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RESEARCH ARTICLE

OXIDATIVE STRESS STATUS OF DIABETIC RETINOPATHY SUBJECTS ATTENDING USMANU DANFODIYO UNIVERSITY TEACHING HOSPITAL SOKOTO, NIGERIA

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ABSTRACT

Background: Diabetic retinopathy (DR) is a complication of diabetes mellitus resulting from increased oxidative stress which affects the retina. Uncontrolled hyperglycemia leads to increase oxidative stress that is associated with development of DR. The study evaluated the oxidative stress status of diabetic retinopathy subjects. **Methods**: Serum fasting blood glucose (FBS), glycated hemoglobin (HbA1C), antioxidants vitamins (A, C, E,), minerals (Manganese, Copper, Zinc and Selenium) and enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) of nine (9) subjects that consist of three (3) apparently healthy individuals serving as control, three(3) newly diagnosed DM and three (3) DR subjects attending Usmanu Danfodiyo University Teaching Hospital Sokoto, Nigeria were evaluated using standard laboratory methods. The DR subjects were recruited based on the result from visual acuity test and funduscopy examination. **Results**: The results of FBS and HbA1C levels revealed significant ($P<0.05$) increase in DM and DR subjects compared with control subjects. Serum vitamin A, C, E, Zinc and Selenium and the activities of GPx, SOD and CAT showed significant ($P<0.05$) decrease among DM and DR subjects compared with the control. Gene expression analysis of supreoxide dismutase 1 (SOD1), catalase 1 (CAT1) and glutathione peroxidase 1 (GPx1) revealed up regulation in DM and down regulation in the DR subjects. **Conclusion**: The results indicated that DM and DR subjects are associated with increased oxidative stress that is associated with decrease activities of antioxidant enzymes and low levels of antioxidants vitamins and minerals.

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INTRODUCTION

Diabetes mellitus (DM) comprises a common group of metabolic disorders associated with hyperglycemia, which result in disrupting normal homeostasis of carbohydrates, lipid, protein and electrolytes (Saidu, 2010), this is the most common endocrine disorder that remains a global challenge. The complications associated with DM are increasing in both developed and developing countries like Nigeria (http://www.idf.org/diabetesatlas; WHO, 2016). The most common ophthalmic problems associated with DM are cataract, glaucoma, corneal abnormalities, iris neovascularization and neuropathies (Sri Hari, 2014). Diabetic retinopathy (DR) is one of the major late retinal complications in patients with both types 1(T1DM) and type 2 (T2DM) DM and occurs due to uncontrolled hyperglycemia associated with increase oxidative stress (Sri Hari, 2014). This is the most common ophthalmic complication among DM patients. It has been reported that DR is the fifth leading cause of blindness in the world, affecting millions of DM subjects and is responsible for 4.8 % of blindness **(**Ganji frockwala, 2015**).** Apart from hyperglycemia and alteration of glucose and lipid peroxidation, T2DM has been rank as one of the free radical diseases with complications associated with increase free radical generation (Desai et al., 2011). The incidence of DR increases with increase in duration of DM, hypertension, hyperglycemia and hyperlipidemia; these factors have been reported to be the risk factors for the development of DR (Nguyen,. 2009). In DR subjects, the retina suffer from oxidative stress (OS); imbalance in oxidant/antioxidant ratio in favor of oxidant (Desai, 2011; Sri Hari, 2014) and this is considered a link between hyperglycemia and metabolic abnormalities important in the development of DM complication (Brownlee, 2001). The commonest forms of DR include Non-proliferative diabetic retinopathy (NPDR); characterized by micro aneurism and Proliferative diabetic retinopathy (PDR); characterized by the growth of new blood vessels in the retina

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(neovascularization) [Aiello, 2003]. When NPDR become severe it develop in to PDR (Aiello, 2003). Hyperglycemia result in alteration of many biochemical pathways in T2DM subjects leading to excessive free radicals (superoxide radical, hydrogen peroxide and the hydroxide radical) accumulation; this affect the antioxidant defense system causing microangiopathic changes in the retina leading to diabetic retinopathy among DM subjects (Aiello, 2003). In view of the above, the present study was design to evaluate oxidative stress status among DR subjects attending Usmanu Danfodiyo University Teaching Hospital, Nigeria.

