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Comparative Study of the Effects of Metformin and Garlic Extract on Hippocampal Na^+/K^+ ATPase, Ca^{2+} ATPase and Glutamine Synthetase Activities in Type II Diabetic Wistar Rat

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ABSTRACT

Diabetes mellitus has not ceased to be on rise in spite of the continuous research on its management. Brain dysfunction associated with Diabetes mellitus especially Type II has been the great concern. The aim of this study was to investigate the effect of insulin sensitizing drug metformin and ethanolic extract of garlic on membrane bound enzymes Na^+/K^+ ATPase, Ca^{2+} ATPase and glutamate-glutamine cycle enzyme, Glutamine Synthetase activities in the hippocampus of streptozotocin-Nicotinamide induced Type II Diabetic rats. Twenty four male wistar rats weighted 120 - 150 g were used and divided into four groups with six rats in each group. Group A was non-diabetic (Control) and Groups B, C and D were diabetic. Group B received no treatment (DNT) while Groups C and D were treated with 1000 mg/kg of ethanolic garlic extract (EGE) and 50 mg/kg of metformin (MET) respectively orally for three weeks. All the groups were fed on standard rat chow with water ad libitum. Blood glucose was monitored weekly. Animals were sacrificed and the brains were removed and hippocampi were carefully excised and homogenate were obtained. Homogenate was analyzed for Na^+/K^+ ATPase, Ca^{2+} ATPase and Glutamine Synthetase (GS) activities. MET and EGE significantly reduced the blood glucose levels. There was a significant increase in the activities of hippocampal Na+/K^+ ATPase, Ca^{2+} ATPase and GS in MET and

EGE when compared to DNT. The results suggest that both MET and EGE increase the activities of hippocampal Na^+/K^+ ATPase, Ca^{2+} ATPase and GS which were reduced by diabetes mellitus, thus garlic and metformin administration exhibiting neuroprotective effect during hippocampal-related disorders associated with diabetes mellitus.

1. INTRODUCTION

Diabetes has become a global epidemic in the general population. The estimated number of African population that would be diabetic by 2030 according to World Health Organisation is approx. 18 million. Diabetes mellitus as a metabolic disease posed neuronal impairment, cognitive deficits and promote progressive neurodegeneration [1]. Insulin is one of the hormones that regulate the activity of Na^+/K^+ ATPase. Complications associated with diabetes has been shown to alter the activity Na^+/K^+ ATPase [2]. Study has shown decreased Na^+/K^+ ATPase activity in animal models of T2DM [3]. Diabetes has been shown to affect neurotransmitter synthesis or release in several brain regions [4]. Ca^{2+} ATPase regulates Ca^{2+} pump activity which acts as a second messenger in the control of cellular processes that plays a central role in mediating neurosecretion. Inhibition of Ca^{2+} ATPase activity has shown to increase intracellular concentration of Ca^{2+} which in turn altered the signal transduction pathways and cellular fluidity and eventually resulted in cell death [5]. A decrease in Ca^{2+} ATPase activity of diabetic brain has been reported [6]. Glutamatergic neurotransmission is important for hippocampal modulation of learning and memory processing [7]. The conversion of glutamate to glutamine by glutamine synthetase that takes place within the astrocytes, represents a key mechanism in the regulation of excitatory neurotransmission [8]. Study has shown that healthy neuronal cells require both intracellular and extracellular glutamine [9]. Development of diabetes-associated cognition decline in db/db mice has been suggested to be due to disturbance of glutamate-glutamine shuttling between neurons and astrocytes in hippocampus [10]. Metformin is an antidiabetic agent that is being widely used in the management of diabetes mellitus especially Type II. Several studies have shown that metformin improved learning and memory behaviours in diabetic model [11, 12], by protecting hippocampal neurons [13, 14], by activating AMPK and Na^+/K^+ ATPase system [15]. Metformin treatment is neuroprotective against the detrimental effects of β-Amyloid and high fat diets on hippocampal synaptic plasticity [16]. Several studies have investigated the anti-diabetic potential of both fresh and aged garlic extract and reported it to have antihyperglycemia, hypolipidemic, renoprotective effects and its ability to scavenging many reactive oxygen species (ROS) in both animal models and humans [17-21]. Fresh and cooked but not aged garlic extracts has been shown to increase both short and long term memory in both diabetic male and female rats [22]. Ethanolic extract of garlic has previously been reported to enhance spatial working memory by increasing hippocampal Na^+/K^+ and Ca^{2+} ATPases activities in wistar rats [23] and improve memory in Type II diabetic rats by increasing Na^+/K^+, Ca^{2+}ATPases and glutamine synthetase activities in the hippocampus [24]. This study aimed to compare the effect of metformin, a standard anti-diabetic drug and ethanolic extract of garlic on hippocampal Na^+/K^+ ATPase, Ca^{2+} ATPase and Glutamine synthetase activities in Type II Diabetic rat model. Our findings showed that both metformin and garlic increased the activities of these enzymes, though increased effect posed by metformin was more than that of garlic thus metformin may be a better anti-diabetic drug, among others in managing hippocampal-dependent dysfunction associated with diabetes mellitus.

