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ORIGINAL ARTICLE

Influence of insulin treatment on the lacrimal gland and ocular surface of diabetic rats

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Abstract Previous studies have observed changes in the lacrimal gland and ocular surface related to diabetes mellitus and related it to insulin resistance or insufficiency and oxidative damage. The aim of this study was to evaluate whether insulin treatment inhibits those changes. Diabetes was induced in male Wistar rats with a single intravenous injection of streptozotocin and a subgroup was treated with insulin. After 5 and 10 weeks, the three groups ($n = 5$–10/group/experimental procedure) were compared for biochemical, functional, and histological parameters. After 5 weeks, changes in morphology and increased numbers of lipofucsin-like inclusions were observed in lacrimal glands of diabetic but not insulin-treated rats. After 5 weeks, malonaldehyde and total peroxidase activity were significantly higher in diabetic rats, but similar to control in insulin-treated diabetic rats ($P = 0.03$, $P = 0.02$, respectively). Our data indicate that diabetes induces histological alterations in lacrimal gland and suggests that hyperglycemia-related oxidative stress may participate in diabetic dry eye syndrome. Prevention by insulin replacement suggests direct hormone action and/or benefit by early sub optimal metabolic control.

Keywords Diabetes mellitus · Dry eye · Insulin · Lacrimal gland · Oxidative damage

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Introduction

Previous studies have suggested that chronic hyperglycemia, oxidative stress, nerve alterations, and disturbance in insulin action may play an important role in the development of ocular surface (OS) alterations and lacrimal gland (LG) impairment in diabetes mellitus (DM) [1–5]. Moreover, experimental work from our laboratory and cross-sectional clinical studies have confirmed clinical evidence that DM is associated with those findings and progress to dry eye syndrome [1, 6–8].

Although the detailed mechanisms are unknown, our group have previously demonstrated that insulin receptor is expressed in LG and OS, that its activation and other steps of the insulin signaling pathways are impaired in the LGs of animals with DM, and that insulin is secreted in the tear film [6, 9, 10]. Therefore, a potential role of the impairment of this pleotropic hormone in the mechanism of the disease is considerable [11, 12].

Reactive oxygen species (ROS) are related to major diabetic complications in various tissues [13]. To monitor these processes, oxidative stress markers as malonaldehyde (MDA) and total peroxidase activity have been used to evaluate the exocrine gland tissue response to ROS [1, 14, 15].

In LG tissue, the peroxidase activity is markedly represented by lactoperoxidase, an enzyme that participate in unspecific immune defense of the ocular surface [16], and

has been used for a long time as a biomarker of lacrimal gland function [17–19]. Other members of peroxidase superfamily, as glutathione peroxidases are responsible for ROS scavenging and therefore their activity has been used as a marker of oxidative stress [20, 21]. Peroxidase activity or expression in exocrine tissues was markedly increased by systemic hormone suppression, not just insulin as in models of DM type 1, but also suppression of sex hormones by gonadectomy [1, 15, 22, 23].

Our hypothesis is that hyperglycemia mediates cellular damage, initially inducing ROS, and that LG may present morphological and functional changes in addition to modified expression of protective mechanisms against markers of oxidative stress.

The aims of the present study were to further investigate the structural, functional, and biochemical mechanisms of diabetic LG and to evaluate the effect of insulin replacement in reducing those effects.

