Wojciech Dąbroś[1], Agnieszka Nikiforuk[2], Anna M. Kordowiak[2]

Morphology and Biochemical Activity of Rat Liver Golgi Complexes after Pretreatment with Bis(kojato)oxovanadium(IV) or Kojic Acid Alone

[1]Department of Clinical and Experimental Pathomorphology, Collegium Medicum,
[2]Department of General Biochemistry, Faculty of Biotechnology, Jagiellonian University, Kraków

The authors studied the effect of a short (2 days) oral treatment of rats with bis(kojato)oxovanadium (IV) [$VO(ka)_2$] as 1.8 mmol liquid solution on the biochemical activity and morphology of liver Golgi complexes (Group pVC). Such a short treatment induced greater changes than a longer (1 week) application of the same vanadium compound, what had been observed previously. Especially the Golgi marker enzyme activity (GalT) was the highest among all the investigated groups, and additionally, the greatest dispersion of result was obtained in this group. This group of animals showed twisted Golgi complexes, which – apart from 2–3 narrow cisterns – often contained 1–2 grossly distended cisterns filled with clear, floccular contents. The fairly long cisterns were irregular in shape and were often bent, forming ring-like structures. In the second experiment, we studied the effect of the ligand alone (kojic acid) (Group C+ka_2) employed in the same way as in the case of the previously used vanadium complex (time and concentration). The biochemical parameters (body and liver weight, liquid and food intake, blood sugar level and GalT activities) were the same as in the untreated control group (C). Contrary to the biochemical findings, the morphology of Golgi complexes changed in effect of 3.6 mmol kojic acid application (the same application, time and concentration as in the whole complex with vanadium) over a 1-week period, manifesting the stimulation of exocytosis (in the *trans* region and directed toward plasma membrane), with the cisterns of Golgi dictyosomes being rounded or oval in more than 85% of cases. In this group, electron microscopy revealed the presence of two types of Golgi complexes, namely 2–3 short, slightly arched cisterns grossly distended at both ends and filled with clear, floccular material, as well as vacuoles with the same contents, which were visible in the vicinity of the cisterns. The other type, which was observed less frequently, was represented by Golgi complexes formed by haphazardly twisted cisterns.

Introduction

Vanadium kojate [$VO(ka)_2$] is a chemical analogue of bis(maltolato)oxovanadium(IV) [BMOV], which is one of more potent and widely investigated organic vanadium complexes with an ability to lower blood sugar level as well as to ameliorate some clinical and experimental symptoms, appearing in different forms of experimental diabetes [1–3, 14–16, 18]. Apart from other researchers, Yuen et al. [20] and McNeill et al. [14] investigated the ability of both vanadium complexes to decrease blood glucose le-vels. In one of our studies, we tested the potency of bis(kojato)oxovanadium(IV) $VO(ka)_2$ in regulating characteristic changes previously observed by us in untreated streptozotocin-induced diabetic rat livers, especially in the Golgi complexes region [8, 10]. Contrary to the BMOV activity investigated in the same experimental model [4, 6], neither a short (7 days) oral treatment with vanadium kojate [11], nor a prolonged treatment with the vanadium compound [5] could not normalize the activity of the liver Golgi apparatus marker enzyme i.e. galactosyltransferase (GalT), and only slightly improved the morphology of this organelle. Additionally, in both models of experiments with $VO(ka)_2$ (a short or prolonged time of vanadium treatment), the level of GalT activities (regardless whether it was expressed as nmoles Gal transferred per h and per g of liver or as total activity in whole liver) was higher in diabetic animals treated with vanadium as compared to appropriate controls, although diabetic animals were exposed to two harmful drugs (streptozotocin and vanadium kojate). The phenomenon was particularly distinct in the prolonged (the so-called

pretreatment with $VO(ka)_2$) experimental model [5]. Therefore, we decided to study only the effect of the short (two days) oral pretreatment with vanadium solution, as well as with the ligand alone (kojic acid) on the morphology and biochemical activities of liver Golgi complexes from control rats.

Material and Methods

Animals

The experiments were carried out in three groups of female Wistar rats c. 6 months old and weighing 200–240g. The animals were fed with peleted food and tap water prior to experiments:

– Group C – control rats, receiving 0.5% (w/v) NaCl as a drinking solution for 7 days (9 rats);

– Group pVC – rats receiving 1.8mmol solution of BKOV in 0.5% NaCl as a drinking liquid for 2 days, followed by a 2–3-week break (when the rats were given only water), and subsequently receiving 0.5% NaCl over the next 7 days (9 rats);

– Group $C+ka_2$ – rats receiving 3.6mmol kojic acid solution (the same ligand concentration as in the complex with vanadium, i.e. $VO(ka)_2$ in Groups C+V and pVC) in 0.5% NaCl for 7 days (9 rats).

