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Original Research Article

Analysis and establish a correlation between serum ceruloplasmin, serum apelin level and thyroid profile in patients with hypothyroidism and healthy controls

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ABSTRACT

Background: Hypothyroidism is an endocrine disorder resulting from deficiency of thyroid hormone. It is often the primary process in which the thyroid gland produces insufficient amounts of thyroid hormone. It can also be secondary, i.e., lack of thyroid hormone secretion due to the failure of either adequate thyrotropin (TSH) secretion from the pituitary gland or thyrotropin-releasing hormone (TRH) from the hypothalamus (secondary or tertiary hypothyroidism). The patient's appearance may vary from asymptomatic to, rarely, coma with multisystem organ failure (myxoedema coma).
Methods: The present observational descriptive, cross sectional study has been conducted on 120 newly diagnosed Subclinical Hypothyroidism patients of 20-50 years age group of both sex attending the OPD of department of medicine, RVRS medical college and associated group of hospitals, Bhilwara from May 2021 to May 2022. Diagnosis of thyroid disorder has been made according to the criteria recommended by the European thyroid association guidelines-2013. The result has been compared with age and gender matched 120 euthyroid subjects acting as controls. Detailed history of participants including age, history of any medications, addictions has been taken. Written consent from all the subjects has been obtained for the study.
Results: The mean serum FT3 level was found to be slightly decreased in Subclinical hypothyroid subjects (group I) as compared to healthy controls (group II) but the difference was statistically significant ($p<0.0001$). The mean serum FT4 level was found to be slightly decreased in Subclinical hypothyroid subjects (group I) as compared to healthy controls (group II) but the difference was statistically significant ($p<0.0001$). A highly significant increase ($p<0.0001$) in mean serum TSH level has been observed in subclinical hypothyroid subjects (group-I) when compared to controls (group- II). In subclinical hypothyroid subjects, mean serum ceruloplasmin levels were found to be significantly lower in comparison to healthy subjects ($p<0.0001$).
Conclusions: The overall findings of the present study thus confirm that serum Apelin level is significantly higher in subclinical hypothyroid subjects and serum ceruloplasmin level is significantly lower in the subclinical hypothyroid subjects. However, further experimental and observational studies are needed to illustrate the role of serum ceruloplasmin and serum apelin in subclinical hypothyroidism.

Keywords: Hypothyroidism, Apelin, Ceruloplasmin, T3, T4, TSH

INTRODUCTION

The role of the thyroid gland is the synthesis and release tetraiodothyronine (T4) and triiodothyronine (T3), which are known as thyroid hormones.[1] Some functions of thyroid hormones are as follows: increasing the basal metabolic rate and oxygen consumption, playing a key role in body temperature homeostasis and energy balance; increasing catecholamine sensitivity by increasing the number of catecholamine receptors in the heart, having positive inotropic and chronotropic effects; inducing osteoporosis by increasing cortisol production and bone turnover; increasing gastrointestinal motility; increasing the contraction and relaxation speed of striated muscle; and controlling the body energy balance and normal growth.[2-4] Thyroid dysfunctions may be classified as hyperthyroidism, hypothyroidism, subclinical hyperthyroidism and subclinical hypothyroidism, according to the level of thyroid homones.[5] Hypothyroidism is the second most common endocrine disease, after diabetes. Typical findings of thyrotoxicosis are weight loss, reduction of fat and muscle mass, discharge of fat stores and the decrease in some serum lipids.[6,7] In hypothyroidism, the oxygen consumption, basal metabolic rate and lipolysis decrease, and the serum triglyceride and cholesterol levels increase. In hypothyroidism, the body weight increases, while thyrotoxicosis is generally associated with weight loss.[8]

Subclinical hypothyroidism (SCH) can be defined as a state of high serum thyroid stimulating hormone (TSH) levels (less than 10 μ IU/L) with normal serum free thyroxine (FT4) levels. Today subclinical hypothyroidism is a common biochemical finding in the general population. Overall, the population prevalence of subclinical hypothyroidism is around 3%-8%. Furthermore, SCH has been found to be more usual in the elderly population[9]. Especially in women and increases with increasing age and is found to be higher in white than in black population.[10] The Whickham survey specifically seems to determine the incidence of thyroid disease in the general population.[11]

Subclinical hypothyroidism (SCH) is asymptomatic in general but it may progress to overt hypothyroidism.[12] It may be associated with manifestations that can be supervised by treatment.[13] However, symptoms suggestive of thyroid hormone deficiency may be present in 30% of patients.[14-16] There may be symptoms like muscle cramps, constipation, puffy eyes, cold intolerance, hoarseness of voice, tiredness, depression, neuromuscular symptoms and menstrual abnormalities.[17]

SCH may be accompanied with a modest increase in the risk of coronary heart disease and mortality, particularly in subjects with higher TSH levels. Some studies reported hyperlipidemia and dyslipidemia in SCH.[18] Dyslipidemia accelerates the atherogenicity and this is seen in the SCH.[19] The condition is also associated with presence of inflammatory marker (CRP). Several studies have demonstrated significantly higher hs-CRP levels in patients with SCH.[20] Ceruloplasmin is a ferroxidase enzyme that in humans is encoded by the CP gene. Ceruloplasmin is the crucial copper- carrying protein in the blood, and in addition plays a role in iron metabolism.

