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Interactions of*Moringaoleifera* Seed (Lam) With Cations Transport Adenosine Triphosphatasesin Protein Energy Malnourished Rats Skeletal Muscle

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Abstract: *The aim of the present study was to evaluate the interactions of dietary supplementation ofdefatted Moringaoleiferaseed (lam) with cations transport adenosine triphosphatases in skeletal muscle of muscle degenerated rats induced by Protein Energy Malnutrition (PEM). PEM has been associated with skeletal muscle degeneration and calcium homeostasis dysfunction. In the course of the experiment, twenty four (24) weanling female albino rats (Rattusnorvegicus) of the Wistar strain (average weight of 49.39±1.73) were used for this investigation. (PEM) was induced in eighteen (18) rats by feeding them with low protein diet and water for twenty eight days while the remaining six rats served as Positive control groupand received commercial feed containing 21% crude protein. These PEM rats were then divided into three groups: A, B and C. Group A was sacrificed while group B and C were fed with recovery diets ad libitum for 28days. Group B received feed containing 20% Fishmeal protein and Group C received feed containing 20% defatted M. oleiferaseed and water . Protein-energy malnourished rats group was associated withsignificant reduced (P<0.05) body weight, total protein and ATPases activities [(Ca^{2+} and Na^{+}/K^{+}- ATPases) with Vmax; 0.308 and 0.241µmol Pi/hour/µg Protein, Km; 10.350 and 35.506mM respectively] and during these experimental days, the animals showed characteristic typical of catabolic states, these include, retarded growth, loss of hair, scaly tail etc. Uponintroduction of recovery diets containing 20% fishmeal protein or 20% M. oleiferain place of 3% fishmeal protein for twenty eight days, the recoverydiet containing 20% defatted M. oleiferaseed caused the most significant (P<0.05) increase in ATPases[(Ca^{2+} and Na^{+}/K^{+}ATPases) with Vmax values of 0.732 and 0.786µmol Pi/hour/µg Protein and Km values of 10.316 and 10.558mM respectively] activities, total protein concentrationand body weight when compared with themalnourished group. The group fed with 20% fishmeal caused no significant difference in body weight, total protein and ATPasesactivities compared with the malnourished group. The effectsof diet containing 20% M. oleifera and 20%fishmeal protein on Ca^{2+}- ATPase activities was not significantly different from each other. The study clearly revealed that ingestion of low protein diet led to skeletal muscle degeneration (reduced ATPases activity and total protein concentration) and dietary supplementation of defattedM.oleiferaseed ameliorates skeletal muscle degeneration by increasing the ATPases activity, total protein concentration and body weight in protein energy malnourished rat models.*

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I. Introduction

Muscle wasting or atrophy is an increased muscle proteolysis or protein degradation resulting to loss of body cell mass due to unintentional loss of body weight (5 to 10%) (Roubenoff*et al*., 1997).Muscle wasting occurs as a resultof distortion in the normal balance between protein synthesis and protein degradation. During atrophy, the protein synthesis pathway is negatively regulated, and an activation of protein degradation (Sandri, 2008). An important molecule that maintains this strict balance in the cell is calcium ion (Ca^{2+}). All muscles use Ca^{2+} as their main regulatory and signaling molecule, and the function of all muscle types is controlled by Ca^{2+} as a second messenger. Inhibition of Ca^{2+}-ATPase (in the case of infection or malnutrition) prevents the pumping of calcium in the muscle cell that would otherwise be used for maintaining overall health of the cell, thus resulting in a wasting and degeneration of muscle cell tissue (Bababunmi, 2002).

Maintenance of electrochemical gradient across the plasma membrane is also calcium dependent (Hoenderop*et al*, 2005). Calcium pumps are therefore important in the sense that they direct the flow of calcium ions through the plasma membrane or organelle membranes and the resulting gradients are used in a variety of signaling systems mediated by gated ion channels (Bautista and Lewis, 2004). Calcium pumps are ATPases that transportions across membranes using energy obtained from the hydrolysis of ATP (Hoenderop*et al*, 2005).

