Biol Trace Elem Res (2010) 134:265–279
DOI 10.1007/s12011-009-8478-7

2010 Pakistan
rvwd 7/2023

Interaction of Copper with Iron, Iodine, and Thyroid Hormone Status in Goitrous Patients

Tasneem Gul Kazi · Ghulam Abbas Kandhro · Hassan Imran Afridi · Naveed Kazi · Jameel Ahmed Baig · Mohammad Balal Arain · Abdul Qadir Shah · Nasreen Syed · Sham Kumar · Nida Fatima Kolachi · Sumaira Khan

Received: 20 June 2009 / Accepted: 17 July 2009 /
Published online: 8 August 2009

Abstract In many developing countries, men and women are at high risk of goiter and iron deficiency. The aim of the recent study is to assess the interaction of (Cu), with iron (Fe), iodine/iodide (I), and thyroid hormones in goitrous patients. Sixty goitrous male (GMPs) and 72 female patients (GFPs) were evaluated for the Cu, Fe, I, and thyroid hormones status in biological samples (serum and urine), and compared to non-goitrous subjects of both genders (M=106, F=120). The biological samples were analyzed for Cu and Fe concentration using atomic absorption spectrometer, while I was measured by the potentiometric method, prior to microwave-assisted acid digestion (MD). Quality control for the method was established with

T. G. Kazi · G. A. Kandhro · H. I. Afridi · J. A. Baig · M. B. Arain · A. Q. Shah · S. Kumar · N. F. Kolachi · S. Khan
National Centre of Excellence in Analytical Chemistry, Sindh University, Jamshoro, Pakistan

G. A. Kandhro
e-mail: gakandhro@yahoo.com

H. I. Afridi
e-mail: hassanimranafridi@yahoo.com

J. A. Baig
e-mail: jab_mughal@yahoo.com

A. Q. Shah
e-mail: aqshah07@yahoo.com

S. Kumar
e-mail: shamlect@gmail.com

N. Kazi
Liaqut University of Medical and Health Sciences, Jamshoro, Pakistan
e-mail: drnveedkazi24@yahoo.com

N. Syed
Nuclear Institute of Medical Radiotherapy (NIMRA), Jamshoro 76080, Pakistan
e-mail: syednasreen07@yahoo.com

T. G. Kazi (✉) · G. A. Kandhro · H. I. Afridi · J. A. Baig · A. Q. Shah · S. Kumar
Center of Excellence in Analytical Chemistry, University of Sindh, Jamshoro 76080, Pakistan
e-mail: tgkazi@yahoo.com

certified samples. Significantly higher mean values of Cu in serum, and urine samples of GMPs and GFPs, while lower value of Fe and I were observed as compared to control subjects ($p<0.015$), respectively. The mean values of free triiodothyronine (FT3) and free thyroxin (FT4) were found to be lower in goitrous patients of both genders than in the age-matched healthy controls ($p<0.006$ and 0.002), respectively, in contrast high mean values of thyroid-stimulating hormone (TSH) were detected in patients ($p<0.009$), as compared to non-goitrous subjects. It was observed that the deficiencies of Fe, I, and thyroid hormone in goitrous patients could be influenced by efficiency of Cu.

Keywords Copper · Iron · Iodine · Thyroid hormones · Goiter

Introduction

Thyroid disorders are the second most common endocrinopathies found in humans and animals. Hypothyroidism is a clinical entity resulting from deficiency of thyroid hormones, and, in hypothyroidism, the basal metabolic rate is decreased [1]. Normal thyroid status is dependent on the presence of many trace elements for both the synthesis and metabolism of thyroid hormones [2]. Thyroid hormones influence the level of essential trace elements in the serum and urine and are also associated with the oxidative and antioxidative status in the human body [3]. Copper (Cu), iron (Fe), and iodine (I) were exposed to irritate the function of the thyroid gland

Iron, an essential element in the body, plays a wide variety of physiological and biochemical roles. Close to two-thirds of the Fe in the body is associated with hemoglobin, found in circulating red blood cells [4]. Iron-dependent physiological functions can become impaired prior to the development of anemia [5]. It was extensively investigated that deficiencies of Fe and I remain major public health problems affecting ≥30% of the global population [6]. Iron deficiency anemia (IDA) could impair thyroid metabolism through anemia and lower oxygen transport [7]. The two initial steps of thyroid hormone synthesis are catalyzed by thyroid peroxidase and are dependent on Fe. The effects of Fe deficiency on the thyroid-stimulating hormone (TSH) axis are equivocal. A low level of dietary Fe or poor biological availability of Fe causes reduced absorption.

Iodine is an essential component of the thyroid hormones that play an important role in human development, growth, and metabolism. The effects of I deficiency are still a major problem in public health in many parts of the world. I intake is important for thyroid function [8]. Adequate levels of I are necessary for proper functioning of a number of body systems, including the eyes, mammary glands, thyroid, salivary glands, and the gastric mucosa [9]. Pakistan is a severely I-deficient country and about 70% of the population was estimated to be at risk for Iodine deficiency disorders (IDDs) in 1993 [10]. However, most of the big cities and towns of Pakistan are located within the Indus Plain such as Swabi, Peshawar, Lahore, Hyderabad, Dadu, Jamshoro, Karachi, and Quetta, which has not been extensively investigated [11].

Copper is an essential nutrient for the production of red blood cells, hemoglobin, and for the activity of various enzymes [12]. Copper is also a component of superoxide dismutase which protects cells against oxidative injury. Besides these, Cu is also required for the synthesis of phospholipids (a class of fats) that are found in the myelin sheaths that insulates nerves to protect them. Phospholipids are required for the stimulation of TSH [13]. Therefore, correct levels of Cu are needed to prevent thyroid problems, and it can be used in the treatment of thyroid disease. Elevation of Cu has been observed to affect the

endocrine system adversely [14]. Vegetarian diets and any medication that has a sedating effect can raise the copper level in the body.

Metal determination in human tissues is the most common application of biological monitoring for screening, diagnosis, and assessment of metal exposures and their risks. Interest in the importance of trace elements to human health has increased considerably during the last decades [15]. Among various biopsy materials, serum and urine may be used as bioindicators for this intention [16]. The determination of trace metals in biological samples requires the use of sensitive and selective technique such as atomic absorption spectrometer. This technique needs solubilization of the analyte and complete or partial decomposition of the matrix using either convective systems or microwave ovens and dry ashing. The main advantage of microwave-assisted samples pretreatment is its requirement of a small amount of mineral acids and a reduction in the production of nitrous vapors [17].

