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Age-Related Changes in Histological and Cytoskeletal Intermediate Filaments of Rabbits Thyroid Glands and the Prophylactic Role of Vitamin E

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Abstract: The present investigation is planned to compare between the histological and immunohistochemical detection of the cytokeratin and vimentin of rabbit thyroid glands of different ages and the prophylactic role of vitamin E on senescent animals. Male New Zeland rabbits were divided into four groups according to age. Group I: represented the young rabbits (one month age, weighing 1 ± 0.4 kg), group II: adult rabbits (6 months age, weighing 4 ± 0.5 kg), group III: aged animals (24 month age, weighing 7.5 ± 0.5 kg and group (IV) aged rabbits treated daily with the therapeutic dose of vitamin E orally at a dose of 10 mg/kg b.w / day for 60 days. The thyroid glands of young rabbits revealed uniform appearance of follicles lined by simple cuboidal epithelia and the lumina of many follicles are filled with vacuolated colloid. In adult studied animals, the follicles are varied in their shapes from oval to round shape seen with numerous vacuolated colloids and lined with cuboidal to columnar cells. In elderly rabbits, the thyroid glands exhibited a numerous number of follicles lost their regular shape lined with a single layer of flattened thyrocytes, colloid empty and fused together to form large irregular vacuoles. Aged animals group treated with vitamin E revealed an obvious improvement of the thyroid structure and restored their simple cuboidal thyrocytes with regular rounded follicles with vacuolated colloid. The intensity of either vimentin or cytokeratin filaments immunoreactivity of the thyroid glands was gradually increased with age. The thyroid glands of young rabbits showed less expression while the adult thyroid rabbits revealed moderate expression of vimentin at the basal part of the thyrocytes and around the blood vessels while cytokeratin immunoreactivity expressed at the apical surface of follicular cells and in the endothelia of the blood vessels. The sections of the thyroid glands of aged animals demonstrated an obvious intense immunopositive reaction to vimentin or cytokeratin. The administration of elderly animals with vitamin E at a dose 10 mg/kg b.w/day for 60 days illustrated a marked improvement and recovery of vimentin and cytokeratin to normal moderate expression almost similar to adult ones. The results indicated that senescent New Zeland rabbits should be used vitamin E to improve the disturbances of the thyroid gland and intermediate filaments.

Key words: Thyroid Gland · Rabbit · Age · Histology · Intermediate Filaments · Vitamin E

INTRODUCTION

Thyroid gland is one of the major and important endocrine gland. It is unique to the vertebrate endocrine system in that it stores its secretory products, thyroid hormones extracellularly. This gland is among the most highly vascularised endocrine glands in mammals. It carries out highly specific functions such as synthesis, accumulation and release of hormones [1]. The gland is composed of two cell populations that secrete two different classes of hormones. Follicular cells secrete the metabolically active iodothyronines, tri-iodothyronine (T3) and thyroxin (T4), whereas the parafollicular cells (C-cells) are concerned with the production of calcitonin [2]. Thyroid hormones affect mammalin metabolism by a thermogenic action in which carbohydrate, lipid and protein metabolism can be accelerated in tissues and thus, increase the amount of heat produced at a given time accelerating body growth and development and increasing cardiac function [3].

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The functional components of the thyroid gland are the individual thyroid follicles, which consists of thin cuboidal and occasionally columnar epithelia, arranged as a single layer surrounding a lumen of colloid separated by connective tissue in which the blood and lymph vessels and nerves are carried. In the normal state, the follicles are filled with homogeneous colloid. The epithelial cells vary in size and number dependant on the activity of the gland [4]. There are an increasing in size and numbers of follicles with increasing age showed in male albino rat [5] and in humped camel *(Camelus dromedarious*) [6].

