

Intravitreal Injection of Corticosteroid Attenuates Leukostasis and Vascular Leakage in Experimental Diabetic Retina

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PURPOSE. Recently, intravitreal injection of corticosteroids has been in wide use as a treatment for diabetic macular edema, and the outcomes have been favorable. However, the exact mechanism remains unclear. The hypothesis for the current study was that intravitreal corticosteroids may improve diabetic retinal edema by amelioration of blood-retinal barrier (BRB) breakdown, by inhibiting leukocyte stasis (leukostasis).

METHODS. Diabetes was induced in 6-week-old male Long-Evans rats by intraperitoneal injection of streptozotocin (75 mg/kg). Three weeks after induction of diabetes, intravitreal injection of dexamethasone (40 μ g/10 μ L) was performed. At 2 days after intravitreal injection, accumulated leukocytes were counted in vivo by acridine orange leukocyte fluorography, and BRB breakdown was evaluated by measurement of retinal vascular permeability. The mRNA expression and protein levels of intercellular adhesion molecule (ICAM)-1 in the retina were also studied.

RESULTS. The number of leukocytes accumulated in the retina, once increased in the diabetic group, was decreased by 31.6% ($P = 0.0001$) after dexamethasone injection. The level of BRB breakdown, also elevated in the diabetic group, was suppressed by 61.1% ($P = 0.0046$) after dexamethasone injection. The level of ICAM-1 mRNA expression and its protein, upregulated in the diabetic group, were downregulated by dexamethasone treatment by 70.0% ($P < 0.0001$) and 56.4% ($P = 0.0003$).

CONCLUSIONS. Intravitreal injection of corticosteroids improves diabetic retinal edema through inhibiting leukocyte recruitment in the diabetic retina. (*Invest Ophthalmol Vis Sci.* 2005; 46:1440-1444) DOI:10.1167/iovs.04-0905

Retinopathy is a major complication of diabetes mellitus (DM) and one of the leading causes of acquired blindness. Diabetic macular edema is most often responsible for reduced visual acuity in these patients. In recent years, intravitreal injection of corticosteroids such as triamcinolone acetonide has been increasingly used as a treatment for diabetic macular

edema, with favorable outcomes.¹⁻³ This type of retinal edema is considered to occur after the breakdown of the blood-retinal barrier (BRB), which is associated with retinal vascular leakage. It is generally believed that intravitreal corticosteroid can ameliorate the BRB breakdown and then improve the macular edema. However, the exact mechanism remains unclear.

Leukocyte stasis (leukostasis) has been implicated in the retinal microcirculation of early diabetic retinopathy.⁴ And leukocytes have been mentioned as a trigger of two major complications of diabetes: retinal vascular leakage and nonperfusion.⁵ More specifically, leukostasis produces free radicals from oxygen molecules and inflammatory cytokines, which increase vascular permeability, or BRB breakdown: Leukostasis leads to BRB breakdown. Leukocytes appear to have a central role in the development of diabetic retinopathy.

In the present study, we tested the hypothesis that diabetic retinal edema may be improved by intravitreal injection of corticosteroid through leukocyte-endothelial cell interactions. We quantitatively determined the inhibitory effects of intravitreal corticosteroid on leukocyte recruitment in the retina and evaluated BRB breakdown in experimental diabetic rats. Leukocyte recruitment in the retina was evaluated by acridine orange (AO) leukocyte fluorography,^{6,7} a technique that allowed us to visualize leukocyte behavior in the retinal microcirculation with minimal invasion. We also examined the effect of intravitreally injected corticosteroid on intercellular adhesion molecule (ICAM)-1 gene expression and its protein levels.

MATERIALS AND METHODS

Animal Model

Animals ($n = 72$) were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. DM was induced in 6-week-old male pigmented Long-Evans rats by intraperitoneal injection of streptozotocin (STZ, 75 mg/kg). The plasma glucose level in each rat was confirmed to be >250 mg/dL 48 hours after injection. Rats injected with an equal volume of saline alone served as nondiabetic (non-DM) control subjects. Three weeks after induction of diabetes, intravitreal injection of dexamethasone was performed. All rats were maintained with free access to water and food in an air-conditioned room on a 12-hour light-dark cycle before the start of the experiments. Plasma glucose levels were measured with a self-monitoring blood glucose system (Dexter Z II; Bayer Healthcare LLC, Tarrytown, NY).

