Morphological Alterations of Submandibular Glands Caused by Cisplatin in the Rat

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Summary: Cisplatin is a platinum-containing antineoplastic agent, widely used in the treatment of various malignant neoplasms including head and neck cancer. In this study we examined ultrastructural changes of rat submandibular glands (SMGs) after cisplatin treatment. Male Wistar rats aged 8 weeks were used in the experiments. Cisplatin was injected intraperitoneally into the rats (5 mg/kg/day) for three consecutive days. After 1, 3, 5, and 7 days, the SMGs were removed and observed under an electron microscope. The terminal portion of the SMG is seromucosal. Acinar cells contained a parallel array of rough-surfaced endoplasmic reticulum in the basal cytoplasm, and the apical cytoplasm was filled with homogeneous secretory granules. Vacuolation of cells was observed on the first day after cisplatin treatment. On the third and fifth days, the rough-surfaced endoplasmic reticulum showed an irregular arrangement. Mitochondrial swelling, and the presence of autophagolysosomes was also confirmed. On the seventh day, the damage to organelles began to decrease and the SMG exhibited a tendency towards improvement.

Key words cisplatin, rat submandibular gland, electron microscopy

INTRODUCTION

Cis-diamminedichloroplatinum (II) (CDDP) is a widely used anticancer drug for the treatment of human testicular [1], ovarian [2], head and neck [3,4], and advanced cancers. CDDP was described by Rosenberg et al. in 1965 [5] as a platinum-containing compound that inhibits *E. coli* cell division. Its anti-neoplastic effect was reported by Rosenberg et al. in 1969 [6]. CDDP is also known to cause side effects in a wide range of tissues such as the kidney, bone marrow and auditory organs [4,7,8]. It has been reported that CDDP has toxic effects on the salivary glands [9], but to our knowledge, ultrastructural changes in the salivary glands have not been reported.

In this study, we observed ultrastructural changes of the submandibular glands (SMGs) in CDDP-treated rats and studied the process of recovery.

MATERIALS AND METHODS

The animals used in the experiments were 8-week-old male Wistar rats (280-320 g). The rats were given solid food and water ad libitum. Animals were sacrificed under deep anesthesia with diethyl ether according to the recommendations of the Kurume University Ethics Committee for Animal Research.

In order to examine the amount of CDDP (Sigma, St. Louis, MO, USA) taken into the submandibular glands, 50 mg/kg CDDP was diluted in normal saline

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Abbreviations: AC, acinar cell; AcPase, Acid phosphatase; CDDP, Cis-diamminedichloroplatinum (II); GD, granular duct; SD, striated duct; SMG, submandibular gland.

and infused intraperitoneally. After 10 and 30 min, the SMG, liver, and kidney were removed from the rats. Each sample taken from the wet tissues (60-200 mg) was prepared by wet ashing, and the amount of CDDP was measured by flame atomic absorption spectrometry with use of a Polarized Zeeman Atomic Absorption Spectrophotometer (Z-8200, Hitachi, Tokyo, Japan).

Additionally, x-ray microanalysis was performed to investigate the intracellular localization of CDDP on epon-embedded sections, freeze-dried sections, and freeze-fractured sections. Ten and 20 mg/kg CDDP were diluted in normal saline and infused intraperitoneally. After 1, 3, and 6 hrs, the SMGs were removed. For epon embedding, the specimens were fixed with 2.0% paraformaldehyde-2.5% glutaraldehyde buffered with Na-cacodylic acid. The specimens were dehydrated in a graded series of acetone, embedded in epon, and cut into approximately 300 nm thick sections. For freeze-dried sections, the specimens were fixed as described above, dehydrated in a graded alcohol series and then freeze-dried using t-butyl alcohol. These specimens were exposed to osmium vapor. For freeze-fractured sections, the specimens were not fixed and fractured. Platinum content in the three kinds of specimens was measured using an x-ray microanalysis system (Model 5916, Oxford, UK) and electron microscope (JEM-2000EX, JEOL, Tokyo, Japan).

