Influence of Thyroid Hormone on Thyroid Hormone Receptor β-1 Expression and Lacrimal Gland and Ocular Surface Morphology

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Purpose. Hormone diseases induce changes in the lacrimal gland (LG) and ocular surface (OS). Thyroid hormone (TH) induces cell proliferation and lipid metabolism through the activation of TH receptors. The aim of the present study was to evaluate the location and comparative expression of TH receptor β-1 (Thrb) in LG of rats with hypothyroidism and in controls and to evaluate the impact of this disease on LG and OS structure and function.

Methods. Hypothyroidism was induced in Wistar male rats by the long-term use of tiamazole. Ten weeks later corneal cells were collected for impression cytology (IC). Rats were humanely killed, and tissues were evaluated by immunoperoxidase staining and Western blot for Thrb. The content of malondialdehyde (MDA) and acetylcholine (ACh) in LG was determined by spectrophotometry ($n = 5$/group in all experiments).

Results. LG weight was significantly lower in hypothyroid rats ($P < 0.05$). Western blot analysis indicated that LGs express Thrb and that hypothyroidism induces a higher expression of this receptor. IC was significantly different and ACh was significantly lower in hypothyroid rats ($P < 0.05$).

Conclusions. Chronically reduced levels of TH lead to biochemical and structural changes and modulate the levels of Thrb in LG. These events confirm that LG is a target organ for TH and may facilitate understanding of the mechanism related to dry eye in hypothyroidism. (*Invest Ophthalmol Vis Sci.* 2007;48:3038–3042) DOI:10.1167/iovs.06-1309

Altered levels of thyroid hormone (TH) are associated with dry eye syndrome; however, the mechanisms that link TH to the lacrimal gland (LG) and to the ocular surface (OS) are unknown.[1–4] TH induces protein synthesis, cell proliferation, and lipid production, which occur through their nuclear receptors á and â (Thra and Thrb), whereas Thrb is the receptor most abundantly expressed in adult epithelial lineage cells.[5–7] In addition, TH regulates oxidative metabolism, and hypothyroidism increases oxidative stress.[6–8]

Previous studies have indicated that hypothyroidism reduces the size of harderian glands, responsible for tear secretion in rodents, and that TH may change the sexual dimorphism of these glands.[9,10] TH also influences lipid secretion and hair follicle homeostasis directly through Thrb.[5,11]

Therefore, the objectives of the present study were to investigate whether LG and OS express Thrb, to evaluate hypothyroidism-induced oxidative stress by measuring reduced glutathione (GSH), malondialdehyde (MDA), and peroxidase, and to determine neurotrophic changes by measuring acetylcholine (ACh) in LG. Moreover, we compared the experimental and control groups regarding the secretory and structural changes in LG and OS related to TH suppression.

Materials and Methods

Animal Model and Tissue Extraction

Eight-week-old male Wistar rats (*Rattus norvegicus*) were obtained from the Animal Breeding Center of the Faculty of Medicine of Ribeirão Preto (Ribeirão Preto, São Paulo, Brazil). Animals had free access to standard rodent chow and water. Hypothyroidism was induced with 500 mg/L thiamazole (Biolab Sanus, Taboão da Serra, São Paulo, Brazil) diluted in drinking water. Controls received regular water. All experimental procedures adhered to the Principles of Laboratory Animal Care (NIH publication no. 85 to 23) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the university's committee on animal experimentation.

After 10 weeks of daily use of thiamazole and after ensuring that corneal and caudal reflexes were abolished, comparative studies of the experimental and control groups were performed under ethyl ether anesthesia.

Schirmer Test and Impression Cytology

Tear secretion was measured in the right eye of each rat in both groups with the use of modified Schirmer test (Ophthalmos, São Paulo, Brazil),[12] by which a 1-mm wide, 20-mm long strip of Schirmer test paper was placed in the cul-de-sac of the eye for 5 minutes.

