

# Tirzepatide Attenuates Diabetes-Associated Testicular Inflammation and Sperm DNA Fragmentation in Obese Diabetic Mice: Potential Pleiotropic Effects Beyond Glycemic Control

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## Article

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# Abstract

## Background

Diabetes is increasingly recognized as a contributor to male infertility. We previously reported that diabetic mice exhibit testicular macrophage accumulation and impaired spermatogenesis, and that glucose-dependent insulintropic polypeptide receptor (GIPR) activation suppresses macrophage inflammatory responses. Here, we investigated whether and how tirzepatide, a dual GIP/glucagon-like peptide-1 receptor (GLP-1R) agonist used for type 2 diabetes and obesity, ameliorates diabetes-associated testicular injury and sperm damage.

## Methods

Obese diabetic KK-Ay mice were treated with vehicle or tirzepatide at a metabolically effective or sub-effective dose (1.00 or 0.16 nmol/kg/day, respectively) for 8 weeks. Testicular histology, macrophage accumulation, inflammatory gene expression, sperm parameters, oxidative stress, and sperm DNA fragmentation were assessed. A separate exploratory experiment with empagliflozin was performed to examine whether a glucose-lowering intervention could affect these testicular and sperm parameters.

## Results

Tirzepatide at 1.00 nmol/kg/day significantly reduced glycated hemoglobin A1c levels, whereas 0.16 nmol/kg/day did not. Tirzepatide at both doses reduced seminiferous tubule vacuolation, testicular macrophage accumulation and oxidative stress, and attenuated sperm oxidative stress and DNA fragmentation. Sperm motility improved only with the higher dose. *Gipr* expression was abundant in the testis, whereas *Glp-1r* expression was minimal, and GIPR immunoreactivity was predominantly localized to testicular macrophages. Empagliflozin improved hyperglycemia and hyperinsulinemia and increased sperm concentration and motility; however, it did not reduce testicular macrophage accumulation or oxidative stress, nor did it attenuate sperm oxidative stress or improve sperm viability.

## Conclusions

Tirzepatide attenuated diabetes-induced testicular inflammation, oxidative stress, and sperm DNA fragmentation in obese diabetic mice, including at a dose that did not improve glycemic control. These findings suggest that tirzepatide may protect against diabetes-associated testicular and sperm injury partly by modulating inflammation in GIPR-expressing testicular macrophages.

## Background

Male infertility has increasingly become an important health issue worldwide, affecting a substantial proportion of couples of reproductive age [1]. Impaired spermatogenesis is one of the major causes of male infertility; however, the underlying mechanisms remain unclear in many patients, and effective pharmacological interventions remain limited [1, 2].

Metabolic disorders, including diabetes, have recently attracted increasing attention as contributors to male reproductive impairment [3, 4]. Given the global increase in diabetes [5], elucidating the mechanisms linking metabolic dysfunction to male reproductive impairment has become an important clinical issue.

Testicular inflammation has been implicated in the pathogenesis of impaired spermatogenesis [6–8]. We previously reported that diabetic mice exhibit testicular macrophage accumulation accompanied by inflammatory changes and impaired spermatogenesis [9]. These findings suggest that macrophage-associated inflammation may play an important role in diabetes-associated male infertility.

Glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are gut-derived peptide hormones that enhance insulin secretion from pancreatic  $\beta$  cells in a glucose-dependent manner. In addition to their pancreatic actions, incretin receptor signaling in the central nervous system contributes to appetite suppression and body weight regulation. More recently, GIP has also emerged as a regulator of inflammation beyond its metabolic actions [10]. We previously demonstrated that macrophages express the GIP receptor (GIPR) and that activation of GIP signaling suppresses macrophage foam cell formation and inflammatory responses [11, 12]. These observations suggest the possibility that GIPR activation may modulate macrophage-associated inflammation in diabetes-induced testicular injury.

Tirzepatide is a dual agonist of the GIP and GLP-1 receptors [13] and has shown marked efficacy in improving glycemic control and reducing body weight in patients with type 2 diabetes and obesity [14, 15]. It is therefore widely used for the treatment of type 2 diabetes and obesity. However, whether tirzepatide improves diabetes-associated spermatogenic dysfunction remains unclear. In particular, it remains unclear whether its potential reproductive effects are attributable solely to improved glycemic control or also involve the modulation of inflammatory processes within the testis.

We therefore hypothesized that tirzepatide could ameliorate diabetes-associated testicular inflammation and sperm abnormalities partly in a glucose-lowering-independent manner. To test this hypothesis, we evaluated the effects of metabolically effective and sub-effective doses of tirzepatide in a mouse model of obesity-associated diabetes. In addition, we conducted a separate exploratory study using empagliflozin, a sodium-glucose cotransporter 2 inhibitor, to examine whether a glucose-lowering intervention could ameliorate diabetes-associated testicular and sperm abnormalities.