In the CDDP-treated group, the CDDP solution (5 mg/kg) was diluted in normal saline to a total volume of 2 ml and infused intraperitoneally once a day for 3 consecutive days. After 1, 3, 5, and 7 days, the SMGs were removed. In the control group, 2 ml/day of normal saline were infused intraperitoneally for 3 consecutive days, and the SMGs were removed one day after infusion. The SMGs were fixed with 2.0% paraformaldehyde-2.5% glutaraldehyde buffered with Na-cacodylic acid. The specimens were sliced and prefixed with the same fixing solution. After a two-hour post-fixation with 2% osmium tetroxide buffered with Na-cacodylic acid, the specimens were dehydrated in a graded series of acetone and embedded in epon. Ultra-thin sections were stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (H-7000, Hitachi, Tokyo, Japan). For light microscopy, 1-2 μ m-thick sections were prepared and stained with 1% toluidine blue on a hot plate.

The presence of lysosomal acid phosphatase was demonstrated using the Acid phosphatase (AcPase) method [10]. The specimens were prefixed with 2%

glutaraldehyde-0.1 M cacodylate buffer (pH 7.2) for 45 min and sections were cut into smaller pieces (the final dimensions were approximately 50 μ m thick). Subsequently, the sections were soaked in reaction solution (1 mM β -glycerophosphate and 2 mM CeCl_3) for 1 hr at 37 $^{\circ}\text{C}$, fixed with 3% glutaraldehyde for 1 hr, post-fixed with 2% osmium tetroxide and embedded in epon. Ultra-thin sections were prepared and stained with uranyl acetate and lead citrate for observation by electron microscopy.

RESULTS

Atomic absorption spectrometry

The absorption of CDDP was evaluated using atomic absorption spectrometry. CDDP absorption was lower in the SMG than in the liver or kidney. Thirty minutes after infusion, the concentration decreased in the liver and kidney but increased slightly in the SMG (Fig. 1).

X-ray microanalysis

In order to investigate the intracellular localization of CDDP, x-ray microanalysis was performed to search for platinum peaks. Platinum peaks were not detected in the epon-embedded sections, freeze-dried sections, or freeze-fractured sections.

Histological findings

Control group

Light microscopic findings: Rat SMG consisted of the acinus and intralobular duct segments. Acinar cells were round or elliptical in shape. The apical cytoplasm of the acinar cells (AC) was filled

Fig. 1. Data from atomic absorption spectrometry of Pt concentrations in SMG, liver and kidney.

with secretory granules and the nucleus was situated at the basal part of the cell. The intralobular ducts consisted of intercalated ducts, granular ducts (GD) with secretory granules, and striated ducts (SD) with basal striations (Fig. 2A).

Electron microscopic findings: Acinar cells are seromucosal cells. The apical cytoplasm of the ACs was filled with numerous homogeneous secretory granules and the nuclei of the ACs were localized in the basal part of the cell (Fig. 2B).

The cytoplasm contained well-developed rough-surfaced endoplasmic reticulum organized in lamellar stacks around the nucleus, Golgi apparatus, and mitochondria. Additionally, myoepithelial cells surrounded the ACs.

The intercalated ducts had somewhat flattened, cuboidal epithelial cells surrounding the lumen. The GDs exist specifically in rodent SMG. The nuclei of these cells were situated in the basal part of the cell, and most of the cytoplasm near the lumen was occupied by secretory granules of different sizes and electron density (Fig. 2C). The SDs were characterized by elaborate infolding of the basal plasma membrane, which was associated with numerous mitochondria (Fig. 2D).

CDDP-treated Group

The first day after CDDP infusion

Light microscopic findings: In the acinar component, vacuoles were scattered in almost all

Fig. 2. Light (A) and electron microscopic findings (B-D) in the control rat.

A; SMG consists of the terminal acinar and ductal system. ($\times 300$)

B; The apical cytoplasm of an AC is filled with numerous homogeneous secretory granules.

C; Most of the cytoplasm of GD cells is occupied by secretory granules.

