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Research Article

Interrelationship of Vitamin B_{12}, Androgens and Cortisol in Chronic Stress and associated Vascular Dysfunction

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Keywords

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- Androgens
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- CVS Dysfunction

Abstract

Stress, both physical and psychological, is attracting increasing attention among neuro researchers. In the last 20 decades, there has been a surge of interest in the research of stress induced manifestations and this approach has resulted in the development of more appropriate animal models for stress associated pathologies and its therapeutic management. These stress models are an easy and convenient method for inducing both psychological and physical stress. To understand the behavioral changes underlying major depression, molecular and cellular studies are required. Dysregulation of the stress system may lead to disturbances in growth and development, and may this may further lead to the development of various other disorders. This article reviews the interrelation of Vitamin B12, androgens and cortisol in chronic stress model and their neurobiology, including the different neurotransmitters and heart function affected. There are various complications associated with stress and their management through various pharmacological and Non-Pharmacological techniques. The use of vitamin b12 in the treatment of stress related problems is in practice in both Indian and Western societies, Examination of the hyper-responsiveness of the Hypothalamic-Pituitary-Adrenal axis, consequent elevated serum cortisol, Androgens plus the effects of this upon brain structure and function, provides a model for understanding how chronic stress may be a causal vector in the development of major organ dysfunction like CVS dysfunction.

INTRODUCTION

The body's principal adaptive responses to stress stimuli are mediated by an intricate stress system, which includes the Hypothalamic-Pituitary-Adrenocortical (HPA) axis and the Sympathoadrenal System (SAS). Dysregulation of the system, caused by the cumulative burden of repetitive or chronic environmental stress challenges (allostatic load) contributes to the development of a variety of illnesses including hypertension, atherosclerosis, and the insulin-resistance– dyslipidemia syndrome, as well as certain disorders of immune function. The brain's limbic system, particularly the hippocampus and amygdala, is also intimately involved in the stress response [1,2]. Chronically elevated corticosteroid levels induced by persisting stress may adversely affect hippocampal structure and function, producing deficits of both memory and cognition. The ability of stress to cause illness in humans is most clearly exemplified by Post-Traumatic Stress Disorder (PTSD), which consists of a predictable constellation of distressing behavioral symptoms and physiological features [3,4]. An appreciable proportion of the observed variance in vulnerability to PTSD is attributable to genetic factors [5]. Vitamin B_{12} has key role in the normal functioning of the brain and nervous system, and for the formation of RBC and blood. Chronic stress leads to decrease the level of vitamin b_{12} in the body by destructing the parietal cells (which secretes intrinsic factor for vitamin b_{12} absorption) in the stomach [6,7]. So, malabsorption of vitamin b_{12} takes place in the absence of intrinsic factor, which leads to vitamin b_{12} deficiency. This lost in functionality of vitamin B_{12} can be measured clinically as an increased in the homocysteine level in vitro. In the metabolism of Homocysteine during remethylation process, Hcy is reconverted into Methionine [8]. This reaction is catalyzed by methionine synthase. Methionine synthase uses Vitamin B_{12} as a co-factor. So.in absence of vitamin b_{12}, the process of Methionine synthesis disturbs and resulted in over accumulation of Homocysteine. Increase the level of homocysteine called as Homocysteinaemiais, which is associated with mostly Cardiovascular Disease (CVD) [9]. HCy increased thrombogenicity, increased oxidative stress, increased inflammatory activation, impaired endothelial function, and finally atherogenesis [10]. Reproductive activity is one of the main functions that becomes altered and inactivates during

the adaptive response to stress .chronic exposure to stressors increases HPA (Hypothalamus-Pituitary-Adrenal) axis activity and concomitantly reduces HPG (Hypothalamus-Pituitary-Gonadal) axis activity [11]. This antagonistic relationship between both these axes has been proposed to underlie the inhibition of reproductive function due to stress. The hyperactivity of the hypothalamic-adrenal axis is involved in mediating the effect of stress on the testes and increased glucocorticoid levels are associated with reduction of testosterone biosynthesis by leydig cells which are the primary site of glucocorticoid binding in the testis [12,13].

