enzyme production is affected in T2DM patients leading to higher risk of cell organ damage [Chandrika et al., 2017]. The low activity of GPX could be directly explained by the low content of GSH found in patients with T2DM, since GSH is a substrate and cofactor of GPX. Enzyme inactivation could also contribute to low GPX activity. GPX is a relatively stable enzyme, but it may be inactivated under conditions of severe oxidative stress. Inactivation of this enzyme may occur through glycation governed by prevailing glucose concentration [Rahbani-Nobar, 1999]. Alteration in GPX activity found in our study can be considered an adaptation of antioxidant defense against increased production of ROS. Reduced GSH level in red blood cells reflects generalized decrease in intracellular content of this compound. Thus the obtained results show that T2DM patients have lower GSH content in

erythrocytes than the healthy control group. The trend found in this study is consistent with the report of Aaseth and colleagues, who found reduced levels of GPX in erythrocytes of subjects with poorly controlled T2DM [Aaseth & Stoe-Birketvedt, 2000, Gonzalez et al., 2016, et al., 2016, Ines G & Elhadj-Ahmed, 2017]. Our result indicates that patients with T2DM have lower activity of GPX, which increases the susceptibility of cells to the damaging effects of ROS.

The observed increase in MDA levels, as shown in Figure 3, may be due to the enhanced production of lipid peroxides and their release in circulation that leads toincreased oxidative degradation of lipid in cell membranes (lipid peroxidation) which is responsible for T2DM progression and its complications and can be a risk factor for development of cancer. The increase in levels of MDA shows that free radicals are formed disproportionately in T2DM by glucose degradation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation, which may contribute to the development of complications in diabetic patients. Late diabetic complications are developed with increased activity of free radical – induced lipid peroxidation and accumulation of lipid peroxidation products. Enhanced MDA level is a prognostic factor for the risk of T2DM progression and its complications. Hence, increased lipid peroxidation with reduced antioxidant activity can be a useful biomarker for OS leading to the risk of T2DM progression and its complications.

In this study, the relationship between the plasma VEGF level and progression of T2DM complications was examined; it was more prevalent in T2DM patients compared to the controls. Table 1 shows the elevated VEGF level in T2DM patients compared to healthy controls and it was found statistically significant. Elevated VEGF level in T2DM leads to the risk factor for the progression of late diabetic complications.

To the best of our knowledge, this is the first cohort study to investigate the association between oxidative stress and VEGF expression in diabetic patients leading to the risk of vascular complications.

Conclusion :

We observed that alteration in VEGF level can be a risk factor for the presence and severity of vascular complications in diabetic patients which was supported by elevated oxidative stress and a strong correlation existed between them. This suggests that it may be a reliable biomarker for evaluating the development and progression of vascular complications.

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Author Statements :

Ethical approval : This study was approved by the Institutional Ethical Committee on 10th May, 2016. Reg No. ECR/62/Inst/WB/2013 issued under Rule 122DD of the Drugs & Cosmetics Rules 1945.

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Conflict of interest: None declared.

References :

1. Orasanu G, Plutzky J. The pathologic continuum of diabetic vascular disease. J Am Coll Cardiol. 2009; 53(5 Suppl) : S35–42.
2. World Health Organization. Global report on diabetes. 2016. Accessed 30 August 2016. ISBN 978 – 92 - 4 - 156525 7 (NLM classification: WK 810).
3. International Diabetes Federation. IDF Atlas. 7th edition. [Last accessed on 2015 Dec 27]. Available from: http://www.diabetesatlas.org.
4. Joshi AR, Pranita A, Phadke AV. Diabesity: curse of development. Int J Med. 2012;12:77–78.
5. Shashank RJ, Banshi S, Muruga V. et al. Prevalence of diagnosed and undiagnosed diabetes and hypertension in India — results from the Screening India's Twin Epidemic (SITE) study. Diabetes Technol Ther. 2012; 12(1):8–15.
6. Kumar, Abbas, Aster. Robbins Basic Paathology, 10th ed. Elsevier; 2018. ISBN: 978-0-323-35317-5; International Edition: 978-0-323-48054-3.
7. Ohkawa H, Ohishi N, Yagi K.1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem., 95: 351-358.
8. Kakkar P., Das B., Viswanathan P N. A modified spectrophotometric assay of SOD. Indian Journal of Biochem Biophys.,1984;21:130-2.
9. MagnaniL, GaydouM, Jean CH. 2000. Spectrophotometric measurement of antioxidant properties of flavones and flavonols against superoxide anion. Anal.Chim.Acta, 411(1-2) 1: 209-216.
10. Sinha KA. Colorimetric assay of catalase. Anal. Biochem. 1972; 47: 389-394.
11. Paglia DE, Valentine WN. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med.,70: 158–69.
12. Kumar, Abbas, Aster. Robbins Basic Paathology, 10th ed. Elsevier; 2018. ISBN: 978-0-323-35317-5; International Edition: 978-0-323-48054-3.
13. Brownlee M.Glycation and diabetic complications. Diabetes. 1994; 43:836–841.
14. Tiwari BK., Pandey KB, Abidi AB, Rizvi SI. Markers of Oxidative Stress during Diabetes Mellitus. 2013. Journal of Biomarkers. Article ID 378790. http://dx.doi.org/10.1155/2013/378790.
15. Asmat, U., Abad, K. and Ismail, K. Diabetes mellitus and oxidative stress — A concise review. 2016. Saudi Pharmaceutical Journal;24(5), pp.547-553.
16. Chandrika R., Sanjana R., Kishan Prasad HL., Vidya B. Catalase and malondialdehyde levels possible markers for type 2 diabetes mellitus. 2017. European Journal of Biomedical and Pharmaceutical sciences; 4(3):232-237.
17. Rahbani-Nobar ME., Rahimi-Pour A., Rahbani-Nobar M. Total antioxidant capacity, superoxide dismutase and glutathione peroxidase in diabetic patients.1999. *Medical Journal of Islamic Academy of Sciences;* 12(4):109–114.
18. Aaseth J., Stoe-Birketvedt G. Trace elements in human health and diseases: an update glutathione in overweight patients with poorly controlled diabetes type 2. 2000. The Journal of Trace Elements in Experimental Medicine; 13:105–111.
19. Gonzalez de VR., Fernandez-Sanchez ML., Fernandez JC., Alvarez Menendez FV., Sanz-Medel A. Selenium levels and Glutathione peroxidase activity in the plasma of patients with type II diabetes mellitus.2016. J Trace Elem Med Biol; 37:44-49. doi: 10.1016/j.jtemb. 2016.06.007.
20. Ines G., Elhadj-Ahmed K. Trace Elements Modulates Oxidative Stress in Type 2 Diabetes. 2017. Diabetes Food Plan.doi:10.5772/intechopen.71172.
21. Gawlik K., Naskalski JW., Fedak D., Pawlica-Gosiewska D., Grudzien U., Dumnicka P, Malecki MT., SolnicaB. Markers of Antioxidant Defense in Patients with Type 2 Diabetes. 2016. Oxidative Medicine and Cellular Longevity. Article ID 2352361. http://dx.doi.org/10.1155/2016/2352361.

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