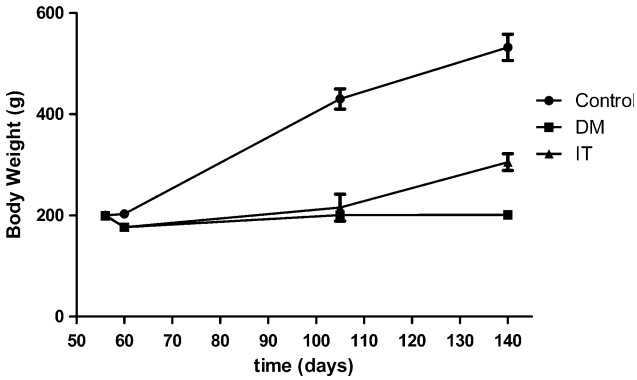

Fig. 1 Body weight progression in rats after 8 weeks of life. The body weight was measured right before streptozotocin injection, 4 days later, and after 5 and 10 weeks. Control (*filled circle*), Diabetic (DM) (*filled square*) and Diabetic insulin-treated (IT) (*filled triangle*) (mean ± SEM)

Results

As previously reported [1], body weight and LG weight were significantly lower than control in the diabetic group and in the diabetic insulin-treated group by the fifth week ($P = 0.009$ for body weight and $P = 0.016$ for LG weight) (Table 1, Fig. 1). Insulin treatment improved body weight ($P = 0.0016$), but not LG weight, after 10 weeks. LG weight was not drastically reduced in the two diabetic groups compared to control ($P = 0.046$) (Table 1, Fig. 1).

Malonaldehyde levels in LG were similar in all groups at the fifth week of treatment, but increased significantly in diabetic rats compared to the other two groups at the tenth week ($P = 0.03$) (Fig. 2a, b). Total peroxidase activity in LG were significantly higher in diabetic rats at the fifth week ($P = 0.02$), but with progression to the tenth week they became similar in all groups ($P = 0.096$) (Fig. 2c, d).

Structural evaluation revealed no differences in IC of corneal epithelia between groups at 5 or 10 weeks (Fisher test, $P > 0.05$) (Fig. 3).

At the fifth week, tear secretion measured by the Schirmer test was 2.5 ± 1.1 mm in diabetic rats, 5.3 ± 0.3 mm in diabetic insulin-treated rats, and 8.0 ± 0.6 mm in controls ($P = 0.03$). At the 10th week, tear secretion was 2.2 ± 0.2 mm in diabetic rats, 1.4 ± 0.2 mm in diabetic insulin-treated rats, and 5.4 ± 0.6 mm in controls ($P = .0.009$). Thus, tear secretion levels, which were not severely reduced in diabetic insulin-treated at the fifth week, became as low as those of untreated diabetic rats after 10 weeks of disease.

Histological observation of diabetic LG 2 days after streptozotocin injection revealed unchanged distribution of epithelial and connective tissue, absence of lymphocyte infiltration, and similar acinar and ductal size and distribution (hematoxylin/eosin staining), and normal nuclear number and distribution (DAPI) (data not shown), as recently observed by our group [24].

Table 1 Comparison of the structural and biochemical parameters of LG between control (C), diabetic (DM) and diabetic insulin-treated rats (IT) after 5 and 10 weeks

	Weeks	C	DM	IT
Body weight (g)	5	430.0 ± 20.0	200.0 ± 28.9	217.5 ± 31.2
	10	522.0 ± 53.7	190.0 ± 10.0*	307.0 ± 17.0
LG weight (mg)	5	140.6 ± 7.0*	61.3 ± 6.1	72.0 ± 2.7
	10	186.1 ± 10.8*	139.1 ± 26.9	136.9 ± 14.3
Serum glucose (mg/dl)	5	96.4 ± 3.7*	435.7 ± 17.2	431.3 ± 32.2
	10	69.5 ± 2.0*	503.6 ± 21.2	390.9 ± 30.7

Data are reported as mean ± SEM

* $P < 0.05$ (Kruskal–Wallis test)

