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RESEARCH ARTICLE

Curcumin induces apoptosis in thyroid cells in rats: possible role of caspase 3

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Abstract

Background: Antioxidants could have a direct positive effect on the thyroid gland.
Aim of the work: This study investigated the possible protective effect of curcumin, an antioxidant polyphenolic compound, against sodium chlorate (NaClO3) - induced hypothyroidism in rats and the possible role of caspase 3 in its mechanism of action.
Materials and Methods: Rats were divided into three groups: group I: control; group II: NaClO3 exposed rats (2g/l in drinking water for three weeks); group III: NaClO3 exposed rats plus curcumin admixed with the diet (0.5% w/w) for one week before NaClO3 exposure and continued for three weeks after exposure. Triiodothyronine (T3), thyroxine (T4), thyroid stimulating hormone (TSH) and superoxide anion (as an index of oxidative stress) levels were determined in plasma. Thyroid sections were subjected to H&E staining, and immunohistochemical staining of caspase 3 as an index of apoptosis.
Results: Curcumin pretreatment in NaClO3 exposed rats produced significant increase in plasma levels of T3 and T4 and decreased significantly TSH and superoxide anion levels in plasma as compared to NaClO3 non-treated rats. Histologically, curcumin pretreated rats showed neither follicular cellular hypertrophy nor hyperplasia as compared to non–treated NaClO3 exposed rats. Moreover, curcumin in NaClO3 pretreated rats significantly increased the mean area percent of caspase 3 in thyroid gland as compared to NaClO3 non-treated rats.
Conclusion: These findings showed an improved effect of curcumin against NaClO3-induced hypothyroidism in rats possibly by its antioxidant property and its apoptotic effect which may be mediated by caspase 3.

Introduction

The thyroid gland is the most important endocrine gland for metabolic regulation (Cunningham, 2002). The thyroid hormones, thyroxin (T4) and triiodothyronine (T3) are necessary for the growth, metabolism and functioning of virtually every cell in the body (Sojka, 1995). Hypothyroidism is a common thyroid disorder which may give rise to goiter, that is, an enlarged thyroid. Hypothyroidism is commonly due to deficient iodine intake or secondarily due to intake of goitrogens (Radostits et al., 2000). Recent studies have shown an increased production of reactive oxygen species in hypothyroidism (Sarandol et al., 2005; Erdamar et al., 2008).

Apoptosis is a particular type of programmed cell death that is characterized by the expression of pro-apoptotic genes and the activation of a family of cystein-proteases called caspases (Taylor et al., 2008). Among them, caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase such as caspase-9. The activated caspase-3 could cleave the poly (ADP-ribose) polymerase which is one protein related to a number of

cellular processes involving mainly DNA repair and programmed cell death (Herceg and Wang, 2001). Apoptosis suppression may contribute to thyroid proliferative diseases including goitre, Grave's disease and cancer (Tanimoto et al., 1995).

Hypothyroidism is commonly treated with iodine supplements or thyroid hormone replacements. However, there are other natural supplements that can support thyroid functioning either by protecting the thyroid gland from injury or by increasing the production of thyroid hormones.

Phytochemicals are naturally occurring substances found in plants. There has been considerable public and scientific interest in the use of phytochemicals esp. polyphenols derived from dietary components to combat human diseases due to their anti-oxidant, anti-inflammatory, anti-bacterial, anti-mutagenicity and anti-cancer properties (Parvathy et al., 2009).

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is among the best characterized natural polyphenols and is the major yellow pigment extracted from turmeric, a commonly used spice, derived from the rhizome of the herb *Curcuma longa* Linn. Curcumin is commonly used as a colouring and flavouring agent in food products (Ammon and Wahl, 1991). Curcumin presents antiviral, hypolipidemic, anti-infectious, antiinflammatory and anticancer properties (Joe et al., 2004; Sharma et al., 2005). It also exhibits antioxidant property and acts as a scavenger of oxygen species, such as hydroxyl radical, superoxide anion, and singlet oxygen (Reddy and Lokesh, 1994). Previous investigations have shown that polyphenol curcumin can enhance the manifestation of hypothyreosis in rats simultaneously treated with propylthiouracil (Papiez et al., 2008). Meanwhile, the mechanism of action of curcumin in hypothyroidism and the role of caspase 3 in its mechanism of action is not well defined.

