Sirtuin 1 and 7 mediate resveratrol-induced recovery from hyper-anxiety in high-fructose-fed prediabetic rats

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Hyperglycaemia in diabetes is either caused by reduced availability of insulin (type 1 diabetes, T1D) or insulin resistance to the cells (type 2 diabetes, T2D). In recent years, the prevalence of T2D has increased to an alarming proportion, encompassing 95% of the total diabetic burden, probably due to economy-driven changes in lifestyle. Recent epidemiological studies show comorbid depression, anxiety and related mental illness. To explore the molecular mechanisms underlying this comorbid conditions, we used Sprague–Dawley rats on high-fructose diet for 8 weeks to induce prediabetic condition. Rats with this metabolic syndrome also showed hyper-anxiety when they were subjected to anxiety-related behavioural assays. Rats were administered with resveratrol, an activator of sirtuins, and metformin, a standard antidiabetic drug, simultaneously with fructose. We observed that resveratrol was more effective in protecting from both the metabolic (prediabetic) and affective (anxiety) disorders than metformin. Molecular studies showed that recovery was associated with the upregulation of few nuclear sirtuins that act epigenetically – Sirt 1 and 7, which were significantly attenuated in the striatum of prediabetic rats. In conclusion, our study showed that hyper-anxiety associated with prediabetic condition is ameliorated by resveratrol through modulation of sirtuins, which is more or less similar to metformin.

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1. Introduction

The global prevalence of diabetes mellitus has gone up to 9% among adults aged 18+ years (Alwan 2011), and a recent report projects that diabetes will be the 7th leading cause of death by 2030 (Mathers and Loncar 2006). The condition of hyperglycaemia, associated with diabetes, arises either due to the lower production of insulin (type 1 diabetes, T1D) or due to insulin resistance to the cells (type 2 diabetes, T2D). Hyperglycaemia in the long run causes serious damage to many organs and systems (Cade 2008). From the Indian perspective, the condition is alarming as there are more people suffering from the T2D (more than 50 million) in India than any other nation (Diamond 2011). Unlike T1D where the cause is probably autoimmune or genetic, T2D, which comprises 95% of the total diabetes burden globally, is largely the result of environmental conditions like less physical activity, inappropriate diet, excessive body weight,

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etc., and is one of many emerging 'lifestyle diseases'. Diabetes and insulin resistance, or the inability of the hormone insulin to properly control blood sugar levels, are also linked to increased risk of mild cognitive impairment and dementia. Earlier reports indicate that people with type 2 diabetes or in the early stages of the disease (metabolic syndrome) appear to be at an increased risk of brain plaque formation of Alzheimer's disease (Luchsinger 2010).

Recent epidemiological studies (Ducat *et al.* 2015; Bernstein *et al.* 2013) show mental health comorbidities of diabetes (Ducat *et al.* 2014). One of the most serious mental illnesses associated with diabetics is major depressive disorder, with or without anxiety disorder (Kesebir 2014). Therefore, it is very important to understand the molecular mechanisms in diabetes-induced depressive and/or anxiety disorders. So far, the animal studies to uncover the molecular mechanisms are lacking on this comorbid mental illness in diabetes. Thus, in order to get an insight into the problem, we tried to determine whether T2D could induce any noticeable mood disorder phenotype other than depression, like anxiety. Male Sprague–Dawley rats were kept on high-fructose diet for 8 weeks, and the biochemical parameters suggested that the fructose-fed animals successfully developed prediabetic condition or metabolic syndrome, the precursor to diabetes (Bagul *et al.* 2012). The rats were then assessed for their mood state and we could see noticeable hyper-anxiety, a mood disorder in the high-fructose-fed group.

Chronic environmental perturbations to brain caused by stress and injury have been shown to induce various neurological and psychiatric disorders through epigenetic mechanisms (Dudley *et al.* 2011). A class of epigenetic regulators, sirtuins, which are NAD-dependent histone deacetylases (HDACs), play an important role in the etiopathology (Tsankova *et al.* 2007). Sirtuins are metabolic sensors that control cellular metabolism, energy homeostasis as well as neural functions (Volmar and Wahlestedt 2015). Therefore, we hypothesized the involvement of the nuclear sirtuins, which are known to act epigenetically, i.e. Sirt 1, 2, 6 and 7, in brain underlying the high-fructose-induced affective hyper-anxiety in rats. In the present study, the rats were treated with resveratrol, a well-known sirtuin activator, which is also a food supplement (Bagul *et al.* 2015; Bagul and Banerjee 2015) for 8 weeks of high fructose feeding. Resveratrol administration led to complete recovery from metabolic as well as anxiety disorders. The resveratrol-induced recovery from the affective disorder appears due to the upregulation of few of these nuclear sirtuins, the ones which were significantly attenuated in the striatum of rat brain in high-fructose-diet-induced disorders. As many researchers have reported that the most commonly prescribed anti-diabetic drug, metformin, protects against diabetes-associated oxidative stress (Correia *et al.* 2008; Chakraborty *et al.* 2011; Esteghamati *et al.* 2013), we included this drug in our study as positive control together with the test drug resveratrol, and for the first time we report here that their therapeutic action appears to be due to the epigenetic mechanism involving a few sirtuins.

