A novel glucokinase activator TMG-123 causes long-lasting hypoglycemia and impairs spermatogenesis irreversibly in rats

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ABSTRACT — The importance of glucose is well known as an energy source in testes. In order to evaluate the effects of long-lasting hypoglycemia on testes, a novel glucokinase activator, TMG-123, was dosed to rats at 5, 20 and 100 mg/kg for 13 weeks. As a result, plasma glucose levels decreased for several hours with increasing doses over the dose range of 5 to 100 mg/kg. No toxicological findings attributable to the test article were observed in clinical observation, measurements of body weight and food consumption, necropsy, and organ weight measurement. Histopathology showed scattered degeneration of seminiferous tubules in testes, and exfoliation of germ cells related to the degeneration of seminiferous tubules was observed in the lumen of both epididymides in the same animals at the end of the dosing period. Similar histopathological findings were noted at the end of the recovery period. In addition, a fertility study was conducted at the same doses for 13 weeks for males and 5 weeks for females. Sperm analysis showed decreases in the sperm concentration and the motility index and an increase in the incidences of sperm malformations. However, there were no abnormalities in the copulation or fertility rate. These results suggest that long-lasting hypoglycemia in rats is harmful to spermatogenesis and the testicular damage does not recover.

Key words: Hypoglycemia, Testicular toxicity, Testis, Sperm, Germ cell, Reproductive organs

INTRODUCTION

We previously reported that testicular toxicity was observed by dosing insulin in rats and this was considered to be caused by long-lasting hypoglycemia (Kobayashi et al., 2015). In this study, we induced hypoglycemic condition by dosing a novel antidiabetic agent and evaluated testicular toxicity and the reversibility of the toxicity. As an antidiabetic agent, a novel Glucokinase (GK) activator TMG-123 was used.

GK belongs to the hexokinase (HK) family and is also known as HK type IV. GK plays important roles in glucose metabolism and facilitates the phosphorylation of glucose to glucose-6-phosphate (Wilson 1995; Massa et al., 2011). GK is mainly located in the major organs like pancreas, liver, brain, and gastrointestinal tract (Matschinsky 2009; Efannov et al., 2005). GK lowers blood glucose concentrations by enhancing glucose uptake and glycogen synthesis in the liver and increasing insulin secretion from pancreatic beta-cells (Matschinsky, 1990, 2009; Ferre et al., 1996).

Tsumura and his colleagues investigated the in vitro and in vivo pharmacological characteristics of TMG-123 (Tsumura et al., 2017). TMG-123 showed selective activation of GK enzyme activity without increasing Vmax. In addition, TMG-123 showed glucose-lowering effects without increasing plasma insulin levels in several animal models of T2DM (Goto-Kakizaki rats, db/db mice and ZDF rats) and, therefore, it is likely that TMG-123 acts primarily on the liver. TMG-123 is assumed to improve
glucose tolerance not by stimulating insulin secretion in pancreatic beta-cells, but mainly by increasing glucose uptake in liver.

We conducted two types of studies to evaluate the effect of hypoglycemia caused by TMG-123 on spermatogenesis. First, we conducted a 13-week repeated dose study and investigated the effect of hypoglycemia on male reproductive toxicity and reversibility of the toxicity by evaluating the organ weights and histopathology of male reproductive organs. Second, we conducted a fertility study and evaluated the effect on spermatic parameters and mating activity.

