

Research Article

Potential for Riboflavin in minimising adverse effects associated with the use of Nucleoside Reverse Transcriptase Inhibitors

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Background: Nucleoside analogues represent the cornerstone of antiretroviral regimens but their long term use is associated with a range of deleterious effects. However due to their potency and cost effectiveness compared to other antiretroviral agents, strategies to minimise the toxicities should be explored.

Objective: To investigate the influence of riboflavin in minimising adverse effects induced by nucleoside analogue reverse transcriptase inhibitors.

Method: Adult Swiss albino mice weighing between 28-30g were orally given stavudine, lamivudine and riboflavin alone and in various dose combinations calculated on weight basis with reference to 70kg normal human dose: In absence of fatality/toxicity drugs were given at three dose levels for up to four months. Blood glucose was assessed periodically followed by animal sacrifice after four months or earlier if signs of toxicity manifested and blood collected by cardiac puncture. Lipid and haematological profiles were measured. Categorical variables were summarised using proportions, means, standard deviations and 95% upper and lower limits. One way ANOVA was used in the data analysis.

Results: Elevated blood glucose levels, prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT) accompanied by deranged lipid profiles were noted in the stavudine and stavudine plus lamivudine treated groups given at standard and double standard doses. The adipose tissue content ranged from diminished to totally depleted in stavudine and stavudine plus lamivudine treated groups given at standard and double standard dose levels respectively. Skin wounds, rashes, red eyes and pale liver were noted in mice treated with stavudine or stavudine plus lamivudine standard doses. Riboflavin was found to protect against these derangements.

Conclusion: Riboflavin has protective effect against adverse effects associated induced by stavudine and lamivudine.

Keywords: Riboflavin, toxicity, lipid profile, blood glucose, haematological changes

Received: October, 2019

Published: August, 2020

1. Introduction

Numerous antiretroviral drugs for HIV have been sanctioned since 2005 with new agents either from the

conventionals or new classes thus broadening treatment options (Bayoumi et.al, 2013). The novel regimens approved in 2005 are more cost-effective in suppressing the viral load and improving the CD4

counts but had a wide range of side effects such as HIV associated lipodystrophy (Mallal and Nolan, 2000), peripheral fat wasting, central adiposity, hyperlipidemia, and insulin resistance (Carr et al, 1999). Other complications include neuropathy, pancreatitis, microvesicular steatosis, and lactic acidosis in a few patients (Mokrzycki et al, 2000) increase the risk of fat wasting and hypertriglyceridemia (Strobel et al, 1999).

Riboflavin is reportedly deficient in people with HIV (Fouty et al, 1998) and has been used to reserve the capacity of the mitochondria (Stankov et al, 2010) and treat severe lactic acidosis in patients presenting with severe mitochondrial toxicities Fouty et al, 1998; Boxwell and Styrt, 1999). The Dutch Association of AIDS Physicians' protocol for lactate acidosis has sanctioned the utility of riboflavin (Brinkman et al, 2000). Whereas riboflavin has been used in management of patients presenting with adverse reaction to treatment, its potential has not been well researched.

The newer agents are relatively safer but costly and inaccessible by low and middle income countries (LMIC) and may deliver marginal gains in patients with multi-drug resistant virus (Bayoumi et al, 2013).

Financially the annual maintenance of patients on ART through the Global Fund is estimated at US\$ 1.7 billion by 2020 for approximately 2.3 million people. (Stover et al, 2011). Global adoption of the 2013 WHO ART eligibility guidelines in place of the 2010 ones will likely cost an additional US\$3.3 billion (Stover et al, 2014). This analysis seems limited by its use of the same unit cost for ART across all countries and its assumption of simultaneous and universal achievement of a target coverage rate. For 2020, UNAIDS estimates that the global HIV response will require US\$9.7 billion, US\$8.7 billion, and US\$17.2 billion in low-, lower-middle-, and upper-middle-income countries, respectively (Joint United Nations Programme on HIV/AIDS, 2014).

