

# Aerobic Exercise Increases Tear Secretion in Type 2 Diabetic Mice

Kokoro Sano,<sup>1</sup> Motoko Kawashima,<sup>1</sup> Akiko Ito,<sup>1</sup> Takaaki Inaba,<sup>1</sup> Kohkichi Morimoto,<sup>2</sup> Mitsuhiro Watanabe,<sup>3</sup> and Kazuo Tsubota<sup>1,3</sup>

<sup>1</sup>Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan

<sup>2</sup>Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

<sup>3</sup>Health Science Laboratory, Keio Research Institute at Shonan-Fujisawa Campus, Keio University, Tokyo, Japan

Correspondence: Motoko Kawashima, Department of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 1608582, Japan; motoko-k@a3.keio.jp.

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**PURPOSE.** To investigate the effects of exercise on tear secretion in type 2 diabetic mice, and to investigate the effect of the adenosine monophosphate-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR).

**METHODS.** Heterozygous controls (db/m; m *Lepr<sup>db</sup>*) and type 2 diabetic mice (db/db; *Lepr<sup>db</sup>*) either underwent forced treadmill exercise training 5 days a week or remained sedentary for 8 weeks. Tear secretion volume was measured by cotton threads for 30 seconds pre- and post intervention. The levels of oxidative stress markers (8-hydroxy-2'-deoxyguanosine [8-OHdG], propanoyl lysine [PRL], and hexanoyl lysine [HEL]) in tears were measured in the postintervention period. Furthermore, C57BL/6Jc1 mice, db/db mice, and db/m mice received a single intraperitoneal injection of AICAR or PBS each day for 5 days, and tear secretion volume was measured.

**RESULTS.** Exercise training for 8 weeks increased tear secretion volume in db/m and db/db mice. The levels of oxidative stress markers in tears were less in the exercise group than in the control group. In C57BL/6Jc1 mice, the tear secretion volumes in both the AICAR 125 mg/kg and AICAR 250 mg/kg groups were significantly larger than in the PBS group ( $P < 0.01$  and  $P < 0.01$ , respectively). Additionally, in db/db mice, tear secretion volume in the AICAR 125 mg/kg group was also significantly larger than in the PBS group ( $P < 0.05$ ).

**CONCLUSIONS.** Exercise training for 8 weeks and a daily injection of AICAR for 5 days increased tear secretion in mice. The results suggest that exercise may be a potential therapy to modulate tear secretion.

**Keywords:** tear secretion, db/db mice, exercise, AICAR

Recently, the incidence of type 2 diabetes mellitus has markedly risen.<sup>1</sup> Behind this increase, there are lifestyle factors such as changes in food intake and lack of exercise.<sup>2</sup> Exercise training is believed to improve the status of patients with type 2 diabetes mellitus, and exercise training is also recommended to increase energy expenditure to assist in weight loss and the treatment of obesity.<sup>3</sup> In experimental models, it has been reported that treadmill exercise significantly decreased diabetes-induced blood glucose and serum corticosteroid levels<sup>4</sup>; exercise training also increased serum adiponectin and decreased serum insulin and glycosylated hemoglobin levels.<sup>5</sup>

There is a potential association between the mechanisms of these effects, such as improved insulin action after exercise, and adenosine monophosphate-activated protein kinase (AMPK), which is a key regulator of energy metabolism and has been proposed as an extremely sensitive indicator of cellular energy status. More recent data strongly suggest that AMPK has a wider role in metabolic regulation.<sup>6–8</sup> AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) is a known activator of AMPK and can be used as an experimental tool to activate AMPK in vivo.

On the other hand, it is widely known that diabetes mellitus leads to major chronic complications, including microvascular diseases such as cardiovascular disease. A number of ocular complications also accompany diabetes mellitus, including

retinopathy and cataracts, as well as dry eye disease. It was reported that tear function is significantly lower in diabetic patients than in controls.<sup>9</sup> It was also reported that the decline in tear film function is more severe in humans with proliferative diabetic retinopathy than in those with nonproliferative diabetic retinopathy.<sup>10</sup> In experimental models, it was reported that the tear secretion of type 2 diabetic Goto-Kakizaki rats was less than that of Wistar rats.<sup>11,12</sup>

Accordingly, we hypothesized that exercise training would affect tear secretion in diabetic patients. Thus, we investigated decreased tear volume in a mouse model of diabetes, and whether its decrease could recover with exercise training. Furthermore, we attempted AICAR treatment, which is a key factor in exercise.

## MATERIALS AND METHODS

### Animals

All procedures undertaken in the present study conformed to the principles outlined in the Guide for the Care and Use of Laboratory Animals published in 2010 by the US National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Keio University School of

TABLE 1. Body Weight and Blood Glucose in db/M and db/db Mice With Exercise Training

	db/m	db/m + Ex	db/db	db/db + Ex
Pre-experimental body weight, g, mean $\pm$ SD	21.8 $\pm$ 1.1	21.9 $\pm$ 1.2	35.0 $\pm$ 2.1*	34.9 $\pm$ 1.7*
Postexperimental body weight, g, mean $\pm$ SD	25.0 $\pm$ 1.9	23.7 $\pm$ 1.1	53.4 $\pm$ 3.7*	48.5 $\pm$ 1.9*†
Pre-experimental blood glucose, mg/dL, mean $\pm$ SD	133.4 $\pm$ 13.7	133.0 $\pm$ 13.8	231.8 $\pm$ 61.3*	265.8 $\pm$ 87.4*
Postexperimental blood glucose, mg/dL, mean $\pm$ SD	131.2 $\pm$ 13.2	130.4 $\pm$ 18.7	404.8 $\pm$ 53.3*	329.0 $\pm$ 89.7*‡

\*  $P < 0.01$  for the db/m group versus db/db and db/db + Ex groups.

