EFFECT OF CHROMIUM ADMINISTRATION ON HYPOTHALAMIC-PITUITARY-THYROID AXIS AND IODINE METABOLISM IN RATS

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EFFECT OF CHROMIUM ADMINISTRATION ON HYPOTHALAMIC-PITUITARY-THYROID AXIS AND IODINE METABOLISM IN RATS

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By

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IN THE NAME OF

ALLAH

THE MOST MERCIFUL
THE MOST BENEFICIENT
THE MOST COMPASSIONATE

CERTIFICATE

This is to certify that the dissertation, submitted by **Mr. Tariq Mahmood** is accepted in its present form by the Department of Animal Sciences, Quaid-i-Azam University Islamabad, as satisfying the requirements for the degree of Doctor of Philosophy in Animal Sciences (Animal Physiology).

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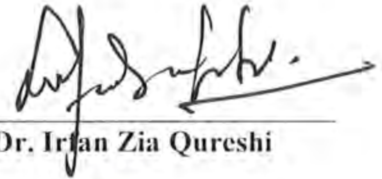

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Dedicated to ....

The dream of my father,

The prayers of my mother,

The sacrifices of my wife,

and

My loving children

Maha Rubab, Rimsha and Shahab

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LIST OF ABBREVIATIONS

°C	degree centigrade
av. wt.	average weight
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
Cr (III)	trivalent chromium
Cr (VI)	hexavalent chromium
dH_2O	distilled water
DPA	diphenyl amine
EDTA	ethylene Diamine Tetra Acetic acid
FT_3	free Triiodothyronine
FT_4	free Tetraiodothyronine
g	gram
H & E	Hematoxylin and eosin
HNO_3	nitric acid
hrs	hours
i.p.	intraperitoneal
$K_2Cr_2O_7$	potassium dichromate
l	liter
LD	lethal dose
LMP	low melting point
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	milli molar
mol. wt.	molecular weight
ng	nanogram
NIBGE	National Institute for Biotechnology and Genetic engineering
NIH	National Institute of Health
nm	nanometer

NMP	normal melting point
PAS	periodic acid Schiff
PBI	protein-bound-iodine
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PFA	paraformaldehyde
pmol	picomole
ppm	parts per million
RNA	ribonucleic acid
rpm	revolutions per minute
sec	second
TBE	tris-borate-EDTA
TEM	Transmission Electron Microscope
TRH	thyrotropin releasing hormone
TRI	trizol reagent
TSH	thyroid Stimulating Hormone
TTE	triton-tris-EDTA
μg	microgram
μl	micro liter
λmax	peak wavelength

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ABSTRACT

Toxicity due to heavy metals is of major concern in Pakistan. Mercury, cadmium, chromium, arsenic, lead, zinc and copper concentrations have increased to alarming levels in the water, soil and air which have become large dumps of industrial effluents. Physiologically, heavy metals can profoundly affect the functioning of the endocrine system. In Pakistan, thyroid related diseases are also very common. Chromium in hexavalent form is highly toxic and a known carcinogen, however as opposed to the trivalent form, its toxic effects on the thyroid structure and function are relatively unexplored. Acute exposure to chromium (VI) can occur in the occupational set up. The present study was designed therefore using laboratory rats as a model system to investigate the thyroid gland structure and function and iodine metabolism following two acute intraperitoneal doses of 30 mg/kg $_{b.w.}$ potassium dichromate given within 48 hours. Pituitary and hypothalamic tissues were also investigated. The study employed diverse methods including: atomic absorption spectrophotometry, solid-phase radioimmunoassay (RIA), RT-PCR, analytical methods for estimation of iodine, light and electron microscopy, DNA ladder assay and acridine orange staining.

Results showed that in the chromium treated animals, hypothalamic chromium concentration increased ($p < 0.05$), while thyroid chromium concentration decreased ($p < 0.01$); however, pituitary chromium was only slightly elevated. Whole blood chromium concentration increased ($p < 0.001$) while serum chromium concentration was slightly raised (p = n.s.). Chromium excretion in the urine increased ($p < 0.05$). Serum FT_4 and FT_3 levels decreased ($p < 0.01$ and $p < 0.001$, respectively), while serum TSH concentration increased ($p < 0.01$). RT-PCR indicated upregulation of TSH mRNA. Both the urine iodine and protein bound iodine levels decreased ($p < 0.01$; $p < 0.05$ respectively). Cells of the anterior pituitary showed hypertrophy with an increase in cell size ($p < 0.001$) but the cellular density decreased ($p < 0.001$). Thyroid gland showed follicular hyperplasia. The number of follicles increased ($p < 0.001$), however the follicular size decreased ($p < 0.001$). A large number of follicles were disorganized becoming irregular in shape, shrunken or regressed, while a few were fused with the others. Necrosis was also evident in a few sections. Interfollicular

spaces widened leading to an increase of stromal tissue. Epithelial cells were desquamated and their heights increased ($p < 0.001$), while the nuclear size regressed ($p < 0.001$). The nuclei were pyknotic ranging from irregular to round, and oval or elongated. Cytoplasmic size increased ($p < 0.01$). Colloid retraction and resorption were frequently observed. Thyroid gland ultrastructural analysis showed disrupted basal laminae of the follicles. The follicles therefore appeared collapsed. The nuclei of the cells were pyknotic with irregular shape. Most cell organelles were deformed. The endoplasmic reticulum and Golgi apparatus were either disrupted or disorganized, while mitochondria were not abundant. Colloid droplets decreased and an increase of collagen fibers was readily noticeable. Acridine orange staining of thyroid cells demonstrated excessive dead cells. DNA ladder assay of the hypothalamus, pituitary and thyroid glands showed fragmentation of the DNA. On quantification, it was found that the DNA damage occurred to the hypothalamus was 56 ± 1.78 %, to the pituitary it was 30 ± 1.26 % and to the thyroid gland it was 55 ± 1.21%.

The present study concludes that chromium in the hexavalent form is highly toxic to hypothalamic-pituitary-thyroid axis of the body. This might lead to severe and persistent hypothyroidism with drastic structural as well as functional alterations and the iodine concentration. It is suspected from the thyroid morphology that a long term or chronic, exposure could possibly result in tumor formation. Further studies are required to elucidate the mechanism of action of hexavalent or trivalent chromium at cellular and molecular levels and a complete understanding of the iodine metabolism in the case of chromium toxicity.

INTRODUCTION

Environmental pollution is one of the most devastating ecological crisis world is facing today. This is also true for most developing countries in the South Asia such as Nepal, India, Bangladesh and Pakistan (Karn and Harada, 2001), where water pollution is more severe and critical near urban stretches due to huge amounts of pollution load discharged by urban activities. About 90 to 95% of all domestic sewage and 75% of all industrial effluent are discharged into surface waters without any treatment (Hinrichsen *et al.*, 1997; Pandey, 2006).

Pakistan is an agricultural country; however, population increase, unplanned urbanization, deforestation and rapid industrialization over the past few decades have all contributed to environmental pollution. Unfortunately, laws as regard treatment and appropriate disposal of industrial wastes and effluents although exist, but despite pressure by the public, national and international environmental agencies, they are not stringently followed. The result is therefore contaminated soil, water and air, which have now posed a serious threat for humans, animals and plant lives (Hamidullah *et al.*, 1997; Iqbal *et al.*, 1998). The main sources of environmental pollution in our country are sewage water, city refuse, fertilizers, pesticides, automobiles, industrial effluents, leather tanning (Tahir *et al.*, 1998; Aftab *et al.*, 2000). Huge amounts of solid sludge and effluent water containing acids, alkalis, sodium chloride, heavy metals mainly cadmium and chromium etc. are released daily in tons of quantities into nullahs and streams; this waste directly flows into rivers and eventually into the ocean (Zaman and Ara, 2000; Tahir and Naseem, 2007).

Severe health effects commonly seen in the general population, besides other reasons, are more likely due to the contaminated environment. In Pakistan, dumping of the industrial waste, effluents and sewage into the soils and wetlands is a common practice that ultimately affects the food chain and the ecosystem. Besides pesticides, herbicides, organochlorines, PCBs, acids, alkalis, heavy metals contamination of lands, streams, rivers, marshes, and nullahs has alarmingly increased (Hanif *et al.*, 1987). Pakistani soils, air and water are heavily polluted with mercury, arsenic, lead, zinc, manganese, lithium, cadmium and chromium (Abbas *et al.*, 2004).

Several heavy metals present in drinking water are known to play important roles in the body provided their levels remain physiologically acceptable and within the specified range as recommended by the environmental protection agencies (US EPA, 1993) and (WHO, 1998). Jaleel *et al.* (2001) collected drinking water samples from different sources and localities of District East of Karachi and found dangerously elevated concentrations of lead and nickel in piped water, hand pump water and tanker water supply as compared to WHO recommended levels. Regarding wildlife, egg, regurgitate and sediment samples collected from two colonies of cattle egret (*Bubulcus ibis*) during the breeding seasons showed that the mean concentration of heavy metals: Pb, Cd, Cr, Co, Ag and Ni in eggs and regurgitates was higher as compared to the maximum residue limit (marl) standards prescribed by the Environmental Protection Agency (EPA) (Bostan *et al.*, 2007).

Of several different physiological systems of the body, endocrine system is of paramount importance. Thyroid gland related hormones are of particular importance with regard to the regulation of the diverse body functions including metabolism (Bentley, 1998).

1.1 Overview of the Thyroid gland

Thyroid gland is a very important endocrine organ of all vertebrates. Its hormones have ubiquitous involvement in growth, differentiation, metamorphosis, reproduction, hibernation and thermogenesis. The thyroid hormones are also present in the invertebrates; monoiodothyronine (MIT) and diiodothyronine (DIT) have been found in coelenterates, annelids, mollusks and insects (Bentley, 1998). Thyroxine in these invertebrates has been linked with seasonal and environmental conditions, in Jellyfish it induces strobilation, and is also associated with skeleton of sponges, corals, with periostracum of molluscan shells, the cuticle and setae of annelids and exoskeleton of arthropods (Kingsley *et al.,* 2001). Thyroid gland maintains optimum level of metabolism in tissues, stimulates oxygen consumption in most cells of the body, helps regulate lipid and carbohydrate metabolisms, and is also necessary for body growth. Absence of thyroid gland causes mental and physical slowing, poor resistance to cold and dwarfism in children. Excess thyroid secretion leads to body wasting, nervousness, tachycardia, tremor and excess heat production. One important function of the thyroid hormones is the regulation of basal metabolic rate (Ganong,

2003). Thyroxine is critical to the regulation of metabolism and growth throughout the animal kingdom. Among amphibians, for example, administering a thyroid-blocking agent such as propylthiouracil (PTU) can prevent tadpoles from metamorphosing into frogs; conversely, administering thyroxine will trigger metamorphosis (Hadley, 1996).

Thyroid gland itself is surrounded by a double connective tissue capsule (Barrington, 1979). Histologically, it is composed of spherical follicles, cells of which selectively absorb iodine as iodide ions (I^-) from the blood for the production of thyroid hormones (Bloom and Fawcett, 1975). Average requirement of iodine for an adult human being is 50 mg per year or 150 μg per day which is fulfilled through food or water. Twenty-five percent of body's iodide ions are present in the thyroid gland (Ganong, 2003).

Inside the follicles, colloid, rich in a protein called thyroglobulin, serves as a reservoir of materials for thyroid hormone production and, to a lesser extent, also acts as a reservoir for the hormones themselves. The thyroid follicles are consisted of a single layer of epithelial cells, which secrete 3, 5, 3′-triiodothyronine (T_3) and 3, 5, 3′, 5′-tetraiodothyronine (T_4), collectively called as iodothyronines (T_3 and T_4), necessary for normal growth, development and metabolism (Kierszenbaum, 2002). Scattered among the follicular cells and in the interfollicular spaces, are parafollicular cells, which secrete a calcium lowering hormone, calcitonin (Stevens and Lowe, 1997) that plays an important role in the regulation of calcium and phosphate metabolism (Sawicki, 1995; Sakai *et al.*, 2000).

Like the steroid hormones and retinoic acid, thyroid hormones cross the cell membrane and bind to intracellular receptors, which act alone, or with the retinoid X-receptor to modulate DNA transcription (Hadley, 1996). The primary site of action of T_3, and to a lesser extent T_4, is the target cell nucleus where they bind to intranuclear receptors. The hormone-receptor complex then forms a heterodimer with retinoid X receptor (RXR) to initiate the gene transcription. The receptors for T_3 (TRs) are encoded by two genes that encode the TRs, cerbA-alpha (TRα) and cerbA-beta (TRβ). Each of these genes is differentially spliced, forming 3 separate receptors (TRs); TRα1, TRβ1, and TRβ2. The effects of thyroid hormones are tissue-, cell-, and developmental stage-specific and it is believed that the relative abundance of different receptors in a specific cell may contribute to this selective action (Lazar, 1993;

Oppenheimer *et al.*, 1994; Mangelsdorf and Evans, 1995; Oppenheimer and Schwartz, 1997).

The half life of tissue stored thyroid hormones is greater than 24 hrs, and a pool of protein-bound thyroid hormones is present that can last for several days (Beard and Nathanielsz, 1976). Interspecies differences in protein binding capabilities, metabolism, reproduction and sex-related differences influence the thyroid function. The half life of both T_4 and T_3 is remarkably longer in humans than in rats. Distinct differences also exist between male and female rats with regard to the thyroid stimulating hormone (TSH), T_3 and T_4 levels. TSH and T_3 occur at higher concentrations in female rats than in male rats, while T_4 occurs at higher concentration in male rats than in female rats (Christian and Trenton, 2003). In rats, T_4 has predominant binding to albumin, with lower amounts bound to pre-albumin and post-albumin while T_3 is bound only to the albumin. This is because rats do not have thyroxine binding globulin (TBG), which is a principal transporter protein for T_4 in humans (Christian and Trenton, 2003).

Any alteration in the concentration of the thyroid hormones results into thyroid malfunction. Low levels of thyroid hormones result into hypothyroidism characterized by a decrease in the basal metabolic rate, hypothermia, cold intolerance, decreased sweating, cutaneous vasoconstriction, reduced cardiac output and slowed pulse. Hypothyroidism in adults leads to myxedema whereas the lack of thyroid hormones in the fetus causes cretinism, a condition observed in iodide-deficient geographic areas (Stubner *et al.*, 1987). Untreated hypothyroidism in children results in mental retardation. Hashimoto's disease is an autoimmune disease associated with hypothyroidism and is caused by autoantibodies targeted to thyroid peroxidase (TPO) and thyroglobulin (TG) (Ganong, 2003).

On the other hand, hyperthyroidism or Grave's disease is an autoimmune disease in which the thyroid gland is hyperfunctional. As a result, thyroid follicular cells become columnar and secrete large amounts of thyroid hormones into the blood circulation. Enlargement of the thyroid gland is called endemic goiter, which is characterized by typical symptoms as tachycardia, hot flushes and bulging of the eyes and is commonly called exophthalmic goiter (Kierszenbaum, 2002).

Relationship between mild iodine insufficiency and autoimmune thyroid disease is poorly understood and it is not clear whether this relationship occurs in non-human mammals, or non-mammalian vertebrates. Moreover, it is not clear whether environmental inhibitors of sodium-iodide symporter (NIS), like perchlorate are related to the autoimmune thyroid disease (Laurberg *et al.,* 2000).

1.1.1 Development of the Thyroid gland

The thyroid gland is the earliest endocrine structure to appear in the mammalian development. In higher vertebrates, the mature thyroid gland has a dual embryonic origin from two distinct regions of the endodermal pharynx. The median anlage arises from a thickening in the midline of the anterior pharyngeal floor (Nunez and Gershon, 1978). The two lateral analgens (ultimobranchial bodies) develop as caudal projections from the fourth or fifth pharyngeal pouch (Merida-Velasco *et al.,* 1989). In lower vertebrates, these two cell populations are not incorporated into the thyroid and exist as independent structures. In higher vertebrates, however, they contain the precursors of the parafollicular cells (Braverman and Utiger, 2000). The medial anlage appears first and becomes visible outpocketing in humans during gestational days 16 and 17. The thyroid diverticulum continues to expand ventrally, with most rapid proliferation at its distal tip, but it remains attached to the pharyngeal floor by a stalk called the thyroglossal duct. Continued proliferation of cells obliterates the lumen of the outpocketing, which becomes filled with cords of cells. The thyroid is essentially pulled to its position near the base of the neck as a consequence of the continuing decent of the heart during these early stages of thyroid formation. This caudal displacement is accompanied by the rapid elongation of the thyroglossal duct, which eventually fragments and incompletely degenerates (Nunez and Gershon, 1978).

1.1.2 Comparative Aspects of the Thyroid gland

Although there is anatomic variation among vertebrate species as regards the location and shape of the thyroid, basic structure of the gland in lower and higher vertebrates demonstrates a follicular pattern. Even though, the macroscopic shape of the thyroid gland is formed by the amalgamation of numerous follicles and can vary considerably among different vertebrate species, yet similarity of follicular cell structure exists at the ultrastructural level. The function of the thyroid gland as a

whole, therefore, is not influenced by its macroscopic shape suggesting that anatomic variation at the species level is not of fundamental evolutionary significance (Gorbman, 1986).

In cyclostomes and teleost fishes, there is no organized thyroid in the adult. Scattered follicles, however, do occur in the subpharyngeal connective tissue in a pattern roughly approximating the ventral aorta and its principal branches into the gills. In a few species of teleosts (parrot fish and swordfish), most thyroid follicles are gathered into an organized gland (Braverman and Utiger, 2000). The thyroid gland in elasmobranch fishes usually is aggregated into a single encapsulated organ near the tip of the lower jaw. In amphibians, thyroid lobes are two round structures, often quite widely separated and associated with branches of the thyroid cartilage. Among reptiles, the shape of the thyroid is variable, with turtles having a large disc of thyroid tissue immediately in front of the heart at the branching of the two systemic aortae, while lizards have a bilobed gland connected by an isthmus that crosses the trachea. Birds have two widely separated, round thyroid lobes, one on each side of the trachea at the level of the clavicles. Mammals are fairly consistent in that the thyroid consists of two lobes located on either side of the trachea and are connected together by an isthmus (Braverman and Utiger, 2000).

In contrast to microscopic and ultrastructural anatomy, total and free serum thyroid hormones reference ranges vary significantly from one mammalian species to another (Ortiz *et al.,* 2000). Within a single species, many different factors such as season, nutritional status, reproductive state, and contaminant load and health condition can influence thyroid hormone activity (Beland *et al.,* 1993; Schumacher *et al.,* 1993; DeGuise *et al.,* 1995; Rolland, 2000; Bhagavan, 2002).

Thyroid hormones are important in the hormonal regulation of seasonal breeders (Dahl *et al.,* 1994; Viguié *et al.,* 1999). Sex-related differences in thyroid hormone concentrations have been found in marine mammals, although results vary, for instance, age and sex-related differences in T_4 and T_3 have been found in the beluga whale (St. Aubin *et al.,* 2001).

1.1.3 Thyroid Histology and Ultrastructure

Thyroid follicular cells are low cuboidal to columnar in shape and their secretory polarity is directed toward the lumen of the follicles (Ericson and Nilsson,

1992). Polarity of the follicular cells is important for iodine uptake, whereas the follicular structure is required for the synthesis of the thyroid hormones (Takasu *et al.*, 1992). There is a great variability in the size of the follicles in human thyroid whereby, small follicles predominate over the large ones. In the rat and guinea pig, the follicles located at the periphery of the gland are larger while the centrally located follicles are smaller in size (Bloom and Fawcett, 1975). The luminal surfaces of the follicular cells protrude into the follicular lumen and have numerous microvilli that greatly increase the surface area in contact with colloid. An extensive network of interfollicular and intrafollicular capillaries provides the follicular cells abundant blood supply (Braverman and Utiger, 2000).

Follicular cells have extensive endoplasmic reticulum (RER) and a large Golgi apparatus in their cytoplasm for the synthesis and packaging of substantial amounts of protein that are then transported into the follicular lumen. Numerous electron-dense lysosomal bodies are present in the cytoplasm, which are important in the secretion of the thyroid hormones. The interface between the luminal side of follicular cells and the colloid is modified by numerous microvilli (Neve and Rondeaux, 1991). The hormonal secretions of the thyroid gland are controlled by the hypothalamus and anterior pituitary gland.

1.2 The Hypothalamic-Pituitary-Thyroid (HPT) axis

The Hypothalamic-Pituitary-Thyroid (HPT) axis and its hormones are of immense physiological importance and any alteration to this axis leads to a general disturbance of whole body metabolism. The hypothalamus is located at the base of the brain, below the third ventricle and just above the optic chiasm and pituitary gland (Krieger, 1980; Braak and Braak, 1992). Hypothalamus is that part of the brain which lies at the floor of the diencephalon. Nuclei or neural clusters contained within this region secrete hormones and are, therefore, considered as neuroendocrine cells. Although these cells are located behind the blood-brain barrier, they secrete their products outside this barrier into the hypothalamic-hypophysial portal blood vessels (Kierszenbaum, 2002).

Neuroendocrine (neurosecretory) neurons are hybrids between neurons and endocrine cells and are found only in the hypothalamus. Like neurons, they contain dendrites, a long axon and develop an action potential when stimulated electrically.

Like the endocrine cells, however, they release their messenger molecules into the blood stream, rather than in a synaptic cleft. There are two classes of neuroendocrine cells, magno- and parvocellular, which differ not only in size but also in function (Braak and Braak, 1992). The neuroendocrine cells of the hypothalamus exert positive and negative effects on the pituitary gland through peptides called releasing and inhibitory hormones or factors (Kierszenbaum, 2002).

Hypothalamus acts as a coordinating center for the endocrine system. It consolidates signals derived from upper cortical inputs, autonomic function, environmental cues such as light and temperature, and peripheral endocrine feedback (Fig. 1.1). The endocrine system is largely under the control of the hypothalamus which receives input from 'higher centres' and controls pituitary function via various releasing factors (e.g. corticotrophin releasing factor) which then stimulate the pituitary gland to release its hormones (e.g. adrenocorticotrophic hormone, ACTH) that stimulate endocrine glands elsewhere, (e.g. adrenal gland). Specifically, the hypothalamic-pituitary axis directly controls or regulates the functions of the thyroid gland, the adrenal gland, and the gonads, and as well as influences growth, milk production, and salt and water balance (Krieger, 1980). Hypothalamus contains several nuclei including the paraventricular nucleus (PVN), supraoptic nucleus (SON), suprachiasmatic nucleus (SC), arcuate nucleus, tuberomammillary nucleus, the lateral hypothalamic area etc. It is the PVN which is immensely important in controlling the hypoyhalamic-pituitary-thyroid axis via the release of a small tripeptide hormone called thyrotropin releasing hormone (TRH). Neurons whose cell bodies reside in the PVN, synthesize TRH (Segersen *et al.,* 1987). The PVN is an important hub where the endocrine and neural input is integrated, and also provides direct output to both the magno- and parvocellular endocrine system and the autonomic nervous system (ANS) (Ganong, 2003). TRH is phylogenetically ancient peptide found in lampreys and even in the snail, an invertebrate. It is widely expressed in central nervous system (CNS) and periphery in amphibians, reptiles and fishes but does not stimulate thyrotropin release in these poikilothermic vertebrates (Barrington, 1979).

Although TRH-secreting neurons are widely distributed throughout the brain (Jackson *et al.,* 1985; Lechan *et al.,* 1986), these neurons project uniformly to the median eminence, a neurohemal organ connected to the anterior pituitary gland by the

hypothalamic-pituitary-portal vessels (Ishikawa *et al.*, 1988; Merchenthaler and Liposits, 1994), and are the only TRH neurons to regulate the pituitary-thyroid axis (Aizawa and Greer, 1981; Taylor *et al.*, 1990). TRH is delivered by the pituitary-portal vasculature to the anterior pituitary gland to stimulate the synthesis and release of TSH or "Thyrotropin" by the pituitary thyrotrophs. TRH selectively stimulates the synthesis of the beta subunit of TSH (Haisenleder *et al.*, 1992) and also regulates post-translational glycosylation of TSH which affects its biological activity (Taylor and Weintraub, 1985; Lipman *et al.*, 1986; Weintraub *et al.*, 1989; Magner *et al.*, 1992; Harel *et al.*, 1993). TSH subsequently stimulates the thyroid gland to release T_3 and T_4 into the blood stream. When T_3 and T_4 levels are too low or too high, the hypothalamus and pituitary glands function to regulate thyroxine production (Hadley, 1996), via regulating the expression of TSH (Franklyn *et al.*, 1987; Mirell *et al.*, 1987; Shupnik *et al.*, 1986) and TRH. It has also been suggested that the functional characteristics of negative feedback must include more than simply the regulation of the gene encoding the secreted protein/peptide (Kollar *et al.*, 1987; Segersen *et al.*, 1987; Zoeller *et al.*, 2002). In this way, thyroid hormones (T_4 and T_3) exert a negative feedback effect (Fig. 1.1) on the release of pituitary TSH and on the activity of hypothalamic TRH neurons (Kollar *et al.*, 1987; Segersen *et al.*, 1987).

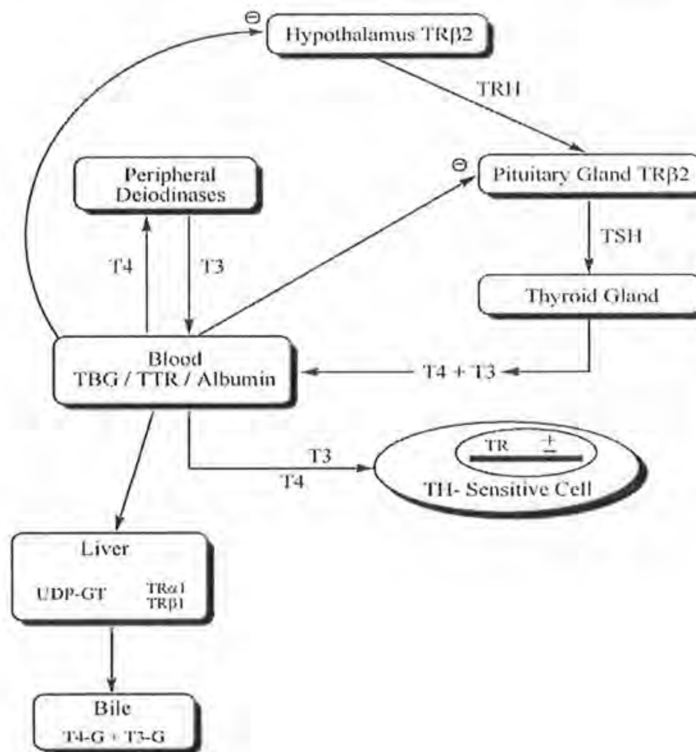


Fig. 1.1 The Hypothalamic-Pituitary-Thyroid Axis and negative feedback system.

