



RESEARCH ARTICLE

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## Analysis of Metformin on Endogenous Antioxidants and Oxidative Stress in Mice Brain Tissue of Alloxan-Induced Diabetes

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### ABSTRACT

Diabetes has become a serious threat to mankind, as it is found in all parts of the world. Alloxan induced diabetic mice were treated by Metformin to study the impact of oxidative stress and endogenous antioxidants. Due to the establishment of diabetes, the oxidative stress indicators were found to be positively correlated with the elevation in the levels of endogenous antioxidants. Whereas, in Metformin-treated diabetic mice, the data revealed the correction of diabetes by lowering of blood glucose level along with body weight. But the levels of endogenous antioxidants were not recorded to increase except GST. It shows that under *in vivo* system level of oxidative stress increases due to the Metformin. The data revealed that Metformin treatment of mice, however, manage the glucose level but not effective in controlling oxidative stress.

**Keywords:** Alloxan, Oxidative stress, Endogenous antioxidants, blood glucose level, *in vivo* system, Metformin.

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### INTRODUCTION

Globally diabetes has become a grave issue to humankind. [1] As a data of 2015, in India 69.2 million people *i.e.*, 8.7% were suffering from diabetes. Diabetic mortality and morbidity have increased worldwide and it is expected that by 2030 diabetes would be at the top to cause death due to non-communicable diseases. [2] Diabetes is classified in three types, type I, type II and gestational DM. Type I diabetes is known as the immune-mediated damage of beta cells that secrete insulin. [3] Presence of islet cells autoantibodies directed against the autoantigens insulin GAD65 and the islet

tyrosine phosphatase IA-2 is the main cause of  $\beta$ -cell damage. Type I diabetes is a T-cell mediated disease associated with lack of immunological tolerance to self-antigens. [4] There is a lack of insulin formation due to the pancreatic cellular destruction. [5] Type II diabetes is a diabetes mellitus, which is insulin-independent [6] and defined as the progressive decline in the function of the  $\beta$  cell as well as chronic insulin resistance. [7] The persistent problems of diabetes contain increased development of cardiovascular disease, renal disease, weak vision and limbic problems. [8] In the diabetic patient insulin is an anabolic hormone because in

diabetes type 1 one has the deficiency of insulin whereas in diabetes type 2 one has to access in insulin level. [9] Gestational DM is defined as glucose intolerance during the initial stage of pregnancy. [10] Hypertension, blindness, stroke, coronary-artery disease and kidney disease as well as peripheral neuropathy are common complications of diabetes. [11]

In several studies, under *in vivo* conditions (experimental mice) alloxan is used for inducing diabetes by disturbing  $\beta$  cells of the pancreas. [12-30] Metformin is one of the commercially available efficient, inexpensive and easily available drugs for the treatment of diabetes. It is clinically most common drug for diabetes but shows some side effects as complications with eyes, kidneys, heart and vasculature, peripheral nerves. The primary action of metformin is to improve insulin sensitivity of the liver as a result of which basal endogenous glucose production decreases. [31] Metformin is a chemical which is extracted from plant *Galega officinalis*. In 1957, Jean Sterne introduced it as a "Glucophage". [32] Earlier report suggests that metformin alters oxidative responses in brain. [33] Primary action of metformin is to improve insulin sensitivity of the liver as a result of which basal endogenous glucose production decreases by activating Adenosine Mono Phosphate activated Protein Kinase (AMPA-PK) [31] (Fig. 1).

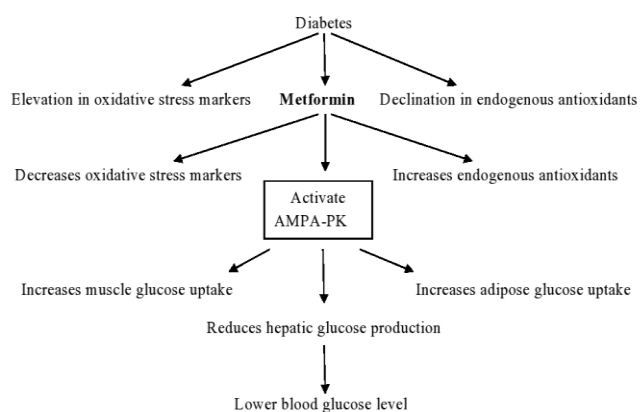


Fig. 1: Cascade of events associated with Metformin action in diabetic response.

