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Metformin-Mg^{2+} adjunct therapy synergistically modulates insulin and PDX-1 gene signatures in STZ-NAD induced diabetic model

Abstract

Diabetes mellitus (DM) is a multi-factorial debilitating disorder of metabolism, usually due to a combination of hereditary and environmental causes, resulting in abnormally high blood sugar levels (hyperglycemia) as a result of defects in either insulin secretion or insulin action in the body. DM is usually accompanied by hypomagnesemia. This study was aimed at investigating the effect of oral magnesium supplementation on pancreatic gene expression of insulin and PDX-1 in type-2 streptozotocin-nicotinamide induced *Sprague dawley* diabetic rats. A total of 24*Sprague dawley*rats (Four groups of six rats each), were used for this study; **Group 1:** Normal rats (CONTROL) given distilled water for 4weeks; **Group 2:** Metformin + Magnesium treated rats (DMM) orally given 100mg/kg and 1000mg/kg body weight respectively for 4weeks; **Group 3:** Metformin treated diabetic rats (DM), orally given 100mg/kg body weight for 4weeks; **Group 4:** Diabetic untreated control rats (DU) given distilled water for 4weeks. Measured data were analyzed statistically. The result revealed that there was significant ($p<0.05$) increase in the feed and water intake of the treated rats but the metformin-magnesium supplement treated group showed more increase when compared with only metformin treated group. PDX-1 and insulin gene expression levels were significantly ($p<0.05$) higher in the control when compared with all the diabetic groups. However, PDX-1 and insulin mRNA levels were significantly ($p<0.05$) higher in DMM, when compared with DM. DMM showed improvements when compared with DM which suggests magnesium supplementation as an adjunct therapy with metformin may help inthe regeneration of the beta cells of the pancreas.

Keywords: *metformin, magnesium supplementation, insulin, pancreatic duodenal homeobox 1 (pdx-1),* and adjunct therapy

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Introduction

Diabetesmellitus (DM) is a constellation of metabolic diseases with characteristic hallmarks including hyperglycemia which is a corollaryof insulindysfunctionseither as insulin secretion deficiency, insulin inaction, or both.[1,2] The World Health Organization (WHO) in 2016, reported that an estimated 346million people lived with DM worldwide and that the number of DM mortality would double by 2030. Hence, DM is an epidemic with burgeoning public health concern.[2,3] DM is classified as insulin dependent (type 1),non-insulin dependent or adult onset (type 2), gestational diabetesand other specific types.[4,5]

Hyperglycemia being an impresa of DM precipitates the conventional symptoms of polyuria, polydipsia and polyphagia. It may also be an antecedent of some micro and macro vascular complicationssuch as neuropathies, nephropathies, and retinopathies, loss of limbs, erectile dysfunction and myopathies.[6] Several oral hypoglycemic agents such as biguanides, sulfonylureas, α-glucose inhibitors, thiazolidinedione, dipeptidyl peptidase-4 (DPP-4) inhibitors have been used for the management of type-2 diabetes however, metformin is the choicest drug in patients with type 2diabetes mellitus presently, as indicated in the established protocols by the European Association for the Study of Diabetes and American Diabetes Association.[4] Metformin (a biguanide derivative) controls glycemic level by reducing the measure of blood sugar absorbed by the stomach or intestine and the quantity of glucose produced by the hepatocytes, restores insulin response, thereby decreasing diabetic complications.[7] Besides its hypoglycemic activity, metformin has prophylactic activity as it helps to prevent diabetes in people who are at high risk of coming down with the disease, when taken with controlled diet and exercise. It is also reportedly used in women with polycystic ovarian syndrome because it is believed to make menstrual cycles more regular and increase fertility.[8]

With all the foregoing benefits, metformin is not with standing associated with certain adverse effects such as hypoglycemia, drug resistance, dropsy, weight gain and toxicity when administered as a monotherapy and combined therapy. Hence it becomes imperative to quest for drugs without side effects, hypersensitivity but with high antidiabetic activities.[9]