Skeletal Muscle Atrophy (SMA) is a phenomenon common in many diseases and disorders such as malnutrition, disuse, denervation, sepsis, acidosis, cancer, diabetes, HIV/AIDS, oxidative stress, and more (Leckere*t al*., 2004). Because SMA is associated with many different disorders, it is possible that there are many different pathways leading to protein degradation. Precise methods to evaluate loss of muscle mass are important to assess both baseline muscle mass and changes over time, particularly in the case of disease processes and interventions intended to reduce muscle wasting (Cohn *et al*., 1983; Kehayias*et al*., 1997).

The loss of body weight with wasting may be associated with low food intake, high levels of energy expenditure, or a combination of both. Protein energy-induced malnutrition is the pure example of the detrimental effect of reduced amino acid supply and loss of muscle mass (Grant, 1983). Skeletal muscle proteolysis increases under conditions of acidosis, up-regulation of branched-chain ketoacid dehydrogenase, the presence of catabolic hormones (glucocorticoids, thyrotoxic states) and catabolic cytokines (interleukin-1 and -6 and tumor necrosis factor), and insulin resistance . Themechanisms of increased protein degradation include activation of the intracellular ubiquitin proteasome ATP-dependent pathway (Mitch, 1996) and the decarboxylation of branched-chain amino acids, both resulting in increased protein catabolism and loss of lean body mass(Gerber and Mitch, 1992). Timely recognition of muscle wasting is critical to intervene successfully while treating the underlying condition. Furthermore, the amelioration of nutritional problems related to wasting may prove to be one strategy for increasing quality of life, enhancing functional independence, and possibly lessening the burden of a specific disease.

Moringaoleifera Lam (*M. Pteriogosperma*) belongs to the monogeneric family *Moringa*ceae and it is one of the best known, most widely distributed and naturalized species (Nadkarni, 1955). It is popularly known as drumstick or horseradish in English and locally known as 'Zogale' in Hausa, 'Eweigbale' in Yoruba and 'Okweoyibo' in Igbo (Dalziel, 1956) and 'Anahuo' in Ebira tribe of Kogi State.Many parts of this plant *i.e*.leaves, immature pods, flowers and fruits are edible and are used as a highly nutritive vegetable in many countries (Anwar and Bhanger, 2003). The phytochemical screening/analysis of this plant showed that theleaves contained β-carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidant due to the presence of ascorbic acid, flavonoids, phenolics and carotenoids. Antihypertensive, antioxidant, antidiabetic, antimicrobial, anticancinogenic and hypolipidemic activities of this plant have been reported(Faizi*et al*., 1994, 1995;Pari*et al*., 2007; Guevara *et al*., 1999; Murakami *et al*., 1998; Ghasi*et al*., 2000;Ndong*et al*., 2007; Jaiswal*et al*., 2009; Caceres *et al*., 1991). However, the possible beneficial effects of *M. oleifera* on skeletal muscle wasting have not yet been reported. This researchwas therefore designed to investigate the effect of dietary supplementation of seeds of this plant on skeletal muscle wasting associated with protein energy malnutrition.

II. Materials and Methods

Plant Materials and Authentication

Moringaoleifera seed was bought from CentralAISAS FARMS LIMITED, Ilorin, Kwara State and it was authenticated at the Plant Biology Department, University of Ilorin, Nigeria with a voucher authentication number I.U.H.V Number 13.Seeds were removed from the seed coats, oven dried at 60^{o}C and pulverized. A known weight of the pulverized seeds wasmeasured into a dry conical flask. The solvent; chloroform: methanol in 2:1 was added and the flask shaken vigorously. The flask was then allowed to stand undisturbed for 24 hours so that the extracted lipids can float out on the solvent. The organic solvent and the aqueous phase were then decanted off and the solvent was recovered using the sohxlet extractor. The procedure was repeated a number of times to improve the efficiency of the extraction process. The defatted seed was then spread on foil paper and air-dried at room temperature.