Sodium chlorate (NaClO3), an oxidizing agent, disrupts thyroid hormone synthesis in rats leading to hypothyroidism and long-term exposure is associated with persistent stimulation of the pituitary-thyroid axis which may lead to hypertrophy and hyperplasia of thyroid follicular cells (Capen et al., 1991).

Hence, this study aims at investigating the protective effect of the antioxidant polyphenol, curcumin, in experimental hypothyroidism-induced by NaClO3 and the possible role of caspase 3 in its mechanism of action.

Material and Methods

Animals

Thirty healthy adult male albino rats of Wistar strain, weighing about 200–250 g were used in the present study. They were obtained from the animal house of Faculty of Medicine, Cairo University. The animals were housed in standard stainless-steel cages at a 12 h cycle of light and dark. Room temperature was kept at 24±2 °C and humidity maintained at 50%. Animals were provided with standard food and water ad libitum. All animal procedures were performed after approval from the ethics committee of the National Research Centre Cairo, Egypt and in accordance with the international regulations for the use and care of experimental animals (Canadian Council on Animal Care Guidelines, 1993).

Chemicals

Sodium chlorate was purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). Sodium chlorate was prepared by dissolving 2g of $NaClO_3$ in 1.0 liter of deionized water (2g/L) as stated by Hooth et al. (2001). The solution was stored at room temperature in a dry cool place away from light. The rats received their respective treatments via clean brown glass drinking water bottles that were fitted with steel sippers and placed on cage tops.

Curcumin was obtained from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA) in the form of powder.

Experimental Design

The animals were divided into three groups, 10 rats each as follows:

Group I (Control group): Rats received standard rat chow diet and water ad libitum for 4 weeks.
Group II ($NaClO_3$ exposed group): Rats received standard rat chow diet and were exposed via their drinking water to $NaClO_3$ (2g/L) for 3 weeks.
Group III ($NaClO_3$ exposed + Curcumin pretreated group): Rats were exposed via their drinking water to $NaClO_3$ (2g/L) for 3 weeks. One week before exposure, rats received a diet enriched with 0.5% w/w curcumin (Arafa, 2005) which continued for 3 weeks after exposure.

At the end of the experiment (4 weeks), blood samples were taken from rat tail vein in collecting heparinized capillary tubes. The samples were centrifuged at 1000 Xg and plasma was split and frozen in polypropylene tubes at -20 ºC until assayed. Animals from all groups were then sacrificed by decapitation and thyroid gland was removed. Part of thyroid gland was kept in 10% formalin for histopathological and immunostaining studies.

Biochemical analysis

I-Detection of thyroid stimulating hormone and thyroid hormones

Thyroid stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) were measured in plasma using ELISA kits provided by Calbiotech Inc, USA according to manufacturer's instruction (Agharanya, 1990; Frank et al., 1996; Sachidhanandam et al., 2010).

II- Detection of Superoxide anion

Superoxide anion was measured in plasma using LumiMax Superoxide Anion Detection Kit supplied by Agilent technologies Canada according to manufacturer's instruction (Stohs, 1995).

Histological examination

Specimens of thyroid gland were fixed in 10% formol saline. Thyroid paraffin blocks were prepared and serial sections of 7µm thickness were obtained and stained with:

-Hematoxylin and eosin (H&E) for routine histological examination (Drury et al., 1988).

-Immunohistochemical staining for detection of caspase 3. Thyroid gland sections were incubated overnight with the primary rabbit polyclonal anti-caspase-3 antibody (Abcam Inc) (Sanii et al., 2012). Caspase-3 immunoexpression was detected in the cytoplasm of epithelial cells lining the follicles and was used as a marker of apoptosis. Tissue sections were counterstained with Mayer's hematoxylin.

Quantitative morphometric measurements

Using 'Leica Quin 500 C' software image analyzer computer system (Leica image system Ltd, Cambridge, England), the mean area percent of caspase 3 immunostaining-positive cells was measured. Measurements were carried out within 10 nonoverlapping fields for each animal at a magnification of × 400.

Statistical analysis

All data were expressed as means ± standard deviation (SD) for the quantitative variable. Analysis of variance (ANOVA) was performed on the means to determine whether there were significant ($P < 0.05$) differences among the groups. When ANOVA indicated statistical significance the Tukey-Kramer test follows up, for intergroup comparisons. GRAPHPAD Software (version 2.0, 1993, Instat, San Diego) was used for all statistical analysis. The results were considered significant when p value <0.05.