**MATERIALS AND METHODS**

**Animal**

Sprague Dawley male and female rats [Crl:CD(SD), 7 (male) and 5 (female) weeks old] were purchased from CHARLES RIVER LABORATORIES JAPAN, INC. (Kanagawa, Japan). In the 13-week repeated dose study, animals were quarantined and acclimatized for 12 days upon arrival at the animal facilities. In the fertility study, males and females were quarantined and acclimatized for 7 days and 28 days, respectively. The rats in both studies were housed individually in hanging-type stainless steel wire cages except during the mating period in the fertility study. The rats were provided ad libitum access to stainless steel feeders for pellet diet (CLEA Japan, Tokyo, Japan or Oriental Yeast Co., Ltd., Chiba, Japan) and water. The animal room was maintained at 22°C to 26°C, 40% to 83% relative humidity, with a 12-hr light/dark regime and an air exchange rate of 10 to 20 times per hr in the 13-week repeated dose study, and 22°C to 24°C, 47% to 68% relative humidity, with a 12-hr light/dark regime and an air exchange rate of 15 to 17 times per hr in the fertility study. Regarding the 13-week repeated dose study, the animals were assigned by stratified randomization to five groups of eight animals for plasma glucose levels and toxicokinetic evaluation (main study group) and four groups (all groups except for negative control) of six animals for plasma glucose levels and toxicokinetic evaluation (satellite group). The vehicle control and high-dose groups in satellite group were also used for reversibility evaluation. Regarding the fertility study, the animals were assigned by stratified randomization to five groups of 20 male animals and five groups of 20 female animals.

All experimental procedures were approved by the Animal Care and Use Committee of testing facilities. All efforts were made to minimize animal suffering.

**Dosing**

TMG-123 was synthesized in KYORIN Pharmaceutical Co., Ltd. (content: 100.4% by high performance liquid chromatography) and used as the test article. Water for injection, JP (FUSO Pharmaceutical Industries, Ltd., Osaka, Japan or Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was used as the negative control article. Gelucire 44/14 (abbreviated as Gelucire, Gattefosse Corporation, Saint-Priest, France) and Polyethylene glycol 400 (abbreviated as PEG400, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were weighed using an electric balance at a ratio of 3:2. This mixture (abbreviated as PEG/Gel) was used as a vehicle. The dosing formulations were heated in a hot water bath at approximately 40°C and sampled in a disposable syringe with a lock attached to a gastric tube for rats while being stirred using a magnetic stirrer. The dosing formulations were administered by oral gavage at 5, 20 and 100 mg/kg/day in both studies. The negative control and vehicle control were administered in the same manner.

**13-week repeated dose study**

The dosing formulations were administered to male rats for 13 weeks which cover the entire sperm cycle, followed by 8-week recovery period. Clinical observation was conducted every day, and body weight and food consumption were measured once a week. The rats were fasted on the day before necropsy and then sacrificed on the day of necropsy. After the rats were necropsied, the testes, epididymides, prostates and seminal vesicles (with coagulating glands), and pituitary glands were weighed. The bilateral testes and epididymides were weighed separately, and the total weights were calculated. The relative organ weights were calculated using the body weight on the day of necropsy. The testes in all toxicity group animals were immersion-fixed in Bouin’s solution and the epididymides, prostates, seminal vesicles and pituitary glands in all toxicity group animals were immersion-fixed in 10% neutral buffered formalin. The tissue specimens were prepared by sectioning the paraffin-embedded tissues, stained with hematoxylin and eosin, and examined microscopically. Blood samples were obtained from 2 or 3 animals of each satellite group at various time points (before dosing, and 0.5, 1, 2, 4, 8, and 24 hr after dosing) on Day 28 and Day 90 of dosing. Plasma glucose levels were measured using the HK method with a 7180 Clinical Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). For toxicokinetics, samples were analyzed by LC-MS/MS and then several toxicokinetic parameters such as maximum concentration ($C_{\text{max}}$), time to maximum
concentration ($T_{\text{max}}$) and area under the plasma concentration-time curve (AUC$_{0\text{-}24\text{hr}}$) were calculated.

**Fertility study**

The dosing formulations were administered to male rats for 13 weeks (from 10 weeks prior to mating, throughout the mating period, until Day 7 of gestation). Males and females in the same group were co-housed on a one-to-one basis from Days 71 and 15 of dosing, respectively, for a maximum of 16 days.

