ORIGINAL ARTICLE

Cyanocobalamin Supresses The Stress Induced Apoptosis in Immune Organs

Abdul Hafeez Dall[*], Ramesh Kumar Tanwani[**], Anjum Naqvi[***], Muhammad Rahib Jamali[****]

ABSTRACT

Objective: The present study has been designed to observe the heat induced changes with Immunohistological markers on splenic tissue of albino rats and the anti - apoptotic role offered by the Cyanocobalamin (Vitamin B12).

Study Design: Experimental study.

Place & Duration: Department of Anatomy, BMSI.JPMC, Karachi from November 2011 to February 2012.

Material & Methods: A total of 45 male albino rats between 60 – 90 days were selected and divided into control A (Subgroups A1,A2,A3), heat-induced B (Subgroups B1,B2 .B3) and protective C (Subgroups C1,C2,C3) groups. Group B animals received heat-stress between 42° C, for 6 hours daily for the 2, 4 and 6 weeks according to the time duration of the subgroups. Group C animals receive protection with Cyanocobalamin (BETOLVEX) 0.8 mg/ kg and heat – induction same as in group B animals for the 2,4 and 6 weeks according to the time duration of the subgroups. Animals then sacrificed and spleens were removed. Formalin fixed paraffin – embedded, splenic tissue sections were obtained on Poly-L – Lysine coated glass slides. Antigens retrieved by HIER techniques and stained with immunostains anti - CD3 and anti- CD 79a for the precise localization of the T and B lymphocytes within all compartments of the splenic white pulp. Five micron thick hematoxylin eosin sections were also prepared and diameter of the white pulp was measured along its maximum size at two perpendicular axes.

Results: Immunostained sections of spleen with anti CD3 and anti CD79a shows a highly remarkable hypocellularity with apoptosis and necrosis of T and B lymphocytes in all compartments of the white pulp in heat induced group B animals. Marked changes seen in subgroups B2 and B3. This decrease cellularity reflects on the size of all compartments of the white pulp when measured in H/E sections, and they significantly decrease in size compare to the animals of the control group A. Whereas the microscopy and measurement of the Immunostained and H/E sections respectively of the group C animals shows a comparable size and cellularity with the animals of the control group A and significantly increase in size and cellularity compared to the group B animals.

Conclusion: Findings of the current experimental study suggest that Cyanocobalamin has substantial immunomodulatory and anti – apoptotic effect in heat –stressed immune organs. In the current wave of the global warming the prophylactic use of the Cyanocobalamin (Vitamin B12) stress induced apoptosis and its consequent morbidity and mortality.

Key Words: Cyanocobalamin, Spleen, Stress, Apoptosis, Anti –CD3, Anti – CD79a.

* *Associate Professor, Deptt. of Anatomy PUMHSW, Nawabshah*

** *Assistant Professor, Deptt. of Pharmacology PUMHSW, Nawabshah*

*** *Associate Professor & Head, Deptt. of Anatomy BMSI, JPMC, Karachi*

**** *Lecturer, Deptt. of Anatomy PUMHSW, Nawabshah*

Correspondence to:

Dr. Abdul Hafiz Dall
Associate Professor,
Depatment of Anatomy
PUMHSW, Nawabshah
Cell: 0301-3558440

INTRODUCTION:

Stress affects our daily lives[1]. Among stressful conditions are also included, intense heat and cold, trauma of any type, infection, surgery and any debilitating disease[2]. Most of the stresses activate counter actions at molecular, cellular or systemic levels that tend to restore the previous state[3]. Response to stressful events is generally regarded as reaction of the organism to accommodate to or compensate for stress[4]. Although activation of these pathways in acute stress is necessary for adaptive processes and

