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PLATINUM-INDUCED TOXICITY AND POTENTIAL MODULATION BY CALCIUM TREATMENT: A HISTOCHEMICAL STUDY

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PLATINUM-INDUCED TOXICITY AND POTENTIAL MODULATION BY CALCIUM TREATMENT: A HISTOCHEMICAL STUDY

By

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ABSTRACT

PLATINUM-INDUCED TOXICITY AND POTENTIAL MODULATION BY CALCIUM TREATMENT: A HISTOCHEMICAL STUDY

By

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Cisplatin (CDDP), Carboplatin (CBDCA) and Taxol (Paclitaxel) are potent anti-neoplastic agents with associated toxicities that are their dose-limiting factors in clinical oncology, especially cisplatin-induced nephrotoxicity. In an attempt to elucidate their mechanism(s) of toxicity, kidney and liver tissues from normal and drug treated wistar rats and dogs were evaluated by analyzing changes in various dehydrogenase and nonspecific lipase enzymes. Specifically, IDH, β-HBDH, GDH, MDH, SDH, LDH, and G-6-PDH were studied using standard tetrazolium salt methods. Nonspecific lipases were localized using the Tween method. Histochemically, cisplatin treatment caused morphological changes in the tissues as well as enzyme inhibition in all the enzymes except G-6-PDH and nonspecific lipases. It is hypothesized that hydrolyzed cisplatin disrupts calcium homeostasis and binds to sulphydryl groups (-SH), resulting in inhibition of ATP synthesis and modified membrane permeability. Further, lipases are activated which strip enzymes from plasma membranes. However, carboplatin treatment results in less toxicity as does cisplatin plus taxol combination therapy. Supplemental treatments of calcium (10 ml/kg of 1.3% $CaCl_2$/day in rats and 0.5 μg rocaltrol/day in dogs) seems to protect against severe toxicity by protecting dehydrogenase enzymes and preserving overall tissue morphology.

Dedicated to My Family

for their Love and Guidance

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Thanks to Dr. SK Aggarwal for being my mentor and friend. Also, thanks to Dr. RA Pax and Dr. A Goyal for their invaluable interest and assistance as guidance committee members. Thanks to Dr. JH Whallon for her help with the LSM and the quantitative analyses. Thanks to Dr. R Nachreiner for his help with the ionized calcium calculations. Thanks to Dr. Wang for his willingness to help and donate his time. Thanks to the Zoology office staff: Chris Keyes, Tracey Barner, Judy Pardee, Lisa Kraft and Janice Mead for all their help. Lastly, thanks to Bristol Meyers and NIH for their samples of Taxol, Cisplatin and Carboplatin.

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INTRODUCTION

Cisplatin (*cis*-dichlorodiammineplatinum II), a heavy metal platinum coordination complex, has received intense scrutiny as to its mechanism of action since its serendipitous discovery by Barnett Rosenberg of Michigan State University in 1965 (Rosenberg et al., 1965). DNA denaturation is one of the accepted methods of its action through its intrastrand and interstrand cross-links interfering with DNA replication and transcription (Roberts and Pascoe, 1972; Rosenberg, 1975; Zwelling et al., 1979, 1981; Fitchinger-Schepman et al., 1987; Reed et al., 1986). It has been shown that proteins containing a high-mobility group (HMG) domain bind specifically to DNA modified by cisplatin, but not to unmodified DNA, and thus prevent excision repair (Huang et al., 1994). Since clinical testing began around 1972, it has become clear that cisplatin is successful in reducing or even eliminating cancers of the head and neck (Jacobs et al. 1983), lung (Ruckdeschel et al., 1985), breast (Cox et al., 1989), ovary (Wiltshaw et al., 1985), bladder (Loehrer et al., 1990) and testicle (Williams and Einhorn, 1980). Coupled to its anti-tumor activity are many severe toxic side effects including peripheral neuropathy (Kedar et al., 1978), ototoxicity (Piehl et al., 1974), myelosuppression (Talley et al., 1973; Ozols et al., 1984), nausea and vomiting (Higby et al., 1974; Yagoda et al., 1976), hypomagnesia (Schilsky and Anderson, 1979), hypocalcemia (Blachley and Hill, 1981), enzyme disruption (Aggarwal, 1993), hepatotoxicity (Cavalli et al., 1978) and