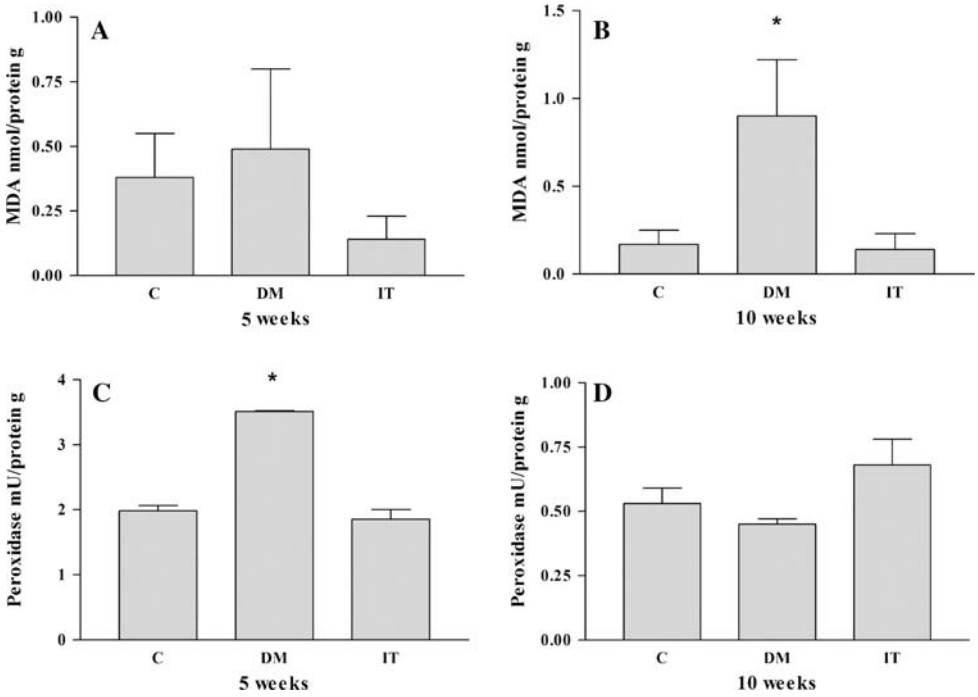

Fig. 2 Lacrimal gland MDA levels at 5 weeks (**a**), and 10 weeks (**b**), peroxidase activity at 5 weeks (**c**), and 10 weeks (**d**), in control (C), diabetic (DM), and diabetic insulin-treated (IT) rats ($n = 5$–10/group). After 5 and 10 weeks, tissues were homogenized and exposed to colorimetric reagents and read by spectrophotometry. Absorbance data were compared to standards and MDA expressed as a ratio of lacrimal gland protein concentration (nmol/g) and peroxidase activity as a ratio of lacrimal gland weight (U/g). * $P < 0.05$ (Kruskal–Wallis test)

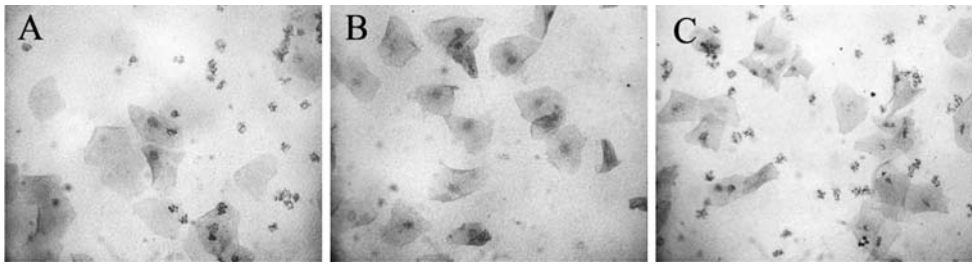

Fig. 3 Representative microphotographs of impression cytology of the cornea from control (**a**), diabetic (**b**), and diabetic insulin-treated rats (**c**) ($n = 5$–10/group) 10 weeks after streptozotocin injection. Grades 0–3 were assigned to each sample in a masked manner, considering cell, shape, nucleus size, and presence of mucus. Data did not differ significantly among the three groups ($P > 0.05$; Fisher test)

After 5 weeks of diabetes, histological comparison between control, diabetic, and insulin-treated rats revealed changes in cytoplasmic content in hematoxylin-stained diabetic LG slides, suggestive of steatosis; although similar DAPI-stained nuclear structures, and higher lipofuchsin-like autufluorescense in diabetic LG (Fig. 4).