The genetic factor is one of the predisposing factors of causing diabetes. Its treatment and complications have a high healthcare expenditure, as it is a multifactorial disease. Age, obesity, genetic predisposition and lifestyle are the major cause of diabetes onset. Hypertension, stroke, coronary-artery disease, blindness, kidney disease as well as peripheral neuropathy are the complications of diabetes. [34] Antioxidants scavenge free radicals to prevent cellular damage. It can reduce the oxidative stress. Oxidative stress is also be defined as imbalance and cytopathological effects of free radicals generation and their cell ability. [35-36] Glucose oxidation can lead to the generation of free radicals, which may cause cell death. Establishment of diabetes can cause damage to

membrane lipids, nucleic acids and cellular proteins. [37] The present study aims to find the behavior of Metformin, most commonly used drug for diabetes by measuring the activities with respect to endogenous antioxidants and oxidative stress also amount of total protein in diabetic as well as Metformin treated diabetic mice.

## MATERIALS AND METHODS

### Experimental Animals

Healthy male mice (*Mus musculus*) of age six weeks were purchased from C. C. S. Haryana Agriculture University and kept in a polypropylene cages bedded by a thick layer of husk, in animal house. They were provided with feed and water *ad libitum*. Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) has approved the mice for study (BV/3523/2017-18).

For the study, mice were categorized into three groups (n=7) as Group I is a control group which was treated with normal saline. Single dose of alloxan (160 mg per kg body weight) was administered to the mice of group II and III that induced diabetes in mice diabetes were validated after 24 hours of administration of alloxan (*i.p.*). Diabetic mice of group III received oral dose of Metformin (180 mg/kg) for 21 days continuously. Tip of mice tail was cut with scissor and pressed to get blood on glucometer strip. [38-40]

### Biochemical Parameters

For study mice were isolated in three different groups: Control (group I), Diabetic (group II) and metformin treated diabetic mice (group III) whole brains of mice were used to determine the level of various antioxidants. On the completion of 21 days the brains were isolated to determine the activities of endogenic antioxidants and oxidative stress of the brain. Total protein amount in the brain of all the group of mice was also calculated.

### Protein estimation

Amount of protein in mice brain was calculated by Lowry method. [41] Concentration of total protein in mice brain was measured by taking a sample volume followed by adding 5 ml of alkaline copper sulphate solution and after 10 minutes of incubation 5 ml of folin-reagent was added; kept for 30 minutes of dark incubation and absorbance read at 660 nm with BSA 0.1 to 1.0 as standard.

### Endogenous antioxidants of brain

**Reduced glutathione (GSH):** It was calculated by Ellman *et al* [42] method. After reacting with DTNB GSH forms a yellow colored 5-thio-2-nitrobenzoic acid, calculated at 412 nm on spectrophotometer. Cuvette contained a reaction volume of 25 $\mu$ l sample added with 2.5 ml phosphate buffer, 800 $\mu$ l EDTA and 100 $\mu$ l DTNB.

**Sodium dismutase (SOD):** SOD activity was measured by the method of Dhindsa *et al.* [43] It is estimated by the inhibition of NBT reduction. Reaction mixture was made by adding 1.5 ml phosphate buffer, 100 $\mu$ l NBT, 50 $\mu$ l of sample, 100 $\mu$ l of sodium carbonate, 200  $\mu$ l

methionine, 100µl EDTA, 1 ml distilled water and 100µl riboflavin. After the completion of 60 minutes incubation spectrophotometric reading was measured at 560 nm against blank.

**Catalase (CAT):** Catalase was measured by [44] method. After decomposition of H<sub>2</sub>O<sub>2</sub> it releases water and oxygen. Its total reaction volume contained 1.975 ml phosphate buffer, 1 ml H<sub>2</sub>O<sub>2</sub> and 25µl samples. Spectrophotometric analysis was done taken at 240 nm.

**Glutathione peroxidase (Gpx):** Activity of GpX was calculated by Mohandas *et al.*, 1984 [45] method. It was determined by the coupled enzyme assay. For its assay 25µl sample, 1.44 ml phosphate buffer, 0.1 ml EDTA, 0.1 ml sodium azide, 0.05 ml glutathione reductase, 0.1 ml GSH, 0.1 ml NADPH and 0.01 ml H<sub>2</sub>O<sub>2</sub> was mixed properly and reaction by mixture was scanned at 340 nm.

