Evaluation of the Effect of the Water Extract of Dried Flowers of Aegle marmelos on Na+/K+ ATPase Activity in Liver, Erythrocytes and Small Intestine of Diabetic Rats

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Abstract- *The water extract of Aegle marmelos (Beli mal Drink) is a popular beverage used by Sri Lankans with many medicinal benefits and the hypoglycaemic and anti-inflammatory effects of this beverage have been scientifically validated. Under many pathological conditions alterations in Na^+/K^+ ATPase activity in tissues are observed and these may lead to many metabolic changes during diseases. The present study evaluated the effect of the water extract of dried flowers of Aegle marmelos on Na^+/K^+ ATPase activity in liver, erythrocytes and small intestine of diabetic Wistar rats. Experimental diabetes mellitus was induced in rats by the intra-venous administration of Alloxan monohydrate (40 mg/kg) and rats with a serum glucose concentration of > 7.00 mmol/L were selected for the experiments. Normal and diabetic control groups received distilled water whereas the Diabetic test group received a 500 mg/kg dose of test extract. After ½ h, each rat was euthanized to collect blood samples as well as the livers and small intestines. The Na^+/K^+ ATPase activity in membrane preparations of liver, erythrocytes and small intestines, was determined in terms of liberation of inorganic phosphate during enzymatic ATP hydrolysis. The Na^+/K^+ ATPase activity in the liver plasma membrane was reduced significantly ($P < 0.001$) by 49.4 % in diabetic rats compared to normal rats. It was increased significantly ($P < 0.001$) by 44.3 % in diabetic rats after administration of the test extract compared to Diabetic control rats. The Na^+/K^+ ATPase activity in the small intestine was increased significantly ($P < 0.01$) by 41.5 % in diabetic rats compared to normal rats. It was further increased significantly ($P < 0.01$) by 24.4 % in diabetic rats after administration of the test extract compared to Diabetic control rats. The Na^+/K^+ ATPase activity in the erythrocytes was reduced significantly ($P < 0.001$) by 29.3 % in diabetic rats compared to normal rats. It was increased significantly ($P < 0.001$) by 35.2 % in diabetic rats after administration of the test extract compared to Diabetic control rats. The treatment with the test extract caused a significant increase in Na^+/K^+ ATPase activity in all three tissues compared to Diabetic control rats and this showed the test extract has an acute effect on the Na^+/K^+ ATPase activity in tissues of diabetic rats. The water extract of dried flowers of Aegle marmelos is a herbal beverage which can be used to revise the short term complications of diabetes associated with impaired Na^+/K^+ ATPase activity in tissues.*

Keywords: Na^+/K^+ ATPase activity, Diabetes, *Aegle marmelos*

I. INTRODUCTION

The Na^+/K^+ ATPase is a plasma membrane bound enzyme complex that plays a fundamental role in cellular functions by maintaining the electrochemical gradient of Na^+ and K^+ ions between two sides of the plasma membrane. It is categorized under the P-type ATPase family of cation pumps that use the free energy of hydrolysis of high energy phosphate bond of ATP to actively transport cations against their electro chemical gradients. The Na^+/K^+ ATPase is an integral protein which transports 3Na+ ions outside and simultaneously $2K^+$ ions inside across the cell membrane. Thus it creates an ion gradient which is associated with the Na-coupled transport of nutrients into cells, osmotic balance, cell volume regulation and maintenance and restoration of the resting membrane potential in excitable cells (Koksoy, 2002).

The Na^+/K^+ ATPase activity in the plasma membrane is altered in many pathological conditions, such as diabetes, cardiac diseases, hypertension, some cancers, etc. The individuals and animals with diabetes have been reported to express changes in Na^+/K^+ ATPase activity in many tissues such as heart, peripheral nerve, kidney, liver, erythrocytes and intestine. The impairment of the Na^+/K^+ ATPase activity may play a role in the development of the chronic complications of diabetes such as retinopathy, nephropathy, neuropathy and premature vascular disease. The intensity and direction of the alterations depend on the duration of diabetes and the tissue involved (Jaitovich and Bertorello, 2006).

