

Antioxidant Enzyme in Blood Test – A Marker for Fructose Metabolism

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Abstract

Excessive fructose consumption can cause metabolic damage. Previous evidence supports that oxidative stress plays a big role in metabolic syndrome (MS)-related manifestations. Recently blood markers like high sensitive-C reactive protein (hs-CRP) and serum lipid profiles are widely used clinical parameters. However, it is unknown if whether antioxidant capacity is related to these changes or not. This study was designed to explore if the concentration of blood markers (triglycerides, cholesterol, glucose, Hs-CRP) is associated to antioxidant capacity in hyperlipidaemia rats. High fructose diet (HFD) was orally given to rats for 80 days to establish hypertriglyceridemia and blood tests were carried on at different time intervals up to 80th day. Serum triglycerides, glucose, glucose, Hs-CRP, SOD, Catalase and LPO were recorded at 50th and 80th days. At last we assessed m- RNA expression of SOD and catalase in WBC. The gradual rise in activities of superoxide dismutase (SOD), catalase and ABTS.+(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)-scavenging potential was recorded without any rise in the serum lipid peroxidation- products, triglycerides (TG), glucose and hs-CRP up to 50th day of HFD feeding. However these changes were reversed on 80th day. The RT-PCR for SOD and catalase mRNA in white blood cells (WBC) also showed similar biphasic. This study suggests that the initial rise in blood antioxidant enzymes to their maximum capability, in early days as adaptive mechanism to encounter the oxidative damage from oxygen free radicals. Thus raised activity of catalase and SOD in blood could be considered as one of the significant biomarkers for early diagnosis of adverse metabolic changes when there is no clinical symptom to MS. This study could be helpful in developing pre-diagnostic parameters to persons, who are likely to develop MS.

Keywords: Antioxidant enzymes; Hypertriglyceridemia; High fructose diet; Metabolic syndrome; Low grade inflammation

Introduction

Urban life-style and western-diet are the major contributors to metabolic syndrome (MS). Earlier, it was frequently observed in developed countries, but now such incidences are very common even in developing countries [1]. Metabolic syndrome encompasses cluster of risk factors for cardiovascular disease which include abdominal obesity, dyslipidemia, hypertension, and hyperglycaemia [2]. The WHO has identified MS as one of the major causes of death and it has warned the medical fraternity to take proper initiatives for its prevention [3]. Most of the disorders associated with MS have no symptoms but the people at early stage of MS have high blood sugar levels, hypertension, high triglycerides and cholesterol levels. Each condition can raise the risk for heart disease, diabetes, strokes (late stage of MS). The western diet consisting of fried food, especially rich in saturated fats (high ratio of n-6: n-3 fatty acids), fructose brings metabolic dysregulation, oxidative stress, and inflammation ultimately causing insulin resistance and fatty liver [4]. In order to reduce the manifestation of MS, its early diagnosis and awareness about its preventive measures in the society can be essential components. Although, serum lipid profile and inflammatory parameters are currently used as diagnostic parameters, but this situation appears to be in late stage of MS manifestation, when it is difficult to reverse. So, more sensitive parameters are required for its early diagnosis. Human body by antioxidants (inside and outside antioxidants) and by different mechanisms harness the oxidative stress. There are complicated antioxidant systems inside cells (SOD, catalase) and blood plasma that prevent from generation of secondary free radicals and protect the body from destructive effects of free radicals. Here, we have hypothesized that feeding of HFD results to hyperlipidaemia and hyperglycaemia due to the decreased antioxidant enzyme activity in blood. The high fructose diet was orally fed for 80 days and blood was collected at different time intervals to assess the activity of SOD, catalase, ABTS, +(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging potential, serum triglyceride

(TG), glucose, lipid peroxidation products and hs-CRP. Until now it was not clear, whether changed enzyme activities are accompanied by altered gene expression we have. Therefore investigated the messenger RNA (m RNA) expression of SOD and catalase in WBC at 50 and 80 days treatment using quantitative reverse transcriptase –polymerase chain reaction (RT-PCR). So in the end of the experiment, gene expressions of these antioxidant enzymes were analysed in white blood cells (WBC).