Given funding constraints for the global HIV response, it is critical to improve resource requirement and funding estimates for ART (Dutta, Barker and Kallarakal, 2015) and also reflect on strategies to improve on the safety profile of the earlier NRTIs like stavudine and lamivudine and salvage their clinical utility. The objective of this study was therefore to investigate the influence of riboflavin in minimising adverse effects induced by nucleoside analogue reverse transcriptase inhibitors represented by stavudine and lamivudine.

2. Methods

2.1 Study animals

Swiss albino mice bred under Good Animal Husbandry conditions and maintained in a clean environment with regular feeding and water *ad libitum* were used in this study. They were kept in solid bottomed cages with hardwood chips beddings for thermal insulation and absorption of faecal and urinary wastes with equal light distribution, controlled environmental noise, room temperature 18° to 24 °C and relative humidity 40% and 70%. Diet consisted of pellets (Unga Farm Care EA limited) with nutritional value of 4-5% fat and 14%

protein. Daily animal inspections to rule out ringtail and swollen and reddened feet were conducted.

2.2 Drug treatment

The experimental drug doses for stavudine, lamivudine (Cosmos Pharmaceuticals (K) Ltd) and the intervention riboflavin (Sigma-Aldrich (UK)) were based on 70kg human therapeutic dose translating to stavudine 40mg twice a day, lamivudine 150mg twice a day, riboflavin is 400mg a day. The average weight for an adult mouse was 30g translating to stavudine 0.0354mg lamivudine 0.13mg and 0.17mg riboflavin daily. These doses are herein after referred to as standard doses.

Groups of six animals each were set and placed in separate marked cages. Animal groups were clustered as stavudine only; stavudine plus lamivudine; stavudine, lamivudine plus riboflavin; stavudine plus riboflavin and riboflavin only. Three drug dose levels set at half standard dose, standard dose and double standard dose of these drugs and their combinations were used. This translated to; half standard doses (stavudine 0.57mg/kg, lamivudine 2.15mg/kg and riboflavin 2.85mg/kg). Standard doses (stavudine 1.14mg/kg, lamivudine 4.3mg/kg and riboflavin 5.7mg/kg) and double standard dose: (stavudine 2.28mg/kg, lamivudine 2.15mg/kg and riboflavin 11.4mg/kg).

The drugs and combinations were orally administered daily to the experimental mice for a maximum of four months with daily monitoring and documentation of body physical changes and other signs of toxicity.

2.3 Specimen collection and laboratory analysis

Whole blood for random blood glucose determination was collected through tail nick. Serum for lipid profile analysis was obtained from whole blood collected through cardiac puncture. Plasma triglycerides, HDL-Cholesterol and total cholesterol concentrations were measured using a lipid profile kit (Human®, Germany) supplied together with the quality control kit. The prothrombin time (PT) and the activated partial thromboplastin time (aPTT) were measured.

The random blood glucose was measured using glucometer (Softstyle®, Hungary) with internal quality control. Equipment calibration was done before sample analysis was undertaken. The random blood glucose levels were determined at three time intervals: pre-treatment, two weeks post treatment and two days before sacrifice for all groups treated with standard and double standard doses of stavudine or stavudine plus lamivudine. However, for groups on half standard doses of the drugs and their combinations, the blood glucose was assessed at intervals of one and four months. Mean group values were calculated and one-way ANOVA used to analyse data on blood glucose levels across treatment groups with 0.05 set as level of significance.

Liver tissues were harvested during animal sacrifice, weighed and slice prepared to assess for fat deposits and cell morphology.

2.4 Ethical considerations

Ethical approval was given by Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (KNH-UON ERC) ref: **KNH-ERC/A/1**.

3. Results

After 14 days of treatment, the mice on either standard or double the standard dose of stavudine alone and its equivalent combination with lamivudine, manifested dose dependent toxicity signs characterized by swollen abdomen, enlarged pale liver, skin rashes, red eyes, swelling at the joints, belly and back and wounds on the back, joints and limbs. The wounds became septic but started healing when pus was drained. The liver weights ranged between 1.3g to 2.5g for the group and very little or complete depletion of adipose tissue. The maximum survival period of the mice in this treatment group was six weeks.

The mice on half standard dose of stavudine either alone or in equivalent combination with lamivudine did not manifest general toxicity described above. The liver

was mildly pale, slightly enlarged with weight between 1.1g and 1.7g. The adipose tissue was present.