MATERIALS AND METHODS

Subjects: Nine (9) subjects attending out-patient department, Usmanu Danfodiyo University Teaching Hospital, Nigeria consisting of three(3) apparently healthy normal, three(3) diabetics and three(3) DR subjects were recruited for this study. All diabetic subjects were diagnosed according to international standard criteria described by American Diabetes Association. The DR assessment was carried out according to the Diabetic Retinopathy Grading reported by Wilkinson *et al.*(2003) Tam *et al.* (2009). Informed consent forms and questionnaires were duly sign by all the subjects recruited including the control subjects. All the subjects included in this study completed a design structured questionnaire for completing the following information: Sex, Age, BMI, hypertension, smoking, exercise, and family history of DM. The approval of ethics and research committee of Usmanu Danfodiyo University Teaching Hospital was obtained for the study.

Chemicals and Reagents: All Chemicals and reagent used for this study were of analytical grade. RNA extraction kit, cDNA kit, SYBR and Accupower cycle script RT premix (dT20) were purchased from Bioneer, USA. Glucose oxidase and HbA1C kit from Randox Laboratory UK. Primers for SOD1, CAT1, GPx1 and ß actin were design using primer3 and supplied by Jena Bioscience Germany. GPx assay kit was purchased from Cayman chemical company USA. Other chemicals used were purchased from Randox Laboratory UK.

Blood sample: Venous fasting blood sample (7 ml) were collected using syringe and needle, 2 ml of the sample were transferred directly into EDTA+flouride oxalate tube while the remaining 5 ml were transferred in to plain tube and allowed to coagulate, both the sample were centrifuged at 4000 rpm for 5 minute, plasma and serum obtained were used for biochemical analysis.

Biochemical assays: Serum FBS were assayed as describe by Barham and Trinder (Barham, 1972), and glycated hemoglobin (HbA1C) as described by Trivelli *et al.* (1971) The assay of antioxidant minerals (Copper, Zinc, Selenium and Chromium) was by Atomic absorption spectrophotometry as described by Bhatti *et al.* (2006), this is based on the absorption of light and excitation by an element, as the element fall back to ground state, it release a photon of light characteristic of that element, the intensity of the color is proportional to the amount of the element in the sample. Vitamin A (Rutkowski *et al.,* 2006) was assayed based on the addition of potassium hydroxide which break up complexes and permit retinol (Vit A) to partition in to xylene which has absorption peak at 335nm and expressed as µg/dl. Vitamin C (Harding *et al.,* 2008) was assayed based on the oxidation of ascorbic acid by copper II to form dihydro ascorbic acid which react with 2, 4- dinitrophenylhydrazine to form a red bis-hydrozone that is measured spectrophotometrically at 520nm and expressed as mg/dl. Vitamin E (Hashim, 1966) was assayed based on the reduction of ferric ion to ferrous ion by serum vitamin E, this form a red colored complex with α, α dipyridyl, the reaction was monitored at 520nm spectrophotometrically and express as µg/dl. Superoxide dismutase activity was measured as described by Beau and Fridorich (Beau Charp, 1971) was assayed based on the competition between pyrogallol autoxidation by superoxide and dismutation of this radical by superoxide dismutase, the reaction was monitored spectrophotometrically at 420nm and expressed as Unit/ml. Catalase activity (Aebi, 1984) was assayed based on the disappearance of hydrogen peroxide produced by SOD, and monitoring the reaction spectrophotometrically at 240nm and expressed as Unit/ml. GPx (Paglia, 1976) was assayed based on indirect couple reaction with glutathione reductase, oxidized glutathione produced open reduction of hydroperoxide by glutathione peroxidase, is recycled to its reduced state by glutathione reductase and NADPH, the reaction was monitored at 340nm spectrophotometrically and expressed as nmol/min/ml.