survival, in chronic stress these pathways have negative physiological effects due to the prolonged exposure to catecholamine and glucocorticoid[5]. Heat stress causes the release of corticosterone and catecholamine and initiates lipid peroxidation in cells membranes[6]. The most detrimental effect of long term stress is the catabolism of structural protein through corticosterone induced gluconeogenesis[7,8]. Mashaly et al[9] reported that increase in mortality during heat stress could be due to inhibition of immune response. The apoptotic process is initiated in physiological as well as in pathophysiological conditions such as oxidative stress, irradiation and anti tumor therapy[10]. Thymus is sensitive to heat induced apoptosis, as demonstrated in the mouse, where hyperthermia was shown to accelerate the normal process of cellular differentiation, causing a decrease in the number of immature thymocytes. Similarly hyperthermia has been shown to induce apoptosis in the rat thymus[11].Oksala et al[12] reported in his experimental work on guinea pig gastric mucosa that caspase-3 is an enzyme responsible for the execution of stress-induced apoptosis. High ambient temperature depletes such antioxidants and induces oxidative stress[13]. Oxidative stability has been improved by antioxidant supplementations for foods of animal origin[6]. The substantial attention has been paid to the role of nutritional additives to minimize the effect of heat-stress[14]. Cyanocobalamin is an indispensable vitamin for sustaining life[15]. Cyanocobalamin is a nutrient necessary for normal DNA synthesis, red cell production and maintenance of the nervous system[16]. Cyanocobalamin (vitamin B12) is exclusively synthesized by bacteria[17] and human being depends on exogenous sources[18]. In the current study spleen is selected among the immune organs as its architecture is similar across species.

MATERIAL & METHODS:

The present study was conducted in the Department of anatomy BMSI, JPMC, Karachi. Forty five male adult albino rats were used in this experimental study. Selected animals were randomly divided into (A) control, (B) heat-induced and (C) Cyanocobalamin treated groups. Each group was further subdivided into three subgroups, A1, A2, A3, B1, B2, B3, C1, C2,and C3 based on the period of the treatment, that was two, four and six weeks respectively, where as each group comprises of 5 animals. Group C animals were protected with Cyanocobalamin (BETOLVEX) manufactured by Alpharma Aps, Denmark at the dosage of 0.8 mg/ Kg of body weight intraperitonealy, two hours before heat–induction[19]. Then animals of group B and C were shifted into a well maintained heat room equipped with 2 double rod electric heaters of 2000 WATT. The temperature was set at 42°C[20], for six hours daily, according to their time duration. Then animals were sacrificed by inhalational anesthetic agent ether. Spleens were removed and fixed in alcoholic formalin for 24 hours. Then tissue were processed in ascending strengths of alcohol from 70 to 100%, cleared in xylene, infiltrated and embedded in paraffin. For immunocytochemistry 4 micron thick sections were affixed on polysine coated glass slides. Endogenous peroxidase activity was quenched with 5% Hydrogen peroxide. Antigen retrieval was achieved by HIER technique in pressure cooker (Kitchen king Telfon Metal ware, Industries; GRW.IRP) slides were heated for 20 minutes in 1o m citrate buffer; pH 6.0 (Ap -9003 -500,thermoscientific;UK) Non specific binding sites were blocked with protein blocking agent (ab.6426 - abcam UK).Then one set of tissue slides incubated with primary antibody, the Rabbit monoclonal anti - CD79a,prediluted (ab-27607, abcam ,UK) and a second set of tissue slides incubated with another primary antibody the Rabbit polyclonal anti.CD3 (ab - 5960, abcam, UK)21,22 for 30 minutes at room temperature. Section then washed and incubated with secondary antibody, the bictinylated goat antirabbit Ig G (ab - 64261, abcam UK) for 10 minutes at room temperature. A complex of streptavidin peroxidase with 3.3'diaminobenzidine (ab - 64261.abcam, UK) as a chromogen substrate was used for detecting antibody binding sites. Finally sections were counterstained with Meyer's hematoxylin Five micron thick hematoxylin eosin sections were also

prepared and diameter of the white pulp was measured along its maximum size at two perpendicular axes with stage micrometer and reticule.

RESULTS: WHITE PULP MEASUREMENT- The mean value of white pulp diameter (µm) in subgroups A-1, A-2 and A-3 were 591.80 ± 3.61, 592.82 ± 2.81 and 591.20 ± 3.39 respectively (Table-1). The mean values of white pulp diameter (µm) in subgroups B-1, B-2 and B-3 were 510.00 ± 4.21, 418.20 ± 2.70 and 388.60 ± 2.90 respectively. The data showed highly significant decrease ($P<0.001$) in white pulp diameter in subgroups B1, B-2 and B-3 compared to the control subgroups A-1, A-2 and A-3 respectively (Table-1).