Similarly treated groups but with riboflavin added to the regimen had significant amount of adipose tissue and liver weight was between 1.0g and 1.2g. The survival period for this group was four months. The results on liver weight are summarised in **Table 1**.

There were no fat deposits on the liver in all treatment groups. The liver from the mice treated with standard dose of stavudine and lamivudine were diffusely enlarged, distinctively pale colour, rounded edges and an accentuated reticulated pattern filling up to one-third of the abdominal cavity and mildly displacing other abdominal organs. This contrasts with the population treated with riboflavin only in which the liver was comparable to that from negative control (**Figure 1A** and **1B**). The liver cell morphology indicated pathology in all treated groups except where riboflavin was included in the drug combinations.

Table 1: Mean weight of liver across all treatment groups

Treatment group	Mean liver weight(g)(SD)		
	Half Standard Dose	Standard Dose	Double Standard Dose
d4T	1.54 (0.18)	1.60 (0.11)	2.00 (1.30)
d4T+3TC	1.20 (0.12)	1.60 (0.14)	1.70 (0.10)
d4T+3TC+R	1.25 (0.10)	1.30 (0.10)	1.50 (0.08)
d4T+R	1.10 (0.13)	1.40 (0.30)	1.50 (0.02)
R	1.01 (0.07)	1.06 (0.09)	1.08 (0.08)
Untreated Control		1.05 (0.09)	

Index: d4T – Stavudine, 3TC – Lamivudine, R – Riboflavin, SD – standard deviation, g – gram. Standard deviation is indicated in parenthesis

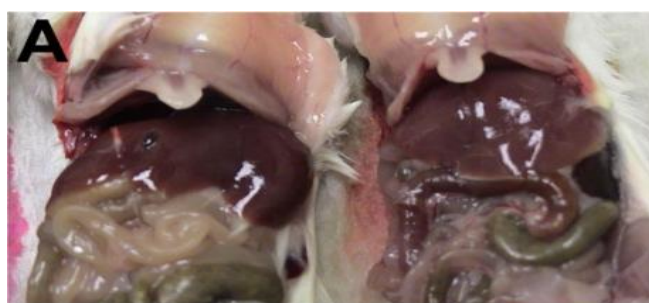


Figure 1A: Liver from untreated control mice. (Appearance; normal in size and colour.)

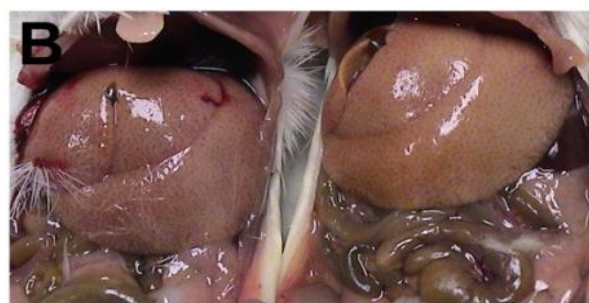


Figure 1B: Enlarged pale liver from mice treated with a daily combination of standard dose of stavudine and lamivudine for two weeks

There was a statistically significant in blood glucose levels between stavudine alone and riboflavin alone ($p=0.00$) as well as between stavudine plus lamivudine at half standard dose compared to riboflavin only group ($p=0.000$). There was no statistically significant

difference between the blood glucose levels of stavudine, lamivudine plus riboflavin group compared to riboflavin only group all given at half standard doses ($p=0.548$) (**Figure 2**).