Corneal samples were obtained from the ocular surface 0.45-μm filter paper (Millipore, Billerica, MA) after anesthesia. Samples were collected from the same area (temporal), transferred to gelatin-coated slides, fixed with 70% ethanol, glacial acetic acid, and formalin, and stained with periodic acid-Schiff (PAS) and hematoxylin. Epithelium staging of squamous metaplasia was evaluated in a masked fashion, according to a four-stage classification scheme ranging from stage 0 (normal morphology) to stage 3 (squamous metaplasia).[13]

Tissue Collection

Next, body weight was measured, tissues were collected under ethyl ether anesthesia, and rats were humanely killed with excess anesthesia. LG and eye globes collected with their lids were fixed in optimum cutting temperature (OCT) compound (Sakura Fine Tek Inc., Torrance,

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TABLE 1. Comparison of the Structural and Biochemical Parameters of LG between Hypothyroid and Control Rats

	Control	Hypothyroid	*P*
Body weight, g	555 ± 17.8	284 ± 8.9	<0.0001
LG weight, mg	65 ± 5.8	26 ± 3.1	<0.0001
Total T4, μg/dL	3.11 ± 0.16	<1.00	—
Free T4, ng/dL	2.03 ± 0.14	<0.30	—

Data are reported as mean ± SEM.

CA) and frozen in liquid nitrogen or homogenized in a buffer of the following composition: 50 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Triton, and protease inhibitor cocktail set III (Calbiochem, San Diego, CA) with a polytron (Virsonic, Biopharma, Winchester, UK). Samples were frozen at −80°C until the time for the experimental procedure.

Thyroxine Analysis

Total and free thyroxine were measured by immunoassay in blood samples from animals of both groups and were developed by chemoluminescence (Immulite 2000; Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer's instructions.

Histologic Analysis of the Meibomian Glands

Paraffin-embedded slides containing the 10th to the 14th sections of the eye globes with lids of both groups were submitted to hematoxylin-eosin staining (five samples per animal; $n = 5$/group). Digital photographs were obtained from the lids (Eclipse E800; Nikon USA, Melville, NY), and the structure and area of the meibomian glands (MGs) were compared in a masked manner by the authors to detect differences between groups.

Assay of ACh and Peroxidase Content in LG

Acetylcholine and peroxidase were measured with the use of an ACh assay kit (Amplex Red; Molecular Probes, Eugene, OR) to compare the amounts of this key neurotransmitter and of enzymes related to oxidative stress in the LGs of both groups ($n = 5$/group), as previously described.[14] For the measurement of ACh, 0.1 mL medium and volumes of tissue homogenates, determined after protein normalization (200 μg), were spotted in duplicate onto 96-well microplates. Standard ACh curves were used in each experiment. A 0.1-mL aliquot of assay buffer (50 mM Tris-HCl, pH 7.5) containing 0.2 M reagent (Amplex Red; Molecular Probes), 2 U/mL horseradish peroxidase, 0.2 U/mL choline oxidase, and 10 U/mL acetylcholinesterase was added to each well. After incubation, absorbance was determined with a spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 530-nm emission wavelength, compared with a standard titration curve, and ACh levels were expressed in millimolar per gram of protein.

For the measurement of peroxidase, a volume of tissue homogenate obtained after protein level normalization and 0.1 mL medium were spotted in duplicate onto 96-well microplates. A 0.1-mL aliquot of assay buffer containing 0.2 M reagent (Amplex Red; Molecular Probes) and 0.2 M hydrogen peroxide was added to each well, and absorbance was determined as described after 30-minute incubation. Peroxidase activity was expressed as units of peroxidase activity per gram of protein against a standard H_2O_2 curve and was compared between groups.

Lipid Peroxidation and GSH Levels

Lipid peroxidation was determined by measuring MDA using the thiobarbituric acid test. Samples of 200 μL LG homogenates of both groups were deproteinized with 20% trichloroacetic acid, gently shaken for 30 minutes, and centrifuged at 5000*g*. The supernatant was exposed to 0.7% thiobarbituric acid, heated to 95°C for 45 minutes, and cooled. Next, absorbance was read at 530 nm (DU 640 spectrophotometer; Beckman Coulter, Fullerton, CA).[15]

GSH was determined by the reaction between LG homogenates and 5,5′-dithiobis (2-nitrobenzoic acid). After protein level normalization and incubation for 5 minutes, absorbance was read at 412 nm (DU 640 spectrophotometer; Beckman Coulter).