†  $P < 0.01$  for the db/db group versus db/db + Ex group.

‡  $P < 0.05$  for the db/db group versus db/db + Ex group.

Medicine (approval number: 08067). All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Seven-week-old female heterozygous control mice (db/m; m Lepr<sup>db</sup>; background strain: C57BLKS/J) and homozygous type 2 diabetic mice (db/db; Lepr<sup>db</sup> background strain: C57BLKS/J) were obtained from CLEA Japan, Inc. (Tokyo, Japan) and housed in an atmosphere-controlled room conditioned with 12 hour light/dark cycles, and fed ad libitum with CE-2 (CLEA Japan, Inc.). After a week-long weaning period, control (db/m) and db/db mice were randomly divided into exercise training (Ex) and sedentary groups. In addition, for the AICAR administration experiments, C57BL/6Jc1 mice, db/db, and db/m mice were obtained from CLEA Japan, Inc. Mice were 10 to 13 weeks of age at the time of the experiments.

## Experimental Protocol

**Exercise Training Test.** Treadmill training consisted of low intensity exercise 5 days a week on an electrically driven treadmill (LE8710M; PanLab, Barcelona, Spain) for a period of 8 weeks.<sup>13,14</sup> The training regimen involved a 2-week graded increase in exercise duration as follows: week 1, 10 minutes at 10 m/min; and weeks 2 through 8, 20 minutes at 10 m/min. Body weight and tear secretion were recorded once a week for all animals. Blood glucose levels were recorded once every 4 weeks. At the end of the exercise training protocol and 24 hours after the last exercise session, mice were dissected after euthanization. Lacrimal gland weights were measured at 16

weeks, and serum samples were collected for biochemical analysis by ORIENTAL YEAST (Tokyo, Japan).

**AICAR Administration Test.** To investigate the effect of AICAR on tear secretion, C57BL/6Jc1 mice were divided into three groups: PBS, AICAR 125 mg/kg, and AICAR 250 mg/kg. The PBS group received a single intraperitoneal injection of PBS, while the AICAR groups received a single intraperitoneal injection of AICAR 125 mg/kg or 250 mg/kg (Toronto Research Chemicals, Toronto, ON, Canada) each day for 5 days.

Next, to investigate the effect of AICAR in diabetic mice, db/db mice and db/m mice were divided into three groups: the db/m (PBS) group, the db/db (PBS) group, and the db/db + AICAR group. The PBS groups of db/m mice and db/db mice received a single intraperitoneal injection of PBS, while the AICAR group of db/db mice received a single intraperitoneal injection of AICAR 125 mg/kg per day for 5 days. The body weight and blood glucose of all animals were recorded on day 0 and day 5. Serum samples were collected for biochemical analysis by ORIENTAL YEAST (Tokyo, Japan).

## Exercise Tolerance Test

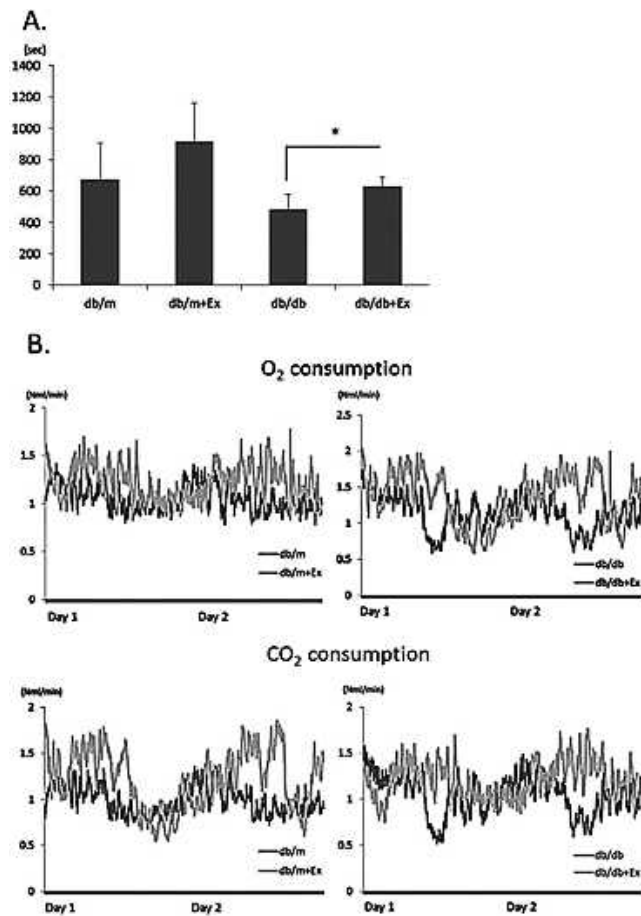
Running endurance was assessed on a treadmill. Mice commenced running at 10 m/min for 2 minutes and the speed was increased by 2 m/min every 2 minutes until exhaustion. Exhaustion was defined as the mouse spending 10 seconds at the base of the treadmill without attempting to recommence running with manual prompting. We measured endurance at 16 weeks.