Aging is a complex biological process leads to gradual loss of ability of an individual to maintain homeostasis. Accumulation of the diverse deleterious changes produced by aging throughout the cells and tissues progressively impairs many cellular functions and can eventually cause irreversible damage [7]. The free radicals play a key role in the pathology of normal aging. They are formed with aging and caused disturbance and damage of many tissues as the reduction in nervous system capabilities, reduced mobility, injury of liver, decreased the reproductive hormones, adrenal gland production and the activity of growth hormone [8, 9].

Da Costa and Rosenthal [10] studies the effects of aging on thyroid gland in old rats of both genders, their results showed that aging induced real changes in rat thyroid gland function and regulation, serum thyroxine (T4) decreased but serum thyrotrophin (TSH) remained unaltered. The young individuals have follicles with a uniform appearance lined with a single layer of cuboidal epithelium with spherical densely stained nuclei but in adult animals the follicular cells appeared columnar in shape. However, with advancing age, the follicles became increasingly irregular and variable in shape and the follicular cells appeared as simple squamous cells with flattened nuclei.-

The thyroid gland was studied in bottlenose dolphins (*Tursiops truncatus)* from the Texas coast of the Gulf of Mexico [11]. They found that the gland tended to compact relatively homogeneous and sometimes partly lobular, but with advancing age it became more lobular, the lobules being defined by fibrous bands. The gland was represented by variation in follicle size and colloid density tended to increase with age. Moreover, Kot *et al*. [12] reported that the size of follicles appeared to decrease in a function of increasing age in the bottlenose dolphin thyroid gland. The large sized follicles were mostly in the peripheral, while small sized follicles were present in the center.

Vitamin E (DL- alpha tocopherol acetate) is an effective antioxidant. It is a lipid- soluble chain breaking antioxidant which protects biological membrane from lipid peroxidation and possibly nucleic acids against oxidative damage [13]. The antioxidants play a role as defense systems for the removal of the excess reactive oxygen species (ROS) and excess hydrogen peroxide produced by thyrocytes during thyroid hormone synthesis and in aged animals [14]. Antioxidants supplementation decrease peroxidation processes and partly restore the thyroid hormones T3 and T4 concentration in serum [15,16] and stimulate the secretion of thyroid stimulating hormone [17,18].

Intermediate filaments (IFs) are one of the three abundant cytoskeletal proteins. IFs include cytokeratin, vimentin, desmin, glial fibrillary acidic proteins, neurofilament proteins, nuclear lamins and nestin [19]. They play an important role in the structural integrity, in movement of organelles and their secretory vesicles and possibly in the transport into and out of the cells. IFs provide flexible intracellular scaffolding whose function is to structure cytoplasm and to resist stresses externally applied to the cell [20,21].

Cytokeratin filamentous proteins are used as diagnostic markers in tumor pathology, particularly for the differential diagnosis of carcinomas at the histologic level [22]. Vimentin is widely distributed in the cells of mesenchymal nature and in stroma [23]. It is used as tumor markers in serum and as means of detecting micrometastases [21]. Miettinen *et al*. [24] studied the expression of intermediate filament proteins in thyroid tumor immunohistochemically and they found the intermediate filament proteins of cytokeratin and vimentin were evaluated in non-neoplastic thyroid glands and in different types of thyroid neoplasms. Follicular epithelia of both normal and goitrous thyroids showed a strong reaction with anticytokeratin antibodies that widely cross-react with various simple epithelia. Only the stromal and interstitial cells reacted with antibodies to vimentin. The provided beneficial and protective actions of antioxidant that modulated the disorganization of intermediate filaments during oxidative stress were studied in hippocampus of adult rats [25-27].

The present study aimed to compare between the histological structure of thyroid glands and detection of the changes in their intermediate filaments, cytokeratin and vimentin, of different rabbits ages (young, adult and old ages) and the prophylactic role of vitamin E on the thyroid glands of elderly rabbits.