Evaluation of the Expression of Thrb in Lacrimal Gland

Thrb expression in whole cell lysates from LGs of rats of both groups was determined by Western blot analysis. After homogenization, protein was quantitated by the biuret dye test. Samples were treated with Laemmli buffer, and equal amounts of protein per sample (200 μg) were subjected to SDS-PAGE (10% Tris-acrylamide) in a miniature laboratory gel apparatus (Miniprotean; Bio-Rad Laboratories, Richmond, CA), in parallel with prestained protein standards and β-mercaptoethanol (Bio-Rad, Hercules, CA). Proteins were then electrotransferred from the gel to

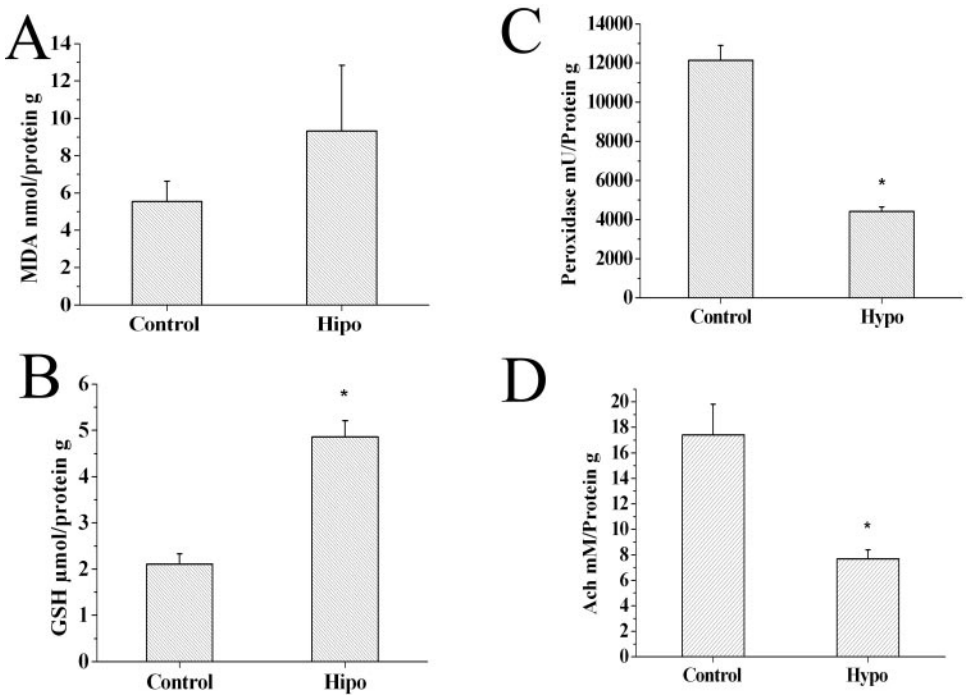

FIGURE 1. Analysis of MDA (nmol/g) (**A**), peroxidase activity (U/g) (**B**), GSH (μM/g) (**C**), and ACh (mM/g)(**D**) content in homogenates of LG of control and hypothyroid rats ($n = 5$ to 8/group). After 10 weeks of tiamazole treatment, tissues were homogenized and exposed to colorimetric reagents. Spectrophotometric analysis was performed, normalized to the protein concentration (g), and compared with standards. $^*P < 0.05$ (Mann-Whitney *U* test).