TABLE 2. Serum Parameters in db/m and db/db Mice With Exercise Training

Serum Parameters	db/m	db/m + Ex	db/db	db/db + Ex
TP, g/dL	4.0 $\pm$ 0.1	4.1 $\pm$ 0.2	5.1 $\pm$ 0.3†	5.0 $\pm$ 0.3†
ALB, g/dL	2.9 $\pm$ 0.13	2.8 $\pm$ 0.3	3.3 $\pm$ 0.2*	3.3 $\pm$ 0.2*
Na, mEq/L	152.0 $\pm$ 3.2	153.8 $\pm$ 3.3	153.3 $\pm$ 2.1	155.0 $\pm$ 3.5
K, mEq/L	4.0 $\pm$ 0.4	4.9 $\pm$ 1.7	4.3 $\pm$ 0.5	5.0 $\pm$ 2.4
Cl, mEq/L	111.6 $\pm$ 3.0	112.2 $\pm$ 2.2	105.0 $\pm$ 2.6†	107.8 $\pm$ 6.3
Ca, mg/dL	8.0 $\pm$ 0.1	7.8 $\pm$ 1.0	8.3 $\pm$ 0.4	8.8 $\pm$ 1.0
IP, mg/dL	6.9 $\pm$ 1.5	8.9 $\pm$ 4.8	10.8 $\pm$ 1.4†	10.9 $\pm$ 2.5*
AST, IU/L	72.4 $\pm$ 58.5	83.8 $\pm$ 40.3	228.5 $\pm$ 130.6*	133.5 $\pm$ 53.3
ALT, IU/L	12.6 $\pm$ 4.2	16.6 $\pm$ 3.5	226.3 $\pm$ 150.2*	124.5 $\pm$ 73.9*
LDH, IU/L	566.4 $\pm$ 595.3	700.8 $\pm$ 512.4	1169.5 $\pm$ 683.8	520.3 $\pm$ 147.0
AMY, IU/L	1501.0 $\pm$ 147.7	1649.4 $\pm$ 260.0	2781.3 $\pm$ 309.2†	2785.5 $\pm$ 339.9†
T-CHO, mg/dL	67.4 $\pm$ 1.7	62.6 $\pm$ 9.2	132.5 $\pm$ 20.6†	125.0 $\pm$ 6.8†
F-CHO, mg/dL	13.8 $\pm$ 0.8	14.0 $\pm$ 1.7	25.3 $\pm$ 5.0†	23.5 $\pm$ 3.1†
E-CHO, mg/dL	53.6 $\pm$ 1.8	48.6 $\pm$ 7.7	107.3 $\pm$ 15.9†	101.5 $\pm$ 3.9†
E/T, %	79.6 $\pm$ 1.1	77.6 $\pm$ 1.8	81.3 $\pm$ 1.3	81.3 $\pm$ 1.5
TG, mg/dL	42.6 $\pm$ 14.3	31.2 $\pm$ 6.4	72.5 $\pm$ 29.9	111.0 $\pm$ 85.0
LDL-C, mg/dL	6.6 $\pm$ 1.8	8.2 $\pm$ 2.3	7.5 $\pm$ 1.3	6.0 $\pm$ 1.6
HDL-C, mg/dL	37.6 $\pm$ 1.5	34.8 $\pm$ 4.3	77.3 $\pm$ 10.3†	76.8 $\pm$ 3.8†
TBA, $\mu$ mol/L	2.2 $\pm$ 0.4	2.2 $\pm$ 0.8	1.8 $\pm$ 0.5	2.3 $\pm$ 1.0

\*  $P < 0.05$ .

†  $P < 0.01$ , mean  $\pm$  SD.



**FIGURE 1.** The exercise ability of db/m and db/db mice with exercise training. The time to exhaustion during treadmill exercise was significantly longer in the db/db + Ex group compared with the db/db group. In db/m mice, the db/m + Ex group tended to demonstrate longer values compared with the db/m group (A). O<sub>2</sub> consumption and CO<sub>2</sub> consumption increased in the exercise groups (B). \* $P < 0.05$ ,  $N = 5$ , mean  $\pm$  SD.

## Measurements of O<sub>2</sub> Consumption (VO<sub>2</sub>) and CO<sub>2</sub> Consumption (VCO<sub>2</sub>)

VO<sub>2</sub> and VCO<sub>2</sub> values were determined using a mouse metabolism measurement system (ARCOSYSTEM, Chiba, Japan) for 2 days. We measured metabolism at 16 weeks.

## Measurement of Tear Secretion Volume (Cotton Thread Test)

A phenol red-impregnated thread was placed on the temporal side of the lower eyelid margin for 30 seconds, as described previously.<sup>15</sup> The length of the moistened fragment was measured.

## Measurement of 8-OHdG, HEL, and PRL Concentrations in Tear Secretions

Tear moisture fragment samples were collected from each group ( $N = 4-5$ ). The 8-hydroxy-2'-deoxyguanosine (8-OHdG), propanoyl lysine (PRL), and hexanoyl lysine (HEL) concentrations of the collection samples were measured using antibody chip methods by Healthcare Systems (Aichi, Japan).

