Metformin Reduces Oxidative Stress Status and Improves Plasma Insulin Level in Streptozotocin-Induced Diabetic Rats

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Abstract: *Background*: Oxidative stress induced by hyperglycaemia plays a crucial role in the development of diabetic complications and metformin is commonly used in treating diabetes mellitus (DM). The aim of this study was to investigate whether metformin at the dose of 100 mg/kg/day could ameliorate oxidative stress and improve plasma insulin level in streptozotocin-induced diabetic rats.

Methods: Twenty one rats (8-10 week old; weighing 190-220 g) were assigned into three groups (n=7 rats per group) i.e. non-DM, DM and DM+metformin (100 mg/kg/day metformin) groups. DM was induced using streptozotocin (60 mg/kg) intraperitoneally and treatments were given daily by oral gavage for four weeks. The levels of plasma biomarkers such as fasting blood glucose, oxidant-antioxidant markers and insulin levels were analysed.

Results: Fasting blood glucose, malonyldehyde and protein carbonyls levels were significantly higher while insulin, total antioxidant capacity, catalase and glutathione peroxidase levels were significantly lower in DM group compared to non-DM group. The levels of fasting blood glucose, malonyldehyde and protein carbonyls were significantly lower while levels of total antioxidant capacity, catalase and insulin were significantly higher in DM+metformin group compared to DM group.

Conclusion: This study may suggest that metformin at the dose of 100 mg/kg/day for 4 weeks reduces oxidative stress status and improves plasma insulin level in streptozotocin-induced diabetic rats possibly through its antihyperglycaemic action.

Keywords: Oxidative stress, rats, diabetes, metformin, insulin.

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized with hyperglycaemia, resulting from defects in insulin secretion, action or both [1]. Gradually it alters normal body metabolism and is reported to significantly reduce important nutrient in the body leading to higher mortality rate associated with DM [2]. Metformin has been the main therapy in DM for many centuries and its inhibition on hepatic gluconeogenesis, anti-glucagon action and insulin sensitising action help in DM management [3]. Moreover, metformin also decreases intestinal glucose absorption and increases peripheral glucose utilization [4]. It belongs to the class of biguanide drug to lower blood glucose level without causing hypoglycaemia [5].

Hyperglycaemia promotes auto-oxidation of glucose to form free radicals and, part of the oxygen taken into the body may produce reactive oxygen species (ROS) and radicals. ROS are toxic in the body and selectively affect specific oxidant-sensitive proteins and tissue in the body [6]. Beta cells of pancreas are sensitive to ROS because they have low antioxidant enzymes [7].

Administration of metformin at the dose of 50 mg/kg/day for 2 weeks significantly reduces blood glucose, improves insulin and reduces malonyldehyde (MDA) levels in fructose fed rat's model of type 2 DM [8]. Another study shows that metformin at the dose of 25 mg/kg/day for 2 weeks significantly improves antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and glutathione reductase] and reduces MDA level in alloxan-induced type 1 diabetic model, with no concomitant measurement of blood glucose and insulin levels [9]. Although, both alloxan and streptozotocin are diabetogenic chemicals, the half-life of alloxan is shorter, and it acts through redox reaction and autoxidation of dialuric acid destroying the whole islet of Langerhans. On the other hand, streptozotocin has longer half-life with alkylating potency, which acts by targeting mitochondrial DNA, specifically leading to beta cells impairment and damage [10].

However, to date, no study has been reported on oxidative stress status with concomitant measurement of insulin and blood glucose levels in animal model using streptozotocin that is commonly used as the diabetogenic agent, treated with metformin at the dose of 100 mg/kg/day that is corresponding to the human dose, and at the duration of more than 2 weeks. Therefore, the aim of this study was to investigate the

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ISSN: 2223-3806 / E-ISSN: 1927-5951/16

effects of metformin at the dose of 100 mg/kg/day for 4 weeks on oxidative stress status, plasma insulin and blood glucose levels in streptozotocin-induced diabetic rats.

2. MATERIAL AND METHODS

2.1. Experimental Animals

Twenty one female Sprague Dawley rats weighing between 190-220 g, aged 8-10 week were purchased from the animal house of the Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia. Animals were housed for a period of one week to acclimatise in a standard cage at 25 ± 2 °C, with 12 hour light/dark cycle. They were maintained with food and water *ad libitum.* The study was approved and performed in accordance with the guidelines by the Animals Ethical Committee, Universiti Sains Malaysia (USM/Animal Ethics Approval/2013/90/503).

