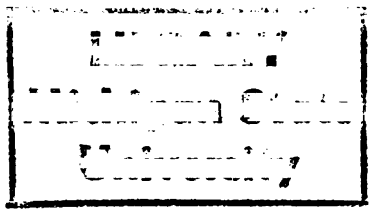

This is to certify that the

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Effects of Cisplatin on the Rat Neurohypophysis

presented by

Philip J. Boyer

has been accepted towards fulfillment
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S. K. Adelmann
Major professor

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EFFECTS OF CISPLATIN ON THE RAT NEUROHYPOPHYSIS

By

Philip Joseph Boyer

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ABSTRACT

EFFECTS OF CISPLATIN ON THE RAT NEUROHYPOPHYSIS

By

Philip Joseph Boyer

Cisplatin treatment (5, 7, or 9 mg/Kg) of young Wistar rats elilcits a dramatically decreased urine output and water intake. Neurohypophysial examination at the light and electron microsopic level either 3 or 5 days after drug treatment reveals two distinct types of abnormal appearing pituicytes (glial cells). 1.) Pituitaries from rats treated with 7 mg/Kg cisplatin have pituicytes which accumulate abnormally large amounts of glycogen and lipids. 2.) Pituitaries from 9 mg/Kg treated rats have numerous translucent-appearing pituicytes. Thus, cisplatin effects on the neurohypophysis appear to be dose-related. While these effects are similar to pituicyte changes elicited by varying lengths of long-term water deprivation of rats, it is concluded that cisplatin treatment affects rat neurohypophyses in ways other than by physiologic dehydration and may in fact have direct toxic effects.

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INTRODUCTION

The anti-neoplastic activity of platinum compounds was first documented in 1969 (38). Cisplatin (cis-dichlorodiammineplatinum(II)) is an effective chemotheraputic agent in the treatment of human testicular (43,10) and ovarian cancers (22) and produces significant results in the treatment of cervical, bladder, prostate, and head and neck (epithelial) cancers (11,15). Cisplatin's promising results are hindered by a broad spectrum of side effects (7,24) of which the most clinically important and dose-limiting is renal toxicity (18,19). Hydration and diuresis therapy as well as timing of treatments have decreased the severity of renal toxicity (13,23,42,43) but high dose therapy still risks considerable renal damage (6,33).

The mechanisms of cisplatin's actions and related toxicities and side effects are not yet clearly understood. Cisplatin's major anti-neoplastic effects are thought to be the result of inhibition of nuclear replication by inter-strand DNA cross-linking (36,46). However, other actions of the drug may be significant too. Cisplatin has been shown, in vitro, to inhibit karyokinesis and arrest cytokinesis (1,2). In addition, cisplatin both *in vivo* and *in vitro* inactivates a variety of plasma membrane-associated enzymes including adenosine triphosphatases, alkaline phosphatase, and 5'-nucleotidase (3,31). This enzyme inactivation leads to cell permeability changes which can alter cell function and may lead to cell death (5).

Much research has gone into the development of analogs of cisplatin in an attempt to duplicate or improve on its anti-neoplastic activity but decrease its side effects (26,28,45). However, each analog has its own problems with solubility, stability, activity, and toxicity (43,45).

Cisplatin is a proven, effective anti-neoplastic drug. Because of its documented success with testicular and ovarian tumors and its potentially broad application in the treatment of other solid tumors (11,15,16) a more complete documentation of cisplatin's side effects would be of considerable significance. Knowledge of treatment's side effects may help ellucidate its mechanism of cytotoxicity and then lead to a means of limiting undesirable side effects.

One side effect of cisplatin treatment in 18, 22, and 38 week old Wistar rats is a marked decrease in urine output (8). While extensive renal tubule damage and decreased glomerular filtration rate by the drug may alter water metabolism (8,12,20), cytotoxic effects on the posterior pituitary and then on the release of vasopressin may be important as well. Various studies have examined cisplatin's renal cytotoxicity (18,24) and some studies have examined plasma vasopressin levels in cisplatin-treated rats (12,20), but no reports of cisplatin treatment's morphologic effect on the posterior pituitary are available.

In this study, the morphologic effects of cisplatin treatment on the neurohypophysis were examined both to evaluate potential cytotoxic or dehydration responses of the

organ and to provide a background for radioimunoassay (RIA) and cytochemical studies of vasopressin in the drug-treated rat. Such studies may help extend the current attempt at delineation of water metabolism disturbances associated with cisplatin treatment.