nephrotoxicity (Leonard et al., 1971). More specifically, it is the nephrotoxicity associated with cisplatin use that has become the dose-limiting factor in its use. It manifests, pathologically, as renal tubular damage which results in elevation of the blood urea nitrogen and serum creatinine levels (Hardaker et al., 1974). Renal damage from cisplatin treatment has been characterized as being similar to that of mercury and other heavy metals which also result in renal tubular necrosis, degeneration, and interstitial edema without glomerular changes (Madias and Harrington, 1978). This severe renal toxicity caused Dr. J. Hill of the Wadley Institutes of Molecular Medicine, who was responsible for the first report of CDDP's clear anticancer activity in human patients, to remark that, "Cis-platinum(II) diamminechloride appears to be too good a therapeutic agent to abandon, yet too toxic for general use (Hill et al., 1974). It was later demonstrated that hydrating patients markedly diminished kidney toxicity associated with cisplatin without a major loss of anticancer activity (Hayes et al., 1977). Slow infusion rates have also been shown to ameliorate kidney toxicity (Merrin, 1976). Further, antioxidants and thiol containing compounds, such as sodium thiosulfate, have become part of the treatment regimen in an attempt to alleviate nephrotoxicity (Powis and Hacker, 1991). Despite these advances, nephrotoxicity is still a major concern and limitation in cisplatin chemotherapy treatment due to the extremely high uptake by the kidneys which is approximately three times the uptake of any other organ (Wolf and Manaka, 1977).

Carboplatin (*cis*-diammine-1,1-cyclobutane dicarboxylate platinum II), is an analogue of cisplatin with a similarly proposed mechanism of action and comparable

effectiveness that has demonstrated reduced toxicity (Alberts et al., 1990). Consequently, carboplatin has been suggested as an alternate to cisplatin use.

Also, cisplatin in combination with many other anticancer agents has proven to be very effective (Woodman et al., 1973). Specifically, cisplatin plus taxol (paclitaxel) has become increasingly prevalent in clinical treatment (Rowinsky et al., 1991). Combination therapy is capable of producing response rates of up to 100% and is seemingly less toxic than the either of the two anticancer compounds given alone (Donehower and Rowinsky, 1992).

Further, it has been shown that calcium supplementation can help protect enzyme function and preserve overall organ function by minimizing the disruption of cellular homeostasis initiated by cisplatin treatment (Aggarwal and Fadool, 1993). Thus, the present study was undertaken to (1) characterize cisplatin-induced enzymatic changes in rat kidney and liver tissues and dog kidney tissues, (2) compare carboplatin-induced enzymatic changes to that of cisplatin treatment, (3) compare enzymatic changes associated with taxol as well as enzymatic changes associated with combination therapy (cisplatin plus taxol), (4) characterize structural and functional changes associated with calcium supplementation and (5) correlate the various changes to the associated toxicities.

MATERIALS AND METHODS

Animals:

Wistar rats (Charles Rivers Laboratory, Wilmington, MA) weighing between 160-200 g were used in the various experiments over a period of 17 months. Animals were kept on a 12 hr light/12 hr dark, cycle with access to laboratory animal food and water ad libitum in accordance with the Guide for Care and Use of Laboratory Animals. Animals received intraperitoneal (ip) injections of freshly prepared cisplatin, carboplatin, taxol, cisplatin plus taxol, cisplatin plus 1.3% calcium chloride or calcium gluconate, or 0.85% sodium chloride or 5% glucose in concentrations and dosages shown in Table 1.

Male dogs weighing 70-95 lbs were kept on a 12 hr light/12 hr dark cycle. The dogs had free access to water and food in accordance with the Guide for Care and Use of Laboratory Animals. Three dogs were twice infused with cisplatin (1.8 mg/kg) in conjunction with saline solution and three received the vehicle alone. The dogs also received 0.5 μg rocaltrol supplements (Roche Laboratories), by oral administration, for five days prior to infusion and every day after infusion to maintain an elevated serum calcium level.

Blood Collection and Analysis:

Blood samples were taken every day for the first week and then every third day from then on and serum was tested for ionic calcium levels as well as blood urea nitrogen (BUN) and creatinine levels.

Tissue Collection:

The rats in the various groups were anesthetized with CO_2 and killed by decapitation at each sampling interval. The tissues (kidneys and livers) were quickly excised and mounted on cryostubs in O.C.T. medium (Miles Laboratories, Elkhart, IN) and frozen at -20°C until use. Sections (10 μm) were cut using a cryostat microtome (Miles Laboratories) for enzymatic analysis.

The dogs were given a lethal dose of sodium pentobarbital (325 mg/kg). The tissues were quickly excised and mounted on cryostubs in O.C.T. medium (Miles Laboratories) and frozen at -20°C. Sections (10 μm) were cut for enzymatic analysis.

Histochemical Studies:

Determination of Optimal pH and Incubation Time. pH levels were varied from 7.0 to 7.6 while incubation time was kept constant. Incubation time was then varied from 20 minutes to 90 minutes while the pH was kept constant.

Dehydrogenase Localization. Frozen sections (10 μm) of normal, cisplatin, cisplatin plus calcium, carboplatin, cisplatin plus taxol, and taxol treated tissues were picked up on standard glass coverslips and allowed to dry at room temperature. Succinate dehydrogenase (SDH), glutamate dehydrogenase (GDH), β-hydroxybutyrate dehydrogenase (HBDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH),

lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6-PDH) were then localized, histochemically, by the standard Nitro BT Method (Burstone, 1962).