To allow for a comparison with our preliminary experimental group $C+VO(ka)_2$ [11], we additionally studied three rats, which received 1.8mmol solution of $VO(ka)_2$ in 0.5% (w/v) NaCl as a drinking liquid for 7 days. The results were analyzed jointly in conjunction with the previously investigated (9 rats).

The kojic acid or vanadium kojate solutions were freshly prepared every 2 days in 0.5% (w/v) NaCl to diminish their toxicity. All animals were weighed each day during the experiment and the amounts of drinking liquids and food consumed by the rats were measured. At the end of the experiment, the rats were exsanguinated under anesthesia before killing. Liver samples were taken for morphological analysis and the livers were immediately used for the isolation of Golgi membrane fractions, followed by estimations of galactosyltransferase (GalT) activity from individual rats [7].

Analytical methods

The galactosyltransferase (GalT) activity was estimated according to the Fleischer method [7]. Protein was estimated by the method of Lowry et al. [13] with crystalline serum bovine albumin as the standard. Free blood sugar level was estimated according to Somogyi and Nelson [17].

Ultrastructural examination

For electron microscopy, 2–4 biopsy specimens of each group were fixed overnight in formaldehyde-glutaraldehyde fixative at 4°C by the method of Karnovsky [9]. The tissue was subsequently postfixed in 1% osmium tetroxide. After dehydration in graded concentrations of ethyl alcohol and propylene oxide, the tissue was embedded in the Spurr medium. Samples were sectioned with an ultramicrotome Reichert Ultracut S using a diamond knife. Semi-thin sections were stained with methylene blue and ultra-thin sections with 8% uranyl acetate dissolved in 50% methanol and then in lead citrate according to Venable and Coggeshal [19]. All the investigations were performed using an electron microscope Zeiss EM 900 operating at 80kV.

Statistical analysis

All the results expressed as mean ±SD were tested for statistical significance by the Student's t-test. Statistically significant ($p<0.05$) values are marked under the table or figure.

Reagents

Sodium cacodylate, serum bovine albumin, TRIS and -mercaptoethanol came from Koch-Light Lab., UDP-Gal, Triton X-100, streptozotocin, kojic acid, uranyl acetate and lead citrate were obtained from Sigma Chemical Co., Dowex 2 × 8 with granulation (200–400 mesh) came from Fluka & Buchs, ^{14}C UDP-Gal with the specific activity of 292mCi/mmol was obtained from Radiochemical Centre Amersham, bis(kojato)oxovanadium(IV) was prepared and determined in the Department of Inorganic Chemistry, Jagiellonian University. All other reagents were purchased in analytical grade from Polish Reagents POChem Gliwice. For electron microscopy, Spurr epoxy resin was supplied by Pelco Co. and formaldehyde, glutaraldehyde and osmium tetroxide were purchased from Polysciences Inc.

Results

In two groups of experimental rats (pVC and $C+ka_2$), similarly as in the case of the untreated controls (C), an increase of body weight during the experiment (approximately 1–3.6%) was obtained. Liquid intake in Group pVC was lower than in Groups C or $C+ka_2$, but in the former, food intake reached the highest value as compared to all the investigated groups. The free blood sugar level in Group $C+ka_2$ was similar to that observed in Group C, but after 2 days of treatment with $VO(ka)_2$, it was statistically significantly higher than in the controls ($p<0.05$). The yield of the Golgi membrane isolation was lower in all the

