ORIGINAL ARTICLE

Restoration of Stress - Related Apoptosis and Altered Splenic Immunoarchitecture with Cyanocobalamin

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ABSTRACT

Objective: To evaluate role of the Cyanocobalamin on the deleterious effects of heat stress on the immunity and immune organs.

Methods: This experimental study was conducted in the department of Anatomy, Basic Medical Sciences Institute, Jinnah Postgraduate Medical Centre, Karachi from January 2013 to April 2013, adult, healthy 45 male and female Sprague-Dawley rats of (180-200 g) were obtained from Animal house of BMSI, JPMC Karachi, randomly divided in 3 groups of 15 rats in each. They further subdivided into 3 subgroups on the basis of treatment duration. Group A animals served as control. Group B animals received Heat-stress. Group C animals received Heat-stress + Cyanocobalmin. The animals were sacrificed, immune organ spleen dissected and fixed in alcoholic formalin for 24 hours. Then processed in ascending strengths of alcohol, cleared in xylene, infiltrated and embedded in paraffin. Five microns thick sections were cut on microtome, and stained with Haemotoxylin and eosin for detailed microscopy and micrometry with stage micrometer and ocular reticule.

Results: Study reveals decreased B and T- cell count, marked reduction in size of all compartments of splenic nodules with characteristic appearance of tingible body macrophages in group B animals received heat stress. Whereas animals of group C who received protection with Cyanocobalamin in addition to heat-stress shows an insignificant decrease in B and T cell count, with few macrophages and prominent mitotic figures.

Conclusion: It is concluded that Cyanocobalamin has expressed itself as an anti-heat stressor and an immunopotentiating agent under heat stress conditions for the individuals who work in warmer environment.

Key words: Global warming, Heat- stress, Immune organs, Cyanocobalamin.

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INTRODUCTION:

Predictions are rising regarding the global warming that it will cause increase in frequency and severity of heat waves including high rate of mortality until and unless strict protective measures are taken[1]. Most of the energy produced in the body during the periods of the heat stress is diverted to thermoregulatory adaptations and energy deficits induces the immunosuppression which predisposes the individuals to severe infectious diseases and high mortality rate[2]. Stress affects our daily lives[3]. Stress is defined as any change in the environment those changes or threaten to change in existing optimal steady state[4]. These changes most often lead to an adaptation, making it possible for the organism to survive in the changing conditions[5]. Recent studies revealed that heat-stress had more effects on pathophysiology of leucocytes, lymphoid organs and immune responses[6,7]. A key factor in a stress

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reaction in both animals and humans is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in a rapid increase in circulating corticotrophins (ACTH) and subseuent rise in glucocorticoids (Corticosterone in rats, cortisone in humans). Both are the stress hormones which are critical for successful adaptation[8,9]. Cells of the immune system express the receptors for glucocorticoids and catechola-mine and there signals alter the several aspects of the functions of the immune cells[10]. The apoptotic process is initiated in physiological as well as in pathophysiological conditions such as oxidative stress, irradiation and chemotherapy[11]. Caspase-3 is an enzyme responsible for the execution of stress-induced apoptosis[12]. Human needs a large number of micronutrients including vitamins, trace elements and other compounds[13]. Nutrition has important role in immune function[14]. Vitamin B12 (Cyanocobalamin) is a nutrient essential for normal DNA synthesis in every living cell, hematopoiesis, myelination and maintenance of the nervous system[15]. The concentration of antioxidant vitamins decrease with heat stress[16,17]. Antioxidant vitamins counteract the free radicals and decrease the ACTH and cortisol levels, protecting the metabolism from the effects of stress[18]. In view of the above facts we designed this study to elaborate the novel anti-stress role of the Cyanocobalamin in potent threats of the global warming.

METHODS:

The forty five growing adult male and female Sprague-Dawely rats (180-200g) were obtained from the anatomy department animal house in the institute of the BMSI, Jinnah postgraduate medical center, Karachi. The animals were kept in a well-ventilated standard laboratory condition in the experimental section of the animal house at a room temperature for a week prior to the commencement of the study. The animals were fed with balanced diet and water was provided ad libitum. Both the experimental and control animals has the free access to both rat chow and water during the experimental period.

Study design: The animals were randomly divided into 3 groups of 15 rats each and, each group further subdivided into 3 subgroups on the basis of treatment duration. Group A animals served as control. Group B animals received Heat-stress. Group C animals received Heat-stress + Cyanocobalmin.

Administration of Cyanocobalamin: Commercially available Cyanocobalamin (BETOLVEX) was obtained from Alpharma Aps, Denmark and administered to the animals of group C only, at the dosage of 0.8mg/kg intraperitoneally, 2 hours before heat-stress induction.