1.2.1 Neurovascular Link between Hypothalamus and Pituitary gland

The pituitary stalk comprises mainly the neural and vascular components. The bulk of the stalk is made up of neural tissue in which lie various coiled capillary vessels on which end the nerve fibers that are derived from cells in the hypophysiotrophic area. The neurohormones coming down these nerve fibers are transferred from the endings of the fibers into the blood passing through the coiled capillaries, and then into the portal vessels. The origin of the vessels which supply them makes an important distinction (Xuereb *et al.,* 1954). The afferent arterioles to the coiled capillaries from which the long portal vessels are derived spring from the arterial ring supplied by the superior hypophysial arteries (arising from the internal carotid arteries above the level of the diaphragma sellae), while those which supply the coiled capillaries that form the short portal vessels are derived from the inferior hypophysial arteries, which leave the internal carotid arteries within the cavernous sinus. The long portal vessels run down the pituitary stalk to supply the larger part of the pars distalis, while the short portal vessels supply a restricted part of the lobe adjacent to that part of the lower infundibular stem which is buried in the pars distalis (Daniel and Prichard, 1975; Vitums, 1975).

1.3 Pituitary Gland

The pituitary gland, or hypophysis, is an endocrine gland which is a protrusion off the bottom of the hypothalamus at the base of the brain, and rests in a small, bony cavity, the *sella turcica*, covered by a dual fold termed diaphragma sellae. The pituitary fossa, in which the pituitary gland sits, is situated in the sphenoid bone in the middle cranial fossa at the base of the brain. It is composed of two lobes: the adenohypophysis and neurohypophysis. The adenohypophysis, also referred to as the anterior pituitary is divided into anatomical regions known as the pars tuberalis, pars intermedia, and pars distalis. The neurohypophysis is also referred to as the posterior pituitary or neurohypophysis (Kierszenbaum, 2002). Since the neurohypophysis and adenohypophysis differ anatomically and physiologically, and mainly because the secretions of the thyroid gland are regulated by thyroid stimulating hormone (TSH) that is secreted by the adenohypophysis or anterior pituitary.

The pituitary gland secretes various hormones which regulate, through their actions on other endocrine glands, diverse functions as homeostasis, growth,

reproduction, milk ejection and uterine contraction etc. It is functionally connected to the hypothalamus by the median eminence or pituitary stalk, through which hypothalamic releasing factors approach the pituitary and in turn, stimulate the release of pituitary specific hormones (Conklin, 1968). There are three distinct types of endocrine cells in the anterior pituitary:

a) "Acidophils", the cells that stain with an acidic dye and are prevalent at the peripheral border of the gland. These cells include somatotrophs and lactotrophs that secrete two major peptide hormones: growth hormone and prolactin, respectively (Kierszenbaum, 2002).

b) "Basophils", the cells that stain with a basic dye and are periodic acid-Schiff (PAS) positive, are predominant in the center of the gland. These cells include gonadotrophs, thyrotrophs and corticotrophs. The thyrotrophs in case of humans constitute only 5% of the anterior pituitary and the diameter of their secretory granules ranges from 120-200 nm. Basophils secrete glycoprotein hormones; gonadotrophins (follicle-stimulating hormone, FSH, and luteinizing hormone, LH, both released by gonadotrophs), thyroid-stimulating hormone (TSH) from thyrotrophs and adrenocorticotrophic hormone (ACTH) from the corticotrophs (Kierszenbaum, 2002). TSH is composed of an alpha (α) and a beta (β) subunit (Wondisford, 1996). The α subunit in case of humans consists of 89 amino acids while β subunit contains 112 amino acids. These two subunits develop association with each other for maximal physiologic activity. The α subunits of TSH, FSH, LH and human chorionic gonadotrophin (hCG) are identical. These are the products of a single gene and have the same amino acid composition. The β subunits, which are produced by separate genes and differ in structure, confer hormonal specificity (Hadley, 1996).

c) "Chromophobes", the cells which are inactive and have depleted their hormone content and also lack cytoplasmic staining (Kierszenbaum, 2002).

The hormones secreted by the posterior pituitary include antidiuretic hormone (ADH) and oxytocin. The intermediate lobe produces melanocyte-stimulating hormone (MSH), although this function is most often attributed to the anterior pituitary (Ganong, 2003).

1.4 Significance of iodine

In areas of the world where there is deficiency of dietary iodine, the thyroid gland can be considerably enlarged, resulting in the swollen necks of endemic goiter (Gutekunst *et al.,* 1986). In humans, children born with thyroid hormone deficiency will have physical growth and development problems, and brain development can also be severely impaired, in the condition referred to as cretinism. Newborn children in many developed countries are now routinely tested for thyroid hormone deficiency as part of newborn screening by analysis of a drop of blood. Children with thyroid hormone deficiency are treated by supplementation with synthetic thyroxine, which enables them to grow and develop normally (Kierszenbaum, 2002).

Because of the thyroid gland's selective iodine uptake and concentration of what is a fairly rare element, it is sensitive to the effects of various radioactive isotopes of iodine produced by nuclear fission. In the event of large accidental releases of such material into the environment, the uptake of radioactive iodine isotopes by the thyroid can, in theory, be blocked by saturating the uptake mechanism with a large surplus of non-radioactive iodine, taken in the form of potassium iodide tablets. While biological researchers making compounds labelled with iodine isotopes do this, however, such preventive measures are usually not stockpiled before an accident, nor are they distributed adequately afterward. One consequence of the Chernobyl disaster was an increase in thyroid cancers in children in the years following the accident (Yalcin *et al.,* 2007).

The use of iodized salt is an efficient way to add iodine to the diet. It has eliminated endemic cretinism in most developed countries, and some governments have made the iodination of flour mandatory. In this regard, potassium iodide and sodium iodide are the most active forms of iodine. Iodine deficiency leads to hypersensitivity to the goitrogenic effects of TSH (Bray, 1968). In humans, mild iodine deficiency can lead to goiter in the absence of elevated levels of serum TSH (Gutekunst *et al.,* 1986), taking into consideration that the population reference range of serum TSH is much broader than the individual variance in serum TSH. Still, goiter development in geographical regions of the world with low iodine correlates better with thyroidal iodine than with serum TSH (Stubner *et al.,* 1987).

1.5 Thyroid Hormone Synthesis

Tetraiodothyronine or thyroxine (T_4) is synthesized by the follicular cells on the tyrosine residues of the protein called thyroglobulin (TG) (Fig. 1.2). The thyroid gland concentrates iodide from the serum and oxidizes it at the apical membrane, attaching it to tyrosyl residues within TG to make diiodotyrosine and monoiodotyrosine. Major players in this process are TG, thyroid peroxidase (TPO), hydrogen peroxide (H_2O_2), pendrin, and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH). Further action of TPO, H_2O_2, and iodinated TG produces thyroxine and triiodothyronine (T_3).

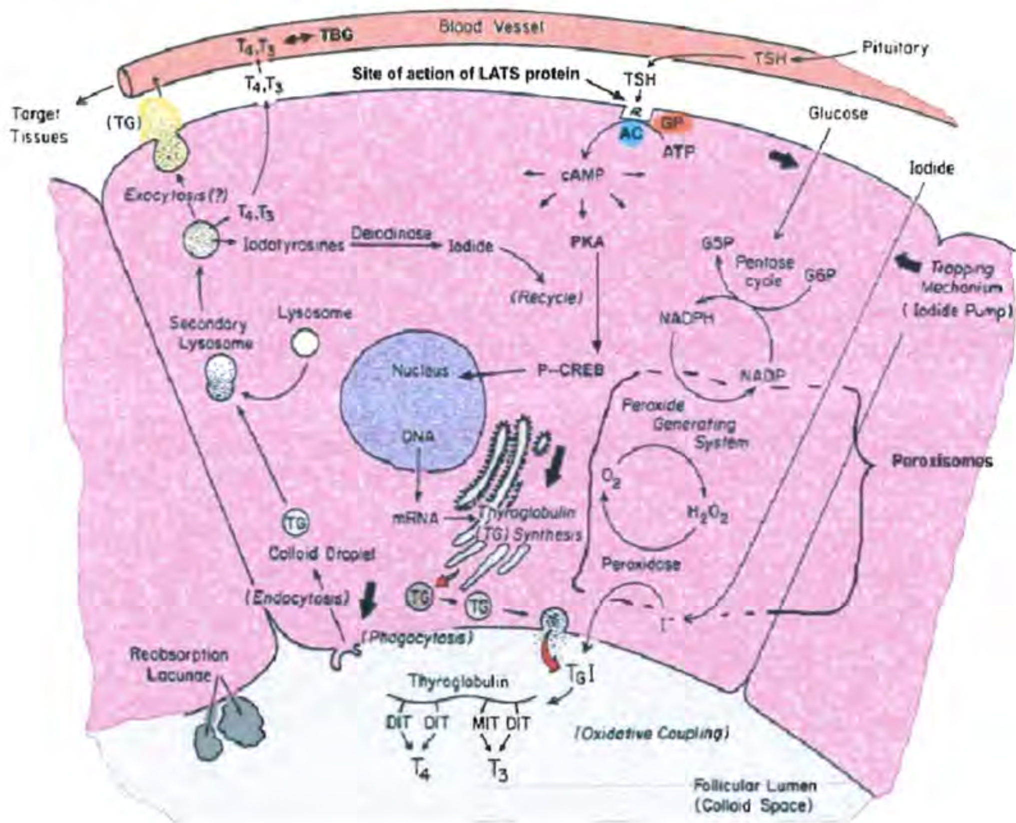


Fig. 1.2 Mechanism of synthesis of thyroid hormones in the thyroid epithelial cells (taken and modified from, "Endocrinology", 4th Ed, Hadley, 1996).

Iodine obtained from food or drinking water enters into the blood and is first converted to iodide, the ionic form of iodine, which is then captured by the process of "iodide trapping". TPO generates H_2O_2 which is linked to the 3' and 5' sites of the benzene ring of the tyrosine residues on TG, and on free tyrosine (Dunn and Dunn, 2001). Hormone-containing TG is stored in the follicular lumen, and then processed, most commonly by micropinocytosis. The lysosomal enzymes cathepsins B, L, and D

are active in TG proteolysis. TG digestion leaves T_4 and T_3 intact, to be released from the cell, while the 3, 5'-diiodotyrosine (DIT) and 3-iodotyrosine (MIT) are retained and deiodinated for recycling within the thyroid (Dunn and Dunn, 2001).
Pituitary TSH binds to receptors on the surface of thyroid follicle cells, stimulating the adenylate cyclase (Taurog *et al.*, 1996; Wondisford, 1996). Consequent increase of cAMP levels then regulate the uptake of iodide into thyroid cells, the iodination of tyrosyl residues on TG by thyroid peroxidase, synthesis and oxidation of TG, TG uptake from thyroid colloid, and production of the iodothyronines, T_4 and T_3. Of the thyroid hormones secreted by the gland, about 90% is T_4 and about 10% is T_3 (Prummel *et al.*, 2000).

Upon stimulation by the TSH, the follicular cells reabsorb TG and proteolytically cleave the iodinated tyrosines from TG, forming T_4 and T_3, finally releasing them into the blood from where they are actively transported into target tissues (Friesema *et al.*, 1999; Moreau *et al.*, 1999). T_4 can be converted to T_3 by the action of outer-ring deiodinases (ORD, Type I and Type II) (St. Germain and Galton, 1997; Bianco *et al.*, 2002). Peripheral conversion of T_4 to T_3 by these ORDs accounts for nearly 80% of the T_3 found in the circulation (Chopra, 1996).

1.6 Mechanism of Hormonal Release

Thyroid hormones are stored in the colloid as part of the iodinated TG molecule and must be released from the peptide linkage within TG before they are released by the thyroid. The process of hormone release from the thyroid gland begins with the activation of the TSH receptor and accumulation of cAMP. This results in the endocytosis of colloid and fusion of the endosome with a lysosome. Following this, the endosomal vesicle fuses with the basolateral membrane of the thyroid follicular cell to release TG, T_4, and T_3 (Hadley, 1996).

The follicular cell responds rapidly to TSH stimulation, with pseudopodia forming on the apical surface into the colloid, followed by numerous colloid droplets inside the cell (Wetzel *et al.*, 1965). Iodinated TG first appears intracellularly inside apical coated vesicles. Immature TG molecules may be recognized and recycled by the thyroid cells. There is evidence that TG binds to membrane preparations made from thyroid cells; binding is pH and temperature dependent, but is not dependent on the degree of TG iodination (Consiglio *et al.*, 1981). In addition, TG binding to

membrane preparations is dependent on the degree of post translational modification of TG including addition of the sialic acid and N-acetylglucosamine (Consiglio *et al.*, 1981; Miquelis *et al.*, 1987; Miquelis *et al.*, 1993). These studies indicate that there occurs selective uptake of TG molecules. Although Kostrouch *et al.* (1991; 1993) found no evidence that TG and albumin were taken up into thyroid cells selectively, they did find that the two proteins exhibited different intracellular fates, further supporting the view of selective sorting process.

1.6.1 Calcitonin

An additional hormone produced by the thyroid gland is calcitonin which contributes to the regulation of blood calcium levels. Calcitonin is produced from the parafollicular cells of the thyroid in response to hypercalcemia. It stimulates the movement of calcium into bones, in opposition to the effects of parathyroid hormone (PTH). However, calcitonin seems far less essential than PTH, as calcium metabolism remains clinically normal after removal of the thyroid, but not the parathyroid. It may be used diagnostically as a tumor marker for medullary thyroid adenocarcinoma, in which high calcitonin levels may be present and elevated levels after surgery may indicate recurrence. It may even be used on biopsy samples from suspicious lesions (e.g. swollen lymph nodes) to establish whether they are metastasis of the original cancer. Calcitonin can be used therapeutically for the treatment of hypercalcemia or osteoporosis (Hadley, 1996; Ganong, 2003).

1.6.2 Thyroglobulin Synthesis and Storage

Thyroglobulin is the substrate upon which thyroid hormones are synthesized (Dunn and Dunn, 2001). In its normal form, TG is a dimer with a molecular weight of 660 KDa. Like the TSH, TG is conserved among vertebrates. Thyroglobulin synthesis is controlled by three transcription factors – TTF-1 (thyroid transcription factor-1), TTF-2, and Pax8 (Damante and Di Lauro, 1994; Kambe *et al.*, 1996; Kambe and Seo, 1996). Experimental evidence shows that hypophysectomy or thyroid hormone treatment can decrease transcription of TG in rats (Van Heuverswyn *et al.*, 1984). The polypeptide chain of TG is synthesized ribosomally bound to rough endoplasmic reticulum. Under normal circumstances, properly folded TG dimers migrate to the Golgi complex to complete the addition of carbohydrate and sulfate moieties (Spiro and Bhoyroo, 1988).

Iodinated TG is stored in the adult rat thyroid gland at a high concentration (>100 mg/ml) (Smeds, 1972) indicating that the rat stores only a few days' worth of thyroid hormone whereas the normal adult human thyroid stores perhaps several months' worth of hormone (Brabant *et al.,* 1992; Dunn and Dunn, 2001). In contrast, fetal and neonatal human thyroid gland contains very little iodinated TG, containing only enough iodinated TG for a single day's worth of thyroid hormone at birth. This is of significance because chemicals that inhibit thyroid hormone synthesis (NIS- or TPO35 inhibitors) would not affect thyroid hormone release until this stored material is depleted (Etling and Larroche, 1975; Etling, 1977; Van den Hove *et al.,* 1999; Savin *et al.,* 2003).

1.6.3 Regulation of Iodine Uptake

The thyroid gland can concentrate iodine 20-40 fold over blood levels under normal physiological conditions. The sodium iodide symporter (NIS) pump mediates the initial step in the thyroid hormone synthesis, the uptake of iodide into the cell (Fig. 1.4). NIS accomplishes this because it is an intrinsic plasma membrane protein

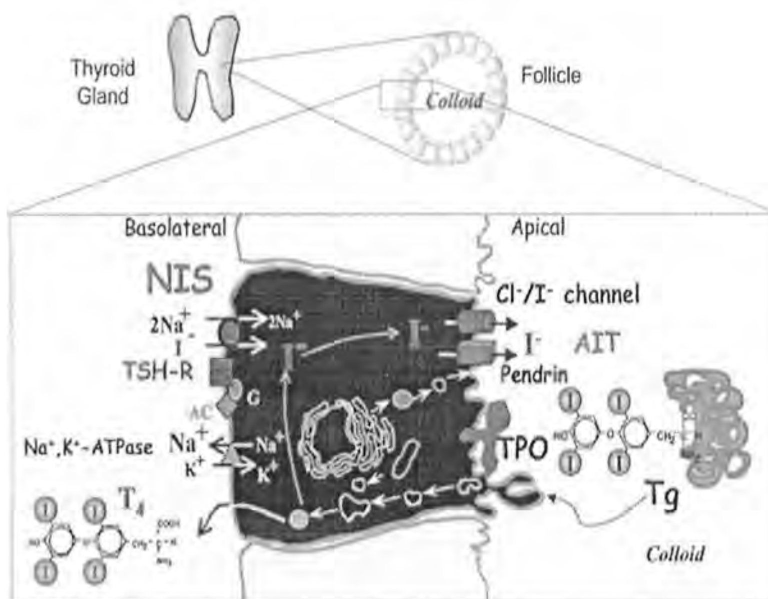


Fig. 1.4 A follicular cell, emphasizing the role of TSH in regulating iodide uptake through the sodium/iodide symporter (NIS), pendrin and the thyroid peroxidase (taken from "Thyroid iodine transport", Thyroid, Spitzweg *et al.,* 2000).

present on the basolateral surface of thyroid follicular cells and it couples the inward "downhill" translocation of Na^+ to the inward "uphill" translocation of I^-. The driving

force for the process is the inwardly directed Na^+/K^+ that generates a large concentration gradient of sodium (35-fold higher outside the cell). Anions such as thiocyanate and perchlorate block this symporter pump. Interestingly, perchlorate does not appear to be transported by the NIS (Eskandari *et al.,* 1997), indicating that it is a blocker of NIS function, not a competitive inhibitor. A number of other environmentally relevant anions such as nitrates, chlorates, also inhibit NIS function (Wolff, 1998). Transcription of the NIS gene is under the regulation of TTF-1, TTF-2 and Pax8 which are activated by protein kinase A (PKA) activity stimulated by the TSH. Thus, the ability of the thyroid gland to trap iodide is enhanced up to 200-fold by the TSH. The NIS pump is also structurally and functionally conserved among vertebrates (Cabello *et al.,* 2003).

1.6.4 Mechanism and Location of Iodine Organification – Thyroid Peroxidase

There are four major sites on the thyroglobulin protein where iodine becomes covalently attached ("hormonogenic" sites designated as A-D). These "sites" are tyrosyl residues that accept an iodine atom as the consequence of thyroid peroxidase (TPO) activity. The utilization of the major and minor (those less frequently used) sites varies under different physiological conditions and among different species. Some of these sites are more important for the formation of T_4 as compared to T_3 (Dunn and Dunn, 2001). Autoradiographic evidence obtained by the electron microscopy indicates that TG iodination occurs at the cell-colloid interface close to the apical membrane (Ohtaki *et al.,* 1982; Yokoyama and Taurog, 1988; Taurog *et al.,* 1990). This process occurs quickly in as much as the appearance of silver grains (in liquid emulsion) concentrated over the apical border of the cell 40 seconds after the injection of ^{125}I in rats. Thus, TG appears to be iodinated on the colloid and the apex of the thyroid follicle cell, and specific tyrosyl residues are coupled within the backbone structure of TG (Ekholm and Wollman, 1975; Wollman and Ekholm, 1981).

Iodide, the form of iodine that enters the cell, must be oxidized to a higher oxidation state before it is transferred to TG. Of the known biological oxidizing agents, only H_2O_2 and O_2 are capable of oxidizing the iodide, a process called organification. Organification of iodine is controlled by the thyroid peroxidase, TPO, which is a heme-containing enzyme and bears two substrate sites. Both substrates are assumed to undergo one-electron oxidation, yielding the corresponding radical, iodine

and tyrosine. Thus, this is a two-step mechanism of iodination, similar to other peroxidases (e.g., lactoperoxidase). However, in the absence of H_2O_2, TPO has no catalytic activity. The stepwise binding of iodide to the tyrosyl residues in thyroglobulin requires oxidation of inorganic iodide (I^-) to molecular iodine (I_2) by the thyroid peroxidase present in the luminal aspect of follicular cells and adjacent colloid. It has been suggested that the glucose-glucose oxidase system produces this important oxidizer (Taurog *et al.*, 1996).

Thyroid peroxidase is also involved in the coupling reaction, the process whereby iodinated tyrosyl residues are coupled together with an ether bond (–O–) (Taurog and Nakashima, 1978). According to the proposed coupling scheme (Taurog, 1996), TPO and H_2O_2 oxidize targeted tyrosyl residues on TG, forming an oxygen radical on one residue and a carbon radical on the other. There is a non-enzymatic coupling forming quinolone ether followed by a rearrangement which removes the amino terminus and forming the iodothyronine. The TPO enzyme is also highly conserved among vertebrates (Taurog *et al.*, 1996). The relationship between TPO inhibition and TG iodination is currently not well understood (Doerge and Chang, 2002; Doerge and Sheehan, 2002).

Excess iodide can inhibit the activity of adenylate cyclase; therefore, as such iodide can block both iodine organification and synthesis. Although there exist no studies which could indicate a functional coupling between iodine organification (i.e., TPO activity) *per se* and hormone synthesis and release, the observation that TPO inhibitors block the autoregulatory effects of iodide is of significance. Within this context, several reports demonstrate that dietary iodine intake changes the vascularity of the thyroid gland. Low dietary iodine content can increase thyroidal vascularity in rats, and this is reversed by an iodine supplement diet. The changes occur within 7 days and remain nearly the same at 133 days (Michalkiewicz *et al.*, 1989).

1.6.5 Thyroid Autoregulation by Iodine

The observation that serum TSH remains within a normal range despite fluctuations in daily iodine intake (in iodine-sufficient regions) of between 50 and 1,000 μg suggests autoregulatory role of iodine. Although children of women treated during pregnancy with the highly iodinated drug amiodarone have hypothyroidism and neurological deficiencies (Bartalena *et al.*, 2001). The mechanism(s) by which

iodide controls thyroid functions are not well understood but it has been suggested that there are iodocompounds other than thyroglobulin and thyroid hormones, produced by the action of TPO which then mediate the inhibitory effects on the thyroid gland. It has been suggested that such compounds are iodolipids, especially arachidonic acid derivatives (Krawiec *et al.,* 1991).

This inhibitory effect of excess iodide on adenylate cyclase is itself blocked by inhibitors of iodide organification, indicating that iodinated intermediates formed by the action of thyroperoxidase play a role in the regulation of cAMP production (Corvilain *et al.,* 1988). Therefore, in both humans and in experimental rodent systems, persistent exposure to excess iodide results in an inhibition of intracellular thyroidal cAMP and all cAMP-mediated events (Van-Sande *et al.,* 1975; Filetti and Rapoport, 1983). The observation that excess iodide inhibits the transport of iodide, uptake of deoxyglucose and amino acids into the thyroid, as well as cAMP formation and Na^+/K^+-ATPase activity in thyroid cells indicates a membrane site of action of iodide (Krawiec *et al.,* 1991).

1.7 Thyroid Hormone Transport

Proteins involved in the transport of thyroid hormones in the blood are serum thyroxine- and triiodothyronine-binding proteins, which are acidic glycoproteins, namely thyroxine-binding globulin (TBG), transthyretin (TTR), formerly called thyroxine-binding albumin and albumin. Transthyretin is a 55 KDa protein in humans, it is non-glycosylated and its plasma concentration is 4 mmol/L (250 μg/ml). Thyroxine binding globulin is a 54 KDa protein, its plasma concentration is 270 nmol/L. Both, TBG and TTR, are synthesized in the liver. In addition, high density lipoproteins transport 3 % to 6 % of the bound circulating T_4 (Benvenga *et al.,* 1993). In humans, about 75% of T_4 is bound to TBG, 15% is bound to TTR and the remainder is bound to albumin (Schussler, 2000). Only a very small fraction of the circulating hormone is free (unbound), of which T_4 is 0.03% and T_3 is 0.3%. It is only the free fraction that bears hormonal activity. Thyroxine binding globulin, the least abundant but most avid T_4 binder, is a member of a class of proteins that also includes cortisol binding protein (CBP) and is cleaved by serine proteases present in the serum. These enzymes are secreted into blood during inflammatory responses and, in the case of CBP, can induce the release of cortisol at the site of inflammation. The

physiological significance of this observation is presently unclear for TBG (Schussler, 2000).

Transport in the blood stream by TBG prevents the thyroid hormones from being metabolized and excreted. The remaining T_4, or free T_4 (FT_4), is biologically active and exerts negative feedback inhibition on TSH secretion, and is capable of entering cells (Thuestad *et al.,* 2000; Hardman and Limbrid, 2001). Low serum thyroid hormone globulin concentrations are observed when hypothyroidism results from athyreosis or a low thyroid reserve (Contempre *et al.,* 1996).

After release into the blood stream, T_3 and T_4 bind to albumin and prealbumin (transporting proteins) through non-covalent bonds and are distributed to peripheral tissues. Most of the circulating T_3 originates from the extra-thyroidal tissues and the peripheral deiodination of T_4 to T_3 takes place mainly in the liver and is dependent on 5′ - monodeiodinase activity (Paier *et al.,* 1993; Chaurasia *et al.,* 1996). Once free T_4 enters the cells it is deiodinated to form T_3 or reverse T_3 (rT_3) (Fig. 1.5). Triiodothyronine is generally being produced during normal circumstances, while rT_3 is generally produced during times of illness, starvation, or excessive endogenous catabolism (Christian and Trenton, 2003). Tetraiodothyronine is primarily metabolized in the liver by removal of the 5′ iodide which yields T_3 or by removal of the 5′ iodide, which yields inactive T_3 (rT_3). Once in the liver, T_3 and T_4 are combined with glucuronic and sulfuric acids via the phenolic hydroxyl group and then are excreted in the bile. Thyroid hormones can also be metabolized locally by target tissues such as the brain (Hardman and Limbrid, 2001).