Intravitreal Injection of Dexamethasone

Animals were anesthetized with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg), and the pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. For additional topical anesthesia, 0.4% procaine hydrochloride (Santen Co., Osaka, Japan) was used. Then, 0.5% of levofloxacin ophthalmic solution (Santen Co.) was applied to the ocular surface, to prevent infection. A single dose of 40 μ g dexamethasone in a volume

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of 10 μL (4 mg/mL; Banyu, Tokyo, Japan) was injected into the vitreous of the right eye with a microinjector (Hamilton Co., Reno, NV) under a dissecting microscope ($n = 6$). A new 30-gauge needle was used to make a punch incision 1 mm posterior to the temporal limbus, and the microinjector 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. For diabetic control, 10 μL physiological saline was injected into the right eye of other animals ($n = 6$).

AO Leukocyte Fluorography

At 2 days after the intravitreal injection, leukocyte behavior was evaluated *in vivo* by AO leukocyte fluorography, which has been described previously.^{6,7} Six rats were used in each group. In this technique, a scanning laser ophthalmoscope (SLO; Rodenstock Instruments, Munich, Germany), coupled with a computer-assisted image analysis system, yields continuous high-resolution images of the fundus of an animal injected with metachromatic fluorochrome AO (Wako Pure Chemical, Osaka, Japan). AO is a widely used probe in biochemical and cytochemical studies. The obtained images were recorded on S-VHS videotape at a rate of 30 frames/s for further analysis.

Immediately before AO leukocyte fluorography, rats were deeply anesthetized with the method just described. In each rat, a catheter was inserted into the tail vein. Arterial blood pressure was monitored with a blood pressure analyzer (IITC, Woodland Hills, CA). The rat was then placed on a movable platform, and AO (0.1% solution in saline) was injected through the tail vein catheter. The fundus was observed with the SLO in the 40° field. The behavior of leukocytes can be observed within several seconds after AO infusion, because AO has a circulation time of <10 seconds and is so membrane permeable that leukocytes are stained with the dye shortly after infusion. AO was injected for 1 minute at a rate of 1 mL/min, to examine the behavior of leukocytes over a few minutes. At 30 minutes after injection of AO, the fundus was observed again to evaluate leukocyte accumulation in the retinal microcirculation.

Image Analysis

The video recordings were analyzed with an image-analysis system consisting of a computer equipped with a video digitizer (Radius, San Jose, CA). The video image was digitized in real time (30 frames/s) to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps. Using this system, we evaluated the number of leukocytes accumulated in the retinal microcirculation.

The number of leukocytes accumulated in the retinal microcirculation was determined at 30 minutes after AO injection, as described previously.⁶ Briefly, an observation area around the optic disc was determined by drawing a polygon surrounded by the adjacent major retinal vessels. This area was measured in pixels on a computer monitor, and the density of leukocytes was calculated by dividing the number of trapped leukocytes, which were recognized as fluorescent dots, by the area of observation. The density of leukocytes was calculated in eight peripapillary observation areas in the fundus. The average density was used as the number of leukocytes accumulated in retinal microcirculation for each rat.

After the experiment, each rat was killed with an overdose of the anesthetic, and the right eye was enucleated to obtain a calibration factor to convert the values measured on a computer monitor (in pixels) into real values (in micrometers).

Quantification of BRB Breakdown

BRB breakdown was evaluated via measurement of retinal permeability with FITC-conjugated dextran (Sigma-Aldrich, St. Louis, MO), according to a method described elsewhere, with slight modification.⁸⁻¹⁰ Two days after the intravitreal injection of dexamethasone or saline vehicle, one eye of each of six diabetic rats was examined. In rats under deep anesthesia, FITC-conjugated dextran (4.4 kDa, 50 mg/mL in phosphate-buffered saline [PBS]), 50 mg/kg body weight; Sigma-