Heating protocol: Animals of group and B and C were shifted in another experimental room for the induction of the heat-stress with double rod electric room heaters of 2000 Watt[11]. The temperature was set at 42° C for 6 hours,[7] daily depending on duration of subgroups. The animals were sacrificed, immune organ spleen dissected and fixed in alcoholic formalin for 24 hours. Then processed in ascending strengths of alcohol, cleared in xylene, infiltrated and embedded in paraffin. Five microns thick sections were cut on microtome, and stained with Haemotoxylin and eosin for detailed microscopy and micrometry with stage micrometer and ocular reticule.

RESULTS:

This experimental study was designed to set up a heat-stressed murine model to observe the alterations in the splenic tissue and protection provided by the Cyanocobalamin. This was done by the microscopic examination and micrometry on the H/E stained sections.

Group B animals: Subgroup B-3, animals showed marked changes. Capsule showed prominent scalloped appearance. The marked "moth eaten" appearance was observed in all compartments of follicle. Tingible macrophages laden with apoptotic bodies and other cellular fragments were numerous. A large number of the pyknotic nuclei were observed (Fig-1).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) This data also showed a highly significant decrease ($P<0.001$) in B-cell

count in subgroups B-1, B-2 and B-3 compared to the control subgroups A-1, A-2 and A-3 respectively (Table-2).The data showed a moderately significant decrease ($P<0.01$) in periarteriolar lymphoid sheath (PALS) thickness in subgroups B-1, B-2 and B-3 compared to control subgroups A-1, A-2 and A-3 respectively (Table-3). The data showed a significant decrease ($P<0.05$) in T-cell count in subgroups B-1 and B-2 compared to control subgroups A-1 and A-2 respectively. There was a moderately significant decrease ($P<0.01$) in T-cell count in subgroup B-3 compared to control subgroup A-3 (Table-4).

Group C Animals: Subgroups C-2 and C-3 showed insignificant architectural changes. Capsular notches disappeared. The size and cellularity of white pulp comparable with the heat-induced subgroups B-2 and B-3. Occasional tingible body macrophages and only few pyknotic nuclei observed. Numerous mitotic figures were also observed (Fig-2). The data showed insignificant decrease ($P>0.05$) in white pulp diameter in subgroups C-1, C-2 and C-3 compared to control subgroups A-1, A-2 and A-3 respectively, and 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). The data showed an insignificant decrease ($P>0.05$) in B-cell count in subgroups C-1, C-2 and C-3 compared to control subgroups A-1, A-2 and A-3 respectively and a highly significant decrease ($P<0.001$) in B- cell count in C1, C-2 and C-3 compared to heat induced subgroups B1,B2 and B3 respectively (Table-2).The data showed an insignificant decrease ($P>0.05$) in PALS thickness in subgroups C-1, C-2 and C-3 compared to the control subgroups A-1, A-2 and A-3, a significant decrease ($P<0.05$) in subgroup C-1, a highly significant decrease ($P<0.001$) in subgroup C-2 and a moderately significant decrease ($P<0.01$) in subgroup C-3 compared with the heat-induced subgroups B-1, B-2 and B-3 respectively (Table-3). The data showed insignificant decrease ($P>0.05$) in T- cell count in PALS region in subgroups C-1, C-2 and C-3 compared with control subgroups A-1, A-2 and A-3, a significant decrease ($P<0.05$) in subgroup C-1, and moderately significant decrease ($P<0.01$) in subgroups C2 and C-3 compared to the heat-induced subgroups B1, B-2 and B-3 respectively (Table-4).

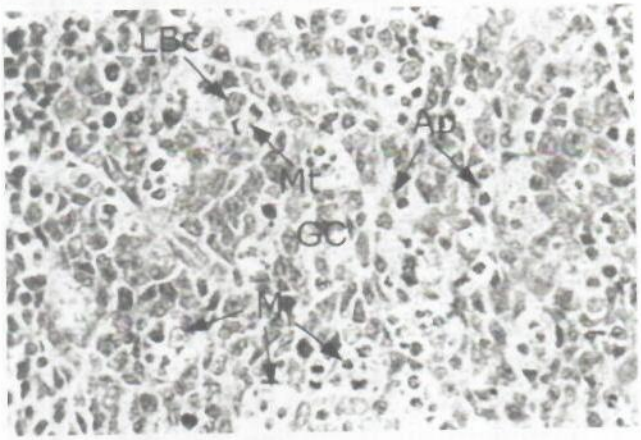

Figure-1: H&E stained section of spleen, in group B animal showing (LBc) large B-lymphocytes in the (GC) germinal center, a large number of (M) tingible body macrophages with cytoplasmic engulfed apoptotic bodies, (Ap) apoptotic cells and few (Mt) mitotic cells.