D; The SD cells were characterized by elaborate infolding of the basal plasma membrane associated with numerous mitochondria. (Bar=1 μ m)

Fig. 3. Light (A) and electron microscopic findings (B-D) in the rat sacrificed the 1st day after CDDP infusion.
 A; Vacuoles scattered in secretory granules were observed in the AC. ($\times 300$)
 B; The secretory granules fused to each other and changed into vacuoles.
 C; Two types of secretory granules with two different electron densities were observed.
 D and E; There were no marked changes in the granular or striated ducts. (Bar=1 μm)

secretory granules. However, there were no changes in the nuclei or cytoplasm. Some narrow zones were observed in the extracellular space between acinar cells. There were no changes in the granular and striated ducts (Fig. 3A).

Electron microscopic findings: In ACs, two types of secretory granules with two different electron densities (biphasic secretory granules) were observed. Some of these secretory granules fused to form vacuoles containing the remaining membrane components. The acinar lumens were enlarged and the nuclei were deformed, probably due to compression by the vacuoles. Moreover, the surrounding cytoplasm was displaced by these vacuolated secretory granules (Figs 3B, C). An irregular arrangement

of rough-surfaced endoplasmic reticulum was observed in the AC. There were no marked changes in the GD and SD (Figs 3D, E).

The third day after CDDP infusion

Light microscopic findings: In the acinar component the ACs were swollen and the extracellular matrix had narrowed. Fewer vacuoles were seen as compared with the first day, and the content of the secretory granules consisted of light areas and dark areas. The nuclei were localized in the basal part of the cells and the cytoplasmic area was decreased. Uneven, irregular surfaces were observed at the basal portions of the GD and SD (Fig. 4A).

Electron microscopic findings: The ACs were

Fig. 4. Light (A) and electron microscopic findings (B-F) on the 3rd day after CDDP infusion.

- A; The acinar cells were swollen and the extracellular matrix had narrowed. Irregular surfaces were observed at the basal portions of the granular and striated ducts ($\times 300$)
- B; The cytoplasm was filled with secretory granules and the granule membranes disappeared.
- C; Enlargement and fragmentation of the rough-surfaced endoplasmic reticulum and a tendency for mitochondrial swelling (arrow) were observed.
- D; The lysosome-like bodies were positive for AcPase.
- E; The basal membrane of the GD was irregular.
- F; The mitochondria were swollen (arrows), and some basal infoldings degenerated or disappeared. (Bar=1 μm)

irregular and swollen. The shapes of the nuclei were also irregular and there was an area of high electron density in the nucleus. The cytoplasm of the ACs was filled with normal secretory granules and large fused secretory granules. The fused secretory granules had light areas and dark areas. Moreover, enlargement and fragmentation of the rough-surfaced endoplasmic reticulum were observed, along with a tendency toward mitochondrial swelling (Figs 4B, C). Swollen and damaged organelles were discharged into some extracellular spaces and the appearance of lysosome-like bodies of high electron density in the cytoplasm was observed. These lysosome-like bodies were positive for AcPase (Fig. 4D). In the GD, irregularity of the basal membrane and

mitochondrial swelling were observed (Fig. 4E). In the SD, the mitochondria were swollen, and some basal infoldings had disappeared due to cell swelling (Fig. 4F).

The fifth day after CDDP infusion

Light microscopic findings: The size of the secretory granules in the ACs had decreased as compared with the third day.

Uneven irregular surfaces of the basal portions of GD and SD were observed (Fig. 5A).

Electron microscopic findings: The shape of the ACs was irregular and there were irregularities of the rough-surfaced endoplasmic reticulum along with mitochondrial swelling. As a whole, the acini were

Fig. 5. Light (A) and electron microscopic findings (B-D) on the 5th day after CDDP infusion.

A; The number of secretory granules in ACs was decreased. ($\times 300$)

B; The acini were atrophic and the secretory granules were decreased. Lysosomes of high electron density were seen around the nucleus and there were numerous autophagolysosomes.

C; Cytoplasm with very low electron density was observed around the secretory granules of the GD.