Animals and experimental design

Twenty four (24) weanling female albino rats (*Rattusnorvegicus*) of the Wistar strain ((average weight of 49.39±1.73)) obtained from the experimental animal unit of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria were used for this investigation. Protein Energy Malnutrition (PEM) was induced in eighteen (18) rats by feeding them with low protein diet according to modified method of Adelusi and Olowokere (1985) and water for twenty eight days. The composition of the diet is as shown in Table 1. These PEM rats were then divided into three groups A to C. Group A was sacrificed while group B and C were fed with recovery diets and water*ad libitum* for twenty eight days. Group B received feed containing 20% protein Fishmeal and Group C received feed containing 20% *M. oleifera* seed while positive control group received commercial feed obtained from Vital Feeds Ltd. As shown in Table 2.

Table 1. Composition of PEM Diet

	3%Fishmeal Protein(g)
Fishmeal/*Moringaoleifera*	15
Maize shaft	510
Sucrose	375
Wheat offal	20
Vegetable oil	50
***Vitamin and Mineral Mix**	30

Table 2. Composition of Recovery Diets.

	20%Fishmeal Protein(g)	20%*Moringa oleifera*Protein(g)
Fishmeal/*Moringaoleifera*	100	100
Maize shaft	425	425
Sucrose	375	375
Wheat offal	20	20
Vegetable oil	50	50
***Vitamin and Mineral Mix**	30	30

Note: Positive control group received commercial feed containing 21% crude protein.

*Vitamin/ Mineral mix: Vitamin A 4,000,000 i.u; Vitamin D3, 800,000 i.u; Tocopherols, 400 i.u; Vitamin K_3 800mg, Folacin, 200mg; Thiamine, 600mg; Riboflavin 1,800mg; Niacin, 6000mg; Calcium pathothenate, 4mg; Biotin, 8mg; Manganese, 30,000mg, Zinc, 20,000mg; Iron, 8,000mg; Choline chloride 80,000mg; Copper, 2,000mg; Iodine, 480mg; Cobalt, 80mg; Selenium, 40mg; BHT, 25,00mg Anticaking agent 6000mg.

Tissue Preparation

The animals were sacrificed one at a time by cervical dislocation then jugular puncture, the rats were then laid on an operating board and dissected in order to isolate tissue of interest (skeletal muscle). The isolated tissue was cleansed with cotton wool to remove blood stains, weighed and immediately immersed in ice cold 0.25M sucrose buffer solution andhomogenized. The homogenates were kept frozen overnight to ensure maximum release of the enzymes (Ngaha*et al*., 1989).

Enzyme and Protein Measurement

Protein Determination

The protein concentration of the homogenates was determined by the Biuret method of Gornal*et al.* (1949).Inalkaline medium, cupric ions form a purple colouredcomplex with compounds containing repeated CONH-links such as protein. The intensity of the colour is ameasure of the number of peptide bonds in solution andhence the protein concentration.

Inorganic Phosphate Determination

The inorganic phosphate liberated was estimated using standard phosphate curve (Stewart, 1974) based on coloured reaction developed using 1.25% ammonium molybdate and 9% ascorbic acid.

Determination of ATPase Activities.

Ca^{2+}- and Na^+/K^+-ATPase assays were carried out by the method of Ronner*et al.* (1977) as modified by Bewaji*et al.* (1985).

Adenosine triphosphate (ATP) is hydrolyzed in the presence of appropriate cation to release inorganic phosphate. The amount of inorganic phosphate released is measured using the ammonium molybdate-ascorbic acid system.

Concentrated sulphuric acid oxidizes ammonium molybdate to molybdic acid, which gives a yellow colour with inorganic phosphate. Ascorbic acid reduces the molybdic acid to give a blue coloration whose intensity is proportional to the concentration of inorganic phosphate liberated into the reaction medium (Fiske and SubbaRow, 1925).

Statistical Analysis

All data are expressed as the mean of six replicates $\pm$ standard error of mean (S.E.M). Statistical evaluation of data was performed by SPSS version 20. Using one way analysis of variance (ANOVA), followed by Duncan's posthoc test for multiple comparisons. Values were considered statistically significant at $p<0.05$ (confidence level = 95%).