Aegle marmelos correa (L) which belongs to family Rutacea, is commonly known as bael fruit and a plant used extensively in traditional medicinal systems of Asian counties. The literature revealed that various parts of this plant have been used to treat complications of diabetes mellitus over centuries (Sharma *et al.,* 2007). A beverage that is prepared by boiling the dried flowers in water is popular among Sri Lankans as it has a refreshing, soothing and calming effect. The hypoglycaemic and anti-inflammatory effect of this beverage is established in rats and humans (Kumari *et al.,* 2013; Kumari *et al,* 2014). The present study is designed to evaluate the effect of the water extract of dried flowers of AM on the Na^+/K^+ ATPase activity in diabetic Wistar rats.

II. METHODOLOGY

Ethical approval

Ethical approval was taken from the Ethics Review Committee of University of Sri Jayewardenepura, Sri Lanka. (Ref no: 432/09). International guidelines and recommendations of Federation of European Laboratory Animal Science Associations (FELASA) were followed for handling of animals. Assays were carried out at the Animal House and the Department of Biochemistry of University of Sri Jayewardenepura, Sri Lanka.

Plant material

Dried flowers were purchased from farmers from different provinces of the country and pooled together. Required amounts were taken from this pool for the preparation of extracts. The plant material was authenticated by the National Herbarium, Peradeniya.

Preparation of the extract

The water extract of dried flowers of *Aegle marmelos* (WEAM) was prepared by boiling 25 g of dried flowers in 500ml of water and reduced to 50 ml. The freshly prepared extract was used for experiments.

Animals

Healthy adult male, Wistar rats weighing 150 - 200 g were purchased from Medical Research Institute, Colombo 8, Sri Lanka. Rats were housed under standard conditions (230 ± 2 0C, 60 % - 70 % relative humidity and 12 h photo period) and fed with standard diet and water *ad libitum.*

Induction of Diabetes

Experimental Diabetes mellitus was induced in rats by the intra-venous administration of 40 mg/kg of Alloxan monohydrate. Fasting blood glucose levels were determined after 72 h and rats with a serum glucose concentration of > 7.00 mmol/L were used for the experiment.

Evaluation of the Na^+/K^+ ATPase activity in diabetic rats

Diabetic rats were randomly categorized in to two groups as the Test and Control groups and another six rats were selected for the Normal control group from the rats that belonged to the same batch but not treated with alloxan. After one week of diabetes induction, the following were administered to the rats using oral feeding needles. Normal control group and Diabetic control group received 2.5 ml of distilled water while the Diabetic test group received a 500 mg/kg dose of WEAM. After 1/2 h each rat was euthanized and a blood sample was collected by a ventricular puncture and the liver and small intestine were removed.

Liver plasma membrane preparation

The removed liver was immediately washed with ice cold saline and frozen in liquid nitrogen and stored at -80 ^{0}C until use. Liver plasma membranes were prepared by the method described by Askari *et al.,* (1997) and the whole procedure was carried out at 4 ^{0}C. Frozen rat liver samples (1 g) were homogenized (Ultra- Turrax T-25) in 5 ml of ice-cold 1 mM $NaHCO_3$ [(pH 7.5) (Himedia, India)] and the homogenates were diluted and filtered through 3 layers of surgical gauze. These were centrifuged (Vision Scientific, VS-550) at 1500 g for 10 min at 4 ^{0}C and the pellets were re-suspended in 1 ml of ice-cold buffer (1 mM $NaHCO_3$, pH 7.5). Then 5.5

volume of 70.7 % sucrose were added and mixed. After transferring into a 50 ml centrifuge tube, 8 ml of 48.2 % and 4 ml of 42.5 % sucrose (HImedia, India) were added. Following centrifugation for 30 min at 19 000 g, the substance collected at the interface of two layers of sucrose [(42.5 % and 48.2 %), (Sigma Aldrich, USA)] was drained and washed. The final pellet was re-suspended in 0.25 M sucrose, 30 mMhistidine (Sigma Aldrich, USA), 1 mM EDTA [(pH 6.8) (Sigma Aldrich, USA)], and stored at −80 °C. These liver plasma membrane preparations were enriched with Na+/K+ ATPase. Total protein estimation was done for unification using total protein kit (Sigma TP0 300) and the test solution was prepared to a final concentration of 1 mg of protein per ml.