D; The basal infoldings of SD had disappeared due to cell swelling. (Bar=1 μ m)

atrophied and the secretory granule content had decreased due to degranulation. There were a few areas indicating fusion of secretory granules or biphasic secretory granules. Lysosomes of high electron density were seen around the nuclei and there were numerous autophagolysosomes that had absorbed swollen mitochondria and fragmented rough-surfaced endoplasmic reticulum (Fig. 5B). In the GD, cytoplasm of very low electron density was observed around the secretory granules (Fig. 5C). In the SD, basal infoldings had disappeared due to cell swelling (Fig. 5D).

The seventh day after CDDP infusion

Light microscopic findings: The secretory

granule content of ACs was increased compared with the fifth day, but the shapes of the cells were still irregular. The surfaces of the basal portions of the GD and SD remained irregular and uneven (Fig. 6A).

Electron microscopic findings: The shape of the ACs was irregular and granular membranes were observed in some parts of the cytoplasm. The structures of the secretory granules were mostly homogeneous (Fig. 6B). Primary lysosomes and autophagocytosis were observed but the number of autophagolysosomes had decreased. Some ACs had completely collapsed. The uneven, irregular areas at the basal membranes of the GD remained; however, recovery was seen in the cytoplasm in very low electron density areas, and in organelles (Fig. 6C). In

Fig. 6. Light (A) and electron microscopic findings (B-D) on the 7th day after CDDP infusion.

A; The number of secretory granules in ACs was increased compared with the 5th day. ($\times 300$)

B; The AC shape was irregular and a granular membrane appeared in the cytoplasm.

C and D; In the GD and SD, irregularities of the basal membranes remained. (Bar=1 μm)

the SD, irregularities of the basal membranes remained (Fig. 6D).

DISCUSSION

The rat SMG consists of seromucous cells composed of secretory endpieces and ductal systems. In the acinar portion, conical-shaped ACs surround the glandular lumen and form round or elliptical acini. In the acinar cells, rough-surfaced endoplasmic reticulum, mitochondria, and Golgi apparatus surround the nucleus, and secretory granules tend to gather in the apical part of the cell above the nucleus. The intralobular ducts consist of intercalated ducts, GD, and SD[11].

CDDP is a platinum (Pt) complex with two chlorines (Cl) and two amines (NH₃) coordinated in a cis fashion. Rosenberg et al. [5] discovered CDDP and reported its antitumor effects [6]. Despite its potent antitumor effects, CDDP is known to cause side effects involving various organs [4,7,8].

We examined changes in rat SMG after cisplatin treatment using atomic absorption spectrometry. Our findings showed that the concentration of CDDP absorbed in the SMG was lower than that in the liver or kidney. Thirty minutes after infusion, the concentration of CDDP was decreased in the liver and kidney, whereas the concentration of CDDP was slightly increased in the submandibular glands. Asanami et al. [12] reported in a study of infused concentration of CDDP in animal tissue that the concentration of CDDP in the SMG is low immediately after infusion and reaches a peak 3 hrs after infusion. On the other hand, the concentration of CDDP in the kidney decreased sharply 30 min after infusion and increased again 3 hrs after infusion. They suggested that the decrease of CDDP concentration in the kidney was caused by excretion of CDDP and that the subsequent increase was caused by re-absorption and accumulation. In addition, there have been other reports on CDDP distribution in various types of tissue [13-15]. Irie et al. [15] reported that the concentration of CDDP reaches its highest level immediately after infusion, and gradually decreases thereafter in most organs except for the kidney and adrenal gland. In the kidney and adrenal gland, the concentration of CDDP is higher at 2 hrs than at 1 hr and remains high 24 hrs after infusion. Therefore, the changes in CDDP concentration in the SMG seen in the present study appear to be similar to those reported in the kidney and adrenal gland. This finding is likely to be due primarily to CDDP re-

absorption and accumulation. Moreover, the reabsorption and accumulation of CDDP in these tissues suggests that the CDDP discharged by ACs remains in the tissue for a long period and causes toxicity.