However, to the best of our knowledge, no study has been performed to evaluate the status of efficiency and deficiency of essential trace elements in biological samples of goitrous patients in Pakistan. The present study was undertaken to investigate the concentrations of Cu, Fe, and I in biological samples (serum and urine) and other biochemical parameters such as, TSH, free triiodothyronine (FT3) and free thyroxin (FT4) among male and female goitrous patients and compared them to age-matched healthy controls subjects of both genders residing in same area (Jamshoro), Sindh, Pakistan. The serum and urine samples were prepared by the microwave-assisted acid digestion method (MD), and the validity of the analytical procedure was checked by corresponding conventional wet acid digestion (CD) of matrix-matched certified reference materials.

Materials and Methods

Study Design and Pretreatment

An epidemiological cross-sectional study was conducted among goitrous patients (n=132) of both genders, age ranged as 20–30 years recruited from the outpatient clinic of the Nuclear Institute of Medicine and Radiotherapy (NIMRA), Jamshoro and age-matched non-goitrous subjects (n=226). Before the start of this study, all the normal and goitrous patients were informed by the administration through a consent form about the aim of the study; all agreed to participate and they signed the form. A questionnaire was also administered to all the normal and goitrous patients in order to collect details regarding physical data, ethnic origin, health, dietary habit, consent age. Physical examinations were performed in the basic health unit of NIMRA to measure participant's weight, height, blood pressure, and biochemical data.

The criteria for the collection of biological samples (serum and urine) of GMPs and GFPs was set that, prior to any treatment, they were not taking any mineral supplement during the last 3 months. The control male and female subjects belong to the same socio-economic status and dietary habits, not suffering from any thyroid problem, mostly the relatives of patients. The preliminary exclusion criteria for patients and controls were hypertension, alcoholism, smoking, diabetes, cardiovascular disease, taking of any vitamins and minerals.

All the venous blood samples (3–5 mL; including control group) were collected after 12-h fasting, using metal-free Safety Vacutainer blood-collecting tubes (Becton Dickinson, Rutherford ®, USA) between 9.30 and 11.00 AM. The blood samples were left standing for 1 h; sera were separated at 2,500 rpm centrifugation and preserved at −20°C until analysis

[18]. For the analysis of other biochemical parameters, up to 5 mL blood samples from the same subjects was sampled by using metal-free Safety Vacutainer blood-collecting tubes containing >1.5 mg K_2EDTA and sent to the pathological laboratories of NIMRA. Morning urine samples were collected in acid-washed, decontaminated 100-mL polyethylene tubes (Kartell1, Milan, Italy). During sampling sessions, the container is wrapped in a clean polyethylene bag. Urine samples were acidified with ultrapure concentrated HNO_3, (l% *v*/*v*) and kept at −4°C. Prior to sub-sampling for analysis, the sample should be shaken vigorously for 1 min to ensure a homogeneous suspension.

Reagents and Glassware

All chemicals were of analytical reagent grade and were supplied by Merck (Darmstadt, Germany). Nitric acid (HNO_3) ≈16 M and 30% hydrogen peroxide (H_2O_2) were used (Merck, Darmstadt, Germany). Ultrapure water was prepared by passing de-ionized water from a Milli-Q system (Bedford, USA) and was used throughout the study. Standard solution of Cu and Fe was prepared by dilution of certified standard solution (1,000 mg/L, Fluka Kamica), while I was prepared from analytical grade potassium iodide (KI) Merck (Darmstadt, Germany). Sodium nitrate stock solution ($NaNO_3$), 0.05% (*w*/*v*), was prepared from sodium nitrate (Merck Darmstadt, Germany). For accuracy of the analytical technique, certified reference samples of human serum (SERO-M10181, Billingstad, Norway) and human urine (Clincheck control-lyophilized®), and certified reference material of human urine NIST SRM 2670a (Gaithersburg, USA) was obtained from the National Institute of Standards & Technology (NIST) were used for validating the determination of Cu, Fe, and I. All glassware and polyethylene bottles were washed thoroughly and then soaked overnight in 2 mol/L nitric acid, and rinsed with ultrapure water before use.

Measurements

A Perkin-Elmer model A. Analyst 700 (Norwalk, CT) atomic absorption spectrometer equipped with deuterium background correction was used in the study. The hollow cathode lamps of Cu and Fe were run under the conditions suggested by the manufacturer. A single-element hollow cathode lamp was operated at 7.0 for Cu while at 7.5 mA for Fe with a spectral bandwidth of 0.7 nm. The analytical wavelengths were set at 324.8 and 248.5 nm for Cu and Fe, respectively. The flow rate of air (oxidant) was 17.0 l/min, while 2.0 l/min for acetylene. Integrated absorbance signals computed by the AA spectrometer were employed throughout. The graphite furnace heating program was set for different steps, drying, ashing, atomization, and cleaning as temperature range °C/time (s) (80–120/15, 300–600/15, 1500–1800/5, and 1800–2000/2) respectively. Iodide-ion selective electrode (ISE) of Metrohm AG, CH-9101 Herisau (Switzerland) with 781 pH/ion meter of Metrohm and Single Junction, Ag/AgCl reference electrode was used. A Pel (PMO 23) domestic microwave oven (maximum heating power of 900 W) was used for digestion of the samples. A WIROWKA Laboratoryjna type WE-1, nr-6933 centrifuge, with speeds ranging from 0–6000 rpm, was used to separate the supernatant from the sample. For preparing and storing solutions, acid-washed plastic (polypropylene) tubs were used.

Biochemical Assays

To assess thyroid function, FT4 and FT3 were measured by using radio immunoassay (Gamma counter, Oakfield, England). Thyroid-stimulating hormone was measured by using

immuno radiometric assay (Gamma counter, Oakfield, England). Serum ferritin (SF), transferrin receptor (TfR), and C-reactive protein (CRP), were also analyzed, as described earlier [19]. The analysis of SF was performed with an IMx analyzer (Abbott Laboratories, Abbott Park. IL, USA) which uses micro particle enzyme immunoassay technology, serum TfR with an enzyme immunoassay and commercial kits (Ramco Laboratories Inc, Houston, TX, USA), CRP by using the Behring Turbitimer System (Behring-werke AG Diagnostica, Germany). Serum albumin (SA) was determined using an automated dye-binding method with bromocresol green [20] and other biochemical parameters were measured by Medonic CA 620, Haematological Counter (Stockholm, Sweden).