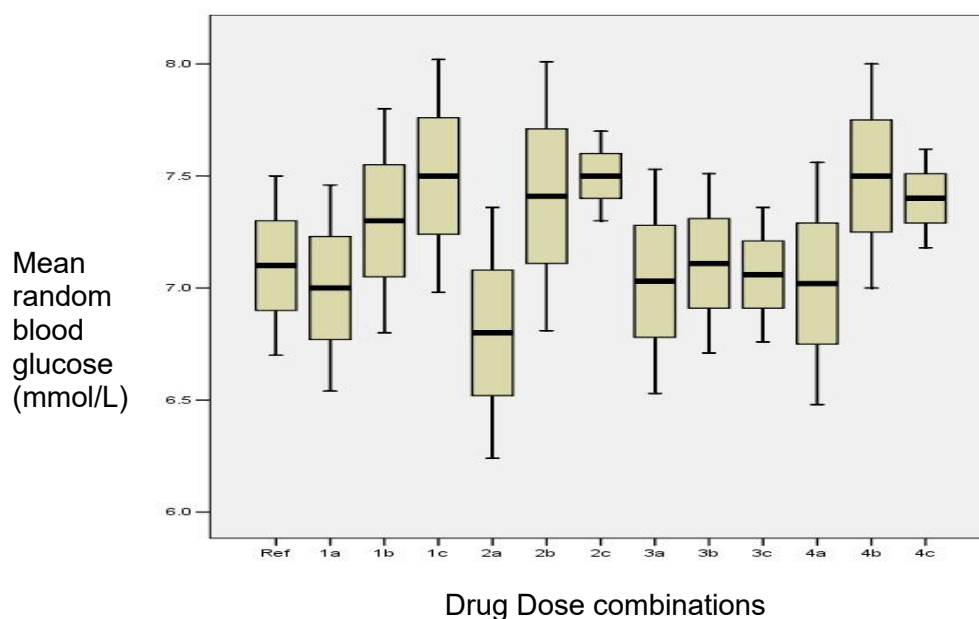


Figure 2: Mean (2SD) of random blood glucose concentration for drugs at half standard dose levels (a= at baseline; b= after 1 month; c= after 4 months. 1= stavudine; 2= stavudine and lamivudine; 3= stavudine, lamivudine and riboflavin; 4= stavudine and riboflavin).

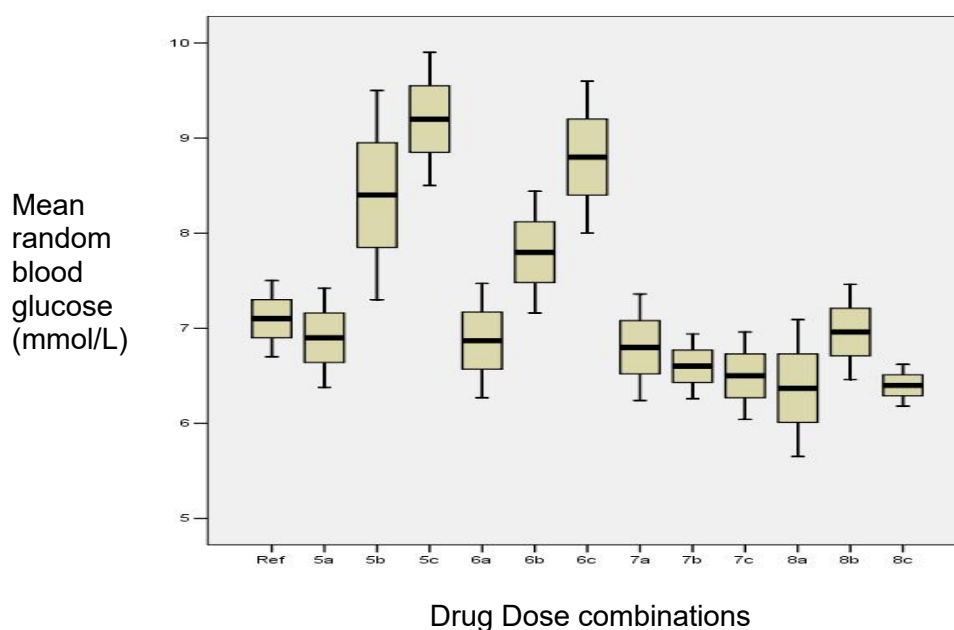


Figure 3: Mean (2SD) of blood glucose concentration for standard doses of drugs (a= at baseline; b= after 2 weeks; c= after 4 weeks. 5= stavudine; 6= stavudine plus lamivudine; 7= stavudine, lamivudine plus riboflavin; 8= stavudine plus riboflavin).

