



Diabetes has an additive effect on neural apoptosis in rat retina with chronically elevated intraocular pressure

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Title: Diabetes has an additive effect on neural apoptosis in rat retina

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慢性緑内障ラット網膜における神経細胞アポトーシス
への糖尿病の影響

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Keywords: diabetes; glaucoma; apoptosis; neural degeneration; rat

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Title page

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Abstract

Purpose: Diabetes mellitus (DM) is known as a risk factor for open-angle glaucoma, although the mechanistic interrelationship of the two opens to debate. The purpose of this study is to test whether DM augments neural apoptosis in rat retina with chronically elevated intraocular pressure (IOP).

Methods: Sprague-Dawley rats became diabetic by intraperitoneal injection of streptozotocin (STZ). At one month after STZ injection, three episcleral veins in one eyes were cauterized to elevate IOP. Rats without STZ injection were treated likewise as diabetic controls. At 2 weeks, 1 month, and 2 months after cauterization, the retina was dissected, flat-mounted, and subjected to terminal dUTP nick end labeling (TUNEL) staining. TUNEL positive cells per unit area of the whole retina were measured.

Results: DM did not affect base line IOP or augment IOP elevation due to episcleral vein cauterization. TUNEL positive cells, which primarily consisted of the neurons and glial cells in the inner retina including retinal ganglion cell (RGC), were counted consistently eight times more in the diabetic retina without IOP elevation than diabetic controls ($n=9$, $P<0.001$). The cauterization significantly elevated significantly elevated IOP up to 28.9 mmHg ($P<0.001$), which was reduced over time, and substantially induced apoptosis in a IOP-dependent fashion ($P<0.001$). Ocular hypertensive retinas with DM had significantly more TUNEL positive cells than those without DM

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despite of the similar time course of IOP changes ($P < 0.001$).

Conclusions: DM has an additive effect on apoptosis induction by chronic elevation of IOP.

Diabetes would act as a risk factor of open-angle glaucoma by increasing susceptibility of RGCs to apoptosis triggered by additional stresses such as elevated IOP.

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Glaucoma is an optic neuropathy characterized by progressive retinal ganglion cell (RGC) death, axon loss, and an excavated appearance of the optic nerve head. Increasing evidence has suggested that apoptotic process is involved in RGC death both in experimental models of glaucoma and in human eyes with glaucoma.¹⁻⁴ Elevated intraocular pressure (IOP) is an established risk factor to mediate glaucomatous optic neuropathy.⁵ By cauterizing the episcleral veins in rats, several independent groups demonstrated 2 to 3-fold increase in RGC loss during 2 to 4 months after IOP elevation.⁶⁻⁸

Apoptosis of the RGCs and inner nuclear layer (INL) cells is also accelerated in diabetic retinopathy even at very early course of disease. Barber and colleagues demonstrated that up to 10% of RGCs were lost 7.5 months after induction of diabetes in rats through the apoptosis process.

⁹ The pro-apoptotic BAX protein was induced in neuronal elements of retina in patients with diabetes.¹⁰ Clinically, retinal nerve fiber layer defect was observable in patients who simple diabetic retinopathy and have no vascular abnormalities yet¹¹.

In addition, glial function is also affected both in patients with glaucoma and those in diabetes, since GFAP immunoreactivity is altered,¹²⁻¹⁸ glutamate metabolism is disturbed,¹⁹⁻²¹ and activated microglia is increased in retina of animal models of glaucoma and diabetes.^{16,22,23} Therefore, we could say that both diseases interfere with neuro-glial interaction in retina.

In clinical settings, diabetes is recognized as a probable risk factor of glaucoma. Diabetes might

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decrease the aqueous outflow facility due to alterations of aqueous humor contents and of extracellular matrix components in trabecular meshwork²⁴, which would give rise to increased intraocular pressure (IOP) and subsequently develop the glaucomatous optic nerve damage. Another possibility is that diabetes might affect metabolism of the RGCs due to aberrant interaction of RGCs and glial cells with no effect on IOP, which would make RGCs susceptible to additional stresses relating to glaucoma such as elevated IOP.

In this study, we hypothesized that diabetes would directly accelerate cell death of neuronal components in glaucomatous retinas with no effect on IOP.

The purpose of this study was to test whether experimental diabetes increases the number of apoptosis of the neuronal cells in the rat retina with chronically elevated IOP induced by cauterization of episcleral veins.

Materials and Methods

Experimental Rat Model

Male Sprague-Dawley rats weighing 225-275g from CREA Japan (Osaka, Japan) were used in this study. All experiments were performed in accordance with the Animal Care Committee of the Kobe University Graduate School of Medicine and with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All animals were housed in an animal housing unit

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with ad libitum food and water and under a 12-h light/dark schedule.

Rats were dosed with intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, MO; 65 mg/kg) in 1mM sodium citrate buffer (pH4.5) to induce diabetes (n=33) or vehicle (n=33).

Developing diabetes was confirmed 1 week later by blood glucose >250 mg/dl, which was measured by the Lifescan meter during late afternoon. At one month after the induction of diabetes,

left eyes of all rats were subjected to induction of ocular hypertension (OH) under anesthesia by intraperitoneal injection of xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (10

mg/kg), essentially according to a method developed by Shareef et al.⁶ In brief, after conjunctival incision, three episcleral veins near the superior and temporal rectus muscles were cauterized by a

disposal ophthalmic cautery. The contralateral right eyes were sham-operated by conjunctival incision. The eyes were flushed with saline, and treated with antibiotic ointment. This model has the

advantage of giving consistent long-term IOP elevation without intraocular inflammation by blocking outflow facility of the aqueous humor.^{6,7} Two days later, IOPs were measured in both

eyes using a calibrated tonometer (Tonopen XL[®]; Mentor, Norwell, MA) under urethane anesthesia (0.6g/kg, intraperitoneally). The IOP measurements were repeated until the average of 4

to 5 consecutive values reached the level with a coefficient of variation less than 5 %. The reason for using urethane was that general anesthesia using xylazine, kaetamine or acepromazine leads to

rapid and substantial decrease in IOP. It was reported that these drugs caused approximately

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40~50% reduction of IOP within 5 minutes.²⁵ In contrast, urethane is reported to have a least effect on IOP reduction (Chen et al, ARVO 2002). The above combination of treatment for induction of diabetes and OH could established 4 groups of retina, which were divided into a totally control group (CNT), a group of diabetes without IOP elevation (DM), a group of OH without diabetes (OH), and a group of both diabetes and IOP elevation (DM+OH). A schema of our experimental design is shown in Fig. 1.

