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ORIGINAL PAPER

Aspirin prevents diabetic oxidative changes in rat lacrimal gland structure and function

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Abstract The aim of this study is to evaluate whether aspirin reduces Diabetis Mellitus (DM) oxidative damage in the lacrimal gland (LG), and ocular surface (OS). Ten weeks after streptozotocin induced DM and aspirin treatment, LG and OS of rats were compared for tear secretion, hidtology, peroxidase activity, and expression of uncoupling proteins (UCPs). DM reduction of tear secretion was prevented by aspirin ($P < 0.01$). Alterations of LG morphology and increased numbers of lipofucsin-like inclusions were observed in diabetic but not in aspirin-treated diabetic rats. Peroxidase activity levels were higher and UCP-2 was reduced in DM LG but not in aspirin treated ($P = 0.0025$ and $P < 0.05$, respectively). The findings prevented by aspirin indicate a direct inhibitory effect on oxidative pathways in LG and their inflammatory consequences, preserving the LG structure and function against hyperglycemia and/or insulin deficiency damage.

Keywords Anti inflammatory drugs · Anti-oxidant capacity · Lacrimal gland · Uncoupled protein · Diabetes mellitus · Oxidative stress

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Introduction

Previous experimental studies have confirmed clinical evidence that diabetes mellitus (DM) is associated with lacrimal gland (LG) and ocular surface (OS) dysfunctions related to dry eye syndrome [1–4].

Although the detailed mechanisms are unknown, previous studies have suggested that chronic hyperglycemia, oxidative stress, nerve alterations, and disturbance in insulin action may play important roles in the development of such alterations [2, 5–8]. In addition, dry eye induced by ocular surface desiccation was recently correlated with ocular surface damage and oxidative stress [9].

We have previously demonstrated that there is insulin receptor expression in LG and OS and its activation and downstream signaling pathways are impaired in the lacrimal glands of animals with DM, and that insulin is secreted into the tear film [1, 10, 11].

It is known that lacrimal gland secretion contains peroxidase activity, due to lactoperoxidase, an enzyme member of the peroxidase-cyclooxigenase super family that reduce hydrogen peroxide producing hypohalous acid and hypothiocianate, and therefore, participate in unspecific immune defense of the ocular surface [12]. Other members of the peroxidase superfamily are responsible for ROS scavenging and tissue protection, and therefore, they can take part in the process of hyperglycemic oxidative damage in lacrimal gland [13, 14].

Peroxidase activity in the lacrimal glands of old rats are reduced; however, in the streptozotocin model of diabetic rats, after 10 weeks of disease, there is a trend of higher levels compared to age-matched controls [2, 15]. Increases in reactive oxygen species (ROS) formation are related to major diabetic complications in other tissues [16]. Therefore, this apparent paradoxal difference in peroxidase

activity in two animal models of impaired secretion may be possible by the action of anti-oxidant enzymes, which may involve two or more members of the peroxidase superfamily other than lactoperoxidase.

Of interest, peroxidase activity in exocrine tissues was markedly increased by systemic hormone suppression, not only insulin as in the models of diabetes mellitus type 1, but also suppression of sex hormones by gonadectomy [2, 17–19].

ROS are produced in a process initiated by hyperglycemia, whereby molecular oxygen used in glucose metabolism is converted in part to free radicals. In order to monitor these processes, antioxidant enzyme levels that scavenge ROS and/or metabolites of lipids, proteins, or DNA damage by oxidation are adopted as oxidative stress markers [20, 21]. Among them, malonaldehyde (MDA), a marker of lipid peroxidation and peroxidase activity, has been used to evaluate the antioxidant defense competence [18, 22].

Hyperglycemia leads to oxidative stress and to the formation of advanced glycation end products (AGE) and activates an intracellular cascade, whereby IkB kinase (IKK) phosphorylates IkB, freeing NF-kB to translocate to the nucleus. NF-kB promotes activation of a large number of genes, including pro inflammatory cytokines and other products that, among other actions, further activate NF-kB [2, 16].

The evidences that hyperglycemia leads to mitochondrial dysfunction and activation of oxidative stress pathways called the attention to mechanisms intended to revert this phenomena. Among them are mitochondrial uncoupling proteins (UCPs), a family of proteins responsible for energy metabolism that would be able to prevent mitochondrial ROS formation in different tissues [23–25]. However, the presence of UCP isoforms in LG and their potential local role with regard to oxidative stress and dry eye syndrome have not been investigated.

