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Intravitreal Administration of Lysine-Acetylsalicylate Could be an Effective Approach to Preserve Retinal Vessels and to Inhibit Leukostasis in Experimental Diabetic Retinopathy

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Abstract

Leukostasis and the loss of retinal capillaries associated with inflammation play a central role in the development of diabetic retinopathy. The oral administration of acetylsalicylic acid (ASA) at high doses has been associated with therapeutic benefits in experimental diabetic retinopathy. Although, its utility in humans remains controversial. We explore the ability of intravitreal injection of lysine acetylsalicylate to preserve retinal vessels and to inhibit the presence of leukocytes in the retinal ganglion cell (GCL) layer and the outer plexiform layer (OPL) using an experimental model of diabetic retinopathy induced with streptozotocin.

The diabetic animals were assigned to two groups, one of them received 2 doses of intravitreal lysine acetylsalicylate (lysine-group) while the other group received 2 intravitreal injections of saline (control-group). The two injections in each group were administered with an interval of 4 weeks.

The immunohistochemical labeling revealed a greater number of intact vessels in the lysine-group in the GCL and in the OPL ($P<0.001$), both in the central and peripheral retina. There was also a lower leukocyte count in the lysine-group compared to the control-group in the GCL of the central retina ($P<0.001$). The correlation study between both variables was significant in the GCL of the central and peripheral retina as well as in the OPL of the central retina.

These results suggest a protective effect of the drug on the capillary network of the retina and a reduction of leukostasis. Intravitreal administration could maximize the action of salicylate and minimize its systemic adverse effects.

Keywords: Intravitreal injection; Diabetic retinopathy; Salicylates; Lysine; Drug therapy

Introduction

Sustained hyperglycaemia, leukocyte stasis (leukostasis), degeneration and loss of retinal capillaries are early pathogenic events described in animal models of diabetic retinopathy [1-3]. The hyperglycaemia induces endothelial effects such as increase of nitric oxide and reactive oxygen species; gene activation and synthesis of mRNAs involves on inflammation and apoptosis including nuclear factor-κB (NF-κB) [4-6]. Inflammatory cytokines, such as prostaglandins and interleukins, attract leukocytes into the retinal vessels which at the same time produces more free radicals from oxygen and cytokines, causing an increase of vascular permeability, endothelial apoptosis and capillary loss [7,8].

Animal models of diabetic retinopathy have permitted a much precise knowledge of pro-inflammatory pathways and their relation with major retinal lesions of this disease. Previous research in animals, especially in rodents, has demonstrated an increase of retinal inflammation markers, such as cytokines, leukostasis and endothelial cells loss among others, 1 to 6 months after diabetes induction [9]. Rodent models have been frequently employed to assay experimental drugs to modulate gene expression or molecular interactions [10,11].

The benefits of oral acetylsalicylic acid (AAS) have been studied several times with different doses in both animals and humans. In humans, the Early Treatment Diabetic Retinopathy Study (ETDRS), [12] performed in patients with mild to severe non-proliferative or early proliferative diabetic retinopathy, concluded that 650 mg/day of oral AAS has no clinically important beneficial effects on the progression of retinopathy. However, the Dipyridamole Aspirin Microangiopathy of Diabetes Study, with 3 years of follow up, showed a 50% less progression of microaneurysms in early diabetic retinopathy patients with an oral aspirin dose of 990 mg/day [13]. In animals, the administration of a dose equivalent to 1.4 g/day for human during 5 years in diabetic-induced dogs, prevented retinal haemorrhages and acellular capillaries formation [14]. Another comparative study between oral clopidrogrel, sorbinil and aspirin in diabetic rats found

that only aspirin protected retinal vessels and avoided endothelial cell apoptosis, suggesting that these benefits were not related with its antiplatelet effect. Authors warned that daily high doses of oral aspirin would be required to achieve this results in humans, increasing the rate serious side effects [15,16].