Aldrich) was injected intravenously. Ten minutes after injection, the chest cavity was opened, and a 20-gauge perfusion cannula was introduced into the aorta. A blood sample was collected immediately before perfusion. After achieving drainage from the right atrium, each rat was perfused with PBS (500 mL/kg body weight) to clear the remaining intravascular dextran. The blood sample was centrifuged at 7000 rpm for 20 minutes at 4°C, and the supernatant was diluted at 1:1000. Immediately after perfusion, the retinas were carefully removed, weighed, and homogenized to extract the FITC-dextran in 0.4 mL of water. The extract was processed through a 30,000 molecular weight filter (Ultrafree-MC; Millipore, Bedford, MA) at 7,000 rpm for 90 minutes at 4°C. The fluorescence in each 300- μL sample was measured (excitation, 485 nm; emission, 538 nm) using a spectrofluorometer (Fluor Imager SI; Molecular Dynamics, Sunnyvale, CA) with water as a blank. Corrections were made by subtracting the autofluorescence of retinal tissue from rats without FITC-dextran injection. For normalization, the retinal FITC-dextran amount was divided by the retinal weight and by the concentration of FITC-dextran in the plasma.

Semiquantification of ICAM-1 Gene Expression by Reverse Transcription–Polymerase Chain Reaction

For semiquantification of ICAM-1 gene expression after 1 day of dexamethasone injection, six eyes per group were enucleated in the following four groups: nondiabetic control rats, DM rats without intravitreal injection, vehicle-treated rats, and dexamethasone-treated rats. Total RNA was isolated from the retina according to the acid guanidinium thiocyanate-phenol-chloroform extraction method.^{8,11} Extracted RNA was quantified, and 2 μg was used to prepare cDNA with a cDNA synthesis kit (Omniscript Reverse Transcriptase; Qiagen, Valencia, CA). Polymerase chain reaction (PCR) was performed under the following conditions: denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute. The reaction was performed for 34 cycles for ICAM-1 and 29 cycles for β -actin. The primers were AGCCTCAGGCCTAAGAGGAC (sense) and AGGGGTC-CGAGAGAGGTCTA (antisense) for ICAM-1 and GGCATCTGACCCT-GAAGTA (sense) and GCCATCTCTTGCTCGAAGTC (antisense) for β -actin. After completion, 10 μL of the reactions were analyzed by agarose gel electrophoresis and ethidium bromide staining to determine the levels of transcript relative to the control transcript β -actin RNA.

Enzyme-Linked Immunosorbent Assay for ICAM-1

The eyes were enucleated after 2 days of dexamethasone injection and an enzyme-linked immunosorbent assay was performed.^{8,11} The retina was carefully isolated, placed in 150 μL of lysis buffer (20% glycerol, 10 mM KCl, 1 mM MgCl_2 , 0.1% Triton, 300 mM NaCl, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 20 mM HEPES [pH 7.9]) and homogenized. The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C, and the ICAM-1 levels in the supernatant were determined with a kit (Quantikine; R&D Systems, Minneapolis, MN) used according to the manufacturer's protocol. Total protein was determined using the bicinchoninic acid (BCA) kit (Bio-Rad, Hercules, CA) and this level was used to normalize the ICAM-1 protein levels.

Statistical Analysis

All data are expressed as the mean \pm SEM. Student's *t*-test was used for statistical analysis between the two groups. ANOVA was used to compare three or more conditions, with post hoc comparisons tested using the Fisher protected least-significant difference procedure. Differences were considered statistically significant at $P < 0.05$.

TABLE 1. Physiological Variables for Each Group

	Control	Untreated DM	Vehicle-Treated	Dexamethasone-Treated
WBC ($\times 10^3/\mu\text{L}$)	8.6 \pm 0.9	7.8 \pm 1.1	7.3 \pm 1.0	7.7 \pm 2.1
Blood glucose (mg/mL)	135 \pm 4	368 \pm 11*	331 \pm 16*	347 \pm 21*
MABP (mm Hg)	103 \pm 4	101 \pm 5	107 \pm 7	102 \pm 6

Data are mean \pm SEM ($n = 6$ at each time point in both groups). WBC, peripheral leukocyte count; MABP, mean arterial blood pressure.

* $P < 0.01$ compared with control levels in each group.

RESULTS

Physiologic Data

Table 1 shows changes in physiologic variables of each group. There were no significant differences among groups in any of the physiological data except blood glucose levels. The blood glucose level was significantly higher in all other three groups than in the control group. No significant difference was found in blood glucose levels between the untreated DM group or the vehicle-treated group and the dexamethasone-treated group.