## Methods

### Animal study

All animal experiments were approved by the Animal Care Committee of Showa University School of Medicine (approval number: 03034, approval date: April 1, 2021) and conducted in accordance with the ARRIVE 2.0 guidelines and the Guide for the Care and Use of Laboratory Animals (8th Edition) [16, 17]. All invasive procedures were performed under general anesthesia using isoflurane, except for plasma glucose measurements, in which blood samples were obtained via a small tail prick. Fifteen male KK-Ay (KK.Cg-Ay/TaJcl) mice, a mouse model of obesity-induced diabetes, were obtained from CLEA Japan (Meguro, Tokyo, Japan) [18, 19]. The sample size was determined based on our previous studies [9, 20] while minimizing the number of animals used. Animals were housed in individual cages and provided standard chow (Labo MR Stock, NOSAN, Yokohama, Japan) with ad libitum access to food and water. All animals were maintained in a specific pathogen-free facility at the Division of Animal Experimentation, Showa Medical University School of Medicine. The rooms were maintained on a 12 h dark/light cycle, 21°C, and 40–60% humidity.

At 7 weeks of age, mice were randomly assigned to the following treatment groups after stratification by body weight to ensure comparable baseline body weights among groups: vehicle, tirzepatide low dose (0.16 nmol/kg/day), or tirzepatide high dose (1.00 nmol/kg/day). Tirzepatide doses were determined based on previous studies [20–22]. In a preliminary study, we confirmed that these doses exerted sub-effective and effective glucose-lowering effects, respectively. Tirzepatide was purchased from Eli Lilly Japan (Minato, Tokyo, Japan) and continuously administered for 8 weeks using osmotic pumps (Model 1004; ALZET, Cupertino, CA, USA) implanted subcutaneously in the dorsal region. The osmotic pumps were replaced after 4 weeks to maintain continuous drug delivery. Animals were monitored, and body weight and food intake were measured regularly throughout the study. No unexpected adverse events were observed, and no predefined humane endpoints were reached during the study. No animals were excluded from the analyses.

After the 8-week intervention, mice were euthanized by overdose of inhaled isoflurane, and the epididymides, testes, and epididymal fat pads were carefully excised. Epididymal tissues were used for sperm collection and analysis. Testicular and epididymal fat pad weights were measured to calculate each organ index as follows: organ weight (mg)/body weight (g). Subsequently, the left testis was fixed in Bouin solution for histological and immunofluorescence analyses, whereas the right testis was snap-frozen in liquid nitrogen and stored for reverse transcription polymerase chain reaction (RT-PCR) analysis. All analyses were performed by investigators blinded to treatment allocation (N.O., H.Y., T.S., D.T., and M.Ogino).

In a separate exploratory study designed to evaluate the hyperglycemia dependency of spermatogenic abnormalities, non-diabetic C57BL/6 mice and diabetic KK-Ay mice were treated with empagliflozin as a glucose-lowering comparator. Details of this experiment are provided in the Additional file 1.

## Measurement of biochemical parameters

Plasma biochemical parameters were assessed as previously described [9, 23]. Blood samples were collected after a 6-h fasting at the end of each experiment. Plasma glucose, glycated hemoglobin (HbA1c), and lipid levels were measured using an enzyme electrode assay, immunoassay, and enzymatic colorimetric assays, respectively. Plasma insulin and testosterone levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Ultra-sensitive Mouse Insulin ELISA Kit, Product ID: M1104, Morinaga Institute of Biological Science, Yokohama, Kanagawa, Japan; Testosterone ELISA Kit, Product ID: ENZ-ADI900065, Enzo Life Sciences, Farmingdale, NY, USA).

## Immunofluorescence staining

Immunofluorescence staining was performed as previously described [9]. Tissue cross-sections were incubated overnight with anti-F4/80 antibody (Product ID: Ab204467; RRID: AB\_2810932; raised in rat; 1:250; Abcam Japan, Chuo, Tokyo, Japan), anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody (Product ID: MOG-020P, RRID: AB\_1106819, raised in mouse, 1:200; Nikken Seil, Fukuroi, Shizuoka, Japan), or anti-GIPR antibody (Product ID: PA5-14408, RRID: AB\_2247512, raised in rabbit, 1:100; Invitrogen, Waltham, MA, USA). The sections were subsequently incubated with appropriate secondary antibodies for 4 h and mounted using VECTASHIELD Antifade Mounting Medium (Vector Laboratories, Newark, CA, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired using a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan), and F4/80-positive cells and 8-OHdG-positive areas were quantified in randomly selected fields using ImageJ software.

## Real-time RT-PCR

Total RNA extracted from tissues was reverse-transcribed into cDNA for quantitative real-time RT-PCR analysis as previously described [9, 23]. Quantitative real-time RT-PCR was performed using a TaqMan Gene Expression Assay and a QuantStudio 3 Sequence Detection System (Life Technologies Japan, Minato, Tokyo, Japan). The following pre-designed TaqMan probe sets were used: *cadherin 2 (Cdh2)*, Mm01162497\_m1; *claudin 3 (Cldn3)*, Mm00515499\_s1; *cytochrome P450 family 17 subfamily A member 1 (Cyp17a1)*, Mm00484040\_m1; *Gipr*, Mm01316351\_g1; *Glp-1 receptor (Glp-1r)*, Mm00445292\_m1; *intercellular adhesion molecule 1 (Icam)*, Mm00516023\_m1; *monocyte chemoattractant protein-1 (Mcp-1)*, Mm00441242\_m1; *occludin (Ocln)*, Mm00500912\_m1; *SRY-box transcription factor 9 (Sox9)*, Mm00448840\_m1; *tight junction protein 1 (Tjp1)*, Mm01320638\_m1; *tumor necrosis factor (Tnf)*, Mm00443258\_m1; *vascular cell adhesion molecule 1 (Vcam)*, Mm01320970\_m1; and *18S ribosomal RNA (18S rrna)*, Mm03928990\_g1. The expression levels of target genes were normalized to those of the internal control 18S rRNA.