Retina preparation and Terminal dUTP nick end labeling (TUNEL) staining

Rats were sacrificed at 2 weeks, 1 month and 2 months after cauterization (n=11, per group) by under deep ether anesthesia. Before sacrifice, all rats were weighed, and blood glucose and IOP were measured under urethane anesthesia. Immediately after sacrifice, either retina was dissected from eyeballs for whole mount preparation or eyeballs were enucleated, embedded in Tissue-Tek OCT compounds (miles Laboratories, Elkhart, IN, U.S.A), snap frozen, and stored at -80°C until use for thin-section histology.

Terminal dUTP nick end labeling (TUNEL) staining was performed with horseradish peroxidase (TUNEL-HRP) detection in whole mount retinas (n=9, per group) or $10\mu\text{m}$ cryosections (n=2, per group) using ApopTag Peroxidase In Situ Apoptosis Detection (Intergen Co., Purchase, NY),

essentially according to the methods by Barber et al.⁹

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Isolated retinas were fixed in 10% normal buffered formalin for 10 min at room temperature, washed in phosphate buffered saline twice, flat-mounted on microscope slides coated with 2% 3-aminopropyltriethoxy silane, and stored at -20°C until use. The retinas were fixed in formalin for 10 min, dehydrated through graded alcohols, and defatted in xylene overnight to allow better penetration across inner limiting membrane of retina. Next day, the retinas were rehydrated, permeabilized with 0.3% Triton for 15 min, and digested with 20 $\mu\text{g/ml}$ proteinase K (Sigma, St Louis, Mo). Endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min. The retina was then catalyzed with terminal transferase to tail single or double strand 3'OH-ends of fragmented DNA molecules with digoxigenin-11-dUTP and dATP, which were recognized with an HRP-bound antidigoxigenin antibody. The HRP was reacted with 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO), a substrate producing an end product red in color.

Cryostat retinal sections of 10 μm -thickness were made, collected onto silanized slides (DAKO, Kyoto, Japan), and fixed in 1% paraformaldehyde overnight. Following fixation in ethanol and acetic acid, the slides were subjected to the TUNEL-HRP staining as described above and were counterstained with 0.5 $\mu\text{g/ml}$ bisbenzimidazole Hoechst 33258 (Sigma, St Louis, Mo).

Data collection and analysis

The comparison of body weight and blood glucose between STZ-injected and control rats at each

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sacrifice point were made by Mann-Whitney nonparametric test (Table 1). Time courses of changes in IOP between cauterized and untreated eyes with (Fig. 2A) or without diabetes (Fig. 2B) were also compared by Mann-Whitney nonparametric test.

Retinal images were captured on a personal computer linked to a digital camera (DP 50, Olympus Optical CO., Tokyo, Japan) mounted on a microscope (AX 80, Olympus Optical CO., Tokyo, Japan) equipped with epifluorescent illumination. For detection of TUNEL positive cells, each retina was visually scanned with a high power (40X) objective under microscope. The total number of TUNEL-HRP positive cells was counted for each whole mounted retina in a masked fashion. The entire retina area was measured by tracing the outline of each retinal image using a image analysis system (Micro Analyzer, Japan poladigital CO, Tokyo, Japan). The number of TUNEL-HRP positive cells was expressed per unit area of 0.5 cm^2 . Time courses of changes in those numbers among 4 differently treated retinal groups were analyzed using two-way non repeated measures ANOVA with Bonferroni/Dunn test.

For the cryosections, TUNEL staining images were superimposed to Hoechst nuclei staining images captured under an ultraviolet filter to localize the apoptotic cells in retinal 10 layers.

All statistical values were judged significant if a P value was less than 0.05.

Results

Effects of streptozotocin (STZ) injection and cauterizaion of episcleral veins on general conditions and IOP in rats

STZ-diabetic rats gained significantly less weight than the age-matched controls (Table). The blood glucose in STZ-injected rats was significantly higher than that in the control rats at all time points tested ($P<0.001$) (Table).

Mean \pm SD of IOP in retina without cauterization was 13.9 ± 2.0 mmHg in non-diabetic rats and 13.4 ± 2.1 mmHg in diabetic rats at induction of OH with no statistical difference ($P=0.433$). These values were kept constant during the entire experimental period. Thus, diabetes alone did not influence base line IOP. In contrast, cauterizaion of three episcleral veins resulted in sustained increase in IOPs up to 8 weeks after treatment irrespective of presence or absence of diabetes (Fig. 2). As soon as 2 days after cauterization, the IOPs were elevated up to 29.0 ± 5.1 mmHg in non-diabetic rats and 28.7 ± 4.1 in diabetic rats (Fig.2). When compared with the IOPs in the untreated eyes, the increase was statistically significant ($P<0.001$) both in non-diabetic and diabetic rats (Fig.2). In comparison, there was no significant difference of IOPs in cauterized eyes between non-diabetic and diabetic rats. Although the elevated IOPs gradually decreased over time, the values were kept significantly higher as compared with untreated eyes during the entire periods tested (Fig. 2). At the end of time course, the IOPs of cauterized eyes in non-diabetic and diabetic rats were 18.1 ± 2.3 and 18.1 ± 2.1 mmHg, respectively, which, again, had no significant difference.

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(Fig2). Thus, diabetes did not affect IOP elevation induced by episcleral cauterization and the characteristic reduction of IOP with time was very similar between in non-diabetic and diabetic rats.