Leukocyte Accumulation

Immediately after AO was infused intravenously, leukocytes were selectively stained among circulating blood cells. At 30 minutes after AO infusion, we identified leukocytes that had accumulated in the retina as distinct fluorescent dots with the highest contrast (Fig. 1). Figure 2 shows the number of leukocytes accumulated in the retina in each group after intravitreal injection. Figure 3 shows time-course changes in the number of leukocytes accumulated in the retina after intravitreal injection of dexamethasone. At 2 days after dexamethasone injection, the number of accumulated leukocytes mostly decreased. Few leukocytes were found in nondiabetic control retinas. However, the number of accumulated leukocytes in the untreated DM rats and in the vehicle-treated DM rats were 1.8- and 2.0-fold as many as in the nondiabetic control rats (both $P < 0.0001$), respectively. There was no significant difference

between these two DM groups. Accumulated leukocyte count was significantly suppressed in the dexamethasone-treated group by 31.6% ($P = 0.0001$), compared with the vehicle-treated group.

BRB Breakdown

Figure 4 shows retinal vascular permeability in each group at 2 days after intravitreal injection. The levels of BRB breakdown were expressed as ratios to the mean level in nondiabetic control rats. Retinal vascular permeability in the untreated DM group and the vehicle-treated group was 4.5- and 5.2-fold as high as in the nondiabetic control group ($P = 0.0049$ and $P = 0.0007$), respectively, and there was no significant difference between these two groups. Dexamethasone treatment compared with the vehicle treatment significantly suppressed vascular permeability ($P = 0.0046$).

ICAM-1 Gene Expression and Protein Levels

The levels of gene expression are shown as ratios to the mean levels in nondiabetic control rats (Fig. 5). At 1 day after intravitreal injection, ICAM-1 mRNA expression was upregulated in the retina of the untreated DM group and the vehicle-treated group (both $P < 0.0001$). There was no significant difference between these two groups. Dexamethasone treatment significantly suppressed ICAM-1 mRNA expression (70.0%; $P < 0.0001$), compared with vehicle treatment. This finding was substantiated by enzyme-linked immunosorbent assay (ELISA, Fig. 6). The ICAM-1 protein levels in the retina of the untreated DM group and the vehicle-treated group at 2 days after dexamethasone injection were 2.1- and 2.3-fold as high as that in the nondiabetic control group ($P = 0.0011$ and $P = 0.0003$), respectively, and there was no significant difference between the two groups. Dexamethasone treatment significantly reduced the ICAM-1 protein level (56.4%; $P = 0.0003$), compared with vehicle treatment.

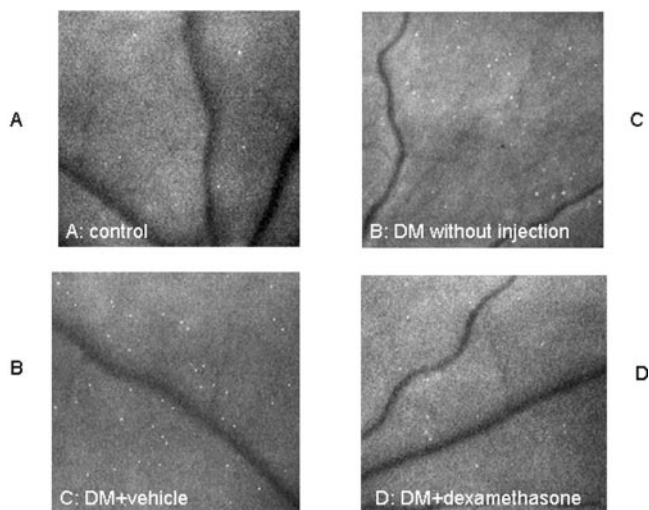


FIGURE 1. Fluorescent dots are accumulated leukocytes after AO injection. (A) A small number of leukocytes were found in the retina of nondiabetic control rats. (B) A large number accumulated in the retina of the untreated DM rats. (C) Almost the same number accumulated in the retina of the vehicle-treated DM rats as were seen in the untreated DM rats. (D) A significant reduction in the number of accumulated leukocytes was seen in the retina of the dexamethasone-treated DM rats.