The substrate or the coenzymes were omitted from the incubation media to serve as controls. Tissue sections were also boiled in distilled water to denature the endogenous enzymes and serve as a control.

Lipase Localization. Nonspecific lipase activity was localized by incubation of tissue sections in a medium according to the methods of George and Ambadkar (1963) and George and Iype (1960). Boiled tissue sections served as a control.

Photomicroscopy:

Slides were viewed and micrographs were prepared of transmitted images using a Zeiss Photomicroscope II.

Qualitative analysis:

Staining intensity was based subjectively on an arbitrary scale from very intense response (+++++) to intense response (++++) to moderate response (+++) to poor response (++) to very poor response (+) to no response (-). Tissue sections were randomly evaluated and the cortex and medulla were considered in the subjective determination of enzyme localization response.

Cell size and general morphology, of the tissues, were evaluated by visual observation and by light micrographs.

Quantitative Analysis:

Approximately four transmitted images from each normal, cisplatin, carboplatin, taxol, cisplatin plus taxol, and cisplatin plus calcium treated tissues, as well as control

slides, were examined by the Zeiss 10 Laser Scanning Confocal Microscope (LSM) and quantitative analyses were made using the 'histogram' program within the LSM computer. Random areas of the tissues were analyzed for staining intensity by the computer and a representative histogram detailing 'gray scale values' was produced for each enzyme. Statistical analysis was performed and gray scale values were then converted to percentages based on normal staining being equivalent to 100% intensity.

Table 1. Experimental Design

Group	Animal	#/Group	Treatment	Interval	Day of Sacrifice
I	Rat	12	CDDP (8 mg/kg)	1 Bolus	2, 3, 4, 5 Days After Last Injection
		12	CDDP (8 mg/kg) plus 1 ml daily injections of 1.3% $CaCl_2$ or Calcium Gluconate		
		12	CBDCA (50mg/kg)		
		6	Control [0.85% NaCl or 5% Glucose (10 ml/kg)]		
II	Rat	12	CDDP (1.8 mg/kg)	5 Consecutive Days	2, 3, 4, 5 Days After Last Injection
		6	CDDP (0.9 mg/kg)		
		12	Taxol (4 mg/kg)		
		6	Taxol (2 mg/kg)		
		6	CDDP (1.8 mg/kg) and Taxol (4 mg/kg)		
		12	CDDP (0.9 mg/kg) and Taxol (2 mg/kg)		
		6	Control [0.85% NaCl (10 ml/kg)]		
III	Dog	3	CDDP (1.8 mg/kg) in 500 ml 0.85% NaCl was infused over a period of 3 hours plus 0.5 µg daily supplements of Rocaltrol	Every 21 Days	40 Days After Last Infusion
		3	Control [0.85% normal saline (500 ml)]		40 Days After Last Infusion

RESULTS

Effects of Drug Treatments on Dehydrogenases and Nonspecific Lipases in Rat Tissues:

Histochemically, of all the dehydrogenases studied, only the G-6-PDH demonstrated an increase in its activity and distribution patterns after various treatments. LDH and MDH resemble the activity and localization of the other dehydrogenases studied: SDH, GDH, HBDH, and IDH. Thus, for the sake of brevity and simplicity, only LDH, MDH, and G-6-PDH are discussed in detail for the rat kidneys and only MDH and G-6-PDH are discussed for the rat livers.

Sections of normal kidney localized for the LDH and MDH showed dark blue granulation with intense, diffuse staining throughout the cytoplasm of the tubules. Specifically, in the normal kidney, staining intensity and localization was approximately the same in both the cortical and medullar regions with the proximal and distal tubules having equally pronounced enzymatic activity. Further, no staining was demonstrated within the nuclei or glomeruli (Figures 1, 2, 4; Tables 2, 3). Similarly, sections of normal liver localized for MDH showed dark and diffuse staining throughout the cytoplasm of the hepatocytes. The nuclei of the hepatocytes were unstained (Figure 4, 5; Tables 2, 3).

In cisplatin treated kidneys, LDH and MDH enzymatic staining was decreased compared to normal, showing indiscriminate and less intense granulation and gray

coloration throughout the cytoplasm of the cells. Further, enzymatic activity was depressed in the medulla compared to the cortex and distal tubule staining was less than that demonstrated within the proximal tubules (Figures 1, 2, 4; Tables 2, 3). Sections from cisplatin treated livers stained for MDH demonstrated a significant decline in enzymatic activity in the cytoplasm of the hepatocytes compared to normal (Figures 4, 5; Tables 2, 3).

