

Virgin olive oil administration improves the effect of aspirin on retinal vascular pattern in experimental diabetes mellitus

Jose Pedro De La Cruz¹, Sergio Del Río², Juan Antonio López-Villodres¹,
Maria Auxiliadora Villalobos³, Nuria Jebrouni¹ and Jose Antonio González-Correa^{1*}

¹Laboratorio de Investigaciones Antitrombóticas e Isquemia Tisular (LIAIT), Department of Pharmacology and Therapeutics, School of Medicine, Campus de Teatinos s/n, 29071 Málaga, Spain

²Service of Ophthalmology, Hospital Costa del Sol, Marbella, Málaga, Spain

³Department of Anatomy, School of Medicine, University of Málaga, Málaga, Spain

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The aim of the present study is to evaluate the possible influence of virgin olive oil (VOO) on the effect of acetylsalicylic acid (ASA) in platelet aggregation, prostanoid and NO production and retinal vascular pattern in rats with experimental type 1-like diabetes. We used 100 male Wistar rats that were distributed into five groups: (1) non-diabetic rats (NDR); (2) untreated diabetic rats (DR); (3) DR treated with ASA (2 mg/kg per d per os (p.o.)); (4) DR treated with VOO (0.5 ml/kg per d p.o.); (5) DR treated with ASA plus VOO. The duration of diabetes was 3 months, and each treatment was administered from the first day of diabetes. Variables that were quantified were platelet aggregation (I_{\max}), thromboxane B₂ (TxB₂), aortic prostacyclin (6-keto-PGF_{1 α}) and NO, and the percentage of retina with horseradish peroxidase-permeable vessels (HRP-PV). Diabetic rats showed a higher I_{\max} (35%) and TxB₂ (63%) than NDR, and a lower 6-keto-PGF_{1 α} , NO and HRP-PV than NDR (–74.6%). ASA and VOO administration reduced these differences and prevented the percentage of HRP-PV (–59.7% with ASA and –46.7% with VOO). The administration of ASA plus VOO showed a strong platelet inhibition (80.2 v. 23.4% for VOO and 50.6% for ASA + VOO, $P < 0.0001$), and reduced HRP-PV differences to –31.6% ($P < 0.001$ with respect to DR and $P < 0.0001$ with respect to DR treated with ASA). In conclusion, the administration of VOO to rats with type 1-like diabetes mellitus improves the pharmacodynamic profile of ASA, and increases its retinal anti-ischaemic effect.

Virgin olive oil: Acetylsalicylic acid: Diabetic retinopathy: Prostanoids: Nitric oxide

Some of the mechanisms involved in the genesis and progression of microangiopathic complications in patients with diabetes mellitus and in experimental diabetic animals are increased platelet activation^(1–3) and increased thromboxane synthesis^(3,4), decreased platelet sensitivity to endogenous platelet substances such as prostacyclin or NO and decreased synthesis of these mediators^(5,6). In diabetic retinopathy, these alterations are closely related with endothelial dysfunction, which is one of the earliest alterations to appear⁽⁷⁾.

Some antiplatelet drugs such as acetylsalicylic acid (ASA)^(3,8,9), dipyridamole⁽¹⁰⁾, diltiazol⁽¹¹⁾, camonagrel and other antagonists of thromboxane synthetase^(12,13) and clopidogrel⁽¹⁴⁾ have shown a prophylactic effect on the development and progression of these retinal vascular lesions in streptozotocin-induced diabetic rats. From these studies, we concluded that these drugs exerted two effects that correlated with the prevention of ischaemic diabetic retinopathy: inhibition of platelet thromboxane and increased vascular production of prostacyclin and NO.

The traditional Mediterranean diet is based on a number of food items that have health benefits, including olive oil as

the main source of fats. The health effects of olive oil on many cardiovascular risk factors have been documented scientifically as beneficial for the lipid and thrombotic profile, insulin-mediated glucose metabolism, blood pressure, haemostasis, endothelial function, inflammation and oxidative stress^(15,16).

Some of the effects of virgin olive oil (VOO) are related with platelet aggregation, prostanoid synthesis and NO production⁽¹⁷⁾. For this reason, the aim of the present study is to evaluate the possible influence of VOO administration on the effect of the most useful antithrombotic drug (ASA) in platelet aggregation, prostanoid and NO production, and retinal vascular pattern in rats with experimental type 1-like diabetes mellitus.