**Glutathione-S-Transference (GST):** Level of GST was calculated by Habig *et al.*, 1974 [46] method. GST detoxify xenobiotics. Reaction mixture contained 0.1 ml sample, 1.675 ml phosphate buffer, 0.2 ml reduced glutathione and 0.025 ml CDNB and sample vial was observed at 340 nm.

#### Oxidative stress markers

**Thio Barbutyric Acid Reactive Substances (TBARS):** Malondialdehyde (MDA) amount was calculated by TBARS which is a reactive compound formed due to the peroxidation of lipid by Ohkawa *et al.* 1979 [47] method. It gives pink color when react with TBA under oxidative stress. In a test tube 25µl sample, 0.2 ml KCl, 0.2 ml SDS. 1.5 ml acetic acid and 1.5 ml TBA was pipetted. Distilled water was used for volumen make up to 4.0 ml. It was kept on water bath at 95°C for 1 hour and cooled at room temperature. In each tube 5ml solution of butanol: pyridine was pipetted and mixed well followed by centrifugation for 10 minutes at 3000 rpm. After discarding the uppermost layer, absorbance was noted at 532 nm.

**Protein Carbonyl Content (PCC):** It is a technique to detect and quantify oxidative modification of protein via carbonyl groups by Levine *et al.* 1990 [48] method. In a tubes 25µl sample, 1 ml phosphate buffer and 100µl of TCA was mixed well and incubated for 15 minutes. Then it was centrifuged for 10 minutes at 8000 rpm. Discard supernatant and pellet was suspended in to 500µl DNPH. After the incubation of 1 hour 500µl TCA was mixed and centrifuged at 5000 rpm for 10 minutes. 1.5 ml ethyl acetate: ethanol (1:1) was added to precipitate and centrifuged at 8000 rpm for 10 minutes to wash excess of DPNH. Washing of DNPH was done 3 times. Spectrophotometric reading was taken at 370 nm after dissolving e final pellet in 2 ml of guanidine hydrochloride.

#### Statistical analysis

To detect the reproducibility of results all biochemical test were performed in sets of three. The results are expressed as means ± SD, n = 7 per group from an independent experiment. A one way ANOVA between the means of 2 groups were evaluated and  $p < 0.05$  were

measured as the analysis of significant data statistically. All graphs are plotted in sigma plot 12.0.

## RESULTS

In current investigation, single dose of alloxan (160 mg/kg) induced diabetes in mice. [49] Glucose level in mice brain was analysed by GlucoOne glucometer. Tail was little pressed to get the drop of blood at the glucometer strip. Diabetic mice had blood glucose level not less than 200 mg/dl. [50] Body weight of all the groups of mice was noted every day. The Body weight of diabetic mice was found maximum (29.333 ± 0.816 g) then in group III (26.166 ± 0.408 g) and minimum in group I (25 ± 0 g). Simultaneously after glucometer testing of mice blood, it was found that glucose level in diabetic mice group II was maximum (384.142 ± 15.093 mg/dl) followed by control group I (145.166 ± 7.44 mg/dl) and recorded minimum in Metformin treated diabetic mice group III (133 ± 16.012 mg/dl) (Fig. 2).

Level of protein in brain was measured highest in diabetic mice (0.7864 ± 0.045 mg/ml) followed by group I (0.5282 ± 0.013 mg/ml) and group III (0.5665 ± 0.041 mg/ml) ( $p < 0.05$ ) (Fig. 3). Similar finding were reported by Maritum *et al.* 2003, indicating the elevation of protein content due to cellular damage in diabetes. [37]