Micronutrients such as vitamin D, vitamin C and supplements such as dietary fibers have demonstrated to be potential diabetes risk modifiers. Magnesium (Mg) supplements was suggested to be an adjuvant therapy in the prevention and management of diabetes.[10] Mg is the most abundant divalent intracellular cation in the cells, the second most abundant cellular ion next to potassium and the fourth cation in general in the human body and it is an electrolyte of chief physiological importance in the body.[11] Type 2 diabetes mellitus (T2DM) as it were, is often chaperoned with the alteration of Mg status as increased prevalence of Mg deficits has been reportedly identified in T2DM patients, especially in those with poorly controlled glycemic profiles, longer duration of the disease and presence of

micro- and macro-vascularcomplications.[12-16] Poor intracellular Mg concentration and increased intracellular free calcium found in T2DM patients, may precipitate insulin resistance. In contrast, higher Mg levels corresponded to a greater degree of sensitivity to insulin. The importance of Mg on insulin sensitivity was suggested in the early 1980s and resulted in the following clinical evidences. Some studies reported the beneficial effects of Mg supplementation on metabolic control in individuals with T2DM while at the same time, other studies showed no significant effects of Mg supplementation on T2DM. Hence, the effects of Mg supplementation remained controversial in the literature.[10] This research investigates the role of Mg supplement on beta cell regeneration and insulin sensitivity by perusing two important genes insulin and PDX-1. PDX-1 is an orphan homeodomain protein and transcription factor essential for the development of the pancreas.[17-19] Hence its upregulation regulation may signal beta cell regeneration in the pancreas.

Experimental

Diabetic models

All protocols related to animal studies were approved by the Animal Ethics Committee of Centre for Research and Development Adekunle Ajasin University Ondo State, Nigeria. Twenty-four (24) male *Sprague dawley* rats (average weight of 150g) were obtained from the Department of Plant Science and Biotechnology, Adekunle Ajasin University Ondo State, Nigeria. They were housed under standardized environmental conditions (well-ventilated room, with 12-hour light-dark cycles and 55±4% at 24±2°C). Animals were allowed to feed*ad libitum.*The models were maintained in line with the US National Institutes of Health's protocolfor the care and use of Laboratory animals.[20]

They were divided into groups (n=6) based on their weight which was used to calculate the dosage of Streptozotocin (STZ, Sigma Aldrich, Hamburg, Germany), Nicotinamide (NAD), Magnesium Sulfate ($MgSO_4$, Sigma Aldrich, Hamburg, Germany), and Metformin (Merck pharma care spoxil)administered. Induction was carried out after three weeks of acclimatization. Administration started when the rats were confirmed diabetic after 72h of induction. The intervention was carried out daily in the following order for four (4) weeks:

Animal design

Four groups of six rats each were used for this study, namely:

a. **Group 1:** Normal control rats (CONTROL) given distilled water;

b. **Group 2:** Metformin+Magnesium treated rats (DMM) orally given 100mg/kg and 1000mg/kg body weight respectively;

c. **Group 3:** Metformin treated diabetic rats (DM), orally given 100mg/kg body weight;

d. **Group 4:** Diabetic untreated control rats (DU) given distilled water;

Induction of diabetes

T2DM was induced as described by[21] with little modifications. Briefly, overnight-fasted rats were given intraperitoneal injection (i.p.) of freshly prepared 60mg/kg STZ (dissolved in a citrate buffer of pH 4.8), 5minutes after the i.p. administration of 110 mg/kg of freshly prepared NAD dissolved in normal saline.

Intervention

Magnesium sulfate and metformin were intubated into the mouth of the diabetic rats in quantity based on their body weights.

Sacrificing and tissue excision

At the end of the treatment, the animals were subjected to fasting overnight for 9hand to cervical dislocation following ethical care and handling of experimental animals' regularities and they were dissected using dissecting set. The rats were sacrificed and the pancreas was excised from each experimental animal. Little quantity of the excised tissues was dropped in eppendorf tubes containing 0.2µl TRIzol across the groups and then spun using laboratory centrifuge.

Gene expression profiling

RNA was isolated from the pancreas using TRIzol Reagent (ThermoFisher Scientific) following manufacturer's guide. Purified DNA-free RNA was converted to cDNA immediately using ProtoScript® First Strand cDNA Synthesis Kit (NEB). PCR amplification was done using the following primer set (Table 1):

Table 1 PCR amplification was done using the following primer set

TARGET GENE	FORWARD 5'- 3'	REVERSE 5'- 3'
GAPDH	TGA AGG TCG GAG TCA ACG GAT TTG GT	CAT GTG GGC CAT GAG GTC CAC CAC
INSULIN	ATGGCCCTGTGGATGCGC	TGCGGGCTGCGTCTAGTTG
PDX-1	GACACATCAAAATCTGGTTCCAAA	TCCCGCTACTACGTTTCTTATCTTC

Representative snapshot of reverse transcription polymerase chain reaction-agarose gel electrophoresis data of all the rats was taken and analysed using the band density (Image-J) which is then plotted as a bar graph (Mean± SEM)

Representative snapshot of reverse transcription polymerase chain reaction-agarose gel electrophoresis data of all the rats was taken and analysed using the band density (Image-J) which is then plotted as a bar graph (Mean± SEM).

