

Concentration-Dependent Effect of Ivermectin on Adenosine Triphosphatase (Atpase) Activities in Human Erythrocytes

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Abstract: Ivermectin remains the drug of choice for Onchocerciasis treatment and previous studies have documented an inhibition of Adenosine Triphosphatase (ATPases) activities in adult *Onchocerca volvulus*. The study is aimed at examining the effects of Ivermectin on ATPase activities of human erythrocytes. Sixty (60) subjects, (30 males and 30 females) residing in Enugu metropolis, were recruited for the study. Venous blood samples (3 mL) were collected from each subject from where erythrocyte ghosts were prepared. Erythrocyte ghosts were prepared via hypotonic lysis, using ice-cold sodium phosphate at pH 7.4. Total ATPase, magnesium (Mg^{2+}) and Calcium (Ca^{2+}) ATPase activities were assayed using standard spectrophotometric methods, before and after Ivermectin administration on the erythrocyte ghosts, whereas Na^+/K^+ ATPase activity was calculated. Data analysis was done by one-way analysis of variance (ANOVA), Turkey's multiple comparison test and Student's t-test. After treatment, the mean Mg^{2+} ATPase activity showed a significant 3-fold increase ($p < 0.0001$; F-ratio = 11.765) compared to the mean basal activity. There was no significant effect on Na^+/K^+ ATPase and Ca^{2+} ATPase activities ($p > 0.05$; F-ratio = 0.6480), ($p > 0.05$; F-ratio = 0.9151), respectively. There was non-significant difference between the mean basal activities of the different ATPases in the different sexes, but Mg^{2+} ATPase activity of the males was significantly higher ($p < 0.05$) than females after administration of 20-40 ng/mL of Ivermectin, whereas Na^+/K^+ ATPase activity was higher in males after the addition of 40-50 ng/mL of the drug. The significant activation of Mg^{2+} ATPase observed might increase its function of energy generation for maintenance of the biconcave shape of erythrocytes. Ivermectin administration affected the activities of Mg^{2+} and Na^+/K^+ ATPases. There is no plausible reason for the observed sex difference.

Key words: Effects, erythrocyte ghosts, Ivermectin, onchocerciasis, trans-membrane

INTRODUCTION

Adenosine triphosphatases (ATPases) make up a family of Adenosine triphosphate (ATP)-driven pumps, which are involved in the trans-membrane transport of charged substrates. They catalyze the hydrolysis of ATP coupled with the function of trans-membrane transport, or to other metabolic processes, such as uptake of amino acids, glucose and other nutrients involved in metabolism. Examples of the charged substrate that they transport include, sodium ions, potassium ions, calcium and magnesium ions (Axelsen and Palmgren, 1998).

Ivermectin is a semi-synthetic macrocyclic lactone, derived from the soil actinomycete *Streptomyces*

avermitilis (Shu *et al.*, 2000). It is the current drug of choice for the treatment of Onchocerciasis, (Continho *et al.*, 1994) which is a parasitic infection of the tropics, especially in developing countries. It works by paralyzing and immobilizing the microfilariae of the parasite *Onchocerca volvulus*, hence, exposing them to the body's defense mechanism, (Goldsmith, 1998), which attacks and destroys them. In considering the possibility of destroying the adult worm itself, there is evidence that repeated courses of ivermectin may have partial destructive effect on them, (Chavasse *et al.*, 1992) hence they may be susceptible to doses of ivermectin greater than 150 ng/Kg body weight, which is the currently used dosage of the drug.

Based on this and considering the fact that membrane associated ATPases participate in important cellular functions, (Shu *et al.*, 2000). The present study is aimed at investigating the in-vitro effects of increasing concentration of Ivermectin on the ATPase activities in human erythrocytes. This will contribute meaningfully to the use of the drug and the study to adjust the dose of Ivermectin administered to patients to achieve an effective concentration on the adult worm without having any profound adverse effect.

