Short Communication

Testicular Mineralization in KK-A\textsuperscript{y} Mice Treated with an Oxovanadium Complex

Takayasu Moroki\textsuperscript{1}, Yutaka Yoshikawa\textsuperscript{1}, Katsuhiko Yoshizawa\textsuperscript{2*}, Airo Tsubura\textsuperscript{2}, and Hiroyuki Yasui\textsuperscript{1*}

\textsuperscript{1}Department of Analytical and Bioinorganic Chemistry, Division of Analytical and Physical Chemistry, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan
\textsuperscript{2}Department of Pathology II, Kansai Medical University, 2-5-1 Shinmachi, Hirakata, Osaka 573-1010, Japan

Abstract: Vanadium has potential for use in diabetes therapy. Many investigators have reported toxic effects of inorganic vanadium salts; however, there are few reports on toxic effects of oxovanadium(VO\textsuperscript{2+}) complexes. Therefore, we studied VO\textsuperscript{2+} toxicity by examining histological changes and measuring the vanadium concentration in the testis after repeated oral administration of bis(1-oxy-2-pyridine-thiolato)oxovanadium(VO\textsuperscript{2+}) (VO(opt)\textsubscript{2}) for 2 or 4 weeks in KK-A\textsuperscript{y} mice. Severe mineralization and degeneration/necrosis of the seminiferous tubules were detected after either 2 or 4 weeks of administration. Vacuolar changes in Sertoli cells and the seminiferous epithelia, and hyperplasia of Leydig cells were observed in the testes of some animals. Vanadium concentrations in the mineralized testis were much higher than those in the testis of untreated KK-A\textsuperscript{y} mice. These results represent the first report of the possibility for seminiferous tubules mineralization induced by VO(opt)\textsubscript{2}, administration. Therefore, our research provides important information about the potentially toxic effects of VO\textsuperscript{2+} complexes. (DOI: 10.1293/tox.26.329; J Toxicol Pathol 2013; 26: 329–333)

Key words: diabetes, Leydig cell, KK-A\textsuperscript{y} mice, mineralization, testis, oxovanadium(VO\textsuperscript{2+}) complex

Vanadium, a trace element in animals and humans, has a wide variety of biological and physiological functions\textsuperscript{1}. The toxic effects of inorganic V\textsuperscript{5+} salts in diabetes model rats include severe diarrhea in life. Histopathologically, hepatocellular necrosis, fatty change and vacuolization, necrosis of renal tubules and necrosis of mucosal epithelial cells in the small intestine have been reported as the toxicities\textsuperscript{2–5}. In addition, testicular toxicity that seems to be related to vanadium-induced oxidative stress during spermatogenesis has been reported\textsuperscript{6}. Thus, inorganic V\textsuperscript{5+} salts have shown significant toxicity, and therefore, several investigators have studied pharmacologic effects of V\textsuperscript{5+} salts with lower toxicity\textsuperscript{7}.

Furthermore, several oxovanadium (VO\textsuperscript{2+}, the +4 oxidation state of the vanadium ion combined with oxygen to form VO\textsuperscript{2+}) complexes have been studied because of their improved bioavailability after oral administration at a relatively low dose, which results from the lipophilicity of VO\textsuperscript{2+} complexes being generally higher than that of either inorganic V\textsuperscript{4+} or V\textsuperscript{5+} salts.

Bis(1-oxy-2-pyridinethiolato)oxovanadium(VO\textsuperscript{2+}) (VO(opt)\textsubscript{2}) has been reported to be a potent, orally active insulin mimic for treatment of diabetes in animal models\textsuperscript{8,9}. However, toxicological studies of VO(opt)\textsubscript{2} have not been described in the literature. Furthermore, although sodium metavanadate (V\textsuperscript{5+}), NaVO\textsubscript{3}, was shown to cause testicular toxicity when delivered by gavage, there are no accounts of testicular toxicity due to administration of VO\textsuperscript{2+} complexes\textsuperscript{5,10}. If VO\textsuperscript{2+} complexes show reproductive toxicity, it would be fatal in humans, because many people take various vanadium (V\textsuperscript{4+} and V\textsuperscript{5+}) salts by gavage as supplements. In this study, we performed a histological assessment of testicular toxicity after repeated administration of VO(opt)\textsubscript{2} in KK-A\textsuperscript{y} mice, a mouse model of type 2 diabetes mellitus (DM)\textsuperscript{11–14}.