2. Materials and methods

2.1 *Experimental animals*

Male Sprague–Dawley rats, 7 to 8 weeks old and weighing 180–200 g were obtained from the National Institute of Nutrition, Hyderabad. Rats were acclimatized in BIOSAFE, an animal quarantine facility at the Indian Institute of Chemical Technology (IICT), for a period of 1 week before start of the study and were maintained as reported earlier (Bagul *et al.* 2012; Padiya *et al.* 2011). Briefly, the experimental room was set to 12 h light/dark cycle at temperature 22±2°C and relative humidity 50±15%. Air changes (15–16 cycles/h) were maintained with 5 μm HEPA filter in a class 10,000 environment. Rats had free access to food [pellet diet supplied from the National Institute of Nutrition (NIN), Hyderabad] and water *ad libitum* during the quarantined period. The study protocol was approved by the Institutional Animal Ethics Committee of the Indian Institute of Chemical Technology, Hyderabad (protocol no. # IAEC/IICT/SKB/SC/2013-14).

Rats were divided into four groups (N=8) for the feeding paradigm, as described in detail in one of our co-authored paper (Bagul *et al.* 2012). Briefly, the control group was fed 65% corn starch diet (Research diet, USA), and the fructose-fed insulin-resistant group, i.e. the diabetic group, was fed 65% fructose diet (Research diet, USA); a third group (Diab + Resv) was fed with 65% fructose along with a single dose of 10 mg/kg/day of resveratrol orally, whereas the fourth group (Diab + Met) was fed with 65% fructose along with a single dose of 300 mg/kg/day of metformin (Sigma, USA) orally, for a period of 8 weeks. The dose of resveratrol and metformin was decided based on previous literatures (Ates *et al.* 2007; Miatello *et al.* 2005). The complete composition of the diet has already been published in our co-authorship (Bagul *et al.* 2012). Resveratrol was supplied by Kumar Organic Products Ltd., Bangalore, India. Resveratrol and metformin were prepared in 25% of DMSO. Accordingly, the Control and Diabetic groups were treated with 25% DMSO to nullify the effects of DMSO.

2.2 *Behavioural procedure*

The rats in all four groups were subjected to behaviour tests that measure anxiety in animals on the very next day of the completion of 8 weeks of experiment which led to the prediabetic condition in high-fructose-fed rats and prevented

the development of this metabolic disorder in the fructose-fed rats treated with resveratrol or metformin.

2.3 *Open-field test*

The rats were transferred to an open-field box measuring 60 × 60 × 60 cm, with the floor divided into 9 equal squares. The central one was considered the center zone whereas the eight peripheral squares were designated as peripheral zone. The open-field session for each animal lasted for 5 min, which was recorded by a video camcorder (Sony handycam) and later scored by an observer not aware of the pharmacological treatments. The number of crossings through the squares and number of visits to the center zone were recorded. This was done manually as described in previous reports (Schmatz *et al.* 2009; Sacharczuk *et al.* 2009). This test was carried out to identify the spontaneous locomotor activity related to the anxiety-like phenotype in a novel environment, which might be influenced by the diabetic condition and its treatments.

2.4 *Light/Dark box test*

The rats were transferred to a box containing two equal compartments, one illuminated aversive compartment and the other dark secure compartment. Animals could cross from one compartment to another through a small hole in the wall separating two compartments. Each rat was introduced at the corner of the illuminated box and the activity was recorded for 5 min and later an observer, who was not aware of the pharmacological treatments, scored the duration of time spent in the dark and light chambers manually (Griebel *et al.* 1997). This test was carried out to measure the anxious phenotype of rats in relation to the diabetic condition and its treatments.

2.5 *Estimation of oxidative stress in striatum*

Striatum, the area of brain highly implicated in anxiety and related mood disorder, from each side of the rat brain were micro-dissected and homogenized in freshly prepared ice cold potassium phosphate buffer saline with 20 times dilution. Tissue homogenate was used for the estimation of thiobarbituric acid reactive substances (TBARS). The remaining volume of homogenate was put for centrifugation at 15,000*g* for 30 min at 4°C. The supernatant was collected and used for the estimation of glutathione (GSH) and catalase as previously reported (Bagul *et al.* 2015; Sojitra *et al.* 2012). TBARS was measured as a marker of lipid peroxidation (Ohkawa *et al.* 1979) and the reduced glutathione and catalase enzymes were estimated for assessing the level of endogenous antioxidants. Similar studies were conducted by using other brain areas like the hippocampus. All of the compounds used for the estimation of different biochemical parameters were obtained from Sigma Inc., USA.

2.6 *Analysis of protein expression by Western blot*

Extraction of whole-cell protein fractions was done with striatal samples. Quantity of protein was estimated by the Bradford method. An equal amount (50 µg) of protein was separated by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to PVDF membranes (Amersham Bio-sciences). The membranes were then blocked by the blocking buffer made of 5% non-fat dry milk in 0.1% phosphate buffered saline Triton X-100 (1xPBS-TX100) for 1 h, and subsequently incubated with primary antibody in blocking buffer [(Sirt1, 2, 6 and 7), with dilution of 1:1000 for Sirt1, Sirt2, Sirt6 antibodies from Millipore and 1:500 for Sirt7 antibody from Abcam] at 4°C overnight. After washing with PBS-Tween 20, membranes were incubated with anti-rabbit horseradish peroxidase conjugated secondary antibody for 1 h with dilutions 1:10000 for Sirt1, 2 and 6 and 1:5000 for Sirt7antibody. The signal was detected by chemiluminescence using the ECL detection system (Amersham). β-actin (Sigma) antibody with a dilution of 1:1000 was used in normalization as loading control. The quantification of bands was performed using the ImageJ software (NIH).