Results

I. Biochemical results:

Effect of curcumin pretreatment on plasma T3, T4 and TSH levels in NaClO3 exposed rats

In the present study, plasma T3 and T4 levels decreased significantly ($p<0.001$) and plasma TSH level increased significantly ($p<0.001$) in rats after 3 weeks of exposure to 2.0 g/L NaClO3 (group II) as compared to control group (Table, 1). Curcumin pretreated rats (group III) showed significant increase in plasma levels of T3 ($p<0.01$) by 58% and T4 ($p<0.001$) by 108% with significant decrease ($p<0.001$) in plasma TSH level by 47% as compared to non-treated NaClO3 exposed rats (Table, 1). Meanwhile, curcumin pretreatment in NaClO3 exposed rats (group III) in this study did not normalize plasma levels of T3 and T4 and TSH to control values as there was significant difference in these parameters in group III as compared to control group (Table, 1).

Effect of curcumin pretreatment on plasma superoxide anion level in NaClO3 exposed rats

The results of the present study showed significant increase ($p<0.001$) in plasma superoxide anion level in NaClO3 exposed rats (group II) as compared to control rats (Table, 1). Curcumin pretreatment in NaClO3 exposed rats (group III) decreased significantly ($p<0.001$) plasma superoxide anion level by 41% as compared to non-treated NaClO3 exposed rats but with significant difference ($p<0.001$) as compared to control group (Table, 1).

Table 1: Effects of Curcumin on plasma T3, T4, TSH & superoxide anion levels in the studied groups.

Groups	Control group	NaClO3 exposed group	NaClO3 exposed + Curcumin pretreated group
T3 (ng/ml)	2.21±0.33	1.02±0.14*	1.61±0.26$^{\$}$**
T4 (ng/ml)	3.53±0.33	1.2±0.18*	2.5±0.33$^{\#}$*
TSH (uU/ml)	0.87±0.03	3.81±0.17*	2.01±0.13$^{\#}$*
Superoxide anion (FU)	1044.5±19.09	2108.8±5.45*	1250.4±13.19$^{\#}$*

Values are expressed as means ± SD (n = 10 each group). **NaClO3**: sodium chlorate, **T3:** triiodothyronine**, T4:** thyroxine**, TSH**: thyroid stimulating hormone. Analysis of variance (ANOVA) followed by Tukey-Kramer analysis was used for the comparison between the groups.

*$p<0.001$, **$p<0.01$ vs control group

$^{\#}p<0.001$, $^{\$}$ $p<0.01$ *vs* NaClO3 exposed group

II. Histological results

A.H&E-stained sections

Sections in the thyroid glands of rats from the control group (group I) showed follicles lined by single uniform layer of flattened to cubical epithelium and contained abundant densely stained colloid material within the lumen (Fig. 1).

Thyroid gland sections from rats exposed to NaClO3 (group II) showed evidence of focal hyperplasia that is characterized by multiple layers of follicular epithelial cells protruding into the lumen. It also shows focal epithelial hypertrophy where the epithelial cells are tall cuboidal to columnar. There are also cytoplasmic vacuoles and the thyroid follicles contain minimal amount of colloid with some follicles containing pale pink, lacy material (Fig. 2).

Thyroid gland sections from rats exposed to NaClO3 and received curcumin pretreatment (group III) showed neither cellular hypertrophy nor hyperplasia where thyroid follicles lined by single layer of flattened to cubical cells with the disappearance of epithelial vacuoles. There was abundant less densely stained colloid substance in the follicles (Fig. 3).

B.Immunohistochemistry-stained sections

Immunohistochemical results of the control group, showed moderately caspase-3 immunopositive cells (brown cytoplasmic deposits) in thyroid sections (Fig. 4). As for NaClO3 exposed rats (group II), thyroid gland sections showed faint few caspase-3 immunopositive cells (Fig. 5). Regarding curcumin pretreatment in NaClO3 exposed rats (group III), there was intense widely distributed caspase-3 +ve cells in thyroid gland sections (Fig. 6).