Another part of the study involved the assessment of endogenous antioxidants parameters as SOD, GSH, CAT, GpX as well as GST, which are used as the markers of the toxicity to determine the level of diabetic neuropathies. [42-44] The level of SOD in brain was measured highest in Diabetic mice (0.087 ± 0.004µg/mg of protein) followed by group I (0.065 ± 0.007µg/mg of protein) and group III (0.06 ± 0.004µg/mg of protein) ( $p < 0.05$ ). GSH level was recorded to be maximum in diabetic mice (2.1413 ± 0.05µM/min/mg of protein) and it differed significantly from group I (1.178 ± 0.13µM/min/mg of protein) and group III (1.403 ± 0.106µM/min/mg of protein) ( $p < 0.05$ ). CAT level was measured notably highest in diabetic group (46.849 ± 1.7530µM of H<sub>2</sub>O<sub>2</sub> consumed/mg of protein) of mice in compared to group I (34.250 ± 3.549µM of H<sub>2</sub>O<sub>2</sub> consumed/mg of protein) and Group III (8.904 ± 0.189µM of H<sub>2</sub>O<sub>2</sub> consumed/mg of protein) ( $p < 0.05$ ). Similar reports were suggested for CAT. Catalase activity increases in the brain of alloxan induce diabetic mice. [36]

Level of Gpx was also noted highest in group II (0.737 ± 0.05µM of NADPH oxidized/mg of protein) whereas in group I (0.5856 ± 0.03µM of NADPH oxidized/mg of protein) and group III (0.577 ± 0.01µM of NADPH oxidized/mg of protein) ( $p < 0.05$ ) it was found very close to each other. But in GST diabetic mice shows minimum activity (1099 ± 20.7 nMCDND conjugate formed/min/mg of protein) in compared to group I (1204 ± 12.4 nMCDND conjugate formed/min/mg of protein) and group III (1269 ± 32.29 nMCDND ( $p < 0.05$ ) conjugate formed/min/mg of protein) (Fig. 4).



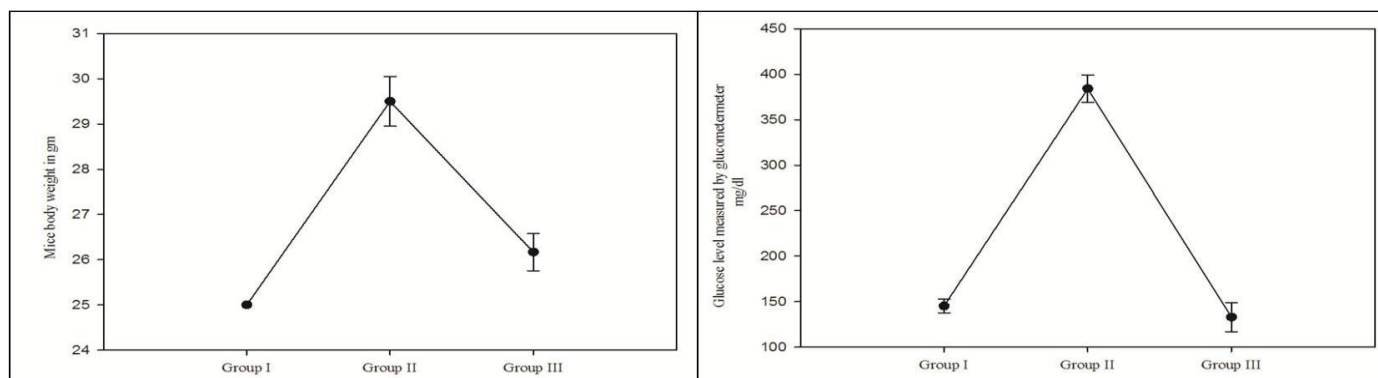


Fig. 2: Body weight and Blood glucose levels of differently treated mice. Group I: Control, Group II: Alloxan treated, Group III: Alloxan + Metformin treated mice.

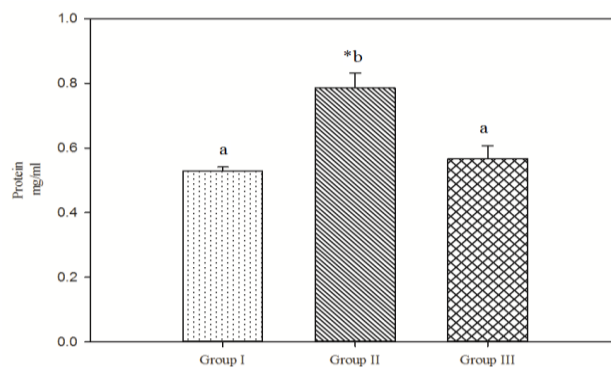


Fig. 3: Total protein concentrations in the brains of differently treated mice. Group I: Control, Group II: Alloxan treated, Group III: Alloxan + Metformin treated mice.