After oral administration, aspirin is secreted in tears in a dose and plasma concentration dependent manner [26]. It has long been known that aspirin (acetylsalicylic acid) improves some metabolic parameters in DM and mechanistic explanations have been recently reported [27, 28]. (Salicylate reduces the expression of oxidants and inflammatory mediators, and tissue inhibiting NF-κB signaling pathway [27–30]). On the other hand, it is known that fatty acids stimulate UCP expression in hepatocytes, but aspirin inhibits eicosapentanoic fatty acid mediated UCP-2 up regulation [31].

Our hypothesis was that hyperglycemia mediates cellular damage, initially inducing ROS and activating the NF-κB signaling cascade and that the LG tissue may respond with up-regulation of UCPs and other protective mechanisms against oxidative stress. This hypothesis also predicts that aspirin treatment may prevent the LG and OS damage induced by DM.

Thus, the objectives of the present study were to further investigate the signaling events and oxidative stress markers of diabetic LG, and to evaluate the effect of aspirin treatment on mitigating the effects of this disease.

Materials and methods

Eight-week-old male Wistar rats (*Rattus norvegicus*) were obtained from the Animal Breeding Centre of the Faculty of Medicine of Ribeirão Preto, 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.

Three days later, diabetic status was verified with a glucose meter test (Accu-check, Roche Diagnostica Brasil Ltda., São Paulo, SP, Brazil) on blood obtained from the caudal vein of 12 h-fasted rats. Fasting hyperglycaemia over 200 mg/dl was considered to indicate the presence of diabetes and on the fourth day part the diabetic group began receiving aspirin diluted in drinking water (500 mg/l). This procedure did not affect the daily volume of drinking water observed throughout the experimental period and lead to an average consumption of 50 mg per kg of body weight/day.

All experimental procedures adhered to the *Guidelines on the Practice of Ethical Committees in Medical Research* issued by the Royal College of Physicians of London and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the University Committee on Animal Experimentation.

Studies on the three groups [i.e., control (C), diabetic (DM), and diabetic receiving aspirin treatment (DM + AAS)] were performed 5 and 10 weeks later to compare body weight by modified Schirmer test as previously described [32]. Epithelial cells were collected from the ocular surface of the three groups for impression cytology analysis under intraperitoneal anaesthesia with a combination of ketamine (5 mg/100 g body weight) (União Química Faramacêutica S.A, Embu-Guaçu, SP, Brazil) and xylazine (2 mg/100 g body weight) (Laboratório Callier S.A., Barcelona, Spain). After sample collection, rats were euthanized with excess anaesthesia.

Histology

LG were collected and weighed, embedded in OCT compound (Sakura Fine Tek Inc., Torrance, CA, USA), and frozen in liquid nitrogen.

OCT-blocked LG 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 tissue was stained with hematoxylin/eosin for histological observation or with DAPI (Vector, Burlingame, CA, USA) for the visualization of the nuclei and cell distribution. Non-stained slides were evaluated with a blue filter (UMWB2) and 460–490 nm excitation light to detect and compare lipofucsin-like inclusions as previously described [33]. Photographic documentation was done using an Olympus light microscope BX41 and an Olympus Q- Color 5 digital camera (Olympus Corp, Japan) at 100 and 400× magnification.

Schirmer test and impression cytology

Tear secretion was measured in the right eye of rats of all the groups by modified Schirmer test as previously described [32].

For impression cytology, corneal epithelia samples were obtained from the ocular surface of the three groups ($n = 5$/group) using a 0.45-μm filter paper ((Millipore Co, Billerica, MA, USA) after general anesthesia described above. 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 haematoxylin. Epithelial staging of squamous metaplasia was evaluated in a masked fashion and epithelial cells were classified into four stages: stage 0 (for normal cell number, morphology, and mucous staining), stage 1 (lower cell number and mucous staining), stage 2 (lower cell number, reduced size of nuclei, and square shape of cells), and stage 3 (squamous metaplasia, whereby lower cell number, higher cytoplasmic volume, and pycnotic or absent nuclei) [34, 35].