The traditional diagnosis of goiter is based on palpation and World Health Organization (WHO) classification [21], while recent developments in ultrasound technology have facilitated the accurate measurement of thyroid volume. In addition, all subjects were examined by thyroid ultrasonography, performed with HS-2000 Honda ultrasound equipment with 7.5 MHz linear probe. The formula we used for calculation of standard thyroid volume was based on a model describing the variation in individual thyroid volume as a function of several covariates such as age, height, and body weight [22].

Microwave-Assisted Acid Digestion Method

A microwave-assisted digestion procedure was carried out, in order to achieve a shorter digestion time. Six replicate samples of certified samples of serum and urine, while duplicate samples of 0.5 mL of each goitrous and control individual were directly placed into Teflon PFA flasks. One milliliter of a freshly prepared mixture of concentrated HNO_3–H_2O_2 (2:1, *v*/*v*) were added to each flask, left for 10 min. The flasks were placed in a covered PTFE container and heated following a one-stage digestion program at 80% of total power (900 W), 2–3 min for serum and urine. After the digestion, the flasks were left to cool and the resulting solution was evaporated almost to dryness to remove excess acid, and then diluted to 10.0 mL in volumetric flasks with 0.1 M nitric acid. The validity and efficiency of the MD was checked with certified values of CRMs of six replicates biological samples and with those obtained from CD [23].

Blank extractions (without sample) were carried out by performing the complete procedures without standard and sample. All experiments were conducted at room temperature (30°C) following the well-established laboratory protocols. The resulted digested solutions were analyzed for Cu by ETAAS and Fe by FAAS. The concentrations were found directly from calibration chart after adjustment of the absorbance for the signal observed from a suitable reagent blank.

Potentiometric Method

Potentiometric analysis for the determination of serum and urinary I was carried out by iodide-ion selective electrode (ISE) [24, 25]. A linear range of I standards was prepared from 12.7 to 127,000 μg/L (10^{-3} to 10^{-7} M) KI, at 30°C and membrane resistance (MΩ) <0.1. The calibration was found linear in the entire range of I concentration with a slope value of −59.8 mV. This showed that the iodide-ISE was very much sensitive for the determination of serum and urinary I. The sample analysis were carried out by taking 1 mL of each serum and urine samples in 10-mL volumetric flasks, added 0.5 mL of 2 M $NaNO_3$ as total ionic strength adjusting buffer and diluted up to the mark. The iodide-ISE was immersed into the sample, stirred gently for a few seconds and after 3 min, I was measured in micrograms per liter directly by using standard addition method in the serum and urine samples of GMPs, GFPs, and controls.

Analytical Figures

Calibration was performed with a series of Cu, Fe, and I standards. The regression equation for Cu, Fe, and I were X=1.156 (Cu)+0.0015, R^2=0.9998, X=(0.0191) (Fe)+0.0007, R^2= 0.9986, and Y=−59.8 (I)−47.073, R^2=0.9998, respectively

Where X is integrated absorbance and the concentration is expressed as milligrams per liter, and Y is integrated electromotive force, (EMF) expressed in millivolts (mV) and the concentration in each case expressed as micrograms per liter. The linear range of the calibration curve used for Cu and Fe from the detection limit up to 100 μg/L. The limits of detection (LOD) and limit of quantification (LOQ) for Cu, Fe, and I was calculated as under, $\mathrm{LOD} = 3 \times {}^{s}/_{m}$ and $\mathrm{LOQ} = 10 \times {}^{s}/_{m}$ respectively, where "s" is the standard deviation of ten measurements of the blank and "m" is the slope of the calibration graph. The LOD of Cu, Fe, and I were 1.5, 64.5, and 6.52 ng/mL, respectively, and LOQ were 5.2, 215.2, and 11.9 ng/mL, respectively.

The validity and efficiency of the MD was checked by the values, obtained from CD [23]. The indicative values for both protocols were calculated as the arithmetic means of 6 replicate of human serum (SERO-M10181, Billingstad, Norway), and human urine (Clincheck control-lyophilized ®), while for I, the certified reference material of human urine NIST SRM 2670a, (Gaithersburg, USA) was used, as shown in Table 1. The MD was efficient and took less than 5 min to complete the digestion of the certified biological samples. The overall recoveries of Cu, Fe, and I in certified biological samples, by using the MD as compared to CD were 97.2–98.8% in CRM of serum and urine, respectively. Mean values of Cu, Fe, and I varied less than 1–2% from the certified values. The coefficient of variation changed less than 2%.

Statistical Analysis

Data processing and statistical analysis were conducted by using the computer program EXCEL (XP 2002; Microsoft Corp., Redmond, WA) and Minitab 13.2 Minitab Inc., State (College, PA) software packages. Normally distributed data were expressed as means ± std, Student's t test and Mann–Whitney test were used to assess the significance of the differences between the variables investigated in goitrous and non-goitrous subjects.

Results

The present study investigated the possible differences in copper concentration among biological samples (serum and urine) and their interaction with iron, iodine, and thyroid hormones in male and female goitrous patients.

The observed results of different biochemical parameter were shown in Table 2. The non-significant differences were observed between weight and body mass index of goitrous and normal subjects. The GMPs and GFPs have lower hemoglobin (Hb) % as compared to recommended range for normal persons (11.5–16.5 g/dL). The hematocrit and serum ferritin levels were significantly lower in GMPs and GFPs than normal subjects (p<0.008 and 0.006, respectively). The levels of serum albumin, TV, CRP and transferrin receptor in goitrous patients of both genders were significantly higher than control subjects (p<0.01), indicating the consequence of thyroid disorder. The other blood parameters such as erythrocyte or red blood cells (RBC), white blood cells (WBC), neutrophils, lymphocytes,

Table 1 Determination of Cu, Fe and I in Certified Samples by Conventional Digestion Method (CD) and Microwave Digestion Method (MD) $N=6$