## Histological assessment of testis

The left testes were fixed in Bouin solution for 24 h at 4°C, followed by further fixation in 10% neutral buffered formalin for several days at 4°C, and subsequently embedded in paraffin blocks. Cross-sections

were obtained from the middle portion of the paraffin-embedded testes and stained with hematoxylin and eosin (H&E) for morphometric analysis of seminiferous tubules. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Histological assessment of seminiferous tubules was performed as previously described with some modifications [9, 24, 25]. At least 20 seminiferous tubules per testis were evaluated, and the averaged value was used as an individual value. The number of vacuoles within each seminiferous tubule was quantified as an additional morphological index of seminiferous tubular injury, as previously reported in diabetic mice [26], and the mean value per tubule was used for analysis.

## Sperm collection

The swim-out method was used to collect spermatozoa from the cauda epididymis as previously reported with some modifications [9, 27]. The left and right sides of the cauda epididymis of each mouse were separately soaked in 1 mL of M2 medium (Sigma-Aldrich Japan) maintained at 37°C. Subsequently, the cauda epididymides were minced within the medium using spring scissors, incubated to allow spermatozoa to swim out for 15 min, and then removed from the medium. Spermatozoa were further incubated in the medium for 45 min to induce their capacitation.

## Sperm analysis

Sperm parameters were assessed as previously reported with some modifications [9, 27]. Sperm concentration was counted using hemocytometers. A 10 µL aliquot of sperm suspension was diluted with the same volume of M2 medium on a glass slide and covered by a 24 mm × 32 mm cover glass for sperm motility assessment. For motility assessment, individual spermatozoa were microscopically classified as having normal motility, defined as progressive movement, or abnormal motility, defined as non-progressive movement or no movement. For morphology assessment, spermatozoa were classified as normal or abnormal; abnormal morphology included a bent or twisted tail, head defect, or tail defect. Sperm motility and morphology were evaluated on at least 100 spermatozoa per epididymis. Spermatozoa that were collected from the left and right epididymides of each mouse were separately analyzed and then averaged as an individual value.

## Sperm staining

Sperm DNA fragmentation and oxidative stress were evaluated using acridine orange staining and 8-OHdG immunofluorescence staining of sperm smears, respectively. For acridine orange staining, sperm were fixed with Carnoy's fixative (methanol:acetic acid, 3:1) at 4°C for 2 h and then rinsed with phosphate-buffered saline. The sperm were stained with a 0.01% acridine orange working solution, prepared by 10-fold dilution of a 0.1% stock solution, for 5 min in the dark, followed by washing with distilled water, with modifications to previously reported acridine orange staining methods [28]. Fluorescence images were acquired immediately using a fluorescence microscope under light-protected

conditions. Immunofluorescence staining was performed as described above after fixation with 4% paraformaldehyde. Sperm DNA fragmentation and oxidative stress were evaluated on at least 50 spermatozoa.

## Statistical analysis

Data are presented as means  $\pm$  standard deviation (SD). Given the small sample size, formal assessment of data normality was not performed. JMP software Student Edition (SAS Institute, Cary, NC, USA) was used for statistical comparison. Comparisons were conducted using one-way analysis of variance with Tukey's post-hoc test. The significance level was defined as a *p*-value of  $< 0.05$ .

## Results

### High-dose, but not low-dose, tirzepatide improved glycemic control

Anthropometric and biochemical parameters are summarized in Table 1. High-dose tirzepatide (1.00 nmol/kg/day) significantly reduced food intake compared with vehicle and low-dose tirzepatide (0.16 nmol/kg/day). High-dose tirzepatide also significantly reduced HbA1c and plasma total cholesterol levels compared with vehicle, whereas low-dose tirzepatide did not. No significant differences were observed in testis weight, epididymal fat pad weight, or plasma levels of glucose, triglycerides, insulin, or testosterone among the groups.