Apelin (also known as APLN) is a peptide that in humans is encoded by the APLN gene. Apelin is the endogenous ligand for the G-protein-coupled APJ receptor that is manifested at the surface of some cell types. It is widely expressed in numerous organs such as the heart, lung, kidney, liver, adipose tissue, gastrointestinal tract, brain, adrenal glands, endothelium, and human plasma. Apelin gene encodes a pre-proprotein of 77 amino acids, with a signal peptide in the N-terminal region. After translocation into the endoplasmic reticulum and cleft of the signal peptide, the proprotein of 55 amino acids may generate several active fragments: a 36 amino acid peptide corresponding to the sequence 42-77 (apelin 36), a 17 amino acid peptide corresponding to the sequence 61-77 (apelin 17) and a 13 amino acid peptide corresponding to the sequence 65-77 (apelin 13).

Conflicting results have been acquired in different studies regarding role of serum apelin and ceruloplasmin in subclinical hypothyroidism. Various Studies have showed changes in serum apelin and ceruloplasmin levels in subclinical hypothyroidism but data showing alliance of serum apelin and ceruloplasmin in subclinical hypothyroidism are not fully elucidated, so further research is required. Therefore, the present study has been undertaken to determine serum apelin and ceruloplasmin levels in subclinical hypothyroidism.

Review of literature

Anatomy and histology of the thyroid gland

The thyroid is one of the largest of the endocrine organs, weighing approximately 15 to 20 g. It is located anterior to the trachea among the cricoids cartilage and the suprasternal notch. The normal thyroid is made up of two lobes joined by a thin band of tissue, the isthmus.

Two pairs of vessels establish the major arterial blood supply, the superior thyroid artery, arising from the external carotid artery, and the inferior thyroid artery, rising from the subclavian artery. The thyroid gland develops from floor of the primitive pharynx during the third week of gestation. The developing gland drifts along the thyroglossal duct to reach its final location in the neck. Thyroid hormone synthesis normally begins at about 11 weeks gestation. The thyroid gland consists of numerous spherical follicals composed of thyroid follicular cells that surround the secreted colloid, a proteinaceous fluid containing high amount of thyroglobulin (Tg), the protein precursor of thyroid hormones. The thyroid follicular cells are polarized-the basolateral surface is opposed to the blood stream and the apical surface, faces the follicular lumen. The follicular

cells differentiate in height with the degree of glandular stimulation, becoming columnar when active and cuboidal if inactive. From 20 to 40 follicles are demarcated by connective tissue septa to form a lobule supplied by a single artery. The endoplasmic reticulum is composed of a network of wide irregular tubules that contain the precursor of thyroglobulin. The carbohydrate moiety of thyroglobulin is added to this precursor in the Golgi apparatus which is located apically. Lysosomes and mitochondria are scattered throughout, the cytoplasm. Stimulation by TSH results in enlargement of the Golgi apparatus, formation of pseudopodia at the apical surface, and the appearance in the apical portion of the cell of many droplets that contain colloid taken up from the follicular lumen.

Thyroid hormone synthesis

Large reduction of iodine cause an inhibition of the formation and release of thyroid hormone, this inhibition is known as the Wolff-Chaik off effect.[21]

Thyroid hormones formations have been divided into the following stages: [22] 1. Iodinetraping, 2. Organification (Iodination), 3. Coupling, 4. Storage and 5. Secretion.

In the thyroid gland iodide is oxidized to iodine and to bound carbon 3 position of the tyrosine residues that are part of the thyroglobulin molecule in the colloid. Thyroglobulin, synthesized in the thyroid cells and secreted in the colloid by exocytosis of granules, also contain thyroid peroxidase, the enzyme that catalyses the oxidation of iodide and its binding.

In the process of hormone synthesis, the first product is monoidotyrosine (MIT). MIT is next iodinated in 5' position to form diiodotyrosine (DIT) molecules then undergo an oxidative condensation to synthesize T4 with the elimination of alanine side chain from the molecule that form the outer ring. T3 is probably formed by condensation of MIT with DIT. A minute amount of reverse T3 is also formed. About 93 per cent of the metabolically active hormones secreted by the thyroid gland is thyroxine, and 7 per cent triiodothyronine. Anyhow, almost all the thyroxine is eventually changed to triiodothyronine in the tissues, so that both are functionally important. The functions of these two hormones are qualitatively the same, but they differ in rapidity and intensity of action. Triiodothyronine is about four times as potent as thyroxine, but it is present in the blood in very smaller quantities and persists for a much shorter time than does thyroxine. To get released into circulation, thyroxine and triiodothyronine must first be carved from the thyroglobulin molecule. The apical surface of the thyroid cells sends out pseudopod extensions that close around small portions of the colloid to form pinocytic vesicles that enter the apex of the thyroid cell. Then lysosomesin the cell cytoplasm immediately fuse with these vesicles to form digestive vesicles containing digestive enzymes from the lysosomes mixed with the colloid. Multiple proteases among the enzymes digest the thyroglobulin molecules and release thyroxine and triiodothyronine in free form. These then circulated through the base of the thyroid cell into the surrounding capillaries. Thus, the thyroid hormones are released into the blood. About three quarters of the iodinated tyrosine in the thyroglobulin never becomes thyroid hormone but resides as monoiodotyrosine and diiodotyrosine.[23]

During the digestion of the thyroglobulin molecule to cause discharge of thyroxine and triiodothyronine, these iodinated tyrosines also are freed from the thyroglobulin molecules. However, they are not released into the blood. Instead, their iodine is cleaved from them by a deiodinase enzyme that makes virtually all this iodine available again for recycling within the gland for forming additional thyroid hormones. In the congenital absence, this deiodinase enzyme, many persons become iodine-deficient because of failure of this recycling process.