TABLE 1

Physiological and biochemical characteristics of investigated group of animals

Characteristics	Investigated groups of rats			
	C n=9	pVC n=9	C+ka$_2$ n=9	C+VO(ka)$_2$ n=9
Weight of rats before sacrifice (g) % change of body weight	233.8 ± 12.9 2.7 %	244.4 ± 25.7 1.1 %	233.9 ± 15.1 3.6 %	210 8 ± 20.6[1] 6.6 %
Weight of liver (g)	7.8 ± 0.5	8.8 ± 1.5	7.9 ± 1.3	6.4 ± 0.5[2]
Liquid intake (ml per rat per day)	25.6 ± 0.3	16.6 ± 1.3[a]	23.1 ± 4.2[b]	15.5 ± 2.5[3]
Food intake (g per rat per day)	18.9 ± 1.8	20.1 ± 2.2	18.6 ± 1.4	15.9 ± 2.8[4]
Free blood sugar level on the last day of experiment (mg/100ml)	115.1 ± 30.9	153.8 ± 38.9[c]	116.8 ± 45.6	103.2 ± 28.3
Yield of Golgi-rich membrane fraction (mg protein/g of liver)	0.661 ± 0.168 (0.446 – 1.012)	0.579 ± 0.156 (0.346 – 0.787)	0.581 ± 0.228 (0.324 – 0.886)	0.401 ± 0.055[5] (0.314-0.474)
Specific activity of GalT (nmoles Gal transferred/h and per mg of protein)	86.0 ± 27.5 (49.2 – 137.7)	231.9 ± 132.0[d] (63.6 – 443.8)	111.4 ± 86.8 (47.1 – 268.3)	89.1 ± 26.3 (65.9 –147.3)
GalT activity (nmoles Gal transferred/h and per 1g of liver)	54.8 ± 15.3 (36.4 – 77.2)	141.3 ± 89.1[e] (43.0 – 258.4)	51.4 ± 21.4 (28.6 – 86.7)	38.8 ± 10.6[6] (17.3 – 53.2)
Total activity of GalT (nmoles Gal transferred/h and per total liver)	430.2 ± 118.1 (258.1 – 563.4)	1266.1 ± 833.3[f] (339.6 – 2584.5)	437.0 ± 258.0 (291.6 – 918.6)	222.6 ± 67.8[7] (114.0 – 331.1)

The data are presented as mean value ±SD (in some cases, the scatter of results is given in round brackets).
As compared to Group C: [a] $t = 22.1453$, $p<0.001$; [b] $t = 3.7589$, $p<0.01$; [c] $t = 2.3439$, $p<0.05$; [d] $t = 3.2537$, $p<0.01$; [e] $t = 2.8837$, $p<0.02$; [f] $t = 2.9806$, $p<0.01$.
The last column C+VO(ka)$_2$ has been included in the Table to compare the presented results with the results obtained previously in part. The following t and p values as compared with the controls have been obtained: [1] $t = 3.110$, $0.001<p<0.01$; [2] $t = 4.866$, $p<0.001$; [3] $t = 7.381$, $p<0.001$; [4] $t = 2.644$, $0.01<p<0.02$; [5] $t = 2.362$, $0.02<p<0.05$; [6] $t = 3.069$, $0.001<p<0.01$; [7] $t = 3.948$, $p<0.001$.

experimental groups as compared to the controls; however, only in Group C+VO(ka)$_2$ was the difference statistically significant ($p<0.05$).

The mean GalT activity values were the highest, the difference being statistically significant, in Group pVC regardless of the calculation method employed (as nmoles Gal transferred /h/mg of protein; nmoles Gal transferred /h/g of liver or nmoles Gal transferred/h/total liver), however a great scatter of the results must be emphasized. The results are summarized in Table1, and particularly in Figure 1. In Group C+ka$_2$, the activity of GalT, the Golgi apparatus marker enzyme, was the same as in the control group. To compare the two investigated groups, appropriate parameters obtained in C+VO(ka)$_2$ Group are given in the last column of Table 1.

Electron microscopy revealed a striking property of Golgi complexes consisting in the prevalence of ring-like or haphazardly twisted structures. In pVC Group, atypically shaped structures were noted in approximately 70% (Fig. 2). Some of the observed Golgi complexes were composed of 3–5 narrow, twisted cisterns with marginal enlargement and with small vacuoles (Fig. 3). In several cases structures resembling the normal Golgi complex form were seen; these were slightly arched cisterns, some of them narrow, with large marginal distensions and filled with clear, floccular material, similarly as numerous vacuoles situated in the vicinity, while other were external, grossly distended, with vacuoles situated inside (Fig. 4). However, both the infrequent normal structures composed of 2–3 narrow cisterns with giant distensions situated along their course or marginally, and the atypical structures consisting of narrow, twisted cisterns with distended segments were highly active, what was confirmed by the presence of vacuoles situated in the close vicinity and almost identical in size, structure and cargo to the marginal distensions. The C+ka$_2$ – kojic acid Group showed characteristic Golgi complexes with unevenly, haphazardly twisted cisterns. In their vicinity, areas of electron-lucid, floccular material were seen, along with small vacuoles and lysosome-like structures (Fig. 5). Such images were frequently documented. Apart from vacuoles, they were accompanied by coated vesicles, and at times by dense core vesicles (Fig. 6). Typically structured Golgi complexes were seen very sporadically; they were character-