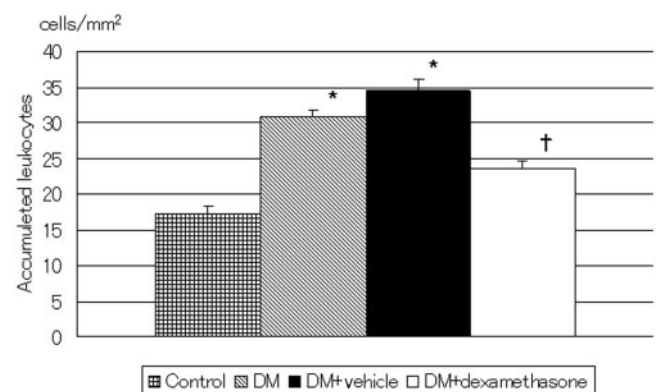


FIGURE 2. The number of leukocytes accumulating in the retina. Data are the mean \pm SEM ($n = 6$ in each group). * $P < 0.0001$ compared with control rats. † $P < 0.001$ compared with vehicle-treated rats.

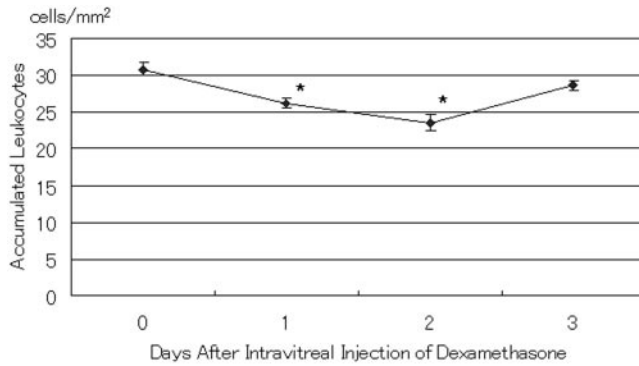


FIGURE 3. Time-course of the number of leukocytes accumulated in the retina of the DM rats after dexamethasone injection. Data are the mean \pm SEM ($n = 6$ at each time). * $P < 0.05$ compared with DM rats before dexamethasone injection.

DISCUSSION

In the present study, the leukostasis and BRB breakdown induced by diabetes were suppressed by intravitreal injection of dexamethasone. This indicates, for the first time, that intravitreal corticosteroid improves diabetic retinal edema by inhibiting leukocyte recruitment in the diabetic retina in vivo.

To date, there have been two major hypotheses for the mechanism of intravitreal corticosteroid action in BRB breakdown: (1) that corticosteroids may reduce retinal capillary permeability by increasing the activity and/or density of the tight junctions in the retinal capillary endothelium¹² and (2) that corticosteroids may inhibit the metabolic pathway of the vascular endothelial growth factor (VEGF), a major vascular-permeability-increasing factor.^{13,14} However, there has been no in vivo study supporting the former hypothesis. The latter hypothesis is not well supported, because there is considerable evidence that corticosteroid does not change VEGF expression.^{15,16} After all, these two hypotheses do not fully explain the mechanism of intravitreal corticosteroid action.

Leukocyte adhesion to the vascular endothelium reportedly leads to junctional disorganization of endothelium.^{17,18} The junctional disorganization may increase vascular permeability, resulting in tissue edema. In diabetic retinas, the leukocytes that are adherent to vascular endothelium have been shown to cause capillary occlusion,⁵ endothelial cell apoptosis,^{19,20} and, finally, BRB breakdown.^{5,20,21} Furthermore, several studies implicate leukocytes as the major source of VEGF.²²⁻²⁴ Because corticosteroids can inhibit leukocyte migration²⁵ and there is a close correlation between retinal leukostasis and BRB break-

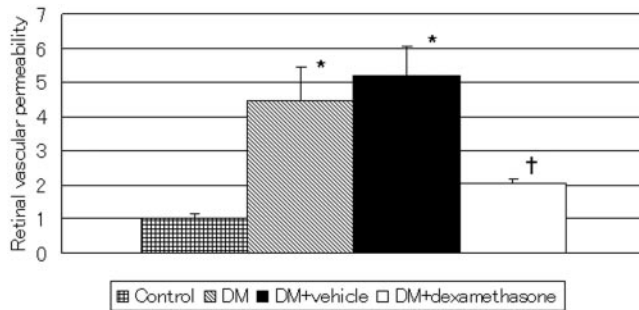


FIGURE 4. Retinal leakage of FITC-conjugated dextran. The levels of retinal vascular leakage are expressed as ratios to the average level in control rats. Data are the mean \pm SEM ($n = 6$ in each group). * $P < 0.005$ compared with control rats. † $P < 0.005$ compared with vehicle-treated rats.