MATERIALS AND METHODS

Twenty eight different ages of male New Zeland rabbits from one to 24 months weighing from 1kg to 8 kg were collected during autumn and housed in environmentally controlled optimal conditions for one week relevant ethics committee and guidelines for the care and use of experimental animals. Diet and water were allowed *ad-libitium.*

The studied animals were divided into four groups according to age. Group I: represented the young rabbits (one month age, weighing 1 ± 0.4 kg), group II: adult rabbits (6 months age, weighing 4 ± 0.5 kg), group III: senescent (aged) animals (24 month age, weighing 7.5 ± 0.5 kg) and group IV: aged rabbits treated daily with the therapeutic dose of vitamin E orally (E-Viton, Kahra Pharm and Chem.Ind.Co) at a dose of 10 mg/kg b.w/day for 60 days. Vitamin E (Alpha tocopheryl acetate) dosage was estimated according to Baydas *et al*. [25].

The animal groups I, II & III were sacrificed after one week, while the animals group (IV) that treated with vitamin E was sacrificed after 60 days. The thyroid glands of all groups were removed carefully and cut into small pieces then fixed in 10% neutral buffered formalin. Specimens were washed and transferred to70% ethanol, dehydrated in ascending ethanol, cleared in xylene, embedded in paraffin wax and sectiond at 5µm thick using a microtome. Paraffin sections were stained with haematoxylin and eosin (H&E) according to Bancroft and Gamble [28] for histolgical study.

For immunohistochemical study, paraffin sections of thyroid and monoclonal antibody either anti- pan cytokeratin (anti-CK AE1/AE3) or anti- vimentin (V9) were used and obtained from Thermo Fisher Scientific Industries. Avidin-biotin immunoperoxidase technique was applied in which a biotinylated secondary antibody reacts with peroxidase conjugated streptavidin molecules. Colour reaction was developed by using diamino-benzidine (DAB) that gives a brown colour [29].

RESULTS

Histological Results: Sections of the thyroid glands of young rabbits (1 month age) revealed uniform appearance of follicles. All follicles are lined by simple cuboidal epithelia with spherical densely stained nuclei and the lumina of many follicles are filled with vacuolated colloid. Blood vessels are frequently present in stroma in between the follicles (Fig. 1).

In adult studied animals (6 months age), the follicles are varied in their shapes from oval to round shape and lined with cuboidal to columnar cells with spherical densely nuclei. The peripheral follicles are more active than the central one due to having numerous vacuoles in colloid of variable sizes (Fig. 2).

In elderly rabbits (2 years age), the thyroid glands exhibited a numerous number of follicles that lost their regular shape and others became dilated. They were lined with a single layer of thyrocytes that seen flattened with darkly stained splayed nuclei. Many follicles were seen empty from colloids and fused together to form large irregular vacuoles (Fig. 3 a&b).

Aged animals group treated with vitamin E at a dose 10 mg/kg b.w / day for 60 days revealed an obvious improvement of the thyroids structure and restored their simple cuboidal thyrocytes shape with spherical densely stained nuclei. Many follicles restored their rounded or oval outlines and the lumina showed vacuolated colloid and others were still empty (Fig. 4a&b).

Immunohistochemical Results:

Vimentin: The expression of vimentin demonstrated as brown filaments at the basal part of the thyrocytes and around the blood vessels. The intensity of vimentin filaments immunoreactivity was changed according to the age of the studied animals. The thyroid glands of young rabbits exhibited less expression of vimentin immunoreaction (Fig. 5).

The adult thyroid rabbits revealed normal moderate expression of vimentin immunorection (Fig. 6) while the thyroid glands of aged animals demonstrated obviously intense dark brown filaments of vimentin (Fig. 7) at the basal part of the thyrocytes and around the blood vessels. The aged studied rabbits treated with vitamin E at a dose 10 mg/kg b.w / day for 60 days illustrated a marked reduction and recovery of immunoreactivity to vimentin and seen with normal moderate expression at the basal part of the thyrocytes and around the blood vessels (Fig. 8) almost similar to adult ones. B- Cytokeratin The cytokeratin immunoreaction demonstrated as brown filaments at the apical surface of follicular cells. In the young individual rabbits, the follicular cells expressed approximately negative immunoreaction to cytokeratin (Fig. 9a&b) while the adult ones revealed a normal moderate immunoreaction to cytokeratin as brown filaments at the apical surface of follicular cells and in the endothelia of the blood vessels (Fig.10). Aged studied rabbits expressed intense cytokeratin immunoreactivity at