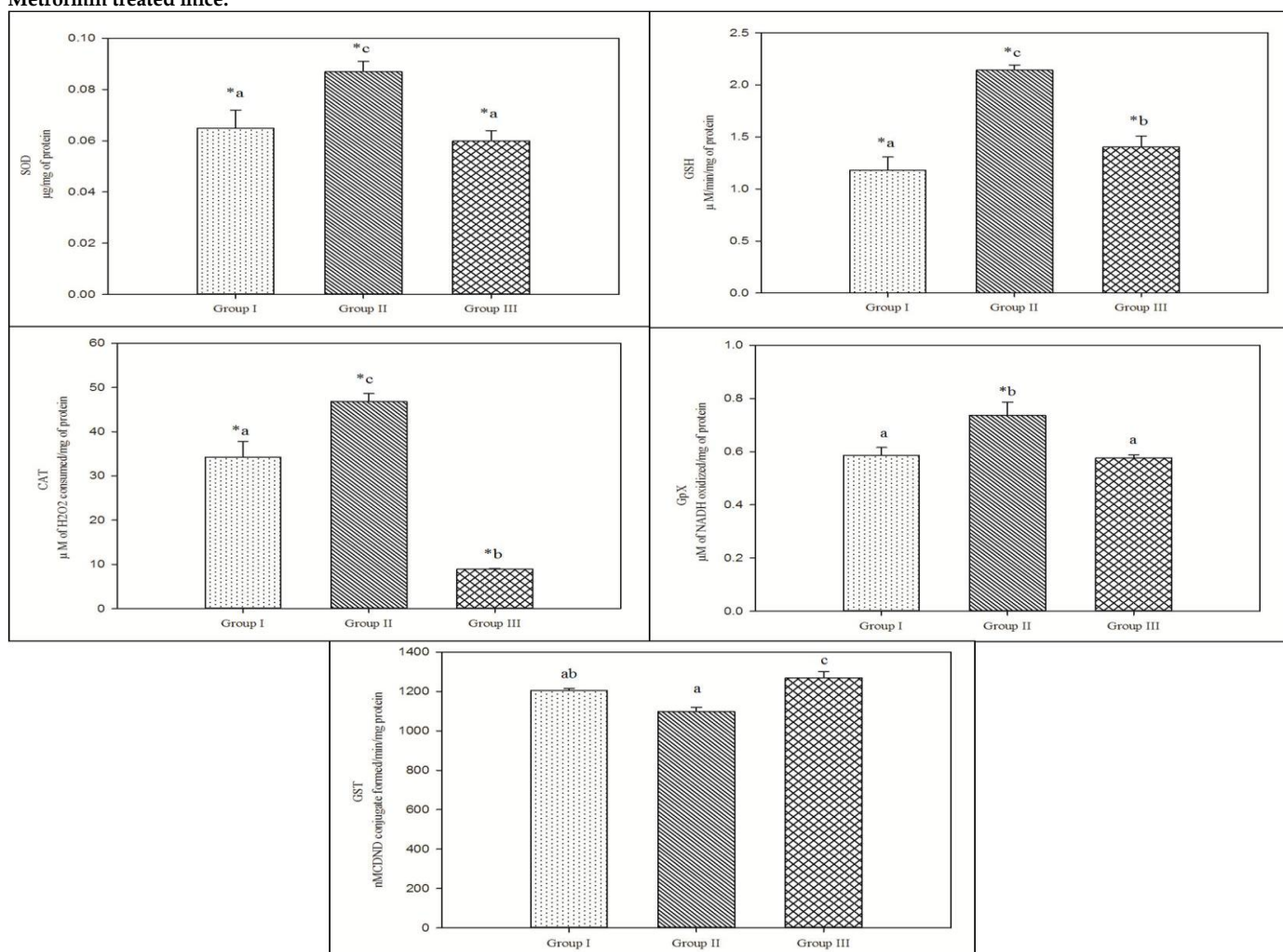


Fig. 4: Levels of Sodium Dismutase (SOD), Reduced Glutathione (GSH), Catalase (CAT), Glutathione peroxidase (Gpx) and Glutathione-S-Transference (GST) in the brain tissue of differently treated mice. Group I: Control, Group II: Alloxan treated, Group III: Alloxan + Metformin treated mice.

