ORIGINAL ARTICLE

Effects of the Hypothalamic Pituitary Adrenal Axis Activation on the Absolute and Relative Weight of the Spleen: An Immunocytological and Morphological Study

Zaheer Ahmed Memon[*], *Abdul Hafeez Dall*[**], *Lal Bux Khaskheli*[***]

ABSTRACT

Objective: To observe the effects of the HPA-axis activation on the absolute and relative weight of the spleen and restoration with the Cyanocobalamin.

Methods: This experimental study was conducted in the department of Anatomy, Basic Medical Sciences Institute, Jinnah Postgraduate Medical Centre, Karachi from December 2013 to March 2014, on healthy 45 Sprague-Dawely rats obtained from animal house of the JPMC, Karachi, divided in control (A), heat-induced (B), and heat-induction with Cyanocobalamin protection group (C). The B and C groups received heat for 6 hours daily. Group C received Cyanocobalamin intraperitoneally 2 hours prior to heat treatment. Spleens and blood sampls were collected for immunostaining with primary antibodies Rabbit monoclonal antibody CD79a and Rabbit polyclonal antibody CD3 for identification of B and T-lymphocytes respectively. Spleens were weighed on an electric balance for absolute and relative weight. Blood samples preserved in vacutainers containing EDTA-K2 as an anticoagulant are analyzed for the plasma ACTH level by using Mouse/ rat adrenocorticotropic hormone (ACTH) ELISA antibody test.

Results: Heat-stressed group B animals show raise levels of plasma ACTH concentration with decrease B and T cellularity in follicular compartments associated with significantly decreased absolute and relative spleen weight. Whereas group C animals show a substantial repair due to treatment with the Cyanocobalamin by decreasing ACTH concentration with increase in absolute and relative spleen weight.

Conclusion: Study shows highly reparative effects of Cyanocobalmin on the weight of the spleen. Cyanocobalamin can be used as a prophylaxis in combinations or as a sole therapy in warmer environments.

Key words: CD79a, CD3, Stress, Absolute weight, Relative weight Immune, Spleen Cyanocobalamin.

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INTRODUCTION:

Several stressors exists in our immediate environment. They activates the hypothalamic-pituitary-adrenal (HPA) axis, and instantly ACTH levels rises with subsequent release of tremendous quantities of cortisone and Corticosterone in circulation [1]. One of the mechanism involved in the activation of the HPA Axis is the release of the interleukins during environmental heat stress [2,3,4]. Acute stress pathways are initially beneficial to maintain the existence of an individual in the environment, most of the stresses activate counteractions at the molecular, cellular or systemic levels that tend to restore the previous state, but when stress mechanisms overshoot then

[*] *Associate Professor, Deptt. of Anatomy, ISRA University, Hyderabad.*
[**] *Associate Professor, Deptt. of Anatomy, PUMHS Nawabshah.*
Assistant Professor, Deptt. of Anatomy, LUMHS Hyderabad.

Correspondence to:
Dr. Zaheer Ahmed Memon
Associate Professor
Department of Anatomy
ISRA University, Hyderabad.
Email: comzaheermemon32@yahoo.com

same produce deleterious effects on body's systems[5,6]. The most harmful effect is Corticosterone induced breakdown of structural proteins of muscles[7]. Stress induces the loss of the appetite, decrease food intake and ultimately leads to the weight loss[8,9,10]. Environmental heat stress mainly affects the white cells of the blood, immunity and lymphoid organs because they express the receptors for the glucocorticoids [11].Stress had negative effects on the immunity via the release of the stress hormones that inhibit the proliferation of the lymphocytes and subsequently leads to decrease in the size and weight of the lymphoid organs [12].Raised levels of the ACTH, Corticosterone, and Heterophil/Lymphocyte ratio are considered as stress markers [13]. Most of the researchers are of the opinion that food stuffs fortified with the micronutrients and antioxidants alleviate the detrimental effects of the environmental stress [14]. Rapidly proliferating cells needs a sufficient amount of the micronutrients including the vitamins and the trace elements to maintain the pace of the cellular turnover. Cyanocobalamin is essential for the cells survival [15], and plays a significant role in the maintenance of the immune system[16]. Studies are not available regarding to the role of the Cyanocobalamin affecting the weight of the immune organs in stress circumstances. The present study is designed to induce the heat stress in a rat model, to analyze the weight of the spleen, plasma ACTH levels and immunocytochemistry of splenic tissue by monoclonal antibodies, CD79a and CD3 for the B and T-lymphocytes to assess the effects of the HPA-axis activation on the spleen and protective effects provided by the Cyanocobalamin.