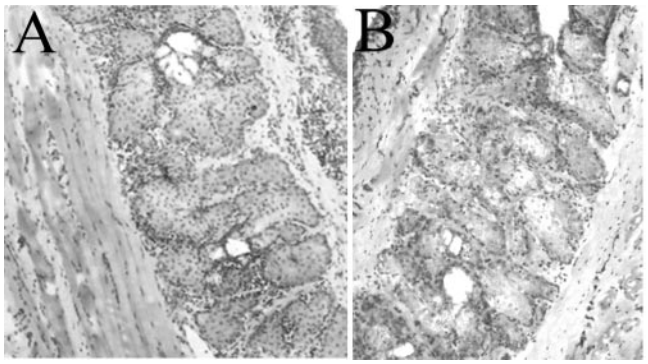

FIGURE 2. Illustrative similarity of MG in control (**A**) and hypothyroid (**B**) rats. Lids were excised, frozen fixed, and stained with hematoxylin and eosin. Digital images obtained from slices of central lids were evaluated, and the MG area was measured and compared between groups. Original magnification, ×100.

an enhanced chemiluminescence (ECL) nitrocellulose membrane (Hybond; Amersham, Buckinghamshire, UK) for 2 hours at 120 V in a miniature transfer apparatus (Miniprotean; Bio-Rad Laboratories). After blocking, the membranes were incubated overnight using rabbit polyclonal anti–Thrb or anti–tubulin (a cytoskeleton protein) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 0.4 μg/μL in a buffer containing 3% bovine serum albumin (BSA) and were washed three times as described. Blots were then incubated with immunoperoxidase and developed by DAB (Amersham, Buckinghamshire, UK). Membranes were scanned, converted to digital files, and analyzed (Scion Image Analysis Software; Scion Corp., Frederick, MD).

Immunochemical Localization of Thrb in the LG, Conjunctiva, and Cornea of Rats

Thrb expression was evaluated by immunohistochemistry in the LG, conjunctiva, and cornea of rats. OCT-blocked LG, conjunctiva, and cornea–OCT compound (Sakura Fine Tek Inc.) were cut into 6-μm sections at –20°C and transferred to poly-L-lysine–precoated glass slides (Perfecta, São Paulo, SP, Brazil).

Slides were incubated in 0.1% H_2O_2 for 5 minutes, washed in PBS (0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.3), and exposed to 2% normal goat serum solution (Vector, Burlingame, CA) for 20 minutes at 4°C. Sections were then overlaid with an aliquot of anti–Thrb rabbit polyclonal antibody (Santa Cruz Biotechnology) at a concentration of 4 μg/μL with 1% BSA (Gibco BRL, Gaithersburg, MD) in PBS. Controls included 0.1% BSA in PBS and preimmune immunoglobulin G (IgG; Sigma, St. Louis, MO).

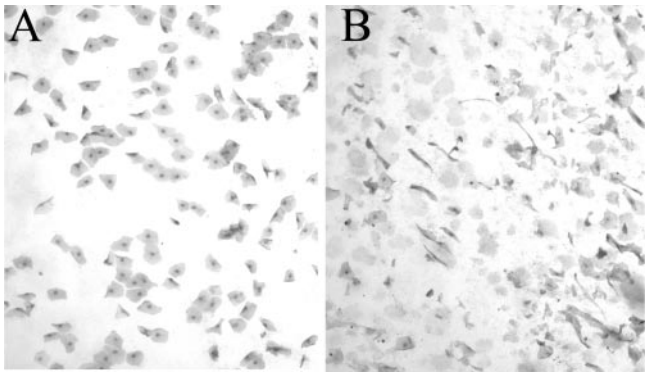

FIGURE 3. Representative microphotographs of impression cytology of the corneas from control (**A**) and hypothyroid (**B**) rats (n = 8/group). Grades 0 to 3 were assigned to each sample in a masked manner, considering the shape of the cell, size of the nuclei, and presence of mucus. Data differed significantly between hypothyroid and control rats (P = 0.002; Fisher exact test).

After incubation with primary antibody for 4 hours in a humidified chamber at 4°C, the sections were washed in PBS and incubated with a biotinylated goat anti–rabbit IgG antibody (Vector). After incubation with the second antibody, sections were again washed in PBS and incubated with an avidin–biotin complex (Vector) for 30 minutes at 25°C before they were developed with a diaminobenzidine (DAB) substrate kit (Vector).