Table 1  
Anthropometric and biochemical parameters of diabetic mice treated with vehicle or varying doses of tirzepatide.

| Number of mice                                                                                                                                                                                                                     | Vehicle     | Low-dose tirzepatide | High-dose tirzepatide   |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|----------------------|-------------------------|
|                                                                                                                                                                                                                                    | 5           | 5                    | 5                       |
| Food intake (g/day)                                                                                                                                                                                                                | 7.2 ± 0.5   | 7.1 ± 0.3            | 6.6 ± 0.2 <sup>ab</sup> |
| Initial body weight (g)                                                                                                                                                                                                            | 31.5 ± 1.7  | 32.1 ± 0.9           | 32.7 ± 0.9              |
| Final body weight (g)                                                                                                                                                                                                              | 44.3 ± 2.0  | 45.4 ± 0.6           | 45.9 ± 0.6              |
| Body weight gain (g)                                                                                                                                                                                                               | 12.8 ± 1.8  | 13.3 ± 1.1           | 13.2 ± 1.3              |
| Testis weight (mg)                                                                                                                                                                                                                 | 106 ± 14    | 104 ± 9              | 117 ± 6                 |
| Testis index (mg/g body weight)                                                                                                                                                                                                    | 2.4 ± 0.2   | 2.3 ± 0.2            | 2.5 ± 0.1               |
| Epididymal fat pad weight (mg)                                                                                                                                                                                                     | 808 ± 165   | 871 ± 42             | 901 ± 70                |
| Epididymal fat pad index (mg/g body weight)                                                                                                                                                                                        | 18.2 ± 3.6  | 19.2 ± 0.9           | 19.6 ± 1.3              |
| HbA1c (%)                                                                                                                                                                                                                          | 9.5 ± 1.3   | 8.3 ± 0.6            | 7.8 ± 0.5 <sup>a</sup>  |
| Plasma glucose (mg/dL)                                                                                                                                                                                                             | 124 ± 14    | 126 ± 20             | 151 ± 13                |
| Plasma total cholesterol (mg/dL)                                                                                                                                                                                                   | 72 ± 9      | 57 ± 18              | 42 ± 14 <sup>a</sup>    |
| Plasma triglycerides (mg/dL)                                                                                                                                                                                                       | 147 ± 38    | 108 ± 27             | 127 ± 28                |
| Plasma insulin (ng/mL)                                                                                                                                                                                                             | 1.23 ± 0.72 | 0.80 ± 0.24          | 0.92 ± 0.46             |
| Plasma testosterone (ng/mL)                                                                                                                                                                                                        | 318 ± 149   | 281 ± 79             | 410 ± 45                |
| Values are presented as means ± SD. <i>p</i> < 0.05: <sup>a</sup> vs. Vehicle; <sup>b</sup> vs. Low-dose tirzepatide. Low-dose tirzepatide, 0.16 nmol/kg/day; high-dose tirzepatide, 1.00 nmol/kg/day; HbA1c, glycated hemoglobin. |             |                      |                         |

### Both low- and high-dose tirzepatide reduced seminiferous tubule vacuolation, macrophage accumulation, and oxidative stress in the testis

Testicular histological findings are shown in Fig. 1 and Additional file 2 (Fig. S1). The epithelial area, lumen area, and perimeter of seminiferous tubules did not differ significantly among the groups (Fig. 1A and Additional file 2: Figs. S1A, B). In contrast, the number of vacuoles per seminiferous tubule was significantly lower in both tirzepatide-treated groups than in the vehicle-treated group (Fig. 1B). Both low-dose and high-dose tirzepatide significantly reduced the number of F4/80-positive cells in the testis (Fig. 1C). In addition, testicular oxidative stress, assessed by 8-OHdG staining, was significantly attenuated in both tirzepatide-treated groups (Fig. 1D).

To further characterize inflammatory changes associated with testicular macrophage accumulation, we analyzed the expression of inflammation-related genes in the testis (Fig. 1E). Tirzepatide at both doses significantly reduced *Icam* expression, whereas a significant reduction in *Mcp-1* expression was observed only in the high-dose group. *Vcam* expression was not significantly altered. *Tnf* expression tended to be lower in both tirzepatide-treated groups, with a significant reduction observed in the high-dose group (Additional file 2: Fig. S1C). No significant changes were observed in blood-testis barrier-related genes or markers of Sertoli and Leydig cell function (Additional file 2: Figs. S1D, E).

## **GIP receptor expression was predominantly localized to testicular macrophages**

*Gipr* expression was highly abundant in the testis, whereas *Glp-1r* expression was barely detectable (Additional file 2: Table S1). Immunofluorescence analysis demonstrated that GIPR immunoreactivity was predominantly localized to F4/80-positive testicular macrophages (Fig. 1F).

## **Tirzepatide attenuated sperm oxidative stress and DNA fragmentation**

As shown in Fig. 2A, sperm concentration was not significantly affected by tirzepatide treatment. In contrast, sperm motility was significantly increased by high-dose tirzepatide (Fig. 2B), whereas the proportion of morphologically abnormal sperm was not significantly altered (Fig. 2C). Both low-dose and high-dose tirzepatide significantly reduced sperm oxidative stress, as assessed by 8-OHdG staining (Fig. 2D). Similarly, sperm DNA fragmentation, assessed by acridine orange staining, was significantly reduced in both tirzepatide-treated groups (Fig. 2E).

## **Empagliflozin improved glycemic control but did not attenuate testicular macrophage accumulation or oxidative stress**

To explore whether the effects observed with tirzepatide were attributable solely to improved glycemic control, diabetic mice were treated with empagliflozin. Plasma glucose, insulin, and HbA1c levels, as well as body weight, were significantly higher in diabetic mice than in non-diabetic controls (Additional file 2: Table S2). Empagliflozin treatment significantly improved hyperglycemia and hyperinsulinemia and reduced body weight gain and epididymal fat accumulation compared with diabetic vehicle-treated mice.