MATERIALS AND METHODS

Animal Preparation

Male Swiss Wistar rats (Crl:(WI)BR--Charles River Breeding Laboratories) weighing between 190 and 290 g and of 55-65 days in age were injected intraperitoneally with a single dose of 5, 7, or 9 mg/Kg cisplatin (Johnson Matthey Research Laboratories, Sonning, U.K.). The cisplatin was dissolved in normal saline (0.75 M) and allowed to stand overnight in a 35 C incubator. Controls recieved saline injections. There were 20 rats in each experimental and control group. All experimental procedures were performed between 9 am and 11 am during consecutive 24 h periods. The day of injection of cisplatin or saline was called day 0.

Physiologic Studies

All drug-treated and control animals were provided, ad libitum, with measured amounts of tap water and Wayne Lab-Blox rat food (Allied Mills). Effective cisplatin treatment in Wistar rats is characterized by significant, steady weight loss and decreased physical activity. For urine output assessment, control and cisplatin-treated animals were placed for 24 h in metabolic cages to which they were acclimated for 2-3 d prior to drug treatment. Water intakes and weight changes were measured daily for a period of a week prior to and during experimental studies.

Tissue Preparation

Experimental and control animals in each treatment group were sacrificed by decapitation 3 days (10 rats) or 5 days

(10 rats) after drug or saline injection. Trunk blood was collected in heparinized tubes, centrifuged, and volumes were recorded. Pituitaries were quickly removed after decapitation, sliced in half longitudinally, and one half of each pituitary was placed in Hollande's Bouin (HB) for 4 h for light microscopy while the other half was processed for electron microscopy. HB-fixed tissues were dehydrated in graded ethanol and embedded in Paraplast. 7 um sections were cut using a rotary microtome (American Optical) and processed routinely. Deparaffinized slides were stained using the periodic acid-Schiff technique and counterstined with Ehrlich hematoxylin (25). Salivary diastase digestion (30 min) prior to periodic acid treatment was performed on negative glycogen control slides (25). For electron microscopic studies, tissues were fixed in 1% osmium tetroxide and 1% glutaraldehyde (both in 0.05 M sodium cacodylate buffer at pH=7.2) for 3 h, dehydrated in a graded acetone series, and embedded in epon. Thin sections were cut on a LKB Ultratome III ultramicrotome and stained with uranyl acetate and lead citrate.

Microscopy

PAS-hematoxylin stained sections were examined and photographed using a Zeiss Photomicroscope II. Thin sectins were examined and photographed using a Hitachi HU11E electron microscope operated at 75 Kv. Sections from near the center of the pituitary were used for all analyses, but each pituitary was examined at various sequential levels of

sectioning for consistancy.

Data Analysis

Blood volumes were standardized by dividing total drained trunk volume (ml) by body weight (Kg) and the percent decrease of drug-treated rat blood volumes versus control blood volumes were calculated. Urine output and water intake volumes were standardized by dividing volume (ml) by body weight (Kg) and graphed (Fig. 1) as mean +/- standard deviation.

RESULTS

Physical, Physiologic, and Necropsy Observations

Cisplatin-treated rats at all dosage levels moved around their cages much less frequently and vigourously than did control rats. Rats treated with 7 or 9 mg/Kg of cisplatin generally sat motionless in a corner of their cage. By day 5, rats in all dosage groups showed considerable cachexia.

A dramatic decrease relative to control volumes is noted in both urine output and water intake at days 1 (not shown), 3 and 5 after cisplatin injection at all dosage levels (Fig. 1). Weight-standardized drained trunk blood volumes decreased significantly (P 0.05) relative to controls by 16%, 28%, and 31% for 5mg/Kg, 7 mg/Kg, and 9 mg/Kg cisplatin-treated rats, respecively. Blood from drug-treated rats appears more viscous, especially at day 5 for the high dosages levels (7 and 9 mg/Kg), than the blood of control rats. At necropsy, all cisplatin-treated rats showed considerable bloating and ulcerations of their stomach and their stomach contents were watery. Few to no fecal pellets were noted in their intestines although a few rats in the high dosage groups had developed diarrhea. Control rats had non-bloated stomachs, moist but compact stomach contents, and numerous fecal pellets in the intestines.