METHODS:

Animal house of BMSI, Jinnah postgraduate medical center, Karachi provided 45 adult Sprague-Dawely rats (180-200g) for this project. The animals were fed with balanced diet and water was provided ad libitum and kept in a well-ventilated room for a week prior to the commencement of the study. 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. Two hours before heat induction, Cyanocobalamin (BETOLVEX-Alpharma Aps, Denmark) was administered at the dosage of 0.8 mg/ kg intraperitoneally. Then heat is induced to animals of group and B and C with double rod electric room heaters of 2000 Watt, at 42° C for 6 hours daily depending on the duration of the subgroups. Animals were sacrificed, spleen dissected and weighed with the help of electronic balance. The relative weight of the spleen was calculated in mg/ 100g body weight. After weight they fixed in alcoholic formalin for 24 hours. Then processed in ascending strengths of alcohol, cleared in xylene, infiltrated and embedded in paraffin, four-micron thick sections were made and, affixed on Poly-L Lysine coated slides, deparaffinized in xylene and rehydrated through gradient alcohols. Antigen retrieval was performed by placing sections in a pressure cooker.Non-specific binding sites were blocked by protein blocking agent (ab-64261, abcam, UK). Then one set of tissue slides incubated with primary antibody, the Rabbit monoclonal anti-CD79a, prediluted (ab-27607, abcam, UK) and other set of tissue slides were incubated with the Rabbit polyclonal anti-CD3, diluted 1:100 (ab-5960, abcam, UK), both for 30 minutes at room temperature for the detection of B- and T-Lymphocytes respectively. The primary antibody is followed by a biotinylated goat anti rabbit 1g G secondary antibody (ab-64261, abcam, UK) for 10 minutes at room temperature, followed by a complex of streptavidin peroxidase with 3,3' diaminobenzidine (ab-64261, abcam, UK) as a chromogen substrate for the antibody binding detection. After immunostaining sections were washed in distilled water, counterstained with Meyer's Haemotoxylin, dehydrated, cleared and mounted. While the animals were still breathing, blood samples about 2 ml were collected from each animal by cardiac puncture, in plastic vacutainers containing EDTA-K2 as an anticoagulant (BD-Franklin Lakes NJ, USA).

Samples are analyzed for the plasma ACTH level by using Mouse/ rat adrenocorticotropic hormone (ACTH) ELISA antibody test Kit (Catalog # 40-109-325002; Gen Way Biotech, INC, CA).

RESULTS:

Animals of heat induced group B were lethargic, with feeble response to stimuli. They were not taking a complete amount of provided diet. On gross examination, the spleen of animals in subgroups B-1 and B-2 were brownish red in color, whereas in subgroup B-3 spleen was dark red in color and they were slightly smaller, atrophic and fragile (Fig-1). Immunostained CD79a and CD3 sections group B-2 and B3, showed marked shrinkage of follicular compartments. Few large sized lymphocytes wide apart from one another found in germinal centers separated by many empty spaces filled with the pyknotic nuclei and fragments of the degenerated B-cells (Figure-3). Similar changes were observed in the PALS, the T-cells rich zone with decrease in size and cellularity when compared to control group on immunostained (Figure-4). Data of the mean value of plasma ACTH level showed a highly significant increase ($P<0.001$) in heat induced subgroups compared to the control subgroups. Data of mean value of absolute spleen weight in animals of group B showed that there was a moderately significant decrease ($P<0.01$) in subgroups B-1 and B-2, and a highly significant decrease ($P<0.001$) in subgroup B-3 compared to the control subgroups A-1, A-2 and A-3 respectively (Table-2). Data of mean value of relative spleen weight in group B showed that there was a moderately significant decrease ($P<0.01$) in all subgroups compared to the control subgroups (Table-3). Animals of group C were healthy and active, with brisk responses to stimuli, taking complete amount of provided food. On gross examination, the spleen of animals in all subgroups were appeared, brownish red in color with smooth surfaces, regular borders and soft in consistency (Figure-2). Immunostained CD79a andCD3 sections shows a substantial mass of B and T-cells in their respective compartments in lymphoid follicles and associated areas. Few tingible body macrophages and pyknotic nuclei were observed (Figuer-5 and 6). The mean value of plasma ACTH data showed a highly significant decrease ($P>0.001$) in all subgroups compared to the group B animals (Table-1). Data of the mean value of absolute weight of the spleen in group C showed that there was an insignificant increase ($P>0.05$) in the absolute spleen weight in all subgroups compared to the control subgroups. When compared to the subgroups B1and B2, there was a moderately significant increase ($P<0.01$) in subgroups C1 and C2, and a highly significant increase ($P<0.001$) in subgroup C3 respectively (Table-2) Data of mean value of relative spleen weight showed that there was an insignificant decrease ($P>0.05$) in relative spleen weight in subgroups C-1, C-2 and C-3 when compared with corresponding control subgroups A-1, A-2 and A-3. The data also showed a significant increase ($P<0.05$) in relative spleen weight in heat and Cyanocobalamin treated subgroups C-1 and C-2 when compared to heat induced subgroups B-1 and B-2 and there was moderately significant increase ($P<0.01$) in relative spleen weight in subgroup C-3 as compared to subgroup B-3 (Table-3).