Materials and Methods

Experimental design

The experiment was performed according to ethical guidelines approved by animal ethics committee of our Institution (IMS, BHU-letter # Dean/2005-06/Animal Ethical Committee/390 dated-18.05.2006). Twelve healthy albino rats of Charles foster (CF) strain of inbred colony, 8-10 week old (weighing 110±10g) were randomly divided in to 2 groups. One was kept on normal diet (untreated rats) and other was supplemented with fructose emulsion that contained 70% of fructose (w/v), 10% lard, 1% vitamin B complex, 1% methionine (w/v) and 18% soya bean-oil in water (20ml/kg bwt/day) along with normal chow diet (high fructose diet rats). On different time intervals (20, 30, 40, 50, 60, 70 and 80 days), blood samples were withdrawn from all the animals via retro-orbital-vein following a

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fasting period of 16 hours from both groups. For the determination of serum lipid profile, glucose, hs-CRP levels, blood samples were collected in plastic tubes without EDTA. The samples were centrifuged at 4°C at the speed of 4000 rpm for 15 minutes and the serum was collected and stored at the -80°C freezer until assayed. The blood was separately collected in the heparinized tube (2% ethylenediamine tetra acetic acid (EDTA) to isolate red blood cells (RBC) for determination of activities of antioxidant enzymes. For termination, all the animals were euthanized with an overdose of ketamine (10 mg/kg, intra-peritoneal injection) and blood were collected. WBC preparations were made by combining 1 ml of red blood cell (RBC) lysis buffer with 0.5 ml of whole blood, rotational mixing for 5 min, centrifugation for 5 min at 2000 g, aspiration of supernatant, and re-suspension of WBC pellet in 0.5 ml of lysis buffer. After an additional 5 min of rotational mixing, samples were centrifuged again at 2000 g for 5 min, the supernatant was aspirated, and WBCs were re-suspended in lysis buffer. Pellet of WBC was used for RNA isolation. The total free radical trapping potential was determined by capacity of plasma to trap ABTS+ radicals [5]. The plasma LPO (Lipid peroxidation) was determined in terms of TBARS (thiobarbituric acid reactive substances) [6] and hs-CRP was determined by immunoassay kit (Diagnostic biochem Canada Inc.). The triglyceride and cholesterol were determined by specific enzymatic kits (Accurex biomedical PVT.LTD Thane, India). Glucose was determined by blood glucose meter (SD Biosensor, Inc, New Delhi). The RBC hemolysate was used to assess the SOD-activity [7] and catalase-activity [8]. The 5 rats, out of 10 rats from each group were sacrificed on 50th day and remaining rats were sacrificed on 80th day after an overnight (16 hours) fast to collect blood.

Reverse transcription-polymerase chain reaction (RT-PCR) of SOD and catalase in WBC

Total cellular mRNA was prepared from the white blood cells (WBC). Five microgram of total RNA was reverse transcribed with superscript II RNase H-reverse transcriptase (RT) using random hexamers according to the manufacturer's instructions (Fermentas) to form c-DNA. For PCR amplification 2 µL of c-DNA was used in a volume of 25 µL containing 2.5 µL from 10 × buffer, 1.0 µL 10 mM dNTP, 35.1 µL (nuclease free water), 1.0 µL of primers (SOD or CAT), and 0.4 µL (5 U/µL) Taq polymerase. This reaction mixture was warmed by a thermal cycler (Bio-Rad) at 94 °C for 3 min and 35 cycles of 45 s at 94 °C, 30 s at 55 °C, 1.3 min at 72 °C, and 72 °C for 10 min. Finally, the temperature was cooled at 4 °C for indeterminate time. The PCR amplified product was analyzed on a 2.0% agarose gel (Hi media). The bands, stained with 10µg/ml ethidium bromide, were photographed by a digital camera and documented by alpha imager software. The bands were revealed with 387 bp (SOD) and 670 bp (Catalase): SOD Primer sequence (5' to 3') forward: TCT AAG AAA CAT GGC GGT CC, Reverse (5' to 3'): CAG TTA GCA GGC CAGCAG AT Primer of catalase Forward (5' to 3'): GCG AAT GGA GAG GCA GTG TAC, Reverse (5' to 3'): GAG TGA CGT TGT CTT CAT TAG CAC TG. AAA CAT GGC GGT CC. The optical density of SOD and Catalase mRNA accumulation was determined with alpha imager (Bio-Rad), and expressed as the ratio against GAPDH.