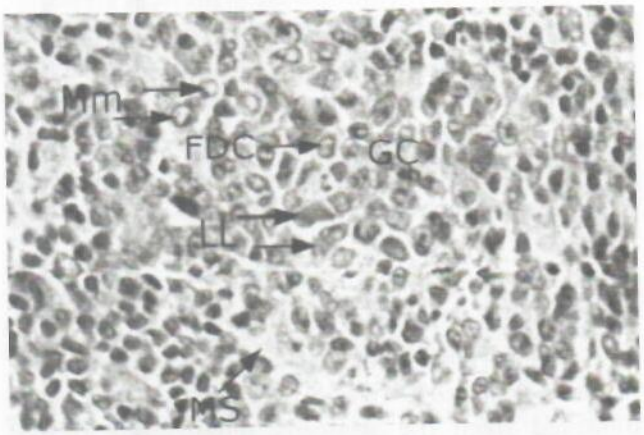

Figure-2: H&E stained section of splenic white pulp, in group C animal showing (GC) germinal center, (LL) large size lymphocytes, (FDC) nuclei of follicular dendritic cell, (MS) marginal sinus and (Mm) Tingible body macrophages macrophages.

DISCUSSION:

In the current study we investigated the anti-stress role of the Cyanocobalmin on the heat-induced architecture of the immune organ, spleen.

Table-1: Mean Diameter of White Pulp (µm) of Spleen in Different Groups of Sprauge-Dawely Rats at variable time intervals.

Group	Sub group	Treatment Given	Diameter of White Pupl		
			2nd Week	4th Week	6th Week
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)			419.20+2.70	
	B3 (n=5)				398.60+2.90
C (n=15)	C1 (n=5)	Heat + Cyanocobalamin	580.00+3.20		
	C2 (n=5)			582.60+3.85	
	C3 (n=5)				582.20+2.43

*Mean+SEM

Statistical Analysis of Mean Diameter of White Pulp of Spleen in Different Groups of Sprauge-Dawely Rats.

Statistical comparison	P-value	Statistical comparison	P-value
B1 vs A1	P<0.001****	C2 vs B2	P<0.001****
C1 vs B1	P<0.001****	C2 vs A2	P>0.05*
C1 vs A1	P>0.05*	B3 vs A3	P<0.001****
B2 vs A2	P<0.001****	C3 vs B3	P<0.001****
		C3 vs A3	P>0.05*

Key: Insignificant* Significant** Moderately Significant*** Highly Significant****

Perhaps the most significant finding is the remarkable degree of loss of B-and T-cells in all compartments of the white pulp. A large number of tingible body macrophages laden with cytoplasmic engulfed fragmentations of apoptotic cells were also found throughout the white nodules. Kearns et al[19], reported that hyperthermia mainly affects the T-lymphocytes, due to impairment of mitochondrial homeostatic regulation by the pore complexes leading to the release of apoptogenic proteins. Khan and Brown[20], suggested that high turnover cells are

Table-2: Mean Number of B-Lymphocytes Count in Germinal Center (/mm²) of White Pulp in Different Groups of Sprauge-Dawely Rats at Variable Time Intervals.

Group	Sub group	Treatment Given	Diameter of White Pupl		
			2nd Week	4th Week	6th Week
A (n=15)	A1 (n=5)	Control	956.80+4.01		
	A2 (n=5)			943.80+4.22	
	A3 (n=5)				955.80+2.65
B (n=15)	B1 (n=5)	Heat	858.20+1.88		
	B2 (n=5)			803.40+3.31	
	B3 (n=5)				758.00+3.86
C (n=15)	C1 (n=5)	Heat + Cyanocobalamin	955.00+3.16		
	C2 (n=5)			947.80+4.52	
	C3 (n=5)				947.20+4.18

*Mean+SEM

Statistical Analysis of Mean Number of B-Lymphocyte Count in Germinal Center of White Pulp in Different groups of Sprauge-Dawely Rats.

Statistical comparison	P-value	Statistical comparison	P-value
B1 vs A1	P<0.001****	C2 vs B2	P<0.001****
C1 vs B1	P<0.001****	C2 vs A2	P>0.05*
C1 vs A1	P>0.05*	B3 vs A3	P<0.001****
B2 vs A2	P<0.001****	C3 vs B3	P<0.001****
		C3 vs A3	P>0.05*

Key: Insignificant* Significant** Moderately Significant*** Highly Significant****

programmed to delete by the apoptosis in response to lethal stimuli. 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. Akberian et al[6], described that splenic atrophy occurred after exposure to heat-stress, similar findings also quoted by Anwar et al[21]. Swaminathan A et al[1], demonstrated widespread apoptosis with disturbed architecture of spleen in a baboon model and detected active caspase-3 in splenic tissue. Findings of the present

Table-3: Mean Thickness of PALS (µm) Region of White Pulp in Different Groups of Sprauge-Dawely Rats at Variable Time Intervals.

