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STUDY OF STRESS ON CYTOKINE MEDIATED AUTOIMMUNE THYROIDITIS

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ABSTRACT

Autoimmune thyroid diseases are the most common organ-specific autoimmune disorders. Approximately 5% of the overall population suffers from this disorder. Hashimoto's thyroiditis (HT) or hypothyroidism is the most common clinical expressions of thyroid dysfunction but differ clinically as well as in pathophysiology. It is the major autoimmune endocrine disorder which is linked to human immune system under psychological stress. The study focuses on the level of thyroid and stress hormone and its impact on the immune system. Two hundred human subjects along with normal healthy population were randomly selected on the basis of clinical examinations. Hormonal and cytokine analysis was conducted of both thyroid and stress physiology, both pro and anti-inflammatory status was performed using serum samples. Statistical analysis was performed of all the parameters.The study depicts significant increase in TSH and decrease in T3 and T4 hormones also rise in stress hormones was observed. Rise in the hormones resulted in significant increase in the level of pro-inflammatory cytokines TNF-α and IFN-γ and decrease in anti-inflammatory cytokines IL-4, IL-6 and IL-10. Individuals' long term effects of relationship between psychosocial and physiological stress conditions (i.e., genetics, constitutional factors) and disease is affected by the biological nature, number, and persistence which may influence the course of chronic disease. Therapies in development of biopsychosocial model provide a basis for understanding and treatment of disease, the impact of illness from a societal perspective. There exists a considerable amount of data on the psychosocial factors related to thyroid disorders.

KEYWORDS: autoimmunity, anti-inflammatory, cytokines, hashimoto's thyroiditis, pro-inflammatory, psychological-physiological stress.

INTRODUCTION

All living organisms maintain a complex dynamic equilibrium, or homeostasis, which is constantly challenged by internal or external adverse effects. Stress is defined as a state in which homeostasis is actually threatened or perceived to be so.[1] Homeostasis is reestablished by a complex repertoire of behavioral and physiological adaptive responses of the organism.[2]

Stress may cause immunodepression but may also exert an immunoenhancing effect on cell numbers same stressor may have a positive effect rather than a negative one, depending on its duration or intensity.[3] According to investigations, stress has been classified as acute laboratory stress and natural stress. Stressor induced neurosensory signals are processed in the paraventricular nucleus (PVN) of the hypothalamus. In response to stressors, hypothalamus secrets corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH-containing neurons have different pathways and projections to noradrenergic centers in the brain stem and spinal cord.[4] CRH activates hypothalamic pituitary axis (HPA), leading to release of peptides from the pituitary and adrenocorticotropic hormone, enkephalins, and endrophins. Adrenocorticotropic hormone induces release of glucocorticoids from the adrenal cortex and CRH and central nervous system (CNS) together stimulate noradrenergic neurons resulting in secretion of norepinephrine (NE) by peripheral sympathetical nervous system (SNS) and release of epinephrine (EPI) from the adrenal medulla. The activation of these two neurochemical pathways and release of hormones and transmitters have profound effects of immune function.[5]

Stress hormones influence numerous physiologic processes; they regulate inflammatory diseases, their effects maintaining balance between cell-mediate and humoral immunity and on neurogenic inflammation in peripheral tissues. The hypothalamic-pituitary-adrenal (HPA) axis and SNS represent the peripheral stress system, its activation occurs in CNS in response to distinct blood-borne, neurosensory signals.[6, 7] Homeostasis within the immune system is largely dependent on cytokines, the chemical messengers between immune cells, which play crucial roles in mediating inflammatory and immune responses, for instance, immune challenges such as blood-borne stressor of infections with bacteria release bacterial lipopolysaccharides (LPS), which induce the nuclear factor (NF) mediated secretion of IL-1

and IL-6 and activates HPA axis and stimulates the hypothalamic stress response.[8] The HPA axis regulates a wide variety of immune functions affecting cell trafficking, migration, maturation and differentiation; this regulation is the result of several neuroendocrine pathways including hormones.