2. MATERIALS AND METHODS

2.1. Chemical

Streptozotocin was purchased from Sigma Aldrich (St. Louis, MO, USA). Nicotinamide and metformin were purchased from Nono Pharmaceutics Ltd. (Kampala, Uganda).

2.2. Ethanol Extraction of Garlic

Extraction was done using cold maceration at the laboratory in the department of Pharmacology, Kampala International University Western Campus Uganda. Peeled garlic weighing 500 g was cut into small pieces and homogenized in 70 ml of cold sterile 0.9% NaCl. The paste material was suspended in 80% ethanol for 48 hours in air tight glass jar using a rubber stopper, and the suspension was shaken periodically for three times a day at 5 minute interval. After 2 days, the suspension was filtered using Whitman filter paper to remove residue. Filtration was repeated 3 times and clear filtrate was obtained. The filtrate was concentrated using rotary evaporator at a bath temperature of 40˚C. The extract concentrate obtained was then transferred to a cornical flask and further evaporated in oven drier at 50˚C to obtain ultimately a gel like mass for the study [23, 25].

2.3. Animals

Male Wistar rats weighing (120 - 150 g) were used in this experiment. The animals were obtained from the Animal House of College of Medicine, Mbarara University of Science and Technology, Uganda. The animals were housed in a well-ventilated room maintained under standard conditions of light, feeding and temperature of research laboratory of Kampala International University Western Campus Uganda. The study was conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals.

2.4. Induction of Diabetes

After acclimatization of animals for five days, diabetes mellitus type II was induced in rats fasted overnight on day six with a single injection of 60 mg/kg streptozotocin dissolved in 0.05 ml of citrate buffer (pH 4.5) intraperitoneally which was followed by administration of 120 mg/kg of nicotinamide dissolved in 0.5 ml of normal saline intraperitoneally 15 min later. Elevated levels of blood glucose (Hyperglycemia) was measured from a drop of tail blood of rats 3 days and a week after injection using One-Touch Ultra-Easy Glucometer (LifeScan, UK). Rats confirmed hyperglycemic with blood glucose concentration (>250 mg/dl) was used as diabetic rats for this study [24, 26].

2.5. Grouping

The rats were divided into 4 groups (n = 6) as follows:
Group A: Non-diabetic Control-received normal saline 1 ml/kg body weight
Group B: Diabetic non-treated (DNT)-received normal saline 1 ml/kg body weight
Group C: Diabetic + garlic (EGE) received 1000 mg/kg body weight of ethanolic extract of garlic
Group D: Diabetic + metformin (MET)-received 50 mg/kg body weight of metformin
Administration orally was carried out daily between 8 - 10 am for period of three weeks.

2.6. Measurement of Na^+/K^+ ATPase Activity

The hippocampal homogenates was analyzed for Na^+/K^+ ATPase according to the method of Tirri *et al.* [27]. Assay medium used consist of (in mM) 30 Tris-HCl buffer (pH 7.4), 50 NaCl, 6 $MgCl_2$, 5 KCl and 50 µg of protein in the presence and absence of ouabain, 0.1 EGTA, in a final volume of 350 µL. The reaction was started by the addition of ATP to a final concentration of 3 mM. After 30 min at 37˚C, the reaction was stopped by the addition of 50% (w/v) trichloroacetic acid (70 µL). The saturating substrate concentrations was used, and reaction was in linear with protein and time. Some controls was included in the assays for non-enzymatic hydrolysis of ATP. The Pi (amount of inorganic phosphate) released was quantified calorimetrically, as described Fiske and Subba [28], using 300 KH_2PO_4 as reference standard. Specific Na^+/K^+ ATPase activity was calculated from the overall activity (in the absence of ouabain) and was recorded as Pi/min/mg of protein in nmol [23].