Mechanism of action of thyroid hormones

The mechanism of action of thyroid hormones at the cellular level are: 1. Mitochondrialactivation, 2. Stimulation of Na+, K+-adenosinetriphosphatase ATPase activity, 3. Stimulation of cell membrane functions, probably through a specific receptor and 4. Interaction with adrenergic system.

Hypothyroidism

Hypothyroidism develop when there are insufficient levels of thyroid hormones to provide metabolic needs at the cellular level. It effects females about 2-8 times as often as males.[24]

Symptoms of hypothyroidism include: Fatigue, weight gain, loss of energy, decreased appetite, cold intolerance, dry skin, hair loss, muscle pain, joint pain, mental impairment, impaired memory and menstrual disturbances.

The physical sign of hypothyroidism include: hypothermia, goiter, bradycardia and myxedema.

Metabolic abnormalities allied with hypothyroidism include anemia, dilutional hyponatremia, hyponatremia reversible increase in creatinine.[25]

Acquired hypothyroidism

Acquired hypothyroidism is a condition where the thyroid gland make too little or no thyroid hormone.[26]

Acquired hypothyroidism can be evolved by thyroid disease (Primary hypothyroidism), a decrease in thyroid hormone secretion caused by a damaged, defective or absent thyroid gland; and (Hypothalamic-pituitary disease) or (Secondary hypothyroidism) and a decline of

the mechanism that stimulate thyroid stimulating hormone (TSH) synthesis, secretion and biologic action. There are considerable causes associated with acquired hypothyroidism: chronic autoimmune thyroiditis, drug lured hypothyroidism, endemic goiter, irradiation of thyroid, surgical excision, subacute thyroiditis; transient phase.[24]

Sub clinical hypothyroidism

Sub clinical hypothyroidism occurs when thyrotropin (TSH) levels elevated but thyroxin (T4) and triiodothyronine (T3) levels are regular.[27]

Although, subclinical hypothyroidism is generally asymptomatic, potential risk which are associated with this condition includes progression to overt hypothyroisim, cardiovascular effects, hyperlipidemia and neuropsychiatric effects.[28]

Other terms for this condition are mild hypothyroidism, preclinical hypothyroidism, biochemical hypothyroidism, and depleted thyroid reserve. The TSH elevation in such patients is modest, with values typically between 5 and 15 mU/L. This syndrome is most often seen in patients with early Hashimoto's disease and is a common phenomenon, occurring in 7% to10% of older women.

Ceruloplasmin

Ceruloplasmin (or caeruloplasmin) is a ferroxidase enzyme that in humans is encoded by the CP gene.

Ceruloplasmin is the major copper-carrying protein in the blood, and in addition plays a role in iron metabolism. It was first described in 1948. Another protein, hephaestin, is noted for its homology to ceruloplasmin, and also participates in iron and probably copper metabolism.

Function

Ceruloplasmin is an enzyme synthesized in the liver containing 6 atoms of copper in its structure. Ceruloplasmin carries more than 95% of the total copper in healthy human plasma. The rest is accounted for by macro-globulins. Ceruloplasmin exhibits a copper-dependent oxidase activity, which is associated with possible oxidation of Fe2+ (ferrous iron) into Fe3+ (ferric iron), therefore assisting in its transport in the plasma in association with transferrin, which can carry iron only in the ferric state. The molecular weight of human ceruloplasmin is reported to be 151kDa.

Regulation

A cis-regulatory element called the GAIT elementis involved in the selective translational silencing of the Ceruloplasmin transcript. The silencing requires binding of a cytosolic inhibitor complex called IFN-gamma-activated inhibitor of translation (GAIT) to the GAIT element.

Clinical significance-Mechanisms of low ceruloplasmin levels, gene expression genetically low (aceruloplasminemia), copper levels are low in general, malnutrition/trace metal deficiency in the food source Zinc toxicity, due to induced copper deficiency, copper does not cross the intestinal barrier due to ATP7A deficiency (Menkes disease and Occipital horn syndrome), delivery of copper into the lumen of the ER-Golgi network is absent in hepatocytes due to absent ATP7B (Wilson's disease).

Like any other plasma protein, levels drop in patients with hepatic disease due to reduced synthesizing capabilities-

Copper availability doesn't affect the translation of the nascent protein. However, the apoenzyme without copper is unstable. Apoceruloplasmin is largely degraded intracellularly in the hepatocyte and the small amount that is released has a short circulation half life of 5 hours as compared to the 5.5 days for the holo-ceruloplasmin.