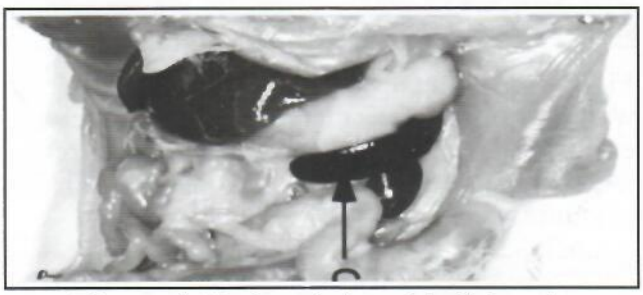

Figure -1. Dark red, Atrophic Spleen in Heat-Iinduced Group B Animal.

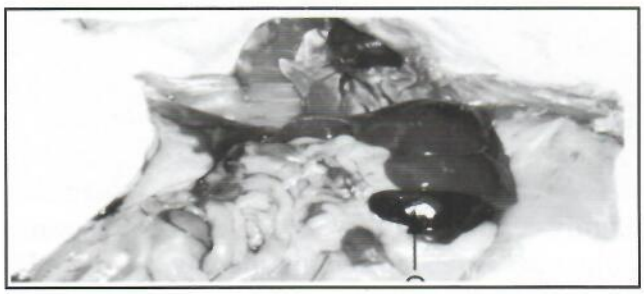

Figure -2. Bright red with normal dimensions spleen In Heat-induced and Cyanocobalamin treated animal

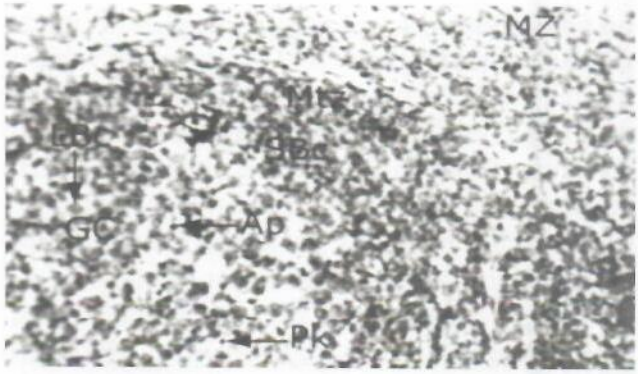

Figure-3: Immunostained anti-CD79a, of splenic white pulp after heat induction, showing few(Lbs) large B-lymphocytes, (GC) in germinal center, (SBc) small B-lymphocytes in (Mtz) mantle zone, (MZ) in marginal zone and with a moderate number of (Pk) pyknotic nuclei of (Ap) apoptotic cells.

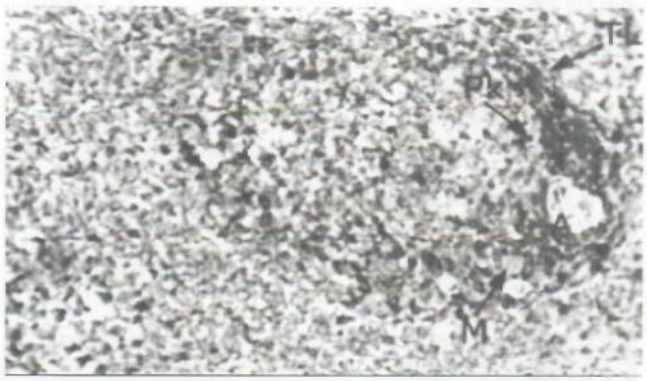

Figure-4: Immunostained anti-CD3 of splenic white pulp after heat induction in group C showing extensive decrease in (TL) T- lymphocytes around (A) central artery with (M) macrophages and (Pk) pyknotic nuclei.