Elements	Certified values	CD	MD	T value[a]	Percent recovery[b]
Certified sample of serum (mg/L)					
Cu	1.2±0.04	1.11±0.031 (2.81)	1.09±0.03 (2.76)	0.320	98.4
Fe	1.3±0.15	1.42±0.19 (13.38)	1.38±0.18 (13.04)	0.015	97.2
I[c]	93.6±1.74	93.3±1.88 (2.02)	92.2±1.77 (1.92)	0.020	98.8
Certified sample of urine (mg/L)					
Cu	0.056±0.01	0.054±0.0009 (1.67)	0.053±0.0008 (1.50)	0.102	98.7
Fe	0.039±0.01	0.036±0.0008 (2.22)	0.0355±0.0006 (1.69)	0.014	98.6
I[c]	88.2±1.24	88.8±1.94 (2.18)	87.5±1.80 (2.06)	0.015	98.5

T (critical) at 95% C.I.=2.57

Values in parenthesis are RSD

[a] Paired t test between CD and MD, DF=5

[b] Percent recovery was calculated according to: $\frac{[MD]\ X}{[CD]}\ 100$

[c] Iodine concentrations measured as for iodide in micrograms per liter

eosinophils, monocyte, basophiles, and platelets show no difference in goitrous and control subjects ($p>0.05$) as shown in Table 2.

The results in Table 3 showed that the concentration of Cu in sera of GMPs and GFPs was significantly higher at 95% confidence interval [CI, 0.90, 1.60] and [CI, 0.91, 1.53] mg/L, respectively, as compared to control subjects ($p<0.006$ and 0.007). The excretion of urinary Cu in controls of both genders was significantly lower [CI, 0.15, 0.47] mg/L, as compared to GMPs and GFPs ($p<0.004$ and 0.005), respectively.

The concentration of Fe in sera samples of GMPs and GFPs was significantly lower [CI, 0.31, 0.91] and [CI, 0.31, 0.87] mg/L, as compared to control subjects ($p<0.003$). The urinary excretion of Fe in controls of both genders was significantly higher [CI, 1.92, 3.26] mg/L, as compared to GMPs and GFPs ($p<0.012$), respectively (Table 3). The levels of serum and urinary I in GMPs and GFPs were significantly lower as compared to control subjects ($p<0.008$), respectively, as given in Table 3.

The concentration of TSH in GMPs and GFPs [CI, 4.62, 5.62] and [CI, 4.99, 6.08] μIU/mL, was significantly higher than controls of both genders ($p<0.009$ and 0.007), respectively, (Table 4). The concentrations of FT3 and FT4 were found to be 1.04–3.40 and 5.66–10.68 pmol/L in GMPs and 0.86–3.38 and 4.67–10.25 pmol/L in GFPs, significantly, lower as compared to controls ($p<0.006$ and 0.002), respectively.

The correlation of biochemical parameters Vs. Cu and thyroid hormone levels (TSH, FT3, and FT4), while correlation of serum and urine Cu levels vs. thyroid hormone levels (TSH, FT3, and FT4) in goitrous male and female patients were statistically analyzed by multiple linear regression equation and Pearson correlation, shown in Tables 5 and 6, respectively

Table 2 Biochemical Parameters in Normal and Goitrous Male (GMPs) and Female Patients (GFPs)

Parameters	Normal	GMPs	Normal	GFPs	Normal range
Weight (kg)	64.3±5.4	61.5±5.1, (0.025)	54.6±4.5	50.4±4.3, (0.022)	–
Height (cm)	157.2±5.8	155.3±5.5, (0.04)	130.4±5.5	126.5±5.2, (0.034)	–
BMI (kg/m^2)	26.03±3.9	25.5±3.7, (0.042)	32.1±4.4	31.5±4.1, (0.041)	–
Hb (g/dL)	13.2±3.4	9.7±3.3, (0.015)	12.3±3.2	9.3±3.1, (0.015)	11.5–16.5
Hct (%)	45.3±2.6	33.5±2.3, (0.007)	42.8±2.4	32.7±2.2, (0.006)	35–55
SA (g/dL)	4.5±0.85	5.8±0.83, (0.01)	4.1±0.82	6.2±0.65, (0.008)	3.4–5.4
SF (μg/L)	46.7±3.8	21.3±5.6, (0.006)	45.2±3.6	20.3±5.1, (0.006)	>30
TV(mL)	14.1±3.9	28.3±5.6, (0.006)	12.4±3.3	22.1±5.1, (0.005)	<18 for F <25 for M
CRP (mg/L)	8.3±1.1	14.5±1.5, (0.005)	7.4±0.97	13.3±1.4, (0.005)	<10
TfR (mg/L)	7.8±2.6	27.9±6.3, (0.001)	6.6±2.3	29.5±6.4, (0.002)	2.9–8.5
RBC (mm^3)	4.1±0.55	3.8±0.35, (0.052)	3.8±0.5	3.5±0.3, (0.05)	3.5–5.5
WBC (mm^3)	8.3±0.6	6.8±0.5, (0.05)	7.6±0.5	6.4±0.4, (0.047)	3.5–10
MCV (μm)	92.3±5.5	67.8±4.8, (0.015)	89.7±5.1	66.9±4.6, (0.011)	75–100
MCH (pg)	32.8±2.2	21.5±1.9, (0.02)	30.8±2.1	20.5±1.7, (0.017)	25–35
Neutophils %	67.9±5.8	51.8±4.4, (0.04)	62.4±5.6	52.3±4.2 (0.037)	35–80
Lymphocytes%	38.2±4.6	29.4±4.3, (0.03)	36.3±4.7	31.6±4.5, (0.043)	15 –50
Eosinophils %	41.6±4.5	34.5±4.4, (0.05)	43.5±4.3	36.2±4.2, (0.026)	15–50
Monocytes %	13.8±2.6	11.1±2.3, (0.052)	12.4±2.3	11.4±2.1, (0.045)	2–15
Basophiles %	12.5±1.8	11.9±1.7, (0.05)	11.9±1.5	11.5±1.6, (0.058)	2–15
Platelets (mm^3)	268±31.3	253±32.1, (0.04)	266±30.2	248±32.7,(0.038)	100–400

Mean ± standard deviations were calculated for all biochemical parameters

Values in parenthesis are *p* value

BMI body mass index, *Hb* hemoglobin, *Hct* hematocrit, *SA* serum albumin, *SF* serum ferritin, *TV* thyroid volume, *CRP* C-reactive protein, *TfR* transferrin receptor, *RBC* red blood cells, *WBC* white blood cells, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin

Discussion

The epidemiological study provided data of Cu, Fe, and I concentrations in serum and urine as well as level of thyroid hormones (TSH, FT3, and FT4) obtained from the GMPs and GFPs and control subjects of developing country, Pakistan. The results of previous studies revealed that goiter still continues to be prevalent in Pakistan [26].