Experimental methods

Material

Thromboxane B₂ (TxB₂) and 6-keto-PGF_{1 α} enzyme immunoassay kits were obtained from GE Healthcare UK Limited

(Little Chalfont, Bucks, UK). The nitrite/nitrate ELISA kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). Collagen was obtained from Menarini Diagnóstica S.A. (Barcelona, Spain). All other reagents were obtained from Sigma Chemical Corporation (St Louis, MO, USA). Olive oil used was purchased from the local department stores; its composition is given in Table 1. A standard commercial chow was used for the daily maintenance of rats (Scientific Animal Food and Engineering, Augy, France).

Study design

We used 100 male Wistar rats with a mean body weight of 200 g at the start of the experiment. The study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals, and the research was approved by the University of Málaga Animal Use Committee.

The rats were distributed randomly into five groups of twenty animals each: (1) a control group of non-diabetic animals studied for 3 months; (2) an untreated group of animals with diabetes followed for 3 months; (3) animals with diabetes treated with ASA (2 mg/kg per d per os) for 3 months; (4) animals with diabetes treated with VOO (0.5 ml/kg per d per os) for 3 months; (5) animals with diabetes treated with ASA (2 mg/kg per d per os) plus VOO (0.5 ml/kg per d per os) for 3 months. In each group, ten animals were used for the retinal vascular pattern, and ten animals for the other quantified variables.

Induction of diabetes

Experimental diabetes was induced with a single intravenous injection of 50 mg/kg streptozotocin. Blood glucose concentration was measured by placing a Glucocard Memory II glucometer (Menarini, S.A.) in contact with blood drawn from a small incision in the tail. Animals were considered to have diabetes if blood glucose was >2000 mg/l for two consecutive days. Rats in the non-diabetic control group

received a single intravenous injection of isotonic saline solution, and blood glucose was measured in the same way as in animals that were made diabetic.

Observation and treatment

During the observation period, diabetic animals were treated with a soluble long-acting basal insulin analogue (Levemir[®]) that was given subcutaneously at a dose of 4 IU/d to reduce mortality due to the high levels of blood glucose. Control animals received the same volume of isotonic saline solution subcutaneously.

ASA and VOO were given from the first day of diabetes as a single oral daily dose via a flexible gastric cannula. In order to prevent any possible pharmacokinetic interaction, VOO was administered at 09.00 hours and ASA was administered at 17.00 hours every day. Non-diabetic control animals received an equivalent volume of isotonic saline solution.

Sample processing

At the end of the third month, all the animals from each group were anaesthetised with pentobarbital sodium (40 mg/kg intraperitoneal). A medial laparotomy was made to withdraw 2 ml of blood from the vena cava; 3% sodium citrate at a proportion of 1:9 was used as the anticoagulant. Then, a segment of the abdominal aorta 0.5 cm anterior to the bifurcation of the femoral arteries was clamped.

Ten animals in each group were used for the retinal study. Sigma type II horseradish peroxidase (1 ml, 180 mg/kg) was injected via the carotid artery. Five minutes later, both eyeballs were removed and placed in a solution of 1.2% glutaraldehyde and 1% paraformaldehyde in 0.2 M-PBS (pH 7.2) for 45 min. The lens and vitreous humour were removed, and the retina was separated from the sclera with a narrow surgical spatula and was immersed in a fixative for 48 h.

Analytical techniques

All techniques were run in a single-blind manner, i.e. the persons who performed the assays were unaware of the origin and nature of the samples.

Platelet aggregometry. Platelet aggregation capacity in whole blood was tested at 37°C with the electrical impedance method (Chrono-Log 540 aggregometer; Chrono-Log Corporation, Haverton, PA, USA). Collagen (10 µg/ml) was used as the inducing agent, and maximum aggregation intensity was determined as the maximum resistance between the two poles of the electrode obtained 10 min after the collagen was added.

Platelet thromboxane B₂. After aggregation was complete, the blood sample was centrifuged at 10 000 g for 5 min, and the supernatant was frozen at -80°C until TxB₂ production was quantified with an enzyme immunoassay.