Morphologic Findings

Figure 2 shows light micrographs of posterior pituitary cross-sections taken from control (A), 7 mg/Kg (B) and 9

mg/Kg (C) cisplatin-treated Wistar rats 5 d after injection. Figure 3 shows electron micrographs from the same dosage groups, also 5 days after injection. With the exception of small amounts of glycogen accumulation in a few peripheral cells, no glycogen is seen in most control pituicytes (Fig. 2A). Virtually all control pituicytes have a dense, basophilic-staining cytoplasm. Electron micrographs (Fig. 3A) of control pituicytes and axons shows large nuclei with dispersed chromatin; normal and evenly distributed Golgi, endoplasmic reticulum (ER), and mitochondria; no marked glycogen accumulation; and a few lipid globuoles. In large cross-sections of pituicytes a variable number of cytoplasmic extensions can be seen which tortuously reach out among, and sometimes encircle, axons. Axons are evenly distributed and a large number of neurosecretory vesicles is seen within them.

Neurohypophyses of 5 mg/Kg cisplatin-treated rats are virtually indistinguishable morphologically from controls, although a few pituicytes have clear cytoplasmic rings surrounding their nuclei. Peripheral glycogen and lipid accumulations are of the same order of magnitude as those in control tissues. No apparent decrease in the number or tortuosity of cytoplasmic extensions is noted.

Neurohypophyses from 7 and 9 mg/Kg cisplatin-injected animals always show one of two distinct, abnormal patterns of pituicyte appearance. At the light microscope level (Fig. 2B), cross-sections of pituitaries from 7 mg/Kg treated animals show striking accumulations of glycogen within some

pituicytes while other pituicytes have less or no accumulation. The pituicyte glycogen granulation in this group of pituitaries is most pronounced around the perphery of the tissues and becomes less intense centrally. The glycogen appears to be exclusively in pituicytes. Glycogen accumulation is approximately of the same magnitude at 3 and 5 d in this group. Also, many pituicytes are seen to have a clear ring of cytoplasm surrounding their nuclei. At the electron microscope level (Fig. 3B), large lipid globules can be seen in cells which have very heavy glycogen accumulations. Such cells tend to retain cytoplasmic extensions among axons. However, the extensions reach neither as far nor as tortuously as do those in control tissues. The distribution of cytoplasmic organelles in glycogen- and lipid-rich pituicytes appears normal although ER and Golgi tend to have a somewhat swollen appearance. Pituicytes loaded with glycogen and lipid can lie in close proximity to a pituicyte with little to no glycogen buildup and normal-appearing cytoplasmic organelles. Axons in this group of pituitaries contain fewer neurosecretory granules than do control axons. The clear cytoplasmic rings observed at the light microscope level cannot be distinguished at the electron microscope level.

At the light microscopic level, cross-sections from 9 mg/Kg cisplatin-treated pituitaries appear to have a third or less of the amount of glycogen seen in 7 mg/Kg treated pituitaries, but roughly three times the amount found in control tissues. At 3 days after drug treatment virtually

all pituicytes have a clear ring of cytoplasm, variable in size, surrounding their nuclei. After 5 days between 15 and 25 percent of pituicytes per mid-pituitary section at low power are seen to have a rounded-up appearing, translucent cytoplasm while the rest of the pituicytes retain the clear ring appearance. At the electron microscope level (Fig. 3C), cells with translucent cytoplasm are seen to have few to no cytoplasmic extensions among axons and their cytoplasm appears centralized. Heterochromatin is marginated along intact nuclear membranes and clumped centrally in these cells. These pituicytes' cellular organelle content appears diminished and the organelles tend to be clumped and appear swollen. Cytoplasmic membranes are intact and no inflammatory cell infiltration is noted. No vascular changes are seen. Lamellated-bodies, between 1 and 4 axons per total area of mid-pituitary sections are seen. Most axons contain fewer neurosecretory granules than do control axons. Non-translucent pituicytes have dispersed chromatin and normal distribution of organelles, yet their cytoplasmic extensions are less far-reaching than those seen in control pituicytes. No cytoplasmic rings can be identified at the EM level.