Photographic documentation was performed with a microscope (Eclipse E 800; Nikon).

Statistical Analysis

Data are reported as mean ± SEM. Comparisons were made using the Mann–Whitney U test for continuous data (StatView software; SAS Institute, Cary, NC) and the Fisher exact test for categorical data (GraphPad 3.0 software; Prism, San Diego, CA). Densitometric values are reported as a percentage of the mean value obtained for the control group, which was defined as 100% in each experimental assay. The level of significance was set at $P < 0.05$.

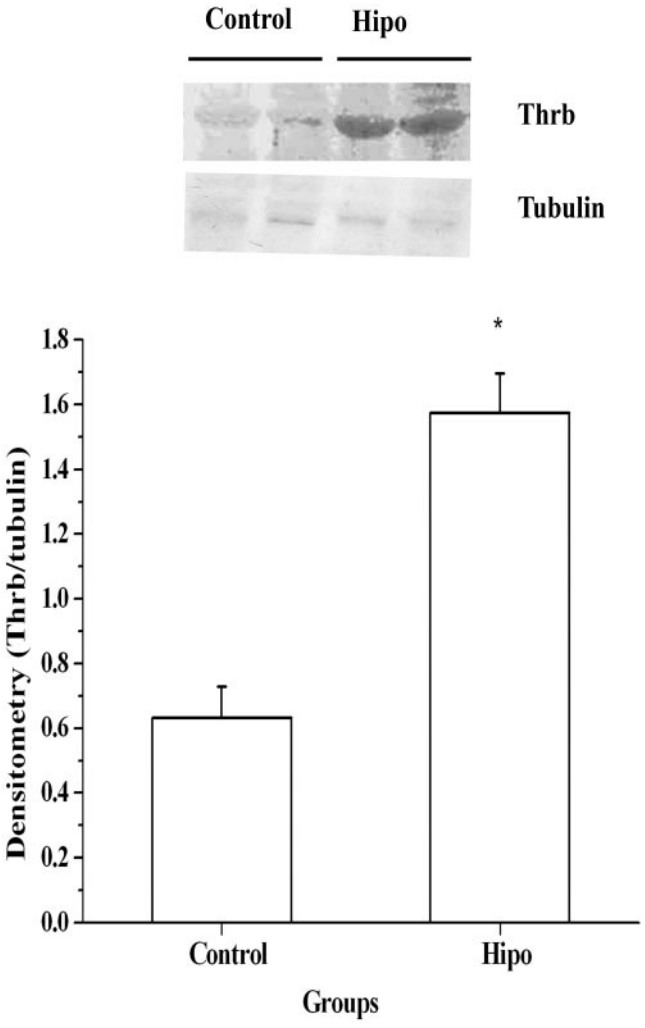

FIGURE 4. Effect of hypothyroidism on Thrb expression in LG. After 10 weeks of tiamazole treatment, LGs were excised, and extracts (n = 5 animals per group) were analyzed by Western blot using anti–Thrb and anti–tubulin antibodies. Data represent the ratio between Thrb/tubulin, reported as mean ± SEM. Values obtained for the control group were defined as 100%, and values obtained for hypothyroid animals are expressed as a percentage of this value. Results are representative of three independent experiments. Higher expression of Thrb was detected in the LGs of hypothyroid animals (*P = 0.02).

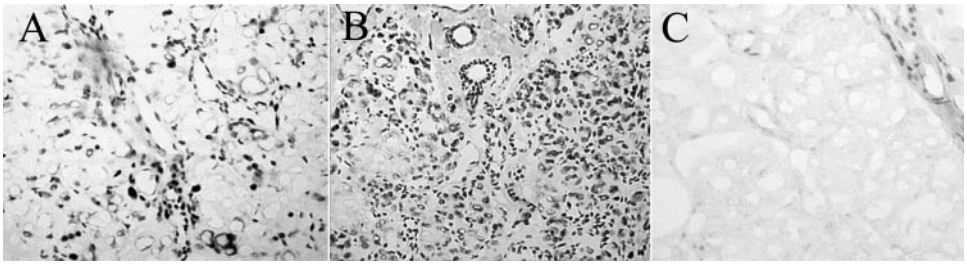

FIGURE 5. Representative immunohistochemical slide of the LG of a normal male rat. (**A**, **B**) Staining shows Thrb in the nuclei of acinar and ductal cells of control and hypothyroid LG, respectively. (**C**) Negative control; only mild background straining was present. Micrographs are representative of three independent experiments with five animals each. Original magnification, ×100.