Thyroid dysfunction is an important cause of depression.[9] Hypothyroidism is considered a potentially reversible cause of depression, and both disorders have symptoms that may complicate studies attempting to clarify the relationship between them.[10,11] The changes occur due to hormone involving both stress and thyroid hormones, among these cortisol, prolactin, thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) are the major.[12]

Stress induces changes in the secretion of several hormones, which affect immune function by either increasing or decreasing immune activity. The thyroid hormones are essential for the maintenance of neurotransmitters associated with stress, and have also a significant impact on the immune response.[13] The changes occurring both in stress and thyroid hormones are the major cause for such changes in the body immune system. Study has been done to analyze these changes in the hormone level and the difference with the euthyroid subjects.[14]

Cytokines encompasses all the immunomodulating agents that trigger inflammation and respond to infections. It includes two classes pro-inflammatory and anti-inflammatory. Stress hormones changes stress system activity through modulating pro or anti-inflammatory cytokines, TNF-α, IFNγ, IL-2, IL-6, IL-4 and IL-10, balance by stimulating or suppressing the progression of this autoimmune disease.[15]

Present study emphasizes on the effect caused due to stress on an autoimmune hypothyroidism and changes in the immune system by the stressors, also study will be a stepping stone in understanding the thyroid–depression interaction, people suffering from hypothyroidism and the one with psychological disorder.

MATERIALS AND METHODS

Study Participants

200 samples, 100 as patients and 100 as normal healthy control, age ranging from 20-60 years from different outdoor patients (OPD) of hospitals of Bhopal. Clinical examination

included height and body weight measurements, and body mass index (BMI). Blood pressure, medical histories, bleeding and smoking habits, were recorded, heart disease, diabetes, stroke or other neurological disorders or depression; significant medication use beta-blockers, inhaled beta agonists, hormonal contraceptives, corticosteroid use within prior three months, psychotropic medication use within prior eight weeks; psychiatric hospitalization within past year; was confirmed at the beginning of the study.

Blood collection and sample preparation

After the Institutional Ethical committee (IEC) cleareance, 10ml blood was withdrawn in serum separation vials from selected subjects after overnight fasting with dry disposable syringe and needle by venous puncture under aseptic conditions. Serum was separated after 30 minutes by centrifuging at 3000 rpm for 10 minutes; this sample was then used for all the assays.

Hormonal Analysis

All the tests were performed using commercially available enzyme immunoassay kits (from Krishgen Biosystems, Mumbai, India). The level of the hormones in serum sample of the subjects was determined by ELISA.

Thyroid hormone analysis

The specific thyroid hormone (TSH, T3, and T4) enzyme linked immunosorbent assay (ELISA) applies quantitative sandwich immunoassay. The microtiter plate was pre-coated with a monoclonal antibody specific for the hormone. Standards, samples and control (25µL) were added to the microtiter plate wells and the hormone if present binds to the antibody pre-coated wells. In order to quantitatively determine the amount of hormone present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for the hormone was added (100 µL) to each well to sandwich the hormone immobilized on the plate. The microtiter plate was incubated (60 minutes), and then the wells were thoroughly washed by working washing solution 5 times (300 µL) to remove all unbound components. TMB (3, 3', 5, 5' tetramethyl-benzidine) substrate solution was then added (100 µL) to each well. The enzyme (HRP) and substrate were allowed to react for a short incubation period in dark (20 minutes). Only those wells that contain the specific hormone and enzyme-conjugated antibody for it exhibit a change in color. The enzyme substrate reaction is terminated by the addition of the stopping reagent (150 µL) (1 N

sulphuric acid solution) and the color change was measured by the ELISA reader at a wavelength of 450 nm.[16, 17]