Lipid peroxidation

Lipid peroxidation as a consequence of ROS accumulation was determined by measuring the presence of MDA by the thiobarbituric acid test [36]. 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*. The supernatant was exposed to 0.7% thiobarbituric acid and heated to 95°C for 45 min and, after cooling, absorbance was read at 530 nm in a Spectra Max 250 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Peroxidase content of LG

Total peroxidase activity was measured using an assay kit (Amplex Red; Molecular Probes, Eugene, OR, USA) compare the amounts of this key enzyme related to oxidative stress in the LG of all groups (i.e., DM, DM + AAS) ($n = 5$/group), as previously described [37].

Volume of lacrimal gland tissue homogenate to obtain 40 μg of protein were 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 sample. Aliquots were collected at 0, 5, 10, 20, 30, 40, and 60 min and absorbance was obtained. This was 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 point of activity was obtained.

Evaluation of the expression of *UCP 1, 2, and 3 and IKKα/β* in lacrimal glands

In order to evaluate the impact of DM and the response to aspirin, the expression of UCP 1, 2, and 3 and IKK *α/β* in whole cell lysates from LG of rats of the three groups was determined by Western blot. After homogenization, protein was measured 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 Bio-Rad miniature lab gel apparatus (Miniprotean, Bio-Rad Laboratories, Richmond, CA, USA), in parallel with pre-stained protein standards and ß-mercaptoethanol (Bio-Rad, Hercules, CA, USA). Proteins were then electro-transferred from the gel to a Hybond ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) for 2 h at 120 V in a Bio-Rad miniature transfer apparatus (Miniprotean). After blocking, the membranes were incubated overnight using goat polyclonal anti-UCP-1, 2, and 3, rabbit polyclonal anti IKK *α/β* antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a concentration of 0.4 μg/μl in a buffer containing 3% bovine serum albumin (BSA), and then washed three times as described above. The blots were then incubated with immunoperoxidase and developed with DAB (Amersham, Buckinghamshire, UK). Membranes were scanned, converted to digital files, and analyzed with the Scion Image Analysis Software (Scion Corp, Frederick, MD, USA).

Statistical analysis

Data are expressed as mean ± 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). Densitometric values are expressed 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 used was $P < 0.05$.

Results

Body weight and LG weight were lower in the diabetic group and were not significantly affected by aspirin treatment after 5 and 10 weeks, despite a trend of improvement, specially in body weight by 5 weeks and LG weight by 10 weeks. In the same way, blood glucose levels were significantly lower in DM + AAS by 5 weeks ($P < 0.006$), but achieved similar levels of untreated DM by 10 weeks (Table 1).

After 10 weeks, peroxidase levels in LG were higher in diabetic rats but similar in controls and DM + AAS ($P = 0.002$), although MDA was not affected after 10 weeks of this treatment ($P > 0.05$) (Table 2).

Structural evaluation revealed no differences in the impression cytology (IC) of the cornea of these groups at 5 or 10 weeks (Data not shown, Fisher test, $P > 0.05$) (Figs. 1 and 2).

Tear secretion measured by the Schirmer test was reduced in diabetic rats at 5 weeks (2.5 ± 1.1 mm) as compared to controls (8.3 ± 0.8 mm) and aspirin-treated animals (8.8 ± 1.1 mm) ($P = 0.001$). This pattern of changes persisted at 10 weeks in diabetic rats (2.8 ± 0.8 mm) compared to controls (11.8 ± 1.5 mm) and aspirin-treated animals (7.4 ± 1.8 mm) ($P = 0.01$).

In a previous study, we observed that after 4 weeks of DM, LG histology revealed a similar aspect to controls, which indicate the absence of damage by streptozotocin [22]. In order to ensure that LG tissue damage was not disrupted by streptozotocin injection and remodeled afterward, histological evaluation of stretozotocin-induced DM was performed. The observation of diabetic LG, 2 days after streptozotocin injection, revealed unchanged distribution of epithelial and connective tissues, absence of lymphocyte infiltration, similar acinar and ductal size, and distribution (hematoxylin/eosin staining), and normal nuclei number, and distribution (DAPI) (Fig. 3).

After 5 weeks, acinar and ductal structures of LG were similar, and nuclei number and morphology were preserved, in comparison with controls and DM + AAS (Fig. 4).