EXPERIMENTAL METHOD AND MATERIAL

Animals

Experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and was cleared by same before beginning the experiment (No. LJIP/ IAEC /13-14/ 01) following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Adult male Sprague–Dawley rats, weighing 250-350 g were used in the study. Animals were divided into 5 groups: 1) normal control, 2) normal control and vitamin b12, 3) chronic stress, 4) chronic stress and vitamin b12, 5)chronic stress, vitamin b12 and testosterone. For all groups of rats were housed per cage in a room with temperature regulated at 22±2°C, with a 12/12 h light/dark cycle (lights on 07:00 h, lights off 19:00 h) [14,15]. Standard chow pellets and water were given ad libitum, except during the experimental period when food or water deprivation was applied.

Drugs

Vitamin B_{12} (500µg/kg) and Testosterone enanthate (0.5mg/100gm) were taken for treatment groups.

Stress procedure

All the rats were acclimatized under laboratory conditions and handled daily for a week prior to the commencement of experiment. Two different stress models were used, acute and chronic unpredictable stress. Acute stress was produced by restraining 12 h fasted rats for 150 min inside a cylindrical steel tube (7 cm diameter, 17.5 cm long, with holes for ventilation). Chronic unpredictable stress procedure includes fasting, tail pinching, restraint, overnight wet cage bedding, isolation, forced swimming, day–night reversal, cold-restraint, water deprivation. Individual stressors and time of exposure during chronic unpredictable stress on every day have been summarized in Table 1 [17-19].

Table 1: Stressor Schedule.

Days	Stressor Schedule	
	Morning	**Afternoon**
Day 1	Tail Pinching – 5 min	Water Depravation 18 hr
Day 2	Tilted cage – 4 hr	Food Depravation 18 hr
Day 3	Restrain – 120 min	Day-Night Reversal 18 hr
Day 4	Forced Swimming - 20 min	Wet Bedding 18 hr
Day 5	Cage Rotation-2 hr	Isolation 18 hr
Day 6	Tail Pinching – 5 min	Food Deprivation 18 hr
Day 7	Restrain – 120 min	Water Deprivation 18 hr

Briefly, rats were subjected to fasting (food deprivation) for 18 h, between 14.00 h to 10.00 h the next day. Tail pinching comprised pinching the tail tip with specially designed steel clips for 5 min. Restraint stress comprised confinement for 150 min inside a cylindrical steel tube (7 cm diameter, 17.5 cm long). Bedding material was soaked with water overnight as a further stressor. For isolation stress, rats were kept alone in a cage for 12 h. For swimming stress, rats were placed in a glass jar (35.5 cm high, 20.2 cm diameter) containing water (depth:25 cm) at 25°C for 30 min. Day and night reversal involved keeping the rats in the dark during the usual day (3 h) and in high intensity light during the night (12 h). During water deprivation, water was removed for 18 h, between 14.00 h to 10.00 h the next day.

Treatment schedule

Animals were randomly divided into various groups as describe above, each group containing 6 rats. The respective groups consist of non-stress, acute stress, chronic unpredictable stress along with Vitamin b12 and testosterone enanthate. Treatment of Vitamin B12 was given daily by oral route and testosterone enanthate was given twice in a week by intra muscular route for 28 days.

Measurement of parameters

After 28 days of stressor schedule various parameters like body weight and physical behaviour were measured. Physical behaviour was measured by: Elevated plus maze model, Open field model, Despair swim test, Sucrose consumption test. Blood was collected by retro-orbital route to check blood parameters like Vitamin B_{12} level, androgen (Testosterone) level, homocysteine level and cortisol level. Animals were sacrificed then, thoracic aorta was isolated to record the Ach induced relaxation of thoracic aorta precontracted with Phenylnephrine.

Statistical analysis

The result was expressed as mean ±S.E.M. where n represents the number of rats. Statistical differences between two groups were checked by unpaired t-test and among the groups were checked by one way ANOVA followed by Tukey's multiple comparison tests. The statistical comparisons were carried out using the Sigma Plot software, version 11. A value of $P<0.05$ was considered as statistically significant. The relationship among normal group and other groups for bio-chemical parameters was assessed by computing Pearson's correlation coefficients. Statistical version 8.0 software (Stat Soft Inc., Tulsa, USA) was used for all statistical analysis. A $p< 0.05$ level was accepted as significant throughout.