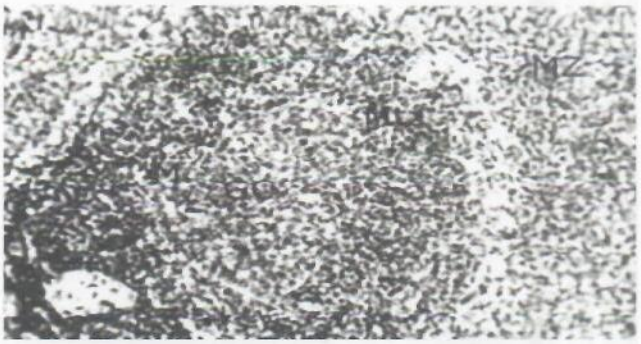

Figure-5: Immunostained anti-CD79a, of white pulp, after Cyanocobalamin therapy, showing B-cells close to control in (GC) germinal center, (Mtz) mantle zone and (MZ) marginal zone with few (M) macrophages.

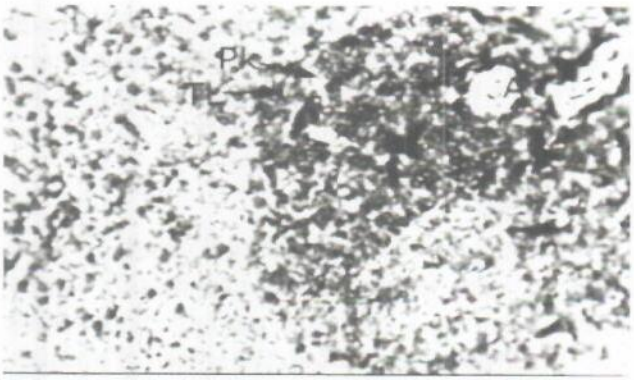

Figure-6: Immunostained anti-CD3, white pulp, after Cyanocobalamin therapy showing increase in (TL) T- lymphocytes around (A) central artery and few (Pk) pyknotic cells comparable to control (Photomicrogr).

DISCUSSION:

One week after commencement of the study period group B animals looks irritable, less interested in feed intake. Heat induced animals shows the criteria akin to the heat -stress such as the raised plasma ACTH and Corticosterone concentrations due to the activation of the HPA-axis related mechanisms[1,17]. Irritability because of the involvement of the nervous system as it is more sensitive to heat-stress Ha[18]. Feeding depletion is mainly due to the activation of HPA-axis that leads to decrease the appetite[4,19], and achlorhydria induced by the nitric oxide which produced in brain-gut axis in response to high ambient temperature[20] or direct gut involvement in which heat stress reduces the splanchnic blood flow which leads to permeability defects, ionic pump dysfunction, hypoxia and necrosis of the enterocytes[21]. The mean concentration of the plasma ACTH found highly significant in this group, it is might be due to the activation of HPA-axis by the cytokines[2,22,23]. Findings of the present study were in accordance with Koko et al[17], who observed a significant rise in plasma adrenocorticotropic hormone (ACTH) in Wistar rats exposed to heat. All of the above downfalls leads to decrease in the body weight. Heat-stress affected feed intake provides only few nutrients for the proper development of the organs including the lymphoid organs. Aengwanich et al[24] demonstrated

Table-1: Mean Plasma Level of ACTH (pg/ml) in different Groups of Sprague Dawely Rats at Variable Time Intervals

Group	Sub group	Treatment Given	Plasma Level of ACTH		
			2nd Week	4th Week	6th Week
A (n=15)	A1 (n=5)	Control	153.20+2.31		
	A2 (n=5)			158.401+1.93	
	A3 (n=5)				162.80+1.77
B (n=15)	B1 (n=5)	Heat	355.60+8.22		
	B2 (n=5)			359.20+2.08	
	B3 (n=5)				361.40+3.01
C (n=15)	C1 (n=5)	Heat + Cyanocobalamin	165.0+4.04		
	C2 (n=5)			166.60+6.45	
	C3 (n=5)				171.80+3.59