The purpose of this study is to evaluate the capability of lysine acetylsalicylate, injected directly into the vitreous cavity, to preserve retinal vessels and reducing the presence of leukocytes in different retinal layers in an experimental model of diabetic retinopathy. The work has been performed in streptozotocin (STZ) induced diabetic rats, which is a widespread experimental model to study diabetic retinopathy. Histologically, diabetic rats show signs of retinal microangiopathy comparable to humans, including vessels degeneration and leukostasis [17-19]. These signs appear even in early stages of diabetic retinopathy [19,20], especially in albino rats (e.g. Wistar, Sprague-Dawley), the first sings of vascular alteration begin only a few days after the induction of diabetes [21]. Several studies indicate the existence of selective damage to the ganglion cell layer, characterized by loss of the capillary network and neuronal apoptosis, due to the activation of caspases related to inflammation [22,23]. Similar damage has been reported in the outer plexiform layer only 1 month after diabetic induction using the streptozotocin model in rats [24]. Based on this knowledge, we focus our work on the specific study of the ganglion cell layer (GCL) and the outer plexiform layer (OPL).

Research Design and Methods

Animal model

The animal and bioethics experimentation committee of the Miguel Hernandez University approved all procedures detailed at this study. Based on previous investigations that tested different intravitreal therapies in induced diabetic rats [21], male Wistar adult rats were assigned into two groups. Both received an intraperitoneal injection of STZ (70 mg/kg body weight) dissolved in citrate buffer (pH 4.5). The development of diabetes (blood glucose 250 mg/dl) was verified 2 days after STZ injection. All rats were maintained with free access to water and food in a room with a 12 h light and dark cycle, body weight was recorded three times a week.

Subcutaneous NPH insulin was administered as needed to prevent weight loss without preventing hyperglycaemia. Plasma glucose levels were measured with a self-monitoring blood glucose system (Dexter Z II; Bayer Medical Co. Ltd. Leverkusen, Germany). Since the induction, on the first day of the fourth and eighth week both groups received different interventions. One of the groups received a total of 2 intravitreal injections of lysine acetylsalicylate (lysine-group) while the other group received a total of 2 intravitreal injections of saline (control-group).

Intravitreal injection of lysine-acetylsalicylate

Animals were anesthetized with inhaled isoflurane at 5% for induction and 2% for maintenance. Pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. For additional topical anaesthesia 0.1% tetracain and 0.4% oxibuprocain (AlconCusi SA, Barcelona, Spain) was used. To prevent infection, a 0.3% of ofloxacin ophthalmic solution (Allergan Pharmaceuticals, Ireland) was applied to the ocular surface immediately before the injection and for seven days after. Based on evidences of injectable intravitreous volumes in rats [25,26] we prepared a 25 µg lysine acetylsalicylate solution in a volume of 5 µl (5 mg/ml) for a supposed vitreous volume of 57 µl [27]. The solution was injected twice into the vitreous in the lysine-group eyes (week 4 and week 8 after STZ-induction) using a micro injector (Hamilton Co., Reno, NV) under a surgical microscope. A 32 gauge needle was used to make a punch incision 1 mm posterior to the temporal limbus, and the micro injector needle was then inserted through the incision, approximately 1.5 mm deep, angled toward the optic nerve. Eyes with injection damaged lenses or retinas were excluded from the study. On the eyes of diabetic control-group a 5 µl of saline was injected using the same technique and protocol.

Histology and immunohistochemistry

Retinas from both groups of rats were fixed in 10% buffered formalin for 2 days and processed for histological analysis. Integrin is a group of adhesion receptors expressed in many cells such as leukocytes and endothelial cells. In case of leukocytes, they shares an identical subunit (CD18; 95 kDa) and is distinguished immunologically by distinct subunits of 170 kDa (CDlla; LFA-l), 160 kDa (CDllb; Mac-1) and 150 kDa (CDllc; p150) in man [28]. We detected retinal leukocytes by using monoclonal antibodies (mAb) against CD18 of the rats (anti-rat CD18) as described by Tamatani et al. [29]. Based on other studies, in order to obtain a differentiated staining of the vessels that make up the capillary network of the GCL and the OPL, we used a specific marker for the adhesion molecule ICAM-1 (anti-rat ICAM-1) present in the endothelial cells [30,31].