III. Results

Morphological Appearance

The characteristics appearance of the protein energy malnourished rats [Rats fed with Isocaloric Protein diet (3% Protein)] in the first 4 weeks includes development of round face**,** Loss of appetite**,** Characteristic loss of fur (severe in the 3rd week),lack of exercise and physical activities,on-set of muscle wasting (atrophy), Scaly tail (more prominent during the 3rd week),presence of sores on the tails,Severe fur loss in the 3rd week**,** Stunted growth with no significant gain in weight**,** Sparse fur on skin**,** Reduced growth rate at the end of 4th weekandMuscle atrophy still persistent

Morphological appearance of the protein energy malnourished rats treated with 20% fish meal based diet shows no significant improved amelioration of hair lose, scaly tails, and no significant weight gain in the animals.

During the experimental period of receiving the recovery diet, the hair lose started growing, there was disappearance of sores on the tail, sparse fur on the skin (pronounced in 3rd week) and little increase in weight (observed in 4th week) in protein energy malnourished rats fed with 20% defatted *Moringaoleifera* seed meal.

Total Protein Determination

The total protein concentration ofPEM control group significantly decrease ($p<0.05$) when compared with positive control group but there was no significant difference when compared with fed with 20% Fish Meal Protein g. During recovery period, there was significant increase ($p<0.05$) in the group that was fed with *M. oleifera* seed supplemented diet as compared with PEM control group.

Table 3: Total Protein Concentration of the PEM group, PEM+20% Fish meal group, PEM+20% Defatted *Moringaoleifera* seed group and control group

Group	Total Protein (mg/ml)
Positive Control	0.083 ± 0.034^{b}
PEM Control	0.013 ± 0.006^{a}
PEM+20% fishmeal	0.019 ± 0.008^{a}
PEM+20% *Moringa* seeds	0.066 ± 0.014^{b}

Values are expressed as means of six(6) replicates ± S.E.M (Standard Error of Mean) and those with different superscripts across the column are statistically different ($p<0.05$).

Determination of Adenosine triphosphatase Activities

Effects of recovery diets on Ca^{2+} -and Na^{+}/K^{+}- ATPase activities in PEM rat skeletal muscle are represented in Table 4. The data revealed that the dietary supplementation of *M. oleifera* seed produced a significant increase ($P<0.05$) in both Ca^{2+} -and Na^{+}/K^{+}- ATPase activities in the skeletal muscle as compared with PEM group. Compared with the control, Ca^{2+} -and Na^{+}/K^{+}- ATPase activities significantly decrease ($P<0.05$) in 20%fishmeal and protein deficient groups. But there was no significant deference between 20%fishmeal diet group and protein deficient (3%protein) group in both enzymes (Table 4).

Table 4: Effects of recovery diets on Ca^{2+} -and Na^{+}/K^{+}- ATPase activities in skeletal muscle of protein energy malnourished rats as compared with control.

Group	Ca^{2+}-ATPase (µmolPi/Hr/µg Protein)	Na^{+}/K^{+}-ATPase (µmolPi/Hr/µg Protein)
Positive Control	0.734 ± 0.052^{c}	0.694 ± 0.041^{b}
PEM Control	0.239 ± 0.018^{a}	0.133 ± 0.015^{a}
PEM+20%Fishmeal	0.441 ± 0.040^{ab}	0.188 ± 0.016^{a}
PEM+20%*M. oleifera*	0.577 ± 0.053^{b}	0.613 ± 0.050^{b}

Values are expressed as means of six(6) replicates ± S.E.M (Standard Error of Mean) and those with different superscripts across the column are statistically different ($p<0.05$).