MATERIALS AND METHODS

Selection and description of subjects: Twenty (20) apparently healthy subjects (10 males and 10 females) aged between 18 and 40 years and residing in Enugu metropolis were recruited for the study. Individuals who were on Ivermectin at the time of study were not included in the study and all the study subjects gave their informed consent before being recruited for the study whereas the ethics committee of the institution gave their approval before the commencement of the study. The study was carried out at the Departments of Chemical Pathology and Pharmacology and Therapeutics, University of Nigeria Teaching Hospital UNTH, Enugu.

Drug preparation: Stock solution of Ivermectin (500 ng/mL) was prepared in 200 mMol/L Tris buffer, pH 7.4. Adequate dilutions were then made from the stock solution, to yield decreasing concentrations of the drug from 100 to 20 ng/mL, using Tris buffer.

Sample collection and preparation: Venous blood samples (5 mL) was drawn from each subject by clean venepuncture and 3 mL was delivered into Ethylene Diamine Tetra Acetic Acid (EDTA) bottles whereas 2 mLs was allowed to clot in chemically plain bottles for serum extraction (protein estimation). As soon as they were collected, erythrocyte ghosts were prepared from them by isotonic lysis (Civenni *et al.*, 1998).

Preparation of erythrocyte ghosts: To prepare the erythrocyte ghosts, 3 mL of whole blood was added to 15 mL of ice-cold 0.9% NaCl and mixed thoroughly by titration, centrifuged at 600 rpm for 10 min and supernatant discarded. The above step was repeated 3 more times before lysis of the red blood cells in 5 mM sodium phosphate pH 7.4. The suspension was centrifuged in a refrigerated centrifuge at 3000 rpm for 20 min and the supernatant was collected into 5 mL EDTA - washed bottles, stored frozen until time for analysis in atomic absorption spectrophotometer. Further lysis and centrifugation was repeated 6 times to remove most of the remaining haemoglobin, until the red colour of the sediments faded to a much milkish-white colour. The sediments were transferred into chemically-clean dry plain bottles, tightly corked and stored frozen at -20°C

until required for use. The erythrocyte ghosts were divided into 5 parts. Whereas 4 parts were administered with increasing concentrations of Ivermectin (20-100 ng/mL), one part was left without drug administration. ATPase activity was assayed for in the erythrocyte ghosts in the absence of and in the presence of increasing concentrations of Ivermectin.

Analytic methods: The serum protein was assayed using Folin Phenol reagent method of Shu *et al.* (2000). The total ATPase was assayed by the method of Bonting (1970), whereas the individual ATPases were assayed by the method of Takeo *et al.* (1980). The inorganic phosphate released was estimated by the method of Fiske and Subbarow (1925). Sample batches served as their own control by the enzyme activities being assayed in the absence and in the presence of increasing concentration of Ivermectin. In all cases, sodium-potassium (Na^+/K^+) ATPase activity was calculated by subtracting the sum of Calcium (Ca^+) and Magnesium (Mg^+) ATPase activities from the total ATPase activity, whereas the specific enzyme activity/unit protein/hour was calculated by dividing the Activity in $\mu\text{mol}/\text{mL}/\text{h}$ by the protein concentration in mg/mL .

Statistical analysis: Data was subjected to one-way Analysis of variance (ANOVA). Differences between means were compared using Turkey's multiple comparison Test. Comparisons between different sexes and different ATPases were done using Students t-test (Nwabuokei, 1986).

RESULTS

Table 1 shows the mean basal activity ($\mu\text{mol pi}/\text{mg protein}/\text{h}$) of sodium-potassium (Na^+/K^+) and calcium (Ca^+) ATPases were both significantly higher than the basal activity of the magnesium (Mg^+) ATPase ($p < 0.05$). There was however no statistical significance ($p < 0.05$) in the difference between the basal activity of the Na^+/K^+ and Ca^+ ATPases.