Statistical analysis

It was carried out by SPSS (VERSION 19). Data were expressed as mean ± standard deviation. Data were analyzed by parametric statistics (ONE WAY ANOVA and t-tests, with Tukey's test used as appropriate) as described for each experiment < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p > 0.05. Asterisks indicate significant differences. P Values of less than 0.05 were considered statistically significant.

Results

Effect of HFD on serum LPO, TG, glucose, cholesterol and hs-CRP

At 50 days HFD feeding was showed slight higher in TG, LPO, hs-CRP, glucose and cholesterol level when compared to its basal value and also with 50 days untreated control group (P<0.001). The mean baseline serum LPO level in animals in HFD group was 0.034 ± 0.002nmol/mg protein this increased to 0.05 ± 0.004 nmol/mg protein (0.68 times), basal TGlevel in animals in HFD group was 52 ± 3.5 mg% this increased to 85 ± 2.8 (0.61 times), basal glucose was 57 ± 2.4 mg/dl this increased to 79 ± 4.1mg/dl (0.72 times), basal hs-CRP level in animals in HFD group was 483.1 ± 25 ng/ml this increased to 523 ± 18.7 ng/ml (0.92 times), basal cholesterol level in animals in HFD group was 62.85mg% this increased to 72.3 mg% (0.09 times) after 50 days high fructose feeding. There is marked increase when compare to basal value. At 80 days mean levels of TBARS reached to 0.63±0.05 nmol/mg protein (18.5 times), mean levels of TG reached to 117 ± 13 mg% (2.25 times) , mean levels of glucose reached to 135 ± 10.5 (2.3 times), mean levels of hs-CRP level reached to 558±15.5 ng/ml (1.15 times). which was significantly higher compared with their baseline and 80 days untreated rats LPO, TG, glucose, Hs-CRP levels (P<0.001). The rise in LPO was highest, followed by rise in TG, but the change in hs-CRP value was lowest. Interestingly no significant change was observed in serum cholesterol. In normal rats no such changes were recorded. Only 50 and 80 days data of untreated rats are shown which is expected to have maximum change (Table 1).

Effect of HFD on antioxidant enzyme activities in blood hemolysate

The HFD feeding showed the rising trend in the activity of SOD (3.8 times) and catalase (1.7 times) up to 50 days when compared with basal value and 50 days untreated rats. The changes were statistically significant (both P<0.001). The ABTS+ scavenging potential was also increased (1.7 fold) in this period when compared with basal value and 50 days untreated rats. (P=0.003 and P=0.0014). At 80th day, HFD feeding induced significant (P<0.001) decline in SOD activity (0.37 times) followed by catalase activity (0.12 times) when compared with its basal value and 80 days untreated rats (both P<0.001). Since ABTS method is known to be a rapid method for the determination of the antioxidant activity in samples. Further ABTS+ scavenging potential also showed similar biphasic pattern. It significantly (P<0.001) declined on 80th day (0.12 times).As expected, rats treated with high fructose diet showed reduced plasma total antioxidant status at 80 day suggesting a diminished antioxidant defence resulting from oxidative stress.

Thus, it could be suggested that changes in activity of SOD, catalase and ABTS+ scavenging potential are some of the sensitive parameter for early detection of HFD feeding related metabolic changes. In normal rats no change was noted on all tested days so all the data have not been shown. Only 50 and 80 days data of untreated rats are shown which is expected to have maximum change (Table 2, Figure 1).

m-RNA expression of SOD and Catalase in WBC at 50 days and 80 days

After normalizing the housekeepers (GAPDH gene) HFD significantly (p < 0.001) raised the WBC m-RNA of SOD (4 times) and catalase (4 times) (p < 0.001) on 50th days as compared with the control diet fed rats (Figure 2a, 2b). After 80th day of HFD feeding, the