Diabetic mice exhibited increased testicular macrophage accumulation, oxidative stress, and *Tnf* expression compared with non-diabetic controls. These testicular inflammatory and oxidative stress parameters were not significantly attenuated by empagliflozin treatment (Additional file 2: Figs. S2A–C).

Diabetic mice also exhibited reduced sperm concentration and motility, both of which were significantly improved by empagliflozin treatment (Additional file 2: Figs. S2D, E). However, sperm morphology, sperm

oxidative stress, and sperm viability were not significantly improved by empagliflozin treatment (Additional file 2: Figs. S2F–H).

## Discussion

In the present study, we demonstrated that tirzepatide reduced seminiferous tubule vacuolation, macrophage accumulation, and oxidative stress in the testis, as well as oxidative stress and DNA fragmentation in sperm, in obese diabetic mice. Notably, these protective effects were observed even at a low dose that did not significantly improve HbA1c levels. These findings suggest that tirzepatide may protect against diabetes-associated testicular and sperm abnormalities, at least in part, through mechanisms independent of glucose lowering.

High-dose tirzepatide significantly reduced HbA1c levels and food intake, whereas low-dose tirzepatide did not significantly reduce either parameter. Therefore, the high and low doses of tirzepatide used in the present study could be interpreted as metabolically effective and sub-effective doses, respectively. Although fasting plasma glucose was not significantly changed even in the high-dose group, HbA1c reflects integrated glycemic exposure over time, including postprandial and diurnal changes. Thus, this discrepancy may reflect the difference between cumulative glycemic exposure and a single fasting glucose measurement.

A key observation of the present study is that low-dose tirzepatide significantly reduced seminiferous tubule vacuolation, macrophage accumulation, and oxidative stress in the testis, as well as oxidative stress and DNA fragmentation in spermatozoa, without significantly improving HbA1c levels. In contrast, low-dose tirzepatide did not significantly improve conventional sperm parameters such as sperm concentration or motility. To further explore the role of glycemic improvement, we conducted a separate exploratory study using empagliflozin, a sodium-glucose cotransporter 2 inhibitor that lowers blood glucose by inhibiting renal glucose reabsorption in the proximal tubules and promoting urinary glucose excretion [29]. Empagliflozin improved hyperglycemia and hyperinsulinemia and was accompanied by partial recovery of sperm concentration and motility; however, it did not attenuate macrophage accumulation or oxidative stress in the testis, nor did it reduce oxidative stress or improve viability in spermatozoa. Taken together, these findings suggest that testicular inflammation and oxidative stress may contribute to sperm oxidative damage in diabetic mice, whereas hyperglycemia may contribute to reduced sperm concentration and motility.

Low-dose tirzepatide reduced testicular macrophage accumulation and oxidative stress in both the testis and sperm, suggesting a potential link between attenuation of inflammatory oxidative injury in the testicular microenvironment and reduced sperm DNA fragmentation. Excessive reactive oxygen species can directly damage sperm DNA and impair chromatin integrity, making oxidative stress a major mechanism underlying sperm DNA fragmentation [30–32]. Because sperm DNA fragmentation reflects an aspect of sperm quality not fully captured by conventional semen parameters such as sperm concentration, motility, or morphology [30–32], its reduction by low-dose tirzepatide may indicate an

improvement in qualitative sperm damage *rather than* merely an improvement in sperm quantity or motility.

In the present study, *Gipr* expression was highly abundant in testicular tissue and was predominantly localized to F4/80-positive macrophages, whereas *Glp-1r* expression was barely detectable. We previously reported that macrophages express GIPR and that GIP signaling suppresses macrophage inflammatory responses [11, 12]. Therefore, although tirzepatide is a dual agonist of GIPR and GLP-1R [13], the present findings suggest that GIPR signaling in testicular macrophages may contribute to the anti-inflammatory and antioxidative effects of tirzepatide in the diabetic testis.

Interestingly, tirzepatide reduced testicular macrophage accumulation at both doses, whereas its effects on recruitment-related and inflammatory genes varied by dose. In particular, tirzepatide at both doses reduced *Icam* expression, while *Mcp-1* and *Tnf* expression were significantly reduced mainly in the high-dose group. This dissociation may reflect the fact that macrophage accumulation, recruitment-related signaling, and downstream inflammatory activation are partially distinct processes [33, 34]. In addition, mRNA expression was assessed using whole testicular tissue, which may be less sensitive to localized changes in macrophage number or activation state. Moreover, reduction of oxidative stress may occur before or independently of detectable changes in inflammatory cytokine expression. These findings suggest that macrophage accumulation, inflammatory signaling, and oxidative injury are related but distinct components of diabetic testicular damage and may together contribute to sperm injury.