## Measurements of Blood Glucose and Serum Insulin

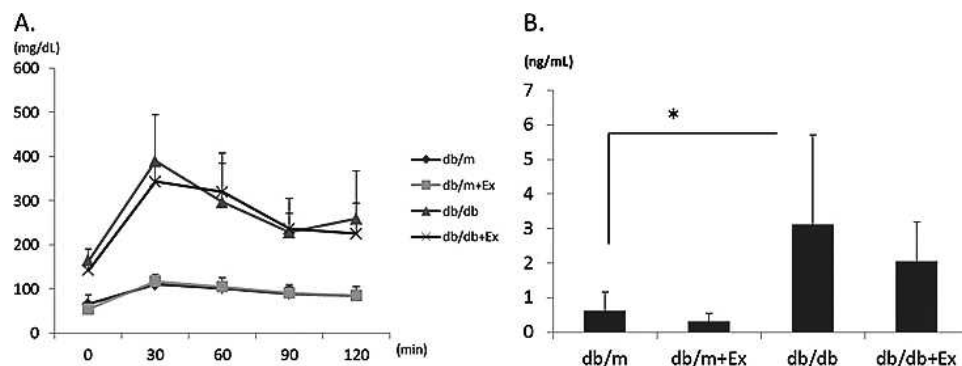
Blood glucose levels were measured from blood samples taken from the tail veins of the mice. Assessments were performed using a glucose meter (EIDIA, Tokyo, Japan). Serum insulin levels were measured from serum samples. Assessments were performed using a Morinaga insulin assay kit (Morinaga, Kanagawa, Japan).

## Glucose Tolerance Test

Oral glucose tolerance testing (OGTT) was conducted at the 8-week experimental time point. Mice were fasted for 20 hours and a blood sample was taken from the tail. Blood glucose concentrations were measured with a glucometer (EIDIA, Tokyo, Japan), after which D-glucose (NACALAI TESQUE, Kyoto, Japan) was injected at a dose of 2 g/kg. Blood glucose was assessed at 30, 60, 90, and 120 minutes after injection.

## Statistical Analysis

Statistical analyses were performed using a commercially available software package, Excel Toukei (SSRI, Tokyo, Japan). A two-tailed Student's *t*-test was used for all analyses. Statistical significance was established at  $P < 0.05$ .



**FIGURE 2.** Physiologic parameters in db/m and db/db mice with exercise training. There was no significant change in the glucose tolerance test between the control and exercise groups. However, blood glucose at the 30-minute point tended to be lower in the db/db + Ex group compared with the db/db group (A). The serum insulin levels of db/db mice were significantly higher than those of db/m mice, and tended to be lower in the exercise groups compared with the control groups (B). \* $P < 0.05$ ,  $N = 4$  to 5, mean  $\pm$  SD.

TABLE 3. Body Weight and Blood Glucose in C57BL/6Jc1 Mice With AICAR Treatment

	PBS	AICAR 125 mg/kg	AICAR 250 mg/kg
Pre-experimental body weight, g, mean $\pm$ SD	23.7 $\pm$ 0.8	23.8 $\pm$ 1.1	23.8 $\pm$ 0.8
Postexperimental body weight, g, mean $\pm$ SD	24.2 $\pm$ 0.7	24.1 $\pm$ 0.8	23.9 $\pm$ 0.7
Pre-experimental blood glucose, mg/dL, mean $\pm$ SD	158.2 $\pm$ 21.8	150.2 $\pm$ 21.4	154.4 $\pm$ 25.4
Postexperimental blood glucose, mg/dL, mean $\pm$ SD	140.7 $\pm$ 14.7	152.1 $\pm$ 18.4	154.2 $\pm$ 15.6

## RESULTS

### db/db Mice as a Model of Type 2 Diabetes

The body weight and blood glucose of the db/db and db/db + Ex groups were significantly higher than those of the db/m and db/m + Ex groups, both pre- and post experiment. Furthermore, the body weight and blood glucose of the db/db + Ex group were significantly lower than that of the db/db group post experiment (Table 1).

In the biochemical serum analysis, the total protein (TP), albumin (ALB), inorganic phosphorus (IP), AST, ALT, amylase (AMY), total cholesterol (T-CHO), free cholesterol (F-CHO), cholesteryl esters (E-CHO), and HDL-cholesterol (HDL-C) levels were significantly higher in the db/db group than in the db/m group (Table 2). No significant differences were observed between the db/m and db/m + Ex group or between the db/db and db/db + Ex group. However, AST and ALT values associated with hepatic function in the db/db + Ex group tended to decrease compared with the db/db group. Moreover, T-CHO, F-CHO, and E-CHO values associated with lipid metabolism also tended to decrease compared with the db/db group. Exercise had a positive impact on hepatic function and lipid metabolism in db/db mice.