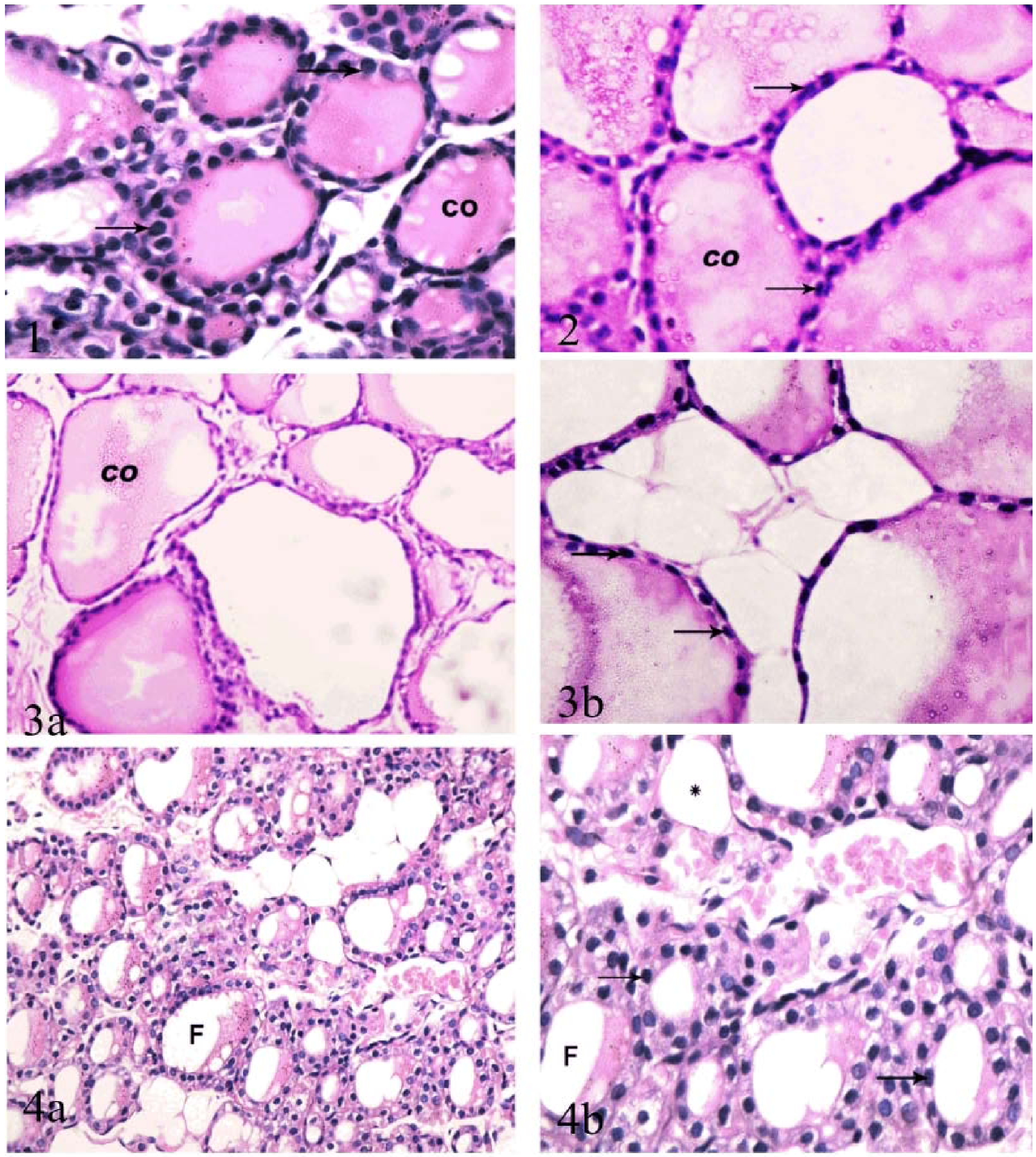


Fig. 1: Section of the thyroid gland of young rabbit(one month age) showing various sizes of follicles lined by simple cuboidal epithelia with spherical densely stained nuclei (arrows) The lumen of many follicles is filled with vacuolated colloid (CO). H&E, X400

Fig. 2: Section of the thyroid gland of adult rabbit (6 months age) showing mostly rounded regular follicles lined by a layer of cuboidal cells with spherical densely stained nuclei (arrows), and the lumens are filled with vacuolated colloid (CO). H&E, X400

Fig. 3: Section of the thyroid gland of aged rabbit (2 years age) showing: a) irregular and dilated follicles lined by flattened cells .Many follicles are empty from colloids and a few of them is seen with vacuolated colloid (CO); b) fusion of many empty follicles lined by flattened cells with darkly flattened nuclei (arrows). H&E, X 200 & X400