Stress hormone analysis

The stress hormones (cortisol, prolactin) immunoassay was performed using competitive microplate enzyme immunoassay. Plate coated with anti-cortisol antibodies was used. Serum reference, patient specimens and control (25 µL) was first added to the microplate well. Enzyme- conjugate (100µL) was added. The conjugate binds with antibody coated microplate to form an antigen-antibody complex. Unbound conjugate was removed by working washing solution 5 times (300 µl each time). The enzyme activity in the antibody-bound fraction is inversely proportional to the native stress hormone concentration. The enzyme activity was revealed by a color change in TMB-Substrate solution (100µL). The plate was incubated for 20-30 minutes at room temperature in the dark. Stop Reagent (150µl) was added into each well at the same timed intervals and absorbance was taken by the ELISA reader at 450nm.[18,19]

Cytokine Analysis

Proinflammatory Cytokines

The procedure is an enzyme-linked immunosorbent assay for quantitative detection of human proinflammatory cytokine (TNF-α, IFN-γ) in cell culture supernatants, human plasma (EDTA, heparin and citrate), serum, cerebrospinal fluid, urine, synovial fluid or other body fluids. Two-fold serial dilution of standards (2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml 125pg/ml, 62.5pg/ml, and 31.3pg/ml) and samples (100 µl) was pipette into the wells. The plate was incubated for 2 hours at room temperature then washed with working washing solution 4 times (300µl each time) to remove unbound labeled antibodies. Detection antibody (100 µl) was pipette to the wells. The plate was incubated for 2 hours at room temperature and were again washed using same working washing solution following same procedure. Streptavidin-HRP (100 µl) was pipette to the wells. The plate was incubated for 30 minutes at room temperature and were again washed using same working washing solution. TMB substrate solution was added (100 µl) to the wells, resulting in color development proportional to the amount of specific cytokine bound. The plate was incubated for 15 minutes at room temperature in dark. The stop reagent changes the color from blue to yellow, and the intensity of the color was measured at 450 nm.

Anti-inflammatory cytokines

Human anti-inflammatory cytokines (IL-4, IL-6, IL-10) ELISA assay employs an antibody specific for human anti-inflammatory cytokine coated on microtiter plate. Two-fold serial dilution of standards (2000 pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml and 31.5pg/m), samples (50 µl) and biotinylated anti-human specific cytokine (50µl) was pipette into the wells. The plate was incubated for 1 hour 30 minutes at room temperature. Cytokine present in a sample is captured by the antibody immobilized to the wells and by the biotinylated specific detection antibody wells were washed with working washing solution 5 times (300µl each time) to remove unbound labeled antibodies. HRP-conjugated streptavidin (100 µl) was pipetted to the wells. The plate was again incubated for 30 minutes at room temperature and were again washed using same working washing solution following same procedure. Following the second wash step, TMB substrate solution (50 µl) was added to the wells, resulting in color development propoportional to the amount of cytokine bound. The plate was incubated for 20 minutes at room temperature in dark. The stop solution (25µl) changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

STATISTICAL ANALYSIS

Statistical analysis were carried out by using the statistical packages for GraphPad Prism 6.0 for Windows (GraphPad Software Inc. California, CA, USA). Mean and standard deviation (SD) were calculated for continuous variables. The group size was small t-test was used to assess the differences of the variables. One tailed p values were considered statistically significant at $p<0.0001$.

RESULTS

Thyroid Hormones

The changes in thyroid hormones of patients are compared with the control. Data is represented in Mean ± SEM (n = 100). Values among thyroid patients are significantly higher in case of TSH ($P< 0.0001$) and significantly not different in case of T3 ($P< 0.1116$) and T4 ($P< 0.0773$) from control Table. 1. The values are illustrated in Fig. 1.

Table 1. Comparative values of thyroid hormones of patients with normal healthy controls.