There was statistical significance between the meanbasal specific activity of Mg^+ ATPase and the mean specific activity after the introduction of increasing

Table 1: Mean \pm SD basal activities of individual ATPases ($\mu\text{mol pi}/\text{mg protein}/\text{h}$)

ATPases	Specific activities	Test of significance between individual ATPases
Mg^+ ATPase	0.21 \pm 0.11	Mg^+ and Ca^+ ATPase ($p < 0.05$)*
Ca^+ ATPase	0.37 \pm 0.20	Mg^+ and Na^+/K^+ ATPase ($p < 0.05$)*
Na^+/K^+ ATPase	0.44 v 0.28	Ca^+ and Na^+/K^+ ATPase ($p > 0.05$)

*: Statistically significant

Table 2: Mean±SD effects of Ivermectin on adenosine triphosphatase (ATPase) activities of erythrocytes

Drug concentration (ng/mL)	Mg ⁺ ATPase	Na ⁺ /K ⁺ ATPase	Ca ⁺ ATPase
0.0	0.21±0.11	0.44±0.28	0.37±0.20
20.0	0.69±0.16	0.45±0.15	0.32±0.20
40.0	0.63±0.22	0.45±0.09	0.29±0.19
50.0	0.66±0.48	0.38±0.18	0.27±0.14
100.0	0.60±0.16	0.40±0.10	0.36±0.19
ANOVA (p-value)	p<0.05*	p>0.05	p>0.05

*: Statistically significant

concentrations of Ivermectin (F-ratio = 11.765, p<0.0001). Individual comparisons between the mean basal activity and the mean specific activities after the introduction of different drug concentrations, yielded significantly increased activities (p<0.001) higher than the basal specific activity (Table 2). The table also showed no statistically significant effect on Na⁺/K⁺ ATPase activity (F ratio = 0.9151, p>0.05) and Ca⁺ ATPases (F-ratio = 0.6480, p>0.05), respectively.

Table 3 compares the activities of the different ATPases (μmol pi/mg protein/h) in the different sexes (males and females). The table shows statistically significant increase (p<0.05) in the activity of Mg⁺ ATPase in males compared to females, after treatment with 20-40 ng/mL of ivermectin whereas treatment with other drug doses yielded no significant difference. Significantly higher activity (p<0.05) was also observed in the Na⁺/K⁺ ATPase activity of the males compared to the females after treatment with 40-50 ng/mL of Ivermectin. Treatment with other concentrations of the drug yielded no statistically significant difference in activity between the two sexes (Table 3). The table also showed no statistically significant difference (p>0.05) in Ca⁺ ATPases activity between the sexes, with treatment with the different doses of Ivermectin.

DISCUSSION

We studied the effects of Ivermectin on the activities of erythrocyte membrane ATPases and our baseline results were similar to previously documented results, but not completely in agreement with some of them. The

study by Niggli *et al.* (1982) showed that calcium (Ca⁺) ATPase activity was higher than both sodium-potassium (Na⁺/K⁺) ATPase and Magnesium (Mg⁺) ATPase activities while Na⁺/K⁺ ATPase activity was higher than Mg⁺ ATPase activity. The results of our study, however, showed that basal Na⁺/K⁺ ATPase activity was higher than that of Ca⁺ ATPase albeit statistically not significant, whereas both activities were higher than that of Mg⁺ ATPase. This seems to have a consistent bio-chemical and physiological background, since Na⁺/K⁺ ATPase is the most important of all the known cationic pumps because of its role in the transport of the major cations in the extra and intracellular fluids in animals, maintenance of electrochemical gradient across the plasma membrane and trans-membrane uptake of solutes such as amino acids via sodium (Na⁺) - symport systems (Jorgensen *et al.*, 2003). Plasma membrane calcium pump cannot be considered to be as diverse as Na⁺/K⁺ ATPase in their roles, so also Mg⁺ ATPase which only catalyzes the hydrolysis of ATP to liberate energy (Cheng *et al.*, 2004).