2.2. Chemicals

Streptozotocin was purchased from Sigma Aldrich Company Ltd., Dorset, UK, and digital glucometer was purchased from Muenster (Accu-Chek, Roche Diagnostic, Mannheim, Germany). Commercial kits for the assays of total protein and plasma antioxidants enzymes [Total Protein, SOD, CAT and glutathione peroxide (GPx) EnzyChrom™ assay kits, Bioassay Systems, California, USA)] and, oxidative stress markers [total antioxidant capacity (TAC) (Total Antioxidant Power assay kit, Oxford Biomedical Research, Michigan, USA); MDA (MDA assay kit, Northwest Life Sciences Specialist, Washington, USA and protein carbonyl (PCO) (PCO Colorimetric Assay kit; Cayman Chemical, Michigan, USA)] and plasma insulin kit (Rat insulin assay kit, Cloud-Clone Corp., Texas, USA) were purchased. All other reagents used for the study were of analytical grade.

2.3. Induction of Diabetes

Experimental diabetes mellitus was achieved by intraperitoneal injection of streptozotocin as a single dose of 60 mg/kg body weight in normal saline, to rats after fasting for 16 hours [10]. The normal control group received similar volume of saline buffer intraperitoneally only as vehicle. After 48 hours of injections, the fasting blood glucose (FBG) was measured using digital glucometer monitoring system based on glucose oxidase principle [11]. Animals that had blood glucose level of ≥ 200 mg/dl were considered diabetes.

2.4. Experimental Design

Twenty one female rats were randomly assigned into three groups (n= 7 rats per group) i.e. non-DM: as a negative control group (on 0.5 ml/day of distilled water), DM: as a positive control group (DM on 0.5 ml/day of distilled water) and DM+metformin: as a treatment group (DM on100 mg/kg/day of metformin). The treatments were given daily by oral gavage for 4 weeks. Blood glucose was estimated on the first day as initial FBG (week 0) and final FBG after the treatments (week 4) were recorded. After the last day of treatments, all the animals were fasted overnight and anaesthetized using 80 mg/kg ketamine and 5 mg/kg xylaxine. Blood sample was taken through cardiac puncture and analysed for FBG, antioxidants enzymes (CAT, SOD and GPx), oxidative stress markers (TAC, MDA and PCO) and plasma insulin levels.

2.5. Analysis of Oxidant-Antioxidant Markers

SOD activity was estimated using EnzyChrom™ SOD assay kit. In this assay, superoxide was provided by xanthine oxidase catalysed reaction. The superoxide reacted with a WST-1 dye to form a coloured change. SOD in the sample scavenged the superoxide thus less superoxide was available for the chromogenic reaction. The colour intensity measured at 440 nm wavelength was used to determine the SOD activity from the standard curve and expressed as U/mg protein [12].

CAT activity was estimated using EnzyChrom™ CAT assay kit. This assay directly measured catalase degradation of hydrogen peroxide using redox dye. The change in colour intensity at 570nm was directly proportional to the catalase activity in the sample which was expressed as U/mg protein [13].

GPx activity was estimated using EnzyChrom™ GPx assay kit. In this assay, NADPH consumption in the enzyme coupled reactions was directly measured. The measured decrease in optical density at 340 nm was directly proportional to the enzyme activity in the sample which was expressed as U/mg protein [14].

TAC was estimated Total Antioxidant Power assay kit in which the reaction of reduced Fe^{+3} to Fe^{+2} was chelated by 2,4,6-tri (2pyridyl)-s-triazine and formed the complex Fe^{+2} –TPTZ. This complex showed an intense blue colour read at 450nm which was directly proportional to the TAC in the sample. The level of TAC was calculated from the standard curve and expressed as nmol/mg protein [15].

MDA was estimated using MDA assay kit based on the reaction of MDA with thiobarbituric acid (TBA) forming an MDA-TBA2 adduct which was read at 514nm. MDA level was calculated from the standard curve and expressed as nmol/mg protein [16].

PCO was measured PCO Colorimetric Assay kit. In this assay, 2,4-dinitrophenylhydrazine reacted with PCO to produce protein-hydrozone which was read at 370nm. The level of PCO was calculated from the controls and expressed as nmol/mg protein [17].

Total protein concentration was estimated using Total Protein Assay kit based on an improved Coomassie blue G method. The dye formed a blue complex specifically with protein and the intensity of colour was measured at 595 nm. The colour change was directly proportional to the total protein concentration in the samples and expressed as mg/mL protein and used for the expression of oxidant-antioxidant markers [18].

3. STATISTICAL ANALYSIS

Data obtained were analysed using InStat 3.1 (Charlesworth Group Ltd, Huddersfield, UK). Result are expressed as mean and standard deviation, and analysed for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc test. The $p<0.05$ was considered statistically significant.

4. RESULTS

4.1. Fasting Blood Glucose Levels

The mean FBG level of non-DM group was almost invariable throughout the experimental study. On the contrary, FBG level in DM only group was significantly higher compared with non-DM group. However, in DM+metformin group treated with 100 mg/kg/day, FBG was significantly lower compared with DM group (Table **1**).