RNA extraction and Reverse transcription of RNA template: RNA was extracted from whole blood as described by Coombs *et al.*, (1999) using RNA extraction kit from Bioneer (USA). The extracted RNA was then reversibly transcribed to cDNA using Accupower cycle script (dT20) according to manufacturer's protocol using oligo dT20 primers. The cDNA was amplified by polymerase chain reaction to detect the gene of choice (SOD1, CAT1, GPx1 and ß actin), 2µl of the cDNA was transferred in to each tube for the gene of interest, followed by 10µl of premix, 2µl each of the respective primers (reverse and forward), a final reaction volume was made with RNase free water. The reaction conditions were 95˚C for 10 minute, 56˚C for 20 second and 72˚C for 15 second for forty circle. The pcr product was analyzed using 1% agarose gel and visualized using ethidium bromide ultra violet transilluminator.

Real time pcr of SOD1, CAT1 and GPx1 Genes: Real time pcr was performed to quantify the expression levels of SOD1, CAT1 and GPx1 using ß actin (internal control/housekeeping gene), the cDNA was amplified by real-time PCR using SYBR Green PCR core reagents. The cDNA (2 µl) was transferred to the reaction tubes followed by adding 2 µl of primers (forward and reverse), 10 µl of premix ($MgCl_2$, dNTPs, DNA polymerase, pcr buffer, SYBR) and 6 µl RNase free water to a final reaction volume of 20 µl. PCR reaction in a thermocycler (Light cycler, Roche Germany) using the following conditions was performed; 95°C for 10 min (denaturation), 56°C for 20 seconds (annealing) , 72°C for 15 seconds (DNA synthesis), 4°C (termination of the reaction). The real time pcr cycle was repeated forty times. The data generated were analyzed using ΔΔct method (relative quantification) with ß actin as the internal control gene (Rozen, 2000).

Statistical Analysis: All the results were expressed as mean±SEM, comparisons between FBS, HbA1C and other parameters was performed using one way ANOVA followed by Duncan tests. Statistical package SPSS version 20 was used for the analysis.

RESULTS

The result of the present study revealed that most of the DM and DR subjects studied (Table 2) are overweight, have hypertension and are physically inactive with family history of DM. Serum FBS and HbA1C (Table 2) revealed significant increase ($P < 0.05$) among DM and DR subjects compared with the control, no significant difference ($P<0.05$) were observed in the serum levels of HbA1C between DM and control subjects. The serum vitamins levels (A, C and E) showed (Table 3) significant decrease ($P<0.05$) among DM and DR subjects compared with the control. Antioxidant minerals (Zn and Se) also revealed significant decrease ($P<0.05$) among DR subjects compared with the control. No significant differences were observed in the serum levels of antioxidant minerals (Table 4) between DM and control subjects. A significant decrease ($P<0.05$) was observed in the activity of serum SOD, CAT and GPx in DM and DR subjects compared with apparently healthy subjects. A significant difference was also observed ($P<0.05$) in the activity of serum SOD, CAT and GPx in the DM subjects compared with DR subjects. The result of gene expression study showed that SOD1, CAT1 and GPx 1 are up regulated among DM subjects relative to apparently healthy subjects. The expression levels of the enzymes (SOD1, CAT1 and GPx1) in DR subjects were down regulated relative to apparently healthy subjects.