For males, clinical observation was conducted every day, and measurement of body weight was conducted twice a week. The rats were sacrificed under ad libitum feeding conditions. At necropsy, 5 μL of semen was collected from the right cauda epididymis and diluted with 625 μL of Dulbecco’s modified Eagle medium. The semen dilutions were incubated for 1 hr at 37°C. Then, the sperm motility index was determined using a Sperm Quality Analyzer II C-P (Medical Electronic System Ltd., Hefa, Israel). The semen dilution was further diluted with saline to a final dilution of 3024-fold. The number of sperms was counted under a light microscope using a Burker-Turk hemocytometer. The sperm concentration in semen from the cauda epididymis was calculated based on the volume of the counting chamber of the hemocytometer and the dilution ratio. The semen dilution was smeared on a glass slide, dried and stained with Giemsa. A total of 200–207 sperms from each animal were evaluated microscopically for sperm morphology.

For females, clinical observation was conducted every day, and measurements of body weight were conducted every day or twice a week. Mated females underwent Cesarean section and necropsy on Day 13 of gestation, and copulation rate and fertility rate were calculated.

**Statistics**

In the 13-week repeated dose study, for body weight and organ weight of the main study group, the data were analyzed statistically as follows. For comparison between the vehicle control and TMG-123-treated groups at the end of the dosing period, numerical data were first analyzed by Bartlett’s test (significance level: 5%). When the group variance was homogeneous, the mean value of all groups was compared by Dunnett’s test, and when the group variance was heterogeneous, the mean value of all groups was compared by Steel’s test (significance levels: 1% and 5%, two-tailed test). For body weight and organ weight of the satellite group, the F test was performed to determine homogeneity of variance between the vehicle control and test article groups (significance level: 5%). If homogeneity of variance was found, Student’s t test was performed; if homogeneity of variance was not found, the Aspin-Welch t test was performed (significance levels: 1% and 5%, two-tailed test).

In the fertility study, the comparison between the vehicle control and TMG-123-treated groups was performed as follows (significance level: 5%). For the copulation rate and the fertility rate, the Chi-square test was used. For body weight, organ weight, pre-coital period (in days), number of corpora lutea, number of implantations, number of live embryos, sperm motility index and sperm concentration, the data were first analyzed by Bartlett’s test. When the group variance was homogeneous, the mean value of two groups was compared by Dunnett’s test, and when the group variance was heterogeneous, the mean value of two groups was compared by a mean rank test of the Dunnett type. For the pre-implantation loss, post-implantation loss and incidence of sperm malformations, a mean rank test of the Dunnett type was used.

**RESULTS**

13-week repeated dose study

There were no treatment-related deaths or moribund states in the test article groups. The mean values of $C_{\text{max}}$ and AUC$_{0\text{-}24\text{hr}}$ increased with the doses ranging from 5 to 100 mg/kg (Table 1). Plasma glucose levels on Days 28 and 90 of dosing decreased with increasing doses over the dose range of 5 to 100 mg/kg (Fig. 1). The changes over time in plasma glucose levels after administration of TMG-123 were similar for Days 28 and 90. No animal had a plasma glu-

| Table 1. Toxicokinetics parameters of TMG-123. |
|-----------------------------------------------|
| Dose | 5 mg/kg | 100 mg/kg |
|      | Day 28  | Day 90    | Day 28 | Day 90    | Day 28 | Day 90 |
| $T_{\text{max}}$ (hr) | 2.0 | 2.0 | 2.0 | 2.0 | 1.0 | 1.0 |
| $C_{\text{max}}$ (ng/mL) | 439 | 357 | 1930 | 1790 | 6400 | 5910 |
| AUC$_{0\text{-}24\text{hr}}$ (ng·hr/mL) | 2430 | 2420 | 8950 | 9780 | 29200 | 28500 |

n=2 or 3
Cose level of 50 mg/dL or lower in the 5 mg/kg group. Some animals had a plasma glucose level of 50 mg/dL or lower up to 4 or 8 hr after dosing in the 20 or 100 mg/kg group, respectively (data not shown). For clinical signs, there were no test article-related changes in the test article groups during the study. During the dosing period, loose stools were sporadically observed in the vehicle control and test article dosing groups.

In body weight, no statistically significant changes were observed during the dosing and recovery periods. In food consumption, there were no test article-related changes during the dosing and recovery periods.