*Mean+SEM

Statistical Analysis of Mean Levels of ACTH in different Groups of Sprague

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****

that the lymphopenia follows the lympholysis in the lymphoid tissue and blood triggered by the steroids. Corticosteroid hormones bind with the lymphocyte receptors, induces the synthesis of the proteins that inhibit the lipid biosynthesis and intracellular glucose transport and activate the endonucleases which breaks the DNA into fragments, these observations are in agreement with the findings of the current study which reports atrophic spleens, raised plasma ACTH level, and hypocellularity of splenocytes. Our study is also in accordance with the two other studies, Swaminathan A et al[25] and Graczyk et al [26] both demonstrate a significant reduction in the

Table-2: Mean absolute Weight of Spleen (G) in different Groups of Sprauge Dawely Rats at Variable Time Intervals

Group	Sub group	Treatment Given	Plasma Level of ACTH		
			2nd Week	4th Week	6th Week
A (n=15)	A1 (n=5)	Control	0.44+0.00		
	A2 (n=5)			0.43+0.00	
	A3 (n=5)				0.45+0.00
B (n=15)	B1 (n=5)	Heat	0.33+0.00		
	B2 (n=5)			0.31+0.00	
	B3 (n=5)				0.30+0.00
C (n=15)	C1 (n=5)	Heat + Cyanocobalamin	0.42+0.00		
	C2 (n=5)			0.43+0.00	
	C3 (n=5)				0.45+0.00

*Mean+SEM

Statistical Analysis of the differences in Mean Absolute Weight of Spleen in the Same Group and between Groups of Sprauge Dawely

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

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

lymphocyte count and subsequent decrease in the weight of the lymphoid organs. The absolute and relative weight of the spleen decreased significantly compared to control group. The causes of decrease in absolute and relative splenic weight are decreased feed intake and decreased body weight as discussed earlier. Doom JR[13] observed a significant decrease in the relative weight of lymphoid organs including the spleen in an experimental study on ACTH treated chickens. The findings of Bartlett and Smith[27] were also in agreement with the results of the present study,

Table-3: Mean Relative Spleen Weight (g/100g) in different Groups of Sprague Dawely Rats at Variable Time Intervals

Group	Sub group	Treatment Given	Relative Spleen Weight (g/100g)		
			2nd Week	4th Week	6th Week
A (n=15)	A1 (n=5)	Control	0.210+0.00		
	A2 (n=5)			0.197+0.00	
	A3 (n=5)				0.193+0.00
B (n=15)	B1 (n=5)	Heat	0.171+0.00		
	B2 (n=5)			0.167+0.00	
	B3 (n=5)				0.162+0.00
C (n=15)	C1 (n=5)	Heat + Cyanocobalamin	0.200+0.00		
	C2 (n=5)			0.203+0.00	
	C3 (n=5)				0.197+0.00

*Mean+SEM

Statistical Analysis of Mean Relative Spleen Weight in Different Groups of Sprague Dawely Rats

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

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

they observed a significant reduction in the weight of all lymphoid organs including spleen by the effects of heat-stress. Group C animals were active and their feed intake was improved. Cyanocobalamin deficit changes the behavior, mood[28], and also associated with the loss of the appetite[29]. Cyanocobalamin therapy improves the behavior and restores the appetite by its growth promoting effects as described by Guerra-Shinohara[30]. Brich et al[31], describe the antioxidant role of the Cyanocobalamin by directly reacting with free radicals and involved in prevention of

cells from the damaging effects of oxidative stress. The absolute and relative splenic weights are increased compared to the heat induced group B. It could be due to the reparative action of the Cyanocobalamin on the cellular D NA and RNA as explained by Guyton and Hall[32] and by antioxidant effect to clear free radicals directly and inhibition of caspases-3 cleavage[31]. This group shows insignificant increase in plasma ACTH concentration compared to control group, might be due to the direct or indirect inhibitory effects of the Cyanocobalamin on the ACTH secretion. Findings of the present study were similar to Lovgren et al[33], who observed the inhibitory effects of the vitamin B12 on the Corticosterone and ACTH. Berg AL[34] demonstrated the effects of the adrenocorticotropic hormone (ACTH) and cortisol on two different groups of the patients and concludes; that increased ACTH concentration or ACTH therapy decreases the Cyanocobalamin. In our study we use Cyanocobalmin and heat in combination to alleviate the detrimental effects of heat-stress, and almost all parameters shifted close to the normality.

CONCLUSION:

Study shows highly reparative effects of Cyanocobalmin on the weight of the spleen by inhibiting the lympholytic effects of the Corticosterone, improving the appetite and restoring the mental irritability. It also inhibit the production of the steroid hormones effectively, It is recommended that Cyanocobalamin can be used as a prophylaxis in combination with other vitamins or as a sole therapeutic agent in warmer environments.

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