2.7 *Real-time PCR*

Total RNA was isolated from striatal tissues using TRIzol reagent (Sigma), further purified with RNeasy mini kit (Qiagen) and quantified by Nanodrop (Thermo). For real-time PCR analysis, cDNAs were synthesized from equal amount of total RNAs from each sample using Verso cDNA reverse transcriptase kit (Thermo) with random hexamer primers, at 42°C for 1h. The resulting cDNAs were subjected to PCR analysis with gene-specific primers (table 1) in the presence of SYBR green (ABIGene). Relative abundance of mRNA was calculated by normalization to the ribosomal protein L32 (RPL32) level and the results were expressed as relative expression levels. The data were quantified by the method of $2^{-\Delta\Delta Ct}$.

2.8 *Histopathology*

Brain samples were fixed in 4% paraformaldehyde for 24 h followed by three consecutive washes in 1X PBS and finally preserved in 20% glycerol made in PBS, for further use. The fixed tissue was mounted on the section stage with appropriate adhesive and then cut into 30µm thin slices using EMS Oscillating Tissue Slicer (Model no. OTS-4500, Harvard Apparatus, USA). Only good selected tissue sections were mounted on positively charged superfrost plus slides

Table 1. List of primers used

Gene	Forward primer	Reverse primer
Sirt1	TGACTTCAGATCAAGAGATGGTAT	TGGCTTGAGGATCTGGGAGAT
Sirt2	CTAGAAAGTCCCCACCTCCTG	GCTCACACAAGCTGCATGTTA
Sirt6	GAGGAGTGCCCCAAGTGTAA	CCCAGTCCAGAATGGTGTCT
Sirt7	GCGTGCAAAAAGGAAGAAAG	AGGCTTTCCACAAGGACAGA
RPL32	AGATTCAAGGGCCAGATCCT	CGATGGCTTTTCGGTTCT

(Fischer scientific, USA). The sections were later stained with Hematoxylin and Eosin (H & E) reagent, dehydrated with graded series of alcohol and mounted with DPX. The stained slides were observed using Axioplan2 Imaging system (MC200, Carl Zeiss Inclusive, Germany), images taken and results were analysed.

2.9 *Statistical analysis*

All values were expressed as mean ± SEM. Data were statistically analyzed using one-way ANOVA for multiple group comparison followed by Newman-Keuls test using Graphpad Prism version 5.0 for windows, Graphpad software, San Diego, California, USA. Difference between two groups was compared by Student's t-test and the level of significance was set at $p \leq 0.05$.

3. Results

3.1 *High-fructose diet for 8 weeks led to the development of prediabetes in rats*

First of all, the rats on high-fructose diet were evaluated for the prediabetic condition. Compared to the control group of animals, the high-fructose-fed animals showed insulin resistance, metabolic syndrome and oxidative stress, all the characteristics of prediabetes (the prediabetic part of the study published (Bagul *et al.* 2012)). Treatment with resveratrol and metformin for the total 8 weeks' period when the animals were on high-fructose diet resulted in significant ($p<0.05$) normalization of all the metabolic parameters studies in liver tissues. Interestingly, resveratrol appears to be more effective than metformin in improving insulin sensitivity and attenuating metabolic syndrome, hepatic oxidative stress in high-fructose-fed animals [data not shown here as this part of the study has been published (Bagul *et al.* 2012), in our co-authorship].

3.2 *High-fructose-diet-induced prediabetes led to hyper-anxiety in rats*

In the light of a few reports (Collins *et al.* 2009) of possible connection of psychological distress such as anxiety and depression to T2D condition, we first performed the 'open-field test' to assess the anxiety state of rats after the cessation of 8 weeks of fructose feeding protocol. The analyses showed significant increase in anxiety level (or hyper-anxiety) in the animals kept on high fructose diet, compared to those on normal chow (figure 1A). The rats on high-fructose diet ventured less in the anxiogenic open or central area of the box in the open-field test (figure 1A). Co-administration of resveratrol or metformin in two separate high-fructose diet groups prevented the prediabetic rats from getting the affective disorder hyper-anxiety ($p<0.05$) (figure 1A). Similarly, in another anxiety measuring assay, the light/dark test, where these prediabetic animals spent less time in the bright side of the box which is anxiogenic, compared to the rats on normal diet, high-fructose-fed rats once again showed hyper-anxiety, as shown in figure 1B. In this behavioural assay too both resveratrol and metformin could significantly ($p<0.05$) attenuate the anxiety level induced in high-fructose-fed group (figure 1B).

The detail analyses of behaviour data showed the heightened level of anxious condition in the diabetic rats as evident by their increased preference for the dark chamber of the light/dark box and increased time spent in the periphery of the open-field box. However, in the open-field test, we also observed that high-fructose-fed rats showed increase in locomotor activity, as assessed by the number of crosses through the whole arena (figure 1C). Interestingly, most of the crosses in prediabetic rats were noticeably restricted to the peripheral zone only (figure 1D), again indicating increase in anxiety, which was not observed in the high-fructose group co-administered with resveratrol and metformin.