Poor nutrition is considered one of the causes of an underactive thyroid; thus, providing optimal nutrition is vital to correcting them, as well as to prevent further decline. Healthy thyroid function is dependent on a range of nutrients, especially Cu, Fe, I, zinc, selenium, and tyrosine [16, 27, 28]. In addition to proper nutrition, fluctuations in hormone levels may also act as a trigger in thyroid dysfunction, resulting in a sub-clinical thyroid imbalance in the body of both GMPs and GFPs. The consumption of a predominantly cereal-based diet, rich in phytate, oxalate, phosphate, and fiber enhance the Cu absorption which increases the Cu bioavailability, which was the main cause of Cu elevation in understudy patients [29]. It was reported that the phytic acid in humans inhibit absorption of Fe, I, Zn, calcium, magnesium, and manganese but not Cu [30].

Table 3 Cu, Fe and I Concentrations in Serum and Urine Samples of Normal and Goitrous Male (GMPs) and Female (GFPs) Patients

	Cu[a]		Fe[a]		I[b]	
	Normal	Patients	Normal	Patients	Normal	Patients
Male						
Serum	1.12±0.23	1.25±0.35 (0.006)	1.52±0.41	0.61±0.30 (0.003)	87.5±11.7	45.8±12.1 (0.007)
Urine	0.18±0.05	0.32±0.15 (0.004)	2.56±0.64	1.90±0.75 (0.01)	119±15.8	60.8±18.4 (0.006)
Female						
Serum	1.14±0.26	1.22±0.31 (0.007)	1.51±0.39	0.59±0.28 (0.002)	90.4±12.9	47.2±12.7 (0.008)
Urine	0.19±0.07	0.29±0.14 (0.005)	2.60±0.66	1.95±0.78 (0.012)	123±17.3	58.2±17.6 (0.005)

Values in parenthesis are p values

[a] Values measured in milligrams per liter

[b] Values measured in micrograms per liter

Table 4 Hormonal Status in Normal and Goitrous Male (GMPs) and Female (GFPs) Patients

	Normal	Patients
Male		
TSH (μIU/mL)	3.47±1.57, [3.16, 3.78]	5.12±1.92, [4.62, 5.62]
FT3 (pmol/L)	4.18±0.67, [4.05, 4.31]	2.22±1.18, [1.92, 2.52]
FT4 (pmol/L)	16.6±2.70, [16.1,17.2]	8.17±2.51, [7.52, 8.82]
Female		
TSH (μIU/mL)	3.91±1.12, [3.71, 4.12]	5.54±2.30, [4.99, 6.08]
FT3 (pmol/L)	4.39±0.79, [4.25, 4.53]	2.12±1.26, [1.83, 2.42]
FT4 (pmol/L)	17.8±3.09, [17.2, 18.3]	7.46±2.79, [6.80, 8.11]

Values in square brackets at 95.0% confidence interval

Both trace elements and thyroid hormones play essential role in the human body. Few studies on serum and urinary Cu concentration in patients of goitrous diseases have been reported [31]. The serum Cu concentrations of the control male and female subjects were lower than that of the hypothyroid patients. However, the previous results about the interaction between these two factors are often controversially given. Iron deficiency anemia may affect thyroid metabolism through several mechanisms. Iron deficiency may influence I deficiency disorders through alterations in the central nervous system of thyroid metabolism or through modifications in nuclear triiodothyronine binding [32]. Also, the initial steps of thyroid hormone synthesis iodide incorporation into tyrosine residues of thyroglobulin and covalent bridging of the residues are catalyzed by heme-containing thyroperoxidases (TPO). Other Fe-containing enzymes [e.g., cytochrome oxidase, myeloperoxidase, and succinate dehydrogenase (ubiquinone)] are sensitive to Fe deficiency [33]. Thyroperoxidase activity in the thyroid, measured by both the guaiacol and the iodide assays, was clearly sensitive to body Fe depletion. Therefore, it was suggested that impairment of TPO activity contributes to the adverse effect of IDA on thyroid hormones and I metabolism.

Iodine deficiency is the most important risk factor for goiter, and the characterization of I status of a population has a central role in thyroid epidemiology. Generally, I status is characterized from serum and urinary I excretion. This is plausible because approximately 90% of ingested I is excreted in the urine whereas the remaining 10% is excreted in feces, however, with large inter-individual differences. Severe I deficiency may result in goiter [29] and cretinism. The results of our study substantiate growing concerns that dietary I deficiency is increasing in understudy area. We need to know whether the recent influx of studies demonstrating I deficiency, indicates a re-emergence, or an ongoing problem of I deficiency in adults. The minimum recommended daily I intake varies with age, ranges from 120 μg/day in children to 150 μg/day in adults [6]. The accumulating facts for I deficiency in subjects of our study suggested that I intake might be lower.

Copper is an essential element for humans and animals, especially in the metabolism of the amino acid tyrosine, which is needed for the production of thyroid hormones [34]. In relation to thyroid health, the imbalance in Cu level associated with development of goiter and other thyroid pathologies including thyroid cancer [35]. Aurthor et al. reported the interrelationships among Cu and thyroid hormones, and they concluded that excess Cu concentration in thyroid patients enhances the effect of hypothyroidism [36]. These investigations are consistent with our study.