Effects of diabetes and chronic elevation of IOPs on apoptosis in retina

In eyes without episcleral cauterization taken from non-diabetic rats (CNT), there were few TUNEL positive cells in the entire area of retina

In contrast, diabetes and/or OH significantly increased the TUNEL positive cells. Figures 3 and 4 show the representative pictures of TUNEL staining in whole mount retina and in cryostat sections, respectively. TUNEL positive cells were indicated by a burgundy precipitate staining nuclei substrated with AEC and were easily recognized against negligible background staining (Fig.3and 4). The majority of these cells were distinct from observable vasculature as previously reported (Fig.3B).⁸ The TUNEL positive cells had a variable size of nuclei. Those having larger bodies were mainly located in superficial planes of microscopic focus in flat-mount retina (Fig.3C) and in the ganglion cell layer (GCL) in the cryostat sections (Fig.4), whereas those having smaller bodies resided in deeper planes of focus in the whole mount (Fig.3C) and in INL in the sections (Fig.4). Thus, TUNEL positive cells included RGCs, other neuronal cells, and possibly Müller cells.

Time courses of changes in the number of TUNEL positive cells in entire retina of each group are shown in Fig. 5. In control, untreated retinas (CNT), there were 4.4 ± 1.7 cells/ 0.5 cm^2 retina,

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whereas diabetes (DM) led to 8-9 fold increase in the number of TUNEL positive cells ($33.3 \pm 15.1 / 0.5 \text{ cm}^2$) 1 month after STZ injection, which were consistent throughout the periods tested without significant fluctuation. Episcleral vein cauterization alone (OH) substantially increased the number of TUNEL positive cells 2 weeks after treatment ($111.5 \pm 36.8 / 0.5 \text{ cm}^2$), which decreased over time comparably with the time course of IOP reduction (Figure 2 & 5). At 8 weeks after cauterization, the number was tapered to similar levels as counted in the untreated retina of diabetic rats (DM), but was still significantly more than control retina (CNT) (one-way ANOVA with Bonferroni/Dunn test, Fig.5).

Retinas taken from cauterized eyes of diabetic rats (OH+DM) had further more TUNEL positive cells than the counterparts of control rats (OH) at any tested points with a statistical significance (two-way non repeated ANOVA with Bonferroni/Dunn test, $P < 0.001$) (Fig.5). The number of TUNEL positive cells in the OH+DM group was reduced over time again along with IOP reduction. The reduction rate of TUNEL positive cell number per 1-mmHg IOP reduction during 2 and 8 weeks of OH induction was calculated to be $11.7 \text{ cells} / 0.5 \text{ cm}^2 / \text{mmHg}$ in the OH group and $13.9 \text{ cells} / 0.5 \text{ cm}^2 / \text{mmHg}$ in OH+DM group. Thus, apoptosis in eyes with OH and DM comprised IOP-dependent and independent mechanisms, reflected the apoptosis process induced by the respective single etiologies.

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Discussion

The current study clearly demonstrates the following four findings. Firstly, sustained elevation of IOP induced by episcleral vein cauterization increased the number of TUNEL positive cells in the rat retina, which was reversed over time along with the IOP reduction. Second, the increased number of the TUNEL positive cells appeared in the retina of diabetic rats by means of streptozotocin injection with a relatively constant rate throughout the entire experiment period. When the cell size and location were considered, these cells were not primarily vascular components in origin, but neurons in the inner retina including the RGCs and, to some proportion, glial cells. These two findings are in a good agreement with previous reports.^{9,26} Third, diabetes did not increase IOP both at base line and after episcleral vein cauterization. The slope of IOP reduction with time in non-diabetic and diabetic rat retinas was remarkably similar. Finally, the amount of apoptosis was augmented by combined inductions of diabetes and ocular hypertension above the amount due to each treatment alone and was reduced over time with a similar rate as seen in retina with ocular hypertension alone.

Thus, diabetes accelerated the cell death in the neuronal components in ocular hypertensive retina with no direct effect on IOP at least in the current experimental paradigm.

There are a couple of possible explanations for the additive effect of diabetes and ocular

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hypertension on apoptosis. First, the two conditions would share the common cell death pathways and augment each other's effect. Second, diabetes and ocular hypertension might induce apoptosis of distinct neural cell types. Third, the two conditions might cause apoptosis of the same cell types but use different apoptotic pathways. To the present, which of these three hypotheses is the case could not be determined, because we don't know the precise mechanism of cell death or specify cells committed to or undergoing to apoptosis due to the two conditions tested. A major reason for this is the technical limitation. We basically used TUNEL staining of whole-mounted retina to evaluate the exact number of dying cells principally through apoptosis mechanism. This methodology is accurate in counting dying cells in entire retina, whereas the precise location and types of cells of interest cannot be specified. Although histological sections are readily prepared and useful to detect the location of dying cells within the retinal 10 layers, it is impossible to calculate the whole number of cell death because of the extremely narrow range of observation area. Retrograde labeling of RGCs by amidine dyes such as fluorogold and fast blue is also widely used to count the degenerative cell number in experimental glaucoma models. Although this method is superior to the TUNEL staining in assessing RGC death, it is still impossible that contamination of activated microglia is eliminated.^{22,27} Co-localization of retrograde labeling and TUNEL staining in flat mount retina was not practically applicable because the extensive exposure to xylene during fixation of the flat-mounted retina washed the amidine dyes out (data not shown).

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Strictly speaking, we cannot rigorously conclude whether the cell death demonstrated in the current experiments was exclusively mediated through the apoptotic process, either, since the TUNEL staining is known to recognize necrotic cells in some situations.

Despite of the above constraints, the current findings have significant implication when the association of diabetes with glaucoma is taken into consideration. Epidemiological studies show no confirmative evidence whether diabetes is a risk factor for the development or progression of glaucoma.²⁸⁻³² Some studies have suggested a higher prevalence of mean IOP among person with diabetes.^{29,33} The current study raised another possibility that co-existence of diabetes and ocular hypertension gives rise to enhanced cell death in the neuronal components in retina. As mentioned earlier, diabetes affects neuro-glial function in the retina even at the very early time course without discernible vascular abnormalities. In this regard, diabetes could be a “risk factor” of glaucomatous optic neuropathy by making RGCs susceptible to additional stresses such as elevated IOP, deprived neurotrophic support,³⁴⁻³⁶ reactive oxygen species,³⁷⁻³⁹ or glutamate toxicity,^{40,41} which are thought to be triggers for RGC apoptosis in glaucoma.