Mutations in the ceruloplasmin gene (CP), which are very rare, can lead to the genetic disease aceruloplasminemia, characterized by hyper-ferritinemia with iron overload. In the brain, this iron overload may lead to characteristic neurologic signs and symptoms, such as cerebellar ataxia, progressive dementia, and extrapyramidal signs. Excess iron may also deposit in the liver, pancreas, and retina, leading to cirrhosis, endocrine abnormalities, and loss of vision, respectively.

Deficiency

Lower-than-normal ceruloplasmin levels may indicate the following: Wilson disease (a rare [UK incidence 2/100,000] copper storage disease). Menkes disease (Menkes kinky hair syndrome) (rare-UK incidence 1/100,000), Copper deficiency, aceruloplasminemia, zinc toxicity.

Excess greater-than-normal ceruloplasmin levels may indicate or be noticed in: copper toxicity / zinc deficiency, pregnancy, oral contraceptive pill use, lymphoma, acute and chronic inflammation (it is an acute-phase reactant), rheumatoid arthritis, angina, Alzheimer's disease, schizophrenia and obsessive-compulsive disorder.

Aims and objectives

To study the serum ceruloplasmin levels in early diagnosed patients with subclinical hypothyroidism. To compare serum ceruloplasmin levels among the groups. To study the serum Apelin levels in early diagnosed patients with subclinical hypothyroidism. To compare serum Apelin levels among the groups

METHODS

It is an observational descriptive, cross sectional, hospital based study. The purpose of this study is to evaluate the status of serum apelin and ceruloplasmin levels in early diagnosed patients with subclinical hypothyroidism and to establish possible relationship between them. The present study has been conducted on 120 newly diagnosed Subclinical Hypothyroidism patients of 20-50 years age group of both sex attending the OPD of department of medicine, RVRS medical college and associated group of hospitals, Bhilwara from May 2021 to May 2022. Diagnosis of thyroid disorder has been made according to the criteria recommended by the European Thyroid association guidelines-2013. The result has been compared with age and gender matched 120 euthyroid subjects acting as controls. Detailed history of participants including age, history of any medications, addictions has been taken. Written consent from all the subjects has been obtained for the study. Blood samples has been collected from antecubital vein by venipuncture in plain vials. Serum has been separated by centrifugation at 2500 rpm for 10 minutes.

Inclusion criteria

Persons suffering from hypothyroidism were included in the study.

Exclusion criteria

Persons with coronary artery disease, diabetes or those with stroke and whomsre Smokers/alcohol users. Persons with Thyroid supplementation and antithyroid agents, drugs that affect Serum apelin and ceruloplasmin level and pregnant women also excluded..

Estimation of serum apelin

Estimation of serum apelin by enzyme linked immunosorbent assay method (ELISA).

Principle

This assay is based on the competitive EIA principle. Each well of the supplied microtiter plate has been pre-coated with an anti- rabbit antibody. An antibody specific to the target antigen (the capture antibody) is then added to each well and binds to the anti-rabbit antibody. Next a biotinylated synthetic antigen peptide is added to each well along with either the non-biotinylated standard peptide or the test sample. The biotinylated synthetic antigen peptide competes with the non-biotinylated standard peptide or native antigen in the sample to bind with the capture antibody. Unbound antigen and peptide is washed away. An Avidin-Horseradish peroxidase (HRP) conjugate is then added which binds to the biotin. Unbound HRP-conjugate is washed away. A TMB substrate is then added which reacts with the HRP enzyme resulting in color development. A sulfuric acid stop solution is added to terminate color development reaction and then the optical density (OD) of the well is measured at a wavelength of 450±2 nm. The OD of an unknown sample can then be compared to an OD standard curve generated using known antigen concentrations in order to determine its antigen concentration. In contrast to typical Sandwich ELISA assays, in competition assays the greater the amount of antigen in the sample, the lower the color development and optical density reading.

Reagents

Reagents used were-Coated 96-well strip plate-1, standard (Lyophilized)-2 vials, assay diluent (5x)-1 vial x 15 ml, capture antibody (Lyophilized)-2 vials, biotinylated peptide (Lyophilized)-2 vials, HRP-Streptavidin Conjugate (100x)-1 vial x 600 µl, positive Control (Lyophilized)-1 vial, wash buffer (20x)-1 vial x 25 ml, TMB Substrate-1 vial x 12 ml, stop solution-1 vial x 8 ml, adhesive plate sealers- 4 and instruction manual.

Reagent preparation

Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.

1x wash buffer: If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved. Prepare 400 ml of working wash buffer by diluting the supplied 25 ml of 20x wash buffer concentrate with 375 ml of deionized or distilled water. Wash buffer can be stored at 4°C once prepared.

1x assay diluent: Prepare 75 ml of assay diluent by diluting the supplied 15 ml of 5x assay diluent concentrate with 60 ml of deionized or distilled water. Assay diluent can be stored at 4°C once prepared.

100x capture antibody concentrate: Briefly spin down the capture antibody and add 55 µl of 1x assay diluent. The capture antibody concentrate can be stored at 4°C for 5 days.

1x capture antibody solution: calculate the volume of capture antibody solution needed for your particular experiment and prepare that volume by diluting the capture antibody concentrate 100-fold (1:100) with 1x assay diluent. Capture antibody Solution must be prepared fresh for each experiment and cannot be stored.