Figure 1 Graph showing water intake and urine output in control versus cisplatin-treated rats at various dose levels 3 and 5 days after treatment

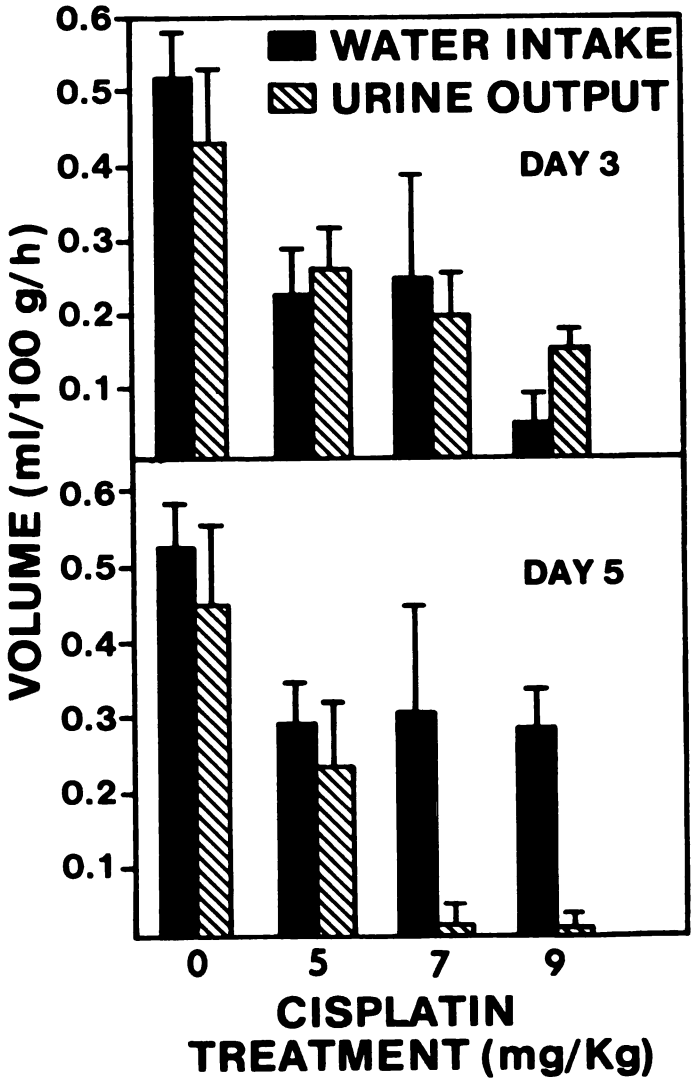

WATER INTAKE
URINE OUTPUT
DAY 3
DAY 5
VOLUME (ml/100 g/h)
0.6
0.5
0.4
0.3
0.2
0.1
0
5
7
9
CISPLATIN
TREATMENT (mg/Kg)

Figure 2 A Control Pituritary. Light micrograph showing pituicyte nuclei (P), and endothelial cell nuclei (E). PAS-Hematoxylin, 1800X.

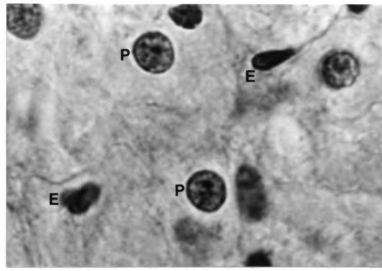

P
E
E
P

Figure 2 B 7 mg/Kg Cisplatin Treated Pituitary, 5d. Light micrograph showing pituicyte nuclei (P), clear cytoplasmic rings (→), and glycogen (→). PAS-Hematoxylin, 1800X.

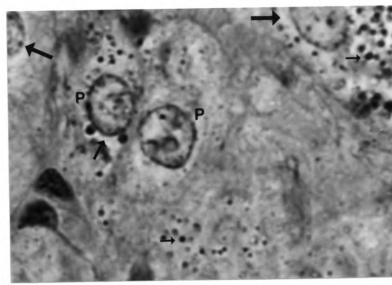

P
P

Figure 2 C 9 mg/Kg Cisplatin-Treated Pituitary, 5d. Light micrograph showing translucent pituicytes (➞), glycogen (➞), and endothelial cell nuclei (E). PAS-Hematoxylin, 1800X.

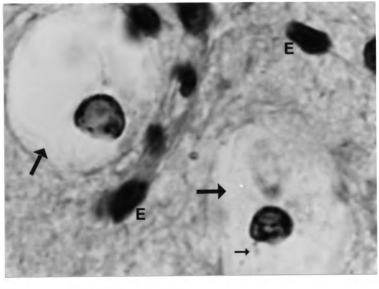

E
E

Figure 3 A Control Pituitary. Electron Micrograph showing axons (A), pituicyte membrane (⟶) and nucleus (N), and lipid globules (L).