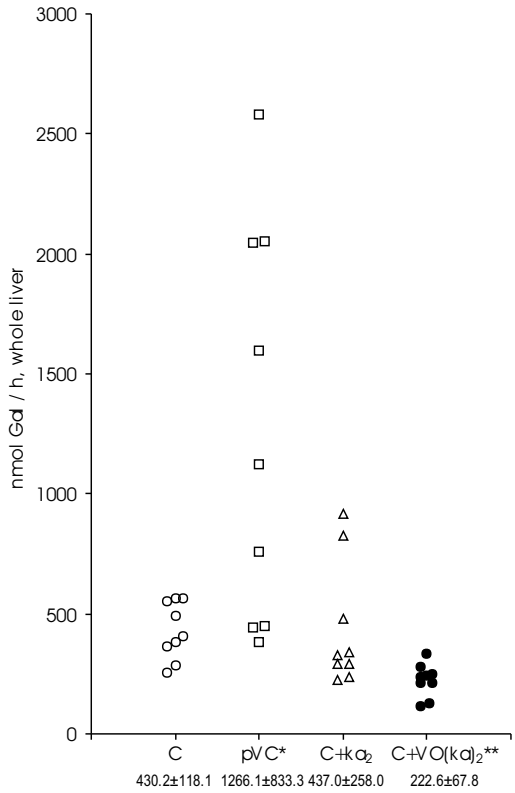

Fig. 1. Total activity of rat liver Golgi marker enzyme i.e. galactosyltransferase (GalT) expressed as nmoles Gal transferred per 1h and per total liver, in the three investigated groups of rats represents the mean value ±SD. The last group is inserted to compare with values previously obtained in the control group treated with the whole vanadium complex [4]. * pVC/C t = 2.9806, $p<0.01$, ** C+VO(ka)$_2$/C t = 3.9480, $p<0.001$.

ized by slightly arched, short cisterns with large, marginal distensions filled with clear, floccular material, similarly as numerous vacuoles situated in the vicinity (Fig. 7).

Discussion

Bis(kojato)oxovanadium(IV) [VO(ka)$_2$] was investigated as a chemical analogue of bis(maltolato)oxovanadium(IV) [BMOV], the organic vanadium compound with the best anti-diabetic activity. As reported by McNeill et al. [14], this complex was less effective than BMOV in decreasing plasma glucose levels and required higher doses in drinking water. The insulin-like effects of vanadium extend *in vitro* to a number of processes involved in carbohydrate (through glucose transport and translocation, glycolysis and glycogenogenesis), lipid (inhibition of lipolysis) and protein metabolism (mitogenesis). Although many *in vivo* effects induced by vanadium compounds in STZ-diabetes were noted [15], the activity of these complexes inside the cell, on the organelle level, is poorly understood. Moreover, some investigators [14, 15, 20] described an increased potency and reduction of toxicity of organic vanadium compounds. We previously studied the VO(ka)$_2$ effect on the biochemical activity and morphology of liver Golgi apparatus, and found that in our experimental model vanadium kojate was less active as an anti-diabetic drug. Thus, our findings were similar to the results of Yuen et al. [20]. This complex did not normalize the biochemical activity of Golgi complexes in STZ-diabetes [4], and only slightly improved the morphology of this organelle, in contrast to the best action of BMOV on the morphology and biochemical activities of liver Golgi complexes [4, 6]. In our previous study, we employed a short-time of application (2 days), i.e. the so called pre-treatment of the rats with VO(ka)$_2$, followed by a one- week treatment with the same vanadium solutions [5]. The first treatment was used to get the animals accustomed to the flavor of this liquid and additionally to test the possible cytoprotective abilities of this compound. Similarly as in the case of rats treated with VO(ka)$_2$ for one week, a prolonged vanadium treatment decreased the GalT activities in the control and diabetic rat livers [5] in a statistically significant manner (as compared to the untreated control C, $p<0.001$ or $p<0.01$, respectively). The morphology of Golgi complexes showed rounded stacks of cisterns, characteristic of untreated STZ-diabetes, but the secretory activity of the organelle was preserved or even stimulated in some cases.

This work presents the effect of pretreatment (2days) of the animals with VO(ka)$_2$ on the investigated parameters. In pVC Group, a lower liquid intake (similar to that noted in C+VO(ka)$_2$ Group), but the highest food intake were observed. Free blood sugar was elevated in comparison with untreated controls (Group C), $p<0.05$. The activity of the Golgi marker enzyme, i.e. galactosyltransferase, was the highest in pVC Group, regardless of the method of calculation, but the greatest scatter of results, never previously observed in any of the investigated groups, must be emphasized. It seemed to depend on the dose of vanadium intake, and especially on response to vanadium characteristic of individual rats.