Immunohistochemistry

Retinal formalin-fixed paraffin sections (20 µm) were immunostained. Retinal sections were washed with a 7.3 pH phosphate-buffered saline solution containing Triton-X100 (0.25%) (PBS-TX). Sections were included during 10 min in hydrogen peroxide (3%) and washed again with PBS-TX before to be included in a PBS-TX with 1% bovine serum albumin (BSA) for 1 h. The monoclonal primary antibodies were mouse anti-rat CD18 (CD-18; 1:250; BD PharmingenTM) and mouse anti-rat ICAM-1 (ICAM-1; 1:250; BD PharmingenTM), which was in contact to the tissues during 15 h. Secondary biotinated anti-mouse IgG (1:200; BD PharmingenTM) was applied for 1 h.

Then, sections were washed once with PBS and twice with 0.1 M hydroxymethyl aminomethane hydrochloride (TRIS hydrochloride). Sections were included then in a solution containing 0.025% diaminobencidine, 0.015% hydrogen peroxide, and 0.1 M TRIS hydrochloride. Finally, tissues were dehydrated and permanent mounted with Entellan® (Merck KGaA, Darmstadt, Germany).

Count of leukocytes and retinal vessels

Leukocytes and preserved vessels were identified and counted at 20x magnification by masked observers. Counts were performed in 2 layers of the retina: retinal ganglion cells layer (GCL) in the inner retina and outer plexiform layer (OPL) in the outer retina. Central and peripheral retina was analysed separately. For viewing and counting we used the Nikon Eclipse 80i microscope (Nikon Instruments Europe B.V., Amstelveen, Netherlands) equipped with the Digital Eclipse DXM1200C 12.6 megapixels camera (Nikon Metrology, Leuven, Belgium) assisted by the ACT-1 capture program (version 2.63) (Nikon Corp., Minato, Tokyo, Japan). Pictures were processed using ImageJ software (version 1.48; NIH, Bethesda, MD, USA).

Statistical analysis

Data are given as mean ± SD for continuous variables, median and range for non-continuous variables such as cells and vessels counting. Body weight and blood glucose between groups were compared using the Student-t test. Counting results from each group were compared in between with the nonparametric Mann-Whitney U test. Differences were considered statistically significant at $P<0.05$. To study the statistical relationship and dependence between the two variables we applied the Spearman correlation test and the coefficient of determination respectively, the results were given using de Spearman's coefficient with the P value and the coefficient of determination expressed as r2 value. The software IBM SPSS Statistics Base 22.0 was used for the analysis.

Results

Physiologic data

Table 1 shows no statistically significant differences among groups in any of the physiological variables measured. The blood glucose level was higher than 250 mg/dl during all the study in the two groups.

	Control-Group (n=10)	Lysine-Group (n=10)
Blood glucose (mg/dl)	292.7 ± 14.88	295.4 ± 12.24
Weight (g)	317.9 ± 22	298.7 ±17.4
P Value	P=0.517	P=0.123

Table 1: Blood glucose and body weight for each group (Data are mean ± SD).

Quantification of preserved vessels in central and peripheral retina

A total of 216 flat-mounted sections of retina were processed, 108 with anti-CD18 staining (56 from lysine-group and 52 from control-group) and the same total number of samples for the anti-ICAM peroxidase positive vessels (56 from lysine-group and 52 from control-group). Four tissue pictures of a 1.1 mm diameter were taken from each retinal paraffin section (2 corresponding to central retina and 2 corresponding to peripheral retina) to be processed afterwards on the analysis program. Administration of intravitreal lysine acetylsalicylate was associated with a higher presence of preserved retinal vessels in each tissue section in the central retina (Figure 1) for both retinal GCL ($P<0.001$) and OPL ($P<0.001$). At the peripheral retina (Figure 2) we found less total preserved vessels compared with the central retina in all sections, but comparison between groups showed more preserved retinal vessels in the lysine-group for retinal GCL ($P<0.001$) as well as OPL ($P<0.001$) (Table 2).

