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Short Communication

Testicular Mineralization in KK-A^y Mice Treated with an Oxovanadium Complex

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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^{2+}) complexes. Therefore, we studied VO^{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^{2+}) ($VO(opt)_2$) for 2 or 4 weeks in KK-A^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^y mice. These results represent the first report of the possibility for seminiferous tubules mineralization induced by $VO(opt)_2$ administration. Therefore, our research provides important information about the potentially toxic effects of VO^{2+} complexes. (DOI: 10.1293/tox.26.329; J Toxicol Pathol 2013; 26: 329–333)

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

Vanadium, a trace element in animals and humans, has a wide variety of biological and physiological functions[1]. The toxic effects of inorganic V^{5+} salts in diabetes model rats include severe diarrhea in life. Histopathologically, hepatocellular necrosis, fatty change and vacuolation, necrosis of renal tubules and necrosis of mucosal epithelial cells in the small intestine have been reported as the toxicities[2–5]. In addition, testicular toxicity that seems to be related to vanadium-induced oxidative stress during spermatogenesis has been reported[6]. Thus, inorganic V^{5+} salts have shown significant toxicity, and therefore, several investigators have studied pharmacologic effects of V^{4+} salts with lower toxicity[7].

Furthermore, several oxovanadium (VO^{2+}, the +4 oxidation state of the vanadium ion combined with oxygen to form VO^{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^{2+} complexes being generally higher than that of either inorganic V^{4+} or V^{5+} salts.

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Bis(1-oxy-2-pyridinethiolato)oxovanadium(VO^{2+}) ($VO(opt)_2$) has been reported to be a potent, orally active insulin mimetic for treatment of diabetes in animal models[8,9]. However, toxicological studies of $VO(opt)_2$ have not been described in the literature. Furthermore, although sodium metavanadate (V^{5+}), $NaVO_3$, was shown to cause testicular toxicity when delivered by gavage, there are no accounts of testicular toxicity due to administration of VO^{2+} complexes[6,10]. If VO^{2+} complexes show reproductive toxicity, it would be fatal in humans, because many people take various vanadium (V^{4+} and V^{5+}) salts by gavage as supplements. In this study, we performed a histological assessment of testicular toxicity after repeated administration of $VO(opt)_2$ in KK-A^y mice, a mouse model of type 2 diabetes mellitus (DM)[11–14].

$VO(opt)_2$ was prepared by mixing 1-oxy-2-pyridinethione and $VOSO_4$ 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[15].

A total of sixteen 5-week-old male KK-A^y mice were obtained from CLEA Japan Inc. (Tokyo, Japan) and allowed free access to solid food (MT, Oriental Yeast Co., Ltd.) and tap water. The animal studies were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University (KPU) and were performed according to the Guidelines for Animal Experimentation at KPU.

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										Leydig cell	
				Degeneration/necrosis #					Mineralization					hyperplasia	
				–	+	++	+++	++++	–	+	++	+++	++++	–	+
Untreated KK-A^y	2	4	421 ± 99	4	0	0	0	0	4	0	0	0	0	4	0
mice (control)	4	4	481 ± 99	4	0	0	0	0	4	0	0	0	0	4	0
$VO(opt)_2$- treated	2	3	364 ± 136	0	1	0	1	1	1	1	1	0	0	1	2
KK-A^y mice	4	5	237 ± 61*	4	0	0	0	1	3	0	0	1	1	3	2

Grading scores: –, none; +, slight; ++, moderate; +++, strong; ++++, severe. *$p < 0.05$ vs. Untreated KK-A^y 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.

KK-A^y 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^y mice and to be much lower than a lethal dose[9].

Blood samples for glucose level analysis were obtained from the tail vein of each mouse and monitored with a Glucocard (Arkray, 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% HNO_3 and 30% H_2O_2 in 50-mL beakers for determination of metals. When the residue turned white, the dried samples were dissolved in 1% HNO_3. Vanadium, calcium and iron concentrations in each sample were then determined using an ICPM-8500 system (Shimadzu Corporation, Kyoto, Japan)[16]. The minimum limits of detection of vanadium, calcium, and iron were 5, 10, and 10 ppb (ng/mL), respectively.

All data are expressed as the means ± standard deviations (SD). Body weight, food consumption, and blood glucose level were analyzed by assessing the variance for homogeneity using the *F*-test. The two-tailed independent Student's and Welch's *t*-test were used for homogeneous and heterogeneous data, respectively. The numbers of animals presenting degeneration/necrosis, mineralization and Leydig cell hyperplasia in the testis were evaluated by Fisher's exact probability test. *P*-values of <0.05 were considered statistically significant.

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^y 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^y mice, as shown in Table 1. These blood glucose level shifts were similar to those reported in a previous study[8].

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. 1c). 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,