S. No.	Groups	TSH (µIU/ml) Mean ± SEM	T3 (ng/ml) Mean ± SEM	T4 (nmol/l) Mean ± SEM
1.	Control	2.11 ± 0.09	0.83 ± 0.02	83. ± 09
2.	Patients	9.30 ± 0.83	0.97 ± 0.11	91.81 ± 5.85

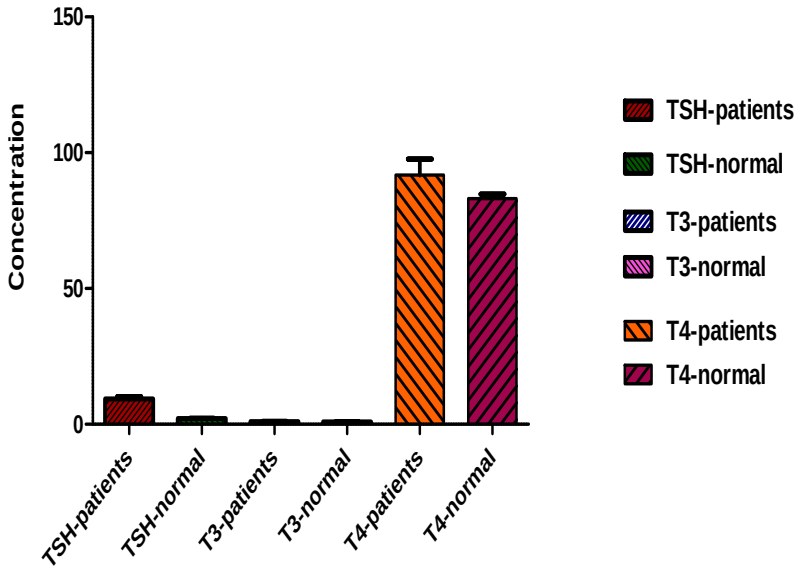

Fig. 1. Mean concentration of thyroid hormones

Stress Hormones

The changes in stress hormones cortisol among patients as compared with normal healthy control in Table. 2. Data is represented in Mean ± SEM (n = 100) are highly significantly higher (P< 0.0001) from normal healthy control. (Fig. 2. represents the mean ± sem of the hormone).

Table 2. Data representing values of stress hormones

S. No.	Groups	Cortisol (nmol/l) Mean ± SEM	Prolactin (mIU/l) Mean ± SEM
1.	Control	289.30 ± 17.12	115.50 ± 19.69
2.	Patients	471.4 ± 29.07	168.90 ± 10.51

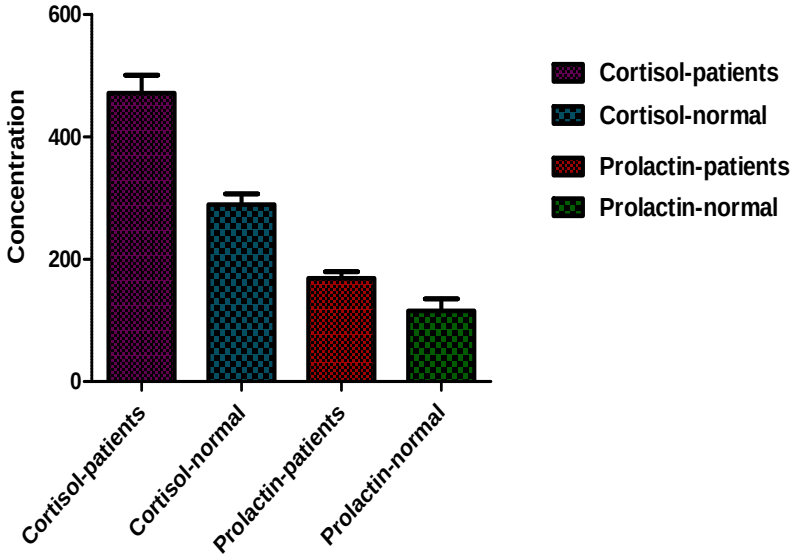

Fig. 2. Mean ± sem values of stress hormones.

Cytokines

Anti-Inflammatory Cytokines and Pro-inflammatory Cytokines

The level of changes in cytokines of thyroid patients as compared with that of the normal healthy control. (Data represented in Mean ± SEM (n = 100)) are highly significantly different (P< 0.0001) from normal healthy control Table. 3.

It has been also observed that the level of pro-inflammatory cytokines value (TNF-α, IFN-γ) was significantly raised as comparison to anti-inflammatory cytokines (IL-4, IL-6 and IL-10) Fig. 3.