Quantification of leukocytes in central and peripheral retina

We evaluated the number of leukocytes marked with anti-CD18 mAb accumulated in the two studied layers using the same image acquisition and analysis protocol employed for vessels counting. At the central retina (Figure 1) we obtained a statistically significant increase of marked leukocytes at both layers, retinal GCL and OPL, in the lysine-group compared with the control-group ($P<0.001$). For peripheral (Figure 2) retina we found more CD18 marked leukocytes in the lysine-group at OPL ($P<0.001$) but there was no statistical difference at the retinal GCL ($P=0.972$) between groups (Table 2).

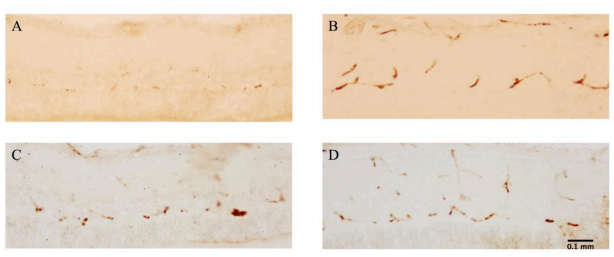

Figure 1: Central retinal sections (20x). Representative photographs of CD18-peroxidase positive leukocytes defined as a brown spot at the retinal GCL and the OPL in the control-group (A) and in the lysine-group (C). Vessels with an intact tubular morphology and cellular content (ICAM-1 peroxidase) in the control-group (B) and in the lysine-group (D).

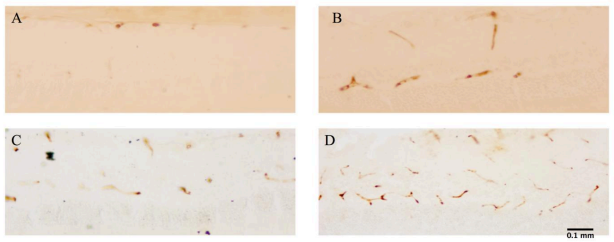

Figure 2: Peripheral retinal sections (20x). Representative photographs of CD18-peroxidase positive leukocytes defined as a brown spot at the retinal GCL and the OPL in the control-group (A) and in the lysine-group (C). Vessels with an intact tubular morphology and cellular content (ICAM-1 peroxidase) in the control-group (B) and in the lysine-group (D).

		Lysine-group (56 sections)	Control-group (52 sections)	P value
Preserved retinal vessels				
Central Retina				
GCL		5.0 (2.8-6.0)*	2.0 (1.0-3.0)	<0.001
OPL		9.0 (7.0-11.0)	3.0 (2.0-4.0)	<0.001
Peripheral Retina				
GCL		3.0 (1.8-4.0)	1.0 (1.0-2.0)	<0.001
OPL		6.0 (5.0-7.0)	1.0 (1.0-2.0)	<0.001
CD18 marked leukocytes				
Central Retina				

GCL		5.0 (3.0-6.0) *	7.0 (4.3-8.0)	<0.001
OPL		16 (14.0-18.0)	12 (9.3-15.0)	<0.001
Peripheral Retina				
GCL		3.0 (2.0-4.0)	3.0 (2.0-4.0)	0.972
OPL		8.0 (6.0-9.0)	4.0 (3.0-5.0)	<0.001

Table 2: Comparative results of the preserved retinal vessels and CD18 marked leukocytes counting in central and peripheral sections (Values are median and interquartile range (IQR). *FMedian of vessels counted in 1.1 mm of tissue).