3.3 *Recovery from T2D-induced hyper-anxiety in rats was independent of the oxidative stress found in liver*

The oxidative stress has been reported to be associated with metabolic disorders, including diabetes (Correia *et al.* 2008; Chakraborty *et al.* 2011). We also observed that 8 weeks of fructose feeding led to the induction in hepatic oxidative stress pathway in liver when the rats developed prediabetes (Bagul *et al.* 2012) and was restored to normal level by resveratrol and metformin administration (Bagul *et al.* 2012). Here, we wanted to check whether the

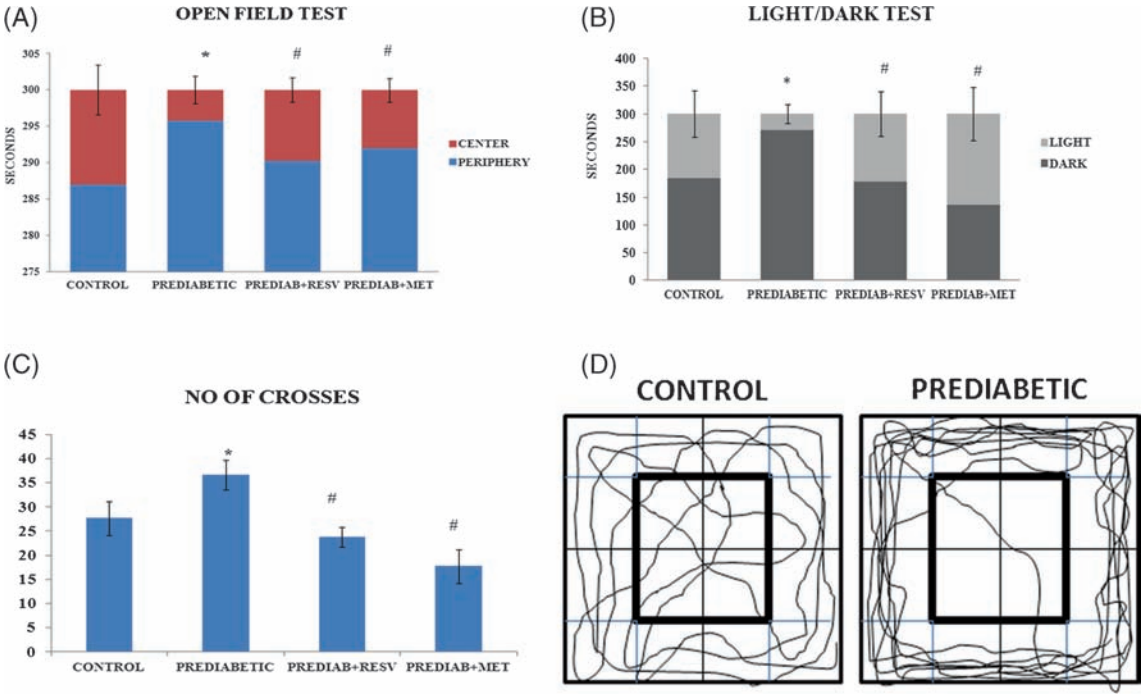

Figure 1. Effect of 8 weeks of fructose-rich diet on in an open-field test (**A**), light/dark box test (B), and number of crosses (C) in Sprague–Dawley rats. Data are expressed as the mean ± SEM (N=8). $^{*}p<0.05$ *vs* Control; $^{\#}p<0.05$ *vs* Prediab group. (D) Digitized video images as a representation of the original tracks of the rats.

oxidative stress pathway was also induced in the striatum of brain, the brain area implicated in anxiety and related mood disorders. Unlike what we found in liver tissue, where it was reported that there was no change in hepatic catalase activity in high-fructose-induced prediabetic condition and the administration of either resveratrol or metformin significantly upregulated the same (Bagul *et al.* 2012), we found that striatal catalase activity was significantly elevated in the prediabetic rats as compared to its level in the striatum of control and its level was significantly attenuated after the administration of either of these two compounds/drugs when compared with the level in prediabetic group (figure 2A). There was no change in striatal GSH (i.e. reduced glutathione) level in the prediabetic group as compared to its level in control striatum. Administration of resveratrol and metformin resulted in the attenuation of GSH level when compared to its level in control striatum (figure 2B). Similar studies were also done with other brain areas like hippocampus, where we found a similar trend of results in most of the parameters like striatum (supplementary figure 1). In contrast to what we reported in our prediabetic liver tissue, where TBARS level was significantly upregulated in fructose-induced prediabetic animals and there was recovery to normal level following resveratrol administration, we did not find any change in the striatal TBARS level in any groups (figure 2C).

3.4 *Resveratrol treatment resulted in recovery from hyper-anxiety through upregulation in metabolic sensors Sirt1 and Sirt7, the NAD-dependent class III HDACs*

Nuclear sirtuins have been implicated in the metabolic disorder (Haigis and Guarente 2006) and also in the affective disorders like anxiety, depression, etc. (Volmar and Wahlestedt 2015). So, the expression level of nuclear sirtuin genes *Sirt1*, *2*, *6* and *7* in striatal tissue was studied using reverse transcriptase real-time polymerase chain reaction (RT-qPCR) in the striatal tissue. Quantitative the analysis of data showed that the mRNAlevel of *Sirt1* and *Sirt7* were severely downregulated in the striatum of prediabetic rats, whereas the levels of *Sirt2* (data not shown here) and *Sirt6* were found unchanged as compared with their transcript level in the striatum of control rats (figure 3A–C). Interestingly, resveratrol administration significantly upregulated the transcription of *Sirt1*, *6* and *7* compared to the level in the prediabetic group. Metformin, on the contrary, failed to induce *Sirt1* and *Sirt6* (figure 3A–C), but it could induce huge level of upregulation in the transcription of *Sirt7* (figure 3A–C).