After 10 weeks, comparison revealed similar distribution of epithelial tissue, absence of inflammatory infiltration, but some disorganization of acinar structure and reduced staining of acinar cell nuclei in diabetic LG and more evident cytoplasmic deposits, suggestive of steatosis compared to controls and diabetic insulin-treated rats (Fig. 5a, d, g). However, nucleus staining with DAPI suggested that nuclear size and distribution were unchanged and acini were hidden in altered cytoplasm content (Fig. 5b, e, h). In addition, lipofuchsin-like autofluorescent granules were more expressed in acini and ductal cells of diabetic rats than in the other groups (Fig. 5c, f, i).

Discussion

Diabetes mellitus is a growing problem in Western cultures, where exogenous insulin, although far from ideal, is a key treatment since the first half of the last century, mostly for DM type 1 [25, 26].

Although, it is widely accepted that most complications are direct conditioned by hyperglycemia and consequent microvascular damage, which could be minimized by insulin treatment, other mechanisms should be considered [26, 27]. For instance, studies indicate that this powerful anabolic hormone is necessary for LG and other tissue proliferation and eye globe growth [28–30].

Insulin is a ubiquitous hormone, responsible for several intracellular and tissue events, mediated by it broad signaling mechanism [6, 27]. Its absence after pancreatic beta cells destruction by streptozotocin leads to dehydratation following glycosuria and a catabolic phenomenon that

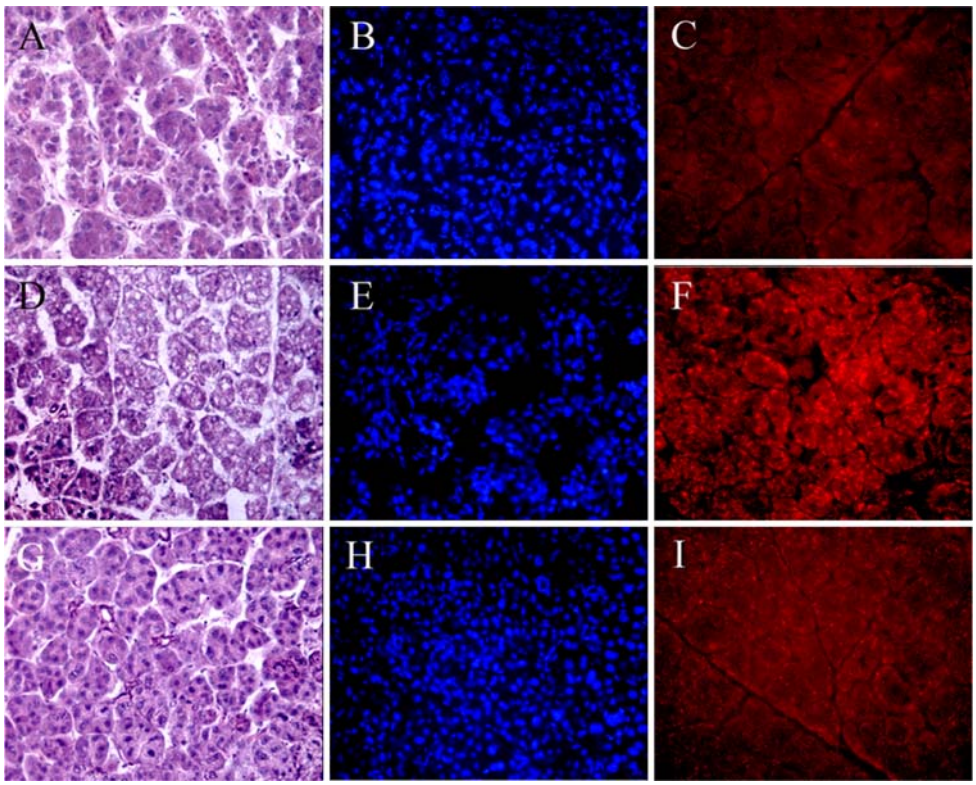

Fig. 4 Histological comparison of LG of control (**a**, **b**, **c**), diabetic (**d**, **e**, **f**), and diabetic insulin-treated rats (**g**, **h**, **i**) after 5 weeks of diabetes induction. The material was stained with HE (**a**, **d**, **g**) or DAPI (**b**, **e**, **h**) and the autofluorescence of lipofuchsin-like inclusions is shown (**c**, **f**, **i**) (magnification 400×)