Working biotinylated peptide solution (100 ng/ml): for use in control, sample, and standard preparation. Briefly spin down the lyophilized biotinylated peptide and reconstitute in 20 µl of DD $_{H2O}$. Next combine this 20 µl solution with 10 ml of 1x assay diluent and mixing gently. The final concentration of the Biotinylated Peptide in this solution is 100 ng/ml.

HRP-streptavidin working solution: Gently mix the stock solution before use. Calculate the volume of HRP-streptavidin working solution needed for your particular experiment and prepare that volume by diluting the HRP-Streptavidin conjugate solution 100-fold (1:100) with assay diluent. HRP- Streptavidin working solution must be prepared fresh for each experiment and cannot be stored.

TMB substrate: Using sterile techniques remove the needed volume of TMB substrate solution for the number of wells you are planning to run. Dispose of unused TMB Substrate Solution rather than returning it to the stock container.

Assay procedure

Keep kit reagents on ice during reagent preparation steps. Prepare all reagents, working standards, and samples as directed in the previous sections.

Add 100 µl of 1x capture antibody solution to each well, cover with a plate sealer, and incubate for 1.5 hours at room temperature, or overnight at 4°C, with gentle shaking (1-2 cycles/sec).

Aspirate the liquid from each well and wash 4 times. Wash by adding approximately 200-300 µl of Wash Buffer using a squirt bottle, multi-channel pipette, manifold dispenser or automated washer. Completely remove all liquid between washes by aspiration. After the last wash, aspirat e to remove any remaining Wash Buffer then invert the plate and tap against clean absorbent paper.

Add 100 µl of standard, blank, positive control, or sample per well, cover with a plate sealer, and incubate for 2.5 hours at room temperature, or overnight at 4 °C, with gentle shaking (1-2 cycles/sec). Use assay diluent only for the blank.

Aspirate and wash the wells 4 times as outlined in step 2.

Add 100 µl of HRP-Streptavidin Working Solution to each well and incubate for 45 minutes at room temperature with gentle shaking (1-2 cycles/sec).

Aspirate and wash the wells 4 times as outlined in step 2.

Add 100 µl of TMB Substrate to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec). Monitor periodically until optimal color development has been achieved.

Add 50 µl of Stop Solution to each well and record the total development time. The blue color will change to yellow. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as the substrate solution.

Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.

Calculation of results

Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance=(B- blank OD)/(Bo-blank OD), where B=OD of sample or standard, Bo=OD of zero standard (total binding)

Typical data

The following standard curve is an example only and should not be used to calculate results for tested samples. A new standard curve must be generated for each set of samples tested.

Normal range: 2.0-7.0 ng/ml

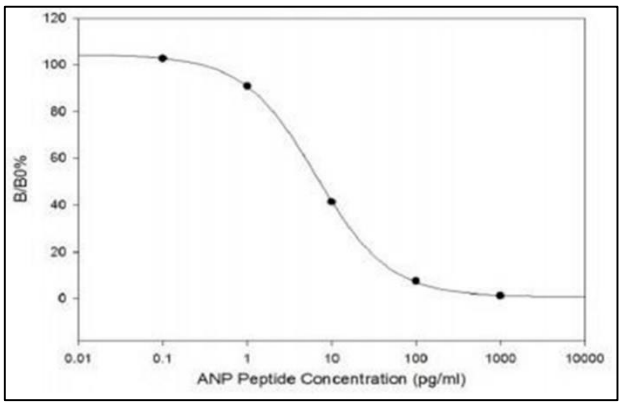

Figure 1: Correlation between percentage absorbance with ANP-peptide concentration.

Estimation of serum ceruloplasmin

Enzyme linked immunosorbent assay method (ELISA) intended use:

This human ceruloplasmin ELISA assay is for the quantitative determination of total ceruloplasmin in human plasma, serum, urine, milk, saliva, and cell culture supernatants.

Principle

Human ceruloplasmin will bind to the affinity purified capture antibody coated on the microtiter plate. After appropriate washing steps, biotin labeled anti-human ceruloplasmin polyclonal antibody binds to the captured

ceruloplasmin. Excess antibody is washed away and bound polyclonal antibody is then reacted with peroxidase conjugated streptavidin. Following an additional washing step, TMB substrate is used for color development at 450 nm. A standard calibration curve is prepared along with the samples to be measured using dilutions of human ceruloplasmin. Color development is directly proportional to the concentration of total ceruloplasmin in the sample.

Reference ranges

Normal blood concentration of ceruloplasmin in humans is 20-50 mg/dL.

RESULTS

Individual with higher BMI, weight and age are more prone to hypothyroidism.

Table 1: Anthropometric parameters of subclinical hypothyroidism and healthy subjects (controls).

Parameters	Group I, subclinical hypothyroidism subjects, mean±SD, (n=120)	Group-II healthy subjects (controls), mean±SD, (n=120)
Age (years)	40.25±11.8	39.50±10.8
Weight (kg)	60.27±3.8	52.58±5.0
Height (cm)	156.0±4.9	154.8±4.5
BMI (kg/m^2)	24.5±2.6	21.9±2.8

Table 2: Biochemical parameters of subclinical hypothyroidism and healthy subjects (controls).