In the kidneys treated with cisplatin and calcium, LDH and MDH staining and localization were maintained similar to normal. Staining was significant throughout the cytoplasm of the cells of the tubules. The cortex and medulla were equally stained as were the proximal and distal tubules. The nuclei and glomeruli showed no significant staining (Figures 1, 2, 4; Tables 2, 3). The cisplatin plus calcium livers stained for MDH again followed a similar pattern to the kidneys. Specifically, MDH staining and localization was maintained similar to the normal tissues with staining being intense throughout the cytoplasm of all the hepatocytes (Figures 4, 5; Tables 2, 3).

Sections of carboplatin treated kidneys localized for LDH and MDH also demonstrated a decline in enzymatic activity, from normal levels, but less than cisplatin-induced enzyme depression. Staining gradually decreased from the exterior of the cortex to the medulla and the proximal and distal tubules declined equally (Figures 1, 2, 4; Tables 2, 3). In livers treated with carboplatin, MDH enzymatic activity also declined in a similar manner to that seen in the kidneys treated with carboplatin. MDH hepatocyte staining was depressed compared to normal but less depressed than after cisplatin treatment (Figures 4, 5; Tables 2, 3).

Cisplatin plus taxol treated kidneys had LDH and MDH enzyme intensities greater than cisplatin treatment alone but less than that seen in normal tissues. Medullar staining was less than cortical and the proximal and distal tubules declined equally (Figures 1, 2, 4; Tables 2, 3). MDH localization in livers treated with cisplatin and taxol followed a similar pattern. MDH hepatocyte staining was less than normal but greater than cisplatin treatment alone (Figures 4, 5; Tables 2, 3).

LDH and MDH after taxol treatment demonstrated a minimal decline in activity from normal. Cortical and medullar staining was approximately the same as was proximal and distal tubule staining (Figures 1, 2, 4; Tables 2, 3). In livers similarly treated with taxol, MDH activity also declined very little from normal levels. MDH staining within the cytoplasm of the hepatocytes was diffuse and only slightly less intense than that seen in the normal tissues (Figures 4, 5; Tables 2, 3).

Sections of normal kidney localized for G-6-PDH were similar to the other dehydrogenases studied. Specifically, G-6-PDH staining was diffuse throughout the cytoplasm of the tubules. Further, staining intensity and localization was approximately the same in both the cortical and medullar regions with the proximal and distal tubules having equally pronounced enzymatic activity. No significant staining was demonstrated within the nuclei or glomeruli (Figure 3, 4; Tables 2, 3). Normal liver localized for G-6-PDH showed dark and diffuse staining throughout the cytoplasm of the hepatocytes (Figures 3,4; Tables 2, 3).

However, in the kidneys and livers of the drug treated animals, the G-6-PDH staining results were reciprocal to those seen with the other dehydrogenases studied,

except for cisplatin plus calcium. Specifically, cisplatin treated kidneys demonstrated increased G-6-PDH staining throughout the tubules compared to normal. The cortical and medullar regions had similarly increased G-6-PDH activity as did the proximal and distal tubules (Figures 3, 4; Tables 2, 3). Liver cryo-sections stained for G-6-PDH also demonstrated an increase in enzymatic activity throughout the cytoplasm of the hepatocytes (Figures 4, 6; Tables 2, 3).

G-6-PDH activity after cisplatin plus calcium treatment was again maintained close to normal levels. In the kidneys G-6-PDH activity closely approximated normal staining with cortical and medullar regions as well as proximal and distal tubules being equally stained (Figures 3, 4; Tables 2, 3). Similarly, cryo-sections of liver demonstrated G-6-PDH hepatocyte staining similar to normal (Figures 4, 6; Tables 2, 3).

Carboplatin treatment also resulted in G-6-PDH enzyme elevation. G-6-PDH elevation was consistent throughout the proximal and distal tubules of the cortex and medulla. G-6-PDH staining after carboplatin was still less than after cisplatin treatment, though both were elevated from normal (Figures 3, 4; Tables 2, 3). Similarly treated liver demonstrated a slight increase in G-6-PDH enzymatic activity in the hepatocytes from normal (Figures 4, 6; Tables 2, 3).

G-6-PDH activity after treatments of cisplatin plus taxol and taxol alone was slightly greater than normal. The increase in G-6-PDH activity in the kidney cryo-sections was approximately the same after both cisplatin plus taxol and taxol alone. Specifically, distribution of G-6-PDH staining was equally elevated in the proximal and distal tubules of the cortex and medulla (Figures 3, 4; Tables 2, 3). Similarly treated

livers demonstrated similar G-6-PDH staining. Specifically, there was a slight increase in G-6-PDH staining from normal after treatment with cisplatin plus taxol and taxol alone with the increase being about the same after both treatments (Figures 4, 6; Tables 2, 3).