Vascular 6-keto-PGF_{1α}. The aortic segment was cut into two parts and incubated at 37°C in a buffer containing (mM): 100 NaCl, 4 KCl, 25 NaHCO₃, 2.1 Na₂SO₄, 20 sodium citrate, 2.7 glucose and 50 Tris (pH 8.3). Segments were placed in 500 µl of fresh buffer, and 10 µl of Ca ionophore A23187 (final concentration 1 µM) were added. Five minutes later, the samples were dried and weighed,

Table 1. Main composition of the virgin olive oil administered to rats

Fatty acids	%
C14:0	0.0
C16:0	15.6
C16:1	2.9
C17:0	0.2
C17:1	0.5
C18:0	2.2
C18:1	76.5
C18:2	9.9
C20:0	0.9
C18:3	0.5
C20:1	0.6
C22:0	0.1
C24:0	0.06
Total	100
Minor components (mg/kg)	
α-Tocopherol	173
Total phenolic compounds	250
Cholesterol	0.002
β-Sitosterol	1.6

and the supernatant was frozen at -80°C until the assay. The production of 6-keto-PGF $_{1\alpha}$ (stable metabolite of prostacyclin) was quantified with an enzyme immunoassay.

Vascular nitric oxide production. NO production was induced with the Ca ionophore A23187 (50 μM), and was measured as nitrite + nitrate concentration in the supernatant of induced aortic rings with an enzyme immunoassay kit. Briefly, aortic rings were incubated for 10 min at 37°C with 200 μl of buffer containing 10 mM-HEPES, 1 mM-EDTA, 1 mM-DL-dithiothreitol, 10 $\mu\text{g/ml}$ leupeptin, 4.8 mM-DL-valine, 1 mM-NADPH, 1 mM-MgCl $_2$, 1 mM-CaCl $_2$ and 20 μM -L-arginine. Basal nitrite + nitrate production was determined first; after this step, Ca ionophore A23187 was added, and after 30 min of incubation at 37°C , the increase in nitrite + nitrate production was evaluated.

Retinal vasculature. The fixed retinas were incubated with a solution of tetramethylbenzidine and sodium nitroferricyanide as the chromogenic substrate, and were then dehydrated in an alcohol gradient, incubated in xylene and mounted in sections for microphotography.

Retinal vessels permeable to horseradish peroxidase were photographed at $\times 40$, and microscopic images were processed in an IBAS Kontron 2000 image analyser (Kontron Bildanalyse, Munich, Germany). The percentage of the retinal surface occupied by peroxidase-permeable vessels was calculated by the system as a standard parameter. Horseradish peroxidase is a high-molecular weight substance that mainly stains erythrocytes; thus in occluded vessels, horseradish peroxidase does not stain the vasculature.

Statistical analysis

All values in the text and figures are the means with their standard errors of the data for all the animals in each group. The data were analysed with the Statistical Package for Social Sciences version 16.0 (SPSS Company, Chicago, IL, USA). Groups were compared with ANOVA followed by the Bonferroni test when the difference between the groups was significant. A P value < 0.05 was taken as the minimum level of significance.

Results

Mean body weight, daily chow ingestion, blood glucose concentration and blood cell counts are given in Table 2. There were no differences in the body weight increments from day 0 to day 90 in any of the diabetic groups. There were no statistical differences in blood cell counts between the groups.

Platelet aggregation that was induced with collagen in whole blood was higher in diabetic rats than in non-diabetic rats (NDR; Table 3). ASA reduced the maximum intensity of platelet aggregation 51.0%, VOO 40.8% and ASA plus VOO 80.5% with respect to untreated diabetic rats (DR). The inhibition of platelet aggregation in the ASA plus VOO group was statistically higher than that in the ASA and VOO groups ($P < 0.05$).

Platelet TxB $_2$ production increased 63.4% in DR with respect to NDR (Table 3). The reduction in the synthesis with ASA reached statistical significance after 3 months of treatment (98% inhibition with respect to DR), and VOO inhibited platelet production of TxB $_2$ after chronic treatment (46.2% inhibition, $P < 0.05$ with respect to the ASA and ASA plus VOO groups). The administration of ASA plus VOO reduced TxB $_2$ production by 98.4% with respect to DR.

Aortic production of prostacyclin, measured as 6-keto-PGF $_{1\alpha}$, was significantly lower in diabetic rats (57.3%) than in NDR (Table 3). Treatment with ASA but not with VOO for 3 months reduced 49.6% prostacyclin production ($P < 0.05$), while that with VOO reduced the difference between NDR and DR to 32.8% ($P < 0.05$ with respect to the ASA group).