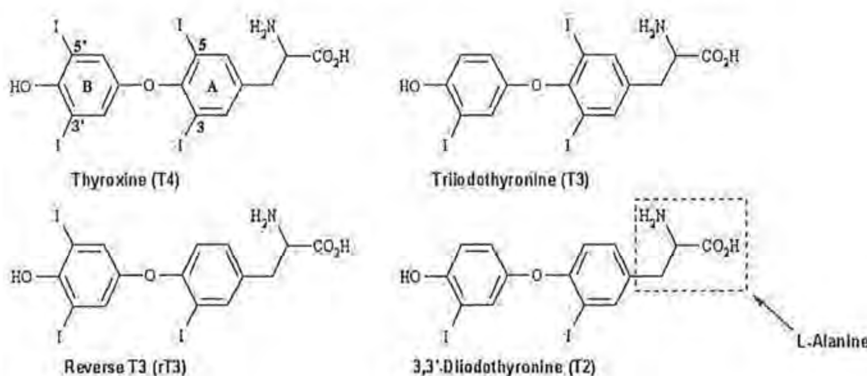


Fig. 1.5 Structures of thyroid related hormones (taken and modified from "Review of Medical Physiology" by Ganong, 2003).

Once the thyroid hormones are cleared from the blood by the liver following glucuronidation via UDP glucuronosyl transferase, these modified thyroid hormones are then eliminated along with the bile. T_4 and/or T_3 are actively concentrated in target cells about 10-fold over that of the circulation (Hood and Klaassen, 2000).

A physiological disturbance such as fasting suppresses the activity of TRH neurons by a neural mechanism that may involve leptin (Fekete *et al.,* 2000). This fasting-induced suppression of TRH neurons results in the reduction of circulating levels of the thyroid hormones. In humans and in rodents, circulating levels of T_4 and T_3 fluctuate considerably within an individual; therefore, TSH measurements are considered to be diagnostic of thyroid dysfunction (Roti *et al.,* 1993; Chopra, 1996).

1.8 Pathophysiology of the thyroid

Although the basic hypothalamic-pituitary-thyroid axis functions in a very similar manner in animals and humans, there are important differences between species that are significant when extrapolating data from chronic toxicity and carcinogenicity studies of new drugs and chemicals under development in animals for human risk assessment (Capen, 1989; Zbinden, 1988).

Chronic treatment of rodents with goitrogenic compounds such as thiouracil and its derivatives, results in follicular cell adenomas (Capen, 1997). Excessive secretion of TSH alone in the absence of any chemical exposure has been reported to produce a high incidence of thyroid tumors in rodents (Ohshima and Ward, 1984). Xenobiotics that induce liver microsomal enzymes and disrupt thyroid function in rats include central nervous system-acting drugs (phenobarbital and benzodiazepines), calcium channel blockers (nicardipine, bepridil), steroids (spironolactone), chlorinated hydrocarbons (chlordane, DDT) and polyhalogenated biphenyls (Capen, 1997).

Similarly, exposure of rats to compounds altering thyroid functions such as ammonium per chlorate causes significant structural and functional changes in the gland (Christian and Trenton, 2003).

A wide variety of chemicals and drugs inhibit the organification of thyroglobulin include thionamides (thiourea, thiouracil, propylthiouracil, methimazole, carbimazole, and goitrin), aniline derivatives and related compounds (sulfonamides, amphenone etc.) and substituted phenols (resorcinol, phloroglucinol, 2, 4-dihydroxybenzoic acid) and other miscellaneous inhibitors (Braverman and Utiger, 2000).

Thyroid toxicants are generally defined as toxicants that alter the circulating levels of thyroid hormones. However, the interpretation of data derived from these studies often rests on an incomplete analysis of the dynamic relationships within the HPT axis (Brucker-Davis, 1998).

Alterations in thyroid function and morphology have been induced by polychlorinated biphenyls (PCBs) in experimental animals (Bryne *et al.,* 1987; Brouwer *et al.,* 1989) and have been related to the effects of organochlorines (OCs) on thyroid hormone transport proteins, receptors, and thyroid hormone activity. Captive harbor seals (*Phoca vitulina*) experimentally fed fish contaminated with PCBs exhibited reductions in serum FT_4 and FT_3 concentrations as compared to seals that ingested fish that were from noncontaminated waters (Brouwer *et al.,* 1989). Plasma T_3 deficiency has also been associated with chlorinated hydrocarbon exposure in spotted seals (*Phoca largha*) and ribbon seals (*Phoca fasciata*) (Chiba *et al.,* 2001). Moreover, there are a number of toxicants that directly interfere with TPO activity (Wolff, 1998). Among other wide variety of toxicants, heavy metals are those elements which have been shown to profoundly affect physiological systems by actions on cells, tissues and biomolecules.

1.9 Metals and the thyroid gland

Metal elements play crucial roles in the maintenance of physiological homeostasis. The roles of sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg), which constitute the macronutrients, are very well established. Similarly, trace elements or micronutrients, such as iodine (I), cobalt (Co), copper (Cu), manganese (Mn), selenium (Se), zinc (Zn) and chromium (Cr), play vital roles as coenzymes or cofactors in various metabolic reactions. Various heavy metals have been found to be uptaken by the tissues causing destructive effects on cells and disruption of metabolic activities. However, not all the metals are toxic as few of them such as zinc (Zn) and selenium (Se) also have protective effects against various toxicants. Type-1 deiodinase, abundant in liver and kidney tissues, is sensitive to Se deficiency, which decreases deiodinase activity and therefore the conversion T_4 into T_3, resulting in a decrease in the T_3:T_4 ratio. On the other hand, type-2 and type-3 deiodinases are less sensitive to Se deficiency, indicating their importance for the maintenance of normal thyroid hormone levels (Arthur *et al.,* 1999). In addition, glutathione peroxidase

(GPx) that may protect the thyroid gland from oxidative damage due to any excess of H_2O_2 produced during the process of thyroid hormone synthesis (Arthur *et al.*, 1999). Therefore, Se deficiency may exacerbate some effects of iodine deficiency and may have a role in the etiology of iodine deficiency disorders (Arthur and Beckett, 1999; Arthur *et al.*, 1999).

The long-term administration of small amount of cadmium (Cd) has been shown to result in significant changes of thyroid gland histomorphology and considerable reduction of serum calcium and triiodothyronine concentration in rats (Pilat-Marcinkiewicz *et al.*, 2002). In addition to its effects on thyroid follicular cells, it also affects the parafollicular cells (Pilat-Marcinkiewicz *et al.*, 2002). Cadmium influences dose dependent structural and functional changes of the thyroid follicular cells in female rats (Pilat-Marcinkiewicz *et al.*, 2003). It also inhibits the conversion of T_4 to T_3 (Pavia *et al.*, 1997, Chaurasia *et al.*, 1996, Gupta *et al.*, 1997 and Gupta and Kar, 1999).

Aluminium (Al), depending on the dose, causes structural tissue degeneration and cellular injury of thyroid follicles. Exposure of rats to Al for a long time period causes degenerative changes in the thyroid gland (Aktac and Bakar, 2002). Isimer *et al.* (1998) and Aktaç (2001) have shown that Al is found in higher concentration in the adrenal and parathyroid glands and causes structural and functional defects in the adrenal glands. Waring *et al.* (1996) reported that plasma T_3 and T_4 concentrations were increased in sub-lethally aluminium-stressed brown trout (*Salmo troutta*).

Lithium (Li) is associated with sub clinical overt hypothyroidism in up to 34 % and 15 % of human patients, respectively and that can reappear abruptly even after many years of treatment (Lazarus, 1998). On the other hand, lithium associated thyrotoxicosis is rare and occurs mainly after the long term use of lithium containing drug (Barclay *et al.*, 1994).

A great concern is about the adverse endocrine effects of several occupational and environmental chemical agents; in particular, heavy metals on human health (Baccarelli *et al.*, 2000). Destructive effects of lead (Pb) and cadmium (Cd) on the function and structure of thyroid gland have been demonstrated at length. Experimental studies performed with laboratory animals have shown that a mixture of lead and cadmium administered for 70 days causes disruption of TSH secretion, slight

alterations in the thyroid gland histomorphology, and insignificant reduction of serum thyroxine level (Wade *et al.*, 2002).

The chronic exposure of human males at work places to small amounts of lead results in a significant reduction of blood T_4 and T_3 levels (Lopez *et al.*, 2000). Exposure to lead has been shown to cause significant toxicity to the hypothalamic–pituitary–thyroid axis resulting in higher concentration of TRH and TSH (Erfurth *et al.*, 2001). In addition to alteration of the endocrine system, the occupational exposure of adults to inorganic lead has been associated with impaired uptake of iodine by the thyroid tissue as well as depressed free thyroxine level and altered morphology of the gland (Siegel *et al.*, 1989).

Administration of the lead solution through gavage results in considerable alterations of thyroid structure and thyroid hormone levels. Lead is well known to influence the TRH synthesis and increases the binding of this hormone to anterior pituitary receptors resulting in consequent increased release of TSH (Lau *et al.*, 1991). As a result, impaired uptake of iodine by the thyroid gland occurs that leads to decreased volume of the follicles (Siegel *et al.*, 1989) and contributing at the same time to the formation of the relative iodine deficiency. It leads to expansion of connective tissue and reduction of the thyroglobulin concentration in the follicles of the gland.

Kobayashi *et al.* (2005) investigated the cellular toxicity and endocrine disrupting action of polyaromatic hydrocarbons (PAHs) and heavy metals on cultured human thyroid follicular cells. They found that cell toxicity was exhibited by cadmium, copper (Cu), nickel (Ni) and zinc; in particular, the influence of cadmium was remarkable. It was also found that the toxicity of cadmium was decreased by the addition of high concentrations of either calcium or selenite.

1.9.1 Chromium

Chromium is an important trace element of the body and is an integral part of glucose tolerance factor (GTF). It is known to play a physiologically significant role in the glucose metabolism via the regulation of insulin secretion (Anderson, 1998). It exists in two valence states in nature: trivalent chromium [Cr (III) or Cr^{3+}] and hexavalent chromium [Cr (VI) or Cr^{6+}]. Chromium (III) is an essential trace element for animal bodies, known to regulate insulin function and is also required for normal

protein, lipid and carbohydrate metabolism (Bagchi *et al.*, 2002). Its deficiency is associated with hyperglycemia which is reversed by chromium supplementation (Vehage *et al.*, 1996). In contrast, chromium (VI) is commonly used in industrial chrome plating, alloys, cast iron and wood treatment and is a proven toxin. Chromium (VI) induces oxidative stress, DNA damage, apoptotic cell death and altered gene expression. Chromium is released in the ambient air through natural resources as industrial end product uses and burning of fossil fuels and wood. The most important industrial sources of chromium in the atmosphere come from ferrochrome production. Ore refining, chemical and refractory processing, cement producing plants, automobile brake lining and catalytic converters for automobiles; leather tanneries and chrome pigments also contribute to the atmospheric burden of chromium. In urban areas, approximately two-third of the chromium in air results from the emission of Cr (VI) from fossil fuel combustion and steel production while the remaining chromium in air is in the trivalent form. Chromium is removed from the air by atmospheric fall out and precipitation (Fishbein, 1981).

1.9.2 Chromium as a Trace element in the Body

Trivalent chromium plays an important role in glucose intolerance, type-II diabetes and gastrointestinal diabetes (Anderson, 1998). In the human body, chromium is absorbed from the small intestine; however, trivalent chromium compounds generally have low toxicity and are poorly absorbed through the gastrointestinal tract (Von Burgh and Liu, 1993). From the site of intestinal absorption, chromium is taken up by plasma-protein fractions. Small physiological doses of chromium bind almost entirely to the iron-binding protein, transferrin (Hopkins and Schwarz, 1964). On the other hand inhaled chromium is bound to albumin rather than to transferrin (Glasser *et al.*, 1984). The chromate ion [CrO_4], the dominant form of chromium (VI), in neutral aqueous solutions can readily cross cellular membranes via non-specific anion carriers (Danielson *et al.*, 1982). Hexavalent chromium readily enters cells through the phosphate and sulfate anion-exchange carrier pathways, although a portion may remain in plasma for an extended period. It is also concentrated in the liver, kidney and spleen tissues. Once in the cell, Cr (VI) may be reduced to Cr (III) which may subsequently interact with cellular macromolecules, including DNA (Wiegand *et al.*, 1985). Chromium is also present in nucleic acids in very high concentrations where it plays a role in nucleic acid

metabolism (Okada *et al*., 1984). RNA synthesis in mouse liver significantly increases due to as little as 1 μmol trivalent chromium in the presence of DNA or chromatin (Okada *et al*., 1981). Trivalent chromium given at the rate of 2.5 mg/kg body weight increases the synthesis of fatty acids and cholesterol in the liver, while lower physiological doses appear to decrease serum cholesterol concentrations in rats (Schroeder and Balassa, 1965).

A number of minerals influence the absorption of chromium. In studies conducted on rats with radioactive chromium (^{51}Cr), it was found that zinc supplementation reduced chromium absorption, while zinc deficiency had the opposite effect, elevating radioactive chromium levels. However, Anderson *et al*. (1996) found no alteration in tissue levels of copper and zinc when mice were fed 5000 ng Cr III/g of feed. In a study on rats fed 5000 ng/g containing a number of organic chromium compounds (chromium picolinate, nicotinate, acetate, glycinate, histidinate etc.), results showed that all compounds tested increased the iron (Fe) content in the liver and spleen while decreasing the iron levels in the heart. The interaction of iron and chromium is thought to be linked to the shared binding sites on transferrin. Iron competes with chromium binding, affecting its transport. This theory is further supported by studies of patients with hemochromatosis who were found to have significantly higher excretion of the unbound plasma chromium as well as a smaller blood pool of chromium due to the saturation of transferrin by iron (Lim *et al*., 1983).

Chromium picolinate, used as chromium supplement, is found to have absorption in humans estimated at 2.8 percent ± 1.14 SD (Gargas *et al*., 1994). Studies on rats have found that 3-8 times more chromium nicotinate was absorbed and retained than was chromium picolinate or chromium chloride. After 6-12 hours, tissues retained on the average 2-4 times more chromium nicotinate than chromium picolinate (Olin *et al*., 1994). Concentrations of chromium picolinate in the liver and kidney were found to be 2-6 times higher than for chromium chloride- or chromium nicotinate-fed rats, with no detectable toxicity (Anderson *et al*., 1985).

1.9.3 Chromium as a Toxicant

Industrial development plays the role of backbone for any country's development and economic prosperity. As Pakistan is a developing country and basically an agricultural country, about 80 % population lives in the rural areas and is

involved in cultivation and farming. Due to the economic pressure people are migrating towards big cities for the sake of employment. Moreover, urbanization is taking place even in the rural areas and agricultural fields as a result are getting short. In the cities, people are becoming associated with industries for earning their living.

Among the industries, tanning industry is one of the most important. In Pakistan, there has been a rapid and uncontrolled mushroom growth of tanning industries in all big cities like Kasur, Faisalabad and Sialkot and metropolitans like Hyderabad, Lahore and Karachi. No safety measures, legislation or laws for the use and disposal of hazardous chemicals exist. It has resulted in gradual deterioration of our environment and health hazards of perceptible magnitude (Hanif *et al.,* 1987). In the leather tanneries, there are used about seventeen different tanning substances but chromium is the most commonly used tanning agent (Venier *et al.,* 1985). About 90% of all the leather produced and exported is tanned with chromium salts (Katherine and Schwedt, 1994). In Pakistan, tanning industry is becoming one of the major causes of environmental pollution in the country. The effluents coming from these industries are a major hazard to the environment which currently is an acute problem in the country (Anonymous, 1998).

There are more than 300 tanning units running only in the Kasur district, the industrial wastes of which are thrown in the open fields or in water bodies and Nullahs located in the vicinity of tanning areas, which is a serious threat to plant and animal life forms and humans. This water then flows into streams, canals and rivers, besides taken up by the plants. The water of nearby ponds is rapidly getting polluted with hexavalent chromium and other heavy metals contained in the effluents. The children of the people living in that area use this pond water to take bath also. Moreover, their cattle also drink this polluted water. Since the soil water of the adjoining areas containing these pollutants is absorbed by the plant roots and in this way, chromium and other heavy metals get straight away into the food chain. Chromium concentration has been determined in the chrome tanning sections in one of the Faisalabad city tanneries and found to be 1600 mg/L (Bhalli and Khan, 2006), which is 3200 times greater than that recommended by the national and international environmental protection agencies (EPA) (Anonymous, 1999).

Hexavalent chromium is highly toxic and causes allergic dermatitis, produces toxic and carcinogenic effects in humans and other animals (Stohs and Bagchi, 1995;

Kawanishi *et al.*, 2002). Acute and chronic neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, immunotoxicity and general environmental toxicity due to chromium (VI) have been extensively demonstrated (Von Burgh and Liu, 1993; Barceloux, 1999). Soluble and insoluble hexavalent chromium salts induce morphologic and neoplastic transformation and mutagenecity in murine and human cells (Patierno *et al.*, 1988). Hexavalent chromium compounds are approximately 1000-fold more cytotoxic and mutagenic than trivalent compounds in cultured diploid human fibroblasts but both the hexavalent and trivalent compounds induce dose-dependent anchorage independence in human diploid fibroblasts (Biedermann and Landolph, 1990).

Trivalent chromium is although an element required for glucose and fat metabolism, it has been suggested that it causes DNA damage in *in vitro* test systems. In addition, it causes DNA damage and inhibits topoisomerase DNA relaxation activity in bacterial cells, probably by preventing the formation of the covalent link between the enzyme and double helix. In addition, Cr (III) decreases the viability and/or proliferation rate of eukaryotic cells such as murine B16 melanoma cells and human MCF-10A neoT ras-transformed human epithelial cells (Plaper *et al.*, 2002).

Glutathionine can reduce hexavalent chromium to trivalent chromium without any cofactors or metabolizing enzymes (Wiegand *et al.*, 1984). Shi and Dalal (1989; 1990) have demonstrated the formation of long-lived chromium (V) intermediates in the reduction of chromium (VI) by glutathione reductase (GR) in the presence of NADPH, and the generation of noxious hydroxyl radicals. Hydrogen peroxide suppresses chromium (V) and enhances the formation of hydroxyl radicals through chromium (V)-catalyzed Fenton-like reaction. Jones *et al.* (1991) have suggested that hydroxyl radicals are generated from a chromium (V) intermediate that is responsible for causing DNA single strand breaks.

Kawanishi *et al.* (1986) demonstrated that chromium (VI) produces noxious reactive oxygen species (ROS) including superoxide anion, singlet oxygen and hydroxyl radicals through the formation of chromium (V) intermediates. Incubation of Cr (VI) with ascorbate generated Cr (IV) and Cr (V). Addition of cumene hydroperoxide generated DMPO/R adduct with an enhancement of Cr (V) signal. Addition of Mn (II), whose function is to remove Cr (IV), caused dose-dependent inhibition of DMPO/R formation, suggesting the possible role of Cr (IV) and its

mediated free radical generation from lipid hydroperoxides in the mechanism of Cr (VI) carcinogenesis (Mao *et al.*,1995). Sidney *et al.* (2001) showed that chromium and cadmium result in time dependent increases in hepatic microsomal and mitochondrial lipid peroxidation and hepatic DNA single strand breaks and as well as enhanced urinary excretion of the lipid metabolites, melanodialdehyde, formaldehyde and acetone.

Izzoti *et al.* (1998) injected Sprague-Dawley rats intratracheal instillations of sodium dichromate (0.25 mg/kg body weight) for three consecutive days, and the day after the last treatment, lung and liver tissues were removed for DNA purification. The results showed a selective localization of DNA lesions in the lung but not in the liver tissue, which can be attributed to toxicokinetics and metabolic characteristics of chromium (VI). DNA alterations included DNA-protein crosslinks, DNA fragmentation, nucleotidic modifications, and 8-hydroxy-2'-deoxyguanosine. The oral administration of the thiol N-acetylcysteine completely prevented any induction of DNA lesions in lung cells.

Hexavalent chromium is genetically active, because of its ability to cross the membranes and enter the cells. However, its genetic activity is suppressed if its reduction takes place outside the cell or even outside the cell nucleus e.g., in mitochondria or microsomes. If the reduction takes place inside the nucleus, near or at, the target DNA molecules, alterations in DNA can occur, depending upon the oxidation power of hexavalent chromium or the formation of trivalent chromium complexes with nucleophilic sites of DNA. Thus trivalent chromium could, therefore, be the ultimate mutagenic form of chromium (Levis and Bianchi, 1982). De Flora *et al.* (1984) have shown that hexavalent chromium causes irritation in nucleotide pools and, due to its oxidizing power, induces single-strand breaks in DNA-protein and DNA-DNA cross links and decreases the fidelity of DNA replication. Russo *et al.* (2005) showed that hexavalent chromium induces DNA single-strand breaks and apoptosis in the p53-transfected cells which is mediated by p53 upregulation of p53-upregulated modulator of apoptosis (PUMA), BAZ translocation to mitochondria, cytochrome C release and caspase-3 activation. In primary human bronchial epithelial cells expressing functional p53, Cr (VI) induces expression of PUMA and Noxa, which promote apoptosis through BAX.

Hexavalent chromium causes reproductive toxicity of the testes in bonnet monkeys. It disrupts spermatogenesis, leading to the accumulation of prematurely

released spematocytes, spermatids and uni- and multinucleate giant cells in the lumen of seminiferous tubules. (Aruldhas *et al*., 2005). Exposure to chromium also affects human sperm because a significant positive correlation is observed between percentages of abnormal sperm morphology and blood chromium levels (Kumar *et al.*, 2005).

Chromium is widely used in the industry but occupational exposure to Cr (VI) has long been associated with increased lung cancer mortality. Moreover, exposure of human beings to chromium compounds in the workplace can result in nephrotoxicity. The administration of a single dose of $K_2Cr_2O_7$ leads to impairement in the function of the renal brush border membrane (Fatima *et al.*, 2005). Chromium is released during several industrial processes and accumulates in some estuarine areas. Chromium (VI), given as chromium oxide intraperitoneally at the rate of 25 μg/kg body weight in winter flounder (*Pseudopleuronectes americanus*), affects several liver genes with potentially altered expression, including an alpha class GST, 1-cis peroxiredoxin, a p-450 2x subfamily member, two elongation factors (EF-1 gamma and EF-2), and complement component C3. Semiquantitative RT-PCR confirms that Cr (VI) down regulates complement component C3, an EST and two potential glutathione peroxidases, $GSTA_3$ and 1-cis peroxiredoxin (Chapman *et al*., 2004).

Serum thyroid hormone levels have been suggested to be useful biomarkers of contaminant exposure and as surrogate measures of health in several species of marine and terrestrial mammals (Schumacher *et al.*, 1993; Beland *et al.*, 1993; DeGuise *et al.*, 1995; Rolland, 2000). The search for indicators of marine mammal health or toxicant exposure has identified many potential candidates, including cytochrome P450 isozyme induction (Hahn *et al.*, 2000; Angell *et al.*, 2004), c-reactive protein (Funke *et al.*, 1997), vitamin A (Simms and Ross, 2000), benzo (a) pyrene monooxygenase (BPMO) activity (Fossi *et al.*, 2003) and serum thyroid hormones (Schumacher *et al.*, 1993; Hall *et al.*, 1998).

Hexavalent chromium is highly toxic, carcinogenic and dangerous to the life forms and bodily tissues. Endocrine system plays vital role in the normal functioning of the body metabolism, homeostasis, growth, reproduction, salt and water balance. Alteration in any endocrine gland can lead to its malfunctioning. In Pakistan, heavy metal pollution is on the rise due to industrial effluents and discharge. As it has been reviewed in the introduction, hexavalent chromium has been studied in relation to

some endocrine glands such as gonads, pancreas and few others. However, there are no such reports indicating the effect of Cr (VI) on the hypothalamic-pituitary-thyroid axis in general, and thyroid gland, in particular.

Study Objective

As thyroid gland is one of the most important glands regulating the body metabolism, the present study was, therefore, designed to investigate the effects of chromium on thyroid gland, using the laboratory rat as a model system.

Plan of Work

The objective was attempted to achieve according to the following plan of work:

- Determination of the accumulation of chromium in the hypothalamus, pituitary and thyroid glands through atomic absorption spectrophotometry.
- Determination of serum thyroid stimulating hormone (TSH), free triiodothyronine (FT_3) and free tetraiodothyronine (FT_4) concentrations.
- Expression of TSH mRNA through RT-PCR.
- Serum and urine iodine concentration through analytical methods.
- Changes in the cellular architecture of the thyroid gland through light and electron microscopy.
- Quantification of DNA damage in the hypothalamus, pituitary and thyroid gland through DNA ladder assay..

MATERIALS AND METHODS

2.1 Animals and Housing

Adult male Sprague Dawley rats (n= 200; av. b.w. 225g ± 1.5) were obtained from the National Institute of Health (NIH) Islamabad in seven batches, each batch contained 30 animals. They were maintained in the animal house facility of the Department of Animal Sciences, QAU Islamabad and were provided standard rat feed, also obtained from the NIH and water *ad libitum* for 15 days prior experimentation. Photoperiods maintained were 12L: 12D hrs controlled through automatic timers. Ambient temperature was maintained at 23 ± 2°C using the air conditioning system. All animal handling and subsequent sacrifice were carried out according to the guidelines provided by the ethics committee of the Department of Animal Sciences, Quaid-i-Azam University Islamabad, Pakistan. The rats were kept in large hygienic steel cages (15"L x 11"W x 9.2"H) having a capacity for 10 rats. To avoid stress due to overcrowding, only five rats were housed in each cage.

2.2 Experimental Design

The rats were divided into control and treatment groups. Because it was not humanly possible to test and analyze each parameter simultaneously, experiments were conducted in different sets to test more closely related parameters at one time. The estimation of hormones, serum protein-bound-iodine, free iodine in the urine and the determination of chromium concentration in the glands were conducted at one time. Similarly, experiments for testing tissue damage at light and ultrastructural level were carried out in the second set of experiments. Investigation of percent DNA damage in the hypothalamus, pituitary and thyroid glands, was investigated in the third set of experiments. Investigation of the expression of TRH in the hypothalamus and TSH in the pituitary gland was carried out in still another set of experiments. All experiments were run either in duplicate or, where possible, in triplicate.

Throughout the experiments, it was ensured that the experimental conditions remained constant. The control and treatment groups of rats used in each set of experiments were of the same age groups and almost similar body weights. Similar

ambient temperature and photoperiods were maintained for each set of experiments. Throughout, standard diet was provided to the rats twice a day, once in the morning and then in the evening except when the treatments had to be made when food was withheld 4 hours (hrs) prior to administering the toxicant (chromium). The rats had full access to water *ad libitum* during the experiments. To eliminate the confounding factor of adding any extra quantity of chromium, the animal feed and water samples were analyzed for chromium content for each batch of animals. The concentrations were found to be in agreement with the Environment Protection Agency (EPA) of Pakistan (1-10 μg/L or μg/kg). To minimize bias that might have occurred due to the handling stress, animals were handled in person during all experiments.