DISCUSSION

DR is a common and highly specific microvascular complication of DM (Antonetti, 2012) and is among the leading cause of blindness in the World (Elia *et al.,* 2017). Major risk factors include long duration of DM, hypertension and poor glycemic control (Mohamed, 2007). The present study revealed that the NDT2DM and DR subjects were associated with hypertension, overweight, physical in activity and family history of DM. The control subjects are however not associated with any of the above factors. All these factors have been reported to be associated with the development of DM and DR. The association of DM and DR subjects with overweight/obesity and physical inactivity is in accordance with earlier studies and is generally accepted that obesity/overweight and physical inactivity are risk factors for the development of T2DM[2]. The association of T2DM and DR with Hypertension has also been reported (http://www.idf.org/diabetesatlas). Hypertension like hyperglycemia is associated with OS; this favor the accumulation of ROS and hence development of DM complications (Saidu, 2010). Due to its morbidity and mortality, hypertension remains a public health concern (http://www.idf.org/diabetesatlas). The study revealed that most of the DM and DR subjects have family history of DM, this is in accordance with the report of Majithia and Florez, 2009; the authors reported that DM is associated genetic factors, it has also been reported that the lifetime risk for developing T2DM is about 7 % in general population, 40 % in offspring of one parent with T2DM, and 70 % if both parents have diabetes (Majithia and Florez 2009). The present study revealed significant increase ($P< 0.05$) in the levels of FBS in the DM and DR subjects compared with the control subjects (Table 2).The significant increase of FBS in DM and DR subjects compared with the control subjects is known, an individuals with FBS above normal level may indicate diabetic status. The significant increase in the levels of FBS in DM and DR subjects may be due poor glycemic control, this led to increase in the levels of FBS in circulation causing many metabolic abnormalities, it has been reported that hyperglycemia is associated with alteration in many biochemical pathways (Brown, 2016) leading to many complication of DM such as DR. Analysis of serum HbA1C levels of DM subjects compared with the control subjects revealed non-significant difference ($P< 0.05$), this may be due to early onset of diabetes, formation of HbA1C has been reported to be correlated with the degree of DM (Sarita et al., 2014) , thus the non-significant difference may be associated with early hyperglycemia. The significant difference ($P< 0.05$) in the serum levels of HbA1C of the DR subjects compared with the control is in accordance with the study of [32] the authors further emphasized that DR subjects tend to have more percentage HbA1c. The probable reason for high HbA1c in DR subjects may be due to persistent hyperglycemia or decrease in the antioxidant vitamins level, It has been reported that antioxidant vitamins supplementation show positive impact on the levels of HbA1c (Stitt, 2001), possibly by donating hydrogen to the glycation reaction intermediates, thereby preventing the formation of Amadori product or by improving overall insulin sensitivity/secretion in response to diet (Stitt, 2001). The present study revealed significant decrease ($P \leq 0.05$) in the serum levels of antioxidant vitamins (A, C and E) (Table 3) in DM and DR subjects compared with conrol subjetcts, this is in accordance with the report of Aliyu *et al.*, (2005). The decrease in the levels of the antioxidant vitamins may be due to continuous use of them in scavenging free radicals. OS among DM subjects and DR subjects has been reported (Aliyu *et al.,* 2005). The low levels of the antioxidant vitamins in DM subjects could lead to secondary complication of DM due to OS. A significant decrease ($P<0.05$) in the serum levels of Zn and Se was observed among DR subjects compared with the control subjects (Table 4) and is similar to the report of Fagbohun *et al.* (2016). This may be due to the duration of DM, it has been reported that subjects with long standing DM tend to have low levels of the antioxidant minerals because of long exposure to OS (Fagbohun, 2016). The decrease in the levels of Zn and Se may be due to OS; because of the impact of the minerals(as cofactor) on the activities of SOD and GPx.

The results also showed significant decrease ($P<0.05$) in the activities of serum SOD, CAT and GPx (Table 5) among DM and DR subjects compared with apparently healthy subjects as earlier reported by Sindhu *et al.*, (2004). The authors reported a significant decrease in the activity of the antioxidant enzymes (SOD, CAT and GPx) in DM subjects with or without retinopathy. The significant decrease in the activity of the antioxidant enzymes in this study may be due to continuous accumulation of free radicals due to poor glycemic control. It has been reported that hyperglycemia causes alteration in many biochemical pathways leading to increased free radical production, thus lowering the activity of the antioxidants defense system (Brown, 2016). The balance between oxidant/antioxidant is crucial in preventing OS induce by hyperglycemia. The probable reason for the decrease in their activity might be due to; (i) down regulation in gene expression of the antioxidant enzymes and (ii) antioxidant enzyme glycation (Bikkad *et al.,* 2014). Enzyme glycation have been reported to be the established mechanism for the development of OS among DM and DR subjects (Bikkad *et al.,* 2014).

Table 1. Primer sequences and expected pcr product size for the antioxidant enzymes; SOD1, CAT1, GPx1, and ß actin (Rozen and Skaletsky, 2000)

Gene	Primer sequence ('5-3')	product size(bp)
SOD1 F	5'GGTTTGCGTCGTAGTCTCCT3'	328
SOD1 R	5'CCCAAGTCTCCAACATGCCT3'	
CAT1 F	5'AAGACTCCCATCGCAGTTCG3'	295
CAT1 R	5'CTGGAATCCCCCGATGACTG3'	
GPX1 F	5'TGGCTTCTTGGACAATTGGG3'	285
GPX1 R	5'TCTTGGCGTTCTCCTGATGC3'	
ß Actin F	5'CTCGCCTTTGCCGATCC3'	
ß Actin R	5'GGGGTACTTCAGGGTGAGGA3'	258

Abbreviation: SOD1: superoxide dismutase 1, CAT1: catalase 1, GPx1: Glutathione peroxidase 1, F: forward primer, R: Reverse primer, pcr; polymerase chain reaction, bp: base pair.