4.2. Plasma Antioxidant Enzyme Levels

Plasma CAT activity in DM group was significantly lower compared with the non-DM group. However, plasma CAT activity in DM+metformin group was significantly higher in comparison with the DM (positive control) group. Plasma GPx activity in DM and DM+metformin groups was significantly lower compared with the non-DM group. However, no significant difference was found for plasma SOD activity among all groups (Table **2**).

4.3. Plasma Oxidative Stress Markers

Plasma MDA and PCO were significantly higher in DM group compared with non-DM group. However, plasma MDA and PCO in DM+metformin group were significantly lower compared with DM group but no significant difference with non-DM group (Table **3**). Plasma TAC was significantly lower in DM group

Table 1: Fasting Blood Glucose Levels for all Experimental Groups

Groups	Initial FBG (mg/dl)	Final FBG (mg/dl)
non-DM	90.55 (1.27)	90.00 (1.29)
DM	424.29 (31.40)[a]	547.57 (61.18)[a]
DM+metformin	467.57 (50.52)[a]	306.00 (67.49)[a, b]

Data are mean (SD), n=7/group, DM: diabetes mellitus, FBG: fasting blood glucose, [a]$p<0.05$ compared with non-DM group; [b]$p<0.05$ compared with DM group (ANOVA followed by Tukey-Kramer post-hoc test).

Table 2: Plasma Antioxidant Enzymes for all the Groups

Groups	CAT (U/mg protein)	SOD (U/ mg protein)	GPx (U/ mg protein)
non-DM	4.414 (0.875)	2.554 (4.187)	241.802 (157.57)
DM	0.090 (0.087)[a]	0.656 (1.464)	79.542 (35.04)[a]
DM+metformin	1.158 (0.538)[a, b]	0.681 (0.202)	102.347 (65.26)[a]

Data are mean (SD), n=7/group, DM: diabetes mellitus, CAT: catalase, SOD: superoxide dismutase, GPx: glutathione peroxidase, [a]$p<0.05$ compared with non-DM group; [b]$p<0.05$ compared with DM group (ANOVA followed by Tukey-Kramer post-hoc test).

Table 3: Plasma Oxidative Stress Markers for all the Groups

Groups	MDA (nmol/ mg protein)	PCO (nmol/ mg protein)	TAC (nmol/ mg protein)
non-DM	0.729 (0.10)	1.184 (0.274)	0.706 (0.142)
DM	2.526 (1.475)[a]	3.663 (1.076)[a]	0.360 (0.094)[a]
DM+metformin	1.040 (0.184)[b]	1.544 (0.488)[b]	0.479 (0.096)[b]

Data are mean (SD), n=7/group, DM: diabetes mellitus, MDA: malonyldehyde, PCO: protein carbonyls, TAC: total antioxidant capacity, [a]p<0.05 compared with non-DM group; [b]p<0.05 compared with DM group (ANOVA followed by Tukey-Kramer post-hoc test).

compared with non-DM group. The plasma TAC of DM+metformin group was significantly higher compared with DM group. However, there was no significant difference in the plasma MDA in DM+metformin group compared with non-DM group (Table **3**).

4.4. Plasma Insulin Levels

The plasma insulin level was significantly lower in DM group compared with non-DM group. However, in DM+metformin group treated with 100 mg/kg/day, plasma insulin was significantly higher than DM group and significantly lower than non-DM group (Figure **1**).

5. DISCUSSION

The lower FBG level in DM+metformin group is in line with previous report on metformin action in comparison with other oral antihyperglycemic drugs glibenclamide and repaglinide although plasma insulin level was not concomitantly measured [9]. This antihyperglycaemic action may be as a result of metformin ability to reduce blood glucose to a good glycaemic control without causing hypoglycaemia due to its mechanism of actions such as inhibits hepatic gluconeogenesis, decreases intestinal glucose absorption and increases peripheral glucose utilization [4]. It has been reported that metformin, via an adenosine monophosphate-activated protein kinase-dependent mechanism, inhibits or suppresses glucose production and gluconeogenic gene expression in primary hepatocytes and in the liver of mice [19].

In this model of DM, treatment with metformin alone at a dose of 100 mg/kg/day for 4 weeks revealed remarkable improvement in the activity of antioxidant enzyme CAT. The improved CAT activity may explain the increased TAC found in the present study. The finding on improved CAT activity is in line with previous report on metformin at the dose of 25 mg/kg per day for 2 weeks. However, no improvement was seen for the activities of SOD and GPx following metformin treatment in the present study although previous study shows an improvement in SOD activity [9]. These contrary findings may be due to the different in the dose and duration of metformin used in the studies.