Group	Sub group	Treatment Given	Diameter of White Pupl		
			2nd Week	4th Week	6th Week
A (n=15)	A1 (n=5)	Control	169.80+1.11		
	A2 (n=5)			169.80+1.98	
	A3 (n=5)				170.60+4.57
B (n=15)	B1 (n=5)	Heat	151.60+2.50		
	B2 (n=5)			152.40+2.34	
	B3 (n=5)				149.00+2.34
C (n=15)	C1 (n=5)	Heat +	166.40+2.37		
	C2 (n=5)	Cyanocobalamin		167.40+2.02	
	C3 (n=5)				168.00+0.70

*Mean+SEM

Statistical Analysis of Mean Thickness of PALS of White Pulp in different Groups of Sprauge-Dawely Rats.

Statistical comparison	P-value	Statistical comparison	P-value
B1 vs A1	P<0.01***	C2 vs B2	P<0.001****
C1 vs B1	P<0.05**	C2 vs A2	P>0.05*
C1 vs A1	P>0.05*	B3 vs A3	P<0.01****
B2 vs A2	P<0.001***	C3 vs B3	P<0.001****
		C3 vs A3	P>0.05*

Key: Insignificant* Significant** Moderately Significant*** Highly Significant****

study were also similar to Sakaguchi et al[22], observed similar findings in spleen of rat models. Elmore[23] find out apoptosis in splenic tissues of rat treated with dexamethasone. He identify apoptotic areas was marked in the B and T-cell rich zones, associated with a large number of tingible body macrophages found between intact cells, also in conformity with the results of the present study. In group C animals, the cellular details and immunoarchitecture of the spleen returns near to the control animals, because of the substantial protection provided by Cyanocobalamin through

Table-4: Mean Number of T-Lymphocytes in PALS (/mm^2) of White Pulp in Different Groups of Sprauge-Dawely Rats at Variable Time Intervals.

Group	Sub group	Treatment Given	Diameter of White Pupl		
			2nd Week	4th Week	6th Week
A (n=15)	A1 (n=5)	Control	2792.80+147.77		
	A2 (n=5)			2861.00+100.54	
	A3 (n=5)				2870.00+65.53
B (n=15)	B1 (n=5)	Heat	2241.00+135.30		
	B2 (n=5)			2198.00+102.23	
	B3 (n=5)				2076.00+89.60
C (n=15)	C1 (n=5)	Heat +	2695.00+160.9		
	C2 (n=5)	Cyanocobalamin		2751+87.91	
	C3 (n=5)				2765+70.72

*Mean+SEM

Statistical Analysis of Mean Number of T-Lymphocytes in PALS(/mm^2) of White Pulp in different Groups of Sprauge-Dawely Rats.

Statistical comparison	P-value	Statistical comparison	P-value
B1 vs A1	P<0.05**	C2 vs B2	P<0.01***
C1 vs B1	P<0.05**	C2 vs A2	P>0.05*
C1 vs A1	P>0.05*	B3 vs A3	P<0.01***
B2 vs A2	P<0.05**	C3 vs B3	P<0.001***
		C3 vs A3	P>0.05*

Key: Insignificant* Significant** Moderately Significant*** Highly Significant****

its growth promoting effects against the apoptosis and induction of lymphocyte proliferation as described by Guyton and Hall[24]. Findings of the present study were also similar with the study of Tamura et al[25], who observed immunomodulatory effects of Cyanocobalamin by restoring the proportion of lymphocytes. Ganong[4], described that glucocorticoids decreases the lymphocyte count by inhibiting the 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. Brich et al[26], suggested in his experimental study that Cyanocobalamin modifying the activity of signaling molecules such as NFkB. It also prevent the apoptosis of cells at molecular level by inhibiting the cleavage of caspases-3, again are in accordance with the findings of the present study.

CONCLUSION:

This study indicated that Cyanocobalamin supplementation during heat-stress had beneficial effects on immunoarchitecture of spleen and an enhancing effect on splenocytes and associated nursing cells of the lymphoid follicles. It is concluded from this experimental study that the Cyanocobalamin has expressed itself as an anti-heat stressor and an immunopotentiating agent under heat-stress conditions for the individuals who work in warmer environment. The result of present study is considered promising enough to warrant further studies on animal models and trials in human subjects.

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