Correlational study between leukocytes and preserved vessels

We performed a correlational study in order to check if the results achieved after leukocyte's quantification were related to the preserved retinal vessels in the treated animals. The Spearman's correlation between the number of CD18 marked leukocytes and the counting results of preserved vessels was positive in all the studied layers. At the central retinal sections we obtained a correlation coefficient of 0.341 (P=0,015) for the OPL (Figure 3) and a coefficient of 0.803 (P<0.001) for the retinal GCL (Figure 4). The statistical Spearman's coefficient between the variables in the peripheral retina sections was 0.005 (P=0.973) in the OPL (Figure 5) and 0.737 (P<0.001) in the retinal GCL (Figure 6). The coefficient of determination in the central retinal sections was r^2=0.116 for OPL and r^2=0.645 for retinal GCL. At the same time, in the peripheral retinal sections the coefficient of determination was r^2=0.000025 for the OPL and r^2=0.543 for the GCL.

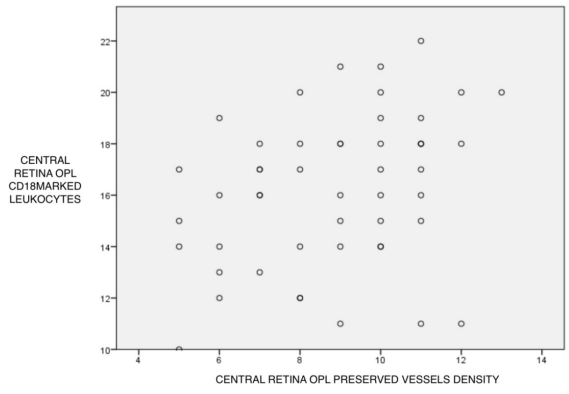

Figure 3: Spearman's correlation between leukocytes and preserved vessels density for the central OPL. Correlation coefficient 0.341 (P=0.015). Coefficient of determination r^2=0.116.

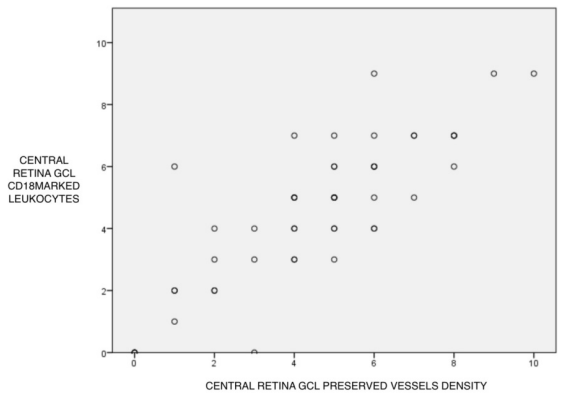

Figure 4: Spearman's correlation between leukocytes and preserved vessels density for the central GCL. Correlation coefficient 0.803 (P<0.001). Coefficient of determination r^2=0.645.

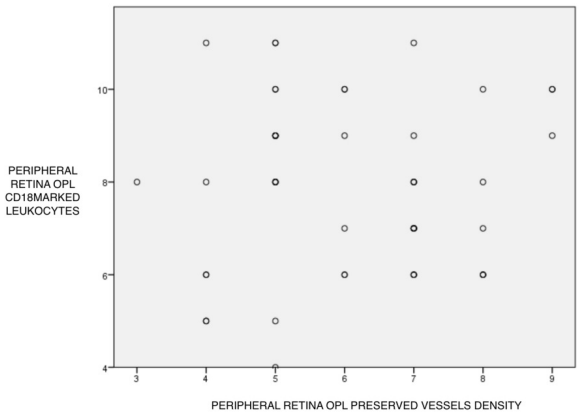

Figure 5: Spearman's correlation between leukocytes and preserved vessels density for the peripheral OPL. Correlation coefficient 0.005 (P=0.973). Coefficient of determination r^2=0.00025.

