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The Influence of Different Levels of Dietary Energy on Lymphocyte Proliferation in *Trypanosoma congolense* Infected West African Dwarf Goats

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Abstract: West African Dwarf (WAD) goats were placed on three different levels of dietary energy (A1 = low; B1 = medium; C1 = high) and subsequently infected with a virulent strain of *Trypanosoma congolense* (*Tc*). The influence of these three different levels of dietary energy on parasitaemia and lymphocyte proliferation were evaluated up to 5 weeks post infection (p. i). The respective high levels of parasitaemia and lymphocyte proliferations were significant ($P < 0.05$) and least pronounced in group A than B or C. Mean lymphocyte count per minute within stimulated infected groups from weeks 1-5 were significantly increased in the order of CI > BI > AI, while unstimulated control cells maintained uniform response over a 72-hour period. Increased dietary energy intake in goats increased their tolerance to *Tc* infection vis-à-vis increased stimulated lymphocyte. It is recommended that dietary energy along with other nutrients be adequately provided for goats to reduce clinical effects of trypanosomosis.

Key words: Dietary Energy · Lymphocyte Proliferation · Trypanosoma Congolense · WAD Goats

INTRODUCTION

The immune system is crucial for the defense against organisms that cause infections and against toxic products that may be released from the infectious agents. A functional immune response requires rapid and extensive cell growth, proliferation and production of effector proteins. A defect in any single component of the immune system can cause a breakdown in this defense and may lead to serious or fatal diseases such as infections, cancers, or autoimmune disorders. In addition, a growing body of evidence suggests that excess inflammation decreases longevity [1, 2] therefore, fine regulation of this system is required to maintain health.

Lymphocytes are part of the adaptive immune response and as such, are crucial for normal immune functions. T or B cell deficiencies are known to result in severe immunodeficiency [3]. T cells use glucose and glutamine as their primary fuel source [4], although ketone bodies and fatty acids can also be used to a small degree. Of these nutrients, glucose appears to be particularly necessary for cell survival, size, activation and cytokine production [5]. Glucose provides much-needed energy for the lymphocyte in the following ways: Glucose can serve as a primary substrate for the generation of ATP; glucose can supply a carbon source for the synthesis of other macronutrients, such as nucleic acids and phospholipids; and glucose can be metabolized by the pentose phosphate pathway to generate NADPH. For ATP generation, glucose can be metabolized via glycolysis or oxidative Phosphorylation [5]. Glycolysis occurs in the cytosol, where one molecule of glucose is broken down into two molecules of pyruvate. The oxidative phosphorylation is a seemingly more efficient way to generate ATP from glucose, although few metabolites remain for biosynthesis. Resting lymphocytes have low-energy needs and derive most of their ATP from oxidative phosphorylation [6] however, activated lymphocytes require a dramatic increase in metabolism upon activation. This is necessary to produce the energy required to stimulate growth and proliferation and produce the protein products expressed by activated immune cells [4, 6]. Glucose metabolism changes by orders of magnitude in an activated T cell and the transition from

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a resting to an activated T cell causes a switch from catabolic to anabolic metabolism, in which ATP is used to produce complex macromolecules from simpler intermediates [7].

Activated lymphocytes generate energy in large part by up-regulating aerobic glycolysis [4, 5, 8], which describes the metabolic program used when a cell continues to convert pyruvate to lactate, despite conditions of adequate oxygen; it is used by many types of transformed or cancer cells [9]. A failure to increase glucose metabolism during lymphocyte activation prevents cell growth [4, 5, 8]. Severe nutritional deficiencies reduces T-cell functions impairing cell mediated responses but sparing B-cell function and humoral immunity [10, 11]. Starving result rapidly in thymic atrophy, a drop in T-cell numbers produced and found in the secondary lymphoid tissues. T and B-lymphocytes are activated by different mitogens. Phytohaemaglutinin (PHA) and Concanavalin (Con. A) stimulate T lymphocyte. Lipopolysaccharide (LPS) mitogen stimulates Bcells. Pokeweed mitogen (PWM) stimulates T and B cells, [12].

There is no information on the influence of different levels of dietary energy on the lymphocyte proliferation in *Tc* infected WAD goats; therefore, this study investigated the influence of different levels of dietary energy on lymphocyte proliferation in *Tc* infected WAD goats.

MATERIALS AND METHODS

Experimental Site: The experiment was carried out at the large animal ward II, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Oyo state, Nigeria.