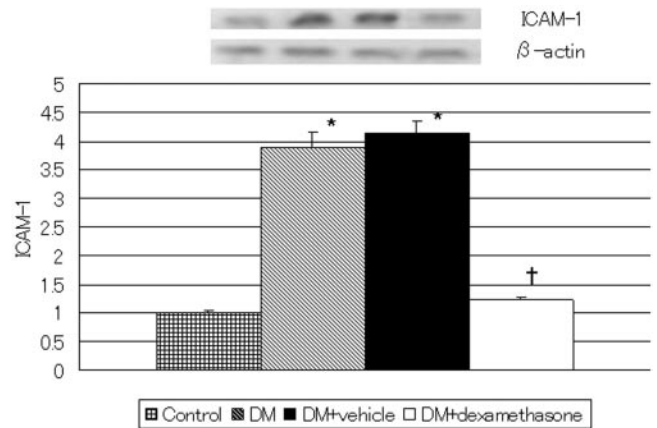


FIGURE 5. Gene expression of ICAM-1. The levels of gene expression are expressed as ratios to the average value of control rats. Data are the mean \pm SEM ($n = 6$ in each group) * $P < 0.0001$ compared with control rats. † $P < 0.0001$ compared with vehicle-treated rats.

down,⁹ it is possible that dexamethasone inhibits leukocyte recruitment in retinal microcirculation and thus reduces BRB breakdown. Given the previous reports about leukocyte functions and corticosteroid's effect on leukocyte recruitment, we hypothesized that intravitreal corticosteroid may improve diabetic retinal edema by amelioration of BRB breakdown by inhibiting leukocyte recruitment in the diabetic retina.

In diabetic retinopathy, the expression of ICAM-1 on vascular endothelial cells is upregulated.^{5,26,27} Upregulated ICAM-1 enhances leukocyte adhesion to the vascular endothelium and leukostasis in the retina, as do other related molecules, including platelet endothelial cell adhesion molecule, vascular cell adhesion molecule, and the selectins.⁵ In the present study, the expression of ICAM-1 and its protein level were downregulated by intravitreal injection of corticosteroid. This indicates that intravitreal corticosteroid inhibits ICAM-1-mediated leukocyte-endothelial cell interaction, a critical step in the development of diabetic retinopathy. In other words, intravitreal corticosteroid exerts beneficial effects in the prevention of diabetic retinopathy through suppressing ICAM-1-mediated leukocyte adhesion to vessel walls.

In the present study, the data show that leukostasis was reduced and BRB breakdown was ameliorated in the diabetic retina by intravitreal injection of dexamethasone. We believe that there is a strong relation between these two observations that supports our hypothesis that intravitreal corticosteroid

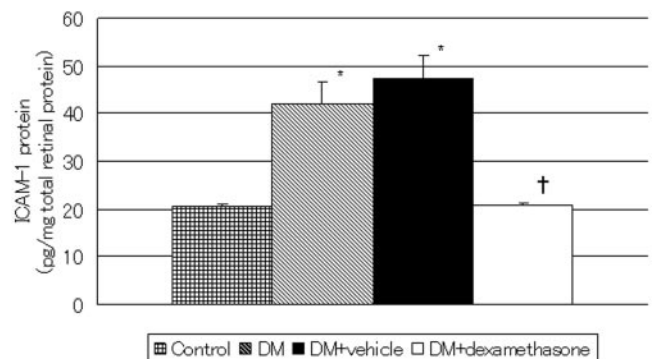


FIGURE 6. ICAM-1 protein levels in the retina were quantitatively measured by ELISA. Data are the mean \pm SEM ($n = 6$ in each group). * $P < 0.005$ compared with control rats. † $P < 0.001$ compared with vehicle-treated rats.

may improve diabetic retinal edema by amelioration of BRB breakdown by inhibiting leukostasis, rather than the other two hypotheses.

In conclusion, in this in vivo study, intravitreal corticosteroid attenuated leukostasis and BRB breakdown in diabetic retinal edema. The findings indicate that intravitreal corticosteroid may improve BRB breakdown and then diabetic retinal edema by inhibiting leukostasis.

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