The mucosal preparations of small intestines

The mucosal preparations of small intestines were prepared by the method described by Barada *et al.,* (1994) and the whole procedure was carried out at 4 ^{0}C. Removed small intestines were flushed with ice-cold 2 M NaCl (Himedia, India) and cut along the anti-mesenteric border. The mucosae were scraped into 5 ml of ice cold 2 M NaCl and centrifuged at 6800 g for 15 min at 4 ^{0}C and washed three times with ice-cold 50 mMtris, 5 mM EDTA (pH 7.2) and two times with 50 mM Tris, 1 mM EDTA [(pH 7.2), (Sigma Aldrich, USA)] and homogenized at 4 ^{0}C in 250 mM sucrose, 30 mMhistidine and 1 mM EDTA buffer (pH 7.2). Total protein estimation was done for unification and the test solution was prepared to a final concentration of 1 mg of protein per ml.

Erythrocyte membrane preparation

Blood samples were collected in EDTA tubes and plasma was removed to store at –80 °C for further assays. Erythrocyte membranes were isolated according to the method described by Kassak *et al.,* (2006) with some modifications. The whole procedure was carried out at 4 0C and whole blood samples were separated by centrifugation at 900 g for 10 min at 4 °C. Excess plasma and platelets were discarded and packed erythrocytes were washed three times with ice cold PBS (10 mM phosphate buffer, 150 mMNaCl, pH 7.4). The pellet was re-suspended in 1 ml of ice cold PBS and five volumes of 20 mM Tris-EDTA-HCl buffer (pH 7.4) was added to initiate haemolysis. Following centrifugation at 19 500 g for 10 min at 4 °C, double washing with 20 mM, 10 mM and 5 mM Tris-EDTA-HCl buffer was done. For unification of the suspensions, protein concentration was estimated using a total protein kit and the ghosts were re-suspended to a final concentration of 1 mg of protein per ml in 5 mM Tris-EDTA-HCl buffer (pH 7.4).

Measurement of Na^{+}/K^{+} ATPase activity

Measurement of the Na^{+}/K^{+} ATPase activity was carried out as previously described by Kassak *et al.* (2006) and it was determined in terms of liberation of inorganic phosphate during enzymatic ATP hydrolysis. Standard curve was drawn for liberation of inorganic phosphate by various concentrations of KH_2PO_4 (8×10^{-5} mol/L – 52×10^{-5} mol/L). The reaction medium was prepared using 100 mM Tris-HCl, 10 mM $MgCl_2$ (Himedia, India), 15 mM KCl (Himedia, India), 85 mM NaCl, 1 mM EDTA and 2 mM ATP [(Sigma Aldrich, USA) (pH 7.4)]. To observe total ATP hydrolysis 15 µl of ghost suspension was incubated with 55 µl of medium at 37 °C for 30 min. As a control for non-enzymatic hydrolysis of ATP another 15 µl of the ghost suspension from the same samples were mixed with 55 µl of medium and incubated at 4 °C for 30 min. To determine the Mg^{2+}ATPase activity, the mixture was incubated in the presence of 0.2 mM Ouabain (Sigma Aldrich, USA), which inhibits the Na^{+}/K^{+} pump. An equal volume of 15 % Trichloroacetic acid (LOBA Chemie, India) was added to stop the reactions. Inorganic phosphate liberated during ATP hydrolysis formed a colored product by the reaction between ammonium molybate (Sigma Aldrich, USA) and amino napthosulphonic acid [(ANS) (Sigma Aldrich, USA)] and absorbance values were estimated spectrophotometrically at 640 nm (Uluwaduge, 2002). The Na^{+}/K^{+} ATPase activity (Na^{+}/K^{+} AA) was evaluated by following equation,

Na^{+}/K^{+} AA = Total ATPase activity – ($Mg2^{+}$ - ATPase activity + non enzymatic hydrolysis of ATP)