Ultrastructural observations demonstrated a predominance of cylindrical, haphazardly twisted structures. The pretreated group showed some Golgi complexes with normal structure, but also in these animals altered structures predominated. A question arises whether the common occurrence of altered structures has resulted from the very experiment (a long-term effect on Golgi complexes) or else it represents the effect of vanadium itself. The latter option would be supported by the fact that in the second group (C +(ka)$_2$ – kojic acid) practically no normal structures have been encountered. In our experiments we additionally investigated the effect of the ligand alone (kojic acid, used in the same concentration and over the same time as in

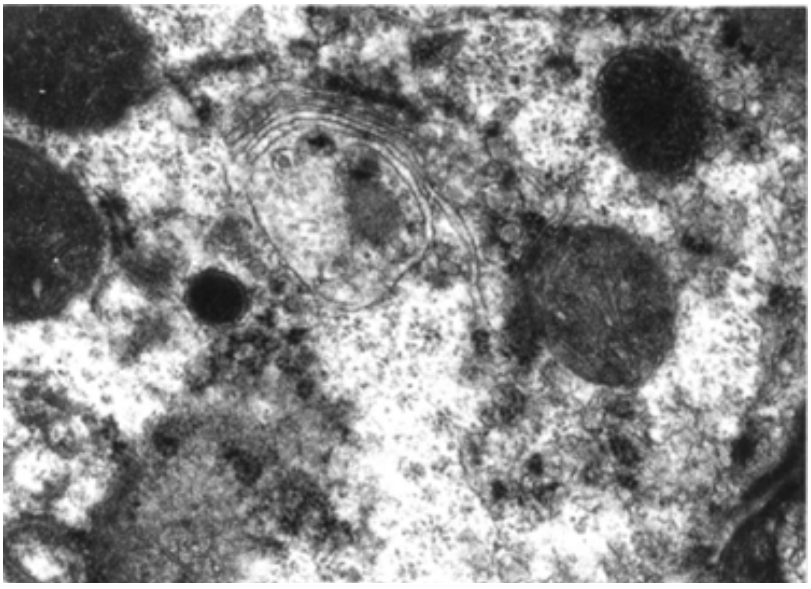

Fig. 2. pVC, pretreatment $VO(ka)_2$. In this group, ring-shaped Golgi complexes predominated. Within relatively narrow, often fully closed cisterns, distended segments were seen, or even vacuoles filled with floccular material. Magn. 25,000x.

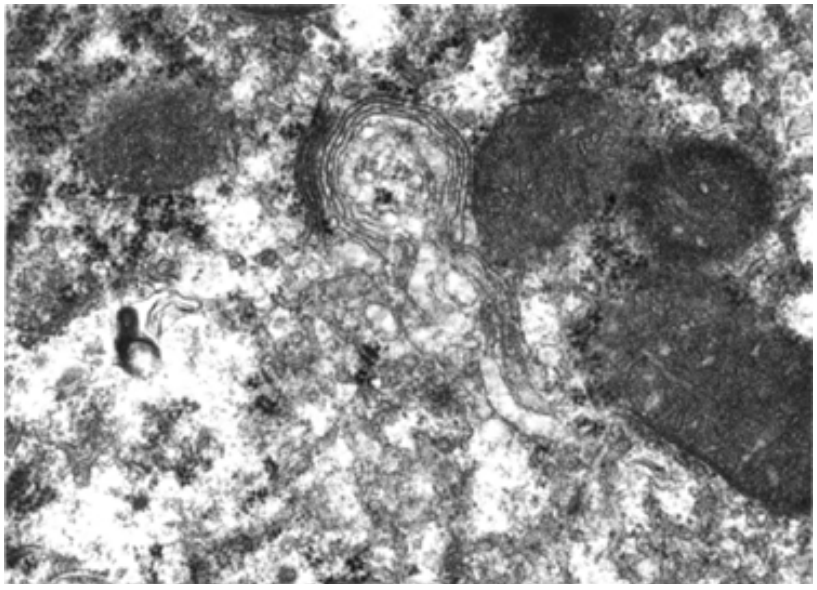

Fig. 3. pVC, pretreatment $VO(ka)_2$. A Golgi complex composed of 3–5 narrow, twisted cisterns. Note the marginal distension and small vacuoles. Magn. 25,000x.