Discussion

In the present study, the intravitreal injection of 25 µg of lysine acetylsalicylate in a 5 µl sample solution is statistically significantly related to a higher density of retinal vessels in the GCL and OPL of the retina. Likewise, the administration of the drug is significantly related to a lower presence of leukocytes in the GCL and with a greater presence of leukocytes in the OPL. We will discuss this paradoxical effect on OPL later.

The correlation analyses showed a positive correlation between both variables in the lysine-group being statistically significant in the retinal GCL and OPL for central retina sections, but only in the retinal GCL for peripheral retina sections (not in the OPL). To explain this lack of correlation in the peripheral OPL we postulate that maybe the lower counting of cells and vessels founded in the peripheral retina compared to the central retina in both groups needs more number of specimens involved to detect statistical differences.

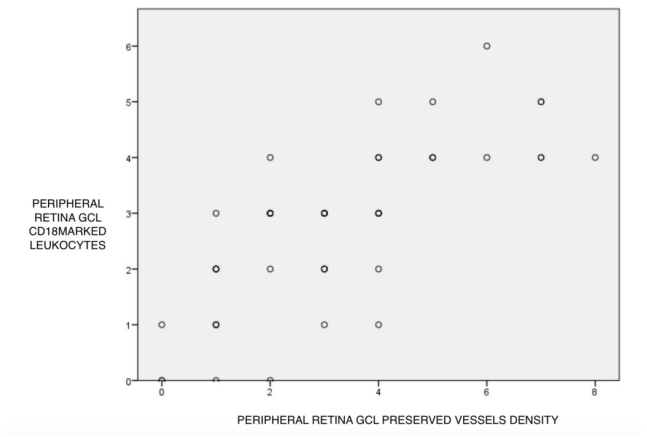

Figure 6: Spearman's correlation between leukocytes and preserved vessels density for the peripheral GCL. Correlation coefficient 0.737 (P<0.001). Coefficient of determination r^2=0.543.

The correlation was highly significant in the retinal GCL at the central and peripheral retina; perhaps the direct contact between the vitreous and the inner retinal layers could explain more concentration and more leukocyte inhibition effect of the solution in the retinal GCL.

Regarding the paradoxical increase in the increased labeling of CD18 in the OPL of the lysine-group we suggest the following explanation. Lysine acetylsalicylate would not, theoretically, prevent arrival of circulating leukocytes to the retina but it would reduce the cascade of events that exert a chemotactic action on them. The significant positive correlation between vascular density and the number of leukocytes allow us to establish a mathematical correlation model capable of predicting the theoretical number of leukocytes expected in relation to each blood vessel present in a histological section:

Central Retina: CD18-marked leukocytes=0.950+0.8 × vessels (P=0.002)

Peripheral Retina: CD18-marked leukocytes=1.066+0.536 × vessels (P<0.001)

At the central retina the model predicts 1.75 leukocytes for each existing vessel, and 1.6 for the peripheral retina. However, in the OPL of the control group for each vessel there is a higher ratio than in the lysine-group, 4 leukocytes per vessel in both the central and peripheral retina. This suggests that although in absolute terms there are more leukocytes in the OPL of the lysine-group, in relative terms to vascular density the proportion of leukocytes is higher in the control-group.

To the best of our knowledge, the use of a lysine acetylsalicylate solution for intravitreal injection has never been reported before our work. Previous studies based on oral aspirin in STZ-diabetic retinopathy has demonstrated a COX-2 inhibition and prostaglandin production blockage at a serum aspirin levels <0.1 μg/μl (0.55 mmol/l) [15,32]. Otherwise, a serum concentration range between 1-5 mmol/l is enough to achieve anti-inflammatory effects of aspirin in rheumatic disorders [33]. Based on the aspirin serum levels previously described, we use a 5 μl solution containing 25 μg of lysine-acetylsalicylate, which is highly soluble and equivalent to 13.88 μg of aspirin.