The change in the level of sirtuin proteins in striatum was also studied in all the groups using Western blot (figure 4). The change in the level of Sirt1 and 7 proteins in high-fructose-induced prediabetic rats (figure 4 A–D)

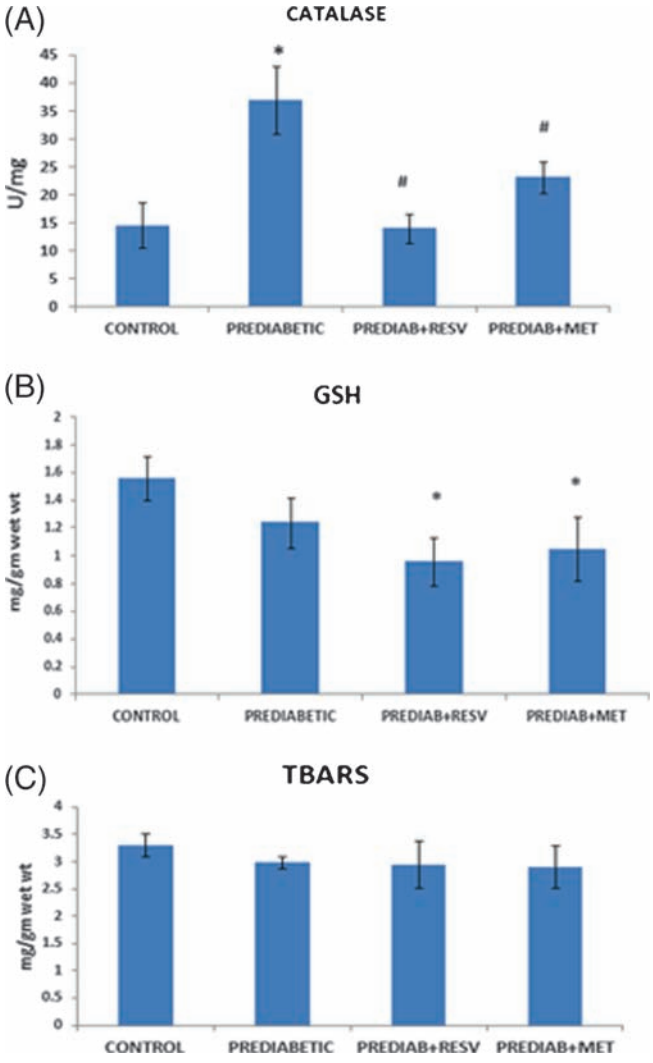

Figure 2. Effect of resveratrol and metformin on striatal catalase activity (**A**), GSH level (**B**) and TBARS level (**C**) after 8 weeks of fructose feeding. Data shown as mean ± SEM (N=8). $^{*}p<0.05$ *vs* Control; #$p<0.05$ *vs* Prediab.

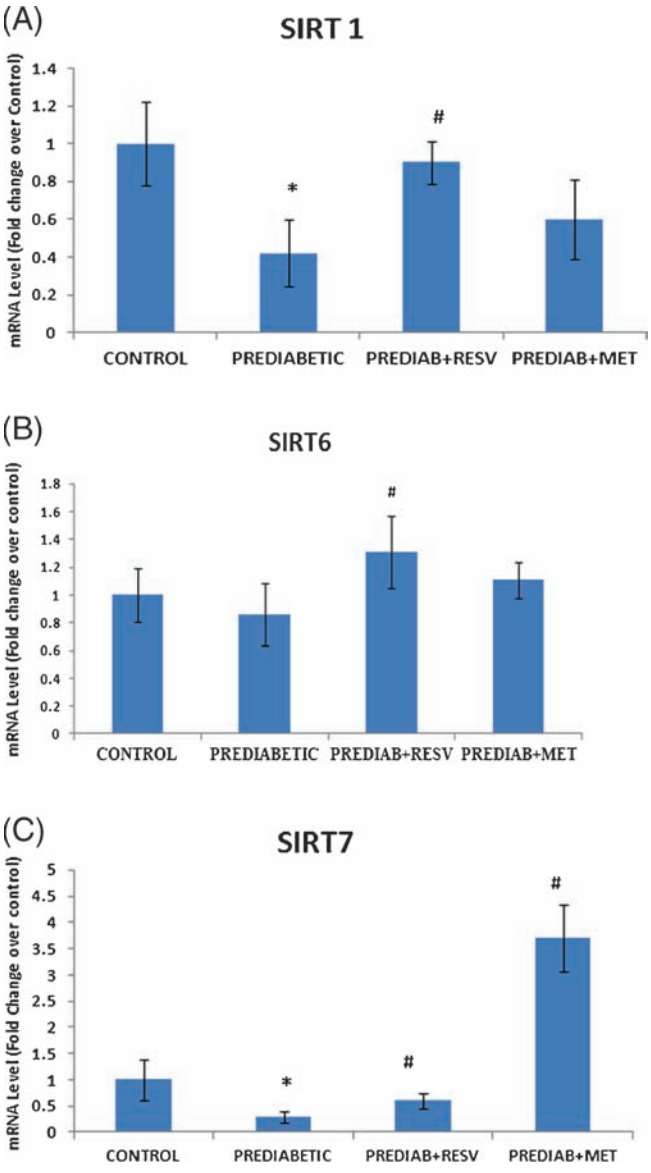

Figure 3. Effect of 8 weeks' fructose-rich diet on the expression level of *Sirt1*, *Sirt6* and *Sirt7* genes in the striatum of the Sprague–Dawley rats (N=8).