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Figure legends

Figure 1: A schematic representation of generating animal models used in this study. *OH*, ocular hypertension; *DM*, diabetes mellitus; *IOP*, intraocular pressure.

Figure 2: Effects of cauterization of episcleral veins on intraocular pressure (IOP)s (mean \pm SD). **A.** diabetic rats **B.** non-diabetic rats. *DM*, diabetes mellitus; *OH*, ocular hypertension; *CNT*, controls; *, $P < 0.001$ (Mann-Whitney nonparametric test).

Figure 3: Representative terminal dUTP nick end labeling (TUNEL) staining of a whole-mounted retina taken from a 2-weeks ocular hypertensive eye of a diabetic rat. **A.** Overview of the flat-mounted retina with low magnification. **B.** A TUNEL positive cell (*black arrow head*) is distinct from the surrounding vasculature (*arrow*). **C.** TUNEL-positive cells with variable sizes residing in different planes of focus. Larger cells (*black arrow head*) in the most superficial focus, while smaller ones (*white arrow head*) in deeper plane.

Figure 4: Representative pictures of cryo-sections of retinas of ocular hypertension alone (**A, B**) and those of both diabetes and ocular hypertension (**C, D**). **A** and **C**; TUNEL staining visualized under light microscope. **B** and **D**; merged images of **A** and **B** with Hoechst 33258 counter staining,

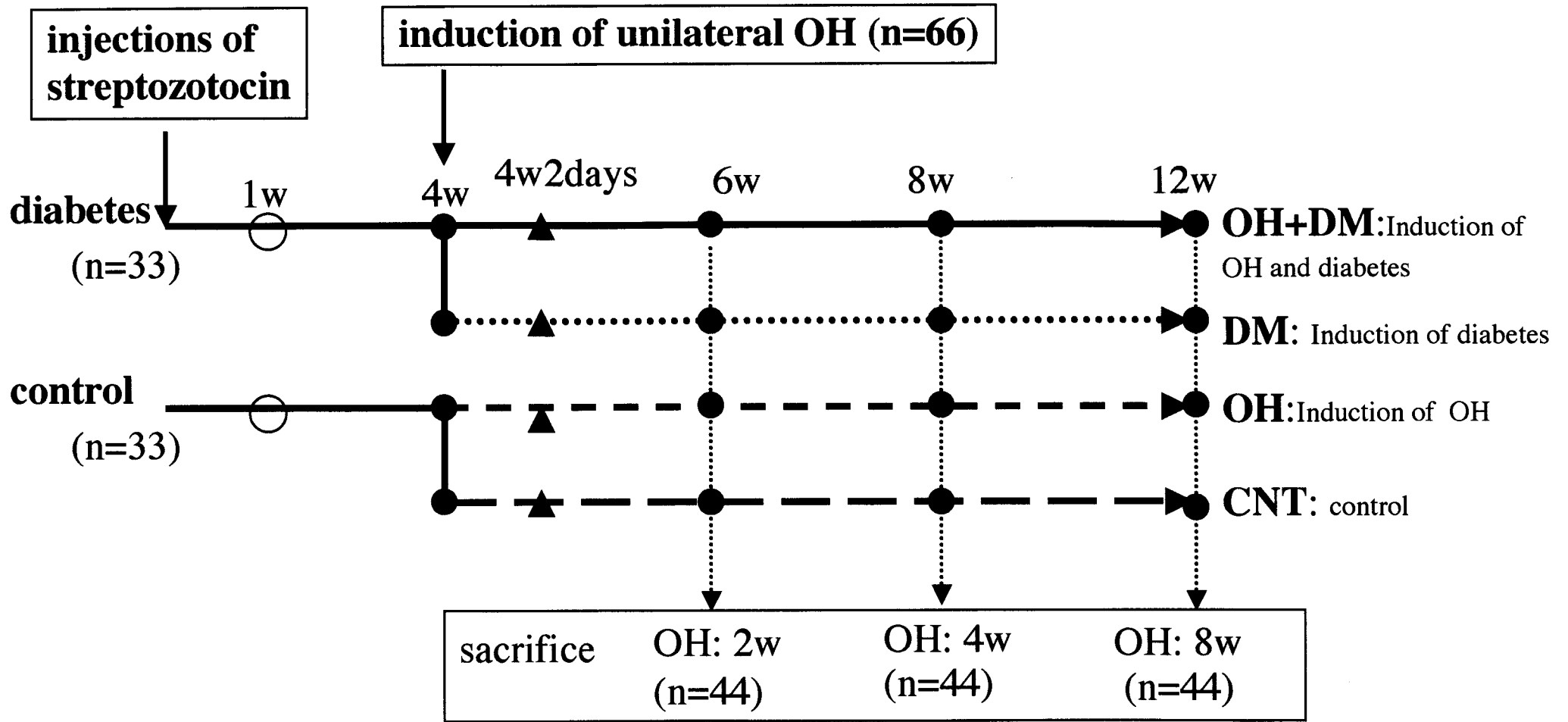
Apoptosis of diabetes and ocular hypertension

respectively, to localize apoptotic cells in the retinal layers. Arrows indicate TUNEL positive cells.

GCL, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer.