However, after 10 weeks of diabetes, histological LG comparison among control, diabetic, and DM + AAS rats revealed similar distribution of epithelial tissues, but some disorganization of acinar structure and reduced staining of the nuclei of acinar cells (Fig. 5a, d, g). In order to verify whether nuclei were damaged, nuclear staining with DAPI was performed and its comparison indicated that the size and distribution of nuclei were unchanged, and hence, cell nuclei were hidden by altered cytoplasm content based on HE staining (Fig. 5b, e, h). In addition, autofluorescence revealed that lipofucsin granules were more expressed in the acini and ductal cells of diabetic rats than in the other groups (Fig. 5c, f, i).

After 10 weeks of diabetes, UCP-1 and UCP-2, UCP-3, and IKK α/β are expressed in LG. Although UCP-2 and -3 were significantly reduced in diabetic LG compared to control ($P = 0.009$ and 0.04, respectively, Mann–Whitney -U test), and UCP-1 and IKK α/β were similarly expressed in all groups ($P = 0.13$ and 0.48, respectively), when compared to the diabetic aspirin-treated group, only UCP-2 was lower in the diabetic group and recovered with aspirin treatment ($P = 0.05$, Kruskal-Wallis test) (Fig. 6).

Table 1 Physiological parameters after 5 and 10 weeks of streptozotocin-induced diabetes among control (C), diabetic (DM), and aspirin-treated diabetic (DM + AAS) rats

	C	DM	DM + AAS
Body weight (g)			
5 weeks	556 ± 57.3	274 ± 21.8*	488 ± 33.1
10 weeks	638 ± 42.9[§]	278 ± 18.6	252 ± 43.3
LG weight (mg)			
5 weeks	137.4 ± 12.1[†]	82 ± 3.8	71.4 ± 5.2
10 weeks	186 ± 10.8	136.9 ± 14.3	169.2 ± 7.15
Serum glucose (mg/dl)			
5 weeks	107.8 ± 11.2	427.4 ± 16.5[‡]	90.8 ± 13.5
10 weeks	88.6 ± 20.0[#]	399 ± 10.7	307.2 ± 71.6

* $P = 0.007$; § $P = 0.008$, † $P = 0.0055$; ‡ $P = 0.0055$; # $P = 0.002$ (Kruskal-Wallis)

Data are expressed as mean ± SEM

Table 2 Comparison of the oxidative stress biomarkers in LG after 10 weeks of streptozotocin-induced diabetes among control (C), diabetic (DM), and aspirin-treated diabetic (DM + AAS) rats

	C	DM	DM + AAS
Peroxidase (μg/ml)	1.70 ± 0.23	2.36 ± 0.05*	1.06 ± 0.17
MDA (nmol/g)	0.25 ± 0.05	0.25 ± 0.113	0.32 ± 0.05

* $P = 0.002$ (Kruskal-Wallis)

Data are reported as mean ± SEM. MDA was evaluated by spectrophotometric analysis, compared to standards, and normalized to protein concentration (g)

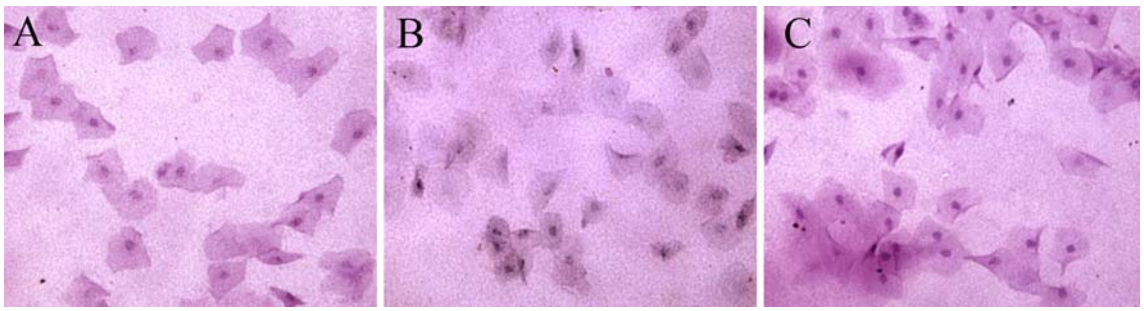

Fig. 1 Representative microphotographs of impression cytology of the cornea from control **a**, diabetic **b**, and aspirin-treated diabetic rats **c** ($n = 5$–10/group) 5 weeks after streptozotocin injection. Grades 0 to 3 were assigned to each sample in a masked manner, considering the shape of the cell, the size of the nuclei, and the presence of mucus. Data did not differ significantly between groups ($P > 0.05$; Exact Fisher test)