Fig. 4: Section of the thyroid gland of aged rabbit treated with vitamin E at a dose of 10 mg/kg b.w / day for 60 days showing: a) the improvement of the follicles that seen lined with a layer of simple cuboidal epithelia and restored the vacuolated colloid in a lot of follicles (F) and epithelia, b) a lot of normal follicles (F) with a layer of simple cuboidal epithelia with vesicular large nuclei (arrows) and the appearance of vacuolated colloids. Some follicles are still devoid the colloid (*). H&E, X200 & X400

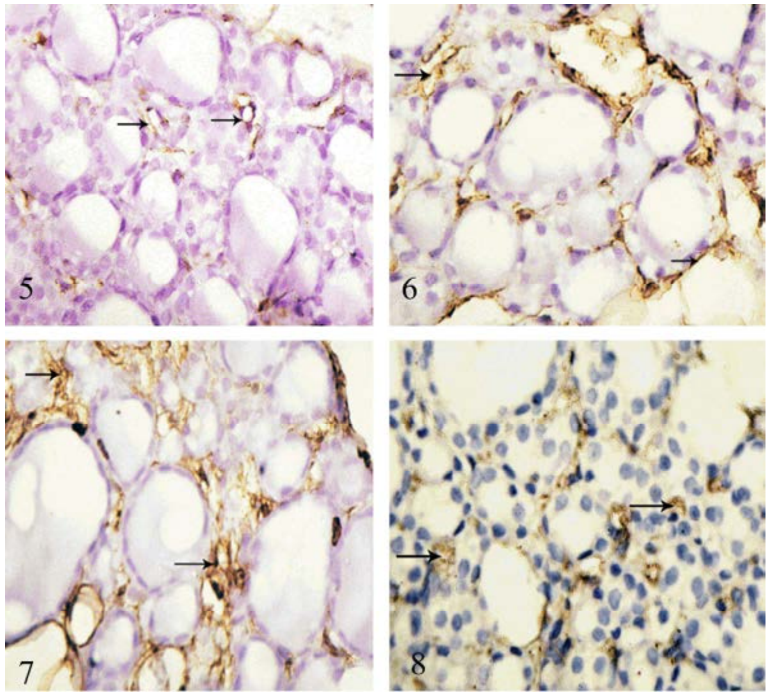


Fig. 5: Section of the thyroid gland of young rabbit showing less expression of vimentin immunoreactivity marked with a little brown filaments present at the basal part of the follicular cells and around the blood vessels(arrows). Vimentin immunostain, X400

Fig. 6: Section of the thyroid gland of adult rabbit revealing normal moderate expression of vimentin immunorectivity marked with dark brown filaments at the basal part of the thyrocytes and around the blood vessels (arrows). Vimentin immunostain, X400