Experimental Animals: A total of four two (42) male West African Dwarf (WAD) goats were used; they were purchased at a local market in Ibadan and were acclimatized for four weeks at the experimental site. They were treated against worm infestation, haemoprotozoan diseases. *Pestes des petit ruminantum* (PPR) vaccine was administered to all goats. Antibiotics, antihelmintics and acaricides dips were also administered. The goats were randomly divided into three experimental groups (A1, B1 and C1) based on weight and sex. Each group contained fourteen goats. The animals were on these rations below for 4 weeks before experimental infections were carried out. This was to create the desired differences in the nutritional status of the animals before experimental infection.

Table 1: Proximate Analysis of ration feeds offered to the WAD Goats

	RATION		
Components	A1	B1	C1
Dry matter %	72.95	75.25	77.57
Crude protein %	13.17	13.26	13.35
Ether extract %	24.64	26.92	29.20
Crude fibre %	16.82	16.82	16.81
Nitrogen free extract %	62.40	54.50	45.80
Total Ash %	3.60	11.60	15.20
Cal. Gross Energ (kcal/k)	2467.29	2548.57	2670.40

Feeding of Experimental Animals: Animals in group A1, B1 and C1 were placed on low, medium and high plane rations respectively based on different dietary energy but isonitrogenous (the crude protein levels were the same). The proximate compositions of the feeds were determined as given in Table 1 [13]. Gross energy values of the feeds were estimated with Gallenkamp ballistic bomb calorimeter. Daily feed offered was based maintenance ration at 4.0% of their body weights [14] and were given in two split doses at 008h and 016h daily and water was provided ad libitum.

Infection with Trypanosomes: The *Tc* parasites used in this experiment was obtained from the National Institute for Trypanosomosis Research (NITR), Vom, Nigeria. *Tc* (Binchi Bassa Strain) was obtained and subjected to six passages in albino mice. The goats were inoculated intraperitoneally with equal number of *Tc* at the rate of 1.0×10^{6}/ ml in sterile normal saline. This experiment lasted for a period of 5 weeks.

Blood Collection Techniques: Five millilitre of blood was taken weekly from the jugular veins of each goats and dispensed inside bijou bottles containing ethylene diamine tetra acetate (EDTA) as anticoagulants.

Separation and Isolation of Trypanosomes from Blood: Trypanosomes were separated and isolated from blood by the modification of method described by [14] (Lanham, 1968). The blood from infected albino rats was centrifuged at 650g for 10 min. at 4^{0C} on a swinging bucket rotor to separate a large part of the components from trypanosomes before purification. The plasma was removed by aspiration of the upper supernatant fluid. The underlying whitish trypanosomal layer was removed with a Pasteur pipette and then resuspended with an equal volume of Phosphoric sodium chloride glucose (PSG) buffer (58mM Na2HPO4,3mM NaH2PO4, 43.6 m

Mnacl, 10mM glucose PH 8.0). Contaminants were then eliminated by further purification by ion-exchange chromatography on (DEAE)-cellulose which had been previously equilibrated with PSG buffer [15]. The purified trypanosomes were eluted with the above buffer and collected when the eluate was milky-coloured. Contamination and counting of trypanosomes were routinely checked under a microscope using an improved Neubauer haemocytometer with a silvered stage. The purified trypanosomes were then pelleted at 600g in a Sorval Rc-5B centrifuge at 4^{0C} using the SS-34 rotor.

Collection from Infected Experimental Animals: 5mls of blood were collected through the Jugular venipuncture of 14 infected WAD goats from Groups A1, B1 and C1 into BD vacutainer CPT TM tube (BD Bioscience) and properly shaken to prevent coagulation of the blood sample. Parasitaemia was determined by haemocytometry described by [16] and scored by [17].

Peripheral Blood Mononuclear Cells (Pbmcs) Isolation: Blood samples collected from infected goats were taken to the laboratory for analysis. The protocol used for the isolation of the Caprine PBMC was the general protocol used for the isolation of human PBMC [18] but with some modifications to suit caprine PBMCs. Briefly, for the isolation of PBMC, each blood samples was diluted with RPMI 1640 in the ratio of 1:1 and then layered gently on 3ml of Ficoll paqueTM with the aid of 10ml sterile pipette and centrifuged at 1800 × g (2800rpm) on a Sorvall RT 6000 centrifuge) for 20minutes at room temperature. After centrifugation the CPT ™tubes were brought to a biological safety cabinet and their tops were carefully opened. The peripheral Blood Mononuclear Cells (PBMCs) were aspirated, transferred to a new 50ml sterile polypropylene conical tube (BD science) and washed 3 times with RPMI 1640. The optimal revolution per minute for washing was determined by varying the speed used between 500rpma and 2000rpm and the time spent for each wash. The optimal revolution per minute was 1000rpm for 5minutes and this was used for the wash. The cells were then resuspended in 10ml of complete medium (RPMI 1640 medium supplemented with L-glutamine, Hepes, Penicilin, Streptomycin and Heat-inactivated fetal bovine serum). 10µl of the cell suspension was added to 10µl of trypan blue (0.4% solution Sigma) in order to determine cell viability and the cells were counted using haemocytometer. Cells were retained for proliferation assay.