Parameters	Untreated rats			HFD treated rats							
	Basal value	50 days	80 days	Basal value	20 DAYS	30 DAYS	40 DAYS	50 DAYS	60 DAYS	70 DAYS	80 DAYS
Plasma LPO (nmol/mg protein)	0.017±0.003	0.013 ± 0.001	0.016±0.003	0.034±0.002	0.038±0.002	0.040±0.003	0.048±0.003	0.05±0.004 ^{***}	0.086±0.007 ^{***}	0.08±0.006 ^{***}	0.63±0.05 ^{***}
TG (mg%)	42±8.1	48±2.0	50±4.0	52±3.5	59.1±1.5	62±2.1	65±4.0	85±2.8 ^{***}	85.3±4.3 ^{***}	115±4.0 ^{***}	117±13 ^{***}
hs-CRP (ng/ml)	483.1±16	490±8.1	495±12.2	483.1±25	490±17.3	493±13.0	493±10.6	523±18.7 ^{***}	539±31.8	549±23.6 ^{***}	558±15.5 ^{***}
Glucose (mg/dl)	65±1.6	72±2.6	75±5.2	57±2.4	63±2.8	68±2.5	70±3.2	79±4.1 ^{***}	120±7.8 ^{***}	123±8.1 ^{***}	135±10.5 ^{***}
Cholesterol (mg %)	60.3±4.9	75±2.8	80±9.7	62.85±4.1	63.5±3.6	68±4.2	68±3.6	72.3±5.3	73±2.6	73.4±2.0	81±3.1

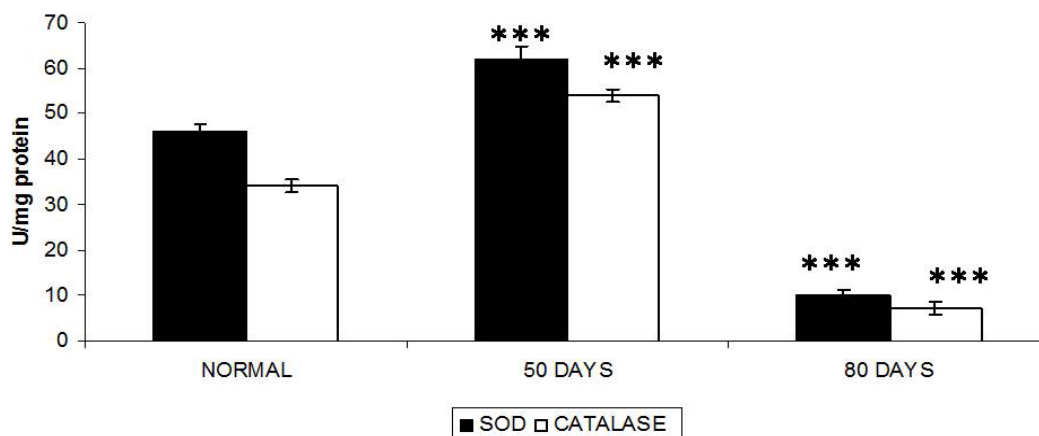
Table 1: Effect of HFD on serum LPO, TG, cholesterol, glucose and hs-CRP.

Data presented as mean± SD. (n = 10.) HFD are compared with its basal value and untreated normal rats of 50 days and 80 days respectively.

Parameters	Untreated rats			HFD treated rats							
	Basal value	50 days	80 days	Basal value	20 DAYS	30 DAYS	40 DAYS	50 DAYS	60 DAYS	70 DAYS	80 DAYS
SOD (U/mg protein)	0.45±0.04	0.45±2.0	0.49±0.04	0.45±0.03	0.82±0.08 ^{***}	0.85±0.06	0.89±0.07	1.75±0.06 ^{***}	0.195±0.01 ^{***}	0.19±0.015 ^{***}	0.17±0.01 ^{***}
Catalase (U/mg protein)	1.1±0.07	1.0±0.22	1.2±0.16	1±0.02	1.6±0.048	1.68±0.03	1.7±0.057	1.7±0.08 [*]	0.84±0.06 ^{***}	0.13±0.01 ^{***}	0.12±0.009 ^{***}
ABTS [*] (%inhibition)	43±1.5	40±2.6	42±1.6	42±1.6	43.2±2.6	48±2.2	50.1±1.7	74.2±3.2 ^{***}	11.3±1.0 ^{***}	9.4±0.032 ^{***}	5.2±0.02 ^{***}

Table 2: Effect of HFD on antioxidant enzyme activities in blood hemolysate.