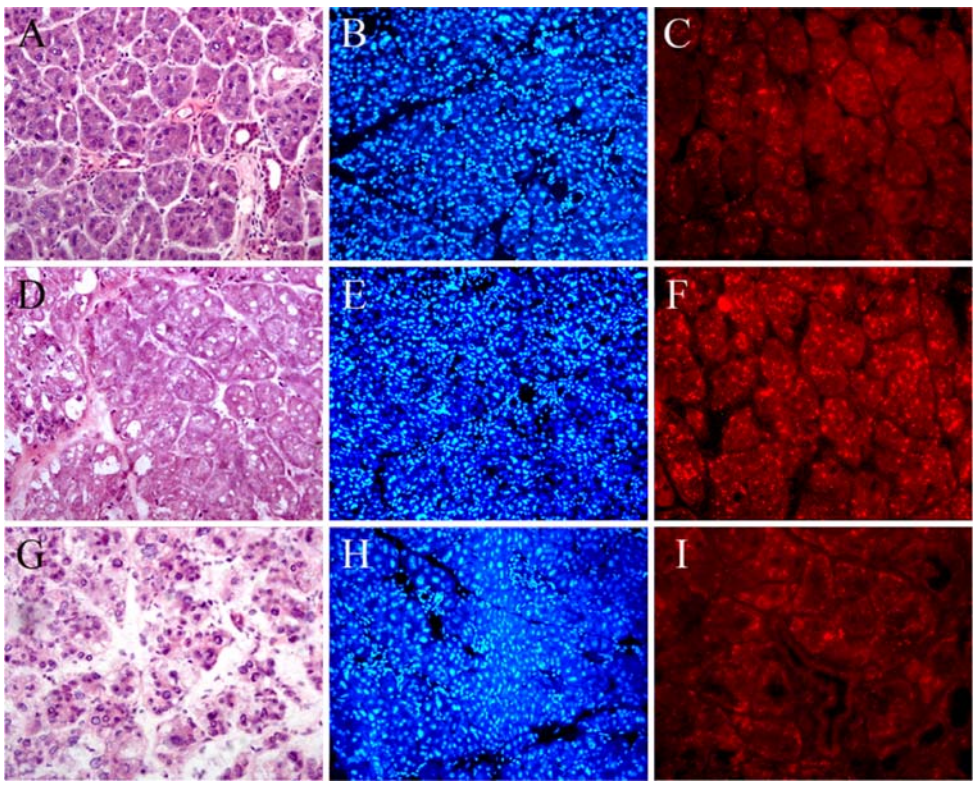

Fig. 5 Histological comparison of LG of control (**a**, **b**, **c**), diabetic (**d**, **e**, **f**), and diabetic insulin-treated rats (**g**, **h**, **i**) after 10 weeks. The material was stained with HE (**a**, **d**, **g**) or DAPI (**b**, **e**, **h**) and the autofluorescence of lipofuchsin-like inclusions is shown (**c**, **f**, **i**) (magnification 400×)

leads to tissue mass reduction. Those events explain the striking differences in body weight among the three groups along the experimental period. Therefore, dehydratation and metabolic impairment may contribute to the difference in lacrimal gland weight and tears secretion in those groups.