Table 3. Values of anti-inflammatory and pro-inflammatory cytokines

S. No.	Groups	†IL – 4 (pg/ml) Mean ± SEM	†IL – 6 (pg/ml) Mean ± SEM	†IL – 10 (pg/ml) Mean ± SEM	*TNF – α (pg/ml) Mean ± SEM	*IFN – γ (pg/ml) Mean ± SEM
1.	Control	545.6 ± 21.64	147.70 ± 8.11	82.03 ± 3.59	553.9 ± 21.75	265.20 ± 10.4
2.	Patients	1006 ± 36.64	73.78 ± 6.56	51.63 ± 5.62	1193.0 ± 32.51	1081.00 ± 33.8

† *Anti-inflammatory cytokines*, * *Pro-inflammatory cytokines*

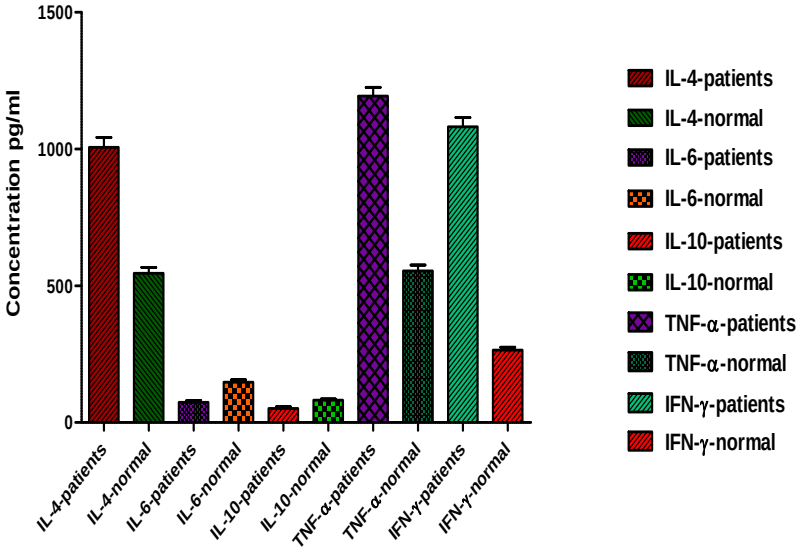

Fig. 3. Concentration of anti and pro-inflammatory cytokines

DISCUSSION

The elevated basal levels of stress hormones like cortisol and prolactin have shown association with chronic stress suppress immunity by directly affecting cytokine profiles. Cytokines are communicatory molecules produced primarily by immune cells.[20] Proinflammatory cytokines mediate acute inflammatory reactions. Th1 cytokines mediate cellular immunity by stimulating natural killer cells and cytotoxic T cells, immune cells that target intracellular pathogens (e.g., viruses). Finally, Th2 cytokines mediate humoral immunity by stimulating B cells to produce antibody, which "tags" extracellular pathogens (e.g., bacteria) for removal. In a meta-analysis of over 30 years of research, intermediate that stressors in life style could promote a Th2 shift (i.e., an increase in Th2 cytokines relative to Th1 cytokines).[21] A Th2 shift has the effect of suppressing cellular immunity in favor of humoral immunity. In response to more chronic stressors (e.g., long-term caregiving for a dementia patient), proinflammatory, Th1, and Th2 cytokines become dysregulated and lead both to suppressed humoral and cellular immunity.[22] Intermediate and chronic stressors are associated with slower wound healing and recovery from surgery, poorer antibody responses to vaccination, and antiviral deficits that are believed to contribute to increased vulnerability to viral infections (e.g., reductions in natural killer cell cytotoxicity.[23]