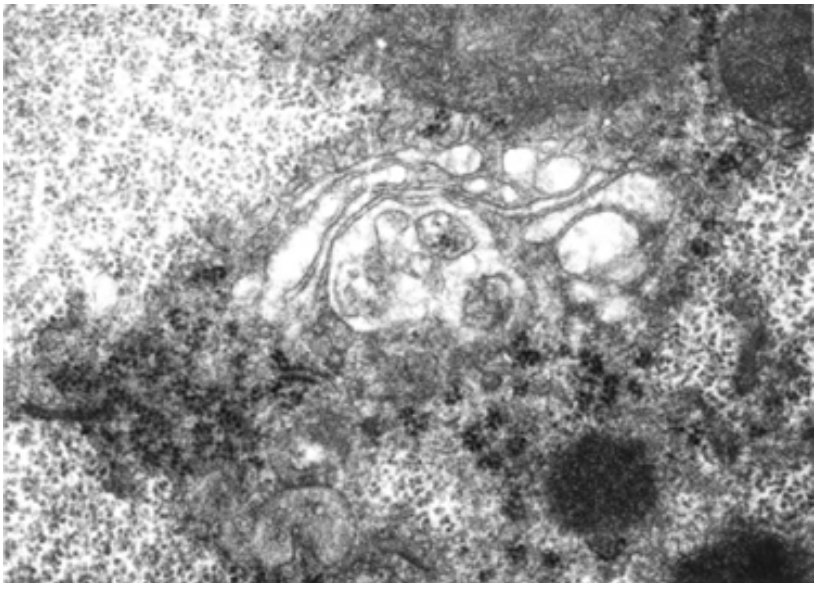

Fig. 4. pVC, pretreatment $VO(ka)_2$. A Golgi complex with an arched, distended cistern on the *cis* side, narrow central cisterns with marginal distensions filled with electron-lucid material, and a balloon-like, distended cistern on the *trans* side containing a vacuole filled with floccular material. Magn. 25,000x.

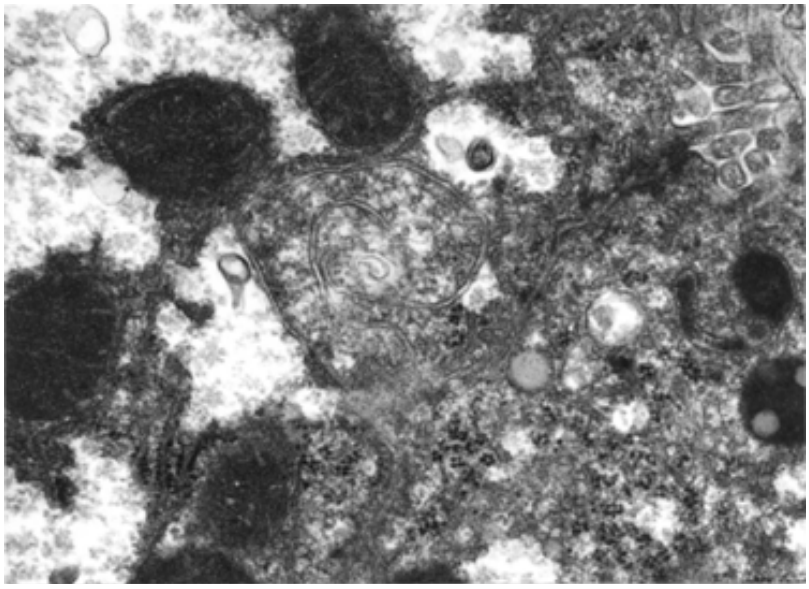

Fig. 5. C+ ka_2 – kojic acid. A striking change, fairly often encountered in this group, consisted in the presence of Golgi complexes with unevenly, haphazardly twisted cisterns. In the vicinity, there were visible areas of electron-lucid, floccular material, as well as small vacuoles and lyzosome-like structures. Magn. 25,000x.