Parameters	Group I, subclinical hypothyroidism subjects, mean±SD, (n=120)	Group-II, healthy subjects (controls), mean±SD, (n=120)
FT3 (pg/ml)	1.820±0.51	2.31±0.60
FT4 (ng/dl)	0.68±0.11	0.80±0.14
TSH (µIU/ml)	8.95±2.0	3.14±1.60
S. apelin (ng/ml)	6.2±1.80	3.1±1.4
S. ceruloplasmin (mg/dl)	14.6±2.1	26.8±6.1

Table 3: Comparison of serum thyroid function (TSH, FT3, FT4 level) of subclinical hypothyroidism subjects and healthy subjects (controls).

Parameter	Group I, subclinical hypo-thyroidism subjects, mean±SD, (n=120)	Group-II, healthy subjects (controls), mean±SD, (n=120)	T statistic 'p' value
FT3 (pg/ml)	1.820±0.51	2.31±0.60	6.816 (<0.0001)
FT4 (ng/dl)	0.68±0.11	0.80±0.14	7.383 (<0.0001)
TSH (µIU/ml)	8.95±2.10	3.14±1.60	24.107 (<0.0001)

*P<0.0001 Highly Significant (HS), p<0.01 significant (S), p>0.05 non significant (NS)

Table 4: Comparison of S. apelin levels of subclinical hypothyroidism subjects and healthy subjects (controls).

Parameter	Group I, subclinical hypo-thyroidism subjects, mean±SD, (n=120)	Group-II, healthy subjects (controls), mean±SD, (n=120)	T statistic, 'p' value*
S. apelin (ng/ml)	6.2±1.80	3.1±1.4	14.89 (<0.0001)

*P<0.0001 highly significant (HS), p<0.01 significant (S), p>0.05 non significant (NS).

Table 5: Comparison of S. ceruloplasmin levels of subclinical hypothyroidism subjects and healthy subjects (controls).

Parameters	Group I, subclinical hypo-thyroidism subjects, mean±SD, (n=120)	Group-II, healthy subjects (ctrl), mean±SD, (n=120)	T statistic 'p' value*
S. ceruloplasmin (mg/dl)	14.6±2.1	26.8±6.1	20.716 (<0.0001)

*P<0.0001 highly significant (HS), p<0.01 significant (S), p>0.05 non-significant (NS).

Patient suffering from hypothyroidism have low T3, T4 and low ceruloplasmin and high TSH and serum apelin level.

The mean serum FT3 level was found to be slightly decreased in Subclinical hypothyroid subjects (group I) as compared to healthy controls (group II) but the difference was statistically significant ($p<0.0001$). The mean serum FT4 level was found to be slightly decreased in subclinical hypothyroid subjects (group I) as compared to healthy controls (group II) but the difference was statistically significant ($p<0.0001$). A highly significant increase ($p<0.0001$) in mean serum TSH level has been observed in subclinical hypothyroid subjects (group-I) when compared to controls (group- II).

In subclinical hypothyroid subjects, mean serum apelin levels were found to be significantly Higher in comparison to healthy subjects ($p<0.0001$)

In subclinical hypothyroid subjects, mean serum Apelin levels were found to be significantly higher in comparison to healthy subjects ($p<0.0001$). In subclinical hypothyroid subjects, mean serum ceruloplasmin levels were found to be significantly lower in comparison to healthy subjects ($p<0.0001$). The overall findings of the present study thus confirm that serum apelin level is significantly higher in subclinical hypothyroid subjects and serum ceruloplasmin level is significantly lower in the Subclinical Hypothyroid subjects

DISCUSSION

In recent years, subclinical thyroidism is unknowingly emerging as a major public health problem in India and it produces an enormous burden on the economy of the country due to high prevalence, risk of progression to overt hypothyroidism and it can lead to adverse cardiovascular consequences.[29] Many studies have shown that serum ceruloplasmin level are affected in thyroid dysfunction like Subclinical hypothyroidism.[30-33]

Mutations in the ceruloplasmin gene (CP), which are very rare, can lead to the genetic disease aceruloplasminemia, characterized by hyperferritinemia with iron overload. In the brain, this iron overload may lead to characteristic neurologic signs and symptoms, such as cerebellar ataxia, progressive dementia, and extrapyramidal signs. Excess iron may also deposit in the liver, pancreas, and retina, leading to cirrhosis, endocrine abnormalities, and loss of vision, respectively.[34]

Ceruloplasmin is a major defence against harmful effects of reactive oxygen species (ROS) in cells, with ahigh capacity to degrade exogenous hydrogen peroxide. Ceruloplasmin is also important in the control of membrane lipid oxidation. Due to oxidative stress and lipid peroxidation serum ceruloplasmin is decreased in subclinical hypothyroidism other than healthy controls.In general, the study found that subclinical hypothyroidism subjects were more likely decrease level of serum ceruloplasmin than the normal healthy subjects. hyroid disorders are very common around the world, and hypothyroidism is the second most common endocrine disease, after diabetes. Thyrotoxicosis occurs less frequently than hypothyroidism, and its prevalence varies between 0.5% and 2%, while both disorders are about 10 times more common in women than in men. The most common cause of hypothyroidism is chronic autoimmune thyroiditis (Hashimoto's thyroiditis). Hypothyroidism due to radioactive iodine treatment and subtotal/total thyroidectomy are next most common causes. The most common cause of thyrotoxicosis is Graves' disease, which has been reported to be responsible for 60-80% of thyrotoxicosis in different societies. Other diseases that cause thyrotoxicosis are toxic nodular goiter (5-10%) and various cases of thyroiditis.[11] The change in the thyroid hormone levels in both directions may affect metabolic, physiological and biochemical processes, and almost all systems of the body.