Table 5 Linear Regression and Pearson Coefficient of (Biochemical Parameters Vs. Cu, TSH, FT3 & FT4) in Goitrous Male (GMPs) and Female (GFPs) Patients

	Cu	TSH	FT3	FT4
Male				
Hb	$0.07x+0.572$	$0.373x+1.50$	$0.197x+0.311$	$0.502x+3.30$
	$r=0.652$, (0.005)	$r=0.642$, (0.015)	$r=0.566$, (0.002)	$r=0.660$, (0.05)
Hct	$0.110x-2.43$	$0.550x-13.3$	$0.347x-9.40$	$0.757x-17.2$
	$r=0.669$, (0.001)	$r=0.618$, (0.001)	$r=0.651$, (0.001)	$r=0.650$, (0.001)
SA	$0.319x+0.233$	$1.63x-0.078$	$0.988x-0.922$	$2.05x+1.65$
	$r=0.751$, (0.01)	$r=0.640$, (0.01)	$r=0.715$, (0.05)	$r=0.679$, (0.01)
SF	$0.041x+0.373$	$0.219x+0.449$	$0.117x-0.272$	$0.276x+2.30$
	$r=0.651$, (0.001)	$r=0.708$, (0.001)	$r=0.571$, (0.001)	$r=0.615$, (0.005)
TV	$0.046x-0.036$	$0.239x-2.13$	$0.127x-2.46$	$0.375x-2.64$
	$r=0.675$, (0.003)	$r=0.64$, (0.002)	$r=0.70$, (0.001)	$r=0.77$, (0.01)
CRP	$0.157x-1.03$	$1.63x-0.078$	$0.553x-5.79$	$1.19x-9.20$
	$r=0.70$, (0.002)	$r=0.624$, (0.01)	$r=0.732$, (0.001)	$r=0.726$, (0.01)
TfR	$0.039x+0.151$	$0.201x-0.494$	$0.116x-1.01$	$0.236x+1.59$
	$r=0.679$, (0.001)	$r=0.660$, (0.001)	$r=0.633$, (0.001)	$r=0.591$, (0.001)
RBC	$0.530x+0.127$	$2.51x-0.189$	$1.50x-0.952$	$3.16x+1.49$
	$r=0.80$, (0.05)	$r=0.592$, (0.01)	$r=0.591$, (0.05)	$r=0.569$, (0.001)
WBC	$0.453x-1.83$	$2.34x-10.8$	$1.43x-7.48$	$3.03x-12.4$
	$r=0.651$, (0.005)	$r=0.620$, (0.05)	$r=0.632$, (0.01)	$r=0.614$, (0.05)
Female				
Hb	$0.065x+0.61$	$0.485x+1.01$	$0.246x-0.113$	$0.778x-17.9$
	$r=0.643$, (0.005)	$r=0.653$, (0.015)	$r=0.666$, (0.002)	$r=0.613$, (0.05)
Hct	$0.094x-1.84$	$0.635x-15.2$	$0.314x-8.07$	$0.583x+2.02$
	$r=0.657$, (0.001)	$r=0.608$, (0.001)	$r=0.603$, (0.001)	$r=0.646$, (0.001)
SA	$0.353x+0.265$	$2.48x-1.16$	$1.20x-1.06$	$2.79x-0.093$
	$r=0.736$, (0.015)	$r=0.707$, (0.01)	$r=0.688$, (0.05)	$r=0.656$, (0.01)
SF	$0.039x+0.415$	$0.279x-0.130$	$0.143x-0.739$	$0.305x+1.26$
	$r=0.643$, (0.001)	$r=0.620$, (0.001)	$r=0.625$, (0.001)	$r=0.557$, (0.005)
TV	$0.040x+0.326$	$0.235x-0.225$	$0.135x-1.30$	$0.423x-2.14$
	$r=0.65$, (0.002)	$r=0.62$, (0.001)	$r=0.74$, (0.001)	$r=0.83$, (0.005)
CRP	$0.147x-0.73$	$1.02x-8.01$	$0.037x+0.792$	$1.25x-9.20$
	$r=0.685$, (0.002)	$r=0.650$, (0.01)	$r=0.671$, (0.001)	$r=0.658$, (0.01)
TfR	$0.034x+0.226$	$0.242x-1.59$	$0.114x-1.21$	$0.271x-0.564$
	$r=0.685$, (0.001)	$r=0.672$, (0.001)	$r=0.627$, (0.001)	$r=0.623$, (0.001)
RBC	$0.470x+0.044$	$3.10x-2.21$	$1.54x-1.67$	$3.70x-1.80$
	$r=0.684$, (0.05)	$r=0.617$, (0.015)	$r=0.614$, (0.05)	$r=0.607$, (0.005)
WBC	$0.506x-2.02$	$3.65x-17.8$	$1.80x-9.33$	$4.34x-20.3$
	$r=0.639$, (0.005)	$r=0.631$, (0.05)	$r=0.623$, (0.015)	$r=0.616$, (0.05)

Values in parenthesis are p values

Table 6 Linear Regression and Pearson Coefficient of (Serum and Urinary Cu Vs. TSH, FT3 & FT4) in Goitrous Male (GMPs) and Female (GFPs) Patients

	TSH	FT3	FT4
Male			
Serum Cu	$3.91x+0.264$	$2.47x+1.94$	$6.14x+0.304$
	$r=0.66$, (0.005)	$r=0.86$, (0.003)	$r=0.80$, (0.002)
Urine Cu	$10.9x+1.36$	$4.92x+0.161$	$17.9x+1.87$
	$r=0.68$, (0.009)	$r=0.76$, (0.007)	$r=0.73$, (0.005)
Female			
Serum Cu	$4.23x+0.186$	$2.57x+1.43$	$5.83x+0.113$
	$r=0.69$, (0.006)	$r=0.87$, (0.004)	$r=0.71$, (0.002)
Urine Cu	$8.13x+2.70$	$5.45x+0.401$	$15.8x+2.19$
	$r=0.72$, (0.010)	$r=0.86$, (0.007)	$r=0.74$, (0.004)

Values in parenthesis are p value

The role of Cu and Fe in the thyroid is less defined but sub-or super optimal dietary intakes of Cu can adversely affect thyroid hormone metabolism [37]. The excess level of Cu may cause an iron deficiency. Copper absorption may be decreased by excess dietary Fe or zinc [38]. Ironically, excess dietary Cu may also cause anemia, which is thought to result from competition between Cu and Fe for absorption sites in the intestine [39]. The deficiency of iodine might occur with high consumption of soybeans (or other beans) which is known to be high in Cu [40].