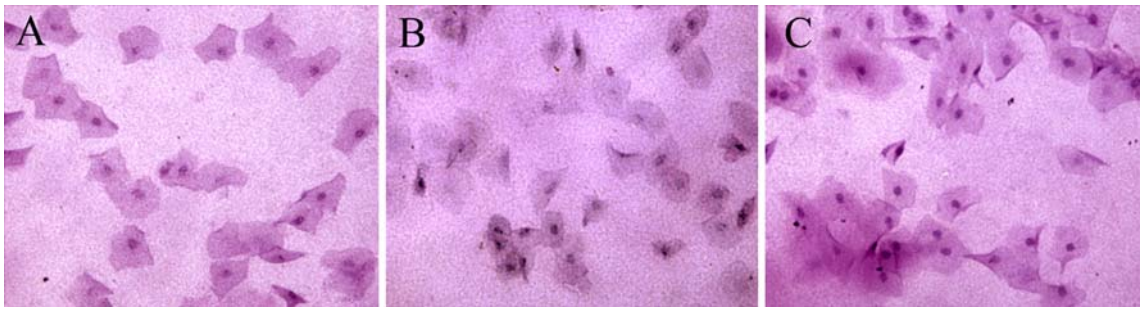

Fig. 2 Representative microphotographs of impression cytology of the cornea from control **a**, diabetic **b,** and aspirin-treated diabetic rats **c** ($n = 5$–10/group) 10 weeks after streptozotocin injection. Grades 0 to 3 were assigned to each sample in a masked manner, considering the shape of the cell, the size of the nuclei, and the presence of mucus. Data did not differ significantly between groups ($P > 0.05$; Exact Fisher test)

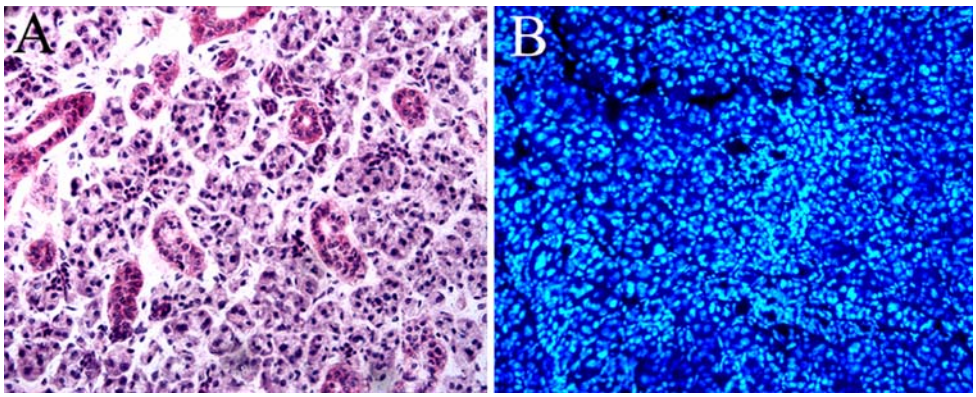

Fig. 3 LG histology 2 days after streptozotocin injection showing **a** preserved acinar and ductal structures (HE) and **b** nucleus number, shape, and distribution (DAPI). (Magnification 400×)

Discussion

Different models of DM in rats have shown LG and ocular surface alterations. These animals are useful for translational studies (i.e., potential clinical and pathological correlations and therapeutic approaches) since they present with reduced tear secretion and impaired corneal epithelial wound healing [2, 7].

A remarkable finding of the present work that opens a potential therapeutical approach is the aspirin prevention of tearing reduction in diabetic dry eye.

In order to further understand the aforementioned inflammatory and ROS-induced oxidant mechanisms, in the present study, we compared in DM rat the effects of aspirin administration on LG and ocular surface appearance. Our data confirmed that DM reduces the weight of LG and tear secretion by 5 weeks and directly affects the morphology of LG after 10 weeks. The unexpected increase in diabetic LG weight by 10 weeks was not explored in detail but it appears to correlate with our histological finding of anomalous material in cytoplasm and previous description of lipid material accumulation after 8 weeks of disease [38].