Statistical analysis

Data areexpressed as mean±standard error of mean (SEM) and analyzed using the ANOVA followed byTukey's Multiple Comparisonpost-hoc test. A p-value below 0.05was considered as statistically significant.

Research outcomes

Feed intake

According to Table 2 there wassignificant ($p<0.05$) decrease in feed intake was observed in DU (48.1±0.51) when compared with

control (49.86±0.51) while DMM (48.7±0.68) and DM (49.0±0.69) were significantly different from DU for the first week. The second week revealed significant ($p<0.05$) decrease in DU (44.4±0.65) when compared with control group (53.71±0.52). However, DMM (46.4±0.37) and DM (45.0±0.82) were significantly ($p<0.05$) higher than DU (44.4±0.65).The third week showed significant ($p<0.05$) reduction between DM (44.6±0.65) and DU (43.6±0.75) when compared, but an increase in DMM (47.0±0.82) and the control group (55.4±0.57). The trend in week 3continued till the end of the intervention period.

Table 2 Table of value for feed intake

Groups	Week 1(g)	Week 2(g)	Week 3(g)	Week 4(g)
Control	49.7±0.51	53.7±0.52	55.4±0.57	55.1±0.51
DMM	48.7±0.68#	46.4±0.37#	47.0±0.82#	52.7±.34#
DM	49.0±0.69#	45.0±0.82#	44.6±0.65#	43.4±0.65#
DU	48.1±0.51*	44.4±0.65*	43.6±0.75*	42.1±0.59*

(*) means significant difference $p<0.05$ when compared with Control

(#) means significant difference $p<0.05$ when compared with DU

Water intake

Table 3 shows that in week 1 there was decrease in DMM (47.7±1.15), DM (48.6±0.48) and DU (48.1±0.63) when compared with control (49.0±0.69). Although, DM (48.6±0.48) and DU (48.1±0.63) were slightly higherthan DMM (47.7±1.15), the difference between DM and DMM was not significant. Second week revealed significant ($p<0.05$) increase in CONTROL (49.3±0.71), DMM (49.7±0.68), DM (49.1±0.51) when compared with DU (46.3±0.29). Third week showed significant ($p<0.05$) reduction in CONTROL (47.9±2.04) and DU (43.6±0.84) when compared with DMM (49.9±0.99) and DM (49.6±0.69). However, there was no significant ($p<0.05$) difference in DMM (49.9±0.99) and DM (49.6±0.69). But there was significant ($p<0.05$) reduction in DU (43.6±0.84) when compared with CONTROL (47.9±2.04). The last week revealed significant ($p<0.05$) reduction in CONTROL (47.0±1.91) and DU (40.3±0.52) when compared with DMM (50.1±1.10) and DM (50.4±0.75). But there was no significant ($p<0.05$) difference between DMM (50.1±1.10) and DM (50.4±0.75). However, there was significant ($p<0.05$) decrease in DU (40.3±0.52) when compared CONTROL (47.0±1.91).

Table 3 Table of value for water intake

Groups	Week 1(ml)	Week 2(ml)	Week 3(ml)	Week 4(ml)
Control	49.0±0.69	49.3±0.71	47.9±2.04	47.0±1.91
DMM	47.7±1.15#	49.7±0.68#	49.9±0.99#	50.1±1.10#
DM	48.6±0.48#	49.1±0.51#	49.6±0.69#	50.4±0.75#
DU	48.1±0.63*	46.3±0.29*	43.6±0.84*	40.3±0.52*

(*) means significant difference $p<0.05$ when compared with Control

(#) means significant difference $p<0.05$ when compared with DU

Insulin gene expression

The result illustrated in Figure 1, shows that there was down-regulation of the relative expression of the insulin gene in group 2, 3 and 4 when compared with the normal control. Group 2showed up-regulations, when compared with groups 3 and 4 and there was no significant ($p<0.05$) difference between group 1 and 2.

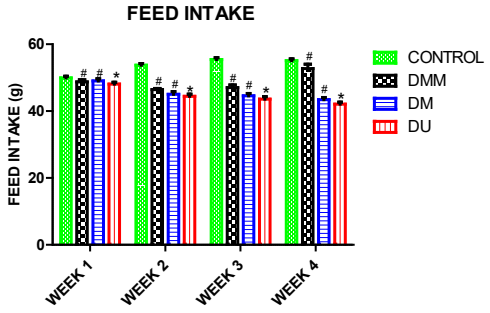

Figure 1 Effect of magnesium supplementation on feed intake.