RESULT

After 28th days of stressor schedule following behavior test were perform:

1) Behavior tests

A) Elevated Plus maze model

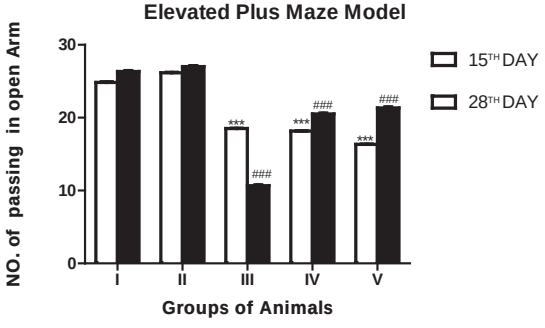

Figure 1 **Shows the comparison of Behavior of rats by elevated plus maze model in normal control rats, stress control and treated rats.** Each point is represented as Mean±S.E.M. n=6. *p<0.05,**p<0.01 and ***p<0.001 compared with normal control rats.### p<0.001 Vs Stress control group. Open arm time spent was significantly decreased in case of chronic stress group rats (p<0.001) than normal controls and drug treated rats.

B) Open field model:

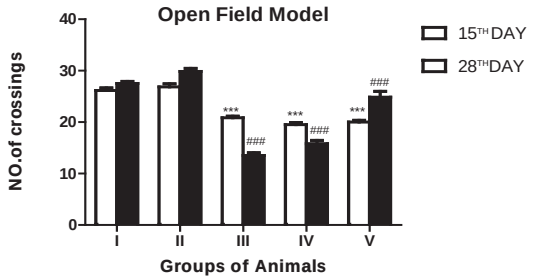

Figure 2 shows **the comparison of Behavior of rats by Open field model in normal control rats, stress control and treated rats.** Each point is represented as Mean±S.E.M. n=6. *p<0.05,**p<0.01 and ***p<0.001 compared with normal control rats.### p<0.001 Vs Stress control group**,** Open field crossings (mobility) were significantly increased in case of normal group rats p<0.001) and drug treated individuals as compare to stress group.

C) Forced Swim model:

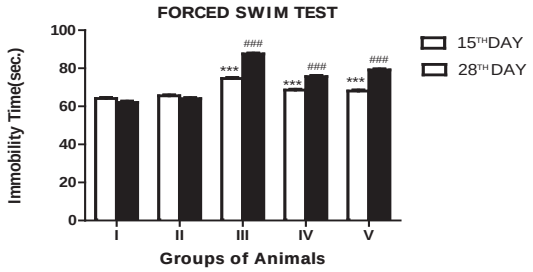

Figure 3 each point is represented as Mean±S.E.M, n=6.15th day indicates the reading at 15th day of experiment while 28th day readings indicate the reading at last day of treatment ***p<0.001 Vs normal group, ###p<0.001Vs stress control group.

Immobility time was significantly increased in case of chronic stressed rats (p<0.001) than normal controls and drug treated individuals.

D) Sucrose Water Intake model:

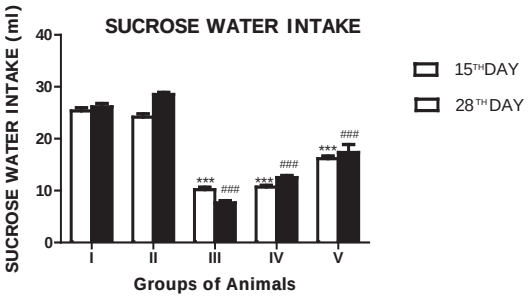

Figure 4 each point is represented as Mean±S.E.M, n=6.15th day indicates the reading at 15th day of experiment while 28th day readings indicate the reading at last day of treatment ***p<0.001 Vs normal group,###p<0.001Vs stress control group. Sucrose water intake was significantly decrease in case of chronic stressed group (p<0.001) as compare to normal control and treated groups.

Co-relation of Behavior Parameters and Blood parameters:

Behavior Parameters	Blood Parameters			
	Vitamin B_{12}	Homocysteine	Cortisol	Testosterone
Elevated plus maze model	0.9003	-0.9625	-0.9615	0.6649
Open Field Model	0.9325	-0.9500	-0.9161	0.6718
Forced Swim Model	-0.9488	0.9573	0.9799	-0.6734
Sucrose Consumption model	0.8959	-0.9389	-0.9590	0.5591
Physical Parameter (Body Weight)	0.9886	-0.9498	-0.9632	0.5899

Negative sign indicates inverse relation between two variables.