Lymphocyte Proliferation Assay: The Cells were diluted to a concentration of $3x10^5$cells/ml and proliferation assays were carried out in triplicate at 37^{0C} in a humidified atmosphere of 5% $C0_2$ in air for 24hours. Each well (total volume of 100µl) contained complete medium with PBMC added at a final concentration of $3 x10^5$cells/ml.

Antigen Stimulation of Lymphocytes: The *Tc* extract (antigen) was allowed to thaw at 4^{0C} and serially diluted 1:100-1:6400 in complete medium and these various dilutions were used to stimulate the PBMC. For the positive control, Phytohaemaglutinin was used at a concentration of 3µl/ml to stimulate the cells and the negative controls were unstimulated cells (i.e. had no antigen added at all). This was incubated at 37^{0C} in a humid atmosphere of 5% $C0_2$ in air for 24hours. 20µl of Alamar Blue dye was added into each well to indirectly measure the proliferation of the cells after the 24hour incubation period and then incubated for another 24hours. Absorbance was measured at 570nm and 600nm with an ELISA plate reader. Alamar Blue assay was developed as a non-radioactive lymphocyte proliferation assay that indirectly measures cell proliferation [19]. The dye is added in an oxidized form (blue colour) and is reduced (red colour) with cell proliferation.

Statistical Analysis: Data collected on parasitaemia, live and dead lymphocytes ratio, Optical density of stimulated, non stimulated lymphocytes and microscopic lymphocyte counts up to 72 hours after stimulation were analyzed by one way analysis of variance and the means were separated using Duncan Multiple Test and significant difference. Results are presented as Means± standard error (SE).

RESULTS

Parasitaemia: Parasitaemia was first detected at week 1 post infection (p. i.) in all the groups with log parasitaemia of 4.0 ± 0.13, 3.6 ± 0.05 and 3.2 ± 0.02 in all infected animals on low (A1), medium (B1) and high (C1) levels of dietary energy respectively. The lowest parasitaemia level was found in the goats placed on a high (C1) level of diet, while the highest level was observed in goats on the low (A1) level of dietary energy as revealed by examination of the buffy coat. The parasitaemia persisted with the peak parasitaemia occurring on week 5 post infections (Table 2).

Table 2: The trypanosomal parasitaemia in goats fed three different levels of dietary energy: Parasitaemia ($\log_{10}$)Trypanosomes ml^{-} blood.

Week (post infection)	Low plane (A1)	Medium plane (B1)	High plane (C1)
1	4.0±0.13	3.6±0.05	3.2±0.02
2	2.9±0.02	3.0±0.04	2.7±0.05
3	6.8±0.02 [a]	6.2±0.03 [b]	6.0±0.03[c]
4	8.0±0.05 [a]	7.0±0.02 [b]	6.8±0.03[c]
5	8.5±0.03 [a]	8.1±0.03 [b]	7.6±0.02[c]

Values are expressed as means±standard errors of mean. a,b,c means in the same column followed by different superscript differ significantly ($p<0.05$)

Table 3: The effect of *Tc* infection on Mean ±S.E Live and dead lymphocytes(%)WAD goats at different levels of dietary energy.

	Live-lymphocytes			Dead-lymphocytes		
Week	A1	B1	C1	A1	B1	C1
1	82.67 ±0.67 [a]	86.50 ±.56 [b]	89.33 ±0.42 [c]	17.33 ±0.67[a]	13.50 ±0.56 [b]	10.67 ±0.42 [c]
2	76.50 ±0.56 [a]	81.83 ±0.65 [b]	84.00 ±0.37 [c]	23.5 ±0.56 [a]	18.17 ±0.65 [b]	16.00 ±0.37 [c]
3	73.67 ±0.43 [a]	78.83 ±0.40 [b]	81.17 ±0.40 [c]	26.33 ±0.4 [a]	21.17 ±0.33 [b]	18.83 ±0.40 [c]
4	73.67 ±0.33 [a]	78.67 ±0.33 [b]	81.17 ±0.40 [c]	26.33 ±0.4 [a]	21.33 ±0.50 [b]	18.83 ±0.21 [c]
5	72.67 ±0.50 [a]	76.50 ±0.50 [b]	79.69 ±0.21 [c]	27.33 ±0.3 [a]	23.50 ±0.50 [b]	20.31 ±0.75 [c]

a,b,c means in the same row followed by different superscript differ significantly ($p<0.05$)in infected A1, B1and C1

Table 4: The effect of *Tc* infection on Mean ±S.E OD of non-stimulated lymphocytes before and after incubation of WAD goats at different levels of dietary energy.