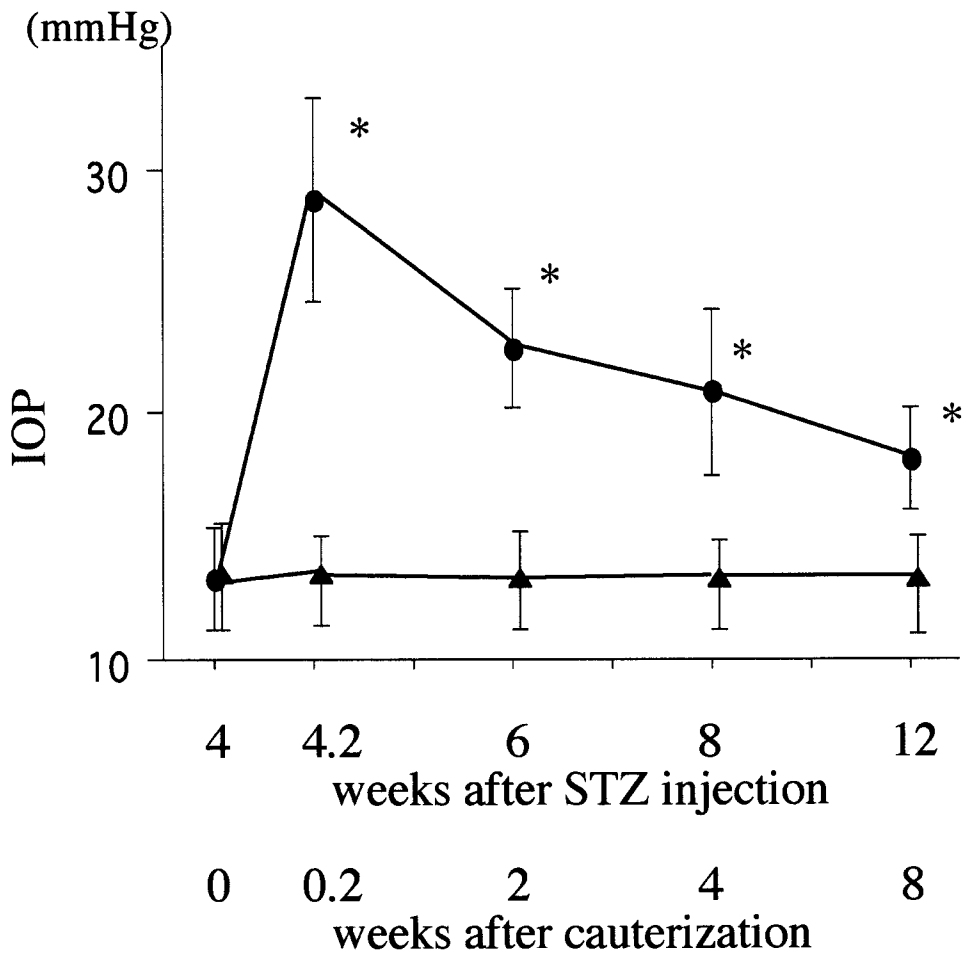
Figure 5: Time courses of changes in the number of TUNEL positive cells per unit of retina in 4 different treatment conditions. As compared with control retinas (CNT), retinas taken from rats with streptozotocin injection (DM) and ocular hypertensive retina (OH) show significantly more TUNEL positive cells (two-way ANOVA with Bonferroni/Dunn, DM;*P<0.001,OH; †P<0.001). Cauterized retinas of diabetic rats (DM+OH) have more TUNEL positive cells than those of non-diabetic rats (OH) (two-way ANOVA with Bonferroni/Dunn, ‡P<0.001). *DM*, diabetes mellitus; *OH*, ocular hypertension.

Table: Summary of blood glucose and body weight in control and diabetic rats.