Data presented as mean± SD. (n = 10.) HFD are compared with its basal value and normal are compared with its initial value.



Antioxidant enzyme gene activity in blood hemolysate of HFD over 50 and 80 days (N=10). Difference between normal vs. HF diets: P< 0.001^{***}.

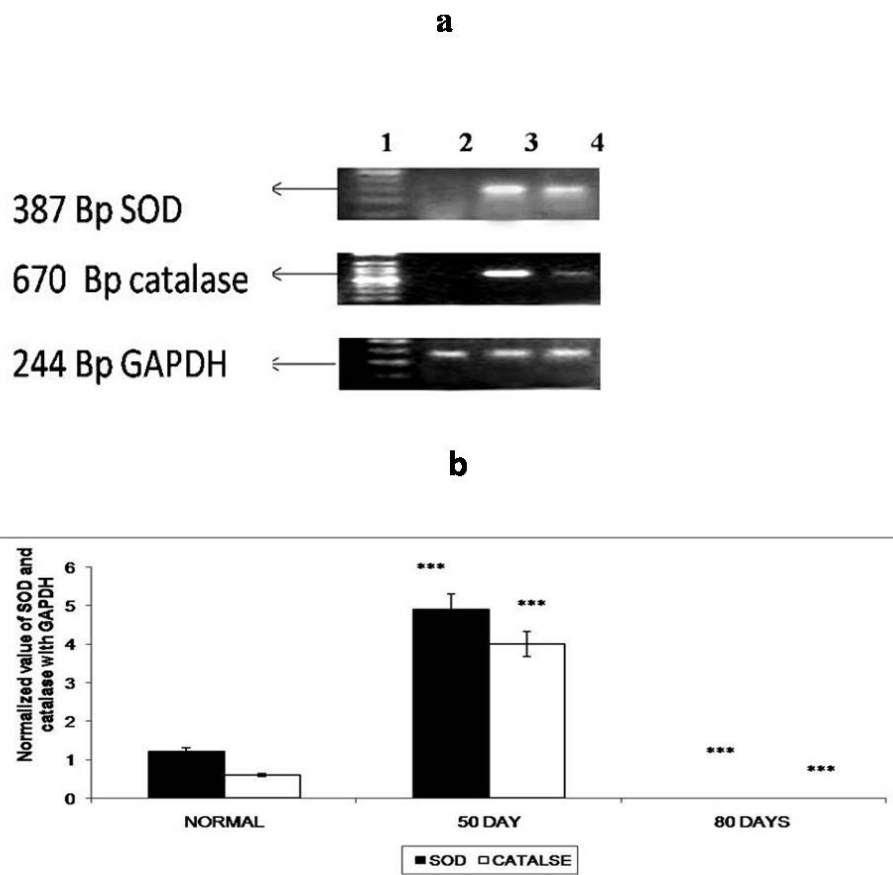
Figure 1: Antioxidant enzyme gene expression in blood hemolysate of HFD.

mRNA accumulation was below the normal value. It was not detectable (Figure 2a, 2b).

Discussion

Uncontrolled metabolic syndrome (MS) is one of the leading causes in overall mortality [9] and contribute high financial burden on the society. Thus, early diagnosis preventive measures can be great concern in the health care sector. The conventional diagnostic biochemical parameters include blood profile for lipid and glucose parameters and recently, inflammatory markers are also included, but assay of antioxidant enzymes are not in routine use. Here we have hypothesized that oxidative stress related parameters could be more sensitive parameter for early diagnosis for metabolic syndrome.