PDX-1 gene expression

The result illustrated in Figure 2, shows that there was down-regulation of the relative expression of the PDX-1 gene in group 2, 3 and 4 when compared with the normal control. Group 2 and 3 showed up-regulations, when compared with group 4. More so, group 2 showed up-regulation, when compared with group 3.

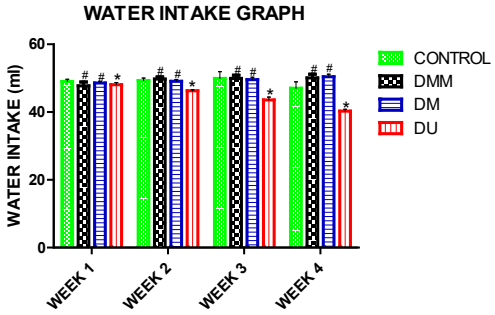

Figure 2 Effect of magnesium supplementation on water intake.

Discussion

Complications are common among diabetic patients and they are responsible for significant morbidity and mortality among these patients as reported by International Diabetes Federation.[22] Hypomagnesemia is a common comorbid condition with T2DM. Hence researches have been carried out to address hypomagnesemia in diabetic subjects. Most of the intervention used over time has been to supplement the diets of diabetic patients with magnesium because it was believed that magnesium is an essential micronutrientand the fourth most abundant ions present in living cells, with several dietary sources including whole grains, green leafy vegetables, legumes, nuts.[11] Magnesium is one of the promising nutritional elements for the management of type 2 diabetes. McCarty,[23] asserted the discrepant outcome of observation studies on the ability of Mg supplement to prevent T2DM and that this preponderance of sequitur necessitates future large scale prevention trials.

This research investigates the role of Mg supplement on beta cell regeneration and insulin sensitivity by perusing two important genes insulin and PDX-1.The data gotten from this investigation clearly demonstrate that Mg supplement present certain changes when compared with other treated groups. The feed intake nose-dived significantly ($p<0.05$) at week 1 in the untreated diabetic group as presented in Figure 1 when juxtaposed with the controlgroup. Meanwhile DMM and DM were significantly ($p<0.05$) different from DU. The second week revealed statisticaldecrease in DU when contrasted with control group. However, DMM and DM were significantly ($p<0.05$) higher than DU. This reduction in feed

intake could have been as a result of the chronic disease condition in the diabetic groupswhich is contrary to the position of,[24,25] The third week showed significant ($p<0.05$) reduction between DM and DU when compared, but an increase in DMM (47.0±0.82) and the control group (55.4±0.57). The trend in week 3continued till the end of the intervention period. The increase in feed intake in DMM could be due to increased excretion of magnesium which might have predisposed the animals to polyphagia which is nonconflicting with the report of Ammerman *et al.,*[26] However, the observation made in DU is contrary with what was reported by Saravanan and Pari,[27] who confirmed increase in feed intake in diabetic rats. The results obtained is consistent with the study documented by Fapohunda.[10]

Figure 2 shows that in week 1 there was decrease in DMM, DM and DU when compared with CONTROL. Although, DM (48.6±0.48) and DU (48.1±0.63) were slightly higher than DMM (47.7±1.15), the difference between DM and DMM was not significant. This reduction in water intake in all the diabetic groups may be due to diabetes type 2 that usually comes with symptoms such as plethoric urination, abatement in feed and water intake as reported by Goldfine,[28] Second week revealed significant ($p<0.05$) increase in CONTROL, DMM, DM when compared with DU. Third week showed statistical reduction in CONTROL and DU when compared with DMM and DM. However, there was no statistical difference in DMM and DM. But there was statistical reduction in DU when compared with CONTROL. The last week revealed statistical reduction in CONTROL and DU when juxtaposed with DMM and DM. But there was no statistical difference between DMM and DM. However, there was statistically significant decrease in DU when contrasted withCONTROL.[28]

The result illustrated in Figure 3, shows that there was down-regulation of the insulin gene expression in all the diabetic groups when compared with CONTROL. This could be probably due to the action of streptozotocin on the β-cell of pancreas which automatically affects the production of insulin from the β-cell of the Langerhans. King,[29] reported that destruction of high percentage of endogenous β-cells result in little endogenous insulin being produced hence hyperglycemia occurs. Marchetti *et al.,*[30] asserted that the reduction in endogenous insulin production precipitates the onset of hyperglycemia. These attributes were observed in this present study and this justifies the observation made in DU, in which there was drastic reduction in the production of insulin.