2.7. Measurement of Ca^{2+} ATPase Activity

The method of Desaiah and Ho [29] was used to assay Ca^{2+} ATPase in hippocampal homogenates. Pi (Inorganic phosphates) was estimated by the method of [28]. The assay medium had a final volume of 200 µL. It consisted of (in mM), 30 Tris-HCl, 100 µg of protein in the presence or absence of 0.4 $CaCl_2$, buffer (pH 7.4), 3 $MgCl_2$ and 0.1 EGTA. The reaction was started by the addition of ATP to a final volume of 3 mM. 60 min after at 37˚C, the reaction was stopped by the addition of 50% (w/v), 70 µL of trichloroacetic acid. Substrate concentrations was used, and reaction was in linear with time and concentration of protein. Some controls was included in the assays to assess non-enzymatic ATP hydrolysis. The Pi (concentration of inorganic phosphate) released was quantified colorimetrically, as described [28], using KH_2PO_4 as a reference standard. The Ca^{2+} ATPase activity was determined by subtracting the activity measured from absence of Ca^{2+} (no added 0.1 mM EGTA and Ca^{2+}) and expressed as Pi/min/mg protein in nmol [23].

2.8. Measurement of Glutamine Synthetase Activity

The method of Rowe *et al.* [30] was used in the enzymatic assay of glutaminesynthetase. In this method, 0.1 mL homogenates solubilized in 140 mM KCl was added to 0.1 mL of the reaction mixture in mM and incubated for 15 min (37˚C). The reaction was stopped by 0.4 mL addition of a solution containing (in mM): 370 ferric chloride, 200 TCA and 670 HCl. The absorbance of the supernatant was measured at 530 nm after centrifugation and standard quantities of ferric chloride reagent treated with c-glutamylhy-droxamate was compared to the absorbance generated. Results were expressed as percentages of the control condition [24].

2.9. Statistical Analysis

All statistical analyses were performed using SPSS version 20 and GraphPad Prism software version 6 was for graphs. All values were presented as means ± SEM (standard error of mean). ANOVA which was followed by Post Hoc Bonferroni test was done and statistically significant differences were accepted at $p < 0.05$.

3. RESULTS

3.1. Effect of Metformin and Ethanolic Extract of Garlic on Hippocampal Na^+/K^+ ATPase Activity in Diabetic Rats

There was a decrease activity of hippocampal Na^+/K^+ ATPase (µmol of pi liberated/min/mg protein) in diabetic control DNT when compared with normal rats. Na^+/K^+ ATPase activity in the hippocampus of diabetic rats in groups C and D *i.e.* EGE and MET were found to be significantly ($p < 0.001$) higher when compared with the diabetic control rats. However, there was a significant ($p < 0.05$) increase in the activity of hippocampal Na^+/K^+ ATPase in MET group when compared with EGE group (**Figure 1**).

3.2. Effect of Metformin and Ethanolic Extract of Garlic on Hippocampal Ca^{2+} ATPase Activity in Diabetic Rats

There was a decrease activity of hippocampal Ca^{2+} ATPase (µmol of pi liberated/min/mg protein) in diabetic control DNT when compared with normal rats. Ca^{2+} ATPase activity in the hippocampus of diabetic rats in groups C and D *i.e.* EGE and MET were found to be significantly ($p < 0.001$) higher when compared with the diabetic control rats. There was also a significant ($p < 0.05$) increase in the activity of hippocampal Ca^{2+} ATPase in MET group when compared with EGE group (**Figure 2**).

3.3. Effect of Metformin and Ethanolic Extract of Garlic on Hippocampal Glutamine Synthetase Activity in Diabetic Rats

Figure 3 shows the significant ($p < 0.05$) decrease in the glutamine synthetase activity (mMol of

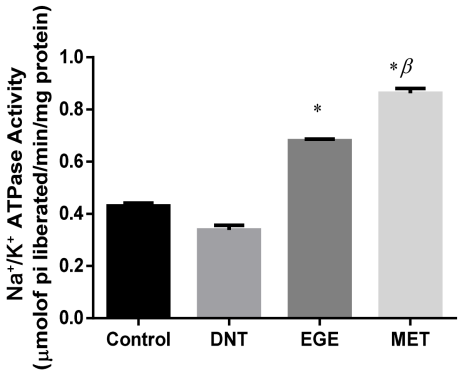

Figure 1. Effect of Metformin (MET) and ethanolic garlic extract (EGE) on hippocampal Na^+/K^+ ATPase activity in diabetic rats. Data are expressed as mean ± SEM (n = 6). *$P < 0.001$ when compared with diabetic non-treated (DNT) group. β denotes significant difference when compared with EGE group.