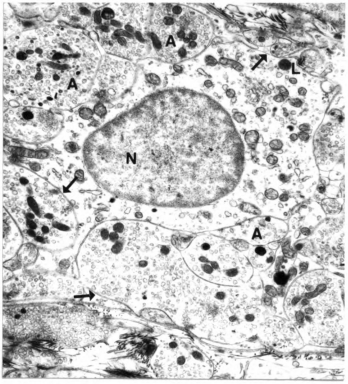

A
A
L
N
A

Figure 3 B 7 mg/Kg Cisplatin-Treated Pituitary, 5d. Electron micrograph showing pituicyte membrane (⟶), glycogen (→), lipid globules (L), and axons (A). 17,200X

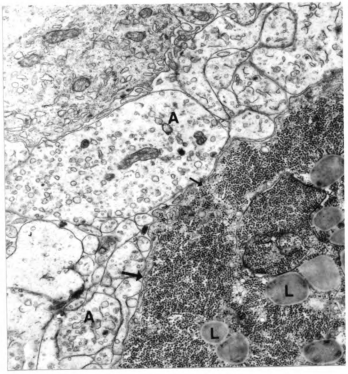
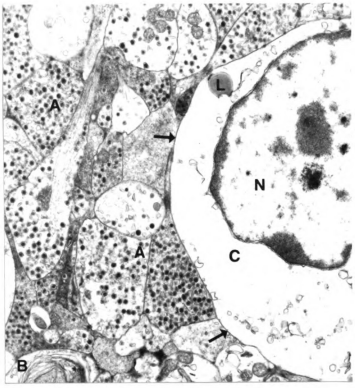

A
A
L
L

Figure 3 C 9 mg/Kg Cisplatin-Treated Pituitary, 5d. Electron micrograph showing pituicyte nucleus (N), translucent cytoplasm (C), and membrane (→), lipid globules (L), axons (A), and lamellated body (B). 13,200X.


A
L
N
A
C
B

DISCUSSION

The highly vascular neurohypophysis consists of axons arising from hypothalamic supraoptic and paraventricular nuclei and of astrocytic glial cells called pituicytes. No easy morphologic distinction exists between vasopressin and oxytocin carrying neurons. Pituicyte cytoplasm is quite extensive and cytoplasmic processes are typically seen, at the electron microscope level, to reach out among and sometimes surround axons in their vicinity in normal, well-hydrated rat pituitaries (21,30,40). It is usually difficult or impossible to trace the pituicyte cytoplasmic extensions in an entire section of a cell because of their tortuous interweaving among and between axons (Fig. 2A). The role of the pituicytes in the neurohypophysis is not entirely clear. They are thought to play an important role in the modulation of vasopressin release (21,40). Further, they help in the removal of necrotic axons (14,27,35) as well as in the development of the neural lobe (17). They probably also play a classic glilal support role in the maintenance of axonal metabolism.

Cisplatin-treated rats are physiologically dehydrated as demonstrated by an alteration of their water metabolism: urine output and drained trunk blood volume are decreased while blood viscosity is increased. A drug-induced dehydration could be contributed to by a number of factors. Most importantly, water intake is significantly decreased beginning at day 1 and extending to day 5 after drug treatment (Fig. 1). A protracted decrease in water intake

probably directly contributes to all other water metabolism changes. In addition, it may be that only part of the water that is taken in by drug-treated rats is effectively absorbed. The stomachs of cisplatin-treated, Wistar rats, at any of this study's dosage levels, undergo dramatic bloating (4,37). The contents of these bloated stomachs are considerably more watery than those of control rats and this water retention may limit the amount of water that reaches digestive tract absorptive surfaces. In addition, although little food passes through the digestive tract of cisplatin-treated rats (4) some 7 and 9 mg/Kg treated rats have a slight degree of diarrhea which would lead to further water loss.

Plasma vasopressin levels during cisplatin treatment have been reported only in Sprague-Dawley rats, and only at 1 day (20) and between 8 and 24 h (12) after drug injection. These rats undergo a diuresis and polydipsia in response to cisplatin treatment. In contrast, cisplatin-treated Wistar rats are dehydrated and vasopressin levels would thus be expected to increase in them and would contribute to a decreased urine volume and increased urine osmolarity and thus conserve water. It is possible that cytotoxic effects on pituicytes could elevate vasopressin levels beyond what woud be expected for the particular dehydration level present and depress urine output still further. Radioimmunoassay studies of plasma vasopressin levels in cisplatin-treated Wistar rats are forthcoming

from this lab. Damage to kidney tubules is severe following single-dose cisplatin treatment at most dosage levels (5,19) and may also contribute to a decreased urine output although Sprague-Dawley studies cite renal damage as possibly leading to increased urine output (18,24).