The blood glucose concentration results for groups treated with stavudine, and stavudine plus lamivudine; and stavudine, lamivudine plus riboflavin at standard dose levels were correlated with the group treated with riboflavin only. The stavudine and stavudine plus lamivudine groups at standard dose level had statistical

significance with $p = 0.011$ and 0.004 respectively. There was no statistical significance for stavudine, lamivudine plus riboflavin; and the stavudine plus riboflavin standard dose level group ($p = 0.60$ and 0.239 respectively) (**Figure 3**).

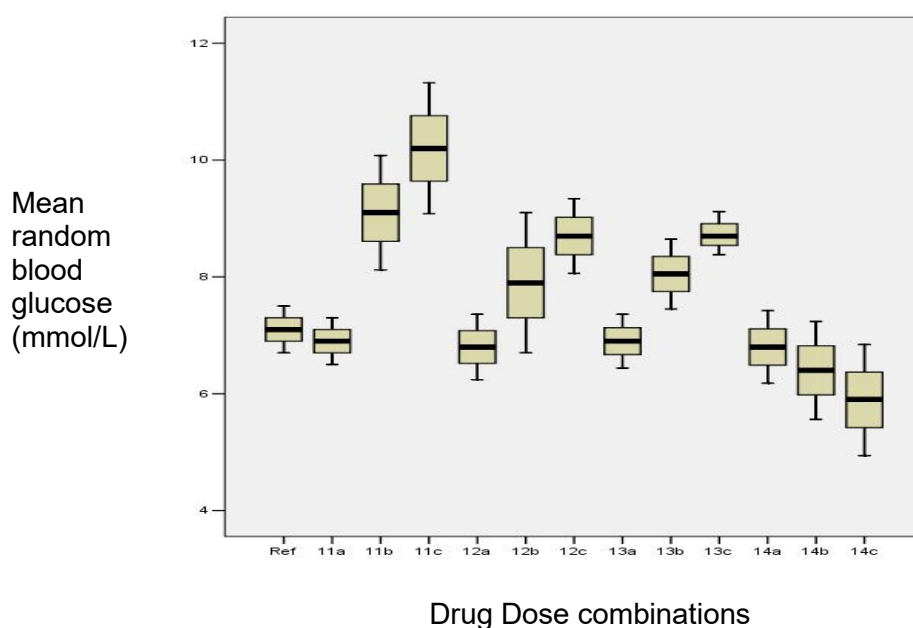


Figure 4: Mean (2SD) blood glucose concentration for double standard doses of drugs (a= at baseline; b= after 2 weeks; c= after 4 weeks. 11= stavudine; 12= stavudine and lamivudine; 13= stavudine, lamivudine and riboflavin; 14= stavudine and riboflavin).

Table 2: Lipid Profile, PT and aPTT values for groups treated with riboflavin only at three dose levels

Parameter	Mean(SD)		
	Half standard dose	Standard dose	Double standard dose
Total cholesterol (mmol/L)	3.7 (0.5)	3.8 (0.4)	4.0 (0.5)
Triglycerides (mol/L)	2.6 (0.5)	2.7 (0.3)	3.1 (0.4)
HDL-C (mmol/L)	1.3 (0.7)	1.5 (0.6)	1.9 (0.5)
LDL-C (mmol/L)	1.3 (0.6)	1.1 (0.5)	1.8 (0.6)
PT (sec)	15.1 (0.4)	15.8 (0.5)	16.3 (0.7)
aPTT (sec)	46.2 (0.3)	47.0 (0.7)	47.1 (0.9)

Index: HDL-C – high density lipoprotein cholesterol, LDL-C – low density lipoprotein cholesterol, PT – prothrombin time, aPTT – activated partial thromboplastin time. Standard deviation is indicated in parenthesis

At double standard dose levels, when correlated with the group treated with riboflavin only, the stavudine only and stavudine plus lamivudine groups showed statistical significance of ($p=0.001$ and 0.004 respectively) whereas stavudine, lamivudine plus riboflavin, and stavudine plus riboflavin groups registered p values at 0.007 and 0.003 respectively. Pre-treatment blood glucose levels were within the reference ranges of $6.7 - 7.5$ mmol/L (Thaimuta et.al *in print*), but gradually increased with continued treatment except for riboflavin only treated groups which maintained normal levels (**Figure 4**).