The mean values of white pulp diameter (µm) in subgroups C-1, C-2 and C-3 were 580.00 ± 3.20, 582.60 ± 3.85 and 582.20 ± 2.43 respectively. The data also showed a highly significant increase ($P<0.001$) in white pulp diameter compared to heat induced subgroups B-1, B-2 and B-3 respectively (Table-1).

MICROSCOPY OF IMMUNOSTAINED SECTIONS:-

Microscopy of the Immunostained annti-CD79a sections showed large sized lymphocytes wide apart from one another found in germinal centers of white pulps. They also contain a large number of tangible body macrophages laden with cytoplasmic engulfed apoptotic fragments of dead B- Lymphocytes giving "moth eaten" appearance.

Table-I: *Mean Diameter of White Pulp (um) of Spleen in Different Groups of Albino Rats at Variable Time Intervals

Group	Sub-groups	Treatment Given	Diameter of White Pulp		
			2nd week	4th week	6th weeks
A (n=15)	A1 (n=5)	Control	591.80 ±3.61		
	A2 (n=5)			592.82 ± 2.81	
	A3 (n=5)				591.20 ± 3.39
B (n=15)	B1 (n=5)	Heat	510.00 ± 4.21		
	B2 (n=5)			418.20 ± 2.70	
	B3 (n=5)				398.60 ± 2.90
C (n=15)	C1 (n=5)	Heat+Cyano -cobalamin	580.00 ± 3.20		
	C2 (n=5)			582.60 ± 3.85	
	C3 (n=5)				582.20 ± 2.43

*Mean ± SEM

A large number of free apoptotic fragments of dead cells also found in all compartments of white pulp (Fig-1-A).These cellular alterations reflect the marked shrinkage of germinal center, mantle and marginal zones of white pulp and they are more pronounced in heat treated subgroups. B-2 and B-3, Microscopy of the Immunostained anti-CD3 sections of the subgroups B-2 and B-3 showed marked changes in the periarteriolar lymphoid sheath (PALS), the T-cell rich zone, and they are same as in the other compartments of the white pulp, but the T-Lymphocytes are more affected than B-Lymphocytes in heat stress and this reflect the marked shrinkage of the T-cell rich PALS zones compared to the other compartments of the white pulp. Microscopy of the Immunostained anti-CD79a sections of Cyanocobalamin protected animals in subgroups C-1, C-2 and C-3 showed few scattered foci of tangible body macrophages and apoptotic fragments. The size and cellularity of white pulp and its compartments returned near to control subgroups A-1, A-2 and, A-3, (Fig-2-A). In Immunostained anti-CD3 sections of subgroups C1, C2 and C3, T-Lymphocyte population and size of the periarteriolar lymphoid sheath returned to thickness comparable to control subgroups A-1, A-2 and A-3 and significantly improved compared to the heat stress induced subgroups B-1,B-2 and B-3 (Fig-2-B).