Tirzepatide also reduced seminiferous tubule vacuolation without significantly altering seminiferous tubule size parameters or the expression levels of blood-testis barrier-related genes and Sertoli or Leydig cell markers. Vacuolation of seminiferous tubules has been described as a histological feature of diabetic testicular injury and may reflect cellular stress or degeneration within the seminiferous epithelium [26, 35, 36]. The absence of marked changes in seminiferous tubule morphometry or cell lineage markers suggests that tirzepatide primarily attenuated injury-related changes rather than inducing broad structural remodeling of the testis during the observation period. This interpretation is consistent with the improvement in oxidative stress and sperm DNA fragmentation despite limited changes in sperm concentration and morphology.

There are several limitations in the present study. First, this study was performed in a single mouse model of obesity-associated diabetes, and the findings should be validated in other diabetic and non-diabetic models of male reproductive dysfunction. Second, although we evaluated conventional sperm parameters as well as sperm oxidative stress and DNA fragmentation, fertility outcomes such as mating success, pregnancy rate, and offspring number were not assessed; therefore, whether these improvements translate into enhanced fertility remains to be determined. Third, macrophage-specific GIP receptor signaling was not directly examined. Future studies using cell-specific GIP receptor deletion or macrophage-targeted approaches will be required to establish a causal role of macrophage GIP receptor signaling in diabetic testicular injury. Fourth, the empagliflozin experiment was conducted as an exploratory study in a separate experimental setting to examine whether glycemic improvement could

ameliorate diabetic testicular and sperm abnormalities. Because the experimental conditions differed from those of the tirzepatide study, including the potential surgical stress associated with osmotic pump implantation, differences in absolute glycemic values between the two experiments should be interpreted with caution. In addition, sperm DNA fragmentation was not assessed in the empagliflozin experiment, precluding direct comparison of this outcome between the two interventions. Fifth, high-dose tirzepatide reduced food intake, and the contribution of altered energy balance or other systemic metabolic changes cannot be completely excluded, although the protective effects observed at the low dose support the involvement of mechanisms not fully explained by glycemic improvement. Finally, consistent with our previous report [9], plasma testosterone levels were not reduced, but *rather* elevated, in this obese diabetic mouse model. This endocrine profile differs from the reduced testosterone levels frequently observed in men with type 2 diabetes and/or obesity [37], and therefore this model may not fully recapitulate the endocrine features of diabetes-associated male reproductive dysfunction in humans.

## Conclusions

Tirzepatide attenuated diabetes-induced testicular inflammation, oxidative stress, and sperm DNA fragmentation in obese diabetic mice, including at a dose that did not improve glycemic control. These findings suggest that tirzepatide may protect against diabetes-associated testicular and sperm injury through mechanisms that are at least partly independent of glucose lowering and may involve modulation of inflammation in GIPR-expressing testicular macrophages.

## Abbreviations

8-OHdG, 8-hydroxy-2'-deoxyguanosine

Cdh2, cadherin 2

Cldn3, claudin 3

Cyp17a1, cytochrome P450 family 17 subfamily A member 1

DAPI, 4',6-diamidino-2-phenylindole

DM, diabetic mice

ELISA, enzyme-linked immunosorbent assay

Empa, empagliflozin

GIP, glucose-dependent insulintropic polypeptide

GIPR, glucose-dependent insulintropic polypeptide receptor

GLP-1, glucagon-like peptide-1

GLP-1R, glucagon-like peptide-1 receptor

H&E, hematoxylin and eosin

HbA1c, glycated hemoglobin A1c

Icam, intercellular adhesion molecule 1

Mcp-1, monocyte chemoattractant protein-1

Non-DM, non-diabetic mice

Ocln, occludin

rRNA, ribosomal RNA

RT-PCR, reverse transcription polymerase chain reaction

SD, standard deviation

Sox9, SRY-box transcription factor 9

Tirz-H, high-dose tirzepatide

Tirz-L, low-dose tirzepatide

Tnf, tumor necrosis factor

Vcam, vascular cell adhesion molecule 1

## Declarations

### **Ethics approval and consent to participate**

All animal experiments were approved by the Animal Care Committee of Showa University School of Medicine (approval numbers and dates: 03034, April 1, 2021; 02072, April 1, 2020). Consent to participate was not applicable because this study did not involve human participants.

### **Consent for publication**

Not applicable.

## Competing Interests

Y.M. received financial support from Boehringer Ingelheim. M.Ohara has received lecture fees from Eli Lilly Japan. S.Y. has received lecture fees from Bayer Yakuhin, Kowa Company, and Novo Nordisk Pharma. The other authors declare that they have no competing interests.

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## Author Contribution

Conceptualization, Y.M.; methodology, Y.M. and T.S.; software, Y.M. and T.S.; validation, Y.M.; formal analysis, Y.M.; investigation, Y.M., N.O., H.Y., T.S., D.T., and M.Ogino; resources, Y.M. and S.Y.; data curation, Y.M.; writing—original draft preparation, Y.M.; writing—review and editing, N.O., M.T., H.Y., T.S., D.T., M.Ogino, M.Ohara, and S.Y.; visualization, Y.M.; supervision, M.T., M.Ohara, and S.Y.; project administration, Y.M.; funding acquisition, Y.M. All authors have read and agreed to the published version of the manuscript.

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Not applicable.

## Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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# Figures

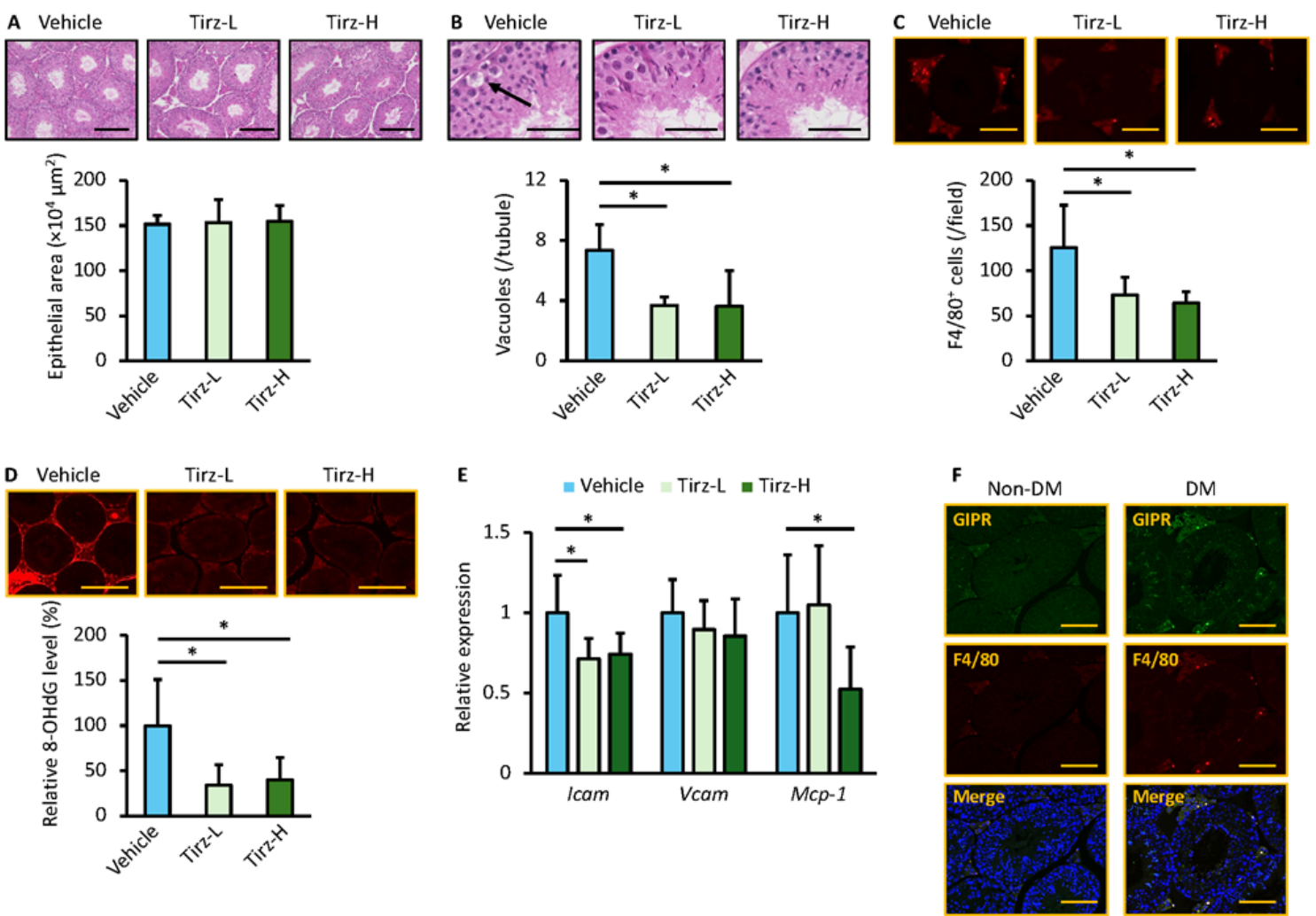


Fig. 1

## Figure 1

**Tirzepatide attenuated seminiferous tubule vacuolation, macrophage accumulation, and oxidative stress in the testis of diabetic mice.**

(A) Epithelial area of seminiferous tubules in the testis. Upper panels show representative H&E-stained testicular sections. (B) Number of vacuoles per seminiferous tubule. Upper panels show representative magnified images of H&E-stained testicular sections. Arrows indicate vacuoles. (C, D) Quantification of F4/80-positive cells and 8-OHdG-positive area in the testis. 8-OHdG levels are presented relative to vehicle-treated mice. (E) Gene expression levels of target molecules in the testis. Gene expression levels were normalized to 18S ribosomal RNA and are presented relative to vehicle-treated mice. (F) Representative immunofluorescence images demonstrating colocalization of GIPR with F4/80-positive macrophages in the testis. GIPR is shown in green, F4/80 in red, and nuclei in blue (DAPI). Tissues were obtained from the Non-DM and vehicle-treated DM groups in the exploratory study described in

Additional file 1. Data are means  $\pm$  SD. \* $p < 0.05$ . (A)–(E),  $n = 5$  per group. Scale bars: (A) and (D), 200  $\mu\text{m}$ ; (B) and (F), 50  $\mu\text{m}$ ; (C), 100  $\mu\text{m}$ . Tirz-L, tirzepatide 0.16 nmol/kg/day; Tirz-H, tirzepatide 1.00 nmol/kg/day; Icam, intercellular adhesion molecule-1; Vcam, vascular cell adhesion molecule-1; Mcp-1, monocyte chemoattractant protein-1; Non-DM, non-diabetic mice; GIPR, glucose-dependent insulinotropic polypeptide receptor.