In humans, DM type 1 dry eye syndrome is more frequent in patients with proliferative diabetic retinopathy (PDR) [31]. Later treatment initiation with insulin accelerates PDR in non-insulin-dependent diabetes mellitus, which is interpreted as an anabolic effect of the hormone in this tissue [32]. Insulin also has proliferative effects on epithelial cells. In an evolutionary perspective, it justifies insulin production by exocrine glands, which embed the hormone in body tissues, as lacrimal gland do by the tear film on the ocular surface. It explains, in part, the beneficial effects of treatment with autologous serum tears or insulin in severe dry eye [10, 33–35].

Previous studies by our group have shown that insulin signaling is impaired in salivary and lacrimal glands since the fourth week of DM in rats and that LG becomes an extra pancreatic source of insulin in a rat model of DM type 1, at least for 4–7 weeks [6, 14].

As observed before, the streptozotocin diabetic model did not show ocular surface damage after 10 weeks, neither in direct exam nor in impression cytology. Histopathology of diabetic LG acinar cells observed here, and in part, prevented by insulin treatment differs from the observations of cornea impression cytology, but agrees with previous biochemical and functional comparisons [1, 36, 37]. The limitations of the conclusions are related to the broad and heterogeneous characteristics of the findings that did not allow quantitative analysis and measurement of intra group variability, as observed in almost all other parameters in DM. However, the histological HE staining findings clearly distinguish the diabetic group from the others and from previous observations in aging models of dry eye, although autofluorescence are comparable between aging and DM [19, 38].

Moreover, despite intact neural connections in LG tissue, after 10 weeks of disease, secretory function is reduced, the NF-kB signaling pathway is up-regulated and markers of inflammation and oxidative stress are present [1].

A recent study demonstrated that the rat model of type 2 DM present at 13–15 weeks of life reduced tear secretion and induced slower corneal wound healing [4], as also observed in diabetic humans. Another report showed that, although several growth factors induced faster keratocyte proliferation in vitro, insulin had a lesser impact in changing the phenotype of these cells [39]. Taken together, these data indicate that not just the insulin or its role in metabolic control, but also its signaling pathways directed at mitosis and tissue proliferation are relevant to cornea homeostasis.

The present study supports the thesis that diabetic damage is dependent not only on hyperglycemia but also hormone deficiency, since partial insulin replacement restored some structural and biochemical parameters of the LG, as observed by histological analysis and evaluation of oxidative damage. Furthermore, the anabolic effect of insulin and its role on tissue proliferation was indicated by previous work showing that the absence of insulin receptors in vascular endothelial cells prevents retinal neovascularization induced by hyperglycemia [40].

The present study also suggest that, at a certain point during the course of the disease, hyperglycemic, vascular, oxidative, and inflammatory damage among other system impairments contributes to the severity and acceleration of the disease. These findings support treatments that might be beneficial to minimize diabetic complications such as antioxidant supplementation and/or anti-inflammatory medications [5, 41, 42].

In this respect and in agreement with the present findings, it was demonstrated that partial insulin replacement in streptozotocin diabetic rats protects brain mitochondria against oxidative damage [43]. Moreover, mild insulin replacement has been used in this model to preserve basal metabolism even in studies of treatment with other medications [44].

It was documented that the acinar histology of the rat parotid salivary glands is altered after 8 weeks of streptozotocin-induced diabetes in a pattern similar to that observed here, suggesting cytoplasmic lipid accumulation, correlating with impaired amylase secretion in response to noradrenalin stimulation and fatty acid content reduction compared to controls [45]. Non-obese diabetic mice also present progressive lipid accumulation in LG acinar cells [46].

After 2 days of streptozotocin injection, the histology of the LG of diabetic rat was not damaged, which agrees with recent works of our group of tissues compared after 2 days and 4 weeks [14, 24]. However, by 5 weeks, mild acinar cells alterations were observed and correlated lipofucsin deposits. Those findings were more evident by 10 weeks, which suggest not a toxic effect of streptozotocin but a progressive lesion, induced by DM and partially minimized by mild insulin replacement.