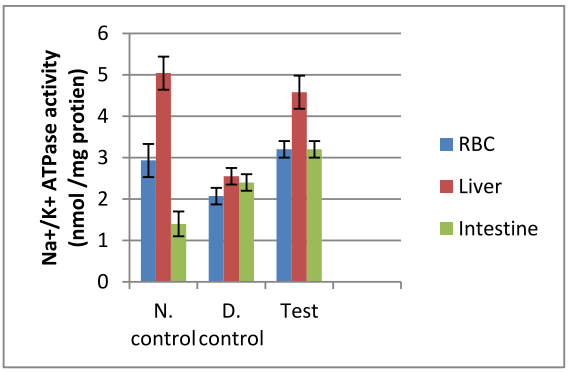
III. RESULTS

The mean Na^{+}/K^{+} ATPase concentration of hepatocyte plasma membranes in Normal control, Diabetic control and Diabetic test groups were 5.04 ± 3.9, 2.55 ± 1.5 and 4.58 ± 3.8 nmol per mg of protein respectively. The Na^{+}/K^{+} ATPase activity in the liver plasma membrane was reduced significantly ($P < 0.001$) by 49.4 % in diabetic rats compared to normal rats. It was increased significantly ($P < 0.001$) by 44.3 % in diabetic rats after administration of the test extract compared to Diabetic control rats (Figure 1).

The mean Na^+/K^+ ATPase concentration of mucosae of small intestine in Normal control, Diabetic control and Diabetic test groups were 1.40 ± 2.6, 2.4 ± 1.6 and 3.2 ± 2.1 nmol per mg of protein respectively. The Na^+/K^+ ATPase activity in the small intestine was increased significantly ($P < 0.01$) by 41.5 % in diabetic rats compared to normal rats. It was further increased significantly ($P < 0.01$) by 24.4 % in diabetic rats after administration of the test extract compared to Diabetic control rats (Figure 1).

The mean Na^+/K^+ ATPase concentration of erythrocyte membranes in Normal control, Diabetic control and Diabetic test groups were 2.93 ± 4.4, 2.07 ± 1.5 and 3.19 ± 2.1 3 nmol per mg of protein respectively. The Na^+/K^+ ATPase activity in the erythrocytes was reduced significantly ($P < 0.001$) by 29.3 % in diabetic rats compared to normal rats. It was increased significantly ($P < 0.001$) by 35.2 % in diabetic rats after administration of the test extract compared to Diabetic control rats (Figure 1).

Figure 1: The mean Na^+/K^+ ATPase concentration of erythrocyte membranes, liver plasma membranes and mucosae of small intestines of the rats in normal control (N. control), diabetic control (D. control) and diabetic test groups.



IV. DISCUSSION

The enzyme Na^+/K^+ ATPase is critical for the maintenance of the electrochemical gradient across the plasma membrane and is associated with regulation of the fundamental functions of the cell (Koksoy, 2002). Scientific investigations have reported that there are significant alterations in Na^+/K^+ ATPase activity in tissues under many pathological conditions and these alterations observed in diabetic subjects may leads to many metabolic changes and play a role in development of diabetes associated complications (Jaitovich and Bertorello, 2006).

The previous investigations have observed the effect of diabetes on Na^+/K^+ ATPase activity of various tissues is different and in some tissues diabetes induces a decrease in Na^+/K^+ ATPase activity which include sciatic nerve, lens, heart, liver and erythrocyte. In some other tissues diabetes causes an increase in enzyme activity such as mucosa of the small intestine and diabetic impairment of Na^+/K^+ ATPase activity could be due to altered enzyme kinetics and/or altered subunit expression (Vague *et al.,* 2004). In the present study the Na^+/K^+ ATPase activity was significantly reduced in erythrocytes and the plasma membranes of liver, while it was significantly increased in the mucosa of small intestine in diabetic rats compared to the normal rats.