### Exercise Training Improved Individual Maximal Running Speed and Metabolism

The times to exhaustion in the exercise groups were longer than those of the control groups (db/m versus db/m + Ex; db/db versus db/db + Ex; Fig. 1A). Especially in db/db mice, the time to exhaustion in the db/db + Ex group was significantly longer than in the db/db group. These results showed that exercise for 8 weeks improved the ability to continue exercise in db/m and db/db mice. Additionally,  $VO_2$  and  $VCO_2$  increased in the exercise groups compared with the control groups (db/m versus db/m + Ex; db/db versus db/db + Ex; Fig. 1B). We demonstrated increases in  $VO_2$  and  $VCO_2$  in both db/m and db/db mice. These results indicate that exercise for 8 weeks resulted in improvements in the metabolism of the mice.

### Exercise Training Improved Physiologic Parameters Related to Type 2 Diabetes

In a glucose tolerance test, we observed no significant differences between the control groups and exercise groups (db/m versus db/m + Ex; db/db versus db/db + Ex; Fig. 2A). However, blood glucose values in the db/db + Ex group tended

to be lower than those in the db/db group at the 30-minute time point. Moreover, the serum insulin levels of db/db mice were significantly higher than those of db/m mice (db/m versus db/m + Ex; db/db versus db/db + Ex; Fig. 2B). No significant differences were observed between the db/m and db/m + Ex groups or between the db/db and db/db + Ex groups. Although the serum insulin levels of the db/db + Ex group tended to be lower than those of the db/db group, we observed the development of resistance to insulin in db/db mice from these results.

### Exercise Training Increased Tear Secretion Volume

At the pre-experimental time point, the tear secretion volume was not significantly different among the four groups: the db/m group (mean  $\pm$  SD, 3.4  $\pm$  2.1 mm), the db/m + Ex group (3.1  $\pm$  1.7 mm), the db/db group (3.3  $\pm$  1.9 mm), or the db/db + Ex group (3.4  $\pm$  1.6 mm). At the 8-week experimental time point, tear secretion volume was greater in the db/m + Ex group (6.5  $\pm$  3.2 mm) than in the db/m group (5.4  $\pm$  2.3 mm). Furthermore, it was also greater in the db/db + Ex group (5.7  $\pm$  1.9 mm) than in the db/db group (4.4  $\pm$  1.7 mm) (Fig. 3A,  $P < 0.05$ ). Tear secretion volume demonstrated a downward trend in the db/db group compared with the db/m group. However, tear secretion volume was not different between the db/m group and the db/db + Ex group. No significant differences were found in lacrimal gland weight (db/m: 9.1  $\pm$  2.3 mg; db/m + Ex: 8.8  $\pm$  2.0 mg; db/db: 9.3  $\pm$  2.5 mg; db/db + Ex: 9.1  $\pm$  1.0 mg) among the four groups (Fig. 3B). Overall, exercise training increased tear secretion volume in db/m and db/db mice.

### Exercise Training Decreased Oxidative Stress Markers in Tear Secretions

We examined oxidative stress markers in tears. The concentration of 8-OHdG was lower in the exercise intervention groups than in the control groups in db/m and db/db mice. Additionally, PRL and HEL concentrations showed the same tendency (Fig. 4).

Oxidative stress markers in tear secretions were influenced by exercise training.

### AICAR Administration Decreased Blood Glucose in db/db Mice

The body weight and blood glucose values were not significantly different among the three groups pre- and post

TABLE 4. Body Weight and Blood Glucose in db/m and db/db Mice With AICAR Treatment

	db/m(PBS)	db/db(PBS)	db/db + AICAR
Pre-experimental body weight, g, mean $\pm$ SD	25.2 $\pm$ 1.5	53.8 $\pm$ 2.6*	54.4 $\pm$ 1.3*
Postexperimental body weight, g, mean $\pm$ SD	24.5 $\pm$ 1.2	54.0 $\pm$ 2.8*	52.9 $\pm$ 3.9*
Pre-experimental blood glucose, mg/dL, mean $\pm$ SD	139.1 $\pm$ 6.5	406.7 $\pm$ 104.5*	410.6 $\pm$ 33.2*
Postexperimental blood glucose, mg/dL, mean $\pm$ SD	129.3 $\pm$ 15.4	480.3 $\pm$ 82.8*	354.1 $\pm$ 163.0*†

\*  $P < 0.01$  for the db/m(PBS) group versus db/db(PBS) and db/db + AICAR groups.

†  $P < 0.05$  for the db/db(PBS) group versus db/db + AICAR group.