VO(opt)\textsubscript{2} was prepared by mixing 1-oxy-2-pyridinethione and VOSO\textsubscript{4} with a molar ratio of 2:1 of ligand:metal ion in an aqueous solution at a pH of 5–6. After mixing each solution for 30 min, precipitates were collected; then, the precipitates were washed several times with pure water and dried\textsuperscript{15}.

Received: 26 March 2013, Accepted: 28 May 2013
*Corresponding authors: H Yasui (e-mail: yasui@mb.kyoto-phu.ac.jp), K Yoshizawa (e-mail: yoshizak@hirakata.kmu.ac.jp)
©2013 The Japanese Society of Toxicologic Pathology
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/3.0/>.
KK-A⁺ mice (aged 10 weeks) received VO(opt)₂ by gavage administration in a PEG400 vehicle (VO(opt)₂-treated group) or received PEG400 (control) daily at about 08:00 for 2 or 4 weeks. The dose of VO(opt)₂ was 3 mg (59 μmol) for the first 2 days and 1.5 mg (30 μmol) for next 12 days; the dose was then adjusted to maintain amounts of 0.38 to 1.5 mg (7 to 30 μmol) vanadium kg⁻¹ of body weight per day for the next 14 days because we needed to adjust the effective dosing schedule to pharmacologically control a normal blood glucose level. The selected dose was the same as that previously shown to lower blood glucose levels in KK-A⁺ mice (aged 10 weeks) receiving VO(opt)₂ by gavage administration in a PEG400 vehicle (VO(opt)₂-treated group) or receiving PEG400 (control) daily at about 08:00 for 2 or 4 weeks. The dose of VO(opt)₂ was 3 mg (59 μmol) for the first 2 days and 1.5 mg (30 μmol) for next 12 days; the dose was then adjusted to maintain amounts of 0.38 to 1.5 mg (7 to 30 μmol) vanadium kg⁻¹ of body weight per day for the next 14 days because we needed to adjust the effective dosing schedule to pharmacologically control a normal blood glucose level. The selected dose was the same as that previously shown to lower blood glucose levels in KK-A⁺ mice and to be much lower than a lethal dose⁹.

Blood samples for glucose level analysis were obtained from the tail vein of each mouse and monitored with a Glucocheck (Arkay, Kyoto, Japan) once a week.

The testes of all mice employed in this study, 3 to 5 mice in each group, were removed after exsanguination under anesthesia at each of the previously described time points, and slides were made and stained with hematoxylin and eosin (HE). In addition, the liver, spleen, kidney, pancreas, gastrointestinal tract and eye of all mice were also examined by the same process as used for the testes. Sequential sections of testes were stained with von Kossa stain to detect tissue mineralization including calcium.

Formalin-fixed testes were divided into halves, and one part was employed for histopathological examination; the other part was weighed and heated repeatedly at approximately 200°C with 60% HNO₃ and 30% H₂O₂ in 50-mL beakers for determination of metals. When the residue turned white, the dried samples were dissolved in 1% HNO₃. Vanadium, calcium and iron concentrations in each sample were then determined using an ICPM-8500 system (Shimadzu Corporation, Kyoto, Japan)¹⁰. The minimum limits of detection of vanadium, calcium, and iron were 5, 10, and 10 ppb (ng/mL), respectively.

Table 1. Histological Changes in the Testis after Administration of VO(opt)₂

| Group | Dosing periods (weeks) | Number of animals | Blood glucose level (mg/dL) | Histological findings |
|-------|------------------------|-------------------|-----------------------------|----------------------|
|       |                        |                   |                             | Seminiferous tubule   |
|       |                        |                   |                             | Degeneration/necrosis | Mineralization       | Leydig cell hyperplasia |
| Untreated KK-A⁺ mice (control) | 2               | 4                 | 421 ± 99                    | 4 0 0 0 0 4 0 0 0 4 0 |
| VO(opt)₂-treated KK-A⁺ mice | 2               | 3                 | 364 ± 136                  | 0 1 0 1 1 1 1 1 0 1 2 |
| VO(opt)₂-treated KK-A⁺ mice | 2               | 3                 | 237 ± 61*                  | 0 0 0 0 1 3 0 0 1 3 2 |

Grading scores: –, none; +, slight; ++, moderate; ++++, strong; +++, severe. *p < 0.05 vs. Untreated KK-A⁺ mice (control). # The number of animals presenting degeneration/necrosis in the 2-week-VO(opt)₂ treatment group was significantly increased compared with the 4-week VO(opt)₂ treatment group.