showed similar trend as shown at the gene level (figure 3A–C). Sirt1 was found downregulated in the prediabetic group as compared to its level in the control group and the treatment with resveratrol or metformin resulted in significant upregulation of this protein in striatum (figure 4A and B). In contrast to our gene expression data (figure 3) where Sirt7 was significantly down in prediabetes, the protein level of Sirt7 was found unaltered; however, the administration of either resveratrol or metformin could significantly increase the Sirt7 protein level in striatum compared to its level in control or prediabetic animals (figure 4C and D).

3.5 *Histopathology observations revealed no remarkable morphological change in the striatum of prediabetic rats*

The sections of striatum (the brain reward region involved in anxiety and related mood disorders) stained with hematoxylin and eosin did not show any remarkable morphological change in fructose-fed prediabetic rats as compared to the striatal sections in control animals. However, all the diabetic groups irrespective of their treatments showed occasional hypercellularity including small nuclei of non-neuronal cells with observable inflammatory bodies (figure 5).

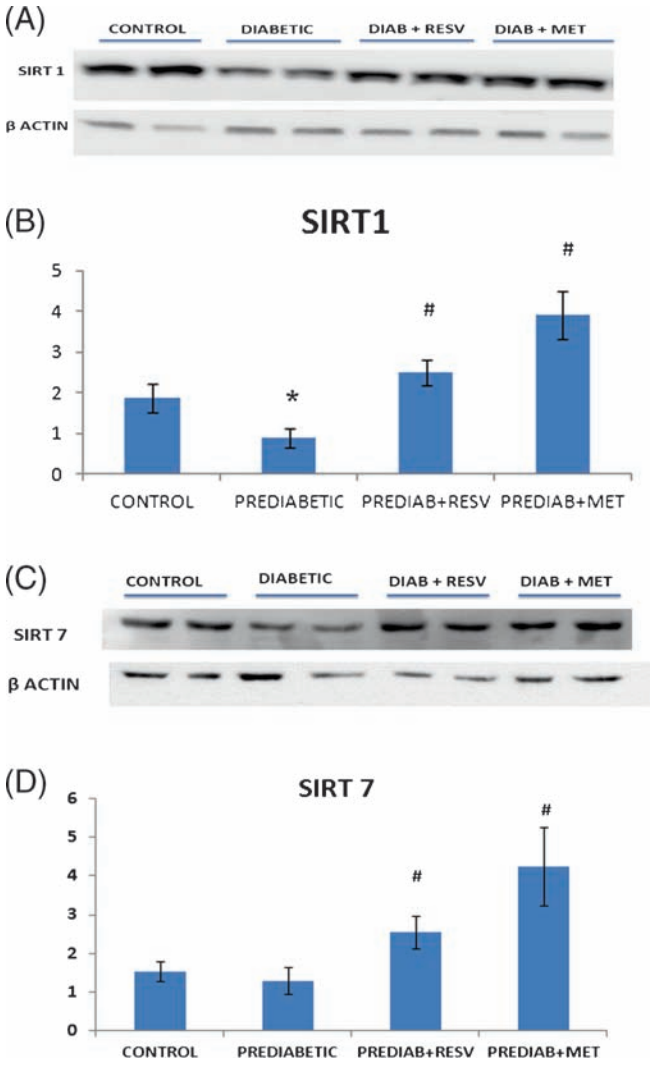

Figure 4. Immunoblots of Sirt1 and Sirt7 expressions in striatum of the brain. (A, C) Immunoblots of Sirt1 and Sirt7 from striatal total protein extract. β-actin was used to document the relative quantity of protein loaded for immunoblotting. (B, D) Densitometry analysis of Sirt1 and Sirt7 levels from total protein extract. Data shown as mean ± SEM. $^{*}p<0.05$ *vs* Control; $^{\#}p<0.05$ *vs* Prediab.

4. Discussion

Type 2 diabetes mellitus (T2D) is a metabolic disorder that appears to be induced by a variety of lifestyle factors including diet, stress, lack of physical activity, alcohol consumption, etc. (American Diabetes Association 2014; Sigal *et al.* 2006). In addition, the current incidence rate of diabetes is higher in the male population than female population (Gale and Gillespie 2001). The recent findings (Farvid *et al.* 2014; McNay and Recknagel 2011) of two-fold incidence of anxiety, depression and related mental illness in diabetic patients is disturbing, and so, uncovering the molecular mechanisms involved in metabolic disorder-induced affective disorders such as depression and anxiety is warranted. Our study is a novel one in that high-fructose diet for brief 8 weeks' period successfully resulted in metabolic syndrome, the precursor to diabetic condition, precisely prediabetes, in Sprague–Dawley rats (already published in (Bagul *et al.* 2012; Padiya *et al.* 2011)) and finally the animals also developed hyper-anxiety, an affective disorder, as shown by the analyses of open field and light/dark tests (figures 1 and 2). The diet used in the protocol to induce metabolic disorder has much higher proportion of fructose than the physiological human dietary fructose, for the hyperglycaemic index, induction of insulin resistance and other features of T2D [this is already published in (Bagul *et al.* 2012)].