Morphometric results

The mean area percent of caspase 3–positive cells was significantly decreased ($p<0.001$) in NaClO3 exposed rats (group II) as compared with control rats. NaClO3 exposed rats pretreated with curcumin (group III) showed significant increase ($p<0.001$) in the area percent of caspase 3 –positive cells as compared to non-treated NaClO3 exposed rats with non significant difference ($p>0.05$) as compared to control rats (Table, 2).

Table 2: The mean area percentage of caspase 3 in the studied groups:

Groups	Caspase-3
Control group	13.319±1.933
NaClO3 exposed group	8.081±1.948*
NaClO3 exposed + curcumin pretreated group	12.562±1.865#

Values are expressed as means ± SD (n = 10 each group). **NaClO3**: sodium chlorate. Analysis of variance (ANOVA) followed by Tukey-Kramer analysis was used for the comparison between the groups.

*p<0.001 vs control group

#p<0.001 *vs* NaClO3 exposed group

Fig. (1): Photomicrograph of a section in the thyroid gland of a control rat (group I) showing follicles lined by single uniform layer of flattened (arrow head) to cubical epithelium (→) and the lumens are filled with densely stained thyroglobulin colloid(C). H&E X 1000

Fig. (2): Photomicrograph of a section in the thyroid gland of a rat that received NaClO3 (group II) showing focal hyperplasia (↑) that is characterized by multiple layers of follicular epithelial cells protruding into the lumen. It also shows focal epithelial hypertrophy where the epithelial cells are tall cuboidal to columnar (arrow head). Also, there are cytoplasmic vacuoles (spiral arrow) and the thyroid follicles contain minimal amount of colloid (F). H&E X 1000

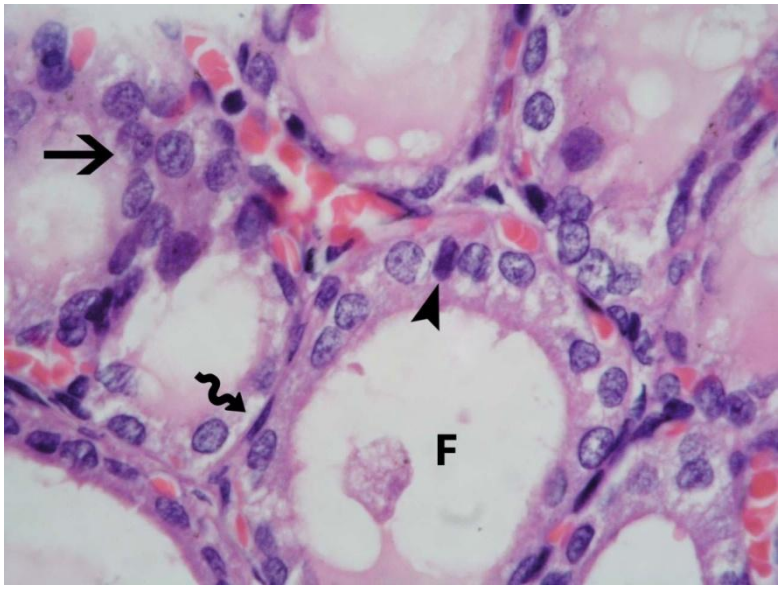

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Fig.(3): Photomicrograph of a section in the thyroid gland of a rat pretreated with curcumin and exposed to NaClO3 (group III) showing thyroid follicles lined by single layer of flattened (arrow) to cubical cells (arrow head) and containing abundant less densely stained colloid(C). H&E X 1000