The measurement of the biochemical parameters such as, hemoglobin, hematocrit, serum albumin, SF, CRP, TfR, and WBC appears to be more useful indicator of Cu status [41]. High intake of dietary Cu was probably the primary dietary factor responsible for disturbance of different biochemical parameters of GMPs and GFPs [42]. Thyroid ultrasonography is a precise and objective method for measuring goiter size that has become feasible for field studies even in remote areas. It is particularly valuable for accurate detection of small goiters in men and women [43]. The reports of understudy

Table 7 Mole Ratio between Cu, Fe, and I in Serum and Urine Samples of Normal and Goitrous Male (GMPs) and Female (GFPs) Patients

	Gender	Mole ratio					
		Cu/Fe	Fe/Cu	Cu/I	I/Cu	Fe/I	I/Fe
Normal							
Serum	Male	0.648	1.54	25.7	0.039	39.7	0.025
	Female	0.663	1.51	25.2	0.040	38.0	0.026
Urine	Male	0.062	16.18	3.02	0.331	48.9	0.020
	Female	0.064	15.57	3.08	0.324	48.0	0.021
Patients							
Serum	Male	1.80	0.555	55.5	0.018	30.8	0.032
	Female	1.82	0.550	51.8	0.019	28.5	0.035
Urine	Male	0.148	6.76	10.5	0.095	71.0	0.014
	Female	0.131	7.65	9.95	0.101	76.1	0.013

patients indicated that thyroid volumes for male and females were higher than normal level [44]. Despite the biochemical evidence of a high risk for Cu, we did not detect any significant relationships between serum Cu and anthropometric status, in hypothyroid patients. The present study showed a positive correlation of hemoglobin, hematocrit, serum ferritin, TV, CRP, TfR, and WBC concentrations with Cu, TSH, FT3, and FT4 levels in both GMPs and GFPs as shown in Table 5.

The relatively strong positive correlations have been found between Cu and TSH, FT3 and FT4 levels [31, 45]. The present study showed a significantly higher urinary Cu excretion in GMPs and GFPs than control subjects and a significant correlation have been found between urinary Cu level and TSH, FT3, and FT4 levels (Table 6). An elevated TSH level may not be a clinically significant adverse effect; it is an indicator for increased risk of emergent clinical hypothyroidism and was therefore selected as the critical adverse effect for Cu [46]. Free triiodothyronine and FT4 needs Cu in order to fulfill its biological activity, and Cu affects the metabolic activity of these hormones in a negative way [47].

The findings of our study are parallel to the above-mentioned reports which claim that Cu has a negative effect on Fe, I, and thyroid functions. It was clearly revealed that the concentration of metals varied in the biological samples of thyroid patients as compared to controls, i.e., Cu concentration increased both in serum and urine, while for Fe and I the reverse pattern was found as shown in Table 3. An increase in Cu and decrease in Fe and I concentrations were observed; these results are consistent with another study [30]. Moreover, a decrease of Fe/Cu ratio, while an increase of Cu/Fe in the serum samples of thyroid patients, but reversed pattern was observed as compared to controls. In the case of Cu/I ratio, increases in serum and urine of both patients and controls were observed (Table 7).

Conclusion

It was concluded that the pathogenesis of thyroid disease has been associated with changes in the balance of certain trace elements especially Cu, Fe, and I in biological samples (serum and urine) of goitrous patients. The increase of Cu in serum and urine samples together with decreases of Fe, I, and thyroid status of goitrous patients may be involved in disturbances of thyroid metabolism.

It was also concluded that inadequate and unbalanced feeding, specifically higher Cu levels in biological samples of thyroid patients, causes absorption disorders in the digestive system and reduction in the feed efficiency which reduces the Fe, I, and thyroid function. The present results revealed that supplementation may be required to improve the efficacy of Fe, I, and thyroid hormones metabolism in goitrous patients.

Acknowledgment The authors would like to acknowledge the Higher Education Commission of Islamabad, Pakistan for funding.

References

1. Laurberg P, Andersen S, Bulow P, Carle A (2005) Hypothyroidism in the elderly: pathophysiology, diagnosis and treatment. Drugs Aging 22:23–38
2. Nazifi S, Mansourian M, Nikahval B, Razavi SM (2009) The relationship between serum level of thyroid hormones, trace elements and antioxidant enzymes in dromedary camel (*Camelus dromedarius*). Trop Anim Health Pro 41:129–134