Lethal dosages for chromium (VI) ($K_2Cr_2O_7$) have been reported by several authors and the doses injected or otherwise given via other routes such as gavage, feed and drinking water to evaluate the toxic effects of hexavalent chromium, are already available in the literature (reviewed by Christian and Trenton, 2003). However, because of the environmental and strain differences of the experimental animals, trial experiments to determine, the lethal dose (LD_{50}) were done. Ten rats were injected with a single dose of 60 mg/kg $_{b.w}$ potassium dichromate ($K_2Cr_2O_7$) intraperitoneally (i.p.) that caused 100 % mortality after 12 hrs. To achieve the sublethal concentration, the original dose of 60 mg/kg $_{b.w.}$ was then split into two doses of 30 mg/kg $_{b.w.}$ i.p. given within 48 hrs. Fifty percent rats (n= 5 out of n= 10) survived for more than 48 hours (hrs) following the split dose thereby reducing the mortality to 50 %. For all subsequent experimentation, therefore, a split dose of 30 mg/kg $_{b.w.}$ was selected.

For all experiments, the treatment group of rats were administered 60 mg/kg $_{b.w}$. $K_2Cr_2O_7$ salt as two split doses of 30 mg/kg $_{b.w.}$ i.p within 48 hours. The rats were injected the first dose of 30 mg/kg $_{b.w.}$ at 09:00 hr in the morning and this was considered the first day. After an interval of 24 hrs (on the 2nd day), they were injected the second dose again at 09:00 hr. The control groups of rats maintained in parallel received 0.9% w/v physiological saline (Geofman Pharmaceuticals, Pakistan) at the same quantity and at the same time.

2.2.1 Dosage Preparation

To achieve hexavalent chromium (Cr VI) toxicity, potassium dichromate ($K_2Cr_2O_7$) was selected because it contains chromium in hexavalent state. A stock

solution was prepared by dissolving 1g $K_2Cr_2O_7$ in 50 ml distilled water (dH_2O) giving a chromium concentration of 0.05 mg/ml.

The actual dose at the rate of 30 mg/kg $_{b.w}$ was injected twice during 48 hours, (totalling to 60 mg/kg $_{b.w}$) which was calculated using the formula:

$$\frac{30}{1000} \text{ x Body weight of rat x } 0.05 = \text{ ml injectible dose of the } K_2Cr_2O_7$$

2.2.2 Collection of Blood and Tissue Samples

The rats were quickly stunned to death and counting from the first day of the injection, sacrificed after 48 hrs post administration of the toxicant. Blood was drawn from the left ventricle of the heart following thoracotomy. It was allowed to stand for 1 hr at room temperature and later centrifuged at 1258 x *g* (Eppendorf centrifuge, 5810 R, Germany) for 15 min to obtain serum for RIA analysis of T_3, T_4 and TSH (section 2.5). Serum samples of control (n=10) and treated rats (n=10) were obtained and aliquoted for the estimation of hormones and serum iodine. For the estimation of iodine (free iodine) in the urine, urine samples of control and treated rats were collected in sterilized Eppendorf tubes by gently pressing the urinary bladder with the thumb.

To determine the chromium concentration using flamed atomic absorption spectrophotometry, due to the small size and hence to obtain sufficient amount of tissues; five tissues each for the hypothalamus, pituitary and thyroid glands of control and experimental rats were pooled to make one sample. Five such samples each for the hypothalamus, pituitary and thyroid glands were obtained, rinsed quickly in freshly prepared phosphate buffered saline (PBS, 0.1g potassium chloride (KCl), 0.1 g potassium dihydrogen phosphate (KH_2PO_4), 1.05g disodium hydrogen phosphate (Na_2HPO_4) and 4.0 g sodium chloride (NaCl) were added to 500 ml of dH_2O, after adjusting the pH to 7.4 for subsequent use), weighed and were subsequently processed for atomic absorption spectrophotometry (section 2.3.2). Data values that appeared odd were excluded and the remaining values were analyzed for the metal concentration in the tissues.

For light microscopy, the pituitary and thyroid glands of control (n=10) and treated rats (n=10) were obtained, rinsed in PBS and fixed in 4% paraformaldehyde

(PFA) solution prepared in PBS for further processing (section 2.7.1). For electron microscopy, the thyroid glands of control (n=10) and treatment rats (n=10) were fixed in 5% gluteraldehyde solution prepared in pipes buffer (section 2.8).

For the investigation of DNA damage and possible cell shrinkage, the hypothalamus, pituitary and thyroid glands of control (n=10) and treated rats (n=10) were collected and processed (section 2.11).

For PCR analysis of TRH and TSH expression, the hypothalamus and pituitary glands of control (n=5) and treated rats (n=5) were collected and immediately frozen in liquid nitrogen and were processed for reverse transcriptase PCR (RT-PCR) analysis (section 2.5).

In all experimental groups, the mortality rate of the treated rats remained between 4 – 10 % while no mortality was recorded in all the control rats.

2.3 Atomic Absorption Spectrophotometry

The chromium concentration was measured in the hypothalamus, pituitary and thyroid glands in order to determine the amount of chromium retained by the tissues minus the amount that was cleared by the body.

2.3.1 Preparation of Stock Solution

$K_2Cr_2O_7$ (0.281 g) of was dissolved in 100 ml dH_2O to make 1000 ppm stock solution. Standard solutions for Cr (VI) were prepared from this stock according to the formula:

$$C_1V_1 = C_2V_2$$

Where, C_1 is the concentration of the stock solution and V_1 is the volume of the stock solution. C_2 is the concentration of the required solution and V_2 is the required volume of the diluted solution.

2.3.2 Estimation of Chromium Concentration

Digests of the tissues and serum samples from the treated and the control rats were prepared according to Fransion (1981) with certain modifications. All glassware used was cleaned thoroughly with nitric acid, detergent and dH_2O and then oven dried and capped. The samples (0.5 g each) were digested with 5 ml HNO_3 (69 % pure,

Merck, Germany) on Microwave Accelerated Reaction System (MARS 1200 W Power CEM Matthews, USA). The maximum temperature was set at 200°C in ramping mode and the power was 1200 Watt. The samples were run for 5 min and then filtered with 0.45 μm filter paper. After digestion, the digests were diluted to 8 ml with dH_2O. The processed samples were subjected to air-acetylene flame in an atomic absorption spectrophotometer (Varian, AA240 FS, USA) for the estimation of chromium. Instrument standards were; peak wavelength (λ max) was 375.9, lamp current was 5 mA and bandwidth was 0.5 nm. Metal concentration (μg/g) in each sample was calculated from the formula:

$$\text{Metal concentration } (\mu g/g) = \frac{\text{Absorbance of the sample (ppm)}}{\text{Weight of the tissue (g)}} \times 8.0 \text{ ml}$$

2.4 Radioimmunoassay (RIA)

Serum TSH, FT_4 and FT_3 concentrations were determined from control and treated serum samples using commercially available rat specific competitive binding radioimmunoassay (RIA) kits (Biocode- Hycel, rue E, Solvay, Liege Belgium, for TSH and Immunotech a.s.-Radiova 1-102 27 Prague 10- Czech Republic, for FT_4 and FT_3). Sample preparations and hormone estimations were according to the manufacturer's instructions for each hormone kit.

2.4.1 Principle of the Assay

The immunoradiometric assay of TSH was a "sandwich" type assay. Mouse monoclonal antibodies are directed against two different epitopes of TSH and hence not competing were used. The samples of calibrators were incubated in tubes coated with the first monoclonal antibody labelled with radioactive iodine (^{125}I). After incubation, the contents of tubes were aspirated and the tubes are rinsed to remove unbound ^{125}I-labelled antibody. The bound radioactivity was then determined in a gamma counter. The TSH concentrations in the samples are obtained by interpolation from the standard curve. The concentration of TSH in the samples was directly proportional to the radioactivity.

2.4.2 Serum TSH

Serum samples were brought to room temperature just before use. A 25 μl aliquot of each of the sample and the control sera were pipetted into respective tubes.

Then 100 μl of antiserum (Ab rat TSH) was added to each tube. The tubes were vortexed and incubated for 3 hrs at room temperature. A 100 μl aliquot of rat TSH I^{125} tracer was then added to each sample and again incubated for overnight at room temperature (20 ± 2°C). The magnetic particles (SORB Ab Fc) were vortexed and 200 μl of these were added to each sample. The samples were again vortexed and incubated for 1 hr at room temperature without further mixing. The contents of all the tubes were centrifuged at 1510 x *g* (3000 rpm) for 20 min. Supernatants were carefully aspirated and finally the bound radioactivity was counted as counts per minute (CPM) in a Gamma counter (Oakfield Sourcerer RIA Counter, No.238, Type SD 16, UK) for at least 60 sec. Mean sensitivity of the assay was 1.03 ng/ml.

2.4.3 Serum FT_4

A 25 μl sample of standard, control and samples were pipetted into antibody-coated tubes. Then 400 μl of tracer and 100 μl of ligand were added and vortexed. The samples were incubated for 90 min at 18 - 25°C with constant shaking (> 350 rpm). The tubes were decanted and the bound radioactivity was counted for 1 min (cpm). Mean sensitivity of the assay was 0.4 pmol/l and the mean intra-assay coefficient of variation was 6.7%.

2.4.4 Serum FT_3

Standard, control and samples (100 μl each) were taken in antibody-coated tubes and 400 μl of tracer was added and vortexed. All the samples were incubated for 120 min at 18-25°C temperature with constant shaking (>350 rpm). The contents were aspirated and the bound radioactivity was counted in a gamma counter as cpm. Mean sensitivity of the assay was 0.5 pmol/l whereas mean intra-assay coefficient of variation was 6.4% .

2.5 Polymerase Chain Reaction (PCR)

2.5.1 RNA Extraction

Rat pituitary tissue samples (50-100 mg) were homogenized in 1 ml of TRI reagent. The tubes were left at room temperature for 5 min. 0.2 ml of chloroform was added and mixed by inverting the tubes. The tubes were left at room temperature for 10 min, and then centrifuged at 13,000 rpm for 15 min at 4°C. Following centrifugation, the colorless upper aqueous phase was separated and transferred to microfuge tubes and 0.5 ml of isopropanol was added to each and left at room

temperature for 10 min. They were again centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded while keeping the pellet and 1 ml of 75% ethanol was added to the pellet but not mixed, centrifuged again at 10,000 rpm for 10 min at 4°C. The supernatant was discarded while keeping the pellets. The tubes were dried by inverting over a tissue paper for 5 min. The pellets were resuspended with 20-30 μl of DEPEC treated water and incubated at 37°C for 20 min and stored at - 20°C. It was run on 2% agarose gel to ensure the RNA.

Complementary DNA (cDNA) Synthesis

Primers were designed using TSH genomic sequence obtained from UCSC Genome Bioinformatics site. The accession number for TSH rat sequence was NM-013116, located at chromosome number 2 at position 197908311-197913186. Genomic size was 4876 bp with Exon Count 3 in which coding Exon count was 2.

Primer sequence for TSH was:

Forward primer: 5′-GGGTATAAAATGAACAGAGTCTGGG-3′,

Reverse primer: 5′-GCCGGTGATCACATTTAACCAG-3′,

while annealing temperature for PCR was calculated as (62+59/2) –5= 55°C.

A 1 μl sample of oligo (dt) primer and 11 μl of DEPEC- treated water were added to 5 μl of template RNA in 0.2 ml capacity eppendorffs. The samples were centrifuged briefly at 5000 rpm for 20 sec and incubated at 70°C for 5 min in a hot block. Samples were chilled on ice and 4 μl of 5x reaction buffer, 1 μl of ribolock RNase inhibitor and 2 μl of 10 mM dNTP were added and centrifuged at 5000 rpm for 20 sec, incubated again at 37° C for 5 min. Then 1 μl of Reverse Aid H Minus M-MuLV reverse transcriptase was added and samples were incubated at 42°C for 60 min and heated to 70°C for 10 min and stored at -20°C.

Reaction mixture for PCR

It consisted of 13.5 μl PCR grade water, 5 μl 1x PCR Buffer, 1.5 μl $MgCl_2$, 0.5 μl dNTPs, 2.5 μl forward primer (50 ng), 2.5 μl reverse primer (50 ng), 1.0 μl taq DNA polymerase, and 1.0 μl template (cDNA) (total 25 μl).

2.5.2 RT-PCR

The microtubes were placed in a thermal cycler (Biometra, Germany) and the loading temperature was set to 106°C. First denaturation was carried out at 95°C for 15 min and then at 94°C for 45 sec. Annealing temperature for TSH was 50°C for 45

sec and first extension was done at 72°C for 2 min and the whole cycle was repeated 40 times and final extension was at 72°C for 10 min. Annealing temperature for TRH was 55°C for 45 sec and first extension was done at 72°C for 2 min and the whole cycle was repeated 40 times and final extension was at 72°C for 10 min. The DNA amplicon was stored at 4°C.

2.5.3 Gel Electrophoresis

A 0.5 gm quantity of agarose gel was dissolved in 25 ml 1x TBE (Tris 890 mM, boric acid 890 mM and EDTA 25 mM) buffer by heating in a microwave oven for 1 min. Ethidium bromide (3 μl) was added and cooled to relatively low temperature. Gel was poured in the electrophoresis tray and submerged in 1 x TBE in the gel tank. DNA amplicon (4 μl) was mixed with 2 μl of loading dye, bromophenol blue, and dispensed in the appropriate well. DNA marker (2 μl) of was mixed with 4 μl of loading dye and dispensed in another well. Samples were electrophoresed at 90 V. After appropriate distance was covered (about 1/3 to ½) by loading dye (0.25% Bromophenol blue, 4g sucrose in water and dH_2O to 10ml), the current was stopped and the gel was observed in gel documentation system (BIORAD, USA).

2.6 Iodine Estimation

For the determination of iodine concentration, free iodine was estimated in the urine while protein-bound iodine (PBI) was measured in the serum.

2.6.1 Free iodine in the urine

Free iodine excreted in the urine was estimated according to the method of Zak *et al.* (1952) with slight modifications. Chloric acid (3 ml) was added to each tube containing 0.5 ml of urine sample. The tubes were placed in an electrically heated sand bath set to a temperature of 105 - 110°C for 1 hr to prepare digests. The tubes were kept under observation for color changes such as orange-yellow to light green until changed to colorless. Chloric acid was added drop-wise wherever necessary to prevent the color changes and to avoid the loss of iodine. The tubes were allowed to cool until red crystals of chromium trioxide appeared. Tubes in which red crystals did not appear, the digestion was allowed to continue until the red crystals appeared on cooling. The samples were analyzed at 420 nm in a UV visible spectrophotometer (Schimadzu, UV-120-01, Japan).

Calculations:

μg iodine found in the sample x 100 = μg iodine / 100 ml

2.6.2 Serum Protein- bound Iodine (PBI)

The protein-bound iodine in the serum samples of control and treated rats was estimated according to the method of Bird and Jackson (1962).

For all experiments, chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri, USA and Merck and BDH, Germany.

Standard Solutions for iodine

Solution A. 168.5 g of dried potassium iodide was dissolved in 1L of dH_2O.

Solution B. 10 ml of Solution A was diluted to 1L with dH_2O. This solution contained 1 μg of iodine per ml.

Working Standard Solutions: Aliquots of solution B were diluted to obtain working standards (I, II and III) which 0.2 ml contained 0.01μg (I), 0.02 μg (II) and 0.03 μg (III) of iodine.

For the estimation of protein-bound iodine (PBI), 0.2 ml of each serum sample was taken in a test tube and 5 ml of 5% TCA (5 g of TCA was dissolved in 100 ml dH_2O) was added to each. The tubes were gently revolved between the palms of the hands for mixing. They were allowed to stand for 30 min and then centrifuged at 1510 x *g* (3000 rpm) for 5 min. The supernatant was poured off and the tubes were decanted on a filter paper (Whattman International Ltd., England) to drain for 5 min. The precipitated protein was washed with 5 ml of 5% TCA, centrifuged again and supernatant was decanted as above. Blank, standards (I, II and III) and samples (0.2 ml each) quantity were treated identically. Chloric acid (2 ml) (125 g of potassium chlorate in 225 ml of dH_2O and heated quickly to obtain solution, 95 ml of 72% perchloric acid, solution cooled in an iced water bath and then placed in a freezer at –20°C overnight, then filtered using a buckner funnel and stored at 4°C in a stoppered glass bottle) and 0.1 ml of sodium chromate (0.4 g of sodium chromate in 100 ml dH_2O) solutions were added to samples which were then allowed to hold in a sand bath at 140 to 150 °C for 1 hour. Two drops of chloric acid were added to each sample at 20, 30, 40, 50 and 55 min times during 1 hr duration after which the tubes were removed. The reaction was complete in those tubes in which red crystals of dichromate appeared on cooling. In those tubes where no red crystals formed, 2 more

drops of chloric acid were added and the tubes were returned to sand bath for an additional 5 min.

When the digestion was complete the tubes were cooled to room temperature and 10 ml of 1% NaCl (10 g of NaCl in 1L of N-H_2SO_4) was added to each tube, followed by 2 ml of 0.2 N arsenious acid (9.891 g of arsenious trioxide and 7 g of sodium hydroxide in 100 ml of dH_2O, volume raised to 400 ml and 42 ml concentrated H_2SO_4, pH 7.0 and the volume was finally raised to 1 L). 0.5 ml of 2% ceric sulphate (4 g of $Ce(SO_4)_2$ in 200 ml of NH_2SO_4) was then added to each sample at 1min. intervals, starting with the 0.03 μg standard, followed by 0.02 μg, 0.01 μg, blank and serum tubes. The tubes were rotated between the palms of the hands for mixing after the addition of 2% ceric sulphate. The time of addition of 2% ceric sulphate to the first tube was noted and reaction was allowed to proceed at ambient temperature for 1 hr. The absorbance of each sample was read in a spectrophotometer at 415 nm. The absorbance values of the standards and blank were plotted on semi-log paper, against the iodine concentration.

Calculation:

μg PBI in 0.2 ml serum x 500 = μg PBI / 100 ml

2.7 Light Microscopy

2.7.1 Histology

Standard methods were followed for fixation and staining. Briefly, pituitary and thyroid glands of the control and treated rats were processed for standard histological procedures. The tissues were fixed in 4% Paraformaldehyde (PFA). For PFA preparation, 33 ml of dH_2O was heated to 60°C to which paraformaldehyde powder (2 g) (Sigma, St. Louis, Missouri, USA) was added and mixed well. NaOH (1 M) was then added drop-wise until the solution became just clear, 5 ml of PBS was then added and pH was adjusted to 7.2, final volume was made up to 50 ml with dH_2O. The solution was filtered with a 0.45 mm filter paper and stored at 4°C until used. Paraffin wax embedded tissues were cut with a rotary microtome (Shandon, Finesse 325, UK) at 5 μm thickness and stained with conventional Haematoxylin and eosin (Bancroft and Stevens, 1990; Riddel, 1996). For haematoxylin preparation, Harris's Haematoxylin crystals (0.5 g), mercuric oxide (0.25 g) and potash alum (10 g) were dissolved in 95 % ethanol (5 ml) and glacial acetic acid (4 ml), final volume

was raised to 100 ml with dH_2O. Eosin solution was prepared by adding 1 g of eosin powder and glacial acetic acid 0.5 ml to 99.5 ml dH_2O. Sections were stained in Harris's Haematoxylin for 2-3 minutes and eosin for 1 min. Sections were dehydrated and mounted in DPX mountant medium (BDH, Germany).

2.7.2 Staining with PAS

Periodic Acid and *Schiff reagent* were prepared to differentially stain the basophils of the anterior pituitary and colloid in the thyroid follicles. PAS was prepared 1% whereas for the preparation of *Schiff reagent,* 1 g basic fuchsin was dissolved in boiling dH_2O. It was allowed to cool to 50°C and then potassium metabisulphate was added with mixing. The solution was further cooled to room temperature and 2 ml concentrated HCl (Merck, Germany) was then added. It was allowed to stand overnight in the dark. 0.2 g activated charcoal was added and shaken for 1 – 2 min. The solution was then filtered and stored in a brown bottle at 4°C until use.

Sections were dewaxed, rinsed in descending grades of alcohol and then in dH_2O. The sections were oxidized in 1% Periodic acid for 5 min, washed with tap water for 1 min and again rinsed in dH_2O again. The sections were then treated with *Schiff reagent* for 15 min and then rinsed first with dH_2O and then with tap water for 10 min. These were then stained with Harris's hematoxylin for 1 min, rinsed in tap water for 5 min, dehydrated through ascending grades of alcohol and mounted with DPX mountant medium. Sections were photographed and

2.7.3 Morphometry

The cells of the anterior pituitary and thyroid follicles were counted according to the method of Abercrombie and Johnson (1946); the cells were counted, following the formulae given therein, for their relative number and size by placing a graticule in the eyepiece of the microscope (Nikon, Optiphot BH-2, Japan). Cell numbers were estimated by counting the number of cells in each box of the graticule. The size of the anterior pituitary cells and thyroid follicles were measured by using standard methods of microscopic measurements. Ten thyroid follicles per section were selected at random for measurement using a labelled grid (Lovin Field Finder, Gurley Precision Instruments, Troy, New York, USA). Sections showing evidence of extensive autolysis resulting in cell or follicle distortion were omitted from the measurements.

Follicular length (FL) was taken at two farthest points in the follicle. Follicular width (FW) was then measured perpendicular to the FL measurement. Four measurements of epithelial cell height (ECH) per follicle were then made at each of the four extremities (from the innermost aspect to the outermost aspect of the follicle lining cell). The nuclear and cytoplasmic sizes were also measured in the same way.

The follicular size (FS) was calculated using the formula:

$$FS = \frac{FL + FW}{2}$$

Once FS was calculated, the epithelium-follicular index was determined using the formula:

$$EFI = \frac{ECH \times 100}{FS}$$

The extent of damage to the thyroid gland was quantified morphometrically.

2.8 Transmission Electron Microscopy (TEM)

Small pieces, approximately 1 mm^3 of control and chromium treated rat thyroid glands were immersed in 5% glutaraldehyde (5 ml of glutaraldeyhde, 10 ml of 0.2 M pipes buffer, pH 6.8, 1 N NaOH, total volume 25 ml with dH_2O) prepared in 0.2 M pipes buffer (6 g pipes, 50 ml dH_2O, 1 N NaOH drop-wise until the solid dissolved, pH 6.8 and final volume raised to 100 ml). These were subjected to vacuum in order to draw the fixative into the cells and vessels. Fixation took place in 18 hrs by placing the samples on a 55° fixed angle specimen rotator at 5 rpm at room temperature. To remove the fixative, samples were rinsed in 0.2 M pipes buffer (pH 6.8) for three times with an interval of 15 min each. Post fixation was achieved with 1% osmium tetraoxide (1ml of 2% aqueous stock solution of osmium tetraoxide in 9 ml of pipes buffer) for 18 hrs at room temperature. The tissues were washed with autoclaved dH_2O twice for 15 min and then shifted to 5% uranyl acetate solution (5 g of uranyl acetate in 100 ml of dH_2O, filtered and sterilized with 0.2 μm cellulose acetate filter and stored in cuvettes covered with aluminium foil) for 16-18 hrs. The tissues were again washed with dH_2O twice for 15 min and then dehydrated in descending grades of ethanol 30 %, 50 % and 70 %, keeping for 15 min in each grade and overnight in absolute ethanol. The tissues were left in absolute acetone for 15 min twice as transitional solvent because the ethanol does not mix well with spur resin. Tissues were infiltrated with a mixture of spur embedding media (10 parts ERL /

VCD 4206, Vinylcyclohexene dioxide; 6 parts DER736, diglycidyl ether of propylene glycol; 26 parts NSA, Nonylsuccnic anhydride and 0.4 parts S1, Dimethylaminoethanol). The ratio of resin to acetone was 1:3 for 18 hrs followed by 1:1 and 3:1 for another 18 hrs each. A 100% resin mixture was added to the samples and vacuum infiltration was carried overnight. The samples were oriented in moulds and resin cured at 70°C for 48 hours.

The polymerized resin blocks were trimmed and faced with fine scalpel blade and glass knife. Semithin sections (2μm) of the control and treated thyroid glands were cut with an ultra microtome (RMC MT 7000, Japan) and stained with toluidine blue. These were observed under light microscope. While ultrathin serial sections of approximately 120 nm size were cut with the same microtome (RMC MT 7000, Japan) and placed on a 200-mesh nickel grid. These sections were stained with 5% uranyl acetate for 30 min, then washed twice with dH_2O, and again stained with lead citrate for 15 min in NaOH chamber. Sections were examined on a transmission electron microscope (JEOL *JEM1010,* Japan) operating at 80 KV at the National Institute for Biotechnology & Genetic Engineering (NIBGE) Faisalabad.

2.9 Nuclear Abnormalities

Nuclear abnormalities of the thyroid epithelial cells were studied by using the slightly modified method of Singh *et al.* (2003).

Preparing Slides

In order to determine the abnormalities of the nuclear size in the thyroid sections, frosted slides were dipped in methanol at least one night before the start of the experiment. Slides were coated with 0.5% normal melting point (NMP) agarose by spreading 1 ml of it on the slide. Before use, agarose was melted at 37 °C in a water bath. 20 mg each of thyroid, pituitary and hypothalamic tissues were homogenized in 1 ml PBS to make the cell suspension. After centrifugation, the supernatant was discarded taking care not to disturb cells present at the bottom. 140 μl of low melting point (LMP) agarose (1%, LMP, 500 mg/100ml added to PBS, heated and then dispensed 2-4 ml aliquots scintillation vials and stored at 4°C till used) was added in the 20 μl cell suspension and mixed well. Two drops of 70 μl were put on to a precoated NMP agarose glass slide (0.5%), and cover slipped. The slides were placed in refrigerator for 20 min. The cover slips were removed and slides were placed in lysing solution and again kept at 4°C overnight in the dark.

Lysis and Electrophoresis

Slides were taken out from the lysing solution and transferred to electrophoresis chamber containing denaturation electrophoresis buffer (0.3 M NaOH (12 g), 10 mM Tetra-sodium EDTA (4.2 g) and 0.1% 8-hydroxyquinoline (1 g) were added to 700 ml dH_2O and 2% DMSO was added while mixing. The pH was adjusted to 13.1 and the final volume of the solution was raised to 1L with dH_2O). Electrophoresis was carried out for 30 min at 25 V and 300 mA.