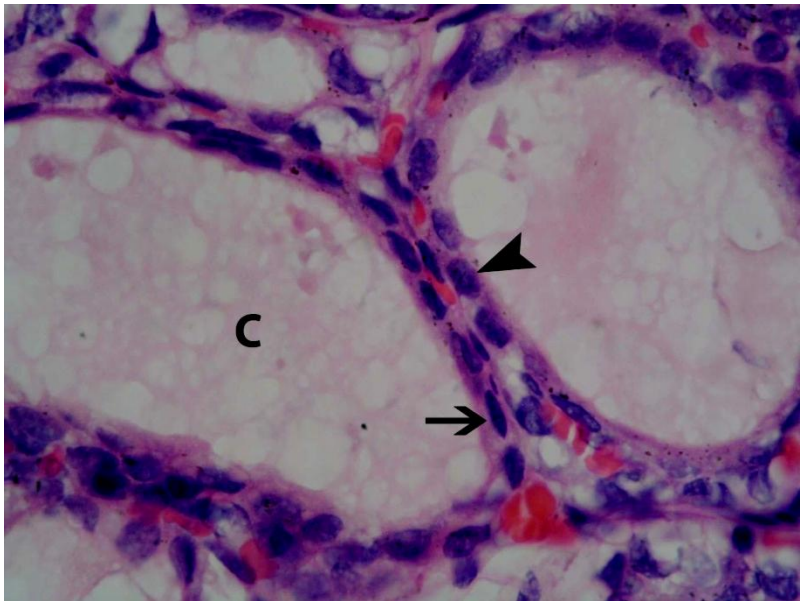


Fig. (4): Photomicrograph of a section in the thyroid gland of a rat from the control group (group I) showing moderately +ve stained caspase-3 immunoreactive cells that appear as brown cytoplasmic deposits (→) in the follicular epithelial cells. Caspase-3 immunostaining X 1000

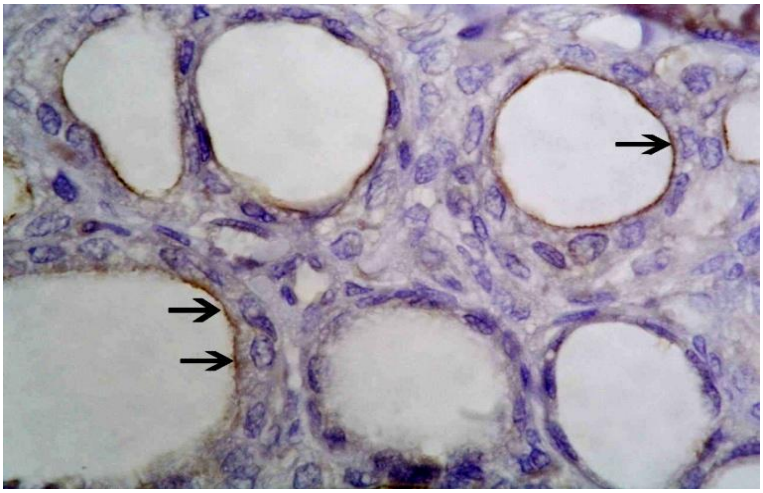


Fig. (5): Photomicrograph of a section in the thyroid gland of a rat that received NaClO3 (group II) showing faint, few +ve caspase-3 immunoreactivity in the follicular epithelial cells (→).Caspase-3 immunostaining X 1000

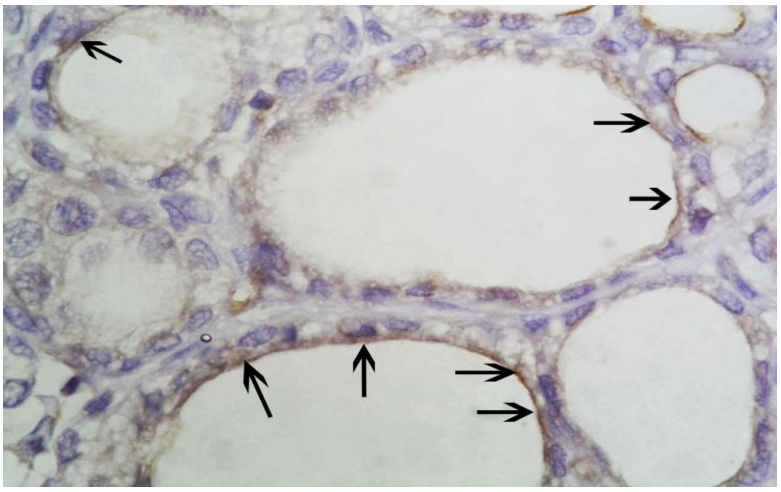
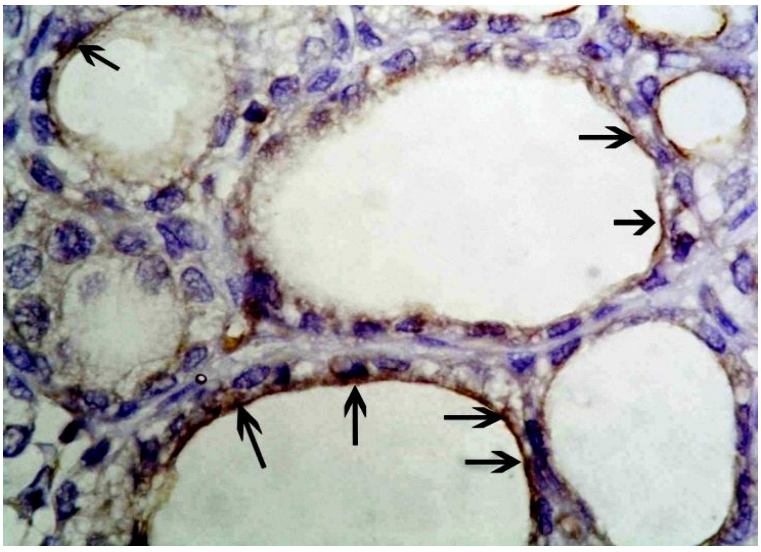