In cases of adipocyte dysfunction, such as that seen in obesity and lipodystrophy, a change in the release of adipocytokines and metabolic and energy disorders occur.[35] These adipose tissue dysfunctions affect the energy metabolism by affecting thyroid functions.[36] In patients with thyroid dysfunctions, changes in weight, thermogenesis and lipolysis in the adipose tissue occur. Patients with hypothyroidism usually gain weight, and have a reduction in body temperature and metabolic rate; however, in hyperthyroidism, there is weight loss despite an increased appetite and increased metabolic rate. Most of these metabolic differences are due to the changes in the adipose tissue. Thyroid hormones and adipocytokines are affected by body weight, body fat mass, body temperature, insulin resistance, and glucose and lipid metabolism, and also affect these metabolic events. It is known that there are TSH and thyroid hormone receptors in adipose tissue, and apelin receptors have been detected in the thyroid gland. Therefore, changes in the thyroid hormones and TSH may affect the release of adipocytokines, so there is a possible relationship between thyroid status, thyroid dysfunction and adipocytokines.[37,38]

Mitochondrial inner membrane carriers, which distribute the protein gradient in mitochondria, make use of energy stored in the mitochondria for heat formation. Uncoupling proteins expressed by the skeletal muscle and brown adipose tissue are thought to play a role in the effects of thyroid hormones on the metabolism. In particular, uncoupling protein 3 expression was increased in rats given T3.[39,40] It has been shown that apelin applied to the mice increases the peripheral energy expenditure markers uncoupling protein-1 and uncoupling protein-3, which regulate the lipid export of skeletal muscle. In this way, apelin increases the body temperature and oxygen consumption, and causes weight loss in mice.[41] In rat models of hyperinsulinemia- dependent obesity, the apelin expression of fat tissue and plasma apelin levels

increase. Apelin expression is strongly inhibited in fasted rats. However, plasma apelin and insulin levels were found to be significantly elevated in obese patients. It has been shown that there is a strong relationship between insulin and apelin secreted by adipocytes. Additionally, it has been shown in rat models that plasma apelin concentrations and adipocyte apelin mRNA levels increase in hyperinsulinemia associated obesity.[42]

In the literature, the only article on apelin levels and thyroid disorders showed that in patients with subclinical hypothyroidism, the apelin levels did not differ from the healthy control subjects.[43]

Studies with apelin-like bioactive peptides, such as resistin, adiponectin, leptin and ghrelin, revealed different results. Leptin activates the thyroid gland by affecting specific receptors in the paraventricular hypothalamic nucleus, thus increasing the release of TRH in humans. In humans, leptin receptor mutations are associated with central hypothyroidism.[44] Negative correlations have been found between the TSH and adiponectin levels in obese women, and a positive correlation was found between adiponectin and free thyroid hormones in healthy euthyroid persons.[45] Another study revealed a positive correlation between serum resistin and FT3 and FT4 levels, but a negative correlation with TSH, while the successful treatment of hyperthyroidism was shown to be associated with decreased resistin levels.[46] Ghrelin levels are lower in hyperthyroidism, and antithyroid medication causes ghrelin levels to return to normal levels. Thyroid hormones also have a direct inhibitory effect on ghrelin.[47]

These conflicting results are thought to be associated with the etiology of hyperthyroidism, because in patients with autoimmune hyperthyroidism (Graves' disease), the serum adiponectin levels were found to be increased; however, in patients with non-autoimmune thyroid disorders, the serum adiponectin levels did not differ.[48,49]

ROS have been reported to induce oxidative damage to membrane lipids, proteins, and DNA and might result in cell death by necrosis or apoptosis.[50] Ceruloplasmin (Cp) is a α2-Globulin that contains approximately 95% of the total copper found in serum. The primary physiological role of Cp involves plasma redox reactions. It can function as an oxidant or antioxidant depending on other factors, such as the presence of free ferric ions and ferritin binding sites. Ceruloplasmin is also important in the control of membrane lipid oxidation-probably by direct oxidation of cations-thus preventing their catalysis of lipid peroxidation.[50]

Ceruloplasmin is a major defense against harmful effects of ROS in cells and in cultured erythrocytes, with a high capacity to degrade exogenous hydrogen peroxide.[51]

The extent of lipidperoxidation marker MDA was significantly increased in hypothyroid patients when compared to healthy controls. Resch et al found that hypothyroidism was associated with enhanced oxidative stress and lipid peroxidation, and supposed that this might lead to the development and progression of atherosclerosis.[52]

It has been suggested that hypothyroidism leads to oxidative stress and to a reduction of antioxidant defenses although the pathophysiological consequences of the decelerated antioxidant levels are not yet elucidated. This biochemical change is thought to be a physiological adaptation and a response to hypothyroidism. In agreement with previous findings, thyroid hormones are involved in combating the toxicity of oxidative stress in humans.[53] Thus, under normal conditions; the protective effect of thyroid hormone against oxidative stress can be explained by the function of antioxidants as a defense system.