Neutralization and Staining of Microgels

Slides were neutralized with neutralizing buffer (1 M ammonium acetate (7.7 g) added to 100 ml of dH_2O and the pH adjusted to 7.0), washed for 10 min and then washed again with dH_2O for 10 min. These were left to air dry overnight. The slides were stained with acridine orange by adding 2 μl per slide. They were cover slipped and scored under fluorescent microscope (Nikon, Optiphot, AFX-II, Japan).

2.10 DNA Ladder Assay

2.10.1 DNA Extraction

DNA was isolated according to the method of Wu *et al.* (2005) and Barone *et al.* (2006). Tissues used for DNA isolation were immediately immersed in liquid nitrogen after extraction and stored at –70 ° C.

Hypothalamus, pituitary and thyroid glands of control and treated rats, about 20-30 mg were washed twice with 1 ml DNA-buffer TE (20 ml of 1 M Tris, pH 8.0, 20 ml of 0.5 M EDTA, Sterile water dH_20, 100 ml). Tissues were ground and 300 μl lysis buffer (1M Tris HCl, pH 8, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.1 mg/ml protein kinase) and 240 μl 10% SDS was added, gently shaken and then incubated for overnight at 45°C in a water bath. Then 200 μl of phenol was added, inverted for 5-10 min, and centrifuged at 3000 rpm for 5 min. Supernatant was pipetted into a new tube, and 200 μl phenol, and 200 μl chloroform/isoamyl alcohol (24:1) was added; inverted for 10 min, and centrifuged at 3000 rpm for 5 min. Supernatant was again pipetted into a new tube, inverted and centrifuged. The supernatant was pipetted into a new tube, and 25 μl 3 M sodium acetate (pH 5.2) and 5 ml ethanol was added, gently shaken until the DNA was precipitated. The solution was pipetted out gently to avoid disturbance to the DNA thread. DNA was washed in 70% ethanol and dried. Then 20 μl TE buffer and 2 μl RNase were added.

Electrophoresis was performed using 1.5% agarose resolving gel in 1xTBE (Tris 89 mM, borate 89 mM, EDTA 2mM) containing 1.0 μg/ml ethidium bromide (10 mg/ml water). 5 μg of total DNA per well was loaded. Electrophoresis was performed for 45 min. at 100V. The gel was viewed under gel doc system (BIO RAD, USA) and photographed.

2.10.2 Electrophoresis

Electrophrosis was done as above for 45 min. at 100 volts. Depending upon the experiment, 5 μg of total DNA per well was loaded. DNA standards 100 bp ladder and 1500 bp ladder (0.5 μg per well) were loaded to separate down to identify the size of the DNA fragments. The gel was viewed under gel doc system and photographed.

2.11 Quantification of DNA Fragmentation

DNA damage was determined according to the method of Wu *et al*. (2005). 30 mg each of hypothalamus, pituitary and thyroid tissues were ground and left in TTE solution (pH 7.4 with 0.2% triton X-100 at 4 °C, 5 mM Tris-HCl, and 20 mM EDTA) for overnight and then centrifuged at 12000 x*g* for 5 min. The supernatant was separated and named as S. To the remaining pellet, 1 ml TTE solution was added and centrifuged at 12000 xg for 5 min. The supernatant was again separated and called as T. To the remaining pellet, 1ml TTE was added and this solution was named as B. Then 600 μl of 5% Trichloroacetic acid (5 g TCA in100 ml dH_2O) was added to all the three S, B and T supernatants and vortexed vigorously. After overnight precipitation at 4°C, DNA was recovered by pelleting for 10 min at 18,000 x *g* at 4°C. Supernatants were discarded by aspiration. DNA was hydrolyzed by adding 160 μl of 5% TCA to each pellet and heating for 15 min at 90°C in a heating block. To each tube 320 μl of freshly prepared diphenylamine (0.1 g DPA into 1 ml H_2SO_4 and 9 ml of acetic acid) solution was added, then vortexed and allowed to develop colour for about 4 hrs at 37°C. Absorbance was read at 620 nm in a spectrophotometer (SmartSpec^{TM} plus Spectrophotometer). Amount of % fragmented DNA was calculated using the following formula:

$$\% \text{ Fragmented DNA} = \frac{T \times 100}{T + B}$$

2.12 Statistical Analysis

The results obtained were analysed and compared through Microsoft Excel 2007 software for Microsoft Windows (Version XP 2008, NY, USA) and also by using the software "Statistica" (Version, Inc. USA). Comparisons between control and treated samples were made using student's unpaired t-test. Correlations among different variables were determined by Pearson's Correlation analysis and values for coefficient of correlation (r) were determined. $P < 0.05$ was considered significant difference.

RESULTS

3.1 Chromium Concentration

Hypothalamic chromium concentration increased ($p < 0.05$), whereas pituitary chromium concentration showed a non-significant increase (p = n.s) as compared to the control rats. Thyroid chromium concentration decreased ($p < 0.01$) compared to that in the control tissue. Whole blood chromium concentration significantly increased ($p < 0.001$), and serum Cr concentration showed a non-significant increase (p = n.s), while the urine chromium concentration significantly decreased ($p < 0.001$) (Table 3.1).

3.2 Hormone Concentrations

Serum FT_4 and FT_3 concentrations decreased ($p < 0.01$ and $p < 0.001$, respectively), while serum TSH concentration significantly increased ($p < 0.01$) in chromium treated rats than in the non-treated control rats. The ratio of FT_4 to FT_3 increased from 4.865 to 5.142 (Table 3.2, Fig. 3.1). RT-PCR analysis showed that the expression of TSH mRNA was upregulated in the treated pituitary tissue (Fig. 3.2).

3.3 Free Iodine in the Urine

Urine analysis in pre- and post-treatment rats showed that the free iodine removal in the urine samples decreased as compared to the control rats ($p < 0.001$). It was also noticeable that the volume of the urine excreted decreased with the passage of time (Table 3.3, Fig. 3.3).

3.4 Serum Protein Bound Iodine (PBI)

The concentration of protein-bound iodine in the serum also decreased as compared to non-treated control rats ($p < 0.05$) (Table 3.3, Fig 3.3).

Table 3.1 Chromium concentrations (μg/g wet weight of tissue) in the hypothalamus, pituitary and thyroid glands, whole blood, serum and urine (μg/ml) of control and treated rats. Values are expressed as mean ± SEM (n = 25 animals in each case).

Tissues	Control	Treated	t – Value	p - Value
Hypothalamus	4.28 ± 0.24	8.59 ± 1.33	3.174	< 0.05
Pituitary gland	10.31 ± 1.59	12.14 ± 1.15	0.933	0.377
Thyroid gland	9.87 ± 1.59	3.28 ± 0.24	4.080	< 0.01
Whole blood	5.98 ± 0.40	161.24 ± 4.16	6.032	< 0.001
Serum	4.34 ± 0.16	4.68 ± 0.27	1.073	0.314
Urine	53.27 ± 7.78	175.18± 2.38	14.97	< 0.001

Table 3.2 Serum FT_4 (pmol/l), FT_3 (pmol/l) and TSH (ng/ml) levels in the control and treated rats. Values are expressed as mean ± SEM (n =10 animals in each case.

Hormones	Control	Treated	t - Value	p - Value
FT_4	19.46 ± 0.57	9.72 ± 2.16	4.33	< 0.01
FT_3	4.00 ± 0.18	1.89 ± 0.29	6.09	< 0.001
TSH	4.52 ± 0.23	15.80 ± 4.91	4.91	< 0.01

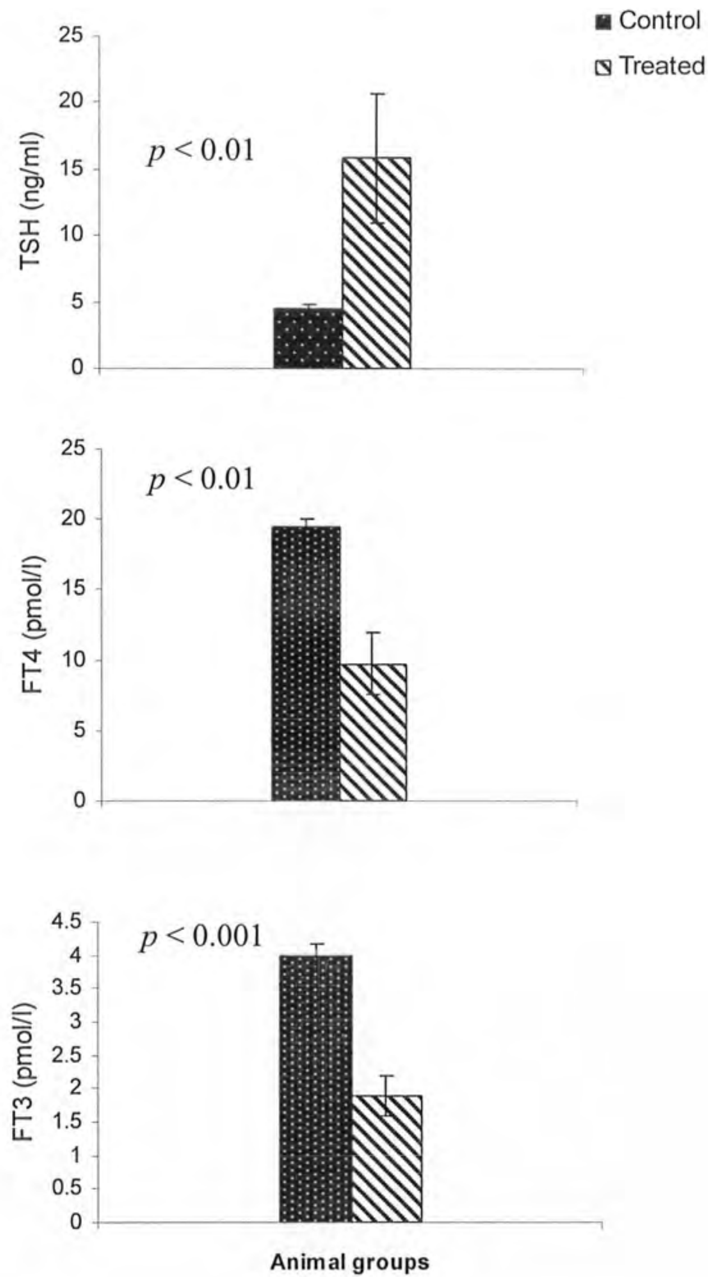


Fig. 3.1 Elevated TSH and decreased FT_4 and FT_3 levels following exposure of the thyroid gland to an acute dose of hexavalent chromium. Values are expressed as mean ± SE.

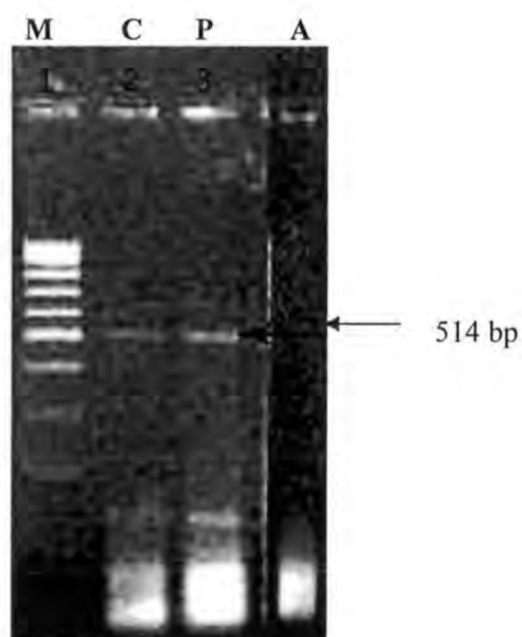


Fig. 3.2 RT-PCR for TSH. 1000 bp DNA ladder, each band= 100bp (lane M); DNA amplicon from control rat pituitary, band size = 514 bp (lane C); DNA amplicon from treated rat pituitary, band size = 514 bp (lane P). A stronger band for TSH in lane P indicates upregulation of TSH mRNA. Negative control (lane A, adrenal gland).

Table 3.3 Concentrations of free iodine (μg/dl) in the urine and serum protein-bound iodine (μg/dl) of control and treated rats. Values are expressed as mean ± SEM (n = 6 animals in each case).

	Control	Treated	*p* Value	*t* Value
Free iodine in urine	16.50 ± 1.81	2.36 ± 0.58	< 0.01	7.41
Serum PBI	18.50 ± 0.91	15.08 ± 0.79	< 0.05	2.82

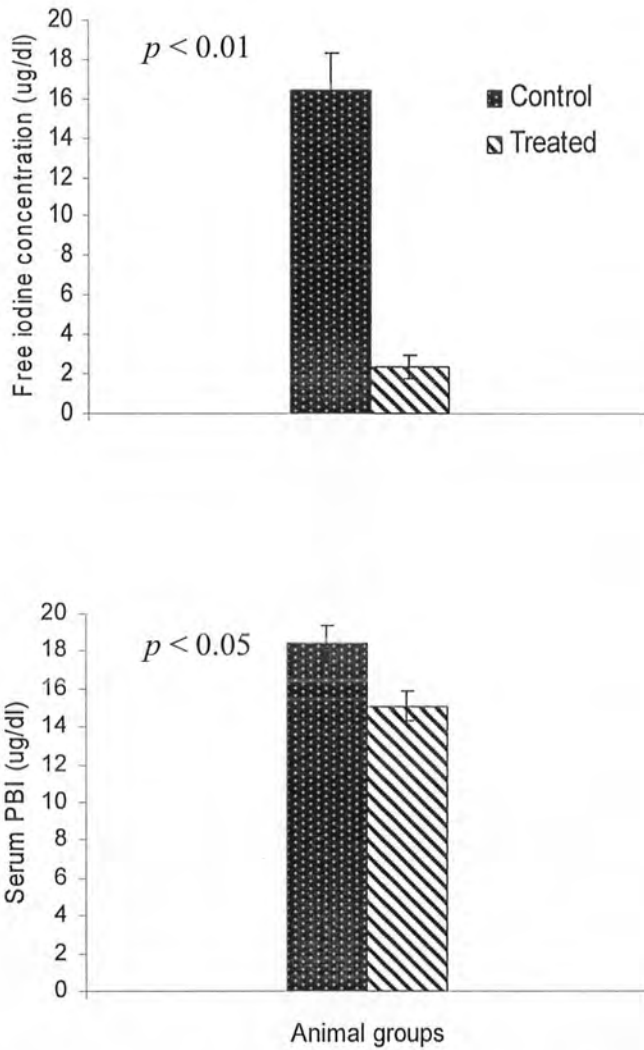


Fig. 3.3 Iodine in serum and urine of rats treated with an acute dose of hexavalent chromium. Values are expressed as mean ± SE.

3.5 Light Microscopy

3.5.1 Pituitary gland

Cellular hypertrophy was noticeable in the anterior pituitary of treated rats as compared to the control animals. Individual cells and their nuclei appeared larger. There was an increase in the number of microvasculature in the treated tissue. The cleft between the *pars distalis* and *pars intermedia* was widened. Cellular density of the anterior pituitary decreased significantly ($p < 0.001$) as compared to the control. In contrast, cell size increased ($p < 0.001$), (Table 3.4, Fig. 3.4). Posterior pituitary did not show any appreciable change in comparison to control rats

Table 3.4 Density ($0.021 mm^2$) and size (μm) of the pituicytes at the light microscopic level. Values are expressed as mean ± SEM (n = 30 in each case).

	Control	Treated	*t*-Value	*p*-Value
Cell density	2441.32 ± 77.30	1276.86 ± 67.40	11.35	< 0.01
Cell size	8.00 ± 0.24	10.79 ± 0.41	5.87	< 0.001

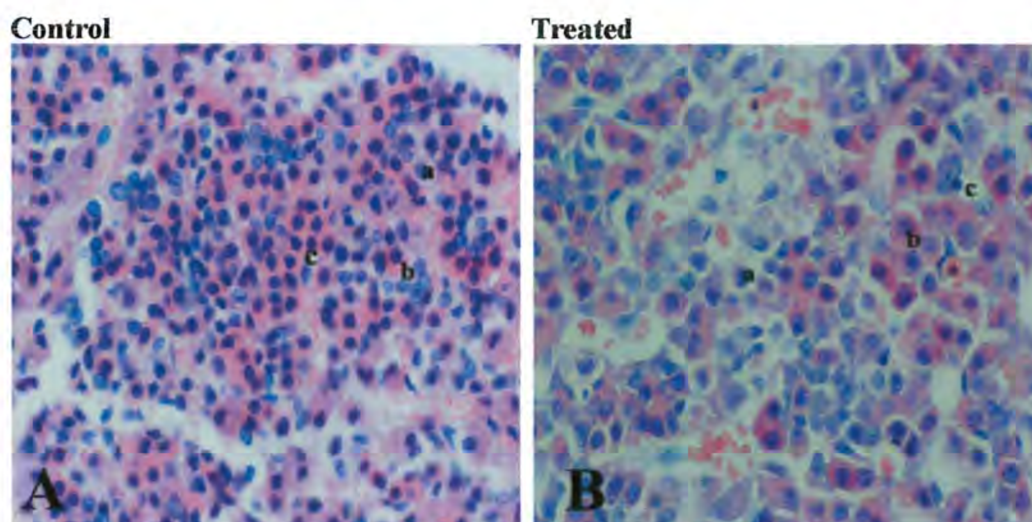


Fig. 3.4 Photomicrographs of rat anterior pituitary showing acidophilus (a) basophils (b) and chromophobes (c)
A Control pituitary showing normal sized cells (x 128).
B Chromium treated pituitary showing hypertrophy of the gland (x 128).

3.5.2 Thyroid gland

Control non-treated Thyroid gland

Control thyroid tissue consisted of small and large variable size follicles with intact basal laminae. Normal secreted and light stained colloid was found near the apical border of cells. Interfollicular spaces were normally formed The cells were desquamated with normal cell height; follicular cell nuclei were spherical (Fig. 3.6 A).

Treated thyroid gland

Thyroid gland showed follicular hyperplasia with darkly stained colloid. The sections overall demonstrated hemorrhagic picture and many red blood cells were frequently observed. Follicular density increased significantly ($p < 0.001$) as compared to control thyroid, while the follicular size showed a significant decrease ($p < 0.001$) (Table 3.5, Fig. 3.6 B). Average size of the largest as well as smallest follicles was significantly decreased as compared to control rats (Table 3.6).

The follicles were disorganized, collapsed and irregularly shaped. Invagination of epithelial cells into the colloid was also noticeable (Fig. 3.6B and 3.7). The connective tissue was also disrupted demonstrating collapsed follicles (Fig. 3.6 and 3.8). Interfollicular spaces enlarged (Fig. 3.6). Colloidal space was reduced in most follicles (Fig. 3.6B). Abnormal nuclear aggregations due to follicular disruption were readily noticeable (Fig. 3.6B and 3.7). Cellular necrosis in the central portion of the tissue was also evident (Fig. 3.7). The apical membranes as well as basal laminae were disrupted (Fig. 3.8 and 3.10).

The epithelial cell height (ECH) was increased as compared to the control ($p < 0.001$) (Table 3.7, Fig. 3.5 and 3.9). There was a significant reduction in the size of the nucleus of the epithelial cells ($p < 0.001$) (Table 3.7). The nuclear shapes were irregular; some nuclei were round and shrunken while others were oval, elongated and pyknotic (Fig. 3.7). On the other hand, the size of the cytoplasm showed a significant increase ($p < 0.01$) (Table 3.7). In some sections of the treated rat thyroid, which were stained with periodic acid Schiff (PAS), colloid retraction was also noticeable whereas in few other sections, colloid showed a significant reduction (Fig. 3.11). Among thyroid follicles of the treated rats, 10% showed colloid retraction whereas 7.6% follicles showed colloid resorption (Table3.8).

The extent of damage to the thyroid gland was quantified morphometrically. The number of follicles with ruptured membranes, desquamated epithelial cells inside the follicles, follicles lined with high-toned epithelium, follicles showing colloid retraction and also follicles showing colloid resorption / reduction, were analysed morphometrically (Table 3.9).

Table 3.5 Mean density (0.021mm^2) and size (µm) of the thyroid follicles at light microscope level. Values expressed as mean ± SEM (n= 30 in each case).

	Control	Treated	*t* -Value	*p* -Value
Follicular Density	64.78 ± 1.64	156.51 ± 4.06	20.93	<0.001
Follicular Size	125.8 ± 3.51	53.6 ± 3.17	15.25	<0.001

Table 3.6 Average size of the largest and smallest follicles (µm) of the control and treated thyroid. Values expressed as mean ± SEM. Sample size in each case was n = 5.

	Control	Treated	*t* -Value	*p*-Value
Largest Follicle	125.8 ± 3.00	112.6 ± 3.41	2.90	< 0.05
Smallest Follicle	69.00 ± 1.81	29.8 ± 1.93	14.77	< 0.001

Table 3.7 Morphometrical parameters (μm) of the control and treated rat thyroid follicular epithelial cells. Values expressed as mean ± SEM. Sample size in each cases was n = 5.

	Control	Treated	*t* -Value	*p* -Value
ECH	4.6 ± 0.14	8.5 ± 0.24	13.96	< 0.001
EFI	3.65	15.80	-	-
Nuclear size	5.46 ±0.16	4.52 ±0.08	5.09	< 0.001
Cytoplasmic size	8.2 ± 0.26	9.74 ± 0.37	3.35	< 0.01
Nuclear / Cytoplasm Ratio	4.52±0.23	15.80±4.91	-	-

ECH: epithelial cell height, EFI: epithelial follicular index

Table 3.8 Colloid retraction (%) and colloid resorption (%) in control and treated rat thyroid follicles. Values are expressed as mean ± SEM. N = 10 in each case.

Group	No. of follicles per 100 squares of the grid	% of follicles showing colloid retraction	% of follicles showing colloid reduction
Control	65	0.00	0.00
Treated	157	10.19	7.64

Table 3.9 Morphometrical analysis of the damage to the thyroid follicles of treated rats. Values are expressed as mean ± SEM. Sample size in each case was n= 10 in all cases.

Follicle Parameters	+	++	+++
Follicles with ruptured epithelium	-	4.72 ± 0.10	-
Desquamated epithelial cells inside the follicles	-	4.60 ± 0.49	-
Follicles lined with high-toned epithelium	2.64 ± 0.24	-	-
Follicles with colloid retraction	2.44 ± 0.11	-	-
Follicles with colloid resorption	2.92 ± 0.32	-	-

+ = Change observed in few follicles, ++ = Change observed in many follicles,
+++ = Change observed in all the follicles.

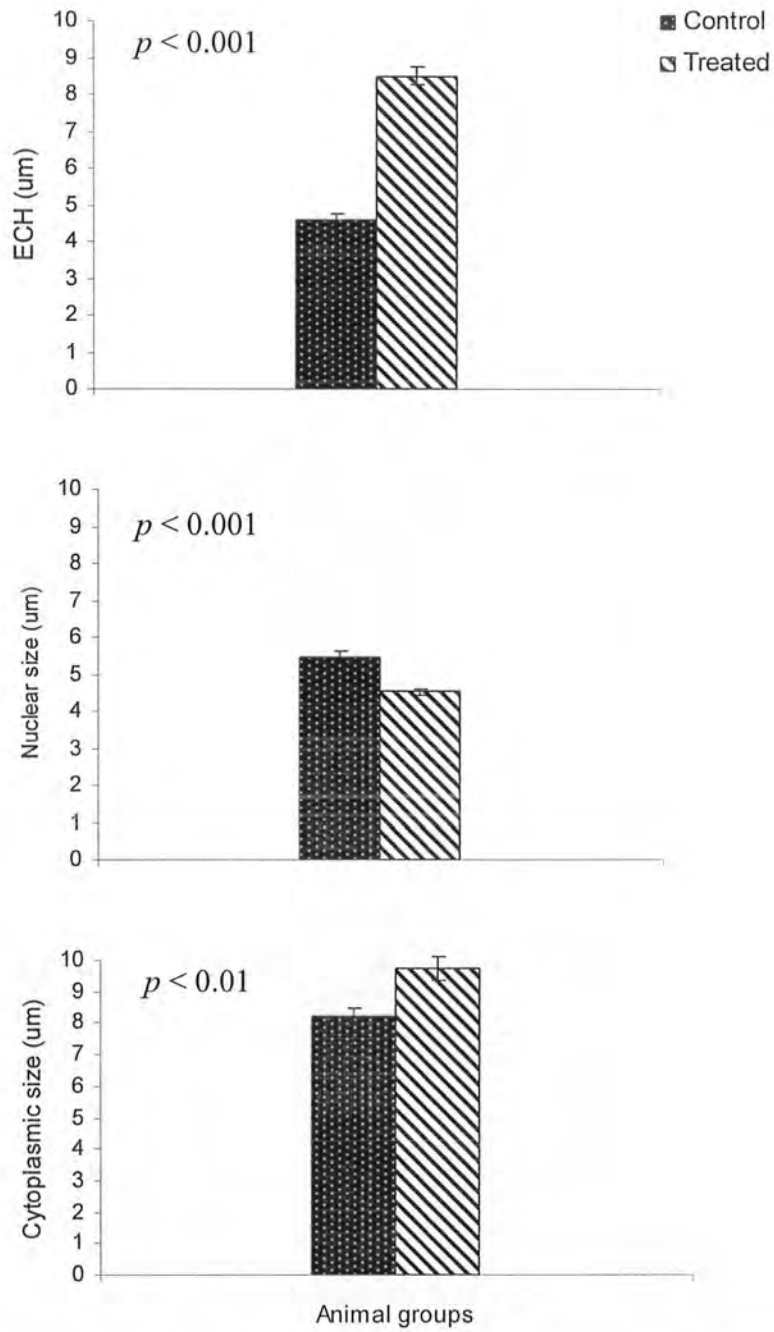


Fig. 3.5 Change in epithelial cell height, nuclear and cytoplasmic size of thyroid follicular cells in response to an acute dose of hexavalent chromium. Values are expressed as mean ± SE.