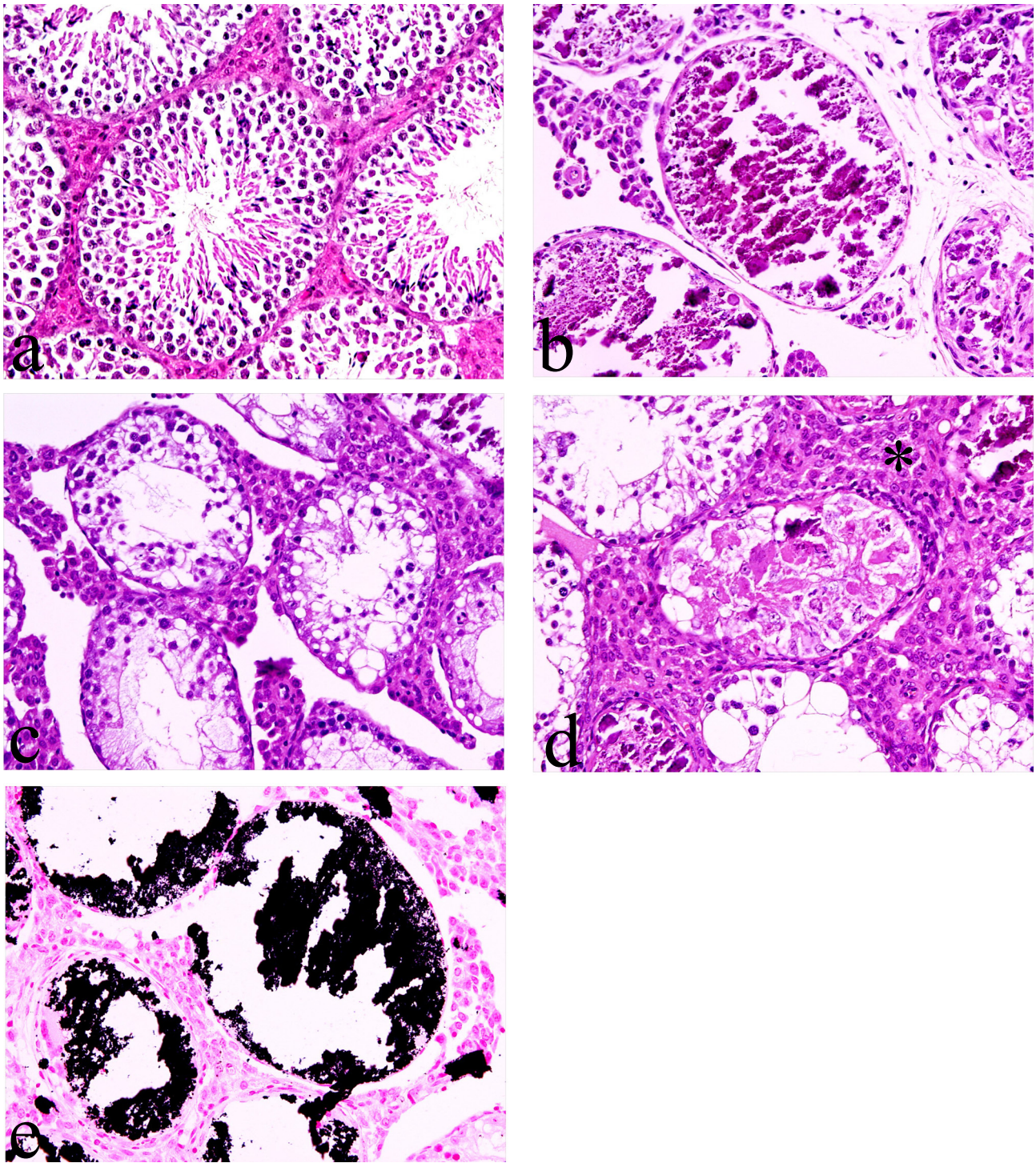

Fig. 1. Testicular morphology in KK-A^y mice exposed to VO(opt)$_2$ for 4 weeks. (a) No abnormal changes in control animals. (b) Mineralization of the seminiferous tubules in KK-A^y mice exposed to bis(1-oxy-2-pyridine-thiolato)oxovanadium(VO^{2+}) (VO(opt)$_2$). (c) Vacuolation of Sertoli cells and the seminiferous epithelium in the seminiferous tubules and Leydig cell hyperplasia. (d) Seminiferous tubular necrosis and Leydig cell hyperplasia (*). Hematoxylin and eosin staining, × 200. (e) Von Kossa staining in the same area as (b), × 200.

fat deposition, kidney, overgrowth of the mesangial matrix, and pancreas, hypertrophy of islet cells, as described in our previous report[14].

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 tes-

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^y 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^y mice	-	1	2	B.L.Q	0.371	0.133
	+	2		0.033	106	0.573
$VO(opt)_2$-treated KK-A^y mice	-	3	4	B.L.Q	0.237±0.207	0.113±0.024
	+	2		0.036	209	0.552

B.L.Q: Below the lower limit of quantification.

tes 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^y mice. Additionally, abnormal changes related to DM were detected in the liver, kidney and pancreas of the KK-A^y mice, but no mineralization was detected in these organs (data not shown)[14]. Therefore, these changes related to DM were not related to mineralization of 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

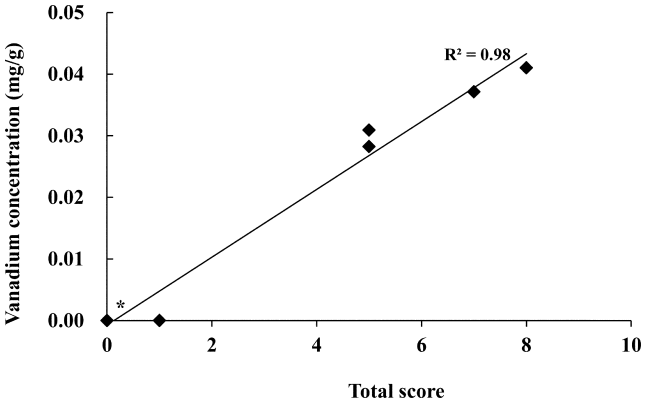

Fig. 2. Correlations between grades of individual histopathological changes and vanadium concentration in the testis. The total score represents the degeneration/necrosis, mineralization or Leydig cell hyperplasia and was graded as + = 1, ++ = 2, +++ = 3 and ++++ = 4, as shown in Table 1 (* 3 animals with no abnormal changes).

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 $NaVO_3$[6], 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 $VO(opt)_2$ 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 $VO(opt)_2$ to normal animals, only diabetic ones, because the original goal of this study was to assess the pharmacological effect of $VO(opt)_2$. 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.

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