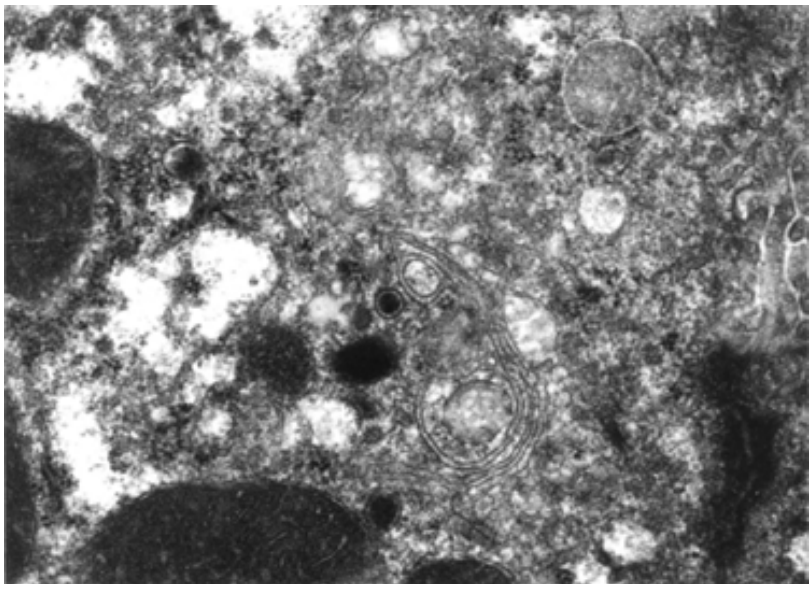

Fig. 6. C+ ka_2 – kojic acid. The image is similar to that presented in Figure 4. Note a structure with haphazardly twisted cisterns, as well as coated vesicles, dense core vesicles, and clear vacuoles. Magn. 25,000x.

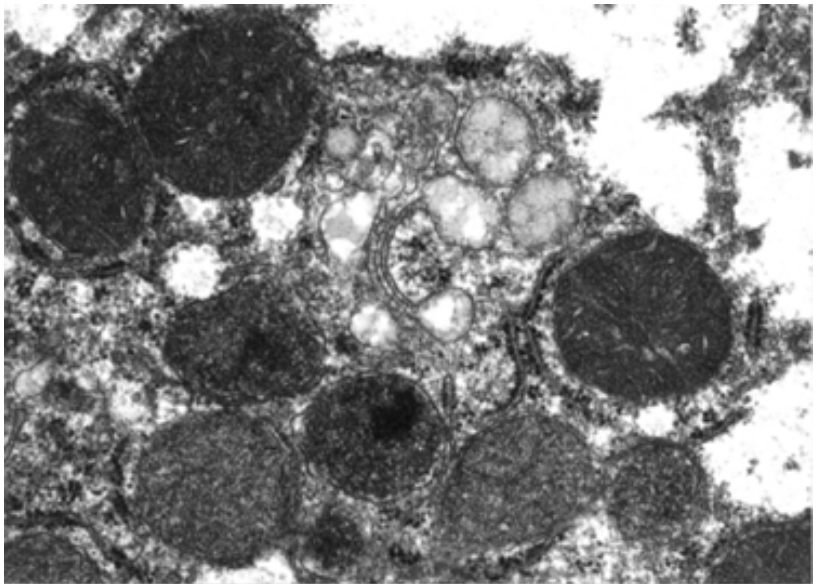

Fig. 7. C+ ka_2 – kojic acid. A normally structured Golgi complex encountered in this group, showing slightly arched, short cisterns with large marginal distensions, filled with clear, floccular material, similarly as numerous vacuoles situated in the vicinity. Magn. 25,000x.

$VO(ka)_2$) on all the investigated parameters. The results, including the GalT activity (regardless of the way of its expression), were similar to those observed in the untreated controls (Group C). In opposite to the results, the great morphological alterations were found. The dictyosome cisterns were destroyed and rounded and the morphology of the organelle was not similar to that noted in Group C. Moreover, the *trans* side of Golgi complexes showed the stimulation of exocytosis (in regions facing towards the plasma membrane) connected with bile canaliculi. The most striking feature was the presence of round or oval Golgi complexes in a vast majority of cases (>85%) There were a lot of vesicles wrapped in a double membrane, which were most probably incorporated in cellular membranes.

Acknowledgements: The authors wish to express their gratitude to Dr. Ryszard Gryboś for his kind supplying bis(kojato)oxovanadium(IV) sulphate, as well as Sławomir Kordowiak Jr. for computer's preparing the graph in Figure 1.