Fig. (6): Photomicrograph of a section in the thyroid gland of a rat that received curcumin with NaClO3 (group III) demonstrating intense widely distributed +ve immunoreaction for caspase-3 (→) in the follicular epithelial cells. Caspase-3 immunostaining X 1000



Discussion

Thyroid hormones are essential for normal body functions including metabolism, growth, maturation, and reproduction. Thyroid hormone levels are controlled by feedback regulation to the pituitary and hypothalamus (Ito et al., 2010), which bears primary responsibility for integrating thyroid function with body needs. When the blood level of thyroid hormone falls the hypothalamus senses the change and secretes thyroid–releasing hormone (TRH). This hormone stimulates the anterior pituitary to secrete TSH (Sukkar et al., 2000) which in turn causes the thyroid gland to increase secretion of thyroid hormone. In the present study, NaClO3 exposed rats showed hypothyroidism which was evidenced biochemically by significant decrease in plasma T3 and T4 levels with significant increase in plasma TSH level as compared to control rats. These biochemical findings were associated with thyroid follicular

cell hyperplasia, cellular hypertrophy and decreased amount of colloid in the follicles as detected histologically in H and E stained sections in NaClO3 exposed rats as compared to control rats.

Sodium chlorate disrupts the hypothalamic-pituitary-thyroid axis in developing and adult mammals. Supportive with the findings of the present work, Hooth et al. (2001) reported that serum T3 and T4 levels decreased significantly followed by a compensatory significant increase in serum TSH levels in male and female F344 rat after 4 days of treatment with 1.0 or 2.0 g/L NaClO3 and after 21 days of treatment with 2.0 g/L NaClO3. They also found that exposure to NaClO3 at 1.0–2.0 g/L for 4–21 days caused histological changes in the rat thyroid gland including depletion of colloid, hypertrophy and hyperplasia of follicular epithelial cells. The histological changes observed in this study were explained by Hood et al. (1999) where they have demonstrated that small increases in serum TSH can be sufficient to stimulate thyroid cell proliferation and thyroid gland growth.

The mechanism by which NaClO3 affect pituitary-thyroid homeostasis was discussed by Carrasco (1993) where he stated that NaClO3 competitively inhibits iodide uptake at the sodium iodide symporter of the thyroid gland, which results in decreased synthesis of thyroglobulin and reduced circulating thyroid hormone. Colloid is rich in a glycoprotein called thyroglobulin which is the precursor of the iodinated thyroid hormones (T4) and (T3) (Pilling et al., 2007) and is synthesized by the follicular cells and stored in the lumen of the follicles (Lin, 2008).

In this study there was significant increase in plasma superoxide anion level in NaClO3 exposed rats compared to control rats suggesting the presence of oxidative stress in this animal model since superoxide anion is a free radical very reactive oxidizing molecule which can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins (Sies, 1997). Our results are in agreement with Hooth et al. (2001) who reported that chlorate is a thyroid toxicants and chemical oxidants similar in structure to bromate and perchlo-rate.

In the current work, rats receiving curcumin pretreatment in diet for 1 week before exposure and for another 3 weeks after exposure to NaClO3 showed significant increase in plasma T3 and T4 levels with significant decrease in plasma TSH level as compared to non-treated NaClO3 exposed rats but with significant difference as compared to the control group. This suggests a partial protective effect of curcumin against thyroid dysfunction which was detected in other studies. Deshpande et al. (2002) found that treatment with turmeric extract reduced the impact of methimazole- induced hypothyroidism in rats with less suppressed T4 and T3 levels suggesting that antioxidants could be having a direct, positive effect on the thyroid gland. Also, Nabavi et al. (2011) indicated that pretreatment with curcumin provides protective effects against sodium fluoride -induced thyroid dysfunction in young adult male rats where serum T4 and T3 levels remained close to normal after exposure to sodium fluoride in the drinking water.