Hematoxylin and Eosin (H & E) Staining

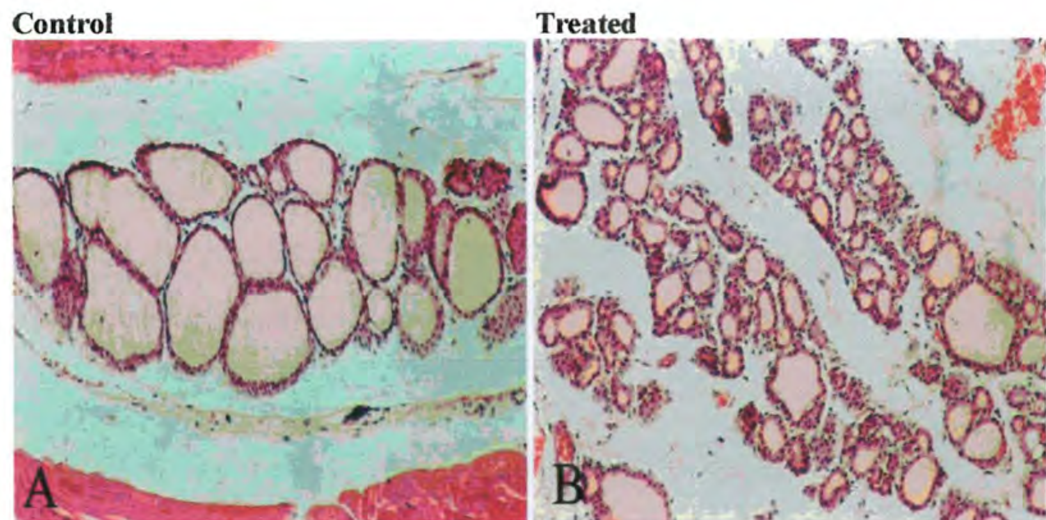


Fig. 3.6 A Photomicrographs of control rat thyroid showing normal follicles with abundant colloid and peripheral positioned epithelial cells (x 32).
B Treated thyroid showing abnormal and disorganized follicular architecture (x 32).

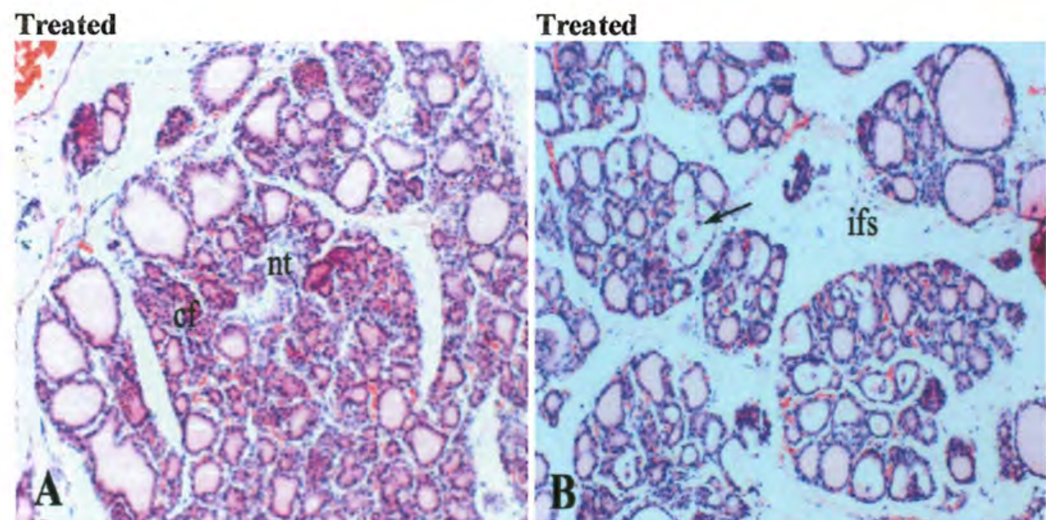


Fig. 3.7 A Photomicrograph of chromium treated thyroid showing follicular hyperplasia, collapsed follicles (cf) necrosed tissue (nt) and abnormal nuclear aggregations in the center (x 32).
B Shows large interfollicular spaces (ifs) due to the disruption of connective tissue and aggregates of follicles in treated thyroid. Arrow showing fusion of follicles. (x 32).

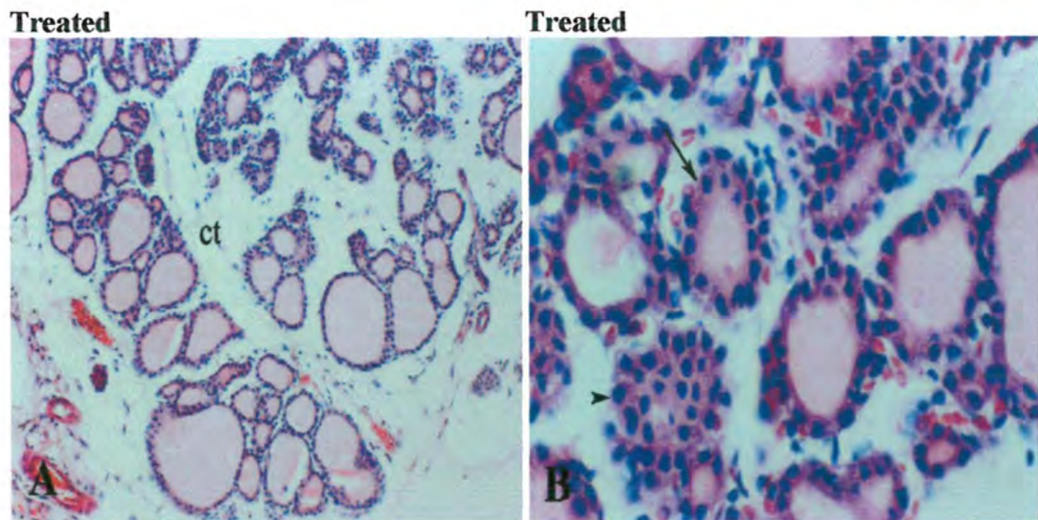


Fig. 3.8 A Chromium treated thyroid section showing dismemberment of the connective tissue (ct) and disorganization of follicles (x 32).
B Shows ruptured basolateral membrane (arrow and arrowhead) (x 128).

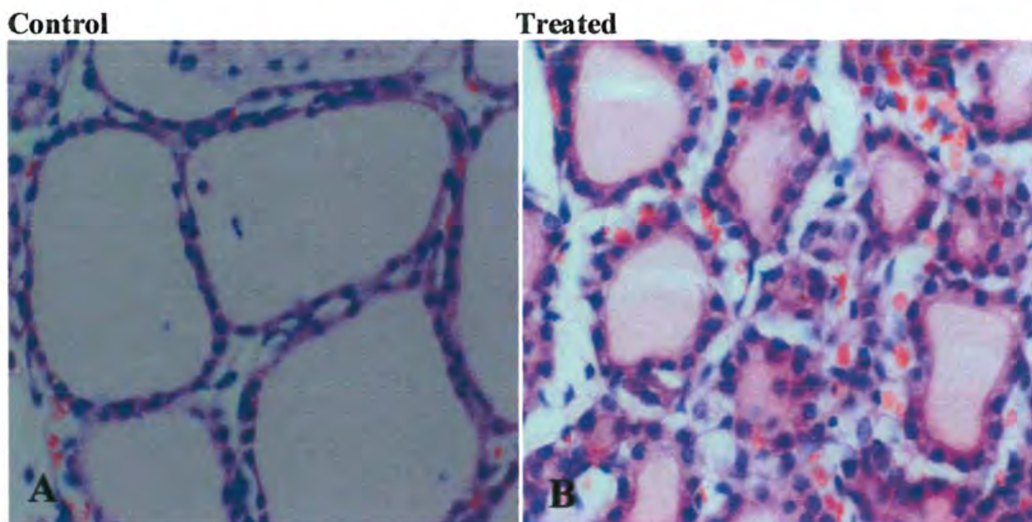


Fig. 3.9 High magnification photomicrographs of control (A) and chromium treated (B) thyroid.
A Control follicles with normal epithelial cell height and colloid (x 128).
B Irregular shape hyperplasic follicles and increased epithelial cell height (x 128).

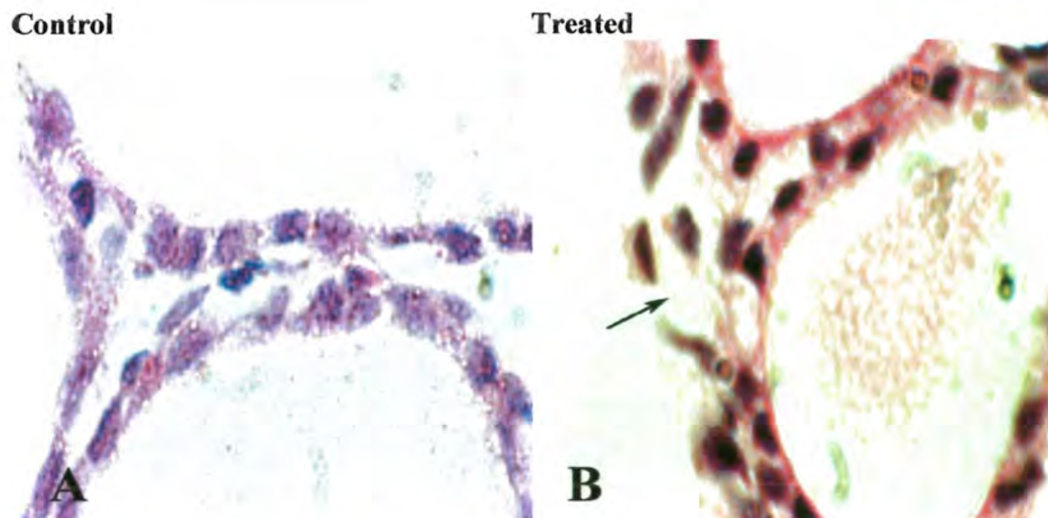


Fig. 3.10 High magnification photomicrographs of control (A) and chromium treated (B) thyroid.

A Control thyroid with normal epithelial cells (x 32).

B Treated thyroid showing ruptured follicular epithelium (arrows) and evasion of nuclei. Nuclei were reduced in size and elongated or pyknotic (x 32).

Periodic Acid Schiff (PAS) Staining

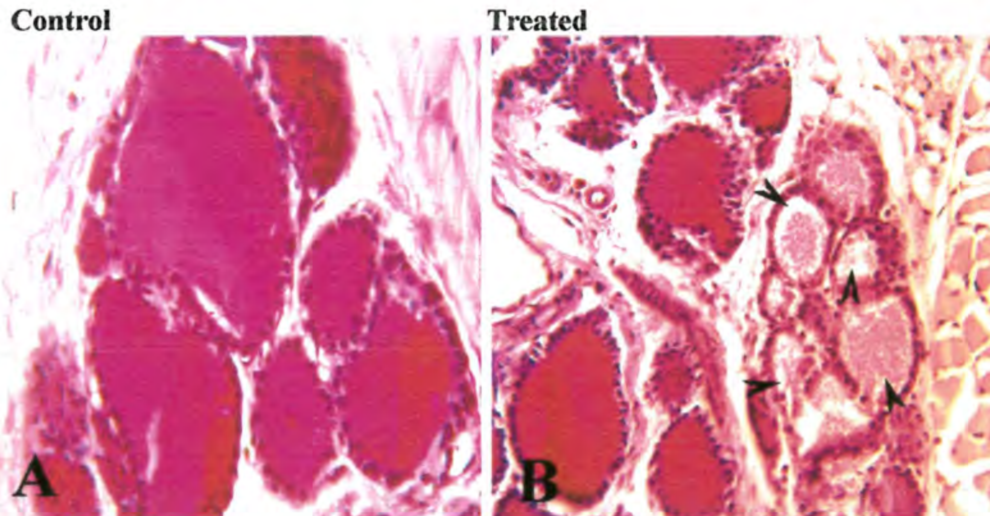


Fig. 3.11 Photomicrographs of rat thyroid sections stained with Periodic acid Schiff (PAS).

A Control rat thyroid showing normal follicles and abundant colloid (x 128).

B Reduced sized follicles showing colloid retraction (arrows), (x 128). Tracheal cartilage is also visible on the right hand corner.

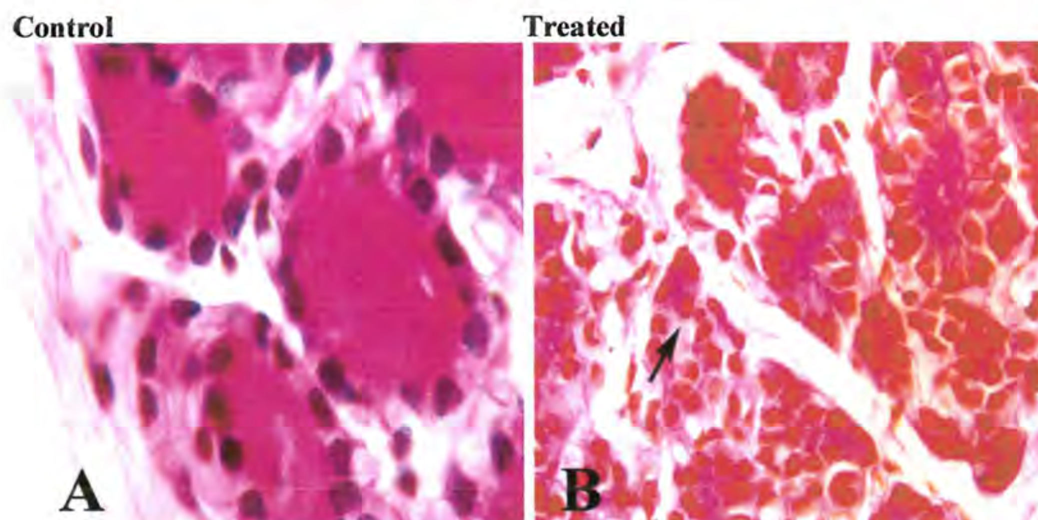


Fig. 3.12 High magnification photomicrographs of PAS stained control (A) and chromium treated (B) rat thyroid sections.
A Follicles having normal colloid content in a control thyroid (x 320).
B Shows reduced colloid (arrows) in collapsed follicles with large interfollicular spaces (x 320).

3.6 Electron Microscopy

Semithin sections

Semithin control sections of non-treated thyroid showed normally formed follicles whereas chromium treated thyroid showed follicular hyperplasia, shrunken and collapsed follicles with thin basal laminae (bl), aggregated towards the center (Fig. 3.13 and 3.14).

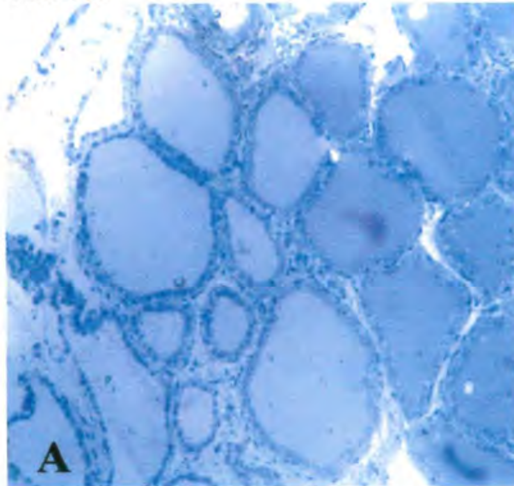
Ultrathin Sections

The control ultrathin sections (120 nm) showed normal follicles with abundant colloid. Basal laminae were intact. The epithelial cells were normally organized and contained round nuclei, endoplasmic reticulum, mitochondria, large number of lysosomes, secretory granules and collagen fibres etc. (Fig. 3.15A, 3.16A and 3.17A).

Chromium treated sections of the thyroid glands showed significant changes as compared to the control.

Toluidine blue Staining

Control **Treated**



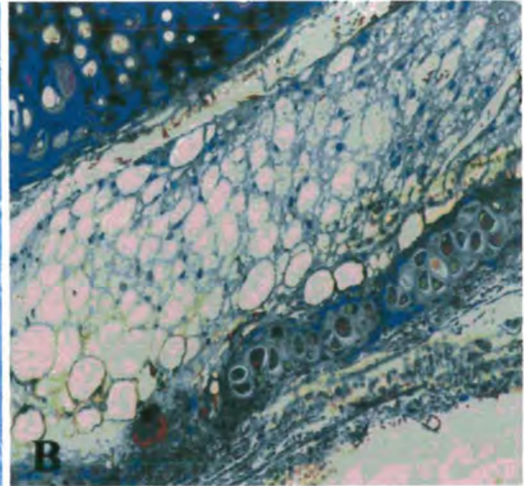
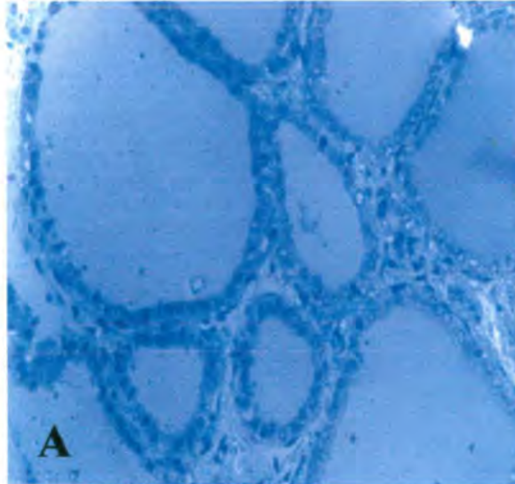


Fig. 3.13 Photomicrographs of semithin sections of control (A) and treated (B) rat thyroid.

A Shows normal follicles and abundant colloid (x 32).

B Shows follicular hyperplasia (x 32).

Control **Treated**



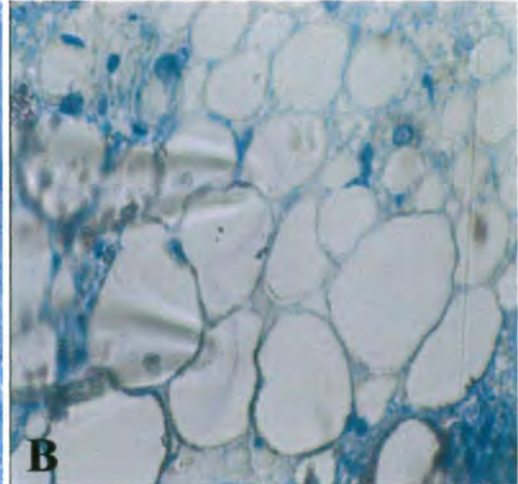


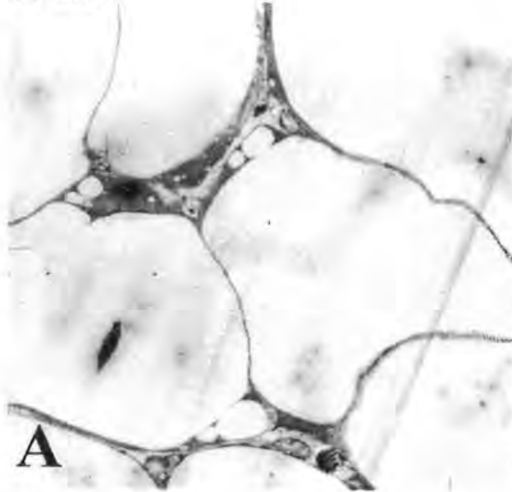
Fig. 3.14 Photomicrographs of semi thin sections of control (A) and treated rat thyroid (B).

A Shows normal follicles at higher magnification (x 128).

B Shows shrunken and collapsed follicles with thin basal laminae (bl). Note aggregations of collapsed follicles at the upper right corner (x 128).

Thyroid Ultrastructure

Control **Treated**



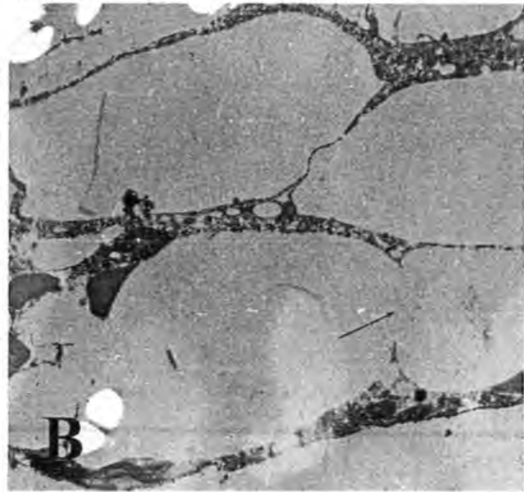
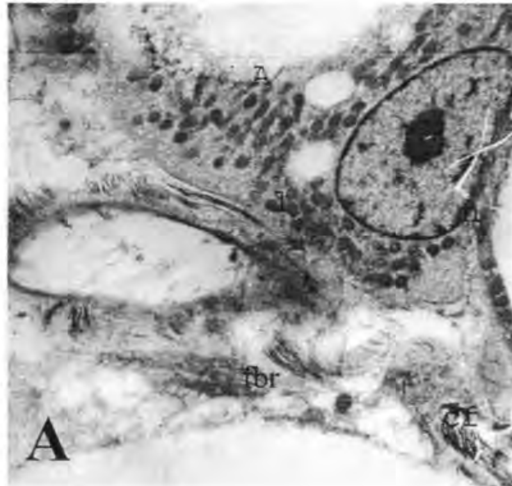


Fig. 3.15 Electron micrographs of control (A) and chromium treated (B) rat thyroid.
A Shows normal follicles and abundance of colloid (x 2,000).
B Shows irregular, elongated disrupted follicles (arrow) (x 2,000).

Control **Treated**



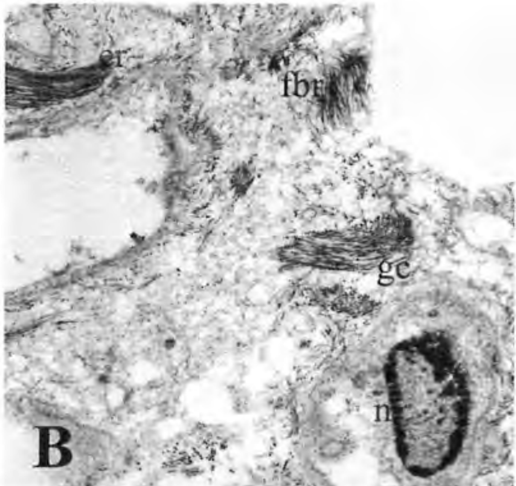


Fig. 3.16 A Electron micrograph of control thyroid showing a nucleus (n), many lysosomes (l), collagen fibers (fbr) and apical surface (A) of the follicle (x 6,000).
B Treated rat thyroid showing pyknotic nucleus (n), Golgi complex (gc) endoplasmic reticulum (er) and collagen fibers (fbr) (x 6,000).

Note: *The magnifications throughout the electron micrographs represent original magnifications.*

a) Follicular organization

In the chromium treated sections, follicular architecture was irregular and damaged. The follicles became more irregular shaped and reduced in size (Fig. 3.15B, 3.16B).

b) Basal lamina

The basal laminae of the follicles were disrupted; follicles were collapsed and fused with one another (Fig. 3.15 B and 3.22 B).

c) Follicular epithelial cells

The epithelial cells showed noticeable shrinkage and appeared disrupted (Fig. 3.17).

d) Nuclei

Nuclei appeared pyknotic as compared to the control. Nuclear shapes were oval while nuclear membranes were irregular and deformed (Fig. 3.17, 3.18 and 3.22).

e) Lysosomes

Lysosomes were less abundant than in control (Fig. 3.16, 3.18 and 3.23).

f) Collagen fibers

Collagen fibers increased in the treated sections (Fig. 3.19).

g) Endoplasmic Reticulum

The endoplasmic reticulum appeared disrupted and highly disorganized Fig. 3.20).

h) Colloid droplets

There was an apparent decrease in the number of colloid droplets of the treated sections compared to control (Fig. 3.19 and 3.20 and 3.21).

i) Golgi apparatus

The Golgi apparatus was either disorganized or depleted (Fig. 3.20).

j) Mitochondria

Mitochondria were less abundant in treated than in the control thyroid sections (Fig. 3.21 and 3.23).

Control **Treated**

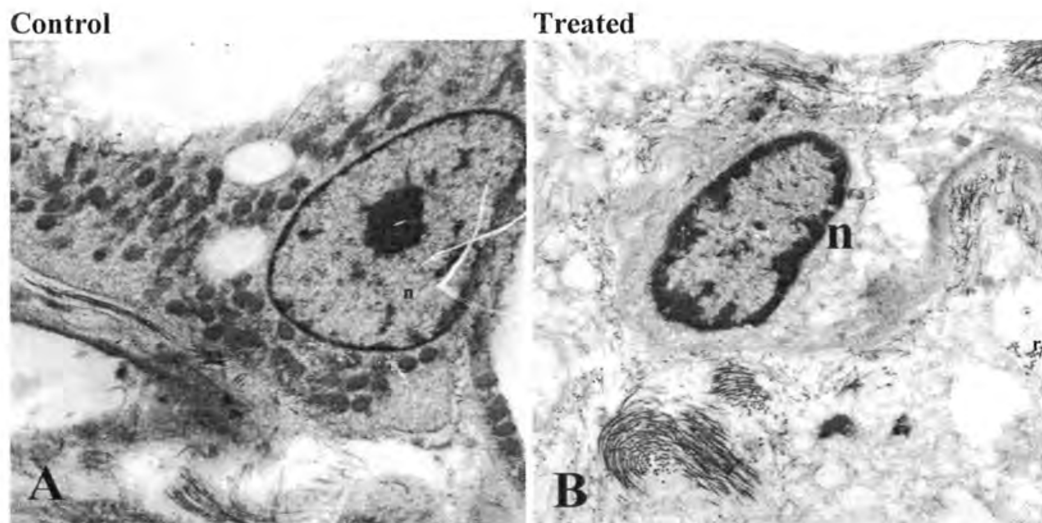


Fig. 3.17 Electron micrographs of control (A) and chromium treated (B) rat thyroid.
A Shows a normal size or normal shaped nucleus (n) (x 6,000).
B Treated rat thyroid epithelial cell showing regressing cell organelles; nucleus, Golgi Complex (Gc), rer and ribosomes (r) (x 12,000).