Immune challenges such as infections with bacteria release bacterial lipopolysaccharides (LPS), which induce the nuclear factor (NF) kB mediated secretion of IL-1 and IL-6, which stimulate the hypothalamic stress response.[6, 7, 24] Immune responses are regulated by antigen

presenting cells (APC), such as monocytes/macrophages, dendritic cells, and other phagocytic cells that are components of innate immunity, and by the helper T-lymphocytes subclasses Th1, Th2, and Treg that are components of adaptive immunity. Homeostatis within the immune system is largely dependent on cytokines, the chemical messengers between immune cells, which play crucial roles in mediating inflammatory and immune responses. It has also been found that increase in cytokine like (IFN)-gamma, TNF-alpha lead to cell-mediated immunity; whereas increase in IL-4, TL-10 leads to the cell stimulation enhance humoral immunity.[25, 26] Naïve T cells (Th0) are precursors of Th1 and Th2 cells, and IL-12 (produced by APCs) is the major inducer of Th1 differentiation and hence, cellular immunity. Thus, IFN-gamma inhibit Th2, whereas IL-4 and IL-10 inhibit Th1 cell activities. IL-4 and IL-10 promote humoral immunity by stimulating the growth and activation of mast cells and eosinophils, the differentiation of B cells into antibody secreting B cells, and immunoglobulin switching to IgE. Importantly, these cytokines also inhibit macrophage activation, T-cell proliferation, and the production of proinflammatory cytokines.[27, 28]

We have found the significant increase in level of TSH and decrease status in T3 and T4 hormone. As thyroid hormones makes and stores hormones that help regulate the heart rate, blood pressure, body temperature, and the rate at which food is converted into energy.[29] It achieves this by manufacturing the hormones, thyroxine (T4) and triiodothyronine (T3) and secreting them into the blood stream. Thyroid stimulating hormone (TSH) secreted by the pituitary gland stimulates the release of other thyroid hormones T4 and T3 which further enhances all the cells of the body to metabolize at a faster rate.[30] On a broad extent two types of hypothyroidism has been studied, primary hypothyroidism caused by decreased production of T4 and T3 due to thyroid dysfunction increase production of TSH; by pituitary (TSH) or hypothalamic (TRH) disease.[31, 1]

Cellular and metabolic processes of the body modulate inflammatory processes of the immune system and can induce major changes in the downstream cytokine. They are effector molecules that can instantly alter the quality of the immune response in the autoimmune diseases. Intervention increase stress hormone levels which interfere beneficial effects of stress-related mediators, such as protection from cytokine-mediated shock syndrome or prevention of autoimmunity.[32]

Hypothyroidism is generally caused by an attack on the thyroid gland it results in inflammation and damage of the thyroid cells.[33] We have seen changes due to hormone

involving both stress and thyroid hormones, among these prolactin, cortisol, thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) are the major.[34] Hypothyroidism is routinely considered in the differential diagnosis of depressive and anergic states, and is screened for with determinations of serum thyroxine (T4), trio dothyronine (T3), and basal thyroid-stimulating hormone (TSH).[35] Thyroid failure with its predilection for behavioral presentation is much more likely to manifest as depression or lack of energy to a psychiatrist.

In HT, cell-mediated immunity promotes the induction of auto-antibodies and self-reactive T cells against Tg, and other auto-antigens, including thyroid peroxidase. HT is characterized by infiltration of lymphocytes and other immune cells, thyroid enlargement and fibrosis, and progressive destruction of thyrocytes that eventually results in hypothyroidism.[36] Upon initiation of the immune response to Tg, thyroid-specific T lymphocytes migrate to the thyroid and through interferon (IFN)-g production induce thyrocyte expression of major histocompatibility complex (MHC) class-II molecules. This results in further expansion of autoreactive T cells and the inflammatory response leading to the accumulation of activated CD4 + and CD8 + T cells, B cells, plasma cells, and macrophages in the thyroid.