Western diet consisting of high fructose and saturated fat is known to induce inflammatory [10] and hypolipidemic changes in the body [11,12]. It is reported that oxidative stress lead to ER stress [13], which is linked to high fat metabolism also [14]. It is also reported that triglyceride accumulation in different organs is type of defence mechanism against fatty acid induced lipotoxicity [15]. The antioxidant system is another defence mechanism against metabolic abnormalities. They are basically meant to prevent the oxidative stress, which are produced during routine metabolic processes [16]. It includes blood Glutathione (GSH) level, glutathione peroxidase and reductases, SOD and catalase [17]. In case of higher free radical (FR) generation, these antioxidants are also stepped up to save the cell [18]. However, this defensive mechanism is compromised during prolonged oxidative

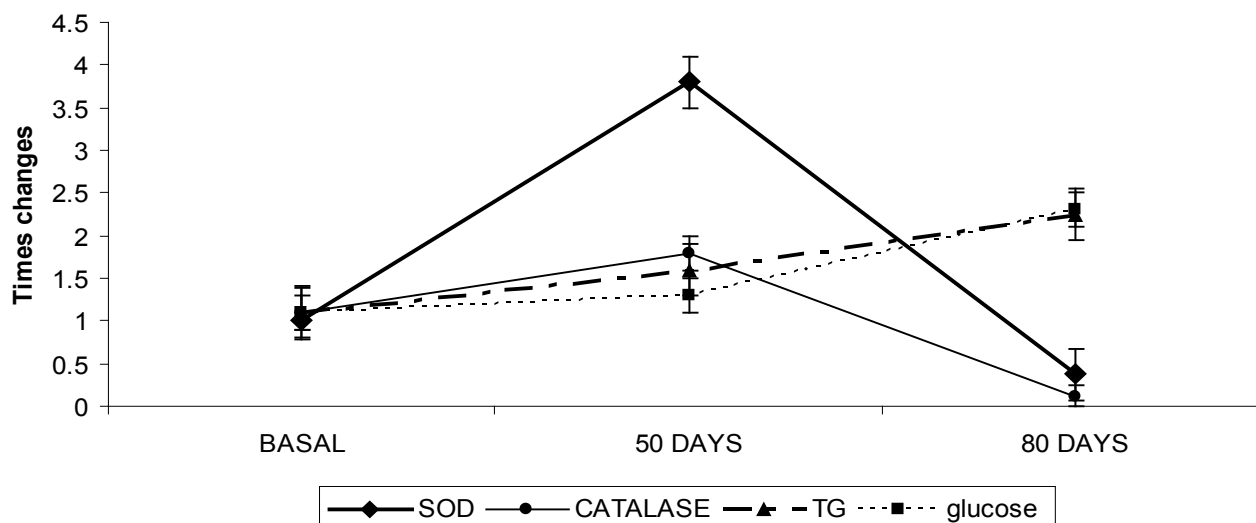


Antioxidant enzyme gene expression in WBC of HFD. (a) m-RNA content of SOD, catalase and GAPDH in WBC of rats fed with HFD (high fructose diet) over 50 and 80 days by RT-PCR: lane1) 100 BP ladder, 2) HFD rats (80 days), 3) HFD rats (50 days), 4: normal rats. (b) The relative level of expression for SOD and catalase in WBC by RT-PCR analysis. (N=10). Difference between normal vs. HF diets: $P < 0.001^{***}$.

Figure 2: Enzyme activities of SOD and catalase in blood haemolysate of HFD in different time interval.

stress, similar to insulin level in pre-diabetic and diabetic conditions. Our results indicated the raised antioxidant enzymes at 50th day in HFD fed rats and it was declined at 80th days. This could be considered as adaptive mechanism to encounter the free radical mediated stress, which is usually generated in pathogenesis of any metabolic stress. Thus, their raised activity would be more prominent parameter than their declined activity, which is often considered in present scenario. Since exact correlation between level of blood antioxidant enzymes and inflammatory/hyperlipidemic status is still lacking, so this aspect has been explored here HFD/ western diet are also known to enhance lipid peroxidation and inflammation in human and animal studies [19]. Our results show significant ($p < 0.001$) rise in activity of blood SOD and catalase in early days of HFD feeding. Such reports have been published earlier also [20], but time kinetics is lacking. Blood samples can be considered as a new approach to the diagnosis as well as the treatment of metabolic disease. We have also found altered expression of SOD and catalase mRNA in WBC by RT-PCR analysis. Furukawa et al have also shown similar results [21], but time dependent changes have not been reported earlier. Our results showed the raised activity of blood SOD to about 3.8 times. Interestingly its expression was also changed to 4 times. Similar pattern was observed in case of catalase activity and its expression in WBC, but their mutual correlation was different than SOD. The activity of catalase in RBC hemolysate was higher to range of 1.7 times but its expression in WBC was raised up to