X-ray microanalysis was performed on fixed tissues, but platinum peaks were not detected, probably because CDDP was lost during fixation or epon embedding. Therefore, fresh freeze-fractured tissue sections were examined with no fixation. However, platinum peaks were again not detected; the reasons for this remain unclear. According to Makita et al. [16] and Daimon et al. [17], platinum peaks were found in microbodies and lysosomes in the liver or kidney. These findings suggest that platinum peaks appear in the liver and kidney where larger amounts of CDDP were absorbed, but do not appear in the SMG because of the small amount of CDDP absorbed.

Morphological alterations in the AC were observed. On the first day after infusion, the granule membranes were fused with each other and intracellular vacuoles were formed. The mechanism of intracellular vacuole formation remains unknown. However, it seems to occur during the process of AC injury. The contents of some secretory granules were aggregated and showed an immature biphasic structure with two different electron densities. On the third day after infusion, fusion of secretory granules became apparent, and the content of the granules was separated into light and dark portions. It has been demonstrated histologically that the main component of the high-density portion (central dense core of the granules) is protein, whereas the low-density portion (peripheral rim) is mainly sialomucin [18,19]. Furthermore, Yoshizuka et al. [20] showed that the secretory granules in the rat submandibular glands begin to show a lamellar substructure (biphasic structure) at 4-8 hrs after bis (tributyltin) oxide treatment. Such biphasic structures in secretory granules in the AC are known to accompany hypersecretion of saliva and are thought to be caused by insufficient fusion between sialomucin and protein [21,22]. Therefore, the biphasic changes in secretory granules observed in the present study are thought to be caused by enhanced salivation in order to eliminate CDDP shortly after infusion. This excretory system may contribute to various morphological changes in the SMG.

In ACs, changes in organelles and basal membranes, such as enlargement and fragmentation of the rough-surfaced endoplasmic reticulum, Golgi disappearance, and basal membrane irregularities were

prominent. The formation mechanism of secretory granules in the SMG is similar to that of many other albumen glands. After secretory proteins are synthesized on ribosomes that are attached to the rough-surfaced endoplasmic reticulum, they are then transferred to the Golgi apparatus where they are concentrated and packed into secretory granules. A damaged rough-surfaced endoplasmic reticulum or Golgi apparatus indicates a malfunction of the AC. Therefore, on the fifth day after infusion, a series of protein synthesis processes were damaged, resulting in a decrease in secretory granules. Generally, the secretory cycle of the salivary glands takes approximately 6 hrs, including time-consuming modification and processing of secretory products [22]. In this study, a decrease in the secretory granules became apparent on the fifth day after CDDP infusion, suggesting that secretory cycle damage in the AC was delayed. This seems to be associated with the total CDDP dose or the amount of CDDP accumulated in the SMG. Morphologically, it may take time for damaged secretory granule formation to appear.

The mechanism of action of CDDP suggests that CDDP absorption into the cell nucleus binds two adjacent guanines in the same DNA strand [23], causing an inhibition of DNA synthesis. The concentrated nuclei in the AC seen on the third day are thought to be caused by cell damage due to inhibition of DNA synthesis. Moreover, swelling, morphological irregularities, and expanded (ballooned) cristae in the mitochondria were observed. These changes in mitochondria have been observed in the kidney [24,25], testis [26], and other organs after CDDP infusion. In particular, Kimura et al. [24], in a study of nephropathy, reported that irregularity of the brush border, ballooned cristae and high electron density of the mitochondria are observed in proximal uriniferous tubular cells. A cytochemical examination of renal tubular cells by Aggarwal et al. [25] suggested the following mechanism of CDDP-induced mitochondrial damage: 1) The damage to the mitochondrial inner membrane occurs due to accumulation of Ca_2^+ in mitochondria by a CDDP or CDDP hydrolysates product. The Ca_2^+ released from mitochondria increases in the cytoplasm, resulting in depolymerization of actin-like microfilaments and microtubules in the cytoplasm, thus damaging the cytoskeleton that maintains cell shape. 2) CDDP hydrolysates inhibit mitochondrial ATPase activity and mitochondrial energy generation is decreased. In our study, such mitochondrial damage and enlarged rough-surfaced endoplasmic reticulum, suggestive of

protein synthesis inhibition, were observed on the third day after infusion. The malfunction of various organelles seen in this study may have been caused by mitochondrial damage. The atrophic changes in the ACs seemed to occur gradually as a result of damage to these organelles.