At necropsy, at the end of the dosing period, retention of liquid contents in the cecum and the large cecum was observed in all animals in the vehicle control and 5, 20 and 100 mg/kg groups. At the end of the recovery period, no changes in the cecum were observed, which were noted at the end of the dosing period.

Fig. 1. Plasma glucose levels on Day 28 (A) and Day 90 (B) of dosing. Blood samples were obtained at 0, 0.5, 1, 2, 4, 8, and 24 hr after administration of vehicle or test article. The data are presented as the mean ± S.D. (n = 2 or 3).
In organ weight, at the end of the dosing period, although the mean absolute testis weights and absolute and relative pituitary weights were statistically significantly lower in the 20 mg/kg group than in the vehicle control group, these lower values were not dose-related and, therefore, considered incidental changes (Table 2). At the end of the recovery period, no statistically significant changes were observed.

In histopathological evaluation (Fig. 2), at the end of the dosing period, scattered degeneration of seminiferous tubules in both testes was observed in 2 (very slight, 1; slight, 1) of 8 males in the 100 mg/kg group and 1 (very slight) of 8 males in the 20 mg/kg group. The histopathological change was diagnosed when more than 5 degenerated tubules were observed in each cross-section surface of testis. The change was graded as “slight” when the scattered degeneration was clearly recognized at low magnification and graded as “very slight” when the scattered degeneration was recognized only at high magnification. The degenerated seminiferous tubules contained relatively large and discrete tubular vacuolation, multinucleated giant cells, germ cells with eosinophilic cytoplasm and nuclear condensation, retention of mature spermatids, disorganization of the germ cells, and germ cell loss. Very slight exfoliation of germ cells was observed in the lumen of both epididymides in these animals. At the end of the recovery period, scattered degeneration of seminiferous tubules in both testes was observed in 2 (very slight) of 6 males in the 100 mg/kg group. Very slight exfoliation of germ cells was also found in the lumen of both epididymides in these animals. There were no treatment-related changes in prostates, seminal vesicles and pituitary glands.

Fertility study

There were no treatment-related deaths or moribund states in the test article groups. Loose and/or muddy stools were observed in the vehicle control and all test article-treated groups. No other clinical signs were observed in any male and female.

In body weight, there were no test article-related changes.

At necropsy, the large cecum was observed in the vehicle control and all test article-treated groups. However, there were no findings related to the administration of the test article in both sexes.

In sperm analysis (Table 3), in the comparison between the vehicle control and each test article-treated group, a statistically significant increase in the incidence of sperm malformations was noted in the 100 mg/kg group. In addition, a tendency to decrease in the sperm concentration (1599 × 10⁶/mL) and sperm motility index (212) was noted in this group, though not statistically significant, and these values were also below the historical ranges of the testing facility (1715 to 2463 × 10⁶/mL for the sperm concentration and 229 to 287 for the sperm motility index). No statistically significant changes or a tendency to increase/decrease was noted in the 5 or 20 mg/kg group.

Regarding mating ability and fertility, no statistically significant differences were noted in the copulation rate, fertility rate or pre-coital period (in days) between the vehicle control and each test article-treated group.

Table 2. Organ weights.