TABLE 5. Serum Parameters in db/m and db/db Mice With AICAR Treatment

Serum Parameters	db/m (PBS)	db/db (PBS)	db/db + AICAR
TP, g/dL	4.1 ± 0.2	4.3 ± 0.2	4.3 ± 0.4
ALB, g/dL	2.9 ± 0.1	2.8 ± 0.1	2.8 ± 0.2
Na, mEq/L	153.3 ± 1.7	149.2 ± 3.6	148.2 ± 4.3
K, mEq/L	3.7 ± 0.4	4.5 ± 0.7	5.0 ± 0.4*
Cl, mEq/L	112.5 ± 1.7	104.4 ± 4.5†	100.8 ± 4.9†
Ca, mg/dL	7.9 ± 0.2	8.0 ± 0.4	8.5 ± 0.8
IP, mg/dL	7.9 ± 0.8	10.8 ± 1.9†	13.0 ± 2.2*
AST, IU/L	42.0 ± 1.8	121.6 ± 50.6†	128.2 ± 60.7†
ALT, IU/L	23.0 ± 2.3	189.8 ± 94.2†	216.4 ± 93.8*
LDH, IU/L	286.5 ± 163.3	865.8 ± 397.6†	774.4 ± 452.0
AMY, IU/L	1439.8 ± 81.6	2422.2 ± 256.2*	2186.2 ± 161.6*
T-CHO, mg/dL	66.3 ± 3.0	119.0 ± 11.2*	114.2 ± 11.0*
F-CHO, mg/dL	15.3 ± 0.5	29.0 ± 3.1*	29.0 ± 2.8*
E-CHO, mg/dL	51.0 ± 3.2	90.0 ± 8.5*	85.2 ± 8.4*
E/T, %	77.0 ± 1.8	75.8 ± 1.1	74.6 ± 1.1
TG, mg/dL	46.0 ± 11.7	138.4 ± 58.4†	161.4 ± 24.7*
LDL-C, mg/dL	8.8 ± 1.7	6.2 ± 1.8	6.6 ± 1.7
HDL-C, mg/dL	35.8 ± 1.7	74.0 ± 6.4*	66.2 ± 5.0*
TBA, μmol/L	3.3 ± 0.5	3.0 ± 0.7	4.0 ± 2

\*  $P < 0.01$ , mean ± SD.†  $P < 0.05$ .

experiment in C57BL/6Jc1 mice (Table 3). The body weights and blood glucose of the db/db (PBS) and db/db + AICAR groups were significantly higher than the db/m (PBS) group pre- and post experiment. Meanwhile, the blood glucose of the db/db + AICAR group was significantly lower than the db/db (PBS) group post experiment (Table 4).

In the biochemical serum analyses, inorganic phosphorus (IP), AST, ALT, AMY, T-CHO, F-CHO, E-CHO, triglycerides (TG), HDL-C, K, and lactate dehydrogenase (LDH) were significantly higher in the db/db (PBS) and db/db + AICAR groups than in the db/m (PBS) group (Table 5). No significant differences were observed resulting from AICAR administration.

### AICAR Administration Increased Tear Secretion Volume

Post experiment, tear secretion volume was significantly larger in the AICAR 125 mg/kg group ( $4.8 \pm 1.7$  mm) and the AICAR

250 mg/kg group ( $4.8 \pm 1.7$  mm) than in the PBS group ( $2.5 \pm 1.0$  mm) in C57BL/6Jc1 mice (Fig. 5,  $P < 0.01$ ).

Pre-experiment, the tear secretion volumes of the db/db (PBS) group ( $3.9 \pm 1.8$  mm) and the db/db + AICAR group ( $3.8 \pm 1.7$  mm) were lower than that of the db/m (PBS) group ( $6.9 \pm 2.3$  mm). Post experiment, the tear secretion volume was significantly larger in the db/db + AICAR group ( $6.3 \pm 2.6$  mm) than in the db/db (PBS) group ( $4.6 \pm 1.8$  mm) (Fig. 6,  $P < 0.05$ ). Additionally, no significant difference was found between the db/m (PBS) group ( $6.5 \pm 3.2$  mm) and the db/db + AICAR group. Overall, AICAR administration increased tear secretion volume in db/db mice as well as in C57BL/6Jc1 mice.

### DISCUSSION

In the present study, we confirmed that tear secretion decreased significantly in a db/db mouse model of type 2

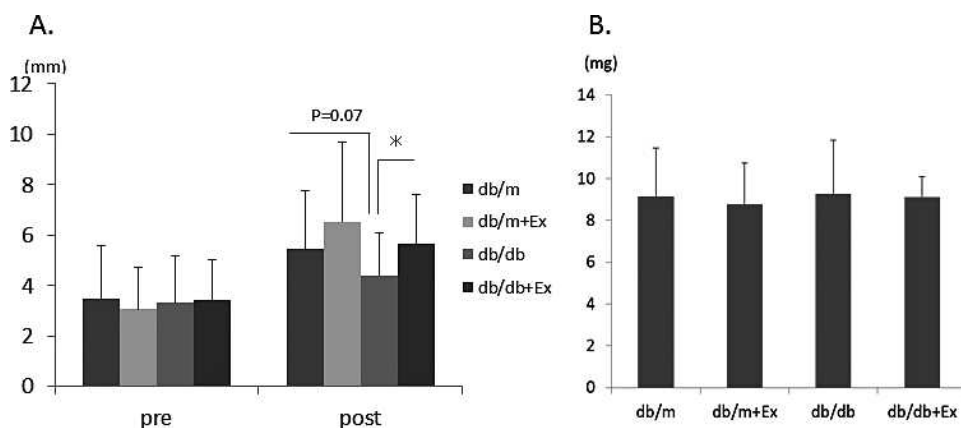


FIGURE 3. Tear secretion volume in db/m and db/db mice with exercise training. Exercise training altered tear secretion in db/m and db/db mice. At the 8-week time point, tear secretion volume was significantly decreased in the db/db group compared with the db/m group. However, it was greater in the db/db + Ex group than in the db/db group, and no significant difference was observed between the db/m group and the db/db + Ex group (A). No significant differences in lacrimal gland weight were found among the four groups (B). (A) \* $P < 0.05$ ,  $N = 13-15$ , mean ± SD. (B)  $N = 5$ , mean ± SD.