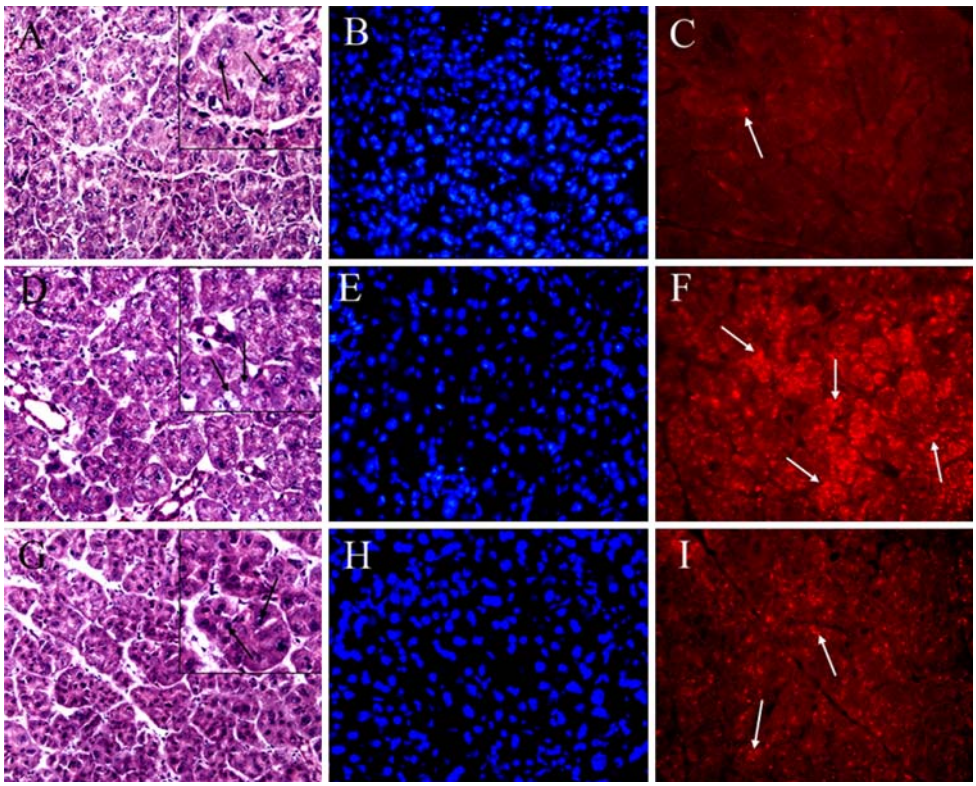

Fig. 4 Histological comparison of LG of control (**a, b, c**), diabetic (**d, e, f**), and aspirin-treated diabetic (**g, h, i**) animals stained with HE (**a, d, g**), with DAPI (**b, e, h**), and autofluorescence of lipofucsin-like inclusions (white arrows) (**c, f, i**) (Magnification 400×) 5 weeks after streptozotocin injection. Insets (**a, d, g**) high magnification images of acinar cells stained with HE, showing acinar cells and their nuclei (black arrows)