Elevated plus maze model and open field model shows direct relation with Vitamin B_{12} level and testosterone level, where as inverse relation with Homocysteine and cortisol levels: This describes that after chronic stress there was significant result seen in all blood parameters.

3) Regression Analysis of Behavior Parameters and Blood parameters:

Behavior Parameters	Blood Parameters			
	Vitamin B_{12}	Homocysteine	Cortisol	Testosterone
Elevated plus maze model	R^2=0.03	R^2= -0.21	R^2= -0.19	R^2=0.05
Open Field Model	R^2=0.75	R^2= -0.22	R^2= -0.19	R^2=0.06
Forced Swim Model	R^2= -0.06	R^2=0.07	R^2=0.05	R^2= -0.07
Sucrose Consumption model	R^2=0.012	R^2= -0.28	R^2= -0.26	R^2=0.01
Physical Parameter (Body Weight)	R^2=0.034	R^2= -0.08	R^2= -0.06	R^2=0.03

Negative sign indicates inverse relation between two variables, According to regression analysis result: positive value of regression coefficient (R^2) shows direct relation between behavior parameters and blood parameters, whereas negative value indicates inverse relation of two variables.

5) Co-relation of Blood Parameters:

Blood Parameters	Vitamin B_{12}	Homocysteine	Cortisol	Testosterone
Vitamin B_{12}	1	-0.9822	-0.9827	0.6657
Homocysteine	-0.9822	1	0.9947	-0.7080
Cortisol	-0.9827	0.9947	1	-0.6782
Testosterone	0.6657	-0.7080	-0.6782	1

Negative sign indicates inverse relation between two variables. According to co-relation result: positive value of parameters shows direct relation between blood parameters, whereas negative value indicates inverse relation of two variables. That shows if vitamin b_{12} level decreases there are significant decrees in testosterone level where as significant increase in the Homocysteine level and cortisol level:

6) Regression Analysis of Blood parameters:

Blood Parameters	Vitamin B_{12}	Homocysteine	Cortisol	Testosterone
Vitamin B_{12}	R^2=1	R^2= -0.17	R^2= -0.15	R^2=0.059
Homocysteine	R^2= -0.17	R^2= 1	R^2=0.077	R^2= -0.34
Cortisol	R^2= -0.15	R^2=0.077	R^2=1	R^2= -0.23
Testosterone	R^2=0.059	R^2=0.077	R^2= -0.23	R^2=1

Negative sign indicates inverse relation between two variables. According to regression analysis result: positive value of regression coefficient (R^2) shows direct relation between blood parameters, whereas negative value indicates inverse relation of two variables

7) Effect on Vascular Reactivity study:

A) Comparison of pD2 values and % Rmax of ONOO- and Acetylcholine

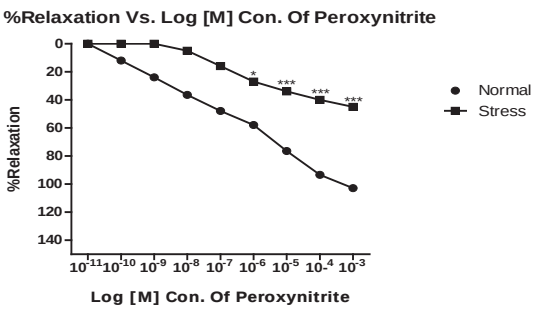

Figure 6 Concentration response curve of $ONOO^-$(10^{-11}M to 10^{-3}M) induced relaxation in control and stressed aortic strips, precontracted with PE (10^{-5}M).Values are expressed in means ± S.E.M., n = 6. *P<0.05 and *** P<0.001 and Vs control

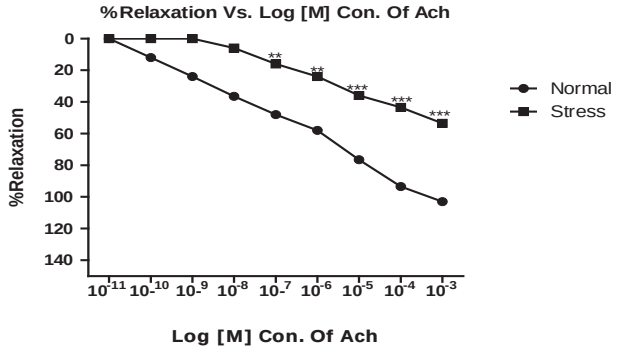

Figure 7 Concentration response curve of Ach (10^{-11}M to 10^{-3}M) induced relaxation in control and stressed aortic strips, precontracted with PE (10^{-5}M).Values are expressed in means ± S.E.M., n = 6. **P<0.01 and *** P<0.001 and Vs control.