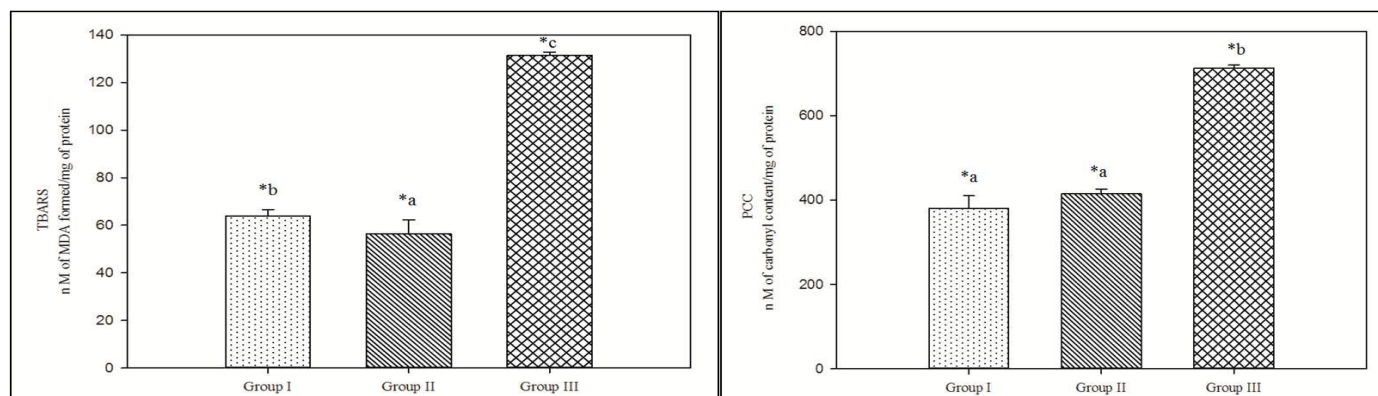


Fig. 5: Thio Barbutyric Acid Reactive Substances (TBARS) and Protein Carbonyl Content (PCC) levels in the brains of differently treated mice. Group I: Control, Group II: Alloxan treated, Group III: Alloxan + Metformin treated mice.

The present investigation revealed the levels of oxidative stress indicated by the markers as TBARS and PCC. TBARS level was measured maximum in group III ( $131.4 \pm 1.368$  nM of MDA formed per mg of protein) followed by group I ( $63.87 \pm 2.801$  nM of MDA formed per mg of protein) and group II ( $56.34 \pm 6.006$  nM of MDA formed/mg of protein) ( $p < 0.05$ ). In PCC oxidative stress was measured highest in group III ( $712.888 \pm 7.298$  nM of carbonyl content per mg of protein) and minimum in group I ( $379.7 \pm 30.8$  nM of carbonyl content/mg of protein); in group II ( $415.2 \pm 11.25$  nM of carbonyl content/mg of protein) ( $p < 0.05$ ) (Fig. 5).

## DISCUSSION

Various studies show that alloxan exerts diabetogenic action upon administration intravenously, intraperitoneally, or subcutaneously to animals. [39] Activity of antioxidant enzyme in diabetic mice is enhanced except GST. Endogenous antioxidants (SOD, GSH, CAT and GpX) levels were found incremented in diabetic mice, with respect to control whereas in Metformin treated diabetic group, levels of endogenous antioxidants were noted positively correlating with the elevated total protein content in the brain tissue. [47] In our study level of endogenous antioxidant enzyme except GST gradually increase in diabetic mice and it declines when treated with Metformin. Resulted in elevation of proteins and endogenous antioxidant in diabetic mice were recorded under *in vivo* conditions. Whereas activity of GST in diabetic mice was increased and it declined when diabetic mice were treated with Metformin. Decline in the activities of antioxidants generates free radicals due to which tissue injury appears. [46] Therefore, Metformin shows protective behavior only in GST. Endogenous antioxidant, enzymes (SOD and GSH) declines in Metformin treated diabetic mice due to inactivation of enzyme protein by ROS that depletes the substrate for enzyme and down regulates the transcription and translation process. [42] The study exhibits that diabetes induced oxidative stress is at parallel with control but when diabetic mice treated with Metformin then oxidative stress generates due to some adverse action of Metformin. Therefore

there is still a quest for alternative medicine for diabetes. Many people rely on herbal medication for diabetes. This leads to the immense need to find a potent antihyperglycemic control system that can eliminate endogenous antioxidants thereby controlling oxidative stress.

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