Nonspecific lipase activity followed the same pattern as that seen in G-6-PDH activity. Normal kidney and livers localized for nonspecific lipase activity demonstrated brown diffuse granulation throughout the tissues. In the kidneys, staining was similar in the proximal and distal tubules of the cortex and medulla. No significant glomerular staining was evident (Figures 7, 8; Tables 2, 3). Nonspecific lipase staining in normal livers was similar to the normal kidneys in that there was diffuse granulation throughout the cytoplasm of the hepatocytes (Figures 8, 9; Tables 2, 3).

Cisplatin treatment caused an elevation in lipase levels throughout the kidney tubules compared to the normal tissues. The increase was consistent throughout the cortex and medulla. No distinction between the proximal and distal tubules was evident (Figures 7, 8; Tables 2, 3). Similarly, hepatocyte staining after cisplatin treatment was also increased compared to normal (Figures 8, 9; Tables 2, 3).

Nonspecific lipase activity was closer to normal after cisplatin plus calcium treatment in both the kidneys and livers. The proximal and distal tubules of the cortex and medulla, in the kidneys, demonstrated equal staining that approximated normal levels (Figures 7, 8; Tables 2, 3). Cytoplasmic staining in liver hepatocytes was also evenly distributed and similar to normal (Figures 8, 9; Tables 2, 3).

Carboplatin treatment was similar to cisplatin treatment in that kidney cryo-sections demonstrated an increase in activity compared to normal. However, lipase levels

increased less than after cisplatin treatment. Nonspecific lipase staining was consistent throughout the cortex and medulla and no difference between proximal and distal tubules was evident (Figures 7, 8; Tables 2, 3). Liver hepatocytes also showed a slight increase in lipase activity compared to normal but the increase was less than that associated with cisplatin treatment. Increased lipase activity in the liver cryo-sections after carboplatin was consistent throughout the tissues with a slight increase in localization evident around blood vessels of the hepatic lobules (Figures 8, 9; Tables 2, 3).

Cisplatin plus taxol treated tissues and taxol treated tissues both demonstrated decreased lipase activity as compared to the normal tissues. Lipase activity after combined treatment (cisplatin plus taxol) and taxol treatment was inconsistently spread throughout the tissues. Though, staining was approximately equal in the cortex and medulla and there was little distinction between the proximal or distal tubules (Figures 7, 8; Tables 2, 3). In the kidneys, taxol staining was slightly greater than seen in the combined treatment. In the livers, cisplatin plus taxol combined treatment and individual taxol treatment also demonstrated a decline in lipase activity throughout the hepatocytes (Figures 8, 9; Tables 2, 3). However, combined cisplatin plus taxol staining was greater than taxol treatment alone which is opposite to that seen in the kidney tissues where taxol staining was slightly greater.

Comparison of Dehydrogenase Activity in Normal and Cisplatin Plus Calcium Treated Dog Kidneys:

Sections of normal kidney localized for MDH and G-6-PDH demonstrated moderate levels of blue granulation with diffuse staining throughout the cytoplasm of the

proximal and distal tubules. Staining was even and consistent throughout the cortex and medulla (Figure 10). In the cisplatin plus calcium treated tissues, the MDH intensity and localization was similar to normal tissues, though slightly depressed. The cytoplasmic staining of the tubules was even, precise, and comparable to the normal tissue dehydrogenase staining. No differences were noticeable in the proximal and distal tubules of the cortex and medulla. Further, enzyme intensity and localization was approximately the same for G-6-PDH as for MDH and the other dehydrogenases studied after cisplatin and cisplatin plus calcium treatments. (Figure 10).

Control Slides for Dehydrogenases and Nonspecific Lipases After the Various Treatments:

Control tissue sections, for all eight enzymes, demonstrated no appreciable staining (Figures 4, 8; Tables 2, 3).

Effects of Drug Treatments on Morphology in Rat Kidney and Liver Tissues and Dog Kidney Tissues:

Normal kidney and liver tissues, stained for all the dehydrogenases and nonspecific lipases studied, were morphologically more distinct due to improved clarity of tissue structure. Specifically, normal tissues showed cylindrical-shaped cell membranes and blood vessels as well as minimal intercellular tissue gaps (Figures 1-3, 7, 9, 10). However, cisplatin treated kidney and liver tissues demonstrated indistinct and amorphous cell membranes and increased intercellular tissue space (Figures 1-3, 7, 9, 10). Further, individual tubules in the kidneys, and hepatocytes in the livers, lacked delineation from adjacent tubules and hepatocytes resulting in histologically indistinct

tissue structures as if the structures had merged together. Tissues treated with cisplatin plus calcium demonstrated increased tissue organization and structure compared to the morphology of the cisplatin treated tissues (Figures 1-3, 7, 9, 10). Cisplatin plus calcium more approximated normal tissues which have clear boundaries between cells. Carboplatin treated tissues were morphologically similar to normal tissues as carboplatin treatment seemed to cause less morphological damage. Cisplatin plus taxol and taxol treated tissues showed tissue morphology more similar to normal than the cisplatin treated tissues (Figures 1-3, 7, 9, 10).