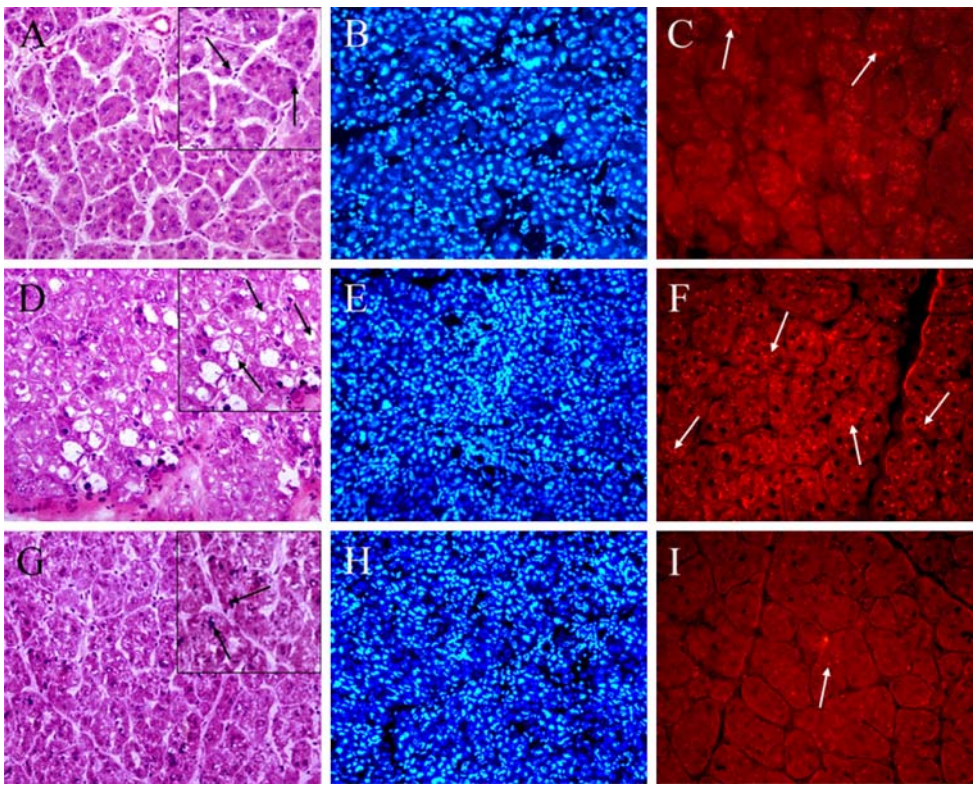

Fig. 5 Histological comparison of LG of control (**a, b, c**), diabetic (**d, e, f**), and aspirin-treated diabetic (**g, h, i**) animals stained with HE (**a, d, g**), DAPI (**b, e, h**), and autofluorescence of lipofucsin-like inclusions (white arrows) (**c, f, i**) (Magnification 400×) 10 weeks after streptozotocin injection. Insets (**a, d, g**) high magnification images of acinar cells stained with HE, showing acinar cells and their nuclei (black arrows)

In addition to reduced tear secretion, diabetic rats show impaired metabolic signaling in LG and reduced mitotic activity in OS, which may help to explain the clinical changes observed in diabetic patients, whose have been benefited by short-term use of anti-oxidants [1, 4, 7, 38–40]. The present findings indicate that using total