Control **Treated**

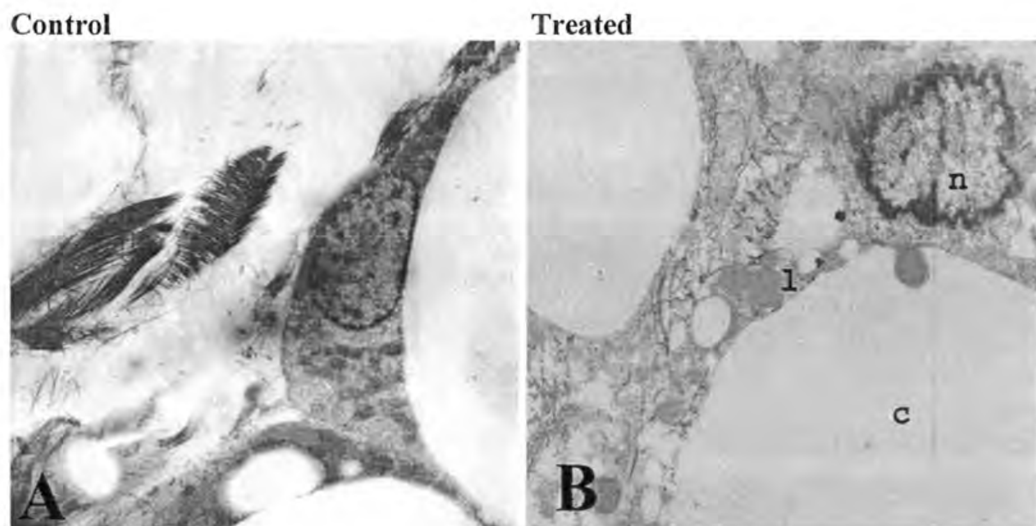


Fig. 3.18 Electron micrographs of control (A) and chromium treated (B) rat thyroid.
A Shows a junction between three follicles with normal prominent nucleus, and other cellular contents (x 5,000).
B Treated thyroid showing a junction between follicles having a deformed nucleus (n), lysosomes (l) and collagen fibers (x 10,000).

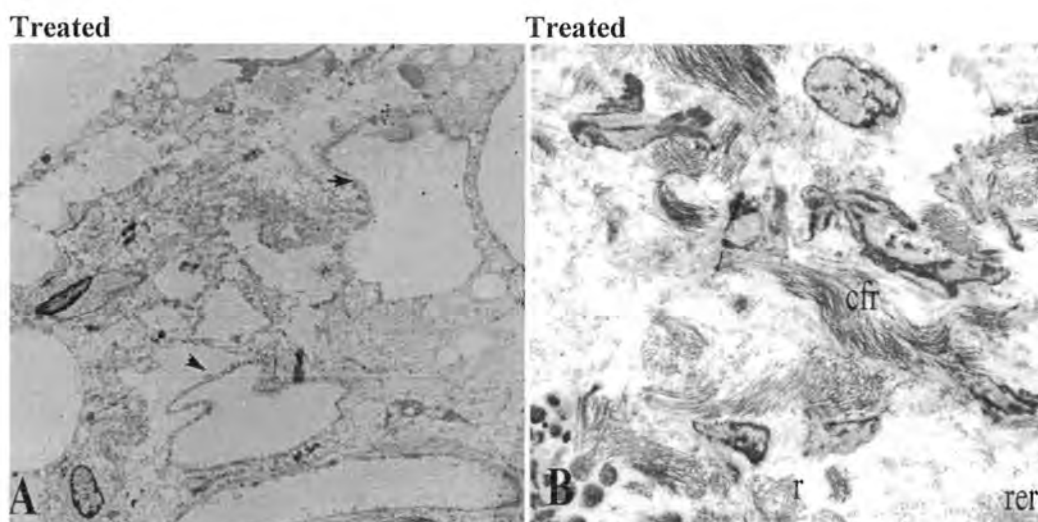


Fig. 3.19 Electron micrographs of treated rat thyroid.

A Shows irregular shaped follicles, deformed or pyknotic nuclei (x 2,000).

B Shows abundant collagen fibers (cfr), a few lysosomes on the lower left side of the section and rough endoplasmic reticulum (rer) are also visible (x 5,000).

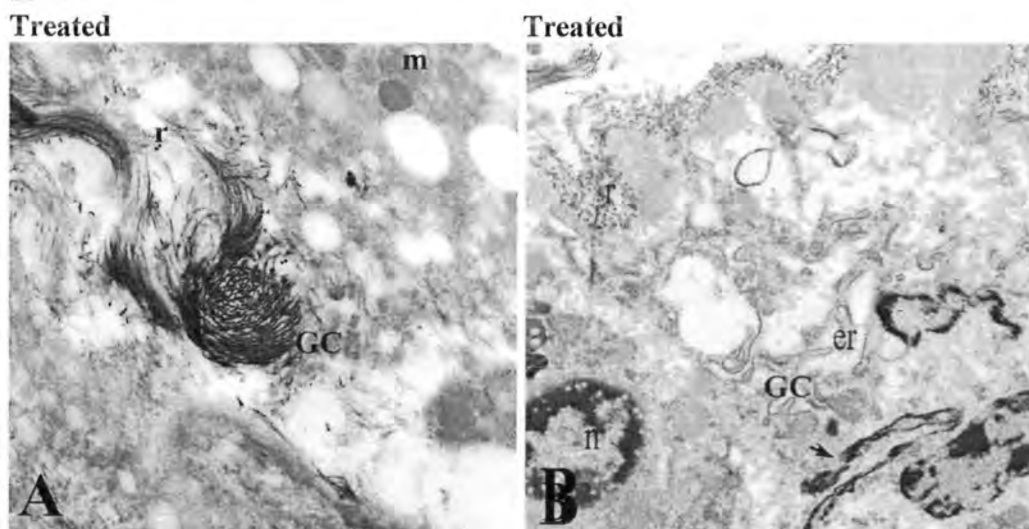


Fig. 3.20 Electron micrographs of control (A) and chromium treated (B) rat thyroid.

A Shows cytoplasm of the cell having Golgi Complex, endoplasmic reticulum, ribosomes and mitochondria (x 10,000).

B Shows shrunken nucleus (n), ribosomes (r) and disruption / depletion of Golgi complex and endoplasmic reticulum (er) (x 6,000).

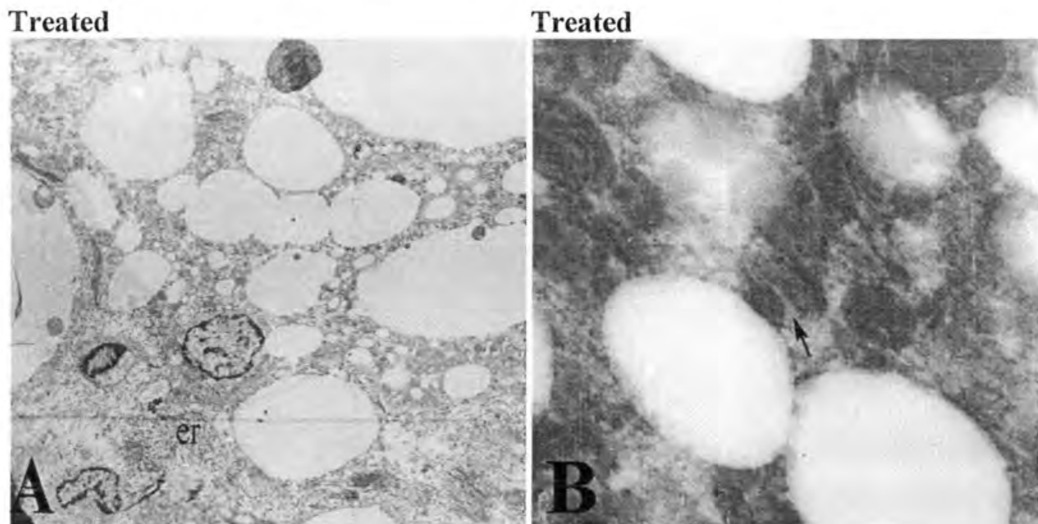


Fig. 3.21 Electron micrographs of treated rat thyroid.

A Shows cytoplasm of the cell having regressing nuclei and colloid droplets (x 2000).

B Shows irregular shape mitochondria visible at higher magnification (x 30,000).

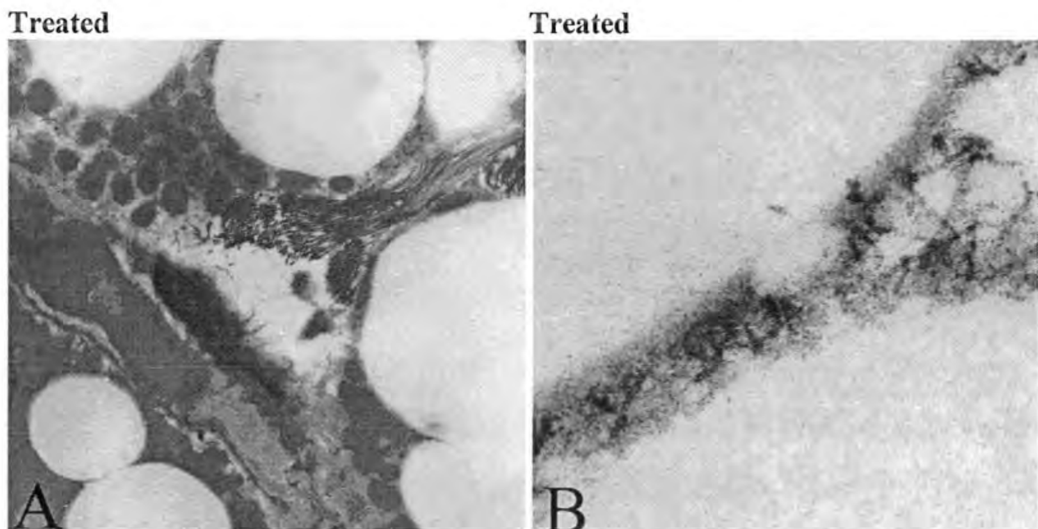


Fig. 3.22 Electron micrographs of treated rat thyroid.

A Shows basal lamina, lysosomes, large vesicles and collagen fibers (x 8,000).

B Border between two follicles showing merger of membranes, one of the two is ruptured (x 40,000).

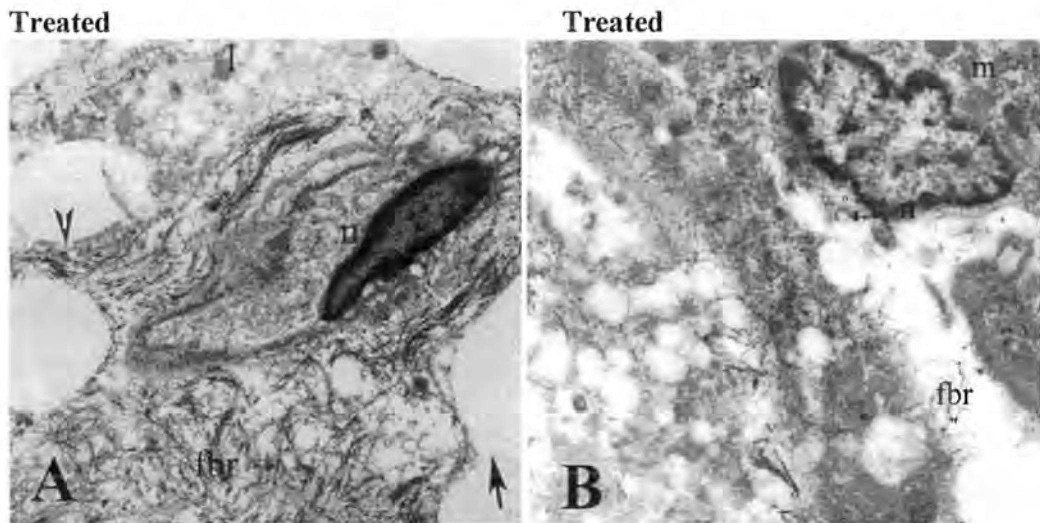


Fig. 3.23 Electron micrographs of treated rat thyroids.
A Showing elongated, squeezed nucleus (n), few lysosomes (l) and abundant clear vesicles (x 8,000).
B Note deformed nuclear membrane of a shrinking nucleus (n), few mitochondria (m), fibers (fbr) and basal lamina (x 10,000).

3.7 Nuclear abnormalities

Acridine orange stained fluorescent photomicrographs demonstrated several dead cells with brightly stained pyknotic nuclei in the treated thyroid sections as compared to the control (Fig. 3.24).

3.8 DNA Ladder Assay

DNA ladder assay demonstrated fragmented DNA in all the three tissues, thyroid, pituitary and hypothalamus (Fig. 3.25).

3.9 Quantification of the DNA fragmentation

In the hypothalamic, pituitary and thyroid tissues of the control and treated rats, DNA fragmentation (%) was quantified which showed that DNA damage (fragmentation) was 56 ± 1.78 % in the hypothalamus, 30 ± 1.26 % in the pituitary and 55 ± 1.21% in the thyroid gland as compared to the control (Fig. 3.26).

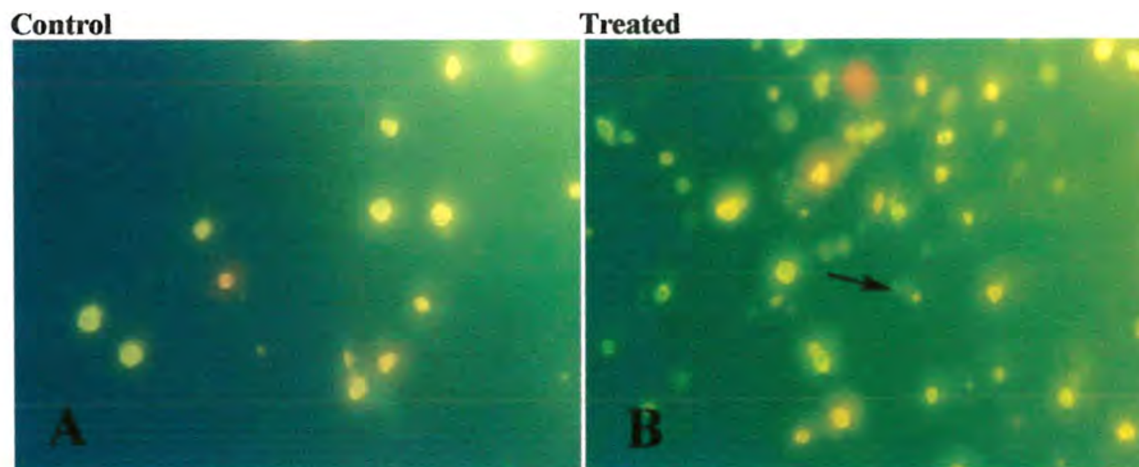


Fig. 3.24 Photomicrographs of rat thyroid gland.

A Showing control thyroid with normal nuclei (x 128).

B. Chromium treated thyroid showing dead nuclei (arrow)(x 128).

Fig. 3.25 Electrophoresis of DNA fragments from rat thyroid, pituitary and hypothalamus. DNA was visualized by UV fluorescence after staining with ethidium bromide. Contents of lanes 2 to 10 are DNA from nuclei of rat tissues as follows: 1, molecular size marker (1500 bp); 2, treated thyroid; 3, treated pituitary; 4, treated hypothalamus; 5-6, control thyroid, 7, blank, 8, control pituitary; 9, blank; 10, control hypothalamus. Arrow on the left indicates DNA fragmentation in the treated samples, while arrow on the right indicates non-fragmented DNA in control samples.

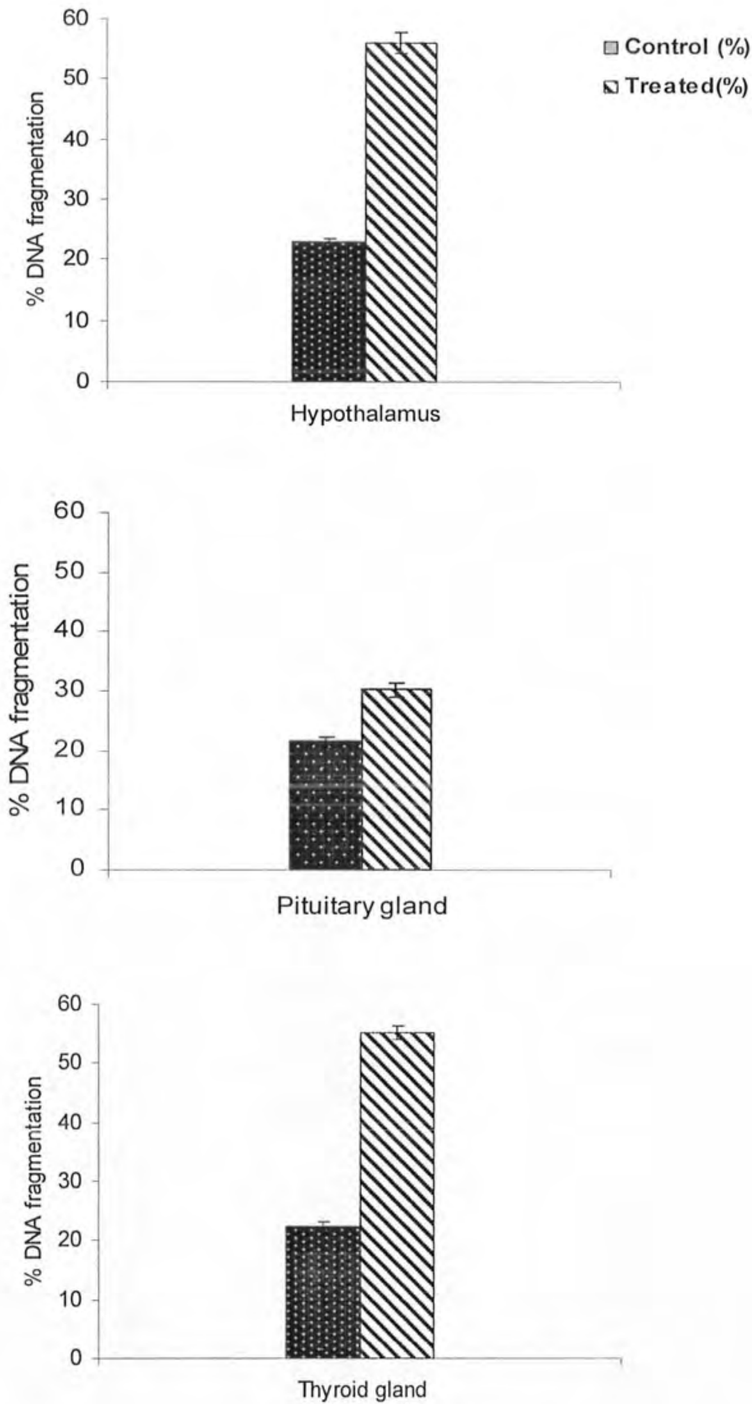


Fig. 3.26 Fragmented DNA (%) of hypothalamus, pituitary and thyroid glands of control and treated rats. Sample size in each case was n = 10 in each group.

Correlation Analysis

A positive correlation was found between thyroid Cr concentration and serum FT_4 and FT_3 concentrations (r = 0.875 and r = 0.925, respectively). In contrast, no correlation was found between thyroid Cr concentration and serum TSH (r = 0.057). Follicular density was negatively correlated with the thyroid chromium concentration (r = – 0.523). However, a weak positive correlation was notable between thyroid chromium concentration and the nuclear size of the cells (r = 0.510).

Pituitary chromium concentration was positively correlated with the serum FT_3 (r = 0.538). Similarly there was a positive correlation between the pituitary chromium concentration and nuclear size of the thyroid follicular cells (r = 0.731). On the other hand, there was negative correlation between pituitary chromium concentration and the chromium concentration in the whole blood as well as the number of thyroid follicular (r = –0.809 and –0.937, respectively).

As regards the chromium concentration in the hypothalamus, serum FT_4 and FT_3 concentrations were negatively correlated with the chromium concentration in the hypothalamus (r = – 0.765 and – 0.575, respectively). Similarly there was a negative correlation between hypothalamic chromium and the nuclear size of the thyroid cells (r = – 0.572).

Chromium concentration in the whole blood was negatively correlated with nuclear size of the thyroid follicular cells as well as chromium excreted through urine (r = – 0.841 and 0.738, respectively). There was found a negative correlation between urine chromium concentration and FT_4 and FT_3 (r = – 0.628 and – 0.575, respectively). Similarly, a negative correlation was found between the urine chromium concentration, whole blood chromium concentration and as well as follicular size of the thyroid gland (r = – 0.738 and – 0.669, respectively).

Serum chromium was negatively correlated with the TSH concentration, thyroid follicular size and epithelial cell height of the thyroid cells (r = – 0.668 and – 0.907, respectively).

DISCUSSION

The focus of the study conducted presently was on investigation of the thyroid function following exposure to hexavalent chromium using laboratory rats as a model system. The study was initiated due to the following reasons: 1) chromium in high oxidation states is extremely toxic to animals and humans, 2) thyroid gland is very important metabolically and can be readily affected by toxicants but the evidence as to the effect of hexavalent chromium on thyroid gland and the HPT-axis, which it makes with that of the pituitary and the hypothalamus is lacking, 3) heavy metal pollution is a major challenge for Pakistan, large amounts of chromium are dumped into the surface waters and soils, and 4) iodine deficiency goitre and other thyroid related problems are very common in this part of the world. The investigation dealt with several aspects of thyroid structure and function.

The study revealed increased accumulation of chromium in the hypothalamus, while pituitary chromium concentration remained unchanged. In contrast, thyroid chromium concentration decreased. Whole blood chromium concentration was increased, serum chromium showed little increase while urine contained high levels of chromium. Serum FT_4 and FT_3 levels decreased, while serum TSH increased. Both the free iodine excreted in the urine and protein bound iodine in the serum were decreased. Structurally, thyroid gland showed follicular hyperplasia with decreased follicle size. Nuclear sizes of thyroid epithelial cells decreased. In contrast, cytoplasmic size and epithelial cell height increased. Follicular collapse, colloid retraction, increased stromal tissue and cellular necrosis were most evident. Pituitary gland was hypertrophied. Ultrastructural examination of the thyroid revealed disruption of basal laminae, pyknotic nuclei with irregular shape nuclear membranes, sparse mitochondria, increased collagen fibers and a disruption of the endoplasmic reticulum and Golgi complex. Quantification of DNA fragmentation demonstrated extensive DNA damage. Acridine orange staining indicated apoptosis-like activity, while the DNA ladder assay demonstrated fragmentation.

Widespread effects of heavy metal pollution on all bodily organs and organ systems and on the endocrine glands have been well documented. Several adverse effects on endocrine systems due to the occupational and environmental chemical

agents in particular heavy metals on human health have also been described (Baccarelli *et al.*, 2000). Thyroid gland is one of the most important endocrine gland of animal bodies (Bentley, 1998). Toxic effects of aluminium (Tanchur *et al.*,1967; Galle, 1987; Zaidi *et al.*, 2001; Aktaç and Bakar, 2002) and cadmium on thyroid have been thoroughly investigated (Pilat-Marcinkiewicz *et al.*, 2003). The current study was concerned particularly with the chromium due to the reason that chromium salts, chromium sulphide and chromium sulphate, are the most common leather tanning agents (Venier *et al.*, 1985), and Pakistan is home to Asia's largest tanneries e.g. "Leather Field" in Sambrial-Wazirabad with an estimated hundred processing units (Bhalli and Khan, 2006). The annual global trade in leather sector as estimated is US $70 billion. The United States, Germany, and other European countries remain major importers of leather products. Countries such as China, India, Pakistan, Thailand, and Indonesia dominate leather and leather products exporters (ITC, 1999). Pakistan is a developing country and earns a major share of its foreign exchange through export of leather goods. According to the recent statistics published by the Ministry of Industries and Production, there are 650 registered leather industries in all major cities: Peshawar, Lahore, Kasur, Sialkot, Wazirabad, Hyderabad and Karachi of the country (Kazmi, 1995). The increase in tanned leather exports, excluding leather garments, from 1990-1995 alone is astounding. The leather products' industry increased its amount of exports from $271 million in the 1990-1991 fiscal years to $349 million in 1994-95 (Zafaruddin, 1995). At present the country produces 7.4 million hides and 36.2 million skins, with an average annual growth of 2.9 and 1.47% respectively (Tahir and Naseem, 2007). The rampant discharge of untreated tanneries effluents is therefore a growing environmental and national problem for Pakistan.

Importantly from the environmental pollution perspective, recent data analysis of effluent samples collected from the chrome tanning section of Saeed Ihsan Tanneries, Faisalabad evaluated for chromium concentration demonstrated a value of 1600 mg L^{-1} (Bhalli and Khan, 2006). This concentration is several times in the order of magnitude of the legal discharge limit of surface waters (0.05-4.0 mg L^{-1}) and sewers (0.5-50 mg L^{-1}) (Alves *et al.*, 1993). Chromium concentration in a wet deposition may vary from 0.004-0.060 $\mu g\ ml^{-1}$ and 0.0006-0.034 $\mu g\ L^{-1}$ for urban and rural areas respectively. Absorption of chromium is inversely proportional to chromium intake, although at any intake level, the body absorbs dietary chromium at

concentrations 0.5-2 % m^{-2} (Vincent, 2000). Although the oxidation state of chromium in the tanning salt is trivalent, discharge norms do not often specify the redox states, because of the concerns of possible conversion of the trivalent state to the more toxic hexavalent form (Bartlett and James, 1979; Fatima *et al.*, 2005). The spent chrome tanning solutions are therefore sources of chromium pollution. In addition to tanneries, exposure to chromium occurs during welding, manufacture of steel, cement and anti-corrosive agents and so on and so forth (Bagchi *et al.*, 2002). Industrial effluents, landfills and sewage sludge contribute maximally to soil and ground water chromium. General population can be exposed to the chromium by using chromium containing compounds, baths, colors, lubricating oils, anti-corrosive agents, wood preservation salts, cement, cleaning materials, textiles and leather tanned with chromium (Polak *et al.*, 1973).

A careful review of literature of the past fifty years as regards heavy metal toxicity revealed only few studies with reference to the effect of chromium on thyroid gland (Goncharov, 1964; Kucher, 1973; Goncharov and Ametov, 1977). Even these studies did not particularly address the toxic effects of hexavalent chromium on hypothalamus-pituitary-thyroid axis, which is one of the very important axes in higher as well as lower animals. However, vast literature exists as regards the role of trivalent chromium as a trace element in lipid and carbohydrate metabolism, chromium picolinate as a supplement and the toxic effects of trivalent chromium on a variety of bodily organs (Von Burg and Liu, 1993; Gargas *et al.*, 1994; Stohs and Bagchi, 1995; Barceloux, 1999; Kawanishi *et al.*, 2002).

A survey of Sialkot and Kasur areas conducted by the author, revealed poor health conditions with a variety of diseases in the people living nearby tanneries and most prominently in tannery workers. Further investigation revealed many cases of thyroid malfunction when local hospitals were consulted. Other problems presented at the hospitals in these cities included respiratory disorders, chronic cough, heart problems, kidney malfunction and failure, contact dermatitis, diarrhoea, frequent vomiting headaches, infertilities, miscarriages and bone and limb disorders. Many families with various sorts of congenital disorders were also encountered.