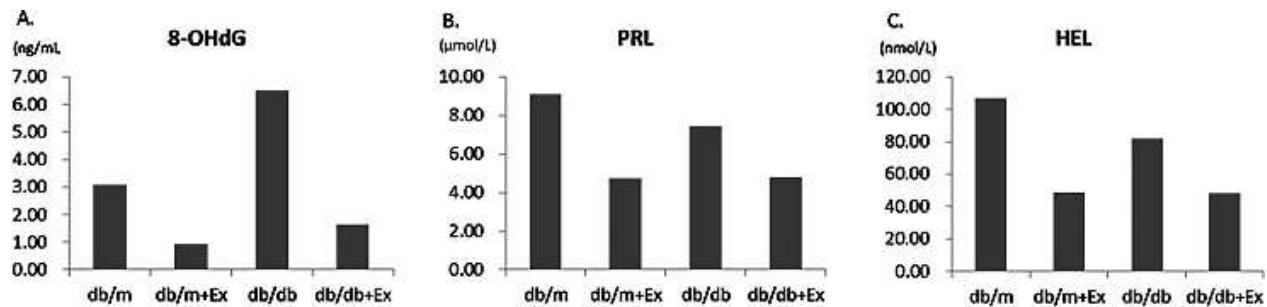


FIGURE 4. Oxidative stress in db/m and db/db mice with exercise training. Exercise training altered the levels of oxidative stress markers from tear secretions in db/m and db/db mice. The concentrations of 8-OHdG, PRL, and HEL were lower in the exercise training groups than in the control groups (A–C).

diabetes, as was previously shown in Goto-Kakizaki rats.<sup>11,12</sup> Furthermore, we found that forced exercise training increased and restored tear secretion in db/db mice. Moreover, we found that a daily intraperitoneal injection of AICAR, which is an AMPK activator and considered a key factor in exercise, increased tear secretion in C57BL/6Jc1 mice and db/db mice.

Clinical studies have revealed clinical manifestations of diabetes mellitus associated with the lacrimal glands and ocular surface dysfunctions related to dry eye syndrome.<sup>9,10</sup> Type 2 diabetes mellitus also influences histology and function.<sup>16,17</sup> In this study, we confirmed similar findings in db/db mice. Evidence of oxidative stress secondary to hyperglycemia has been explored for a long time, and is suggested to contribute to cell damage and exocrine gland dysfunction.<sup>18–20</sup> Environmental factors, especially diet, physical activity, and age, interact with genetic predisposition to affect disease prevalence. It was previously reported that exercise training can attenuate oxidative stress and increase mitochondrial DNA content in the skeletal muscle of rats with type 2 diabetes mellitus.<sup>5</sup> As we expected, we found that exercise training can attenuate oxidative stress markers in tear secretions. Exercise training may provide improvements in the quality of tear secretions as well as increase tear secretion volume. Thus, several mechanisms may be indicated from the present observations. The first is the direct influence of exercise on the lacrimal glands, and secondly, the indirect influence from the systemic effects of exercise. We expected that lacrimal glands in db/db mice would demonstrate reduced function compared with those in db/m mice. However, we were unable to observe a difference in lacrimal gland structure between db/m mice and db/db mice at 16 weeks (hematoxylin-eosin staining and 8-OHdG immunostain-

ing, data not shown). Prior to morphologic changes, functional damage may occur. Observations over a longer duration should be investigated in future studies. Additionally, the conjunctiva may indicate increases in tear secretion volume in addition to the lacrimal glands. An analysis of the conjunctiva and studies using ocular instillation could be used to research the mechanism of action in the future. Moreover, we believe that the indirect involvement of exercise is visible through metabolic improvements in the muscles and liver. They are also visible through central nervous system functions in the brain controlling the lacrimal glands and conjunctiva.

As we speculated that AMPK is related to the effects of exercise in db/db mice, we investigated whether AICAR administration affects tear secretion. Our data show that AICAR increased tear secretion volume both in db/db mice and in C57BL/6Jc1 mice. Adenosine monophosphate-activated protein kinase is an energy-sensing molecule relevant to metabolism. We confirmed decreases in the body weights and blood glucose levels of db/db mice resulting from exercise training and AICAR injections. We determined that the metabolism of db/db mice was improved by the intervention. The function of AMPK in the lacrimal glands has not yet been clearly defined. We assume there is a relationship between AMPK activity, ER, and mitochondrial morphology in the lacrimal glands, considering the findings that AMPK largely prevented alterations in ER or mitochondrial morphology in  $\beta$ -cells. Changes and alterations in ER and mitochondrial morphology in LG should be investigated in future studies. There are other possibilities regarding the increase in tear secretion volume by exercise training and AICAR injection. In a recent study, adiponectin increased saliva secretion by