	OD of non-stimulated lymphocyte before incubation			OD of non-stimulated lymphocyte after incubation		
Week	A1	B1	C1	A1	B1	C1
1	0.40±0.01	0.40±0.02	0.42±0.01	0.40±0.01	0.40±0.01	0.42±0.02
2	0.38±0.01	0.40±0.01	0.41±0.02	0.38±0.01	0.40±0.02	0.42±0.02
3	0.35±0.02	0.38±0.03	0.39±0.03	0.34±0.02	0.39±0.03	0.40±0.02
4	0.34±0.01	0.35±0.02	0.39±0.02	0.35±0.08	0.36±0.02	0.40±0.03
5	0.33±0.04	0.32±0.02	0.38±0.05	0.34±0.02	0.33±0.02	0.39±0.04

Table 5: The effect of *Tc* infection on Mean ±S.E OD of stimulated lymphocytes before and after incubation of WAD goats at different levels of dietary energy.

	OD of PHA stimulated lymphocytes before incubation			OD of PHA stimulated lymphocytes after incubation		
Week	A1	B1	C1	A1	B1	C1
1	0.42±0.01	0.43±0.01	0.44±0.02	0.75±0.01	0.78±0.02	0.79±0.01
2	0.39±0.01	0.41±0.02	0.42±0.02	0.67±0.02	0.75±0.03	0.77±0.01
3	0.36±0.02	0.39±0.01	0.39±0.02	0.53±0.03	0.72±0.04	0.74±0.03
4	0.36±0.02	0.37±0.01	0.39±0.04	0.50±0.03	0.71±0.02	0.74±0.03
5	0.34±0.02	0.35±0.02	0.39±0.02	0.45±0.04	0.59±0.03	0.70±0.04

Table 6: The effect of *Tc* infection on Microscopic cell counts Mean ±S.E of PHA-P stimulated lymphocytes before and after incubation of WAD goats at different levels of dietary energy.

		Post-infection period			
Week	GROUPS	0HR MLCPM $\times10^3$	24HRS MLCPM $\times10^3$	48HRS MLCPM $\times10^3$	72HRS MLCPM$\times10^3$
1	A1	0.8±0.02	1.2±0.02	1.4±0.01	1.5±0.02
	B1	0.8±0.04	1.6±0.03	2.1±0.02	2.1±0.03
	C1	0.8±0.03	2.0±0.02	2.7±0.01	3.0±0.05
2	A1	0.7±0.06	1.0±0.03	1.1±0.01	1.3±0.04
	B1	0.7±0.01	1.5±0.01	1.9±0.02	2.0±0.03
	C1	0.8±0.20	2.0±0.02	2.5±0.01	2.7±0.01
3	A1	0.6±0.01	0.9±0.03	1.0±0.03	1.1±0.02
	B1	0.7±0.02	1.4±0.04	1.8±0.03	1.9±0.02
	C1	0.8±0.03	1.9±0.02	2.4±0.03	2.6±0.01
4	A1	0.56±0.01	0.86±0.01	0.9±0.04	0.9±0.02
	B1	0.66±0.91	1.34±0.02	1.74±0.03	0.9±0.02
	C1	0.70±0.03	1.80±0.03	2.36±0.02	2.48±0.01
5	A1	0.50±0.01	0.76±0.02	0.80±0.04	0.90±0.03
	B1	0.66±0.01	1.30±0.01	1.70±.0.06	1.70±0.01
	C1	0.70±0.02	1.76±0.01	2.30±0.02	2.42±0.01

Table 7: The effect of *Tc* infection on Microscopic cell counts Mean ±S.E of unstimulated lymphocytes before and after incubation of WAD goats at different levels of dietary energy. Unstimulated control cells maintained a uniform response over the 3-day period between (0.4 to 0.8 mlcpm).