Although it is not possible to link the results of the present study with thyroid abnormalities reported by the inhabitants of these areas, there is every possibility that chromium strongly interferes with the thyroid function. Recent data analysis has

shown that chromium concentration within the tannery and the effluent is 3200 times greater than recommended by the national and international EPAs (Anonymous, 1999; Bhalli and Khan, 2006). Notably, chromium that is contained in the tannery waste is in the trivalent form and is much less toxic than the hexavalent chromium (Dahbi *et al.,* 2002) but it readily oxidizes to hexavalent form by the soil microbes (Reddy *et al.,* 2003). In addition, absorption of hexavalent chromium is on the several order of magnitude than the trivalent chromium, but once absorbed by the living beings, it is again converted into trivalent form (Wiegand *et al.,* 1985; Aldrich *et al.,* 2003) which, if present in a quantity greater than required by the body is usually highly toxic (Stohs and Bagchi, 1995; Kawanishi *et al*., 2002).

Thus, the presence of large amounts of hexavalent chromium in the environment may lead to acute exposure. Presently, a heavy dose of hexavalent chromium in the form of potassium dichromate salt was administered. It appears though the chromium dose selected presently was several times greater than the possible exposure that can occur to humans or animals, however, it should be noted that the amount of chromium contained in 30 mg $K_2Cr_2O_7$ is ≅ 7 mg. Chromium doses in the range of few micrograms to 20 mg/Kg $_{b.w.}$ are justified from earlier studies (US NAS, 1974a,b). The toxicity of hexavalent chromium varies with the route of entry into the body. Rats tolerated hexavalent chromium in drinking water at 25 mg L^{-1}, for 1 year. Larger doses of hexavalent chromium are highly toxic and may cause death especially when injected intramuscularly, subcutaneous or intragastrically. The LD_{50} of $K_2Cr_2O_7$ administered orally (stomach tube) to rats was 177 mg/kg $_{b.w.}$ in males and 149 mg/kg $_{b.w}$ in females (Hertel, 1982). For mice, LD_{50} of chromium carbonyl was 30 mg/kg $_{b.w}$ (IARC, 1980). Lethal oral doses of soluble chromates (acute toxicity) are considered to be between 50-70 mg/kg $_{b.w.}$. Clinical features include vomiting, diarrhoea, haemorrhage and diathesis, and blood loss in the gastrointestinal tract causing cardiovascular shock. Other major effect observed is liver and kidney necrosis (Langard, 1980).

As people working in the chrome plating and stainless steel industries can at times be accidently exposed to huge concentrations of chromium directly (Stern *et al.,* 1987), in these occupational set ups, an acute dose was quite logical. To achieve sub-lethal exposure and at the same time thorough absorption of chromium by the tissues, intraperitoneal way of administering the toxicant was the method of choice.

It is very well known that the hexavalent chromium irrespective of its route of entry in the body, is readily taken up by the red blood cells in much larger quantities than the trivalent chromium (Wiegand *et al.*, 1984). Presently also, whole blood chromium concentration was significantly increased while serum chromium showed only a slight increase. In the serum, it competes with the blood for binding to plasma transferrin (Hopkins and Schwarz, 1964). Small phosphorylated doses of ^{51}chromium have been shown to bind almost entirely to the iron-binding protein transferrin, whereas from the site of intestinal absorption, chromium is taken up by plasma protein fractions (Hopkins and Schwarz, 1964). Moreover, soluble chelated forms have more affinity for the bone marrow, liver and spleen and clear more slowly. The blood clearance of hexavalent chromium, such as chromate, is slow, because of irreversible binding within the red blood cells (Visek *et al.*, 1953).

Once in the blood, chromium is immediately transported to the tissues where it is reduced in sequential steps to its trivalent form, in which form it then resides in the body (Wiegand *et al.,* 1985; Anderson, 1998; Bagchi *et al.,* 2002). Also, chromium (III) compounds are cleared rapidly from the blood and more slowly from the tissues (Aghdassi *et al.,* 2006). Presently, non-significant increase of chromium concentration in the serum indicates that as the chromium burden increased in the blood, it was soon transported to the tissues. Ingested or injected chromium leaves the blood rapidly; hence, blood chromium levels do not reflect the overall chromium content of tissues, except after a glucose load (Anderson *et al.,* 1985).

The absorption of ingested chromium compounds can be estimated by measuring the amount of chromium excreted in the urine, as almost all of the intravenously injected chromium is excreted via the urine and only 2% is found in the faeces (Donaldson and Barreras, 1966). Hence, urine analysis can provide better information as to the removal of the toxicant or the level of toxicity.

In the current study, a significant increase in urine chromium concentration is not surprising because the kidney removes maximal load of chromium to get rid the body of chromium burden (Behari and Tandon, 1980); however, tissue concentration of chromium increases non-linearly with dose and concentrations in the kidneys increase with duration (Tandon *et al.,* 1979). The kidney in so doing fails due to tubular necrosis and low-molecular-weight proteinuria (Appenroth *et al.,* 1994; Abdulkader *et al.,* 2008; Hanji *et al.,* 2008).

Barrera *et al.* (2003) demonstrated that $K_2Cr_2O_7$ induced renal damage in rats 24 hour after treatment. Noticeably, the decreased urine volume and urine color that varied from pale yellow to dark brown to red indicates damage to the renal capillary.

Hepatorenal syndrome can follow large inhalation doses of dichromate. Cardiogenic shock and renal failure were most possibly the reasons behind limited survival of the experimental animals. A woman who ingested 400 ml of tanning solution containing 48 g of basic chromium sulfate died of cardiogenic shock complicated by pancreatitis and gut mucosal necrosis and haemorrhage (De Flora, 2000).

The voluminous literature on analysis for chromium reviewed by US EPA (1978) recognize two categories of analyses a) methods for measuring large potentially toxic concentrations of chromium as a contaminant, b) methods for analyses of chromium as an essential nutrient. The first category requires reliable determination of chromium at the μg/kg level; the second requires greater sensitivity, to determine accurately the chromium level in urine at several hundred μg/litre. Chromium concentration was although determined using flamed atomic absorption spectrophotometer, the lower detection limit of the instrument for chromium was 1 μg L^{-1}. Chromium concentrations in the blood of unexposed human beings range from 2-70 mg L^{-1} in serum and plasma, and 5-54 μg L^{-1} in red blood cells (US EPA, 1978) while in the urine they range from 1.8-11 μg L^{-1} (Imbus *et al.*, 1963). As current analysis fall in the first category, chromium concentrations measured presently appear validated.

Elevated hypothalamic chromium concentration and a decreased thyroid chromium concentration and at the same time no change in the chromium concentration of pituitary gland indicates different tissues bioavailability of the chromium. Significant uptake or accumulation of chromium by the hypothalamus indicates transportation by the blood due possibly to the damage caused to the blood-brain barrier. On the other hand decreased chromium concentration in the thyroid gland likely attributes to a depletion of the chromium content. How could this has possibly occurred is although not clear from the present study but the most plausible explanation is selective disruption of the thyroid follicles and follicular cell membranes, which is evident from the histological examination of the thyroidal tissue. Thus, chromium would most likely have leaked into the interstitial spaces.

Although, in the present study there was no indication of goitre-like problem, increased chromium concentrations in goitre-affected thyroid glands in humans have been reported (Sorkina and Pavlyuchenkove, 1963). A significant accumulation of chromium in most tissues, brain, kidney, intestine, spleen, lungs, heart, skin, and blood on both 24 hours acute and 90 days chronic exposure to potassium dichromate (unpublished observations from the lab) and is also known from earlier studies (Jacobsen *et al.*, 2007; Rubio *et al.*, 2008), suggesting that chromium readily enters into most of the body tissues.

Radike *et al.* (2002) assessed the distribution of various metals in female mice after ingestion of a metal mixture in either water or feed. They demonstrated highest levels in the small intestine and kidneys of those mice that had received the metal mixture in water. The levels of As, Cd and Cr were greater in small intestine, while the levels of As and Cd were higher in the pancreas. Similarly, the levels of Cr and V were significantly greater in the femur than the controls, at 4, 8, 12, 16 and 24 weeks. According to Solis-Heredia *et al.* (2000), subcutaneous injections of hexavalent chromium ($K_2Cr_2O_7$) into rats follows a dose dependent pattern of the metal. They showed that the levels of Cr were lower in the pancreas and liver but higher in the kidneys, which suggests different tissue bioavailability and accumulation pattern. Similarly, Sutherland *et al.* (2000) investigated the Cr concentrations in various tissues of rats after chronic ingestion of hexavalent chromium in drinking water and found that testicular chromium concentrations were elevated while in the brain, ovaries and blood, chromium concentrations were below detection limits in all exposure groups.

Currently, decreased FT_4 and FT_3 and elevated TSH levels typically suggest hypothyroid state of the gland, which is a significant finding of the present study. However, thyroid weight of treated animals was not changed. Circulating T_4 and T_3 levels act as useful biomarkers of contaminant exposure and as surrogate measures of health in species of marine and terrestrial mammals (Beland *et al.*, 1993; Shumacher *et al.*, 1995; De Guise *et al.*, 1995). Serum levels of T4 and T3 act as reliable indicators of the thyroid function in both human and experimental animals. Any change in their levels reflects disturbance in the glandular synthesis and/or secretion as well as disorders in the extrathyroidal metabolism (Rolland, 2000).

Unavailability of the iodide may account for the decreased FT_4 and FT_3 concentrations. There is a possibility that iodide was available to the thyroid gland but

it was probably unable to interact with the tyrosine residues leading to an impairment of the process of organification. Sodium iodide symporter pump (NIS), however, appears not affected because protein-bound iodine in the serum was decreased indicating that at least iodine uptake took place normally whereas decreased iodine in the urine was quite possibly due to the kidney failure (Chmielnicka *et al.*, 2002).

Chromium combines actively with globulins present in the animal body it is possible that it enhances the synthesis of thyroglobulin; however, it hinders at the same time the process of proteolysis of thyroglobulin and consequent decrease of serum FT_4 concentration (Goncharov and Ametov, 1977). Thus, failure of thyroglobulin proteolysis because of binding with the chromium might be another possibility for decreased levels of concentrations FT4 and FT3. At the ultrastructure level, damage to lysosomes and other cellular organelles of the chromium treated thyroid epithelial cells further support this hypothesis.

Thyroid chromium concentration showed a strong positive correlation with FT_4 and FT_3 (r = 0.875 and r = 0.925, respectively), but there was a weak positive correlation between thyroid chromium content and serum TSH (r = 0.057). These observations indicate that a certain amount of chromium might be necessary for normal functioning of the thyroid gland. Goncharov and Ametov (1977) have shown that in terms of morphology and function of the thyroid gland a chromium dose of 3 $\mu g/kg_{b.w.}$ is considered physiological dose but higher chromium doses (30 - 2250 $\mu g/kg_{b.w.}$) are non-physiological. These doses facilitate a considerable increase in the weight of the thyroid gland and fall in the protein bound iodine level in it. However, contrary to these observations, currently, weight of the thyroid gland was unaltered.

In the case of endemic goitre, when there is deficiency of food related iodine, chromium accumulates in the thyroid gland, giving rise to perceptible changes in its morphology and raising its function (Goncharov, 1964). Goncharov (1968) reported also a close interaction between the chromium and dietary iodine. In iodine-deficient white rats, addition of chromium to the diets in amounts 06-600 μg/animal/ day stimulated thyroid function, as initiated by morphological and functional changes. Addition of daily doses of 0.2, 0.5 and 5.0 $mg/kg_{b.w.}$ potassium dichromate to rabbit diet leads to parenchymatous goitre after 60 and 545 days (Kucher, 1973).

Collins and Capens (1980) and Gerber *et al.* (1985) have demonstrated that in response to long term stimulation of the follicular cells by TSH, as occurs with

chronic iodine deficiency, both lateral lobes of the thyroid are uniformly enlarged due to intense hypertrophy and hyperplasia of follicular cells. Endocytosis of colloid usually proceeds at a rate greater than synthesis, resulting in progressive depletion of colloid. During the present study, small size thyroid follicles and a partial collapse of follicles might have occurred due to the lack of colloid.

Such data are also available for other metals as cadmium and lithium (Gupta and Kar, 1999). Cadmium interferes with the thyroid function at the glandular level as well as at the peripheral level by inhibiting the conversion of T_4 to T3 (Chaurasia *et al*., 1996; Gupta and Kar, 1997; Gupta *et al*., 1999; Gupta and Kar, 1999). Cadmium at the dose of 50 mg/l does not influence T_3, but lead to a decreased T_4 concentration and increased T_3/T_4 ratio and non-significantly increased TSH concentration (Pilat-Marcinkiewicz *et al.,* 2003). In spite of low retention in the thyroid, exposure to cadmium causes serious damage to the thyroid follicular cells (Gupta and Kar, 1997; 1999). It interferes with the thyroid function at the glandular level as well as at the peripheral level by inhibiting the conversion of T_4 to T_3. Since only thyroid gland synthesizes T_4 and T_3, the decrease in the serum level of these hormones in the cadmium treated rats, suggests that it influences the production and secretion of T_4 and T_3 by follicular cells. In this respect, effects of chromium observed presently are very similar to cadmium.

T_3 generation by extrathyroidal deiodination of T_4 occurs mainly due to the liver, 5/- monodeiodinase activity. Furthermore, cadmium can inhibit 5/-D activity through binding to sulfahydryl groups of this enzyme (Chaurasia *et al*., 1996). Evidently, type-1 deiodinase, abundant in liver and kidney is essential to Se deficiency, which decreases deiodinase activity and therefore T_4 to T_3 conversion, resulting in a decrease in the T_3:T_4 ratio. Type-2 and type-3 deiodinases are less sensitive to selenium deficiency, indicating their importance for the maintenance of normal thyroid hormone levels (Arthur *et al*., 1999). Waring *et al.* (1996) also, have reported that plasma thyroxine (T_4) and triiodothyronine (T_3) concentrations increase in sub-lethally aluminium stressed brown trout (*Salmo trutta*).

According to Christian and Trenton (2003), "despite some modulation of metabolic rate by hormones such as testosterone, growth hormone, and norepinephrine, T_4 and T_3 are the most important metabolic rate modulators. In rodents, decreased levels of serum T_4 and T_3 and increased levels of serum TSH, with sustained release of TSH and resultant follicular cell hypertrophy/hyperplasia, are

typical hormonal and histopathological findings attributable to compounds altering thyroid function". Lopez *et al.* (2000) and Singh *et al.* (2000), have shown that depending on duration of adverse influence, chronic exposure of humans at the work places to the small amounts of metals like lead can result in significant reduction of blood thyroxine and triiodothyronine level as well as marked reduction of blood TSH level. The present study in rats appears similar to that of Erfurth *et al.* (2001), who demonstrated that human exposure to lead causes significant toxicity to the hypothalamic–pituitary–thyroid axis resulting in higher concentration of thyrotrophin-releasing hormone and TSH. Presently, thyrotropin (TRH) releasing hormone concentrations were not determined due to certain limitations, raised TSH levels indicate similarly elevated TRH levels. Among other metals, lithium has been associated with hypothyroidism. The inhibitory effect of lithium occurs mainly at the level of hormone secretion although effects on iodide trapping, release and coupling have also been described. However, no significant interference of lithium on thyroid hormone metabolism or action has been described (Lazarus, 1998).

Thyroid hormones have been studied with reference to some other metals also. For example, Pilat-Marcinkiewicz *et al.* (2003) have shown that cadmium influences dose dependent structural and functional changes of the thyroid follicular cells in female rats. At low exposure, only structural changes in the thyroid follicular cells occur whereas at the highest exposure, cadmium causes both structural and functional damage to these cells. Similarly, Yoshizuka *et al.* (1991) have shown that cadmium accumulates in the mitochondria of the thyroid follicular epithelial cells and it can inhibit the synthesis and release of thyroid hormones influencing the oxidative phosphorylation of these organelles. Presently, marked histopathological findings as collapsed and disintegrated follicles, colloid retraction or absorption, hyperplasia, alteration of the cellular architecture, damaged or reduced number of cell organelles indicate severe impairment of the thyroid gland both at the structural and functional level.

In the current study, depressed levels of thyroid hormones and hyperplasia of the thyroid strongly indicate that the chromium administration induced suppression of the thyroid hormone synthesis and release in turn stimulated the pituitary to secrete more TSH because of the negative feedback mechanism. Decreased protein-bound iodine in the treated rats indicates that thyroid gland, in order to synthesize T_4 and T_3 perhaps attempted to trap normal or more quantity of iodine from the blood under the

influence of the hypothalamus and the pituitary through increased TSH concentration. Since the concentrations of FT_4 and FT_3 were depressed, this suggests that iodide was possibly trapped in the thyroid gland and was not available for the synthesis of the hormones. These results indicate that the primary target of chromium is the thyroid gland. Wolff (1998) has also demonstrated that the presence of excess iodide inhibits the thyroid gland function by multiple mechanisms and as a result, thyroid activity slows down. Similar to the present study, Siegel *et al.* (1989) demonstrated that apart from alteration of the endocrine system occupational exposure of adult human males to inorganic lead is associated with impaired uptake of iodine by the thyroid tissue as well as depressed free thyroxine level and altered morphology of the gland.

Histopathology of the anterior pituitary gland showed a generalized hypertrophic condition, which was obvious because the gland was under stress of the toxicant and was trying to cope with this condition by secreting greater quantities of TSH. The signal for the pituitary and quite possibly for the hypothalamus to release more TRH employed both the short and long negative feedback loops. Although the products could not be quantified due to certain limitations, RT-PCR analysis indicated an up-regulation of TSH mRNA in the chromium treated animals. This observation is supported by a parallel increase of TSH concentration determined by radioimmunoassay.

Similar effects due to the stressors have been shown by Bailey (1984) who demonstrated that depending upon the intensity stressors act indirectly on the pituitary gland and stimulate it to release adrenocorticotropic hormone (ACTH). If the stress continues, the adrenal cortex enlarges and maintains the production of corticoids. At this resistance stage, adrenal cortex is large but not depleted. Continuation of excessive levels of stress causes the exhaustion phase in which adrenal cortex is both large and depleted. This stage is associated with hypoglycaemia, with kidney damage and enteritis or ulcers and many animals die of these problems.

Presently also, the hexavalent chromium acted as a stressor on the pituitary gland to make it hyperactive. It appears that the continued stress (second dose after 24 hours) led to hypertrophied pituitary gland. Observations like, increased epithelial cell height, decreased nuclear size and increased cytoplasmic size in the thyroid follicles all indicate state of acute stress. Exposure of rats to 3.0 and 30 mg/kg/day doses of ammonium perchlorate in drinking water led to an increase of relative thyroid weights, hypertrophy, hyperplasia, and statistically significant differences in

TSH, T4 and T3 in the 30 mg/kg/day dosage group (Christian and Trenton, 2003). Aktaç and Bakar (2002) demonstrated similar findings in rats exposed to aluminium for a longer period. They showed that most abundant degenerative changes in the thyroid gland occurred in the 5 % $AlCl_3$ dosage group. Except thyroid weight of chromium-treated animals that remained unaltered, similar degenerative changes were currently noticeable.

Degenerative changes as, follicle destruction, hypertrophy and hyperplasia, nuclear, cytoplasmic and organelle abnormalities suggest accumulation of chromium in the cellular organelles. Yoshizuka *et al.* (1991) have shown that cadmium accumulates in the mitochondria of the thyroid follicular epithelial cells and it can inhibit the synthesis and release of thyroid hormones influencing the oxidative phosphorylation of these organelles. Cobo and Castineira, (1997) have also demonstrated oxidative stress-induced mitochondrial dysfunction. However, a lower thyroid chromium concentration seen at present apparently does not bring these observations closer to the above studies. Meager numbers of mitochondria with morphological abnormalities were readily noticeable in ultrathin sections of thyroid gland. Thus, altered thyroid morphology is quite possibly attributed to the toxicant as no such changes were visible in the control thyroid.

Currently, ultrastructural analysis of the thyroid gland demonstrated regressed and collapsed follicles with disrupted basal laminae; pyknotic nuclei; disorganized Golgi complex and endoplasmic reticulum; less abundant mitochondria; abundant collagen fibers and lysosomes in the thyroid stroma all indicating abnormal status of the gland. Possible decrease of thyroglobulin synthesis can also be attributed to these cellular changes. Severe alterations were evident in the nuclei. It can be safely assumed because of the appearance of a large number of acridine orange positive pyknotic nuclei that the process of transcription of mRNA was either very much reduced or halted eventually leading to an abnormal working of the thyroid. Aluminium produces similar effects as damaged nuclei within follicle lumen and increased fibers within dispersed stroma of rats exposed to aluminium (Aktac and Bakar, 2002).

Deckwerth and Johnson (1993) have demonstrated that maintenance of structural homeostasis requires active function of all structures of thyroid gland and therefore long-term stimulation is accompanied by dystrophic changes: accumulation of thyrocytes with pyknotic nuclei, destruction of thyrocytes in follicles adjacent to

collapsed capillaries and in the lumen of follicles with preserved blood supply. Thyrocyte destruction (pyknosis of thyrocyte nuclei, lysis of the cytoplasm, fragmentation and condensation of cytoplasmic structures) and their appearance in the follicular lumen correspond to morphological signs of apoptosis described for other cell structures.

Determination of the Percent DNA fragmentation demonstrated greater damage in the chromium treated sections of hypothalamus, pituitary and thyroid glands. From the DNA ladder assay it appears as if chromium interacted with the DNA and possibly caused single and double strands breaks. Since hexavalent chromium is readily reduced to its trivalent form, there is every possibility that the tissue concentration of chromium (III) was elevated. Hexavalent chromium is genetically active, because of its ability to cross the membranes and enter the cells. However, its genetic activity is suppressed if its reduction takes place outside the cell (or even outside the cell nucleus) e.g., in mitochondria or microsomes). But if the reduction takes place inside the nucleus (near or at, the target DNA molecules) alterations in DNA can occur, depending upon the oxidation power of hexavalent chromium or the formation of trivalent chromium complexes with nucleophilic sites of DNA. Thus, trivalent chromium could be the ultimate mutagenic form of chromium (Levis and Bianchi, 1982; De Flora *et al.*, 1984).

Although trivalent chromium is required in trace amounts for proper sugar and fat metabolism, it has been suggested that it causes DNA damage in *in vitro* test systems. It inhibits topoisomerase DNA relaxation activity in bacterial cells and decreases the viability and/or proliferation rate of eukaryotic cells such as murine B16 melanoma cells and human MCF-10A neoT ras-transformed human epithelial cells (Palper *et al.*, 2002). Izzoti *et al.* (1998) injected Sprague-Dawley rats intratracheal instillations of sodium dichromate at the rate of 0.25 mg/kg $_{b.w.}$ for three consecutive days. They showed localized DNA lesions in the lung but not in the liver tissue, they ascribed this to toxicokinetics and metabolic characteristics of chromium (VI). DNA alterations included DNA-protein crosslinks, DNA fragmentation, nucleotidic modifications, and 8-hydroxy-2'-deoxyguanosine.

The current study indicates that exposure to hexavalent chromium may lead to hypothyroidism. Hypothyroidism besides other physiological disturbances leads to infertility (Elbetieha and Al-Hamood, 1997; Aruldhas *et al.*, 2005). Thus, if people living in those areas where there is greater environmental concentration of hexavalent

or other oxidative forms of chromium are exposed to such high concentrations, would likely present infertility risk. Moreover, dangerously elevated levels of even trivalent form are now being recognized as highly toxic. Selenium as the antioxidant enzyme glutathione peroxidase (Gpx) may protect the thyroid gland from oxidative damage due to any excess H_2O_2 produced during thyroid hormone synthesis (Thomson *et al.*, 2005). Several methods have been suggested and are on trial to remove environmental chromium (Tahir and Naseem, 2007).

Hexavalent chromium is a known human carcinogen (Kimura, 2007). Increased cancer mortality caused in particular from stomach cancer is associated with exposure to high concentration of hexavalent chromium in well water in Liaoning Province, China (Beaumont, 2008; Smith, 2008). Presently, there was no direct indication of tumor formation in the thyroid or pituitary gland, probably due to the very short exposure, however, severe hyperplasia of the thyroid and pituitary hypertrophy suggest that if such a stress had continued, hyperplasia would have transformed into tumor formation during the shortest exposure. Molecular damage caused by chromium may be due to its intracellular reduction to the even more highly reactive and short-lived chemical species chromium (III) and chromium (V). Exposure to chromium (VI) can result in point mutations in DNA and to chromosomal damage as well as to oxidative changes in proteins and to adduct formation (Paine, 2001).

Excess iodide alone can be toxic to thyroid cells in culture and cause thyroid hypertrophy and changes in colloid *in vivo* in the rat (Capen, 1997). However, iodide excess does not induce follicular cell hyperplasia and thyroid tumors in rats but more commonly cause hypothyroidism (Backer and Hollowell, 2000). Trivalent chromium as chromium picolinate causes renal failure after its ingestion (Wasser and Feldman, 1997). If TSH levels remain elevated chronically, there is an association of such high levels with an increased risk of thyroid tumors in the rat, which may be due, in part, to the high turnover rate of circulating T_3 as compared to humans who have a lower T_3 turnover rate (Capen, 1997). Synthesis and secretion of thyroid hormones is either disrupted or enhanced at one or more steps on exposure to different chemicals and drugs, resulting in decreased of T_4 and T_3 levels and a compensatory increase in the TSH levels. Most chemicals and drugs when tested in rats and mice lead to follicular cell hypertrophy / hyperplasia and increased thyroid weights. Long-term exposure to such compounds leads to a secondary oncogenesis associated with hormonal

imbalances (Capen, 1994; De Sandro *et al.*, 1991; Saito *et al.*, 1991; Smith *et al.*, 1991).It has already been shown that heavy metals have a negative impact on ovarian and pituitary function and that heavy metal-induced hormonal and immunological changes are important factors in the pathogenesis of repeated miscarriages (Gerhard *et al.*, 1998).

The present study could not address some issues because of certain limitations; however, the study bears significance as to the high level of heavy metal pollution in Pakistan. Accumulation of the chromium can be localized in the thyroid and other tissues using radioactive Cr. Functioning of the glands should be thoroughly checked by radioactive iodine. Immunocytochemical localization should be used for identifying and morphometrical examination of pituitary thyrotrophs and TRH secreting neurons. Indication of apoptosis emphasizes the need to perform quantitative Tunnel-assay. It is pertinent to further investigate the hexavalent chromium toxicity using low but chronic doses. Moreover, evaluation of thyroidal enzymes TPO and 5′-deiodinase should be investigated. There is also a need to conduct large-scale comprehensive studies on humans as regards the effect of chromium (VI) on human and animal thyroid. Whether exposure to hexavalent or trivalent chromium actually leads to thyroid abnormalities of humans remains to be established.

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