4 times. This data suggest that change in activity of blood SOD could be considered as more sensitive diagnostic marker for oxidative stress. The overall FR scavenging potential of blood was evaluated by its ABTS scavenging potential. It was about 1.7 times higher than basal value (HFD rats), on 50th day of HFD feeding, but significantly ($P < 0.05$) low (0.12 times) at 80th days. This is also in agreement with biphasic change in activity of SOD and catalase as discussed above. Further, we assessed the level of lipid peroxidation products (LPO-products) and hs-CRP in blood samples of these animals. They showed contradictory results, means low on 50th day and higher on 80th day. These changes were similar to the change in serum TG (triglycerides). This observation is in agreement with earlier reports indicating triglycerides induce rise in hs-CRP [22] and fructose induce lipid peroxidation [23]. On the 80 days of HFD feeding, there was sharp decline in both activity and expression ($p < 0.001$) of SOD and catalase. This conclusion is supported by earlier finding where it has mention HFD induces oxidative stress [24]. It is also reported that oxidative stress induces expression of SOD and catalase [25,26]. This is the 1st report indicating the direct correlation in serum TG, glucose and hs-CRP with that of antioxidant enzymes in context of HFD feeding. Similar report is also available with other animal models of streptozotocin induced diabetes and cisplatin induced nephrotoxicity, where changes in activity of SOD and catalase have been reported as early diagnostic parameters [27]. Our data clearly indicate the biphasic changes in these parameters but in opposite direction. The



Time changes (fold change) of antioxidant enzyme activity, triglycerides and glucose over 50 and 80 days.

Figure 3: Correlation between antioxidant enzyme activity, triglycerides and glucose in WBC of HFD.

same has been graphically presented in (Figure 3). Thus, it appears that body initially tries to adjust with oxidative stress by rising the activity of available enzyme in the body may be due to both translational and post translational control [28]. Later on it induces the expression of these enzymes. The enzyme activity changes were not caused by mRNA level, which in part might be enzyme inactivation/activation or degradation/synthetic process. However due to perpetuating stress condition, the antioxidant defence mechanism of body fails to cope up with oxidative stress thereby allowing the adverse reaction of metabolic stress such as hyper triglyceride state and low grade systemic inflammation. Our result also suggests that when degree of oxidation stress due to HFD keeps on rising up to 80 days. From 10 day to 50 days antioxidant enzyme play protective role but on later days, this protection cover is compromised and high triglycerides synthesis and its accumulation in adipose tissue starts to play a protective role against the rise in blood FFA, which is more injurious to endothelial cells, finally resulting low grade inflammation, a cause of endothelial dysfunction, insulin resistance and overall systemic-inflammation. These changes further take the body to vascular disorders. Thus, low activity of blood antioxidant enzyme even with pre-diabetic or pre hyperlipidemic conditions could be considered as alarming situation for manifestation of MS. The concerned person should be warned to change life style and food habits. When fructose is consumed in whole fruit, the whole fruit also contains water, fiber, antioxidants, and nutrients so our body can tolerate it quite well. The problem is the amount of fruit we consume (3-5 pieces a day), addition of sugars (50% fructose) and high fructose corn syrup. Based on this study, first time we are reporting that high fructose diet induces hypertriglyceridemia, hyperglycaemia, oxidative stress and low grade inflammation only when antioxidant defence system is compromised. Thus, rise in antioxidant enzymes could be used as early diagnostic marker for identification of MS prone persons. It also indicates towards the new targets for further management of diet induced metabolic changes.

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