During the course of the study, abnormal clinical signs were not observed. Body weight and food consumption of VO(opt)₂-treated animals were not significantly different from those of the untreated mice in either the 2- or 4-week administration groups (data not shown). Blood glucose levels after VO(opt)₂ administration in KK-A⁺ mice were slightly lower in the 2-week treatment group and significantly lower in the 4-week treatment group than in the untreated KK-A⁺ mice, as shown in Table 1. These blood glucose level shifts were similar to those reported in a previous study⁶. The histopathological findings in the testis of the control and VO(opt)₂-treated group are summarized in Table 1. Testicular sections from the control animals showed no abnormal features at any time point (Fig. 1a). Two of 3 mice in the 2-week treatment group and 1 of 5 mice in the 4-week treatment group showed slight to severe mineralization. Furthermore, all mice in the 2-week treatment group, and 1 of 5 mice in the 4-week treatment group showed slight to severe degeneration/necrosis in the seminiferous tubules (Fig. 1b). The severity grading was used to provide an index of the numbers of germ cells and tubules affected. In the severe cases, diffuse coagulative necrosis and depletion of all types of germ cells with Sertoli cell vacuolation were seen in most tubules. The mineralized areas were strongly positive for von Kossa stain (Fig. 1e), indicating mineral deposition. Slight degeneration/necrosis of the seminiferous tubules was accompanied by vacuolar changes (Fig. 1e). Moderate to strong degeneration/necrosis of the seminiferous tubules was accompanied by coagulative necrosis, along with Leydig cell hyperplasia in 2 of 3 mice in the 2-week treatment group and 2 of 5 mice in the 4-week treatment group (Fig. 1d). The number of animals presenting degeneration/necrosis in the 2-week treatment group was significantly increased compared with the 4-week treatment group; however, significant differences in other changes were not detected.

The related changes in other main systemic organs, such as the liver, spleen, kidney, pancreas, gastrointestinal tract and eye were also examined histopathologically. Abnormal changes related to DM were detected in the liver,
fat deposition, kidney, overgrowth of the mesangial matrix, and pancreas, hypertrophy of islet cells, as described in our previous report.

The vanadium, calcium and iron concentrations in the testes are presented in Table 2. The vanadium concentrations in the testes of the control and VO(opt)$_2$-treated mice without mineralization were under the minimum limit of detection. The calcium and iron concentrations in the testes...
Testicular Toxicity Induced by VO(opt)$_2$

Table 2. Measurement of Element Concentrations in the Testes after 2 or 4 Weeks of Repeated Administration

| Group                  | Mineralization in the testes | Number of animals | Dosing period (weeks) | Vanadium (mg/g dry weight) | Calcium (mg/g dry weight) | Iron (mg/g dry weight) |
|------------------------|-----------------------------|-------------------|-----------------------|---------------------------|---------------------------|-----------------------|
| Untreated KK-A$^+$ mice (control) | -                           | 4                 | 2                     | B.L.Q                     | 0.472±0.775               | 0.121±0.020           |
|                         |                             | 4                 | 4                     | B.L.Q                     | 0.322±0.136               | 0.125±0.024           |
| VO(opt)$_2$-treated KK-A$^+$ mice | -                           | 1                 | 2                     | B.L.Q                     | 0.033                     | 0.133                 |
|                         |                             | 2                 | 4                     | B.L.Q                     | 0.237±0.207               | 0.113±0.024           |
| B.L.Q: Below the lower limit of quantification.

Testes of the control and VO(opt)$_2$-treated mice without mineralization were similar between the groups. In contrast, the vanadium concentrations in the testes with slight to severe mineralization were detectable, as shown in Table 2. Furthermore, the calcium and iron concentrations in these groups were higher than the corresponding concentrations in the control and VO(opt)$_2$-treated mice without mineralization. In addition, the correlations between the grades of individual histopathological changes and the vanadium concentration are presented in Fig. 2. Histopathological changes were well correlated with the vanadium concentration in the testis.