Table 5: Summary of Kinetic Parameters of Ca^{2+}-ATPase activity in skeletal muscle homogenates

Groups	Km value (mM)	Vmax value (µmol pi/hour/µg protein)
Positive Control	8.6942	0.912
PEM Control	10.350	0.308
PEM+20% Fishmeal	10.151	0.559
PEM+20%*M.oleifera*	10.316	0.732

Table 6: Kinetic parameters of Na^+/K^+-ATPase activity in homogenates of rat skeletal muscle

Groups	Km value (mM)	Vmax value (µmol Pi/hour/µg Protein)
Positive Control	6.704	0.826
PEM Control	35.506	0.241
PEM+20% Fishmeal	11.164	0.243
PEM+20%*M.oleifera*	10.558	0.786

IV. Discussion

The symptoms of protein energy malnutrition include oedema, diarrhoea, weight loss, alopecia, retinopathy, opportunistic infections (Oyagbemi*et al*., 2008).Other complicationsare; muscle wasting, reduced exercise,physicalactivities and decreasedtotal protein concentration. The results of this study showed that muscle wasting with significant ($p<0.05$) reduced Ca^{2+}-ATPase, Na^+/K^+-ATPase activity, body weight and total protein concentration developed in the protein malnourished ratscompared with the normal control.. The significant reduction in body weight when PEM group was compared with positive control may be due low protein intake, inadequate exercise and physical activities. In Protein insufficient intake, the endogenous amino acids generated mainlyfrom skeletal muscle proteolysis become the source of amino acids for protein synthesis and the obligatory nitrogen losses (WHO/FAO/UNU, 1985).Butterfield *et al*.(1992) reported that exercise and physical activity enhance protein utilization and contribute to the prevention of and recovery from wasting.The result also agreed with the work of Keys *et al.* (1950) and Winick, (1979) who reported that in prolonged malnutrition, a significant decrease in body weight is accompanied by reduced total protein concentrations.

The significant decrease ($p<0.05$) observed in the specific activity of Ca^{2+}-ATPase and Na^+/K^+-ATPase (Table 3) of the skeletal muscle when protein energy malnourished (PEM) control group was compared with positive control group, may be due to the inhibition or degradation (proteolysis) of these enzymes (membrane bound protein) during infection resulting from low-protein intake or an increase in the permeability of the sarcolemma for Ca^{2+} due to damage or opening of Ca^{2+} -Conducting Channels. Data obtained from this study supported the work of Bababunmi (2002) that theinhibition of Ca-ATPase (in the case of infection or malnutrition) prevents the pumping of calcium in the muscle cell that would otherwise be used for maintaining the overall health of the cell, thus resulting in a wasting and degeneration of muscle cell tissue.

Insulin is an important regulator of protein synthesis (Kimball *et al*., 1994) and proteolysis (Tessari*et al*., 1987) in skeletal muscle. Insulin resistance or deficiency results in impaired muscle protein turnover (Garibotto*et al*., 1994) and muscle wasting (Kaysen, 1996).In skeletal muscle, circulating plasma IGF-I concentrations stimulate intracellular amino acid and glucose transport as well as protein synthesis while suppressing protein degradation (Musey*et al*., 1993). It was observed that a low-protein diet adequate in energy resulted in atrophy of type I muscle fibers associated with significant declines in plasma IGF-I levels.Metabolic acidosis stimulates the intracellular ubiquitin proteasome ATP-dependent pathway, which catalyzes the breakdown of abnormal and short-lived proteins (Mitch, 1996).Increases in cytosolic calcium concentration $[Ca^{2+}]c$ may result in activation of Ca^{2+}-activated neutral proteases (calpains), activation of PLA2, an increased production of ROS, and/or mitochondrial Ca^{2+} overload. Reactive oxygen intermediates play a role in the formation of reactive aldehydes (i.e. 4 hydroxy-2, 3- trans-non-enal) which decreases activity of plasma membrane calcium ATPase activity (Powers *et al*., 2005). The inactivity of this enzyme hinders the movement of calcium in and out of the cell.