Vascular NO production induced via the Ca-dependent pathway was significantly lower (45.0%) in diabetic rats than in NDR after 3 months (Table 3). Treatment with ASA for 3 months reduced the differences between DR and NDR by 3.4%. Treatment with VOO for 3 months reduced the differences between DR and NDR by 19.5%. Diabetic rats treated with ASA plus VOO showed 12.1% higher NO production than NDR. There are no statistical differences between the treated groups.

Table 2. Body weight, percentage of change in body weight, blood glucose and blood cell counts in non-diabetic rats (NDR), diabetic rats without treatment (DR) and diabetic rats treated with 2 mg/kg per d per os acetylsalicylic acid (ASA), 0.5 ml/kg per d per os virgin olive oil (VOO) or ASA plus VOO at the same doses (n 20 rats per group)

(Mean values with their standard errors)

	NDR		DR		DR + ASA		DR + VOO		DR + ASA + VOO	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)	309	15	313	25	294	12	339	18	314	21
Change in body weight (%)†	+104	15	+119	16	+107	14	+106	14	+101	20
Rate of chow ingestion (g/d)†	39.8	4.1	41.6	3.2	38.5	1.0	39.4	2.1	40.3	2.2
Blood glucose (mg/l)	962*	86	4630	210	4520	570	4270	650	4170	900
Erythrocytes ($\times 10^{12}$ per litre)	6.1	1.5	6.8	0.1	6.3	0.6	6.6	0.2	6.7	0.3
Leucocytes ($\times 10^6$ per litre)	2.8	0.5	2.3	0.5	2.4	0.5	2.9	0.8	2.3	0.7
Platelets ($\times 10^6$ per litre)	691	124	757	54	606	188	752	145	754	188
Hb (g/l)	13.2	0.7	13.5	0.7	13.7	1.1	13.9	0.4	13.7	0.6
Haematocrit (l/l)	39.2	4.7	37.1	1.5	36.6	3.5	37.2	0.9	36.6	0.3

* Mean values were significantly different with respect to all the diabetic groups ($P < 0.0001$).

† This variable was calculated as follows: (body weight at day 90/body weight at day 1) $\times 100$.

Table 3. Maximum intensity of platelet aggregation (I_{max}) and thromboxane B₂ (TxB₂) production in whole blood induced with collagen, aortic 6-keto-PGF_{1 α} and nitrite + nitrate (NO₂⁻ + NO₃⁻) production induced with calcium ionophore A23187, and percentage of retinal surface covered with peroxidase-permeable vessels in non-diabetic rats (NDR), diabetic rats without treatment (DR) and diabetic rats treated with 2 mg/kg per d per os acetylsalicylic acid (ASA), 0.5 ml/kg per d per os virgin olive oil (VOO) or ASA plus VOO at the same doses (*n* 10 rats per group for retinal study and 10 rats per group for all the biochemical variables)

(Mean values with their standard errors)

	NDR		DR		DR + ASA		DR + VOO		DR + ASA + VOO	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
I_{max} (Ω)	6.1	0.3	8.1*	0.3	4.0**	0.3	6.2**†	0.4	1.6**†	0.2
TxB ₂ (pg/ml)	60.3	1.2	98.0*	11.0	3.62**	0.2	43.3**‡	3.6	3.5**	0.2
6-Keto-PGF _{1α} (pg/mg protein)	67.0	5.2	29.2*	2.0	14.6**	0.3	45.7**†	3.5	35.5**†	3.0
NO ₂ ⁻ + NO ₃ ⁻ (nmol/mg tissue)	41.5	3.1	25.6*	0.5	40.1**	0.7	33.2**	0.4	46.2**	0.6
Retinal surface covered with HRP-stained vessels (%)	13.8	0.5	3.5*	0.2	5.6**	0.6	7.4**	0.3	9.5*†	0.4

HRP, horseradish peroxidase.

* Mean values were significantly different with respect to NDR ($P < 0.05$).

** Mean values were significantly different with respect to DR ($P < 0.05$).

† Mean values were significantly different with respect to ASA ($P < 0.05$).

‡ Mean values were significantly different with respect to VOO ($P < 0.05$).

Diabetes induced with streptozotocin in rats reduced (74.4 %) the retinal surface occupied by peroxidase-permeable vessels (Table 3) after 3 months of evolution. Treatment with ASA reduced the differences between DR and NDR to 59.1 %. Treatment with VOO for 3 months reduced the differences between DR and NDR to 46.3 %. Treatment with ASA plus VOO reduced this difference to 41.3 % ($P < 0.05$ with respect to the ASA group). Fig. 1 shows the representative images of retinal vascular patterns from a NDR and diabetic rats with and without treatment.