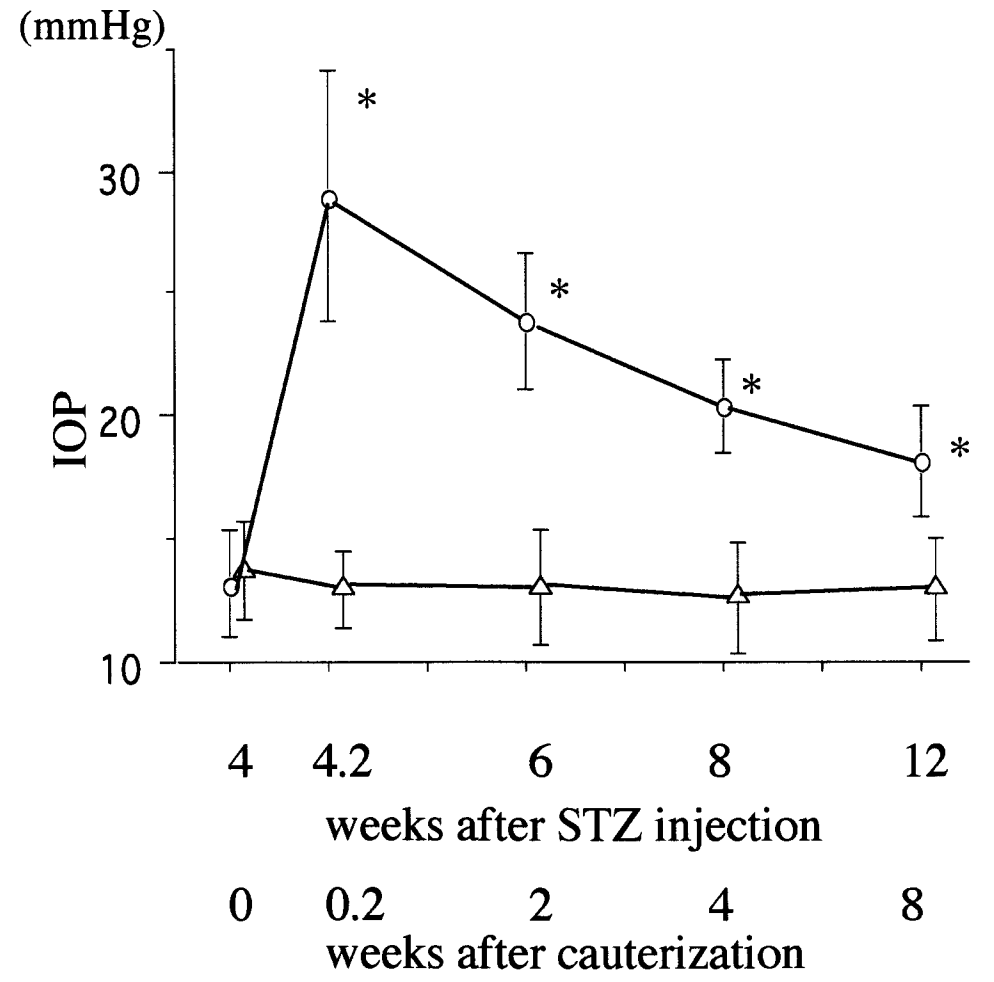


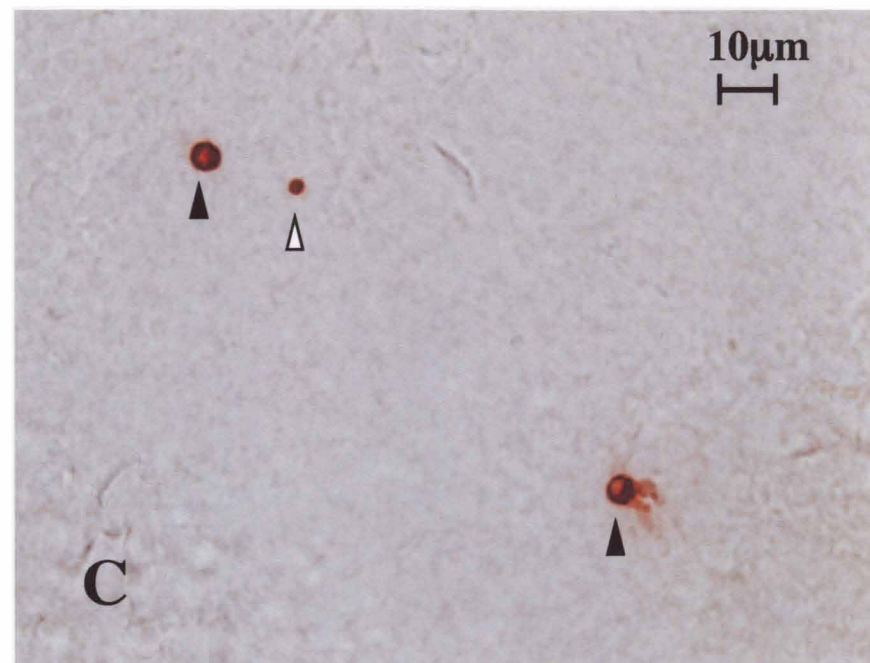
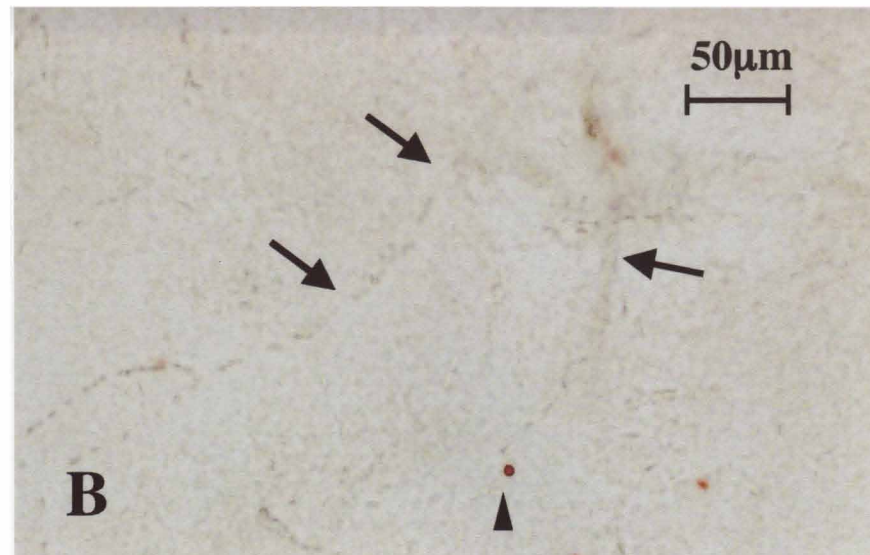
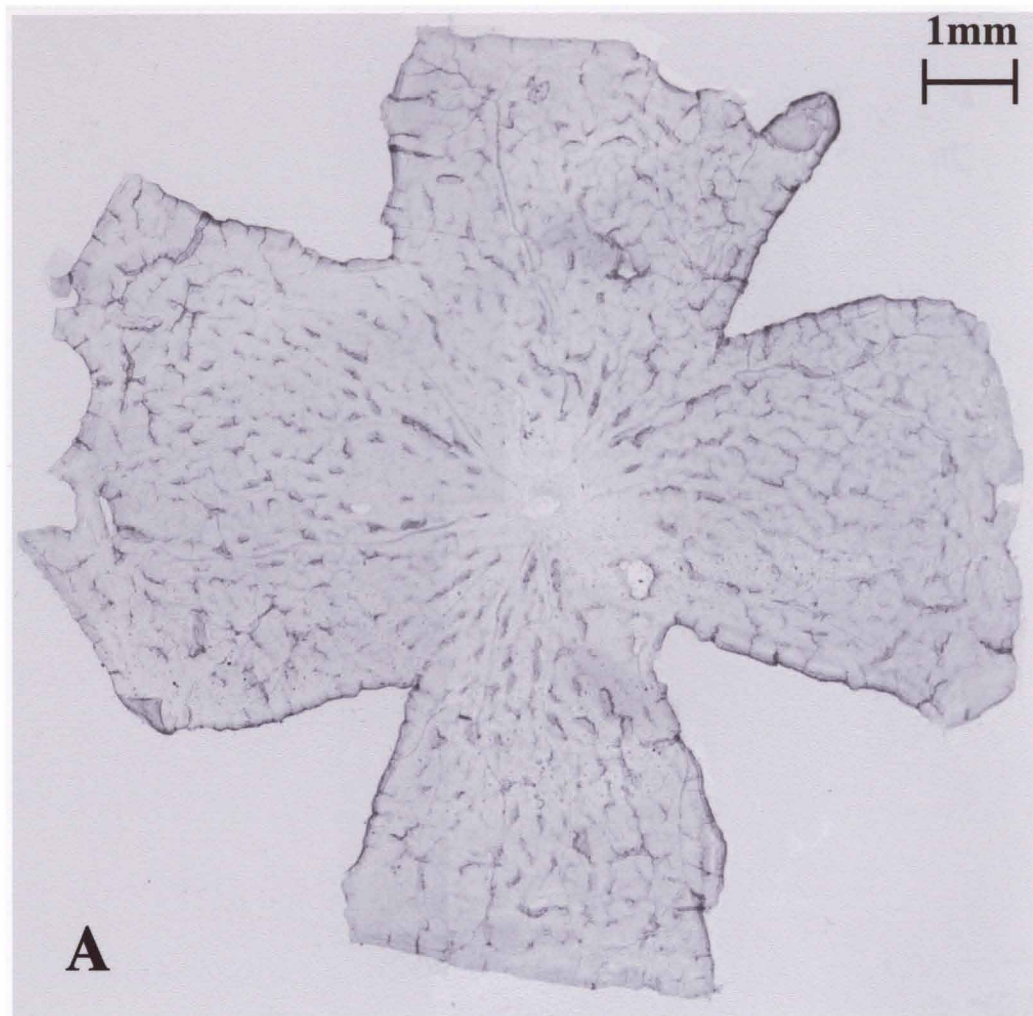
- measurements of blood glucose
- ▲ measurements of IOPs
- measurements of blood glucose, body weight and IOPs