3. Al-Sayer H, Mathew TC, Asfar S, Khourshed M, Al-Bader A, Behbehani A et al (2004) Serum changes in trace elements during thyroid cancers. Mol Cell Biochem 260:1–5
4. Kazi TG, Afridi HI, Kazi N, Jamali MK, Arain MB, Jalbani N et al (2008) Copper, chromium, manganese, iron, nickel, and zinc levels in biological samples of diabetes mellitus patients. Biol Trace Elem Res 122:1–18
5. Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochim Biophys Acta 1275:161–163
6. World Health Organization (2001) Assessment of iodine deficiency disorders and monitoring their elimination. A guide for programme managers, second ed. WHO, Geneva
7. Zimmermann MB, Kohrle J (2002) The impact of iron and selenium deficiencies on iodine and thyroid metabolism: biochemistry and relevance to public health. Thyroid 12:867–878
8. Laurberg P, Nohr S, Pedersen KM (2000) Thyroid disorders in mild iodine deficiency. Thyroid 10:951–962
9. Venturi S, Donate FM, Venturi M, Venturi A, Grossi L, Guidi A (2000) Role of iodine in evolution and carcinogenesis of thyroid, breast and stomach. Adv Clin Pathol 4:11–17
10. WHO, UNICEF, ICCIDD (2002) In: Azizi F (ed). Iodine deficiency disorders in the Middle East and Eastern Mediterranean region, Pakistan. Tehran Endocrine Research Centre of Shaheed Beheshti: University of Medical Sciences, 30
11. Mayrides J, Khan MA (1995) Iodine deficiency disorders in urban areas of Pakistan. The Med Spec 16:22–23
12. Olivares M, Uauy R (1996) Copper as an essential nutrient. Am J Clin Nutr 63:791–796
13. Aihara K, Nishi Y, Hatano S, Kihara M, Yoshimitsu K, Takeichi N et al (1984) Zinc, copper, manganese, and selenium metabolism in thyroid disease. Am J Clin Nutr 40:26–35
14. Valko M, Morris H, Cronin MT (2005) Metals, toxicity and oxidative stress. Curr Med Chem 12:1161–1168
15. Kazi TG, Jalbani N, Kazi N, Arain MB, Jamali MK, Afridi HI et al (2009) Estimation of toxic metals in scalp hair samples of chronic kidney. Biol Trace Elem Res 127:16–27
16. Polkowska Z, Kozlowska K, Namiesnik J, Przyjazny A (2004) Biological fluids as a source of information on the exposure of man to environmental chemical agents. Crit Rev Anal Chem 34:105–119
17. Mesko MF, De Moraes DP, Barin JS, Dressler VL, Knapp G, Flores EMM (2006) Digestion of biological materials using the microwave-assisted sample combustion technique. Microchem J 82:183–188
18. Zowczak M, Iskra M, Paszkowski J, Manczak M, Torlinski L, Wysocka E (2001) Oxidase activity of ceruloplasmin and concentrations of copper and zinc in serum of cancer patients. J Trace Elem Med Biol 15:193–196
19. Thurlow RA, Winichagoon P, Green T, Wasantwisut E, Pongcharoen T, Bailley KB et al (2005) Only a small proportion of anemia in North East Thai school children is associated with iron deficiency. Am J Clin Nutr 82:380–387
20. Tanphaichitr V, Leelahagul P, Suwan K (2000) Plasma amino acid patterns and visceral protein status in users and nonusers of monosodium glutamate. J Nutr 130:1005–1006
21. Perez C, Scrimshaw NS, Munoz JA (1960) Technique of endemic goiter surveys. WHO Monograph series 44: endemic goiter. WHO, Geneva 369-383
22. Adibi A, Sirous M, Aminorroaya A, Roohi E, Mostafavi M, Fallah Z et al (2008) Normal values of thyroid gland in Isfahan, an iodine replete area. JRMS 13:55–60
23. Kandhro GA, Kazi TG, Afridi HI, Kazi N, Baig JA, Arain MB et al (2009) Effect of zinc supplementation on the zinc level in serum and urine and their relation to thyroid hormone profile in male and female goitrous patients. Clin Nutr 28:162–168
24. Abraham GE, Handal RC, Hakala JC (2006) A simplified procedure for the measurement of urine iodide levels by the ion-selective electrode assay in a clinical setting. Orig Internist 1:125–135
25. Abraham GE (2004) Serum inorganic iodide levels following ingestion of a tablet form of Lugol solution: evidence for anenterohepatic circulation of iodine. Orig Internist 11:29–34
26. Ghayur S, Siddiqui S, Alam MM, Shaukat A, Khan A (2004) Spectrum of iodine deficiency in school children of Rawalpindi. Pak Armed Forces Med J 51:27–32 Elahi S (2001). Nutr Res 24:1005-1010
27. Iftekhari MH, Eshraghian MR, Khosravi HM, Saadat N, Shidfar F (2007) Effect of iron repletion and correction of iron deficiency on thyroid function in iron deficient Iranian adeloscent girls. Pak J Biol Sci 10:255–260
28. Ordookhani A, Pearce EN, Hedayati M, Mirmiran P, Salimi S, Azizi F et al (2007) Assessment of thyroid function and urinary and breast milk iodine concentrations in healthy newborns and their mothers in Tehran. Clin Endocrinol 67:175–179
29. Kandhro GA, Kazi TG, Afridi HI, Kazi N, Arain MB, Sarfraz RA et al (2008) Evaluation of iron in serum and urine and their relation with thyroid function in female goitrous patients. Biol Trace Elem Res 125:203–212

30. Egli I, Davidsson L, Zeder C, Walczyk T, Hurrell R (2004) Dephytinization of a complementary food based on wheat and soy increases zinc, but not copper, apparent absorption in adults. J Nutr 134:1077–1080
31. Kucharzewski M, Braziewicz J, Majewska U, Gozdz S (2003) Copper, zinc, and selenium in whole blood and thyroid tissue of people with various thyroid diseases. Biol Trace Elem Res 93:9–18
32. Smith SM, Finley J, Johnson LK, Lukaski HC (1994) Indices of in vivo and in vitro thyroid hormone metabolism in iron-deficient rats. Nutr Res 14:729–739
33. Murakawa H, Bland CE, Willis WT, Dallman PR (1987) Iron deficiency and neutrophil function: different rates of correction of the depression in oxidative burst and myeloperoxidase activity after iron treatment. Blood 69:1464–1468
34. Zaichick VY, Tsyh AF, Vtyurini BM (1995) Trace elements and thyroid cancers. Analyst 120:817–821
35. Theophanides T, Anastassopoulou J (2002) Copper and carcinogenesis. Crit Rev Oncol Hemat 42:57–62
36. Aurthor KA, Kirchgessner M, Eder K (1996) Concentrations of thyroid hormones in serum and activity of hepatic 5 monodeiodinase in copper- deficient rats. Z Ernährungswiss 35:288–291
37. Aurthor JR, Beckett GJ (1999) Thyroid function. Brit Med Bull 55:658–668
38. Minerals for health, http://www.drlera.com/MINERALS/copper.htm
39. Cooper LB. Nutrients and nutrient requirements. Veterinary Nursing 221-222
40. Ikeda T, Nishikawa A, Imazawa T, Kimura S, Hirose M (2000) Dramatic synergism between excess soybean intake and iodine deficiency on the development of rat thyroid hyperplasia. Carcinogenesis 21:707–713
41. Judith RT, Joseph MD, William RK (2004) Zinc, iron, calcium, and magnesium metabolism with high copper intake. J Trace Elem Exp Med 17:172–181
42. Liel Y, Harman-Boehm I, Shany S (1996) Evidence for a clinically important adverse effect of fiber-enriched diet on the bioavailability of levothyroxine in adult hypothyroid patients. J Clin Endocrinol Metab 81:857–859
43. Vitti P, Martino E, Aghini-Lombardi F, Rago T, Antonangeli L, Maccherini D (1994) Thyroid volume measurement by ultrasound in children as a tool for the assessment of mild iodine deficiency. J Clin Endocrinol Metab 79:600–603
44. Akarsu E, Akcay G, Capoglu I, Unuvar N (2005) Iodine deficiency and goiter prevalence of the adult population in Erzurum. Acta Med (Hradec Kralove) 48:39–42
45. Liu N, Liu P, Xu Q, Zhu L, Zhao Z, Wang Z et al (2001) Elements in erythrocytes of population with different thyroid hormone status. Biol Trace Elem Res 84:37–43
46. Institute of Medicine Iodine (2002) In dietary reference intakes for vitamin a, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. DC National Academy Press, Washington, pp 258–289
47. Freake HC, Govoni KE, Guda K, Huang C, Zinn SA (2001) Actions and interactions of thyroid hormone and zinc status in growing rats. J Nutr 131:1135–1141