Discussion

We report that the administration of VOO to rats suffering from type 1-like diabetes mellitus could improve the beneficial effect of low-dose ASA in platelet aggregation, prostacyclin production and retinal vascular pattern, which are clearly altered in diabetes.

Enhanced platelet aggregation in diabetes has been widely reported in both animal models and human subjects. As earlier studies have shown^(18,19), this increase in platelet aggregation capacity is accompanied by an evident imbalance in prostanoïd synthesis, i.e. an increase in platelet thromboxane A₂ production and a decrease in vascular prostacyclin synthesis.

With respect to NO production, sustained high blood glucose levels in diabetes mellitus are assumed to stimulate oxidative stress mechanisms, which may be one of the causes of the inhibition of endothelial prostacyclin synthetase and the reaction of free radicals with NO to produce peroxynitrites^(20,21). Both the effects are manifested as the endothelial dysfunction reported in diabetes. Both prostacyclin and NO are vasodilators and antiplatelet compounds, and a deficit in these substances has been linked to early vascular alterations in diabetic retinopathy⁽²²⁾. In our experimental model, vascular NO synthase activity (measured indirectly as NO production after stimulation with a Ca ionophore) was clearly reduced in diabetic animals.

The alterations summarised above may contribute to the reduction that we found in the retinal surface area occupied by peroxidase-permeable vessels (Table 3). However, these

are not the only mechanisms that can condition retinal ischaemia in diabetes. It is now accepted that the appearance of ischaemia may reflect the sum of these and other factors such as increased endothelin synthesis, anomalies in protein kinase C and increased activity of the polyol pathway^(22,23). However, the synthesis of both prostacyclin and NO is altered as a result of the endothelial dysfunction in diabetes.

The antiplatelet effect of ASA has been widely reported, and the results reported here are similar to values found in earlier studies in type 1-like diabetic rats^(24,25). The antiplatelet effect of ASA is directly related with two fundamental mechanisms: inhibition of platelet thromboxane synthesis⁽²⁶⁾ and increased Ca-dependent NO production^(27–29).

The effect of ASA on overall inhibition of aggregation *in vivo* has been shown to involve stimulation of Ca-dependent NO^(27–29), which inhibits platelet functioning. In our experimental model, ASA almost prevented the decrease in NO production in diabetic animals, similar to the previous results in the same experimental model⁽⁹⁾.

Our results show that the daily oral administration of VOO in type 1-like diabetic rats modified platelet and endothelial biomarkers. The antiaggregatory effect of VOO in diabetic rats confirms the same data described in normoglycaemic rats⁽¹⁷⁾. Animals treated with VOO showed not only inhibition of platelet aggregation but also reduced production of thromboxane, which has also been demonstrated in human subjects⁽³⁰⁾. This effect was responsible for the beneficial action of VOO on the prostacyclin/thromboxane ratio (7.85 (SEM 0.69)), which is an important factor in the prevention of thrombosis; this ratio reached the value in non-diabetic animals.

Treatment with VOO and ASA produces inhibition of platelet aggregation that is greater than that produced by the two compounds separately, as well as it prevents the inhibition of vascular synthesis of NO in experimental diabetes. With respect to thromboxane synthesis, there are no statistical differences between the effects of aspirin alone or in combination with VOO; this is due to the well-known rapid and potent inhibition of platelet cyclooxygenase by aspirin which is near to 95 %, and for this reason, VOO did not support