The lysosome-like bodies of high electron density observed on the third day were confirmed to be lysosomes by positive reaction for AcPase. Some of these lysosomes were found to be autophagolysosomes that engulfed secretory granules, mitochondria and other organelles. On the fifth day, the number of autophagolysosomes tended to increase. Autophagocytosis in ACs is considered to be the first step toward recovery through digestion of cellular contents that were damaged by CDDP. On the other hand, organelles were discharged extracellularly due to the collapse of membranes on the fifth day. The presence of these collapsed cells indicates that CDDP may induce necrosis.

On the seventh day, the shape of the ACs remained irregular, but the number of secretory granules increased. Although some ACs were collapsed, a granular membrane was identified in most cells. The irregularities of the rough-surfaced endoplasmic reticulum and mitochondrial swelling were decreased. These findings show that the acinar portion generally tended to recover.

Morphological changes observed in SD cells included mitochondrial swelling and the disappearance of infoldings due to cytoplasmic edema on the third day after CDDP infusion. The SD is known for its striated structure formed by complicated infoldings of the basal plasma membranes. Mitochondria are located between these foldings and active ion transport takes place here. The SD is known to play a role in reabsorption of electrolytes from the primary saliva [27]. Therefore, CDDP may have been reabsorbed in the SD from primary saliva containing CDDP secreted from the ACs. This may then have caused mitochondrial damage and disappearance of the basal infoldings. Similar findings have been reported for heavy metals such as bis (tributyltin) oxide [20]. The damaged basal membranes of ductal components were still observed on the seventh day. This may suggest that the damage was not caused when CDDP was transported through the basal membrane shortly after infusion, but rather was caused when the CDDP was reabsorbed. The reabsorption of CDDP induced a rapid degeneration of the SD leading to cellular atrophy and thickened or curved basal membranes.

In this study, CDDP-induced morphological changes in the rat SMD were observed in both ACs and in the ductal system. Changes showing early recovery appeared on the fifth day and a tendency toward further recovery was shown on the seventh day. Prolonged observation is needed, however, to confirm complete recovery of rat SMG after treatment with CDDP.