Cytokines are involved in common endocrine diseases, such as diabetes mellitus and autoimmune thyroid disease (ATD). The hormones mediate the differentiation of Th0 (naïve T Helper cells) towards the Th2 humoral immune response to the detriment of the Th1 cell-mediated response. APC's secrete cytokines that mediate Th1 differentiation, however the presence of bacterial products such as LPS that bind to Toll-like Receptors induce the production of IL-1 and IL-6, which cross the blood-brain barrier and trigger the hypothalamic CRH-stress response.[37] In this manner, a blood borne stressor of infectious nature can activate the HPA axis. Th1 effects are mediated by the cytokines IL-12,18,2 and γInterferon and T cells and Macrophages. Th2 responses are mediated by IL-4,6,13 and B Cells, Eosinophils and Mast Cells. CRH: Corticotropin releasing Hormone; NE: Norepinephrin; Th0: Naïve Helper cells; APC: Antigen Presenting Cell; LPS: Lipopolysaccharide; HPA: Hypothalamic-Pituitary-Adrenal Axis.[38]

The defense system is an example of subtle autoregulation which is intervened with medications. The therapeutical use of this intervention is called 'immunomodulation'.[39] Variation in the hormones of one of the complete body system affects its metabolism as well as the body's complete immune system. In early 1930s, evidences has supported that

prolactin and cortisol play a prominent role in stimulation and modulation of immune function. An immediate immune response occurs through small cell signaling by numerous cells of the immune system. Cytokines encompasses all these cells and a large and diverse family of regulators produced throughout the body by cells. Cytokines refer to immunomodulating agents, trigger inflammation and respond to infections.[40]

Cytokines include two classes of it pro and anti inflammatory. We have found stress hormone changes stress system activity through modulating pro or anti inflammatory cytokines. Increase in significant level of pro-inflammatrative cytokines of TNF-α and IFN-γ and significant decrease in IL-4 and IL-10. Stress hormones changes stress system activity through modulating pro or anti-inflammatory cytokines, TNF-α, IL-6, IFN-γ, IL-4 and IL-10, balance by stimulating or suppressing the progression of autoimmune diseases.

Studies from humans and animal models have revealed significant new insights into the complex role of cytokines in the pathogenesis of AITD. Modulating cytokine responses have yielded highly encouraging results and they hold considerable promise in the treatment of autoimmune diseases. Pro-inflammatory cytokines such as GM-CSF and IL1b can contribute to Foxp3 + Treg expansion, whereas a regulatory suppressor cytokine such as TGF-b can initiate a pathogenic Th17 T cell response. These observations highlight the paradoxical effects of cytokines and their critical roles in maintaining a delicate balance between health and disease. Therefore, additional studies to understand the complex interplay between different cytokines and their effects on the different components of the immune system in the context of a particular disease are essential.

CONCLUSION

Present era is full of stress that to of environmental, psychological, physiological and physical stress and all of it compound to produce severe impact. Any of above if dominates leads to affect our body physiology directly. Surprisingly out of the above psychological stress, life style pattern and physiological interaction leads to progressive disturbance in producing disease syndrome.

Study interprets a significant rise in stress hormones like cortisol and prolactin, leading to immune regulation of body homeostasis. We have found that stress hormone after recognizing the immune cells which promote the mother T and B cells and Th cells type Th1 and Th2 are the most affected. The balance of Th1 and Th2 has been disturbed leading to

autoimmune process. Increase in cytokines alpha-TNF and gamma interferon attacked on thyroid cells destruction and predominates for Th2 mediated immune response promoting antigen specific B cells. These B cells produce anti-TSH receptor antibodies leading to hypothyroidism.

In addition to above we also found that compound effect of stress, immunity and endocrine system, they are directly linked and in adverse conditions these leads to autoimmune diseases like HT. Due to above combination a lower response of T4 hormone leads to low BMR and glucose absorption rate in digestive tract, due to low BMR and inappropriate glucose absorption has lead to hyperlipidemia posing high response blood pressure and other cardiovascular diseases.

The study concludes that to regulate the hypothyroidism like autoimmune disorder one has to take proper care of compound stress increasing day by day particularly in immune suppression autoimmune conditions. Supportive and suggestive combination therapy in addition to regular treatment will benefit the patient of hypothyroidism because stress, immunity and infection play a major role not only hypothyroidism but may lead to other autoimmune diseases.

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CONFLICT OF INTEREST

There are nonfinancial competing interests (political, personal, religious, ideological, academic, intellectual, commercial, or any other) to declare in relation to this manuscript.

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