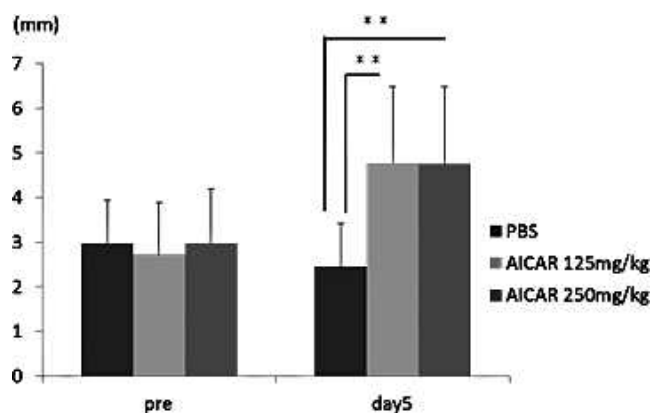
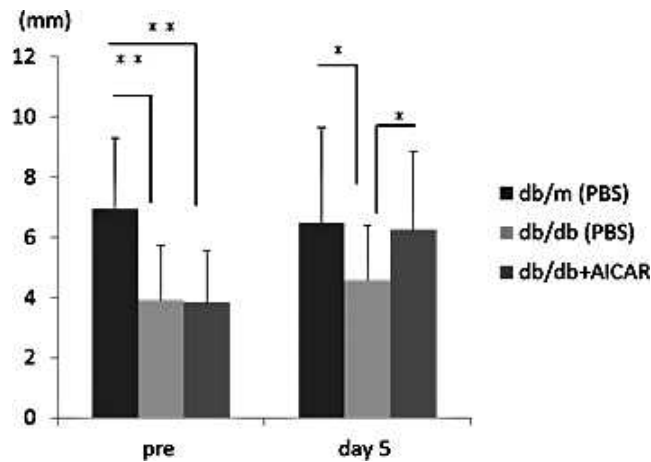


FIGURE 5. Tear secretion volume in C57BL/6Jc1 mice with AICAR treatment. Tear secretion volume was significantly larger in the AICAR 125 mg/kg group and AICAR 250 mg/kg group compared with the PBS group in C57BL/6Jc1 mice.  $**P < 0.01$ ,  $N = 9$  to 10, mean  $\pm$  SD.



**FIGURE 6.** Tear secretion volumes in db/m and db/db mice with AICAR treatment. Tear secretion volumes were significantly lower in db/db mice compared with db/m mice. However, the tear secretion volumes of db/db mice injected with AICAR for 5 days showed no significant difference compared with db/m mice. Furthermore, tear secretion volume was significantly larger in the db/db + AICAR group than the db/db group. \* $P < 0.05$ , \*\* $P < 0.01$ ,  $N = 9$  to 10, mean  $\pm$  SD.

modulating the structure and function of tight junctions through binding to the receptors for adiponectin (AdipoRs) and through the activation of AMPK. It was also reported that AICAR increased saliva secretion.<sup>21</sup> Tear secretion is mainly controlled by the parasympathetic and sympathetic autonomic nervous systems, similar to saliva secretion.<sup>22,23</sup> In fact, a connection between adiponectin and tear volume has been reported. Mice treated with adiponectin showed a significant improvement in tear volume and corneal irregularity when compared with the experimental dry eye control group. A topical application of adiponectin markedly improved clinical signs and decreased inflammation of the ocular surface and lacrimal glands in dry eye experiments.<sup>24</sup>

Additionally, there are some reports about increases in tear secretion volume. Some reports have focused on epithelial sodium channels (ENaC) as the mechanism by which tear secretion volume is increased following AICAR injection. It was previously reported that tear quantity showed significant increases at 15 and 30 minutes compared with the pre-instillation values in eyes receiving amiloride eye drops, which block ENaC in rabbits.<sup>25</sup> It is known that ENaC exist in many tissues throughout the body. These channels mediate the first step of active  $\text{Na}^+$  reabsorption and play a major role in the maintenance of electrolytes and water homeostasis in all vertebrates.<sup>26</sup> A recent study has confirmed the role of AMPK in the regulation of ENaC in vivo.<sup>27</sup> Adenosine monophosphate-activated protein kinase-sensitive ion channels include ENaC and the delayed, outwardly rectifying, voltage-gated  $\text{K}^+$  channel.<sup>28</sup> The existence of ENaC in the rat conjunctival epithelium has already been reported.<sup>29</sup> Similarly, the expression of ENaC was evaluated in lacrimal glands in rats by RT-PCR.<sup>30</sup> Furthermore, it is reported that the activity of AICAR is related to ENaC. AICAR has been shown to alter the fluidity and surface charge of phospholipid membranes, which could have additional effects on the functioning of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, and ENaC.<sup>31,32</sup> Further studies are required to elucidate the mechanism.

It has been reported that exercise in a clinical setting resulted in risk reductions for a number of diseases such as hypertension, hypercholesterolemia, and diabetes mellitus.<sup>33</sup> Additionally, it has been reported that exercise also affects various eye diseases, including glaucoma, cataract, and macular degeneration.<sup>34–36</sup> Furthermore, our research group demonstrated preliminary evidence that a lower level of physical activity was significantly associated with a lower value in

Schirmer's test.<sup>37</sup> Exercise for the treatment of dry eye disease may be a clinical application, in addition to treatment with eye drops.<sup>38–40</sup>

In summary, our experiments demonstrated that tear secretion changes with exercise training in db/db mice and a daily intraperitoneal injection of AICAR in C57BL/6Jc1 mice and db/db mice. Although further investigations are needed to clarify the mechanism of the decrease in tear secretion in db/db mice and the increase in tear secretion resulting from exercise training, these findings will improve our understanding of the mechanisms involved and may provide a potential therapeutic strategy to modulate tear secretion.

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