Recent research implicates epigenetic mechanisms in the etiopathology of metabolic disorders such as diabetes (Jiang *et al.* 2008; Urdinguio *et al.* 2009; Bagul and Banerjee 2013) as well as affective or mood disorders such as depression, anxiety and related disorders. Epigenetic mechanisms refer to change in chromatin, induced by diverse classes of epigenetic regulators that affect the function of genes without bringing any alteration in gene sequence. Sirtuins are epigenetic regulators known as class III HDACs and out of seven sirtuins, Sirt1, 2, 6 and 7 are nuclear sirtuins that act at chromatin level, i.e. epigenetically (Chakravarty *et al.* 2014). Sirt1 has been shown to be cell metabolism regulator and its level is attenuated in cells showing high insulin resistance and its upregulation has been shown to increase insulin sensitivity. In addition, Sirt1 can de-acetylate and affect the activity of both members of the PGC1-alpha/ERR-alpha complex, critical transcription factors regulating the cell metabolism (Sun *et al.* 2007; Rodgers *et al.* 2005; Nemoto *et al.* 2005; Lagouge *et al.* 2006; Liu *et al.* 2008; Canto *et al.* 2009). Only Sirt1 and Sirt6 are reported to be involved in the regulation of metabolism (Liu *et al.* 2011). However, in our study we report for the first time that not Sirt6 but Sirt 1 and Sirt7 appear to be significantly attenuated in the striatum of rats when they developed metabolic and anxiety disorders (figures 3 and 4).

Sirtuin activator resveratrol was quite effective in our study in inducing recovery from both metabolic syndrome (published in (Bagul *et al.* 2012) and hyper-anxiety (figure 1) and the recovery from the hyper-anxiety appears to be due to the upregulation of Sirt1, Sirt6 and Sirt7 in striatum, the brain reward region, at mRNA (figure 3) as well as protein level (figure 4). This is quite an interesting finding that the anxious phenotype of the Sprague–Dawley rats induced due to high-fructose-fed prediabetic condition recovered completely by the treatment with resveratrol for 8 weeks.

As mentioned before, we have used metformin as a standard drug to compare the activity of resveratrol, and we have observed similar anxiolytic role shown by metformin in