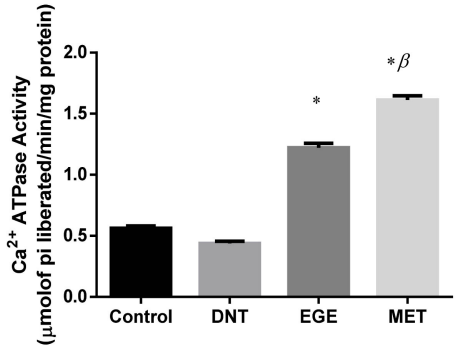

Figure 2. Effect of Metformin (MET) and ethanolic garlic extract (EGE) on hippocampal Ca^{2+} ATPase activity in diabetic rats. Data are expressed as mean ± SEM (n = 6). *$P < 0.001$ when compared with diabetic non-treated (DNT) group. β denotes significant difference when compared with EGE group.

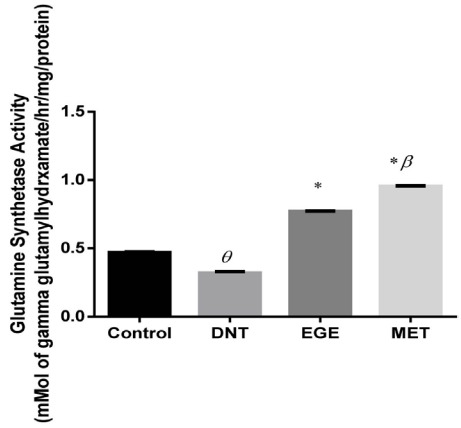

Figure 3. Effect of Metformin (MET) and ethanolic garlic extract (EGE) on hippocampal Glutamine Synthetase activity in diabetic rats. Data are expressed as mean ± SEM (n = 6). *$P < 0.001$ when compared with diabetic non-treated (DNT) group. β denotes significant difference when compared with EGE group. θ denotes significant difference when compared with non-diabetic rats (Control).

gamma glutamylhydroxamate/hr/mg/protein) of hippocampus of diabetic non-treated group when compared with normal control group. Hippocampal glutamine synthetase activity was significantly ($p < 0.001$) higher in EGE and MET groups when compared with diabetic control group. Activity of glutamine synthetase in hippocampus was found to be significantly ($p < 0.05$) higher in MET group when compared with EGE group.

4. DISCUSSION

In the present study, we investigated the effect of metformin, an insulin sensitizing drug and ethanolic extract of garlic on hippocampal membrane bound enzymes Na^+/K^+ ATPase, Ca^{2+} ATPase activities and glutamate-glutamine cycle enzyme, glutamine synthetase activity in Type II diabetic rat model. Both metformin and garlic increased the activities of Na^+/K^+ ATPase, Ca^{2+} ATPase and glutamine synthetase in the hippocampus of diabetic rat. Diabetes mellitus showed reduction in the activities of these enzymes which is in line with the study of Vague *et al.* [3] for Na^+/K^+ ATPase, Kamboj *et al.* [6] for Ca^{2+} ATPase and Zheng *et al.* [10] for glutamine synthetase [24]. Administration of metformin and garlic to diabetic rats raised the level of activities of these enzymes in the hippocampus more than non-diabetic rats. However, metformin posed higher effects compared with garlic extract. Our previous studies reported increased hippocampal Na^+/K^+ ATPase and Ca^{2+} ATPase activities in normal rats [23] and in Type II diabetic rats [24] following garlic extract consumption. Diabetes in pregnancy has been shown to induce neuronal cell apoptosis in offspring hippocampus which could be normalized by controlling the maternal glycemic state with insulin treatment [31]. Study has shown that garlic administration during pregnancy and lactation protected the rat offspring against lead-induced neuronal damage in the hippocampus [32]. Metformin has been reported to reduce the decrease loss of neurons in the hippocampus of diabetic animals [14]. The increased activities of Na^+/K^+ ATPase, Ca^{2+} ATPase and Glutamine synthetase in the hippocampus by metformin revealed by our present study could be mechanism through which loss of neurons in hippocampus is prevented in diabetic rats. Metformin has been shown to recruit neural stem cells and enhance neural function by activating an a PKC-CBP pathway [33]. Neuroprotective properties of glucagon-like peptide-1, alone and in combination with metformin, in Type II diabetes has been shown by Lennox *et al.* [34] where metformin combination enhanced learning and memory, improved long-term potentiation in the hippocampal CA1 region with reduced hippocampal levels of 8-oxoguanine and glia fibriallary acidic protein. The results of our present study could be added to the established mechanisms through which metformin mediates its neuroprotective functions especially hippocampal–dependent cognitive functions.

5. CONCLUSION

In conclusion, the present study suggested that the neuroprotective effect of metformin could be found by increasing the activities of Na^+/K^+, Ca^{2+} ATPases and glutamine synthetase in the hippocampus of Type II diabetic rats, thus makes the metformin a better drug in the management of Type II diabetes that is associated with complication of cognitive dysfunction.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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