ONOO- and Ach induced relaxation was impaired in stressed aortic strip as compared to control. This may indicate the development of vascular and endothelial dysfunctions in stressed rats respectively

B) Effect of treatment on aortic strips of various groups:

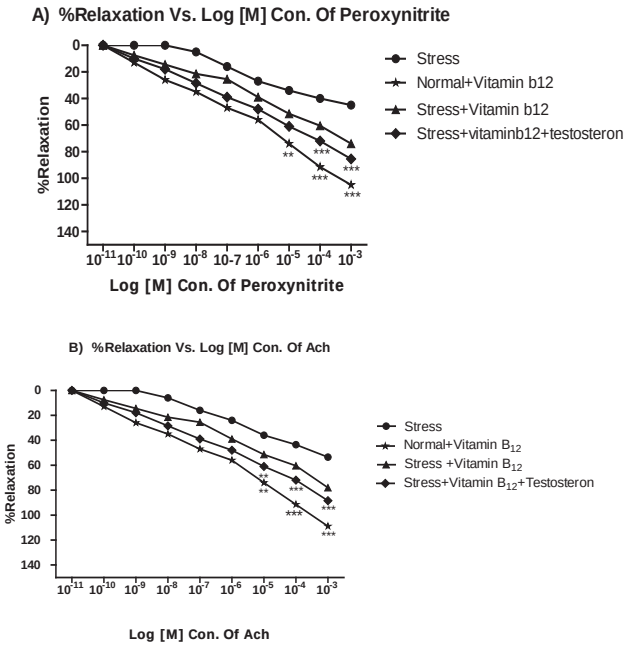

Figure 8 Cumulative concentration response curves (CRCs) of $ONOO^-$(A) and Ach (B) on endothelium intact aortic spiral preparations obtained from stressed rats and treated rats. Each point is represented as Mean ± S.E.M. n = 6. * p<0.05 ** p<0.01 and ***p< 0.001 Vs respective stressed group.

Treatment showed significantly increased (p<0.01) the $ONOO^-$and Ach induced relaxation as compared to stressed rats.

DISCUSSION

Chronic stress influences the Sympathoadrenal system and the Hypothalamic Pituitary Adrenocortical (HPA) axis, which are, in turn, mediated by the hippocampus .Stress stimulates the release of Corticotropin-Releasing Factor (CRF), from the hypothalamic Paraventricular Nucleus (PVN), into the hypophysial-portal

circulation, where it induces the release of Adrenocorticotropin Hormone (ACTH) from the anterior pituitary and glucocorticoids (cortisol in humans; corticosterone in rodents) from the adrenal glands [1].

Activation of this axis, results in glucocorticoid release into systemic circulation. Stress and glucocorticoids have specific effects on cognitive functions in humans and in animal models. These hormones trigger physiological "fight-or-flight" mechanisms, which include increases in heart rate, respiration rate, fat and carbohydrate breakdown, and blood pressure.

Chronic stress leads to decrease the level of vitamin B_{12} in the body by destructing the parietal cells (which secretes intrinsic factor for vitamin b_{12} absorption) in the stomach. So, malabsortion of vitamin B_{12} takes place in the absence of intrinsic factor, which leads to vitamin B_{12} deficiency [6,7].

Stress disrupts the circadian rhythmic secretion of cortisol. An effective method to phase-shift circadian rhythm is a combination of bright-light exposure and methylcobalamin. Methylcobalamin is thought to assist bright light in resetting the circadian rhythm by enhancing the light sensitivity of the circadian clock. Methylcobalamin also appears to generate the right quality of sleep activity by both reducing sleep time and improving sleep quality, resulting in feeling refreshed upon waking [9].