Fig. 7: Section of the thyroid gland of aged rabbit showing an obvious intense immunopositive reaction to vimentin at the basal part of the follicular cells and around the blood vessels (arrows). Vimentin immunostain, X400

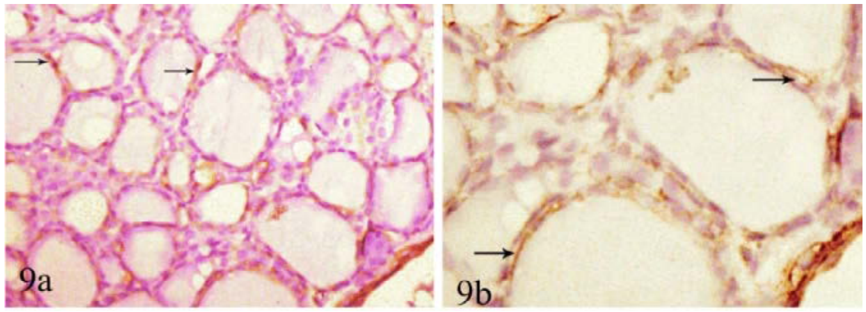
Fig. 8: Section of the thyroid gland of aged rabbit treated with vitamin E at a dose of 10 mg/kg b.w / day for 60 days illustrating a marked reduction and recovery of immunoreaction of vimentin (arrows) almost similar to adult one. Vimentin immunostain, X400

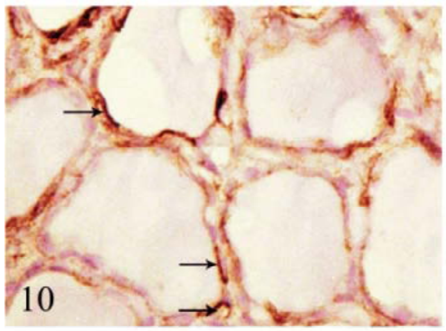
the apical surface of the flattened thyrocytes (Fig.11). After treatment of aged animals with vitamin E at a dose 10 mg/kg b.w/day for 60 days, the glands elucidated an improvement and a noticeable reduction in the cytokeratin immunoreaction in the thyrocytes and demonstrated almost like normal form (Fig. 12).

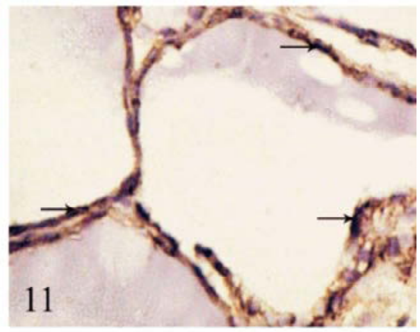
DISCUSSION

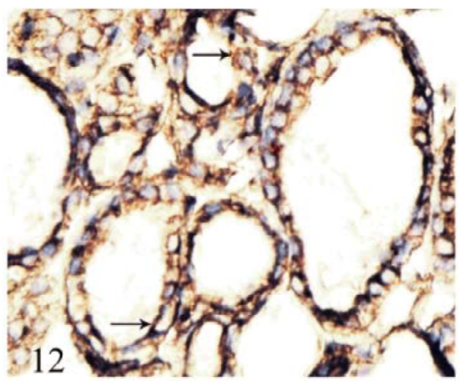
The mammalian thyroid gland maintains basal metabolism in tissues for optimal function and synthesizes thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) under the influence of thyroid stimulating hormone (TSH) secreted from pituitary gland [30]. The normal adult thyroid follicle consists of thin cuboidal to columnar epithelia, arranged as a single layer surrounding a lumen of colloid and separated by connective tissue in which the blood and lymph vessels and nerves are carried. The epithelial cells vary in size and number dependant on the activity of the gland [1, 31].