In this study, curcumin pretreated NaClO3 exposed rats showed significant decrease in plasma superoxide anion level compared to non-treated NaClO3 exposed rats which suggests an antioxidant effect of curcumin. Dietary curcumin is reported to inhibit superoxide anion generation and hydroxyl radical generation through preventing the oxidation of Fe^{2+} in Fenton's reaction, which generates ·OH radicals (Reddy and Lokesh, 1994). Curcumin is considered to be an effective antioxidant against oxidative tissue damage. It can significantly inhibit the generation of reactive oxygen species both in vitro and in vivo (Okada et al., 2001; Biswas et al., 2005). Curcumin has been reported to show antioxidant properties by one or more of the following interactions: Scavenging or neutralizing free radicals by oxygen quenching and making it less available for oxidative reaction and/or inhibition of oxidative enzymes like cytochrome P450, interacting with oxidative cascade and preventing its outcome and chelating and disarming oxidative properties of metal ions such as iron (Soudamini et al., 1992; Unnikrishnan and Rao, 1995).

In the current study, histological examination of thyroid gland sections from rats exposed to NaClO3 and received curcumin pretreatment revealed neither cellular hypertrophy nor hyperplasia as compared to non-treated NaClO3 exposed rats which suggest an antiproliferative action of curcumin. Meanwhile, there was less densely stained colloid in curcumin pretreated group as compared to control group and this agrees with the biochemical findings where curcumin did not normalize plasma thyroid hormones levels as there was significant decrease in plasma T3and T4 levels in curcumin pretreated group compared with their respective control group.

Regarding immunohistochemical results, the current study revealed faint, few +ve caspase-3 immunoreactivity in the follicular epithelial cells in NaClO3 exposed rats where the mean area percent of caspase 3 -positive cells was significantly decreased as compared with control rats. Meanwhile, there was intense widely distributed +ve immunoreaction for caspase-3 by immunohistochemical analysis together with significant increase in the mean area percent of caspase 3 -positive cells in NaClO3 exposed rats pretreated with curcumin compared to non-treated NaClO3 exposed rats. Caspase-3 is a member of interlukein converting enzymes and it is the most commonly one involved in the execution of apoptosis in various cell types (Cohen, 1997).

Thus the previous results suggest an apoptotic effect of curcumin in experimental hypothyroidism which was demonstrated by several authors in other studies. Curcumin is known to inhibit proliferation of cancer cells by arresting them at various phases of the cell cycle and to induce apoptosis in tumor cells (Su et al., 2006). Curcumin was found to significantly inhibit cell viability and promoted cell apoptosis in a dose-dependent manner in papillary thyroid cancer cells (Song et al., 2012). The mechanisms responsible for apoptosis induction by curcumin were discussed previously including: inhibition of Akt dephosphorylation, NF-κB activation, down-regulation of the levels of Bcl-2 and Bcl-XL, increase of cytochrome *c* release, up-regulation of growth arrest and DNA damage gene (GADD153), and activation of p38 and caspase-3 (Pan et al., 2001; Woo et al., 2003; Shishodia et al., 2005).

Thus the findings of the present work suggest that the antiproliferative action of curcumin may be secondarily, to the presence of apoptosis as detected by caspase 3 in immunohistochemical studies and also to the significant decrease in plasma TSH in NaClO3 pretreated rats. Other studies proposed that curcumin exerts antiproliferative action through its inhibitory influence on the activity of protein kinases including protein kinase C and the kinase of the epithelial growth factor (Korutla and Kumar ,1994) since these enzymes play a key role in signal transduction which leads to thyreocyte proliferation.

In conclusion, the results of the present study showed that the protective effect of curcumin pretreatment in NaClO3 induced hypothyroidism in rats may be mediated by its antioxidant effect and its apoptotic effect which is mediated by caspase 3. Curcumin exerts anti-proliferative and pro-apoptotic effects in an experimental model of hypothyroidism. Thus, curcumin as a health-promoting food ingredient could prevent thyroid proliferative diseases including goiter and Grave's disease.

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