Optimal Enzyme Intensity and Localization at Variable pH Levels and Time Schedules:

pH levels ranging from 7.0 to 7.2 demonstrated optimal dehydrogenase staining over variable time schedules ranging from 20 minutes to 90 minutes (Figure 11). Intensity was optimal in conjunction with localization. At pH levels above 7.2, staining increased but was indiscriminate.

The average optimal incubation time for dehydrogenase staining was 45 minutes (Figure 11). Staining for less than 45 minutes demonstrated incomplete staining in most instances, however variability in incubation time occurred depending on the enzyme of study. Incubation for more 60 minutes or more was also unsatisfactory as staining became indiscriminate and occasionally excessive as the time increased.

A pH between 7.0 and 7.2 and an incubation time between 35 and 50 minutes was the optimal dehydrogenase localization protocol. However, variation in optimal enzymatic staining conditions occurred, but overall, staining anomalies, such as no

reaction or 'nothing dehydrogenase' formation were prevented and staining was optimal following the aforementioned optimal localization protocol (Figure 11).

Blood Collection and Analysis:

Serum calcium, blood urea nitrogen (BUN), and creatinine levels were maintained at high normal physiological levels after calcium supplementation in cisplatin treated dogs (Figure 12).

Figure 1. Light micrographs of cross-sections of rat kidney showing the localization of lactate dehydrogenase (LDH). (A) Normal histological section showing strong presence of LDH activity within the cytoplasm of the epithelium cells of the tubules (arrows). (B) Cross-section of kidney tubules from cisplatin treated rat showing a remarkable decline in LDH activity as compared to normal. Note the lack of staining in the glomeruli (g). (C) LDH staining intensity after CDDP plus calcium treatment, demonstrating increased enzyme activity, in the renal tubules, as compared to CDDP (arrow). (D) Cross-section of carboplatin treated kidney showing slightly elevated LDH tubule staining compared to cisplatin treated tissue though still less than normal. (E) Section of CDDP plus taxol treated kidney demonstrating decreased LDH activity, in the cytoplasm, compared to normal tissue, though greater than cisplatin treated kidneys. (F) Kidney tubules after taxol treatment. Note that staining is comparable to the CDDP plus taxol treated tissue section, though less than normal. Original magnifications: A-F: X 420. Bars = 40μm.

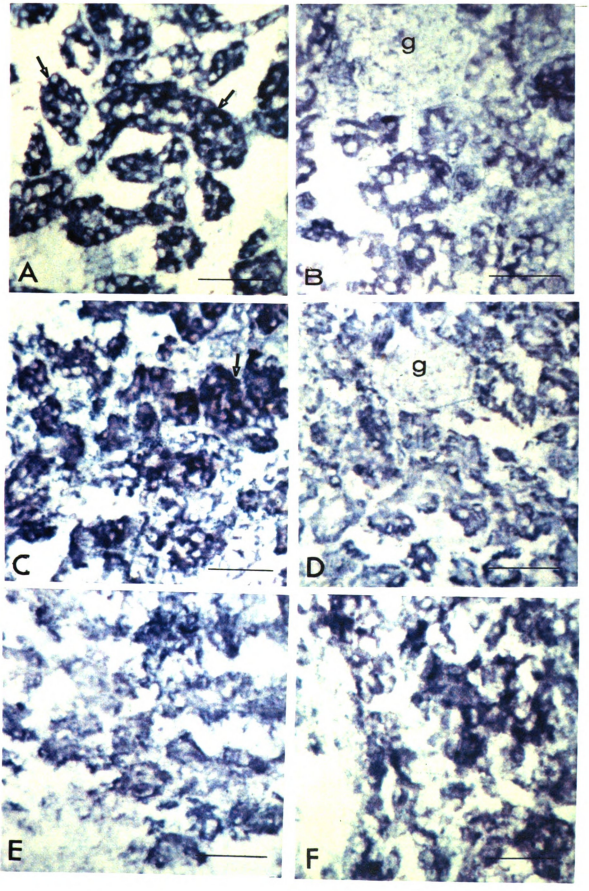
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Figure 2. Light photomicrographs from normal and treated rat kidneys showing localization of malate dehydrogenase (MDH). (A) Normal histological section showing intense MDH activity throughout the cytoplasm of the cells of the rat kidney tubules. (B) Cross-section of cisplatin treated kidney showing a significant decline in MDH activity throughout the tissue compared to normal. (C) Section of CDDP plus calcium treated kidney demonstrating cortical tubules with preserved MDH activity similar to that of normal, especially when compared to the cisplatin tissue section (arrow). (D) MDH staining after carboplatin treatment is decreased as compared to normal but slightly increased as compared to CDDP treatment. (E) Cross-section of CDDP plus taxol treated kidney approximates the LDH activity of normal kidneys. (F) MDH activity in taxol treated tissue section is comparable to normal. g, glomerulus. Original magnifications: A-F: X 420. Bars = 40μm.