There was no significant difference ($p<0.05$) in the specific activity of these enzymes (Ca^{2+}-ATPase and Na^+/K^+-ATPase),body weight, and total protein concentration when protein energy malnourished (PEM) group was compared with20% fishmeal protein based diet fed group. This suggests that increase the protein content of the diet cannot reserve the damage caused by protein energy malnutrition. This also suggests that the observed significant decrease seen in the activities of these enzymes in Protein Energy Malnutrition is not only due to the deficiency of protein but an interaction of different reactions leading to degradation of these proteins (membrane bound enzymes). This may involve calcium hemostasis dysfunctions that result from calcium overload in the cell following infection. Calcium overload activates some reactions [Ca^{2+}-activated neutral proteases (calpains), increase production of free radicals (ROS), activation of PLA2] resulting to the degradation of these enzymes or causing peroxidation of membrane lipid that allow the enzymes to leak out of the cell. These might be the reasons for insignificant increase in the activities when the protein content of the diet was increased.

Studies have led to the conclusion that reduced Calcium adenosine triphosphatases (Ca-ATPase) activity in protein-energy malnutrition (PEM) may be linked with the generation of reactive oxygen species. Moreover, damage to skeletal muscle by different stresses has been found to occur by three common pathways or reactions (McArdleand Jackson, 1997):

(i) Loss of energy supply to the cell;

(ii) Loss of intracellular calcium homeostasis; and

(iii) Over-activity of oxidizing free radical-mediated reactions(McArdleand Jackson, 1997; McArdle*et al.,* 1999).

Significant increase ($p<0.05$) in the specific activity of Ca^{2+}, Na^{+}/K^{+}- ATPases and total protein concentrationwas observed when the group that was fed with 3% protein fishmeal based diet (3%protein) was compared with the group that was fed with 20% defatted *M. oleifera* seed based diet four weeks post treatment of malnutrition. This suggested that the *M. oleifera* seeds based diet activates the synthesis of these proteins (enzymes) .It has been reported that *M. oleifera* plant is a natural rich source of all essential amino acids(Siddhuraju and Becker, 2003).This is in accordance with Olorunsogo*et al*. (1989) who reported differences in protein concentration of membrane of healthy children when compared with children having kwashiorkor.

Kerstetter*et al*.(2003; 2003) reported that protein rich diets may lead to increased absorption of calcium from the gut, hypercalcuria and subsequent loss of calcium in urine. This is because excess protein increases the body's acid load which is buffered or neutralized in part by the bony skeleton, thus releasing calcium into the general circulation (Barzel and Massey, 1998; Kerstetter*et al.,*2003). This extra serum calcium is eventuallyexcreted by the kidneys into the urine, thus causing hypercalcuria. Acid loading also directly inhibits renal calcium reabsorption, resulting in an increase in urinary calcium excretion (Kerstetter*et al.,* 2003; 2003). Hypercalciuria has been reported to stimulate an increase in the activity of the Ca2+ ATPase (Bianchi *et al*., 1988; Heguilen*et al*., 2009). The observed increase in the total protein concentration and activity of the Ca2+ ATPase may therefore be connected to the hypercalciuria caused by high protein diet.

It may also be due to the antioxidant agents of the plant that mop or scavenge the free radicals (ROS) generated during malnourishment. *M. oleifera* plants are used in folk medicine for their free radical scavenging effect. Several biological properties ascribed to various parts of *M. oleifera* tree have been reviewed in the past (Anwar *et al.,* 2007) part of this includes its use as an antioxidant (Verma*et al.,* 2009), The leaves have been reported to be a rich source of β-carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidant due to the presence of ascorbic acid, flavonoids, phenolics and carotenoids. Numerous epidemiological studies suggest that herbs/diets rich in phytochemicals and antioxidants execute a protective role in health and disease (Vinson *et al*., 2001). Polyphenols inhibit lipid peroxidation by acting as chain breaking peroxyl radical scavengers and can protect membrane lipid from oxidation (O Byme*et al*., 2002).

V. Conclusion

The present study reveals that defatted *M. oleifera* seed has some obvious therapeutic implications against Protein Energy Malnutrition, calcium homeostasis dysfunction and their prevention in skeletal muscle degeneration of protein energy malnourished animal models. Defatted *M. oleifera* seed with its multiple beneficiary properties would seem useful as an adjuvant for the prevention and/or management of skeletal muscle degeneration.

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