Agarwal *et al.,* (1985) also observed a reduction of the Na^+/K^+ ATPase activity in the erythrocyte membrane in diabetic rats, compared to the normal rats. The diabetes-induced impairment in Na^+/K^+ ATPase activity could be related to the defect in myo-inositol metabolism leading to altered lipid metabolism and lipid order in the membrane (Yorek *et al.,* 1988). The increase in oxidative stress, the formation of advanced glycation products, the nerve growth factor metabolism (Sima and Sugimoto, 1999) and the disturbance in essential fatty acid metabolism leading to an abnormal ω6/ω3 ratio in red blood cell membrane (Djemli-Shipkolye *et al.,* 2003) are the other metabolic changes induced by diabetes which can also down-regulate the Na^+/K^+ ATPase activity in erythrocytes.

A significant decrease in the Na^+/K^+ ATPase activity in hepatocytes was observed by Mishra *et al.,* (1995) in alloxan induced diabetic mice and Carnovale *et al.,* (1991) in streptozotocin induced diabetic rats. Diabetes induced alterations in lipid composition of cell membranes cause a decrease in the fluidity of the hepatocyte membranes (Clandinin *et al.,* 1985; Dang *et al.,* 1989). This may lead to down regulate the Na^+/K^+ ATPase catalytic a1-subunit expression in hepatocytes (Ng *et al.,* 1993; Barada *et al.,* 1994) and a decline in Na^+/K^+ ATPase activity. The decreased enzyme activity reflect the reduced glucose transport across the cell membranes and hence leads to reduced glucose uptake by the liver during diabetes which exacerbates the hyperglycaemia.

Studies done on intestines of diabetic rats revealed that the Na^+/K^+ ATPase activity was increased in mucosa of the small intestine (Gnanaprakasam and Srivastava, 1978) and mucosa basolateral membrane (Luppa and Muller, 1986) while there was no change of sodium pump activity in mucosa brush border region (Luppa and Muller, 1986). The increase in Na^+/K^+ ATPase activity is considered due to the up regulation of synthesis of mRNA levels of α1 and β1 isoforms (Fedorak *et al.,* 1991; Barada *et al.,* 1994) in mucosal cells. The increase in Na^+/K^+ ATPase activity in turn increases the Na^+ dependent glucose absorption in the small intestine which leads to hyperglycaemia.

In the present study the diabetic rats were treated with the test extract and after ½ h, Na^+/K^+ ATPase activity was measured in erythrocytes, liver membrane and small intestine. The Na^+/K^+ ATPase activity in all three tissues was increased significantly compared to Diabetic control and this showed the test extract has an acute effect on the Na^+/K^+ ATPase activity in tissues of diabetic rats.

The test extract may act directly on the enzyme protein components or indirectly via stimulation of the secretion of insulin. According to the results of the study done on hypoglycaemic mechanisms, the test extract significantly increased the serum concentration of insulin. Insulin has a direct effect on the Na^+/K^+ ATPase activity and when insulin is present in high serum concentration, it increases Na^+/K^+ ATPase activity in tissues (Sweeney and Klip, 1998).

Previous studies have observed the direct action of insulin on isolated plasma membranes of liver and erythrocyte (Fehlmann and Freychet, 1981; Luly *et al.,* 1981). In vitro studies done on cell membranes showed the hormone-receptor interaction could affect membrane fluidity, resulting in an alteration of the membrane microenvironment which is responsible for variations in activity of membrane-bound enzymes such as Na^+/K^+ ATPase (Hyslop *et al.,* 1984). Thus the effect of elevated level of insulin may leads to the enhancement of Na^+/K^+ ATPase activity in diabetic rats after consumption of the test extract and the direct effect on the enzyme components should be studied further.

Insulin mediated translocation of enzyme subunits from intracellular pool to the plasma membrane is a short term regulatory mechanisms of Na^+/K^+ ATPase activity (Sweeney and Klip, 1998) and this may be responsible for the acute effect of the test extract on the Na^+/K^+ ATPase activity in diabetic rats. The results suggest the test extract is a herbal beverage which can use to revise the short term complications of diabetes associated with impaired Na^+/K^+ ATPase activity in tissues and further investigations should be carried out to evaluate the long term effect on the Na^+/K^+ ATPase activity.

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