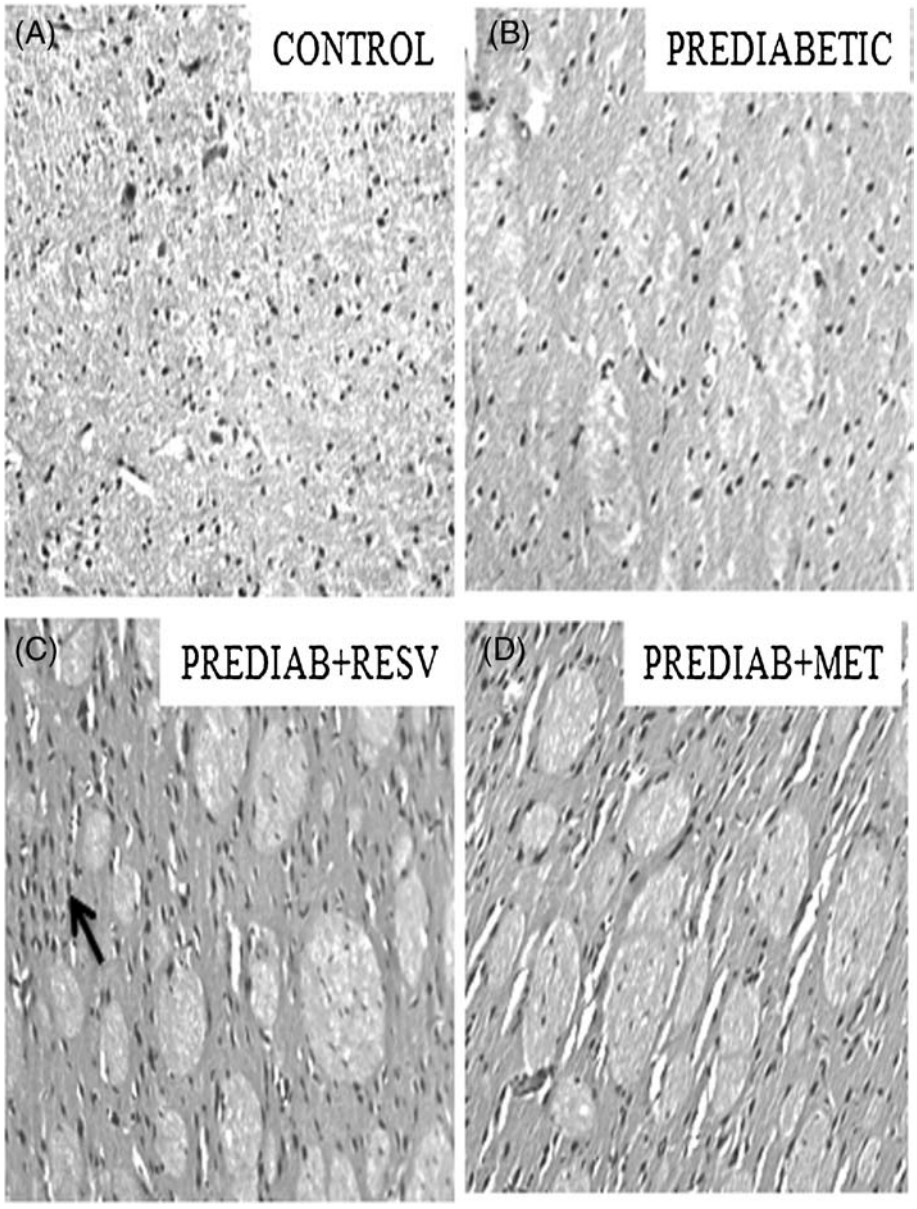

Figure 5. Photomicrograph of control and prediabetic brain section, H&E, 10X illustrating striatal area of the control group showed normal morphology with no observable cellular degenerative changes (**A**). Prediabetic groups also looked normal except in the few occasions of hypercellularity (marked by arrow) with inflammatory changes.

these prediabetic rats as resveratrol. Metformin induced recovery from the affective disorder was also due to the upregulation of Sirt1 and Sirt7 at mRNA (figure 3) as well as protein level (figure 4). AMPK activation, a well-known mechanism of metformin, also contributes to the learning and memory, and enhanced brain function as depicted previously (Pintana *et al.* 2012). However, metformin was not as good as resveratrol in inducing Sirt6, which has been reported to be protective to cells in stress conditions (Mao *et al.* 2011).

Oxidative stress is implicated in the development of diabetes and its complications (Giacco and Brownlee 2010; Maritim *et al.* 2003). We have reported the expected low level of the protective endogenous antioxidant enzymes in liver (Bagul *et al.* 2012) in fructose-fed group that indicates severe oxidative damage caused by free radicals in diabetes. However, in this study the level of few of these enzymes were contradictory to the levels found in liver. For example, catalase shows upregulation in the striatum of the high-fructose-induced prediabetic group compared to its level in

the striatum of the control group of animals. Interestingly, treatment with resveratrol and metformin resulted in the attenuation of catalase activity in the prediabetic group. This is unusual since catalase is known for its protective effect against free radical damage. However, there was no change in GSH activity in striatum in prediabetic animals compared to control ones, unlike what was observed in liver tissue. Interestingly, the level of GSH was attenuated in striatum after the resveratrol and metformin treatment, when compared to its level in control rats. There was no change in the level of TBARS in the prediabetic striatum, unlike in liver tissue, and this suggests that not much oxidative damage is there in brain in this affective disorder. So, our study suggests that unlike the oxidative damage caused by free radicals in liver (Bagul *et al.* 2012), the brain, in particular the striatal area, is not affected by the oxidative stress associated in diabetes. There might be a possibility of the oxidative damage in full blown diabetes.

Resveratrol, an activator of sirtuins particularly sirt1 and 2, is known for its neuroprotective activity and anti-aging effect (Albani *et al.* 2010). The results of our study that resveratrol also increases sirt7 level in the straitum of prediabetic rats suggests that sirt1 and sirt7 actions might be synergistic in providing the neuroprotection as reported earlier (Vakhrusheva *et al.* 2008). Induction of Sirt7 mRNA by metformin might be the reason why it improves the prediabetic condition in spite of increasing the not so neuroprotective Sirt2. Thus, Sirt7 appears to have adaptive advantage in the brain and. resveratrol seems to be better choice in the comorbid mood disorder as doesn't induce Sirt2 in our prediabetic model.

Similar to other recent reports (Li *et al.* 2010; Jiang *et al.* 2014) we also found that metformin has neuroprotective action in our model. The anti-diabetic action of metformin appears to be through the epigenetic mechanism as it induces the Sirt1, Sirt7 mRNA (figure 3) and proteins (figure 4) in the rat striatum thereby improving the diabetic condition. This gives a hope of trying re-purposing the metformin for the brain disorders such as affective disorders as well as cognitive disorders such as Alzheimer's, dementia, etc., due to the epigenetic action it has shown in our study.

All together our data suggest that Sirt 1, Sirt6 and Sirt 7 act against cellular stress associated with the metabolic syndrome and the induced affective disorder and significant recovery by resveratrol appears to be through these three nuclear sirtuins which might be acting epigenetically at chromatin and transcription regulation level. The recovery by metformin was also good but not better than resveratrol as metformin could not induce Sirt 6 at mRNA (figure 3) and protein level (figure 4).

The histological study of striatum by Hematoxylin and Eosin also confirms mild striatal damage in the prediabetic condition-induced hyperanxiety, which is followed by recovery seen with resveratrol and metformin treatments, where metformin was showing perfect recovery almost back to the control condition. This suggests protective role of metformin even in neurological complications of T2D through epigenetic regulation of Sirt1 and 7.

5. Conclusion

This study implicates, for the first time, Sirt7, in addition to Sirt1, in metabolic disorder-induced affective (hyperanxiety) disorder in fructose-fed prediabetic animal model. The sirtuin activator resveratrol's effectiveness in treating the anxiety disorder in addition to its treatment of metabolic syndrome is another interesting finding. We report here that sirtuins, specifically, Sirt1 and Sirt7, appear to play an important role in the etiopathology of T2D-associated psychological distresses like anxiety. Furhter detailed studies are warranted. The most novel finding in our study is the epigenetic action (activating Sirt1 and Sirt7) of a non-epigenetic drug used to treat diabetic patients, metformin.

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