All the correlations for the treatment groups were statistically significant except for stavudine plus riboflavin, and stavudine, lamivudine plus riboflavin groups at half standard dose levels. Addition of riboflavin positively moderated the blood glucose levels. Groups treated with standard doses of stavudine only and stavudine and lamivudine combination registered a rise in blood glucose whereas a decrease was observed in riboflavin only standard dose group that had levels comparable those from groups treated with half

standard dose combinations of stavudine-riboflavin and stavudine-lamivudine plus riboflavin.

There was decreased total cholesterol and triglycerides in the groups treated with stavudine, lamivudine plus riboflavin; and stavudine plus riboflavin at half standard dose combinations. Statistically significant difference between total cholesterol and triglycerides was shown in the stavudine plus riboflavin group ($p=0.034$ and 0.02 respectively) but not for stavudine, lamivudine plus riboflavin ($p=0.38$ and 0.228 respectively).

Standard dose of stavudine plus riboflavin induced elevated HDL-cholesterol and low levels of triglycerides ($p=0.038$ and 0.125 , respectively). The lipid profile for the group treated with riboflavin only (**Table 2**) was within reference ranges (Thaimuta et. al *in print*).

The cut off values for PT and aPTT were determined using control adult Swiss albino mice. These results were used to interpret PT and aPTT values obtained in the experimental animal groups. There was prolonged coagulation time as expressed by both PT and aPTT

($p=0.139$ and 0.093 respectively in all groups treated with stavudine only and stavudine plus lamivudine half standard dose levels. The group on stavudine, lamivudine plus riboflavin combination at half standard dose reported prolonged PT and aPTT ($p=0.139$ and 0.093) respectively. The aPTT was significantly prolonged for the group on half standard dose of stavudine plus riboflavin.

Levels of statistical significance for groups treated with standard doses were; Stavudine only, [significantly prolonged coagulation time for PT and aPTT ($p=0.03$ and 0.016)] respectively; stavudine plus lamivudine, [significantly prolonged PT and aPTT ($p=0.0248$ and 0.008) respectively]; stavudine, lamivudine plus riboflavin, [prolonged PT and PTT ($p=0.603$ and 0.375) respectively], stavudine plus riboflavin, [shortened coagulation time for PT and aPTT (p values 0.116 and 0.123)] respectively.

The levels of significance of PT and aPTT from animals treated with double standard doses were; Stavudine, prolonged coagulation time both PT and aPTT ($p=0.00$ both); stavudine plus lamivudine ($p=0.128$ and 0.615 respectively); stavudine, lamivudine plus riboflavin combination, prolonged PT and aPTT ($p=0.158$ and 0.221 respectively), and stavudine plus riboflavin PT and aPTT was within the reference range ($p=0.00$ for both). The PT and aPTT levels for the group treated with riboflavin only were within reference ranges (**Table 2**) (Thaimuta et. al, *in press*.)

4.0 Discussion

Antiretroviral treatment derangements such as fat depletion and adipose tissue redistribution associated particularly with thymidine analogues: stavudine and zidovudine (Gaou et al, 2001; Metzler et al, 2001) pose both psychosocial and medical challenges characterised by inferior self-image and poor adherence to treatment. This has led to decline in clinical favour for these agents posing a big blow to LMIC because the drugs are cost effective and hence accessible to the poor.

The pathogenesis of this phenomenon has been associated with mitochondrial dysfunction, impaired fatty acid metabolism, reduced activity of adiponectin, leptin resistance, decrease in growth hormone activity and effects of immune reconstitution (Leung and Glesby, 2011).

In this study, animals treated with half standard dose of stavudine and/or lamivudine did not show any sign of ill health whereas those treated with standard and double standard doses of the drugs and their combinations registered significant toxicities. However these toxicities were ameliorated by addition of riboflavin to the treatments. The groups in which riboflavin was added were found to have body weight within the normal reference ranges, adequate adipose tissue, healthy smooth skin texture, alertness and active.