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REFERENCES

1. Bosl GJ, Gluckman R, Geller NL, Golbey RB, Whitmore WF et al. VAV-6: an effective chemotherapy regimen for patients with germ-cell tumors. *J Clin Oncol* 1986; 4:1493-1499.
2. Bruckner HW, Wallach R, Cohen CJ, Deppe G, Kabakow B et al. High-dose platinum for the treatment of refractory ovarian cancer. *Gynecol Oncol* 1981; 12:64-67.
3. Kish J, Drelichman A, Jacobs J, Hoschner J, Kinzie J et al. Clinical trial of cisplatin and 5-FU infusion as initial treatment for advanced squamous cell carcinoma of the head and neck. *Cancer Treat Rep* 1982; 66:471-474.
4. Weaver A, Flemming S, Kish J, Vandenberg H, Jacob J et al. Cis-platinum and 5-fluorouracil as induction therapy for advanced head and neck cancer. *Am J Surg* 1982; 144:445-448.
5. Rosenberg B, VanCamp L, and Krigas T. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. *Nature* 1965; 205:698-699.
6. Rosenberg B, VanCamp L, Trosko JE, and Mansour VH. Platinum compounds: a new class of potent antitumor agents. *Nature* 1969; 222:385-386.
7. Robert S, Jonathan W, Marvin G, Donald M, Steven D et al. Cisplatin Nephrotoxicity. *Am J Kidney Dis* 1986; 8:356-367.
8. Schaefer SD, Post JD, Close LG, and Wright CG. Ototoxicity of low-and moderate-dose cisplatin. *Cancer* 1985; 56:1934-1939.
9. Chiba Y, and Kano Y. Dose effects on vital parameters of guinea pig by continuous administration of cisplatin. *Gan To Kagaku Ryoho* 1994; 21:525-530. (in Japanese)
10. Robinson JM, and Karnovsky MJ. Ultrastructural localization of several phosphatases with cerium. *J Histochem Cytochem* 1983; 31:1197-1208.
11. Tamarin A, and Sreebny LM. The rat submaxillary salivary gland. A correlative study by light and electron microscopy. *J Morph* 1965; 117:295-352.
12. Asanami S, Ikeuchi S, Ohsaka F, Okada Y, Ohtaishi M et al. Study on distribution of Cis-platinum in oral tissues in animal tumor bearing. *J Jpn Stomatol Soc* 1984; 33:371-374. (in Japanese)
13. Litter CL, LeRoy AF, and Guarino AM. Disposition and distribution of platinum following parenteral administration of cis-dichlorodiammine-platinum (ii) to animals. *Cancer Treat Rep* 1979; 63:1485-1492.
14. Kurose T, Sasaki T, Mochizuki M, Yasuda M, Yamazaki T et al. Pharmacokinetic evidence for the renal reabsorption of CDDP. *J Jpn Soc Cancer Ther* 1989; 24:1229-1237.
15. Irie T, Hashimoto Y, Irie A, Ishibashi M, Miyazaki H et al. Absorption, Excretion and Distribution of Cisplatin in dogs and rats. *IYAKUHIN KENKYU* 1983; 14:384-410. (in Japanese)
16. Makita T, Itagaki S, and Ohokawa T. X-Ray microanalysis and ultrastructural localization of cisplatin in liver and kidney of the rat. *Jpn J Cancer Res* 1985; 76:895-901.
17. Daimon T, Furuta N, and Maruyama T. Subcellular localization of platinum in the Guinea pig after treatment with cisplatin. *Med Electron Microsc* 1994; 27:340-342.
18. Simson JAV, Spicer SS, and Hall BJ. Morphology and cytochemistry of rat salivary gland acinar secretory granules and their alteration by isopreterenol. *J Ultrastr Res* 1974; 48:465-482.
19. Ichikawa M, and Ichikawa A. Light and electron microscopic histochemistry of the serous secretory granules in the salivary glandular cells of the Mongolian gerbil (*Meriones meridianus*) and rhesus monkey (*Macaca irus*). *Anat Rec* 1977; 189:125-140.
20. Yoshizuka M, Hara K, Nagata N, Doi Y, Yokoyama M et al. The role of the rat submandibular gland in the excretion of Bis(tributyltin) oxide: electron microscopy, X-ray microanalysis and atomic absorption analysis. *Tissue cell* 1992; 24:725-733.
21. Yamashina S, and Mizuhira V. Postnatal development of acinar cells in rat submandibular gland as revealed by electron microscopic staining for carbohydrates. *Am J Anat* 1976; 146:211-236.
22. Ichikawa A, and Ichikawa M. Fine structural analyses on the secretory process of the salivary gland. *J Electron Microsc* 1978; 13:106-114. (in Japanese)
23. Zwelling LA, and Kohn KW. Mechanism of action of cis-Dichlorodiamineplatinum (II). *Cancer Treat Rep* 1979; 63:1439-1444.
24. Kimura S, Nakazono M, and Tazaki H. Electron microscopic observation of the renal tubular damage after administration of cis-diamminedichloroplatinum (II). *Jpn J urol* 1985; 76:1439-1453. (in Japanese)
25. Aggarwal SK, Whitehouse MW, and Ramachandran C. Ultrastructural effects of cisplatin. In: *Cisplatin current status and new developments*, ed. Prestayko AW, Academic press INC, New York, pp70-111, 1980.
26. Ohara M. Ultrastructural study of testicular toxicity. Effects of CDDP on the rat seminiferous tubules. *Jpn J urol* 1988; 79:788-798. (in Japanese)
27. Schneyer LH, Young JA, and Schneyer CA. Salivary secretion of electrolytes. *Physiol Rev* 1972; 52:720-777.