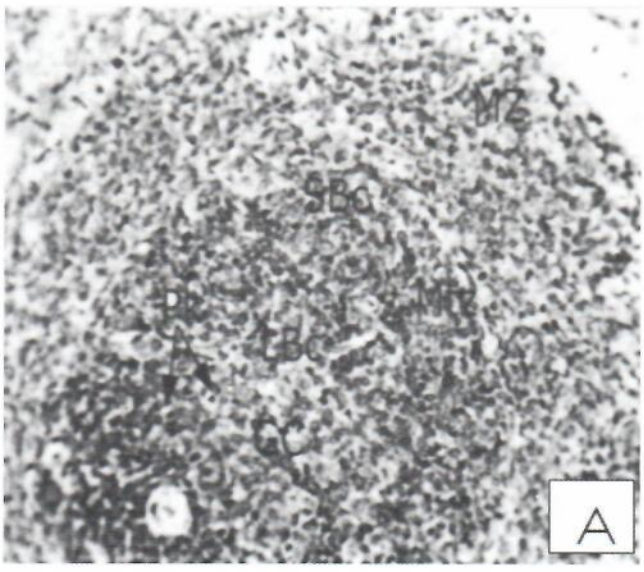

Fig. 1-A

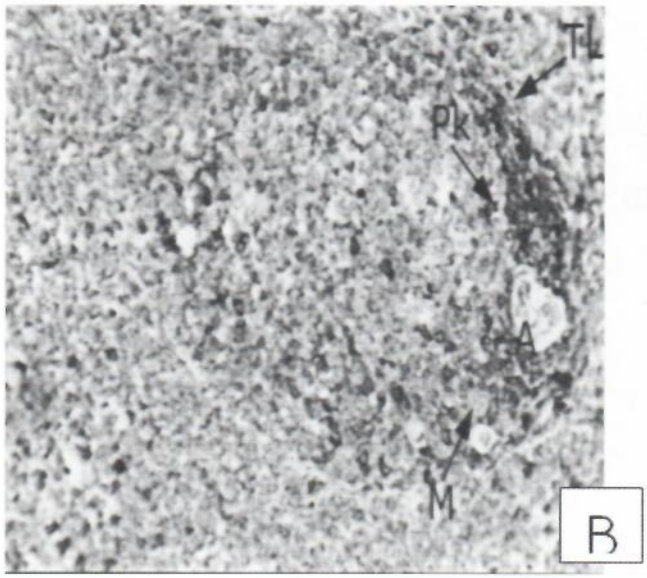

Fig. 1-B

Figure-1-A and B:-Immunostained anti-CD79a and antiCD3, 4 µm thick paraffin section of splenic white pulp, after 6 weeks of heat treatment, showing a moderate decrease in (LBc) large B-lymphocytes, (GC) in germinal center, (SBc) small B-lymphocytes in (Mtz) mantle zone, in (MZ) marginal zone and with few nuclei of (Pk) pyknotic cells and a more marked decrease in T-Lymphocytes around central artery(A) in periarteriolar lymphoid sheath (PALS) (Photomicrograph x400).