		Post-infection period			
Week	GROUPS	0HR MLCPM $\times 10^3$	24HRS MLCPM $\times 10^3$	48HRS MLCPM $\times 10^3$	72HRS MLCPM$\times 10^3$
1	A1	0.8±0.02	0.8±0.02	0.8±0.01	0.8±0.02
	B1	0.8±0.04	0.8±0.03	0.8±0.02	0.8±0.03
	C1	0.8±0.03	0.8±0.02	0.8±0.01	0.8±0.05
2	A1	0.7±0.06	0.7±0.03	0.7±0.01	0.7±0.04
	B1	0.7±0.01	0.7±0.01	0.7±0.02	0.7±0.03
	C1	0.8±0.20	0.8±0.02	0.8±0.01	0.7±0.01
3	A1	0.6±0.01	0.6±0.03	0.6±0.03	0.6±0.02
	B1	0.7±0.02	0.7±0.04	0.7±0.03	0.7±0.02
	C1	0.8±0.03	0.8±0.02	0.8±0.03	0.8±0.01
4	A1	0.56±0.01	0.56±0.01	0.56±0.04	0.56±0.02
	B1	0.66±0.91	0.66±0.02	0.66±0.03	0.66±0.01
	C1	0.70±0.03	0.70±0.03	0.70±0.02	0.70±0.01
5	A1	0.50±0.01	0.50±0.02	0.50±0.04	0.50±0.03
	B1	0.66±0.01	0.66±0.01	0.66±.0.06	0.67±0.01
	C1	0.70±0.02	0.70±0.01	0.70±0.02	0.70±0.01

The ratio of live and dead lymphocytes was shown in (Table 3). The live and dead lymphocytes decreased significantly ($P<0.05$) as from one week post infection in all infected groups but the decline was more pronounced in goats on low level of dietary energy.

The optical density (OD) of non stimulated lymphocytes before and after incubation did not shown any significant changes in their proliferation ($P>0.05$).although there was a significant difference ($P<0.05$) between infected A1, B1 and C1. (Table 4). The significant difference was more pronounced in goats on low dietary energy level. The OD of stimulated lymphocytes before and after incubation in all infected goats showed a significant changes in their proliferation ($P<0.05$) and the rate of proliferation was more pronounced in goats on high level of dietary energy.(Table 5). The effects of *Tc* infection on Microscopic cell counts Mean ±S.E of PHA-P stimulated and non-stimulated lymphocytes before and after incubation of WAD goats at different levels of dietary energy were significantly different ($P < 0.05$) (Table 6 and 7).

DISCUSSION

The results obtained from this study indicated that dietary energy had a marked influence on parasitaemia as well as lymphocyte proliferation in all goats infected with Tc. The different levels of dietary energy influenced the susceptibility of goats to Tc infection and the infected goats in group A fed low levels of dietary energy had significant high parasitaemia and low lymphocyte proliferation than infected goats in group B and C fed medium and high levels of dietary energy. The groups of infected goats fed different levels of dietary energy became parasitaemic at the same time during infection period. It was observed that differences in dietary energy had no effect on the prepatent period to parasitaemia, this agreed with observation made by [20], who found no differences in the prepatent periods in N'Dama cows inoculated with bloodstream forms of Tc when on either a low or a high plane of nutrition. Moreover, the present study indicated that addition of alamar blue to cultured cells did not alter their viability unlike that which occurs during monitoring with trypan blue. The infection of WAD goats with Tc and on different levels of dietary energy led to a significant changes in the ratio of live-dead lymphocytes. As infection was in the progress the number of live cells declined while dead cells increased but more significant in group A than groups B and C. lymphocyte proliferation by PHA mitogen had significant changes than non stimulated lymphocytes. The significant changes might be associated with different level of dietary energy, It is well known fact that PHA is a potent polyclonal cell activator [12]. The magnitude of the proliferative response induced by PHA mitogen in this study could be attributed to the fact that the isolated cells contained both B and T-lymphocytes as well as nutritional energy. The observation that infected WAD goats on low energy diet tended to develop lower ratio in live-dead lymphocyte level and low level of lymphocytes proliferation than their better fed counterparts suggest that cellular immunity may be modulated by nutritional energy and that adequate feeding may assist in ameliorating the deleterious effect of trypanosomosis.

T cells require glucose for proliferation and survival. In the absence of glucose, T cells will not proliferate, even if adequate glutamine is present. Protein synthesis during lymphocyte growth also depends on glucose metabolism for ATP and biosynthetic substrates and thus, cells deprived of adequate glucose levels cannot produce the immune products required for effector function, such as IFN-g [6, 8, 21, 22]. Therefore, the growth, function and survival of an activated lymphocyte depend on a dramatic increase in glucose metabolism that is not simply responsive to energy demands but is directly regulated and has a profound impact on T cell survival and function. In conclusion, it was observed from this work that if goats were given adequate feed with required energy level, this will boost cellular immunity and reduce the susceptibility to infection.

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