The present investigation revealed that the sections of thyroid glands of young rabbits have follicles with a uniform appearance lined with a single layer of cuboidal epithelium with spherical densely nuclei. In adult studied animals, the follicular cells are seen cuboidal to columnar in shape. However, with advancing age, the follicles became increasingly irregular and variable in shape. The aged studied animals showed irregular and dilated follicles and the follicular cells appeared as simple squamous flattened cells with splayed nuclei. Similar results were observed in European bison glands [2]; in rabbits [31]; in dog [32] and in camel [6, 33]. In current study, the lumina









Figs. 9a&: Section of the thyroid gland of young rabbit demonstrating weak expression of cytokeratin immunoreactivity at the apical surface of follicular cells (arrows). Cytokeratin immunostain, X200 & X400

Fig. 10: Section of the thyroid gland of adult rabbit showing a moderate immunoreaction to cytokeratin at the upper surface of the thyrocytes and the endothelia of blood vessels (arrows). Cytokeratin immunostain, X400

Fig. 11: Section of the thyroid gland of aged rabbit showing a marked increase of cytokeratin immunoreactivity at the surface of flattened thyrocytes (arrows). Cytokeratin immunostain, X400

Fig. 12: Section of the thyroid gland of aged rabbit treated with vitamin E showing an improvement and a noticeable reduction of the immunoreactivity to cytokeratin at the apical surface of the thyrocytes (arrows). Cytokeratin immunostain, X400

of some follicles of the thyroid glands of young rabbits were filled with vacuolated colloid near the margin of follicles indicating the sign of activity and others with no vacuoles indicating weak activity of the thyroid glands. In adult animals, the thyroid gland exhibited signs of secretory activity more than the young ones, the lumina of follicles were filled with vacuolated colloid. In elderly studied animals, the majority of follicles were empty from colloids indicating the signs of no activity in the thyroid follicles. Breifly, the activity of thyroid gland was decreased with increasing age. In accordance, Farag [5] demonstrated the colloid vacuolations in the thyroid follicles were found near the luminal margin of the follicular cells of male rats at one- month age. The same results were observed by da Costa and Rosenthal [10] and Wollman *et al.* [34] who explained that during the follicular cell activities, dissolution of some colloid by acid phosphatase secreted from lysosomes with the hormonal release were occurred. However, Torres *et al.* [32] recorded the colloid of old age was high viscosity and they explained this viscosity to the reduction of water content. Pasupathi and Latha [35] recorded a high production of reactive oxygen species (ROS) and oxidative stress with enhanced lipid peroxidation and concomitant failure of antioxidant defense mechanisms in hypothyroidism patients.

Aging is an intricate phenomenon characterized by progressive decline in physiological functions and increase in mortality that is often accompanied by many pathological diseases. Many theories of aging have been proposed, including the free-radical and mitochondrial theories of aging. Both theories speculate that cumulative damage to mitochondria and mitochondrial DNA (mtDNA) caused by ROS and it is one of the causes of aging. Oxidative damage affects replication and transcription of mtDNA and results in a decline in mitochondrial function which in turn leads to enhanced ROS production and further damage to mtDNA. Increasing intracellular oxidants by altering ambient oxygen concentrations or lowering antioxidant levels accelerates the onset of senescence [8].

In the present results, the elder animals treated with vitamin E at a dose of 10 mg / kg b.w / day for 60 days revealed an obvious improvement of the thyrocytes and they returned to a round shape and lined with simple cuboidal to columnar cells with vacuolated colloid approximately as in adult ones. Ramanathan *et al*. [36] recorded the vitamin E is the most important lipophilic antioxidant and resides mainly in the mitochondria thus helping to maintain membrane stability and decreased the cell death which is due to the oxidants and free radicals in different cells. The antioxidants play an important role in preventing free radicals damage associated with age by interfering in the generation of radicals or by scavenging [14].

Moreover, Kim *et al.* [18] recorded the antioxidants (melatonin & Thiol) stimulated the secretion of TSH, which subsequently stimulates the follicular cells activity to produce thyroid hormones [17]. Antioxidants (selenium or vitamin E) supplementation decrease peroxidation processes and partly restore the thyroid hormones T3 and T4 concentration in serum [15,16] and stimulate the secretion of TSH [37].