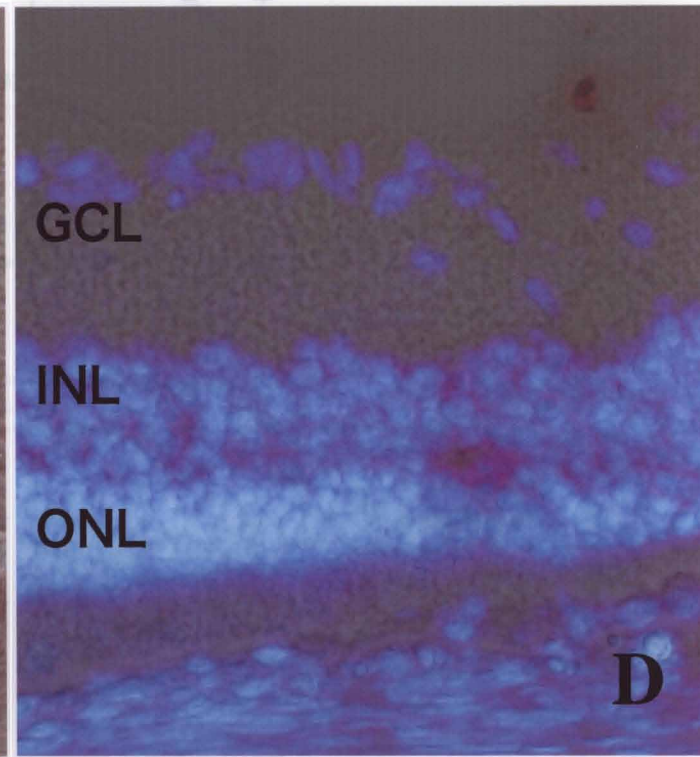
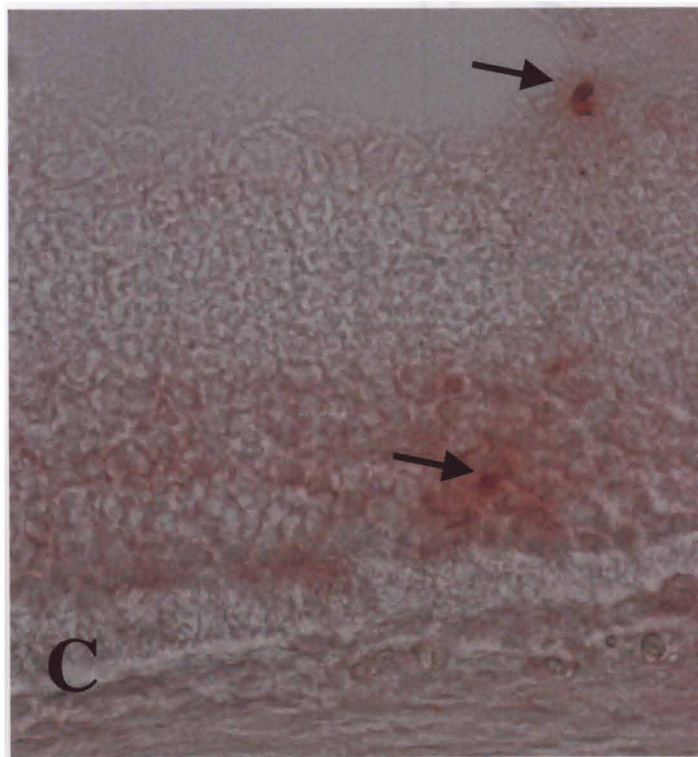
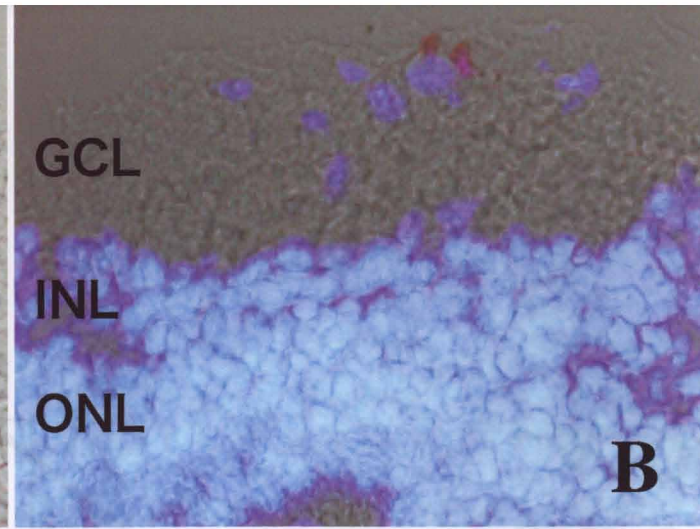
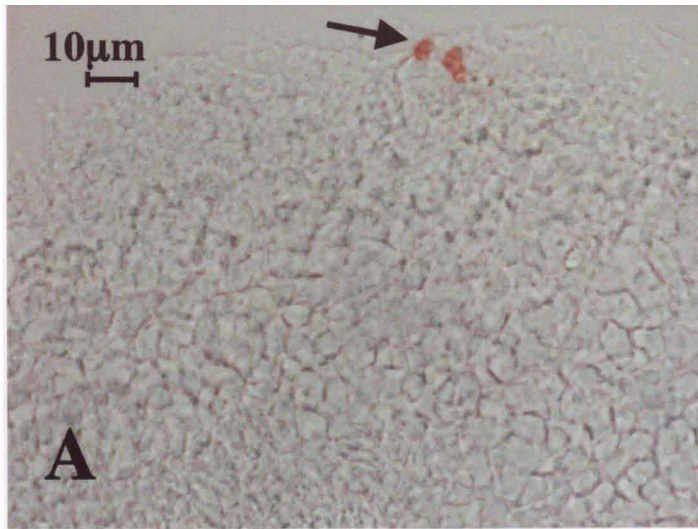
A ● cauterized eyes (DM+OH)
 ▲ contralateral eyes (DM)