In the present study, VO(opt)$_2$ induced seminiferous tubular degeneration and coagulative necrosis, followed by dystrophic mineralization. There are no reports of either mineralization or degeneration/necrosis of the seminiferous tubules in previous studies.$^{2-6}$ Vanadium concentrations in the testes with slight to severe mineralization were higher than those in the testes without mineralization, and the testes with mineralization also showed higher calcium and iron concentrations. After damaged tissues received dystrophic calcification, divalent mineral cations were distributed to them from the blood circulation, like calcium and iron. Oxovanadium(VO$^2+$) would also be distributed to the dystrophic calcification because of divalent mineral cation. Therefore, vanadium was not measurable in the testis when mineralization was not detected. To evaluate this association, the correlation between grades of histological changes with vanadium concentrations was examined (Fig. 2). In normal mice and rats, mineralization of the seminiferous tubules has often been observed. However, almost all autogenetic mineralization represents a focal change, and its occurrence is relatively infrequent. Therefore, the mineralization of the seminiferous tubules described in the present study may be related to VO(opt)$_2$ exposure of KK-A$^+$ mice. Additionally, abnormal changes related to DM were detected in the liver, kidney and pancreas of the KK-A$^+$ mice, but no mineralization was detected in these organs (data not shown)$^{14}$. Therefore, these changes related to DM were not related to mineralization in the testis. However, there was no significant incidence of mineralization and Leydig cell hyperplasia mainly because of the small number of animals examined in this study. The grades of degeneration/necrosis and mineralization in the group treated with VO(opt)$_2$ for 4 weeks did not increase time-dependently in the dosing period compared with the group treated for 2 weeks. The dosage of VO(opt)$_2$ was much higher for the first 2 weeks, 1.5 to 3 mg vanadium kg$^{-1}$ of body weight, compared with the latter 2-week period, 0.38 to 1.5 mg vanadium kg$^{-1}$ of body weight. Therefore, we assume that the testes of five of the eight VO(opt)$_2$-treated mice might have been severely damaged in the first 2 weeks and that the damage might be not enhanced largely with time over a longer period.

Leydig cell hyperplasia occurs in response to increased levels of the luteinizing hormone from the pituitary or in response to the release of stimulatory paracrine factors within the testes as a compensatory response to decreased spermatogenesis.$^{17}$ Although the plasma testosterone concentrations decreased after administration of NaVO$_3$, the role of Leydig cells in the production of testosterone is known, and Leydig cell hyperplasia is not induced. This suggests that the Leydig cell hyperplasia observed in our study might be associated with primary damage to the seminiferous tubules induced by VO(opt)$_2$.

In conclusion, VO$^{2+}$ complexes may induce degeneration/necrosis of the seminiferous tubules, followed by dystrophic mineralization. The changes observed were associ-
ated with the accumulation of vanadium in the testes. These same remarkable findings after VO\(_{\text{opt}}\)\(_2\) administration were also observed in the testes after bis(ethylmaltolato) oxovanadium(VO\(_2^+\)) administration (data not shown). These results suggest that testicular toxicity may be induced by administration of other VO\(_2^+\) complexes. However, only 2 complexes were tested in this study. Even if testicular toxicity can be induced by VO\(_2^+\) complexes, it is not always observed in all the animals treated. Because there are many other similar compounds, it will be necessary to study other VO\(_2^+\) complexes. In addition, we have not administrated VO\(_{\text{opt}}\)\(_2\), to normal animals, only diabetic ones, because the original goal of this study was to assess the pharmacological effect of VO\(_{\text{opt}}\)\(_2\). Therefore, if we wish to investigate the mechanism of testicular toxicity of vanadium, we will have to use many more animals to accumulate a large amount of background data and observe further experiments. Despite the limitations of the present study, there are currently no reports of testicular toxicity describing mineralization induced by VO\(_2^+\) complexes, and thus, our study may provide important information regarding the effects of VO\(_2^+\) complexes in diabetes.

Acknowledgments: This study was supported financially in part by a grant from the MEXT of Japan-Supported Program for the Strategic Research Foundation at Private Universities (2012–2016, S1201008).

References

1. Crans DC, Smee JJ, Gaidamauskas E, and Yang L. The chemistry and biochemistry of vanadium and the biological activities exerted by vanadium compounds. Chem Rev. 104: 849–902. 2004. [Medline] [CrossRef]

2. Domingo JL, Gomez M, Sanchez DJ, Llobet JM, and Keen CL. Toxicology of compounds in diabetic rats: the action of chelating agents on vanadium accumulation. Mol Cell Biochem. 153: 233–240. 1995. [Medline] [CrossRef]

3. Imura H, Shimada A, Naota M, Morita T, Togawa M, Hasegawa T, and Seko Y. Vanadium toxicity in mice: Possible impairment of lipid metabolism and mucosal epithelial cell necrosis in the small intestine. Toxicol Pathol. 41: 842–856. 2013.