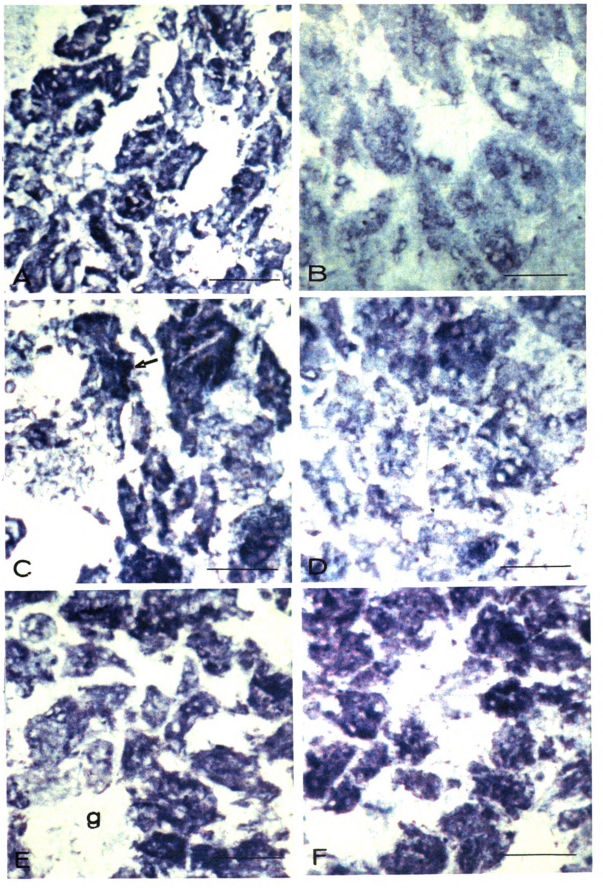
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Figure 3. Light micrographs of normal and treated rat kidneys showing localization of glucose-6-phosphate dehydrogenase (G-6-PDH). (A) Normal histological section showing G-6-PDH activity. (B) Cross-section of cisplatin treated kidney showing a significant increase in G-6-PDH activity, compared to normal, in the cytoplasm of the cells of the tubules (arrows). (C) G-6-PDH levels after CDDP plus calcium treatment demonstrating enzymatic activity approximating the normal tissues. (D) Section of carboplatin treated kidney showing a slight G-6-PDH increase from normal, though less than with CDDP treatment. (E) Section of CDDP plus taxol treated kidney showing LDH activity, in the tubules, comparable to normal. (F) G-6-PDH staining after taxol treatment demonstrating enzymatic activity similar to normal. g, glomerulus. Original magnifications: A-F: X 420. Bars = 40 μm.

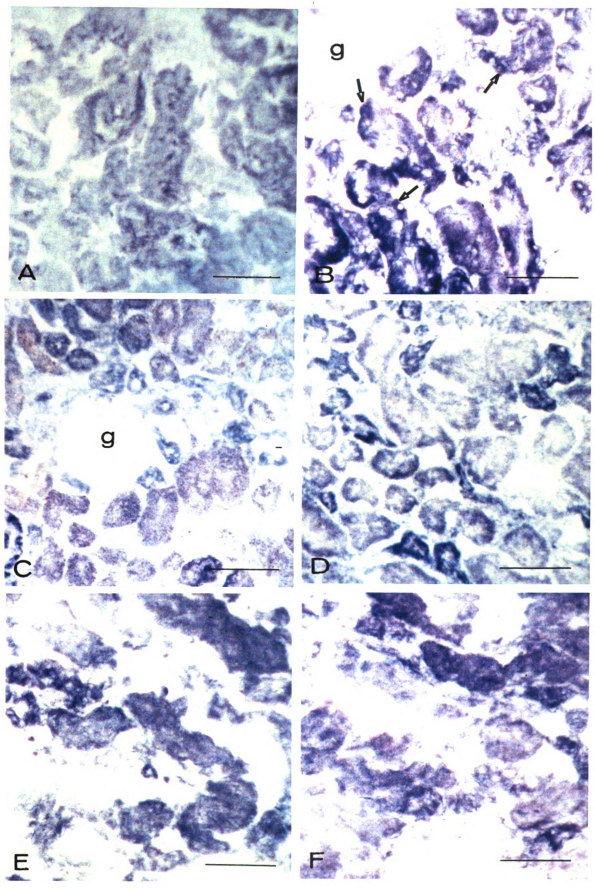
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Figure 4. Bar graph showing comparative enzymatic activity of MDH and G-6-PDH in normal and treated kidney and liver tissues. Note that MDH resembles SDH, GDH, HBDH, IDH, and LDH activity. Note that 100% staining is the baseline and represents normal tissue enzyme activity.