| Group                  | Negative Control | Vehicle Control | TMG-123 5 mg/kg | TMG-123 20 mg/kg | TMG-123 100 mg/kg |
|------------------------|------------------|-----------------|-----------------|------------------|-------------------|
| No. of animals         | 8                | 8               | 8               | 8                | 8                 |
| Testis (g)             | 3.4594 ± 0.3580  | 3.5817 ± 0.0822 | 3.2390 ± 0.3464 | 3.2282 ± 0.3411  | 3.4735 ± 0.2462  |
| (%)                    | 0.6518 ± 0.0608  | 0.6562 ± 0.0906 | 0.6076 ± 0.0518 | 0.5857 ± 0.0431  | 0.6372 ± 0.0784  |
| Epididymis (g)         | 1.493 ± 0.1409   | 1.4480 ± 0.0719 | 1.4071 ± 0.2292 | 1.4127 ± 0.1106  | 1.4127 ± 0.1579  |
| (%)                    | 0.282 ± 0.0319   | 0.2654 ± 0.0407 | 0.2633 ± 0.0344 | 0.2570 ± 0.0197  | 0.259 ± 0.0372   |
| Prostate and seminal   | 3.0300 ± 0.4823  | 3.1775 ± 0.4474 | 3.0313 ± 0.4966 | 3.0265 ± 0.3936  | 2.9201 ± 0.2332  |
| vesicles (%)*          | 0.5702 ± 0.0808  | 0.5748 ± 0.0468 | 0.5696 ± 0.0933 | 0.5541 ± 0.1006  | 0.533 ± 0.0361   |
| Pituitary gland (g)    | 0.0133 ± 0.0018  | 0.0147 ± 0.0024 | 0.0135 ± 0.0017 | 0.0124 ± 0.0014* | 0.0147 ± 0.0015  |
| (%)                    | 0.0025 ± 0.0003  | 0.0027 ± 0.0002 | 0.0025 ± 0.0002 | 0.0022 ± 0.0002**| 0.0027 ± 0.0003  |

Values are mean ± S.D.

a: The sum of right and left organ weights.
b: Ratio of organ weight to body weight (relative organ weight).
*: p < 0.05, **: p < 0.01 (Significantly different from Vehicle Control)
Fig. 2. Representative histopathological images (hematoxylin and eosin staining) of testes and epididymides. Images of a control testis (A) and an epididymis (B) at low magnification. No abnormalities were observed in the control tissues. Images of a testis at low (C and D) and high magnification (E), and an epididymis at low magnification (F) from an animal treated with TMG-123 100 kg/kg after a 13-week dosing period. Degenerated seminiferous tubules were observed sporadically. The tubules contained germ cells with eosinophilic cytoplasm and nuclear condensation (arrowheads in C), relatively large and discrete tubular vacuolation (arrows in C), multinucleated giant cells (arrows in D), retention of mature spermatids (arrows in E), germ cell loss, and disorganization of the germ cells. The epididymis exhibited exfoliation of germ cells in the lumen (arrows in F). Images of a testis (G) and an epididymis (H) at low magnification from an animal treated with TMG-123 100 kg/kg after an 8-week recovery period. Degeneration of seminiferous tubules and exfoliation of germ cells were still observed. The scale bars represent 200 µm.
Glucokinase activator causes hypoglycemia and impairs spermatogenesis

**DISCUSSION**

In this study, we evaluated the relationship between hypoglycemia and testicular toxicity by using a GK activator. At first, we dosed the compound to rats for hypoglycemia and testicular toxicity by using a GK about 50 mg/dL (Rosenstock et al., 2001; Bonds et al., 1995; Ueda et al., 2011). Spermatogonia may utilize glucose as the major energy substrate. Spermatocytes and spermatids require lactate/pyruvate, which is converted from glucose in Sertoli cells. Therefore, hypoglycemia caused by the GK activator was considered to lead to testicular toxicity.

Moreover, large and discrete vacuolation in the seminiferous tubules is one of the most common findings when Sertoli cells are injured (Creasy, 2001). Therefore, taking into consideration histopathological features in this study, there is the possibility that Sertoli cells were also injured and resulted in the failure to support spermatogenesis (Creasy, 2001). Since it is generally reported that testicular damage noted in spermatogonia and Sertoli cells tend to be irreversible (Nolte et al., 1995), our results indicating no reversibility of testicular changes are consistent with the reported data.