The distinct morphologic responses of pituicytes to cisplatin treatment seems to be dose-related and may be indicative of varying degrees of pituicyte response to injury. Rats deprived of water for between 1 and 15 days show moderate to heavy accumulation of glycogen and lipids (29,32). This experiment's water deprivation studies, while carried out for only 3 and 5 days, showed both pituicyte glycogen and lipid buildups considerably greater than control levels. However, these levels of accumulation come nowhere near the amount of the substances found in 7 and 9 mg/Kg cisplatin-treated rats. Large glycogen and/or lipid accumulations are also found in a variety of physiologic and experimental situations including during development of the neural lobe (17), during aging (35), after hypophysial stalk transection (14,27) and after reserpine treatment of rats (34). All of these reports claim that the appearance of glycogen and/or lipid accumulations is indicative of the "activation" of pituicytes involved with increasing the amount of vasopressin released from axons or in stimulating the growth of, maintaining, or phagocytizing axons (i.e., in support roles). At least for the water deprivation experiments, a correlation exists between dehydration (which should elicit increased vasopressin release) and glycogen

and lipid accumulation. The massive glycogen and lipid accumulations seen in cisplatin-treated rat pituitaries may be associated with an activation of the cells in response to both dehydration and injury.

Electron micrographs of well-hydrated rat neurohypophyses show a large percentage of axonal processes to be in contact with or surrounded by a pituicyte's or pituicytes' cytoplasm (40). The number of glial enclosures of and contacts with axons is seen to be significantly decreased by water deprivation of between 4 h and 4 d or by increased osmotic pressure as induced by raising ionic content in vivo or in vitro (21,40,44). The reduction of pituicyte-axon contact is thought to result from retraction of pituicyte cytoplasmic processes (41). The formation of translucent pituicytes in 9 mg/Kg cisplatin-treated pituitaries is probably a function of retraction of cytoplasmic processes, but to a point much greater than is elicited by the 4 h through 4 d water-deprivation. That this is probably the case is supported by the finding of numerous "degenerating" pituicytes in 15 day water-deprived pituitaries (32), the electron micrographs of which are indistinguishable from cisplatin-induced translucent pituicytes.

The clearness of translucent cells may be due, at least in part, to the dilutional effects which result from the rounding up and centralization of these pituicytes' cytoplasm. The clear rim of cytoplasm seen surrounding most

pituicyte nuclei in 7 and 9 mg/Kg cisplatin-treated rats, only at the light microscopic level (Fig. 2B,C), may be the result of the moderate cytoplasmic process retraction noted in these cells.

The pituicyte changes seen in 7 and 9 mg/Kg cisplatin-treated rats, like those seen in water deprivation studies, may be distinct pituicyte responses to different levels of cellular injury. With slight or moderate injury the pituicyte may respond with varying degrees of cytoplasmic process retraction and accumulation of glycogen and lipid. With severe injury the pituicyte may respond by totally retracting its cytoplasmic processes and accumulating some lesser amount of glycogen and lipid. There may also be a point where long-term, moderate injury culminates in total retraction of cytoplasmic processes.

The mechanism of action of cisplatin which might lead to the hypothesized pituicyte injury is not clear. Cisplatin-induced dehydration cannot by itself account for the rapidity or magnitude of change in water metabolism that is seen. The neurohypophysial effects of cisplatin treatment bring about morphologic responses similar to, but more extensively and rapidly than, long-term water deprivation. Relative to the anterior pituitary, cisplatin treatment has been shown to decrease serum levels of the hormones LH and prolactin in pregnant rats by 39% and 63%, respectively (9). Cisplatin is not found to accumulate in general brain tissue (39), but accumulation specifically in the hypophysis has not been investigated. The blood-brain

barrier not withstanding, the degree of vascularity of the gland suggests at least some contact with the drug is probable, if only at low levels, but especially with high-dosage cisplatin treatment. Cytotoxic effects would than possible.

While the huge stores of glycogen and lipid in otherwise healthy, although somewhat retracted, pituicytes can almost certainly return to normal levels, it is not clear whether the translucent cells are irreversibly injured or whether they can recover with time. The translucent cells are certainly damaged, however the integrity of the plasma and nuclear membranes, the lack of pyknosis of the nuclei, and the lack of inflammatory cell infiltration indicate that such cells are not yet necrotic and that recovery is possible. An examination of neurohypophyses from 7 and 9 mg/Kg treated rats at 7, 10, 15, and 30 days after treatment will be undertaken to watch the course of both the glycogen and lipid accumulations and the translucent cells.

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