Curiously, the weight of LG of diabetic rats, significantly lower than controls by 5 weeks, was similar by 10 weeks and relatively higher, compared to body weight. A possible explanation is the gain of mass by lipid cytoplasmic deposition as suspected by histological findings in this and previous work with rat parotid gland [45].

As shown here in lacrimal glands, lipid peroxidation and peroxidase activity were also influenced by diabetes mellitus in salivary glands. Submandibular and parotid glands presented different response and large intra group variation. MDA, reduced, and oxidized glutathione were higher in submandibular glands, and catalase and glutathione peroxidase were higher in parotid glands of diabetic, compared to control rats after 4 weeks of disease [15]. The

present work revealed an initial increase in total peroxidase activity and MDA in diabetic lacrimal gland, prevented by insulin treatment, as a response to higher oxidative/hyperglycemic damage in this group at 5 weeks. The lipid peroxidation (MDA) persisted higher after 10 weeks, but total peroxidase activity reduced to the levels of the other groups (despite of a trend to maintain higher in diabetic insulin-treated) probably because of a failure of the anti-oxidant mechanism. It is possible that higher doses of insulin replacement and complementary therapies would promote better anti-oxidant results [47].

Considering the structural and functional impairment of diabetic lacrimal gland in the present and previous studies, the unexpected result of higher total peroxidase activity in lacrimal glands of diabetic rats at 5 weeks of disease rises the question whether lactoperoxidase alone would be responsible for that and/or increased levels of ROS induced a temporary higher expression of anti-oxidants as other members of peroxidase superfamily in this tissue [1, 6]. To clarify that, a comprehensive study of specific peroxidase expression and activity in lacrimal gland in normal conditions and diseases is necessary, with careful attention to non-specific and cross-reaction between different enzymes [48, 49].

In summary, the present study confirms that DM induces structural and functional changes in rat LG, followed by changes in oxidative parameters. It was also observed that functional and biochemical parameters change in parallel to LG morphology, although it is possible that the method for assessing corneal epithelial changes (i.e., impression cytology) may be less sensitive to the histological and biochemical measures of the lacrimal gland status.

In conclusion, the present study provides evidence that DM contributes to LG dysfunction and that low doses of systemic insulin may be able to reverse some of these changes. Moreover, the findings indicate that using total peroxidase activity, as a marker of function and/or ROS scavenger in exocrine glands must be taken carefully due to double function (i.e., secretory and anti-oxidant) of peroxidase enzymes in those tissues.

Materials and methods

Eight-week-old male Wistar rats (*Rattus norvegicus*) were obtained from the Faculty of Medicine of Ribeirão Preto Animal Breeding Centre, Ribeirão Preto, SP, Brazil. Animals were given free access to standard rodent chow and water. Food was withdrawn 12 h before the experiments and diabetes was induced with a single dose of streptozotocin (Sigma, St. Louis, MO, USA), 60 mg/kg body weight, diluted in 1 ml 0.01 M citrate buffer administered through the caudal vein. Controls were injected with citrate buffer alone.

Two days later, diabetic status was verified with a glucose meter test (Accu-check, Roche Diagnostica Brasil Ltda., São Paulo, SP, Brazil) in blood obtained from the caudal vein of 12 h fasted rats. Integrity of lacrimal tissue was compared by histology as described below, between diabetic and control rats after euthanasia ($n = 2$/group). Fasting hyperglycemia over 200 mg/dl was considered to indicate the presence of diabetes and on the fourth day part of the diabetic group initiated human recombinant insulin treatment with a dose of 1 IU administered every other day by subcutaneous injection (Humulin, Lilly, Mexico). This doses was not sufficient to maintain adequate glycemic control, but was enough to avoid body weight loss and growth retardation [44].

All experimental procedures adhered to the "Principles of laboratory animal care" (NIH publication no. 85-23) and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the University Committee of Animal Experimentation.