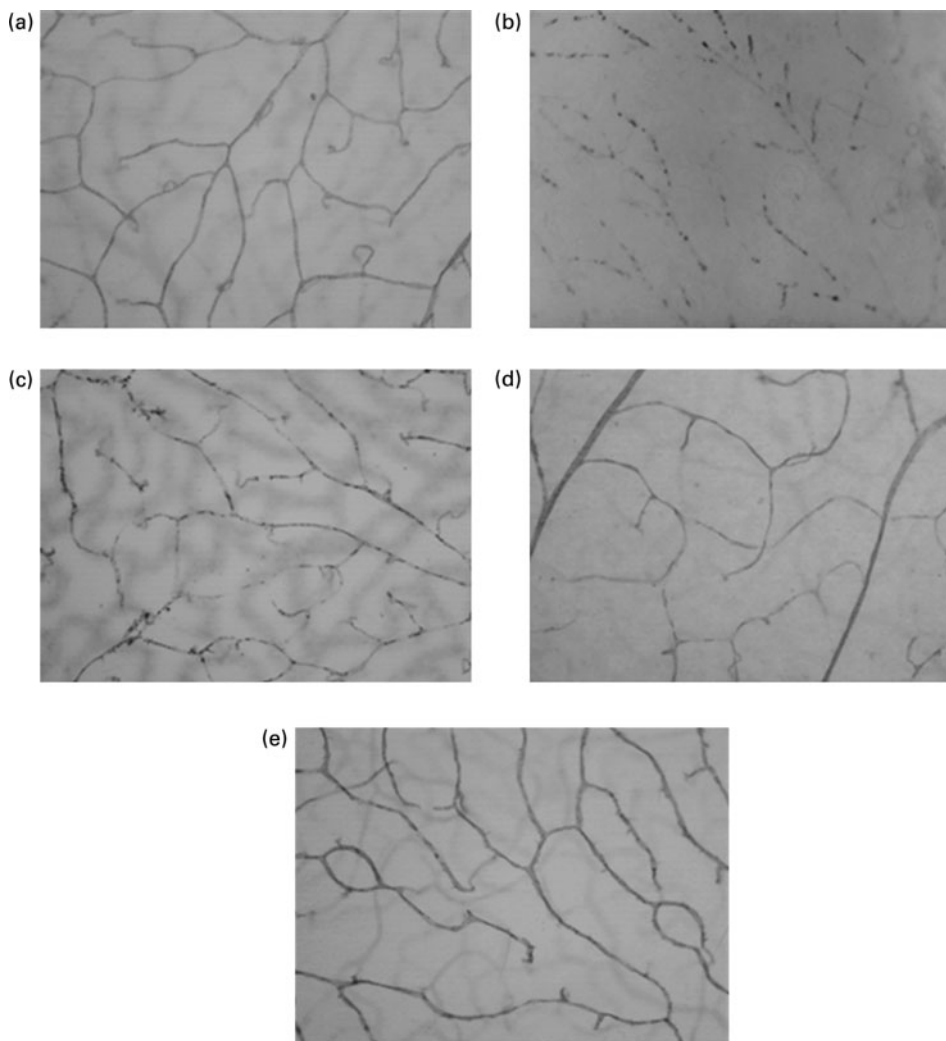


Fig. 1. Representative images of retinal horseradish peroxidase-permeable vessels from non-diabetic rats (a) and diabetic rats after 3 months without treatment (b) or treated with 2 mg/kg per d per os acetylsalicylic acid (ASA) (c), 0.5 ml/kg per d per os virgin olive oil (VOO) (d) or ASA plus VOO (e). Magnification 40 ×. For processing methodological details, see text.

any additional effect of aspirin in the platelet prostanoid synthesis. Similar results have been described after the treatment of ASA with other antioxidants such as vitamin E⁽³¹⁾, albeit at relatively high doses in relation to the dosage of vitamin E that is administered to human subjects.

Some studies have shown a relationship between changes in the thromboxane/prostacyclin ratio and the degree of retinal ischaemia in this experimental model^(8–14). This ratio was 1.12 (SEM 0.07) in NDR, 0.29 (SEM 0.02) in non-treated diabetic rats, 3.75 (SEM 0.49) in diabetic animals treated with ASA and 7.85 (SEM 0.69) in those treated with ASA plus VOO. Despite the methodological differences in the measurement of both prostanoids, we measured TxB₂ and 6-keto-PGF_{1α} production after induction with the same stimulus, Ca ionophore A23187. ASA alone and in combination with VOO disrupted this ratio in favour of a proportionally greater inhibition of platelet thromboxane production.

The importance that NO owns in the mechanism of action of ASA in the prevention of retinal ischaemia has also been demonstrated⁽⁹⁾. The administration of VOO has shown a beneficial effect on these parameters, especially in

the prevention of endothelial damage caused by diabetes (Table 3). All this may contribute to the higher effect of ASA plus VOO administration on the percentage of retinal peroxidase-permeable vessels.

We conclude that the administration of VOO to rats with type 1-like diabetes mellitus improves the pharmacodynamic profile of ASA, especially in terms of platelet aggregation and vascular production of prostacyclin and NO. Moreover, the administration of VOO increases the retinal anti-ischaemic effect of ASA in this experimental model.

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