References

1. *Bhanet S, Girn J, Poucheret P, McNeioll JH:* Effects of bis(maltolato)oxovanadium(IV) on protein kinases in skeletal muscle of streptozotocin-diabetic rats. Mol Cell Biochem 1999, 202, 131–140.
2. *Crans DC, Mahroof-Tahir M, Keramides AD:* Vanadium chemistry and biochemistry of relevance for use of vanadium compounds as antidiabetic agents. Mol Cell Biochem 1995, 153, 17–24.
3. *Crans DC:* Chemistry and insulin-like properties of vanadium(IV) and vanadium(V) compounds. J Inorg Biochem 2000, 80, 123–131.
4. *Dąbroś W, Dziga D, Gryboś R, Kordowiak AM:* Biochemical and morphological alterations in rat liver Golgi complexes after treatment with bis(maltolato)oxovanadium(IV)[BMOV] or maltol alone. Path Res Pract 2000, 196, 561–568.
5. *Dąbroś W, Gryboś R, Miarka A, Kordowiak AM:* Biochemical and morphological study on liver Golgi complex in streptozotocin-diabetic and control rats treated with bis(kojato)oxovanadium(IV) $[VO(ka)_2]x2H_2O$. Part II. Prolonged treatment with vanadium compound. Pol J Pathol 2000, 1, 17–24.
6. *Dąbroś W, Kordowiak AM, Dziga D, Gryboś R:* Influence of bis(maltoloto)oxovanadium(IV) on activity of galactosyltransferase (GalT) and morphology of rat liver Golgi apparatus in control and streptozotocin diabetes. Pol J Pathol 1998, 49, 67–76.
7. *Fleischer B:* Isolation and characterisation of Golgi apparatus from rat liver. In: Methods in Enzymology. Fleischer S, Packer L, eds. Acad Press Inc N York, London 1974, vol. 31a, 180–191.
8. *Kaczmarski F, Kordowiak A, Sarnecka-Keller M*: Influence of insulin on galactosyltransferase activity and morphology of rat liver Golgi apparatus in control and streptozotocin diabetic rats. Path Res Pract 1981, 172, 130–137.
9. *Karnovsky MJ:* A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 1965, 27, 137A–138A.
10. *Kordowiak A, Turyna B, Kaczmarski F, Sarnecka-Keller M:* Comparison of rat plasma glycoprotein composition with biochemical activity and morphology of liver Golgi apparatus in streptozotocin-diabetes treated with insulin. Folia Histochem Cytochem 1981, 19, 181–188.
11. *Kordowiak AM, Nikiforuk A, Dąbroś W:* Biochemical and morphological study on liver Golgi complex in streptozotocin-diabetic and control rats treated with bis(kojato)oxovanadium(IV) $[VO(ka)_2]x2H_2O$. Part I. One week treatment with vanadium compound. Pol J Pathol 2000, 1, 9–16.
12. *Kordowiak AM, Dziga D, Dąbroś W:* Streptozotocin-induced alterations of rat liver Golgi complexes are ameliorated by BMOV [bis(maltolato)oxovanadium(IV)] activity. Horm Metab Res 2004, 36, 148–154.
13. *Lowry OH, Rosebrough NJ, Farr AL, Randall RJ:* Protein measurement with Folin phenol reagent. J Biol Chem 1951, 193, 265–275.
14. *McNeill JH, Yuen VG, Dai S, Orvig C:* Increased potency of vanadium using organic ligands. Mol Cell Biochem 1995, 153, 175–180.
15. *Poucheret P, Verma S, Grynpas MD, McNeill JH:* Vanadium and diabetes. Mol Cell Biochem 1998, 188, 73–80.
16. *Reul BA, Amin SS, Buchet JP, Ongemba LN, Crans DC, Brichard SM:* Effects of vanadium complexes with organic ligands on glucose metabolism: a comparison study in diabetic rats. Brit J Pharmacol 1999, 126, 467–477.
17. *Somogyi MJ, Nelson N:* Determination of reducing sugars and carbohydrates. In: Methods in Carbohydrate Chemistry. Whistler R, Wolprom R, eds. Acad Press N York, London 1962, 1, 380–394.
18. *Tsiani E, Fantus IG:* Vanadium compounds. Biological actions and potential as pharmacological agents. Trends Endocrinol Metab 1997, 8, 51–58.
19. *Venable JH, Cogeshall RA:* Simplified lead citrate stain for use in electron microscopy. J Cell Biol 1965, 25, 407–408.
20. *Yuen YG, Caravan P, Gelmini L, Glover N, McNeill JH, Setyawati IA, Zhou R, Orvig C:* Glucose lowering properties of vanadium compounds. Comparison of coordination complexes with maltol or kojic acid as ligands. J Inorg Biochem 1997, 68, 109–116.

Address for correspondence and reprint requests to:
W. Dąbroś M.D.
Department of Pathomorphology
Collegium Medicum Jagiellonian University
Grzegórzecka 16, 31-531 Kraków
e-mail: mpdabros@cyf-kr.edu.pl