Raised blood glucose levels were noted in all treatment groups except in the group treated with combination of stavudine and riboflavin. Accumulation of glucose in the blood may lead to anaerobic metabolism causing accumulation of lactic acid. The accumulation may be due to loss of liver function, pancreatitis, exocrine pancreas dysfunction (Brinkman et al, 1998) and

alteration to oxidative phosphorylation function resulting to rise in mitochondrial redox state and shift to pyruvate/lactate equilibrium (Munnich et al, 1992). Lipodystrophy and increased blood glucose levels present with a clustering of abnormalities parallel to metabolic syndrome. Metabolic syndrome significantly increases the risk of incident cardiovascular disease and mortality (Ford, 2005), with waist circumference being increasingly recognized as an independent risk factor for myocardial infarction (Gami et.al, 2007)

Low total cholesterol levels were registered for the groups treated with stavudine and stavudine plus riboflavin at standard doses.

The groups treated with stavudine and: stavudine, lamivudine plus and riboflavin at half standard doses were found to have total cholesterol and triglycerides below reference range.

Metabolic processes of adipocytes are insulin resistant, resulting from defects in the insulin signaling pathway and secreting cachectic factors like $TNF\alpha$ that promote adipocyte loss and oppose insulin action (Weiner, et.al, 1991). Besides the diabetic state, numerous global hormones such as glucagon, which promotes an increased cyclic AMP (cAMP) level in adipocytes, and glucocorticoids are abnormally elevated (Dubuc et al, 1984). Both elevated cAMP and glucocorticoid promote cell death (Loftus, Kuhajda and Lane, 1998). In this study, the mice treated with standard and double standard doses of stavudine, and those on stavudine plus lamivudine developed weight gain, hyperglycaemia and hepatomegaly.

Half standard dose of stavudine induced significant prolongation of PT and aPTT whereas similar dose of stavudine plus lamivudine showed significantly shortened PT and aPTT but the values normalized with the inclusion of riboflavin to the regimen.

The PT and aPTT were significantly prolonged for the groups on standard dose of stavudine but the values fell to within reference range after addition of riboflavin. However this protective effect of riboflavin was not seen with standard dose of stavudine plus, lamivudine.

The groups treated with double standard dose of stavudine and double standard dose combination of stavudine plus lamivudine were found to have PT and aPTT significantly prolonged. However this effect was neutralized when riboflavin was added to the combination with the values falling to within reference range.

Prothrombin time measures the quality of the extrinsic pathway (and the common pathway) of coagulation. The speed of the extrinsic pathway is greatly affected by levels of functional factor VII in the body. One of the factors leading to prolongation of prothrombin time is deficiencies in vitamin K (Fritsma, 2002).

The activated partial thromboplastin time (aPTT) test reflects the activities of most of the coagulation factors, including factor XII, pre-kallikrein [PK] and high molecular weight kininogen [HMWK]; and factors XI, IX, and VIII in the intrinsic pro-coagulant pathway, as well as coagulation factors in the common pro-coagulant pathway that include factors X, V, II and fibrinogen

(factor I). The aPTT depends on phospholipid (a partial thromboplastin), ionic calcium and an activator of the contact factors (like silica), but reflects neither the extrinsic pro-coagulant pathway that includes factor VII and tissue factor, nor the activity of factor XIII (fibrin stabilizing factor) (Miletich, 1995).

Prolongation of the aPTT can occur as a result of deficiency of one or more coagulation factors (acquired or congenital in origin), or the presence of an inhibitor of coagulation such as heparin, a lupus anticoagulant, a nonspecific inhibitor such as a monoclonal immunoglobulin, or a specific coagulation factor inhibitor (Greaves and Preston, 2001). Shortened APTT has no clinical significance (Korte et al, 2000).

5.0 Conclusion

This study has documented evidence of the protective effect of riboflavin against derangements induced by nucleoside analogues transcriptase inhibitors namely stavudine and lamivudine. Protection includes normalisation of blood glucose levels, improvement in the blood coagulation, restoration of morphological characteristics of tissues and containment of the notorious lipodystrophy induced by the nucleoside analogues during long term use. Clinical trials are recommended to establish the same.

Acknowledgements

The authors are grateful to Mr. Faustus Mmbaya of the Department of Biochemistry for providing technical support in experimental and laboratory animal handling.

Conflict of Interest declaration

The authors declare no conflict of interest.

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