In the fertility study, the sperm analysis showed decreases in the sperm concentration and the motility index and an increase in the incidences of sperm malformations. On the other hand, there was no change in the fertility rate. The rat is well known to be fecund. It is reported that even when rat sperm concentration was

| Group          | Negative Control | Vehicle Control | TMG-123 5 mg/kg | TMG-123 20 mg/kg | TMG-123 100 mg/kg |
|----------------|------------------|----------------|-----------------|-----------------|------------------|
| No. of animals | 20               | 20             | 20              | 20              | 20               |
| Sperm motility index | 269 ± 21 | 256 ± 45 | 271 ± 24 | 269 ± 27 | 212 ± 87 |
| The sperm concentration (×10^6/mL) | 1953 ± 225 | 1895 ± 233 | 1989 ± 264 | 1911 ± 245 | 1599 ± 516 |

The incidences of sperm malformations (%)

- Total: 0.4 ± 0.5, 0.8 ± 0.6, 0.6 ± 0.6, 0.7 ± 0.5, 2.7 ± 1.9**
- Head: 0.2 ± 0.3, 0.2 ± 0.3, 0.2 ± 0.3, 0.2 ± 0.3, 0.7 ± 0.8*
- Neck: 0.2 ± 0.3, 0.5 ± 0.6, 0.3 ± 0.4, 0.5 ± 0.5, 1.6 ± 1.6**
- Middle piece: 0.0 ± 0.0, 0.0 ± 0.1, 0.0 ± 0.0, 0.0 ± 0.0, 0.0 ± 0.0
- Tail: 0.1 ± 0.2, 0.1 ± 0.2, 0.1 ± 0.2, 0.1 ± 0.2, 0.4 ± 0.7

Values are mean ± S.D.

- a: The incidences were calculated in each region: head, neck, middle piece, and tail.
- b: Number of animals in TMG-123 100 mg/kg group. One male was excluded since sperm morphology could not be evaluated because of incidental changes.

*: p < 0.05, **: p < 0.01 (Significantly different from Vehicle Control)
reduced by 90%, it can still be fertile (Robaire et al., 1984; Bieber et al., 2006). The sperm concentration in the 100 mg/kg group in our study decreased by 16% compared with the vehicle control group and, therefore, there is no wonder that the fertility rate was not affected.

Although testicular changes could be affected by stress and the disruption of sex hormones, there is little chance of the secondary changes caused by these factors in our study. This is because the only finding in the clinical signs was a soft stool, which was also noted in the vehicle group, and no changes were observed in the organ weight of prostate and seminal vesicles, which is known as the highly sensitive parameter to the changes of sex hormone levels (Creasy, 2001; OECD, 2009). In addition, there were no treatment-related changes in the organ weight and histopathology of the epididymis and abnormalities in the sperm. In the previous report (Kobayashi et al., 2015), we reported the effects of insulin on spermatogenesis. The histopathological examination revealed that the insulin-treated animals exhibited degeneration of seminiferous tubules in the testes and exfoliation of germ cells in the lumens of epididymides. Sperm analysis of the group receiving 400 IU/kg insulin indicated that the sperm concentration tended to decrease and the incidences of sperm malformations tended to increase.

In this report, we used a GK activator compound, which has a unique characteristic. It is reported that TMG-123 did not increase plasma insulin levels in model animals, and it is assumed that TMG-123 improves glucose tolerance not by the stimulating insulin secretion, but mainly by enhancing hepatic glucose uptake (Tsumura et al., 2017). Two types of antidiabetic agents, which have different mechanisms, were confirmed to cause long-lasting hypoglycemia and similar testicular findings. Therefore, these results indicate that long-lasting hypoglycemia impairs spermatogenesis. Moreover, this impairment is not considered to be reversible taking into consideration the findings at the end of recovery period in this study.

There is just another report which conducted histopathological examination of the testes under hypoglycemic conditions in rats. Mancine et al. (1960) induced hypoglycemic coma by dosing insulin or tolbutamide in rats and revealed lesions of germinal epithelium such as vacuolation and multinucleated masses in testes. This report also supports our opinion that hypoglycemia damaged spermatogenesis.

In conclusion, our results suggest that hypoglycemia, induced by a compound that activates GK and mainly enhances hepatic glucose uptake, causes histopathological changes in testis and abnormalities in the sperm. In addition, the histopathological findings were not likely to be reversible. Since similar findings were reported when treated with insulin, it is strongly suggested that hypoglycemia could secondarily induce testicular toxicity. Therefore, very careful attention must be paid when observing hypoglycemic condition in preclinical studies.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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