Assuming a monocompartmental pharmacokinetic model and a vitreous volume of 57 μl [27], it was calculated that the achieved concentration of vitreous lysine acetylsalicylate should be 0.438 μg/μl after each injection. This is equivalent to 0.24 μg/μl (1.34 mmol/l) of aspirin. Based on the serum concentration ranges described in other studies, mentioned previously, we assumed that the administered dose of lysine acetylsalicylate should be effective.

Effects mediated by inhibition of COX-2 are not the only mechanism by which acetylsalicylates may be useful in diabetic retinopathy. Salicylates can inhibit the phosphorylation of the IκB kinase complex by preventing the activation of NF-κB [34]. In diabetic rats induced with STZ, this blockade of NF-κB has been observed after an intermediate daily oral dose of aspirin, even with low serum levels of aspirin (0.037 ± 0.023 mmol/l) [32]. The increase in factor activity Transcriptional NF-κB has been associated not only with diabetic retinopathy but also with a number of cancers such as prostate, colon, breast, Hodkin disease, among others [35,36]. NF-κB suppresses apoptosis in tumor cells, regulates the synthesis of various cytokines, chemotactic proteins and matrices involved in inflammation, in immune responses and cell proliferation [37]. In diabetic retinopathy, overexpression of NF-κB has been associated with increased adhesion of leukocytes and of its adhesion capacity to the endothelium of the capillary network of the retina [38,39].

Another inflammatory pathway that can be inhibited by salicylates is the phosphorylation of the transcriptional factor C/EBPβ (CCAAT-enhancer-binding proteins) [40,41]. Increased levels of C/EBPβ have been reported in the kidney of diabetic rats [41]; also the up-regulation of this transcriptional factor has been associated with specific damage on the GCL after an ischemic-induced retinal injury in mice. These studies found that a C/EBPβ knockout protects against loss of ganglional cells and preserve function in ischemic retina [42]. *In vitro* studies has demonstrated a cytoprotective action of aspirin (3-30 μmol/l), up to 95% compared with control cultures, by increasing the viability of endothelial cells in a concentration-dependent manner [43].

Our results seem to be consistent with other evidences about the effects of aspirin and other salicylates protecting retinal vessels and avoiding leukostasis even in early stages of diabetic retinopathy in rats [14,15,32]. Others non-steroidal anti-inflammatory drugs (NSAID's) (e.g. nepafenac [44], meloxicam [45]), have demonstrated to be useful preventing the same early vascular and inflammatory events in the pathogenesis of the STZ-induced diabetic retinopathy as salicylates do.

Although the results obtained suggest that intravitreal lysine acetylsalicylate could be a novel therapeutic approach for diabetic retinopathy, futher investigations will be necessary to complement and confirm our results. Future experiments should verify the effects of the administration of acetyl salicylatelysine not only in diabetic animals but also in healthy animals to establish a comparative analysis between both groups. We consider that integrate other variables such as vascular permeability, retinal neurodegeration, performing *in vivo* tests (retinography, optical coherence tomography, fluorescein angiography) and the examination of whole-mounted retinas preparations would be very useful. For an eventual future development of the drug, studies of pharmacokinetics, safety and toxicity will be required in other animal species such as pigs and rabbits, among others.

Based on this first experience with the intraocular use of lysine acetyl salicylate, we consider that the intravitreal administration of

salicylates deserves to be explored as a therapeutic option for diabetic retinopathy and perhaps for other inflammatory eye diseases.

Acknowledgement

This study has been a collaborative work. C.F-M is the guarantor author who designed the study, methods, examined tissues, did the statistical analysis and manuscript writing; JM.R-M, JJ.M-T, performed the intravitreal injections, eyes dissection and fixation. E.C. developed the immunohistochemical assay and tissues preparation; A.M-L did the pharmaceutical methodology.

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