4. Obianime AW, Gogo-Abite M, and Roberts II. The effects of ammonium metavanadate on biochemical, hormonal, haematological and histopathological parameters of the female Wistar rats. Niger J Physiol Sci. 24: 187–194. 2009. [Medline]

5. Wei CI, Al Bayati MA, Culbertson MR, Rosenblatt LS, and Hansen LD. Acute toxicity of ammonium metavanadate in mice. J Toxicol Environ Health. 10: 673–687. 1982. [Medline] [CrossRef]

6. Chandra AK, Ghosh R, Chatterjee A, and Sarkar M. Amelioration of vanadium-induced testicular toxicity and adrenergic corticomedullary hyperactivity by vitamin E acetate in rats. Mol Cell Biochem. 306: 189–200. 2007. [Medline] [CrossRef]

7. Shukla R, Barve V, Padhye S, and Bhonde R. Reduction of oxidative stress induced vanadium toxicity by complexing with a flavonoid, quercetin: a pragmatic therapeutic approach for diabetes. Biometals. 19: 685–693. 2006. [Medline] [CrossRef]

8. Takeshita S, Kawamura I, Yasuno T, Kimura C, Yamamoto T, Seki J, Tamura A, Sakurai H, and Goto T. Amelioration of insulin resistance in diabetic ob/ob mice by a new type of orally active insulin-mimetic vanadyl complex: bis(1-oxy-2-pyridinethiolato)oxovanadium(IV) with VO(S\(_2\)O\(_2\)) coordination mode. J Inorg Biochem. 85: 179–186. 2001. [Medline] [CrossRef]

9. Sakurai H, Sano H, Takino T, and Yasui H. An orally active antidiabetic vanadyl complex, bis(1-oxy-2-pyridinethiolato)oxovanadium(IV), with VO(S\(_2\)O\(_2\)) coordination mode; in vitro and in vivo evaluations in rats. J Inorg Biochem. 80: 99–105. 2000. [Medline] [CrossRef]

10. Chandra AK, Ghosh R, Chatterjee A, and Sarkar M. Effects of vanadate on male rat reproductive tract histology, oxidative stress markers and androgenic enzyme activities. J Inorg Biochem. 101: 944–956. 2007. [Medline] [CrossRef]

11. Iwatsuka H, Shino A, and Suzuki Z. General survey of diabetic features of yellow KK mice. Endocrinol Jpn. 37: 23–35. 1990. [Medline]

12. Diani AR, Sawada GA, Hannah BA, Jodelis KS, Connell MA, Connell CL, Vidmar TJ, and Wyse BM. Analysis of pancreatic islet cells and hormone content in the spontaneously diabetic KK-A\(^+\) mouse by morphometry, immunocytochemistry and radioimmunoassay. Virchows Arch A Pathol Anat Histopathol. 412: 53–61. 1987. [Medline] [CrossRef]

13. Diani AR, Sawada GA, Zhang NY, Wyse BM, Connell CL, Vidmar TJ, and Connell MA. The KK-A\(^+\) mouse: a model for the rapid development of glomerular capillary basement membrane thickening. Blood Vessels. 24: 297–303. 1987. [Medline]

14. Moroki T, Yoshikawa Y, Yoshizawa K, Tsubura A, and Yasui H. Morphological characterization of systemic changes in young adult KK-A\(^+\) mice as type 2 diabetic model animal. In Vivo., 27: 465–472. 2013. [Medline]

15. Yoshikawa Y, Murayama A, Adachi Y, Sakurai H, and Yasui H. Challenge of studies on the development of new Zn complexes (Zn(opt)\(_2\)) to treat diabetes mellitus. Metalomics. 3: 686–692. 2011. [Medline] [CrossRef]

16. Fujimoto S, Yasui H, and Yoshikawa Y. Development of a novel antidiabetic zinc complex with an organoselenium ligand at the lowest dosage in KK-A\(^+\) mice. J Inorg Biochem. 121: 10–15. 2013. [Medline] [CrossRef]

17. International Harmonization of Nomenclature and Diagnostic Criteria (INHAND): Proliferative and Non-Proliferative Lesions of the Male Reproductive and Mammary Systems of the Rat and Mouse: A Joint Publication of the American, British, European, and Japanese Societies of Toxicologic Pathology. 528–538. 2012.