Table 2. Life style and clinical characteristic of the apparently healthy, DM and DR subjects

	AHS	DM	DR
M:F	2:1	1:2	1:2
AGE (years)	27.67±1.45	37.33 ±1.45	51.33±4.91
BMI (kg/m^2)	20.94±060	28.83±2.89	29.7±6.40
Hypertension (%)	0(0 %)	2(66.67 %)	2(66.67 %)
Smoker (%)	0(0 %)	0(0 %)	1(33.33 %)
Exercise(%)	100 %	1(33.33%)	1(33.33%)
DM History (%)	(0%)	2(66.67%)	2(66.67%)
FBS (mmol/L)	4.13±0.15^c	9.13±2.60^{ab}	11.80±4.11^a
HbA1c (%)	5.70±0.0.40^c	7.40±0.80^{ab}	9.90±3.80^a

Data are expressed as mean±SEM. values with different super script in the same column are statistically different ($p<0.05$) using one way ANOVA. M: Male, F: Female, Kg/m^2: kilogram/meter square, AHS: apparently healthy subjects, DM: Diabetes mellitus, mmol/L: millimol/liter, NDT2DM: Newly diagnosed type 2 diabetes mellitus, DR: Diabetic retinopathy, BMI: body mass index, FBS: Fasting blood sugar, HbA1c: glycated hemoglobin, n=3 per group.

Table 3. Serum Antioxidant vitamins levels of apparently healthy, NDT2DM and DR subjects

Group	Vit. A (µg/dl)	Vit. E(µg/dl)	Vit. C (mg/dl)
AHS	19.50±4.30^a	1.670±0.47^a	1.85±0.10^a
NDT2DM	11.00±1.13^b	0.798±0.25^b	0.71±0.04^b
DR	6.12±0.66^c	0.297±0.06^c	0.44±0.70^c

Data are presented as mean±SEM. Values with different super script in the same column are statistically different ($P< 0.05$) using one way ANOVA. AHS: apparently healthy subjects, NDT2DM: Newly Diagnosed Diabetes Mellitus, DR: Diabetic Retinopathy, Vit A: Vitamin A, Vit E: Vitamin E, Vit C: Vitamin C, µg/dl: microgram per deciliter, Mg/dl: milligram per deciliter, n=3 per group.

Table 4. Serum antioxidant minerals levels of apparently healthy, NDT2DM and DR subjects

Group	Zn (µmol/L)	Cu (µmol/L)	Mn (mg/dl)	Se (mg/dl)
AHS	13.90±2.06^a	17.07±2.19^a	0.0006±0.0003^a	0.642±0.06^a
NDT2DM	11.23±0.23^b	15.60±0.60^a	0.0005±0.00012^a	0.340±0.17^b
DR	7.00±0.75^c	20.97±1.97^a	0.0006±0.00013^a	0.160±0.0.06^c

Data are presented as mean±SEM. Values bearing different superscript in the same column are statistically significant ($P< 0.05$) using one way ANOVA. AHS: apparently healthy subjects, NDT2DM: Newly Diagnosed Diabetes Mellitus:, DR: Diabetic Retinopathy, Cu: copper, Zn: zinc, Cr: chromium, Mn: manganese, Se: selenium, µmol/L: micromole per liter, n=3 per group

Table 5. Serum antioxidant enzymes activity of apparently healthy, NDT2DM and DR subjects

Group	SOD (U/ml)	CAT (U/ml)	GPx(nmol/min/ml)
AHS	159.16±15.12^a	0.927±0.038^a	21.63±5.17^a
NDT2DM	97.91±34.60^b	0.640±0.059^b	11.03±2.25^b
DR	15.47±1.909^c	0.440±0.045^c	7.65 ±1.47^c

Data are presented as mean±SEM. Values bearing different superscript in the same column are statistically significant ($P< 0.05$) using one way ANOVA. AHS: apparently healthy subjects, NDT2DM: Newly Diagnosed Diabetes Mellitus: DR: Diabetic Retinopathy, SOD1: superoxide dismutase, CAT1: Catalase, GPx1: glutathione peroxidase, U/ML/E: Unit per mill of enzyme, SEM: standard error of mean, n=3.