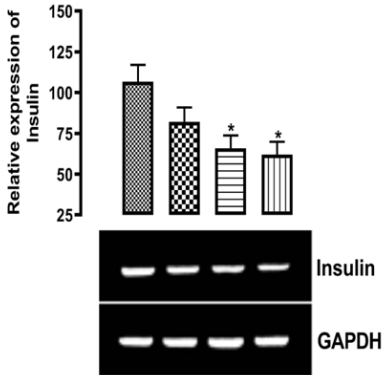

Figure 3 Relative expression of Insulin gene.

(*) means significant difference $p<0.05$ when compared with Control.

(#) means significant difference $p<0.05$ when compared with DU.

Group 1: Normal control; Group 2: Metformin and magnesium sulphate (DMM); Group 3: Metformin (DM), Group 4: Diabetic untreated (DU).

There was deregulation of insulin gene in all the rats treated with STZ-NAD. This could possibly have resulted from the partial destruction of the β-cell of the pancreas responsible for the secretion of insulin.[31] However, this pattern was reversed after 28 days of intervention. DMM showed higher expression level of insulin gene, when compared with DM and DU, no significant ($p<0.05$) difference was envisaged between CONTROL and DMM according to Figure 3. This could be as a result of the magnesium supplementation, through mechanisms that are yet to be discovered. When the level of blood glucose increases, the insulin expression level increases to regulate glucose level and when the level of blood glucose subsides, insulin secretion is inhibited.[32]

PDX-1 is also referred to as insulin promoter factor.[19,33] Several literatures reported that PDX-1 is a key factor with specific roles in the differentiation, post-natal function, survival of β-cells and development of the pancreas. They reported that downregulation of this gene could possibly form the basis of beta-cell nonfeasance and T2DM.[18,34-44] PDX-1 was also reported to undergo downregulation in cases of DNA damage, oxidative stress and advanced glycation end-products (AGEs).[45-47] From the result presented in Figure 4, there was downregulation of PDX-1 gene after the administration of STZ-NAD. This is a pointer to that fact that this combinational diabetogenic agents perpetrate their action via partial DNA damage and oxidative stress[48-50] which is consistent with Busineni *et al.,*[51] leading to the downregulation of PDX-1 as expected of type 2 diabetes animal model in agreement with Wier *et al.,*[39] Kakkar.[52] The intervention of Metformin and Magnesium supplementation upregulates PDX-1 gene more than metformin monotherapy. This upregulation suggests β-cells survival and pancreatic development in the STZ-NAD assaulted rats. This mechanism of action potentiates the increased level of insulin gene expression in Met-Mg^{2+} treated rats. This result is consistent with the pattern in literatures.[31,35,54,55] However, it is not yet known whether Met-Mg^{2+} therapy controls PDX-1 at genetic level only or also at epigenetic, transcriptional and post-translational (phosphorylation and sumolyation) levels.[40,46,47,56-66]

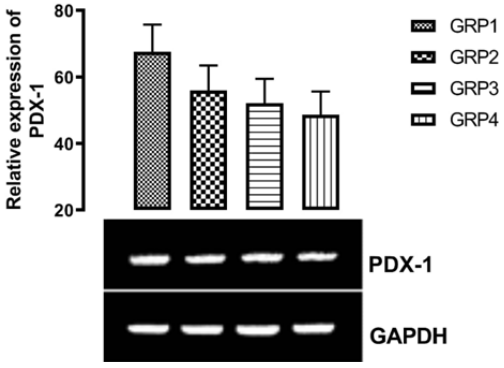

Figure 4 Relative expression of PDX-1 (pancreatic and duodenal homeobox 1).

(*) means significant difference $p<0.05$ when compared with Control.

(#) means significant difference $p<0.05$ when compared with DU.

Group 1: Normal control; Group 2: Metformin and magnesium sulphate (DMM); Group 3: Metformin (DM); Group 4: Diabetic untreated (DU).

Conclusion

This study investigated the underlying mechanisms of magnesium supplement using *in vivo* experiments. This supplement showed increased insulin and blood GLP-1 release when used as adjuvant

therapy with the standard anti-diabetic drug metformin. Intriguingly, the research outcome substantiates that magnesium supplement represents an important adjunct for the management of T2DM.

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None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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None.

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