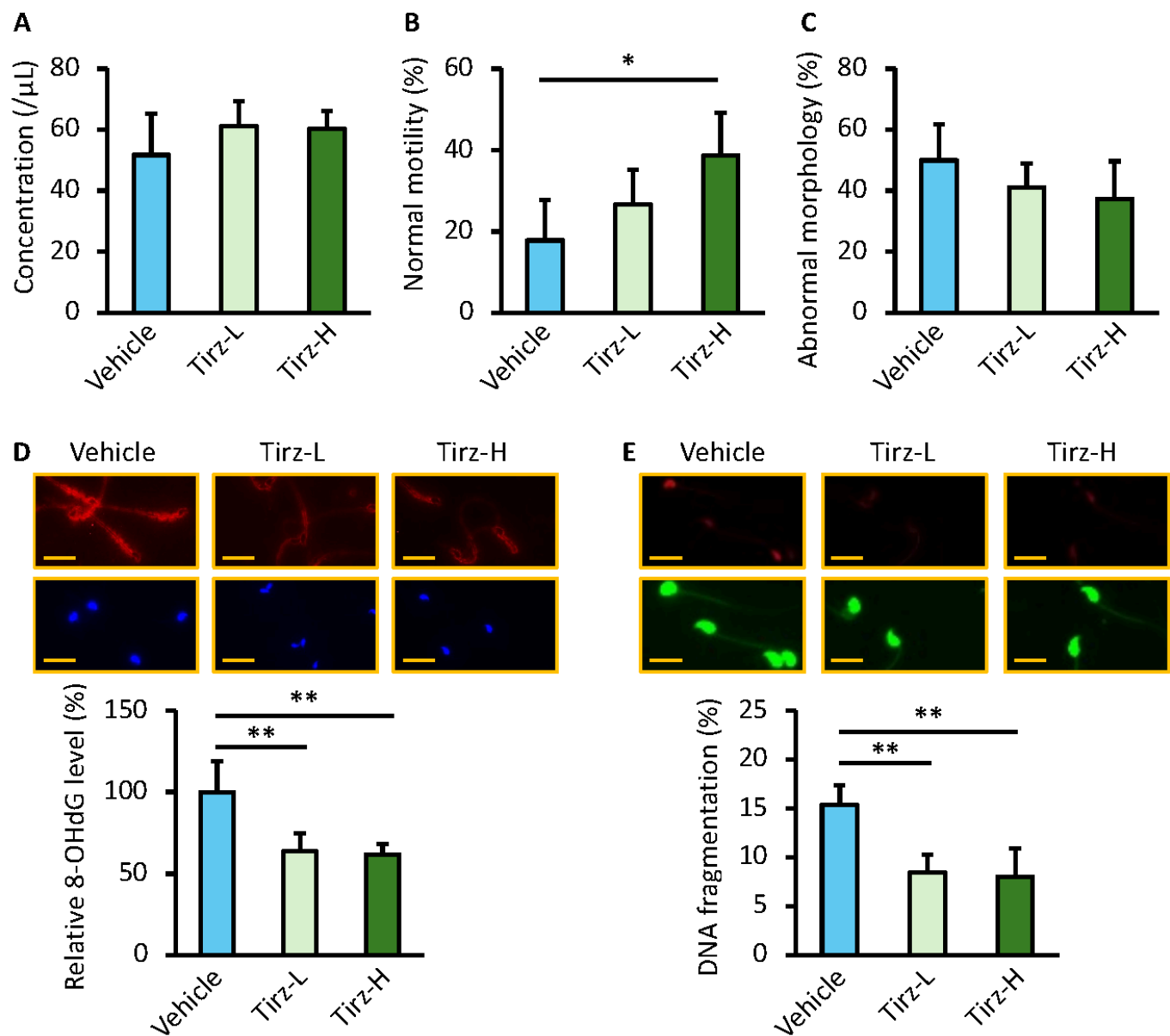


Fig. 2

Figure 2

## **Tirzepatide attenuated sperm oxidative stress and DNA fragmentation in diabetic mice.**

(A) Sperm concentration. (B) Sperm motility. (C) Abnormal sperm morphology. (D) Sperm oxidative stress assessed by 8-OHdG staining. Upper panels show representative images of 8-OHdG staining (red), and lower panels show DAPI nuclear staining (blue). 8-OHdG levels are presented relative to vehicle-treated mice. (E) Sperm DNA fragmentation assessed by acridine orange staining. Upper panels show sperm with fragmented DNA, indicated by red fluorescence, and lower panels show sperm with intact DNA, indicated by green fluorescence. Data are means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ .  $n = 5$  per group. Scale bars: (D), 25  $\mu\text{m}$ ; (E), 50  $\mu\text{m}$ .

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