Antioxidant enzymes glycation led to inactivation of the enzymes allowing free radicals to accumulate leading to OS there by damaging tissue and organs. Analysis of expression levels (Figure 1) of the antioxidant enzymes (SOD1, CAT1 and GPx1) further support the decrease in the activity of the antioxidant enzymes in DR subjects, the expression of SOD1, CAT1 and GPx1 were down regulated relative to apparently healthy subjects. However, the expression levels of SOD1, CAT1 and GPx1 in the NDT2DM subjects relative to apparently healthy subjects were up regulated. The upregulation of the enzymes in NDT2DM subjects may be due to early onset of diabetes as part of compensation in response to high free radicals levels produced by hyperglycemia (Bonnefont-Rousselot *et al.,* 2000). It has been reported that hyperglycemia up regulate the synthesis of these enzymes at early onset of diabetes, the authors stated that this up regulation is against persistent hyperglycemia (Bonnefont-Rousselot *et al.,* 2000). It has also been reported by Limaye *et al*.(2003) that the expression of the antioxidant enzymes were up regulated at an early stage of hyperglycemia (Limaye *et al.,* 2003) and later (six weeks after induction of diabetes in rats), the expression levels were down regulated. It can be concluded from the results of this study that that oxidative stress is one of the features of not only newly diagnosed diabetic subjects but also diabetic subjects that

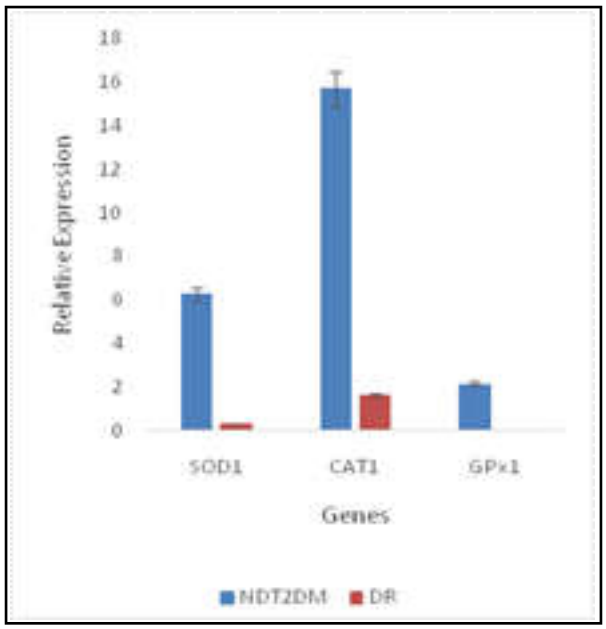

Figure 1: Relative quantification of SOD1, CAT1 and GPx 1 normalized to ß actin relative to Normal subjects of the NDT2DM and DR subjects. The result shows that SOD1, CAT1 and GPx 1 are upregulated among DM subjects relative to apparently healthy subjects. The expression level of the enzymes (SOD1, CAT1 and GPx1) in DR subjects were down regulated relative to apparently healthy subjects.CAT1: catalase 1, SOD1: superoxide dismutase 1, GPx1: glutathione peroxidase 1, DM: Diabetes Mellitus, DR: Diabetic retinopathy

already develop some of the complications of diabetes (in this case diabetic retinopathy). Antioxidant supplementation should be consider in the overall management of diabetes mellitus.

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Conflicts of interest: None declared

Key points

- The work further establish the existence of oxidative stress not only in early diabetic state but also at later stages of diabetes when late complications ensue (Diabetic retinopathy)
- The work also provide insight at genetic levels that early diabetic state is characterized by up regulation of genes for antioxidant enzyme (SOD,CAT GPx) but at later stage there is down regulation of these genes.
- Antioxidant supplementation should be considered in the overall management of diabetes mellitus and in delaying the development of late complications considering the oxidative stress associated with diabetes.

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