RESULTS

Body weight, LG weight, and both forms of T4 (total and free) were lower in the hypothyroid group (Table 1). Control and hypothyroid animals had similar levels of MDA ($P = 0.7$) but significantly higher levels of GSH ($P = 0.008$), and ACh and peroxidase levels were significantly lower in the hypothyroid group ($P = 0.01$ and 0.008, respectively; Mann-Whitney U; Fig. 1).

The Schirmer test showed 8.0 ± 1.9 mm in controls and 3.2 ± 0.3 mm in the hypothyroid group ($P = 0.004$). Structural evaluation revealed no differences in MG area or structure (Fig. 2); however, IC of the cornea indicated a higher level of epithelial cell alteration but no metaplastic keratinization in the hypothyroid group ($P = 0.002$; Fisher exact test; Fig. 3).

Expression of the Thrb/tubulin protein ratio was detected in the LG of rats, and higher levels of expression were detected in extracts of hypothyroid animals compared with controls ($n = 5$ per group; $P = 0.001$). These results were confirmed in three independent experiments (Fig. 4). No significant differences were found in Thrb between normal male and female LG (data not shown).

Immunohistochemistry demonstrated Thrb expression in the nuclei of acinar and ductal cells of LG (Fig. 5). In addition, Thrb expression was observed in corneal and conjunctival epithelia, with higher density in basal layers (Figs. 6A, 6B, 7A, 7B).

DISCUSSION

As previously indicated, TH regulates the structure and function of LG, and its deficiency may predispose to OS structural changes and dry eye syndrome. The present study showed the presence of Thrb predominantly in the nuclei of epithelial cells of LG acini, cornea, and conjunctiva. These findings, in addition to higher expression of Thrb in hypothyroid LG, indicate that this tissue is a direct target organ for TH and for other hormones previously investigated.

After 10 weeks of TH deprivation, Thrb upregulation was observed in LG tissue compared with controls and was interpreted as a compensatory effect, though it was unable to reverse the changes in LG and ocular surface. Similar findings were obtained specifically with Thrb in fish retina after 4 to 6 weeks of hypothyroidism.[16] In contrast, 10 to 16 days after castration or hypophysectomy, reduced expression of androgen receptors was observed in the LG of male rats. After 4 weeks of streptozotocin-induced diabetes mellitus type 1, reduced insulin receptor (IR) activity was observed in the retina and LG.[17–19] These differences—related to the deprivation of these hormones and of their influence on their receptors—indicate variable receptor regulation, depending on tissue and hormone specificity, which may affect the hormone signaling, tissue function, and time course of disease manifestation.

Confirming previous epidemiologic, clinical, and experimental data regarding hypothyroidism and dry eye syndrome, our study revealed that hypothyroidism has an impact on tear secretion and on the epithelial cells of the cornea because it reduces the Schirmer test values and alters the impression cytology findings.[4]

The hypothesis that autoimmune damage leads to LG and thyroid gland dysfunction has been raised in clinical studies. However, a synergic effect combining tear film dysfunction resulting from inflammation and lower TH stimulation of exocrine glands and epithelial tissues of the ocular surface is also appropriate because, in the present study and in previous studies, TH suppression was the adopted model, and no lymphocyte infiltration was observed in LG, ocular surface, or MG.[2,3,9]

A mechanistic explanation tested here to explain the LG changes was the impaired inhibition of reactive oxygen species. In our study, significantly higher GSH levels and a trend to increased MDA and peroxidase levels suggested that oxidative stress may play a role in dry eye related to hypothyroidism, as observed in other organs.[8] MDA levels varied widely in the LG of the hypothyroid group in different assays, which was not observed in other tissues or in the LG of other animal models. Although our data may confirm our hypothesis, the wide variations of these parameters, mostly MDA, also suggest a limitation of the assays performed in the present model.