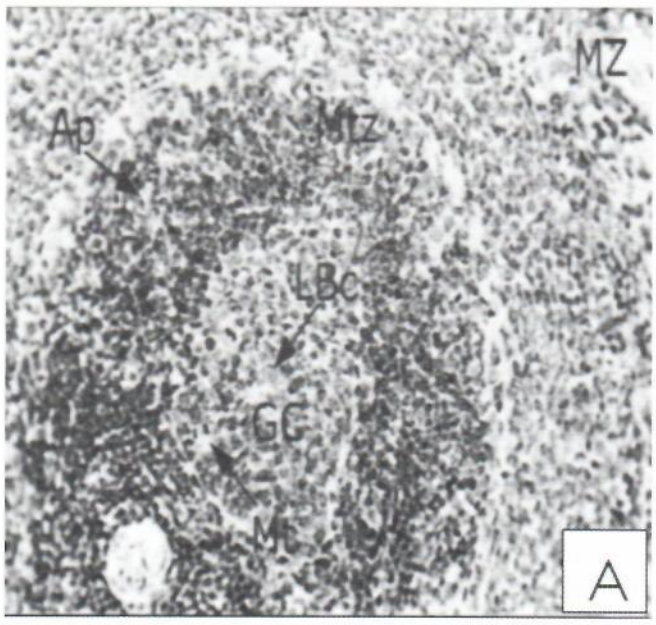

Fig. 2-A

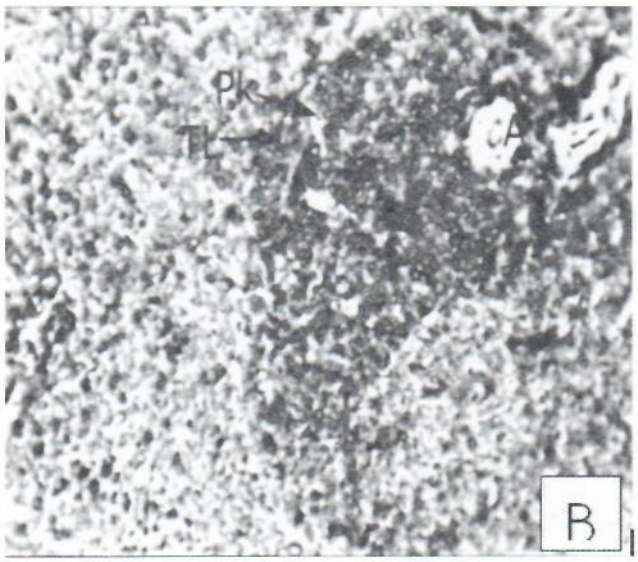

Fig. 2-B

Figure-2-A and B:-Immunostained anti-CD79a and antiCD3, 4 µm thick paraffin section of splenic white pulp, after 6 weeks of Cyanocobalamin therapy , showing a significant amount of large size B-lymphocytes (LBc) in (GC) germinal center, (Mtz) mantle zone , (MZ) marginal zone with few foci of (Mt) mitotic figures and apoptotic cells and a significant increase in T- lymphocytes (TL) around a central artery(A) in periarteriolar lymphoid sheath. (Photomicrograph x400).

DISCUSSION:

Perhaps the most significant finding in the present study is the remarkable degree of loss of B-and T-cells in spleens of heat-induced animals of B group and an equal restoration in Cyanocobalamin treated models. In spleens the loss of B-lymphocytes concentrated in lymphoid follicles and marginal zones was observed equal to the loss of T-cells associated with periarteriolar lymphoid sheaths (PALS). Kerans et al[23] reported that hyperthermia mainly affects the T-lymphocytes. In an experimental study Bloom et al[24] reported that B-cells are more resistant to apoptosis due to increases in the levels of Bcl-2 proteins. The possible mechanism involves impairment of mitochondrial homeostatic regulation by the pore complexes leading to the release of apoptogenic proteins. Van der Zee et al[25] reported that hyperthermia-artificial raising of temperature to 40-45°C is an effective method of killing cells, especially for cells in hypoxic, nutrient deprived and low-pH environments. Khan and Brown[11] suggested that cells which are in high turnover state, are programmed for apoptosis and thus easily activate this mode of cell death in response to lethal stimuli. The heat-stress activate protein kinase-C Jun and –Terminal Kinase pathway. This in turns triggers activation of the caspases cascade, which target several proteins, to bring about apoptotic cell death. Heat has also induces changes such as a drop in mitochondrial membrane potential and release of cytochrome C, which activates caspases-9 and in turn caspases-3. Roberts et al[26] demonstrated widespread apoptosis with disturbed architecture of spleen in his study on a baboon model to evaluate the effects of heat stroke. They detected active caspase-3 in splenic tissue and established apoptosis as a cause of cell death in heat stroke. The findings of the present study were also similar to Sakaguchi et al[27], who also observed similar findings in spleen of rat models in his study on apoptosis in tumor and normal tissues induced by whole body hyperthermia. Group C animal's morphology showed that cellularity and size of the spleen and its microscopic Immunoarchitecture returns near to the animals of control group, because of the substantial protection provided by Cyanocobalamin through its growth promoting effects against the apoptosis and induction of lymphocyte proliferation as described by Guyton and Hall[2], is also in agreement with our study. Findings of present study were also similar with the study of Tamura et al[28], who observed immunomodulatory effects of Cyanocobalamin by restoring the proportion of lymphocytes. Gonong[3] explained that glucocorticoid decrease the lymphocytes by inhibiting lymphocyte mitotic activity. Their ability to reduce secretion of cytokines by inhibiting the effect of NFkB on the nucleus and reduce the secretion of the cytokine interleukin-2 leads to reduce proliferation and cells undergo apoptosis. Our findings are in accordance with Brich et al[29] who suggested in his experimental study that Cyanocobalam in

modulates the expression of certain cytokines and growth factors, it is possible that this occurs as a consequence of Cyanocobalamin modifying the activity of signaling molecules such as NFkB. Cyanocobalamin also prevents the apoptosis of cells at molecular level by inhibiting the cleavage of caspases-3 and also prevent the hydrogen peroxide mediated cell death by reacting directly with free radicals.

CONCLUSION:

Heat stress is a major problem encountered when exposed to warmer environment. Most commonly it affects the immunity of an individual. Antioxidants are currently under consideration to prevent and fight of the consequences of the heat induced stress. Cyanocobalamin (Vitamin B12) has expressed itself as an immunopotentiating agent under heat stress by restoring the architecture and cell count of immune organs. Based on these results, we suggest the use of the Cyanocobalamin as an anti-apoptotic agent in individuals who are exposed to warmer environment.

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