Plasma Homocysteine concentrations accumulate with B_{12} deficiency thus providing a functional biomarker of vitamin B_{12} status. Elevated total plasma Homocysteine (tHcy) is an independent risk factor for peripheral vascular, cerebrovascular, and Coronary Artery Disease (CAD).

In general, there is a great increase in serum adrenal steroids for a short period after acute stress. With the prolonged exposure to extreme stress, serum adrenal steroids concentrations become subnormal and appear to affect directly or indirectly the depression of serum reproductive steroids like cortisol and testosterone [20,21].

The increased body weight of the rats is especially responsive to the administration of androgens Testosterone enanthate, at a dose of 0.5mg/kg, produced a significant increase in body weight. The analyses of the organs of rat treated with testosterone demonstrated that the hormones definitely stimulated the synthesis of protein independently of each other. These studies are solely based on chronic stress induced depressive behavior. Results obtained from EPM test clearly indicates that an increase tendency of rodents to enter in the open-arm and their time spent in that arm is an index of anti-anxiety behavior of rodents. Similarly, the observations of OFT demonstrates the tendency of rodents to avoid brightly illuminated areas, and this avoidance is confused as a symptom of anxiety. Normal rats generally show increased ambulation and rearing in a novel open field. Whereas, stressed rats display decreased ambulation and rearing in a novel open field Considering immense validity and reliability of depressive-like states in Learned Helplessness (LH) nature of depression.

Stressed animals have loss of weight, agitation, decreased locomotors activity, sleep disturbances, decreased libido, reduced learning in some tests but not in spatial learning tests, and alterations in the HPA axis, with elevated corticosterone. Anhedonia has been postulated as a central symptom of depression, which can be monitored in animals using sucrose preference tests. An anhedonic state generated on the rats by the CMS was reflected as a reduction in their sucrose consumption of at least 2 g.

Vascular dysfunction was shown from the increase Homocysteine level, the mechanism behind the vasorelaxation effect of ONOO on rat cerebral arteries. The ONOO exerted vasodilation due to activation of potassium channel, myosin phosphates and elevation of sGC [22].

Endothelial dysfunction is considered to represent reduction of bioavailability of nitric oxide. So with special emphasis of ONOO induced relaxation in stressed aorta, study was carried out to elucidate potassium channel dysfunction.

Present experiment showed that, in stressed rat thoracic aortic strips, ONOO and Ach induced relaxation was impaired as compared to normal control rat thoracic aortic strips. Ach induced relaxation in rat aortic strip were significantly reduced in stressed rats indicating endothelial dysfunction, while ONOO induced relaxation in rat aortic strips of stressed rats were significantly impaired indication vascular dysfunction .Treated groups significantly increase the ONOO and Ach induced relaxation as compared to stressed rats showed endothelial and vascular protective effect.

Chronic stress and vitamin b_{12} level thereby increases serum homocysteine levels and which produced vascular endothelial dysfunction. Also over expression of adrenal gland promotes Na^+ retention, osmotic retention of H_2O, and increasing blood pressure by increasing blood volume.

CONCLUSION

At the end of the study, it was observed that treatment with vitamin B_{12} and testosterone has shown significant change in stress responses on physical parameters as well as on blood parameters. Vitamin B_{12} and testosterone treated group's shows significant response in elevated plus maze model, open field model, despair swim test and sucrose consumption tests. In elevated plus maze model no. of entries increases in open arm by normal control groups and treatment control groups as compare to disease control group. Same expected responses were seen in open field model, sucrose consumption test and despair swim test, also significant decreases were measured in vitamin B_{12} level and testosterone level increases the level of homocysteine and cortisol in blood parameters. By doing correlation co-efficient analysis there was good correlation seen between all stress parameters (behaviour parameters) and blood parameters, which justify the title of thesis that there is significant interrelation occur between Vitamin B_{12}, androgens and cortisol in chronic stress and associated vascular dysfunction. By dose response curve it had been noted that vascular relaxation property was reduced in chronic stress condition as compare to normal control and treatment treated groups.

Thus from the above result it can be concluded that there is significant interrelationship present in vitamin B_{12},androgens and cortisol in chronic stress conditions and associated vascular dysfunction . Elevation in either of these three blood parameter may lead to potential biomarker parameter for chronic stress condition and related vascular dysfunction.

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