Reactive oxygen species are suggested to be among the important factors that alter metabolism and beta cells function [18]. Both streptozotocin and hyperglycaemia increase free radicals formations which

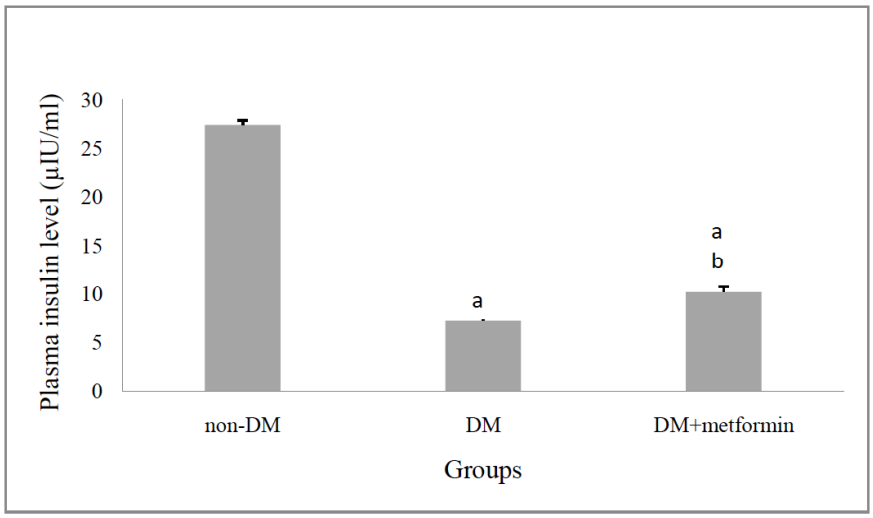

Figure 1: Plasma insulin levels in all experimental groups. Data are mean (SD), n=7/group, DM: diabetes mellitus, [a]p<0.05 compared with non-DM group; [b]p<0.05 compared with DM group (ANOVA followed by Tukey-Kramer post-hoc test).

can compromise antioxidant enzyme activities [19]. This may lead to the imbalance between the levels of oxidant and antioxidant enzyme activity as seen in the present study. In the present study, plasma MDA and PCO were significantly higher while TAC and CAT were significantly lower in DM group compared with non-DM group may suggest an increase in oxidative stress status in DM group. On the other hand, the plasma levels of MDA and PCO were significantly lower while CAT was significantly higher in DM+metformin group compared with DM group showing the indirect antioxidant effect of metformin possibly by its antihyperglycaemic effect. The decreased MDA level is in line with previous study using metformin at the doses of 25 [9] and 50 mg/kg per day [8]. Similarly, recent study has shown that metformin at a dose of 500 mg/kg per day for 7 days significantly reduces oxidative stress and prevents lipid peroxidation in gastrocnemius muscle of diabetic rats [20]. This is further supported by previous study that any substance with antihyperglycaemic effect potential may have indirect effect on oxidative stress by reducing the formation of free radicals from the auto-oxidation of glucose which in turn may protect the tissue from damage [21].

The significant decreased level of plasma insulin in DM group compared with non-DM and significantly higher plasma insulin in DM+metformin group compared with DM group are in accordance with previous study on metformin (50 mg/kg per day for 2 weeks) in fructose fed-diabetic animal model [8]. It is also found that metformin lowered fasting blood glucose and improved insulin action in a pregnant woman with obesity [22]. Furthermore, the ability of metformin to ameliorate oxidative stress-induced damage associated with streptozotocin-induced diabetes could be secondary to its antihyperglycaemic effect and improve insulin action in both overweight patient and high fructose-fed rats [23]. Therefore, it is possible to suggest that the significant increase in plasma insulin level found in the present study may be due to antihyperglycaemic effect of metformin which can ameliorate oxidative stress and subsequently may improve the pancreatic beta cell function or may give a regenerative effect on the pancreatic beta cells that may potentiate the production or secretion of insulin by the beta cells which needs further studies. Moreover, the reduced oxidative stress following metformin treatment may also possibly enhance cellular response to insulin which in turn further contributes to its antihyperglycaemic effect.

CONCLUSION

In conclusion, this study suggests that metformin at the dose of 100 mg/kg/day for 4 weeks reduces oxidative stress status as well as improves plasma insulin level in streptozotocin-induced diabetic model possibly through its antihyperglycaemic effect. Nevertheless, further studies are suggested to further evaluate the possible beneficial effects of metformin in protecting or reducing end organ damage.

ACKNOWLEDGMENT

The authors would like to thank Universiti Sains Malaysia for the financial support [USM Research University Grant (1001/PPSP/813072)].

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Received on 27-10-2016 Accepted on 08-11-2016 Published on 28-11-2016

DOI: http://dx.doi.org/10.6000/1927-5951.2016.06.04.1