The three-fold activation of magnesium ATPase and the statistically non-significant effect on Na⁺/K⁺ ATPase and Ca⁺ ATPase activities, by increasing concentration of Ivermectin, does not agree with the findings of Shu *et al.* (2000) which showed an inhibition of the activity of Mg⁺ ATPase in the adult female *Onchocerca volvulus* and also an inhibition of the activity of Na⁺/K⁺ ATPase in both male and female worms over similar range of Ivermectin concentrations (0-100 ng/mL). This is probably not unexpected, since they worked on ATPases from a different source. These differences are in line with a report by Zinchuk *et al.* (1997) which stated that various ATPases differ in various ways and that the most meaningful of these differences, are specific transporting properties and diverse inhibitor and activator sensitivities. They also stated that P-type ATPases exhibit subtle varieties, even if they translocate the same ion.

Mg⁺ ATPase is a membrane-bound protein, which catalyzes the hydrolysis of Adenosine Triphosphate (ATP), with a release of free energy needed to power metabolic activities in the body (Ranjan *et al.*, 2000). If

Table 3: Mean±SD effects of ivermectin on adenosine triphosphatase (ATPase) activities of erythrocytes in the different sexes

Drug conc. (ng/mL)	Mg ⁺ ATPase activities		Na ⁺ /K ⁺ ATPase activities		Ca ⁺ ATPase activities	
	Male (n = 10)	Female (n = 10)	Male (n = 10)	Female (n = 10)	Male (n = 10)	Female (n = 10)
0.0	0.23±0.11	0.22±0.16	0.54±0.31	0.34±0.22	0.45±0.23	0.29±0.13
20.0	0.75±0.16	0.62±0.13*	0.49±0.14	0.41±0.15	0.42±0.22	0.22±0.13
40.0	0.73±0.22	0.54±0.17*	0.50±0.09	0.40±0.06*	0.32±0.22	0.26±0.16
50.0	0.74±0.60	0.57±0.34	0.46±0.20	0.29±0.10*	0.29±0.14	0.26±0.15
100.0	0.62±0.14	0.58±0.18	0.41±0.18	0.38±0.10	0.36±0.23	0.35±0.15

*: Statistically significant compared to the males

Ivermectin stimulates Mg^{+} ATPase, the net effect will be an increase in the rate of production of free energy of hydrolysis of ATP which is utilized for metabolic processes. This will lead to an increased production of ATP, to replenish the ATP stores and restore the state of the system. Since previous studies show that once ATP concentration is adequate and red cells have their normal biconcave shape, phosphorylation of spectrin and other cytoskeletal proteins, from exogenous sources (or by an induced increase in the activity of non-pump ATPases contained in spectrin) does not alter the shape of the cells (Ranjan *et al.*, 2000). Hence, the 3-fold increase in activity of Mg^{+} ATPase by Ivermectin will not lead to alteration in shape of red cells which could affect the activities of red blood cells adversely.

Our study also showed that administration of 20-40 and 40-50 ng/mL of Ivermectin stimulated the Mg^{+} ATPase and Na^{+}/K^{+} ATPase activities respectively in the males, compared to the females. This is likely to be as a result of the well-known sex differences found in almost every physical variable, including body build, gross and fine anatomy, physiological function and biochemical composition. However, the actual reason for the difference was not clear.

CONCLUSION

In the light of these findings, we conclude that increasing concentrations of Ivermectin up to 100 ng/mL will induce a 3-fold activation of spectrin Mg^{+} ATPase, but this induction is safe since it does not result in alteration in the shape of the cells. Also the baseline ATPase activities are not significantly different between the sexes but such differences could possibly manifest under stress. We therefore support the previously advocated increase in the dosage of the drug as recommended by the study of Shu *et al.* (2000) for an improvement in the chemotherapy of Onchocerciasis.

Finally, we recommend that ATPases be taken more serious by modern researchers, considering their profound physiological and biochemical importance in the body. Efforts should also be geared towards establishing their reference ranges, standardizing their methods of assay and including them in routine tests carried out in clinical biochemistry laboratories, especially in developing countries.

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