Comparative studies of the three groups, i.e., control (C), diabetic (DM) and diabetic with insulin treatment (IT), were performed 5 and 10 weeks later.

The animals were weighed, the modified Schirmer test was performed as previously described [50] and epithelial cells were collected under intraperitoneal anesthesia consisting of a combination of ketamine (5 mg/100 g b.w.) (União Química Faramacêutica S.A, Embu-Guaçu, SP, Brazil) and xylazine (2 mg/100 g b.w.) (Laboratorio Callier S.A., Barcelona, Spain). After sample collection, rats were euthanized with excess anesthesia.

Histology

Lacrimal Gland were collected, weighed, embedded in OCT compound (Sakura Fine Tek Inc., Torrance, CA, USA), and frozen in dry ice. Blocks from the three groups ($n = 5$–10/group) were cut into 6 μm sections at −20°C and transferred to poly-L-lysine pre-coated glass slides (Perfecta, São Paulo, SP, Brazil). The material was then stained with hematoxylin/eosin for histological examination or with DAPI (Vector, Burlingame, CA, USA) for the visualization of nuclei and of cell position in relation to the adjacent cells. Non-stained slides were evaluated with a UMWG filter and excitation filter BP510-550 nm to detect and compare the autofluorescence of lipofuchsin-like inclusions, as previously described [19]. Photographic documentation was done using an Olympus BX40 light microscope (Olympus Corporation, Tokyo, Japan) and a digital camera (Olympus Q-color 5) at 100× and 400× magnifications.

Impression cytology

Corneal samples were obtained from the ocular surface of the three groups ($n = 5$/group) using 0.45 μm filter paper ((Millipore Co, Billerica, MA, USA) after anesthesia. The samples were collected from the same area (temporal) and transferred to gelatin-coated slides, fixed with 70% ethanol glacial acetic acid and formalin, and stained with periodic acid-Schiff (PAS) and hematoxylin. Squamous metaplasia of epithelial cells was staged in a masked fashion according to a four-stage classification scheme from 0 (normal morphology) to 3 (squamous metaplasia) [51, 52]. The images were recorded with the light microscope described above.

Lipid peroxidation and total peroxidase activity in LG

After collection, LG were 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 with a polytron instrument (Virsonic, Biopharma, Winchester, UK). Protein was quantitated by the biuret dye test and samples were frozen until the time for the experimental procedure.

Lipid peroxidation as a consequence of ROS accumulation was determined by measuring the presence of MDA using the thiobarbituric acid test [53]. Samples of 200 μl of LG homogenates from the three groups were deproteinized with 20% trichloroacetic acid, gently shaken for 30 min, and centrifuged at 5,000*g*, and the supernatant was exposed to 0.7% thiobarbituric acid and heated to 95°C for 45 min. After cooling, absorbance was read at 530 nm in a Spectra Max 250 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Total peroxidase activity were measured using an colorimetric assay kit (Amplex Red; Molecular Probes, Eugene, OR, USA) in order to compare the amounts of total peroxidase activity in the LG of the three groups ($n = 5$/group), as previously described [54]. Volume of LG tissue homogenate to obtain 40 μg of protein was spotted in duplicate onto 96-well microplates. H_2O_2 (0.2 M), 0.1 ml medium and 0.1-ml aliquot of assay buffer containing 0.2 M reagent were added to each experimental and control samples. Aliquots were collected at 0, 5, 10, 20, 30, 40, and 60 min and absorbance was obtained and compared to a standard H_2O_2 titration curve with known amount of horseradish peroxidase. Peroxidase activity was expressed as units/ml g^{-1} and the time point used for comparison was 30 min, when the steady state of activity was obtained for all groups.

Statistical analysis

Data are expressed as means ± SEM. Comparisons were made using the Kruskal–Wallis test for continuous data and the Fisher test for categorical data (Graphpad 3.0 software, Prism, San Diego, CA). The level of significance used was $P < 0.05$.

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