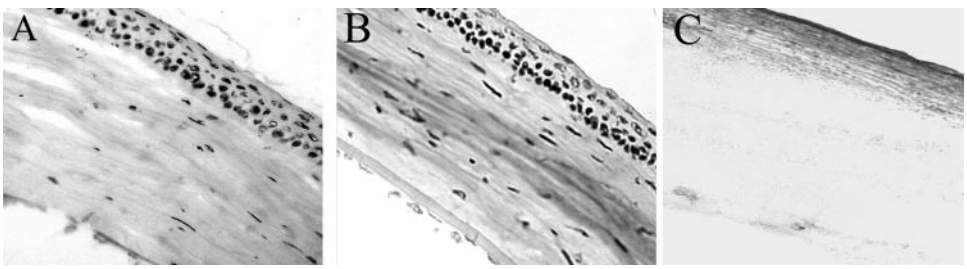

FIGURE 6. Representative immunohistochemical slides demonstrating Thrb staining in the corneas of normal rats (**A**), hypothyroid rats (**B**), and (**C**) negative control samples. Selected micrographs are representative of three independent experiments with five animals each. Original magnification, ×400.

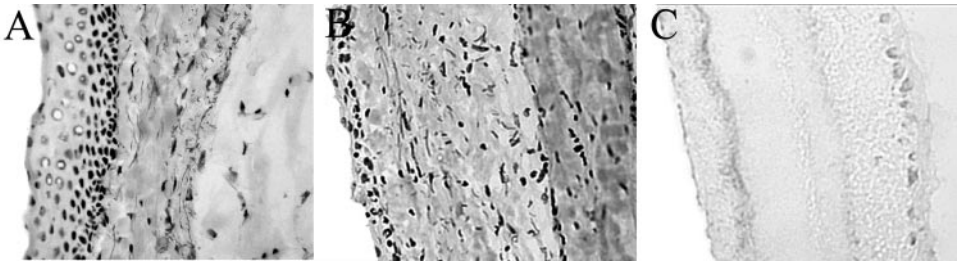

FIGURE 7. Representative immunohistochemical slides demonstrating Thrb staining in the conjunctiva of normal rats (**A**), hypothyroid rats (**B**), and (**C**) negative control samples. Selected micrographs are representative of three independent experiments with five animals each. Original magnification, ×400.

Given the well-known impact of hypothyroidism in the early phases of neural and retinal development,[16,20] we investigated the possibility of neurotrophic impairment of LG. Reduced levels of ACh in the LG of the hypothyroid group indicate that reduced neuronal inputs may contribute to the reduced tear secretion occurring in this condition.

Sexual dimorphism in LG related to TH suppression has been observed in previous studies[9]; however, thyroxine was unable to reverse the reduction on LG weight or tear volume caused by the reduction in testosterone levels by orchiectomy.[21] We compared the expression of Thrb in LG of normal age-matched males and females and did not observe a sex difference, in disagreement with other sex-related differences in hormone receptors in LG (e.g., androgen and insulin).[22,23] Further studies are necessary to determine whether and how female sex or sex hormones and hypothyroidism are related factors in dry eye syndrome.

Moreover, the present data support the idea of an investigation of ocular discomfort in subclinical thyroid disease, another condition more prevalent in women, defined by higher levels of TSH and normal levels of TH, with a characteristic clinical presentation.[24] The possible association of subclinical hypothyroidism and dry eye syndrome may offer an explanation for several undetermined cases of dry eye in the population.

In conclusion, our study indicates that LG has specific thyroid hormone receptor and that hypothyroidism impairs LG function and may be directly driven. Future studies that conclusively prove an association between hypothyroidism and dry eye would contribute to the understanding of ocular discomfort in hypothyroid patients and may lead to future research in more specific treatments for these patients.

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