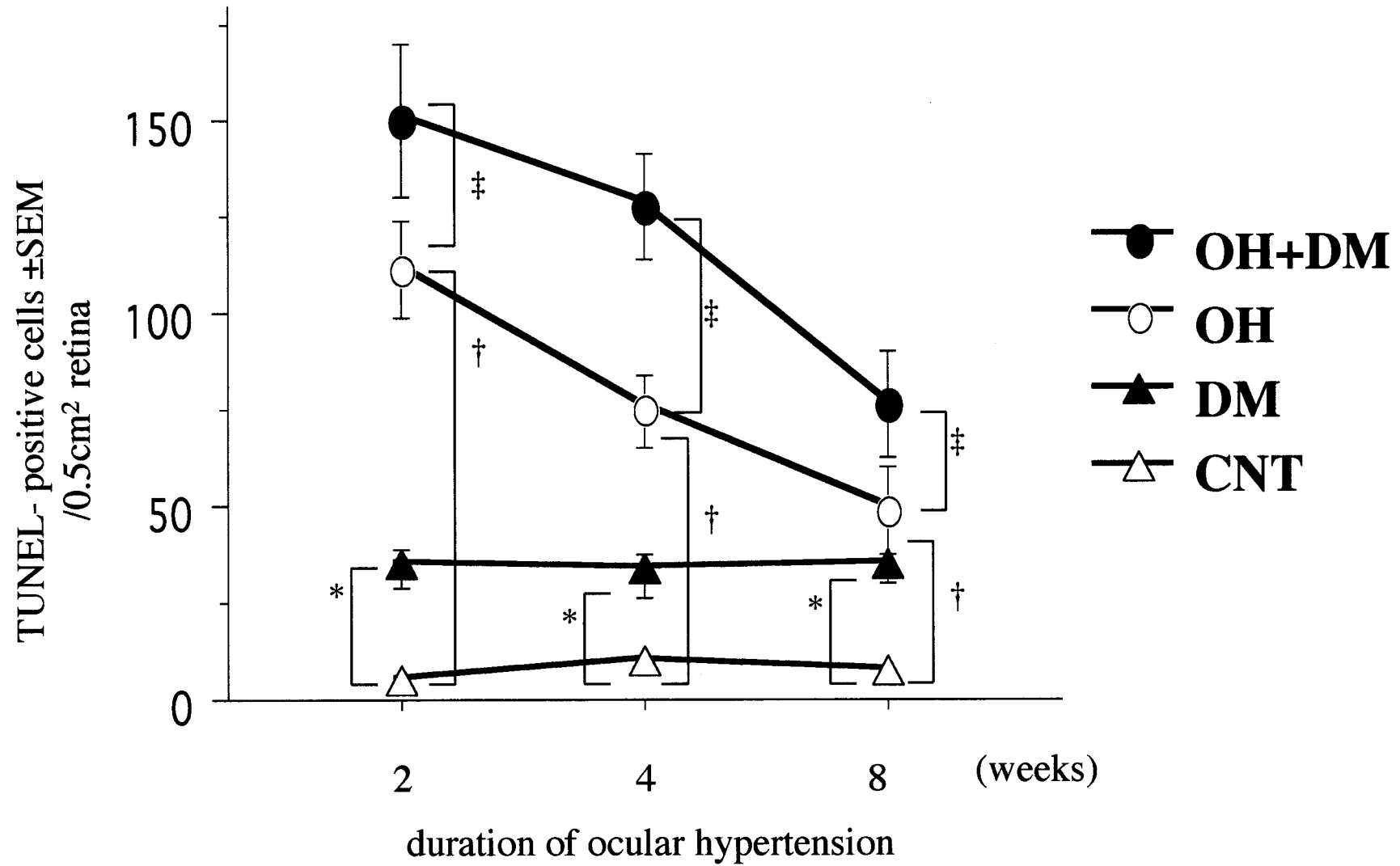


B ○ cauterized eyes (OH)
 ▲ contralateral eyes (CNT)









Duration of diabetes (weeks)	Duration of OH (weeks)		n	Blood glucose \pm SD (mg/dL)		Body weight \pm SD (g)
6	2	diabetes	11	398.6 ± 95.6] *	359.5 ± 35.8
		control	11	112.4 ± 8.1		465.6 ± 65.8] *
8	4	diabetes	11	457.3 ± 78.7] *	365.2 ± 40.5
		control	11	114.0 ± 10.3		556.3 ± 51.1] *
12	8	diabetes	11	495.1 ± 30.0] *	384.0 ± 79.4
		control	11	113.5 ± 9.4		643.4 ± 41.9] *

OH:ocular hypertension

*, P<0.001; Mann-Whitney U-test