The depletion of antioxidants observed in hypothyroid individuals may reflect the increased free radical production in the electron transport chain in the mitochondrial inner membrane. The increase of free radicals is not compensated, as one would expect, due to a decrease of antioxidants like ceruloplasmin. A high oxidative state in hypothyroid people has metabolic and biochemical characteristics such as increased mitochondrial enzyme activity. Thus, it is likely that patients' cells are damaged by prolonged oxidative stress that far exceeds the capacity of the patients' organs to synthesize antioxidant molecules or to synthesize them from extracellular sources.[54]

The present study was undertaken in two groups viz group-I and group-II i.e., subjects subclinical hypothyroid subjects and normal healthy controls subjects respectively. Basic anthropometric parameters of all subjects subclinical hypothyroid subjects and normal healthy controls subjects in are summarized in Table 1. The anthropometric parameters viz, age in years was (40.25±11.8), (39.50±10.8) in group-I and group-II respectively, BMI mean±SD in kg/m^2 in the group-I subjects, Subclinical Hypothyroid subjects and normal healthy controls subjects group-II was (24.5±2.6) and (21.9±2.8) respectively (Table 1).

Table 2 shows the biochemical parameters viz, serum fT3 (pg/ml), serum fT4 (ng/dl), serum TSH (μIU/ml), serum apelin (ng/ml) and s. ceruloplasmin (mg/dl) levels in subjects subclinical hypothyroid subjects and normal healthy controls subjects respectively.

Table 3 shows statistically significant difference between fT3, fT4 and TSH levels in group I and group II). The mean serum TSH level was found to be significantly high in group I (8.95±2.0 μIU/ml) as compared to group II (3.14±1.60 μIU/ml; $p<0.0001$). The mean serum fT3 level was found to be significant in group I (1.820±0.51 pg/ml) as compared to group II (2.31±0.60pg/ml; $p>0.05$). The mean serum fT4 level in mean±SD was also

found to be significant in group I (0.68±0.11 ng/dl) as compared to group II (0.80±0.14 ng/dl; p>0.05) (Figure 1).

In Table 5, comparison of mean serum ceruloplasmin levels in normal healthy controls and subclinical hypothyroid subjects is shown. The mean serum Ceruloplasmin level in subclinical hypothyroid subjects (14.6±2.1 mg/dl) is found to be lower than healthy controls (26.8±6.1 mg/dl; p<0.0001) (Table 5).

In contrast, Bhattacharya et al studied serum ceruloplasmin and serum malonyl dialdehyde (MDA) levels in hypothyroid patients. Their study shows that the mean malonyl dialdehyde (MDA) was high in hypothyroid patient compared to control group similar to that seen instudy by Dumitriu. While ceruloplasmin was significantly lower than in controls.[25]

The limitations of this study include problems of diagnostic accuracy, its only observational and analytical. Another limitation of this study was that the impact of genetics and ethnicity that was not specified or controlled. Secondly, subclinical hypothyroidism incidence has low prevalence that due to the low number of samples to be restricted. Therefore, prospective studies with larger sample sizes would allow for a better understanding of the relationship between apelin and ceruloplasmin with hypothyroidism progression. The drawbacks of the present study are that it included a small group of population and it cannot be applied on a global scale, as the study group included was mainly people in and around Bhilwara.

CONCLUSION

The present study was conducted on 120 Subclinical hypothyroid subjects (group-I) and 120 healthy controls subjects (group-II). The results were compared with age matched 120 healthy controls of either sex (group-II). Serum FT3, serum FT4, serum TSH, serum apelin and serum ceruloplasmin were estimated. The mean Serum FT3 level was found to be slightly decreased in subclinical hypothyroid subjects (group I) as compared to healthy controls (group II) but the difference was statistically significant (p<0.0001). The mean serum FT4 level was found to be slightly decreased in subclinical hypothyroid subjects (group I) as compared to healthy controls (group II) but the difference was statistically significant (p<0.0001). A highly significant increase (p<0.0001) in mean Serum TSH level has been observed in subclinical hypothyroid subjects (group-I) when compared to controls (group-II). In subclinical hypothyroid subjects, mean serum apelin levels found to be significantly higher in comparison to healthy subjects (p<0.0001). In subclinical hypothyroid subjects, mean serum ceruloplasmin levels found to be significantly lower in comparison to healthy subjects (p<0.0001). Overall findings of present study thus confirm that serum apelin level is significantly higher in subclinical hypo-thyroid subjects and serum ceruloplasmin level is significantly lower in subclinical hypo-thyroid subjects.

However, further experimental and observational studies are needed to illustrate the role of serum apelin and serum ceruloplasmin in subclinical hypothyroidism. Confirmation of the level of serum apelin and serum ceruloplasmin in subclinical hypothyroidism may allow its use in initiating treatment in special patient and disease groups including atherosclerosis and cardio-vascular disease concomitantly with advance age hypo-thyroidism.

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