Also, the present observations also agreed with the finding of El-Desouki [38] who demonstrated that the treatment of vitamin E improved many disturbancs in the thyroid glands of diabetic rats that accompanied with many atrophied follicles with irregular outlines and the majority of follicular cells displayed low epithelia and shrunken nuclei and the follicles restored their round outline and the lumina showed vacuolated colloid. Recently, vitamin E protects against toxicity on the cerebellar cortex of the albino rat brain [39].

In the current work the immunohistochemical study covered both cytoskeletal protein filaments, vimentin and cytokeratin, the intensity of either them was gradually increased from adult to elder rabbit thyroids. Sections of thyroid glands of young, adult and aged studied animals expressed the vimentin immunoreactivity in the connective tissue at the basal part of the thyrocytes and around the blood vessels in a less expression in young, moderate in adult and an obvious intense immunopositive expression in aged animals. The cytokeratin immunoreactivity was expressed at the apical surface of follicular cells and in the endothelia of blood vessels of the thyroid glands. It was weak in young, moderate in adult and intense in aged animals.

In accordance, Kameda [23] reported that the vimentin is widly distributed in cells of mesenchymal nature and he found on his studies in immature thyriod gland of dog that typical thyroid follicles showed no immunoreactivity for vimentin and observed when thyroid follicles stored considerable amounts of colloid in the follicular lumina (active follicles). Thus, the author concluded that the vimentin filaments may participate in increased cellular activities of the thyroid, i.e. thyroglobulin synthesis and folliculogenesis. Moreover, Fonseca *et al*. [40] found that in normal thyroid, the cytokeratin was expressed in simple epithelial cells and was stronger in thyroid lesions than normal thyroid.

The cytoskeletal intermediate filament proteins are typically used in animals as an indirect marker of induced thyroid and other tissue injury [27,41]. Oxidative stress associated with tissue lesions directly affects the intermediate filament proteins, glial fibrillary acidic protein (GFAP) in the hippocampus of rats [26]. Cytokeratin and vimentin are increased in atrophied thyroid gland of diabetic rats [38]. Moreover, Follicular thyroid carcinomas shared the expression of cytokeratin immunoreactivity in simple epithelial is frequently stronger and more widely distributed within each particular tumour in follicular carcinoma. Cytokeratin and vimentin coexpression are found in thyroid follicular carcinoma, endometroid adenocarcinoma and ovarian serous adenocarcinoma [42]. However, the immobilization stress could be induced disturbances in the intermediate filaments of stomach, a marked intense immunoreactivity to cytokeratin is seen at the apical part and lateral borders of the mucosal cells and to vimentin in the lamina propria and around the blood vessels [43].

Administration of vitamin E in the present study to aged rabbits showed an improvement and a noticeable reduction of the vimentin and cytokeratin immunoreactivity of the thyrocytes of thyroid gland and observed approximately like normal form. Sakamoto *et al.* [17] found that the secretion of TSH was increased in rat treated with antioxidant (melatonin) and they discussed that the secretion of TSH decreasing the vimentin synthesis. Additionally, increased in GFAP immunoreactivity in retina during diabetes or in hippocampus after induced free radicals and neurotoxicity was significantly reduced in rats by using antioxidants (vitamin E or melatonin) treatment [25, 26,28].

In conclusion, the structure of the thyroid glands changed with age and the thyrocytes turn from active cuboidal cells to inactive flattened cells and their efficiency was decreased. The intermediate filamentous vimentin and cytokeratin structural proteins have a role in this transformation progresses until the animal's elder. Vitamin E administration improved the disturbances of the thyrocytes and intermediate filaments vimentin and cytokeratin of the senescent animals. The results indicated that the elder rabbits should be used vitamin E to improve the disturbances of the thyrocytes and intermediate filaments

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