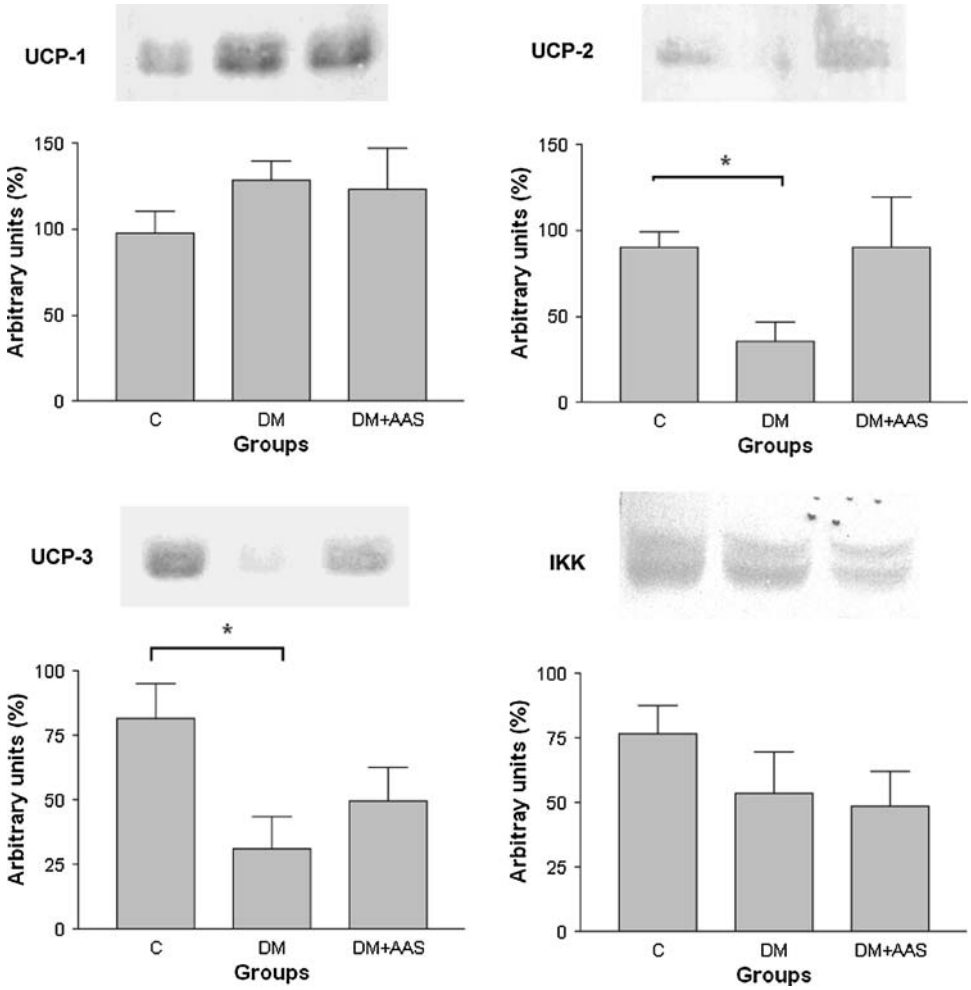

Fig. 6 Effect of DM on UCP-1, 2, and 3, and IKK α/β expression in LG. After 10 weeks of DM, LG were excised and extracts ($n = 5$ animals per group) were analyzed by Western blot using anti-UCP-1, -2, -3 and IKK α/β antibodies. Results are representative of three independent experiments. Membranes were scanned, converted to digital files, and analyzed with the Scion Image Analysis Software

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 [14, 18, 41, 42].

In contrast to our recent observations related to hypothyroidism in rats [43], after 10 weeks of DM, there were no significant corneal epithelia histological alterations. Previous studies demonstrated that corneal nerve damage in those animal models are apparent after 16 weeks of disease; therefore, ocular surface epithelial damage may be worsened at this time [5]. Evaluation of goblet cell density, collected with a biopsy, as recently recommended, would be an interesting possibility for further clarification of the effects of DM on rat OS [44].

Chronic oxidative stress induced by persistent hyperglycaemia is a causative mechanism of the lacrimal complications, as observed in other DM tissues [45]. The role of salicylates and aspirin in particular was identified and the pro-inflammatory NF-κB signaling pathway was implicated in the mechanism of action due to inhibition of IKK-β activity [30]. Moreover, salicylates may contribute to blocking the increase in ROS through a mitochondrial mechanism, since, after 10 weeks, in the DM + AAS (i.e., aspirin treated DM group), the levels of lipofucsin and peroxidase were lower and UCP-2 and −3 levels were higher than in untreated DM.

We demonstrated here for the first time the presence of UCPs in LG tissue. Their role in protecting against oxidative damage has been indicating, but continues to require clarification [46]. In our study, lower levels of UCP 2 and 3 in diabetic LG, associated with changes in functional parameters and oxidative stress markers, indicating that hyperglycaemic cellular damage induced by ROS caused not only overexpression of peroxidase but also altered expression of some UCP isoforms. Although it is not clear whether the increase in UCP induced by aspirin was an anti-oxidant effect, it indicates a distinct response observed in hepatocytes, where ex vivo studies demonstrated that aspirin inhibited fatty acids and prostaglandins induced over expression of UCP 2 [31].

In agreement with the above-mentioned mechanism of action of aspirin on NF-κB signaling, a recent study found in retina tissues of streptozotocin diabetic rats that salicylates are able to inhibit NF-κB activation, apoptotic events,

and pro-inflammatory protein expression [27, 30]. Those findings were not confirmed in our study, since IKKα/β, a precursor of NF-κB, was mildly but not significantly reduced in DM LG, and it was not reverted by aspirin treatment.

In conclusion, the present study advanced the understanding of the physiopathology and anti-oxidant reaction that correlate DM with dry eye, which may have variability among individuals and time-length of observation. However, it unequivocally confirms previous findings of diabetic induced damage to LG, reinforces the perception that this damage is mediated by oxidants mediators, and indicates that aspirin therapy can reduce tissue damage and functional impairment.

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