Comparative Dehydrogenase (MDH, SDH, GDH, HBDH, IDH, LDH, G-6-PDH) Activity in Normal and Treated Kidney and Liver Tissues

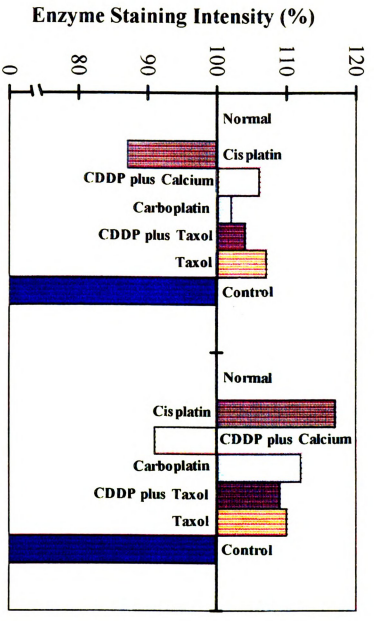

Figure 5. Light photomicrographs from normal and treated rat livers showing localization of malate dehydrogenase (MDH). (A) Normal liver section with hepatocytes with unstained nuclei. Enzymatic staining is predominantly in the cytoplasm surrounding the nuclei (arrow). (B) Cross-section after cisplatin treatment. Note the decline in enzymatic activity in the cytoplasm of the hepatocytes. (C) Cross-section of cisplatin plus calcium treated liver demonstrating MDH activity similar to normal tissues (arrows). (D) Section of carboplatin treated liver showing decreased enzymatic staining throughout the cytoplasm of the hepatocytes. Note that the enzymatic staining is greater than demonstrated with cisplatin treatment. (E) MDH activity after cisplatin plus taxol treatment demonstrating a slight decrease in enzymatic staining compared to normal and (F) section of taxol treated liver showing slightly less staining than normal. bv, blood vessel. Original magnifications: A-F: X 420. Bars = 40 μm.

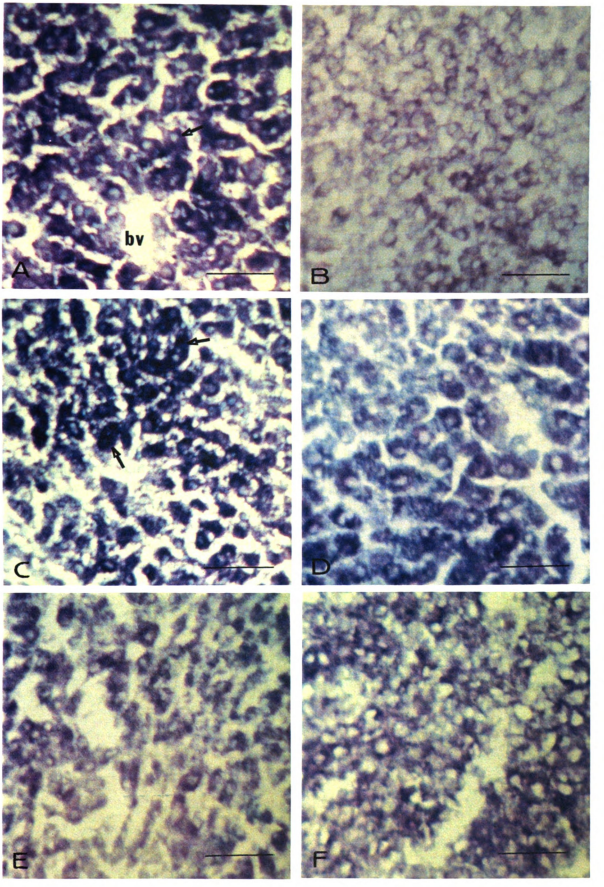
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Figure 6. Light micrographs from normal and treated rat livers showing localization of glucose-6-phosphate dehydrogenase (G-6-PDH). (A) Normal histological section showing G-6-PDH activity. (B) Cross-section of cisplatin treated liver showing a significant increase in G-6-PDH activity in the cytoplasm of hepatocytes (arrows) compared to normal. (C) G-6-PDH levels after CDDP plus calcium treatment demonstrating enzymatic activity approximating the normal tissue section. (D) Carboplatin treatment shows a slight G-6-PDH increase from normal, though less than the increase in CDDP treated tissue. (E) CDDP plus taxol treatment demonstrates enzymatic activity, in the hepatocytes, comparable to that of the normal tissues, though slightly less. (F) G-6-PDH activity after taxol treatment demonstrating G-6-PDH staining slightly less than normal. Original magnifications: A-F: X 420. Bars = 40 μm.

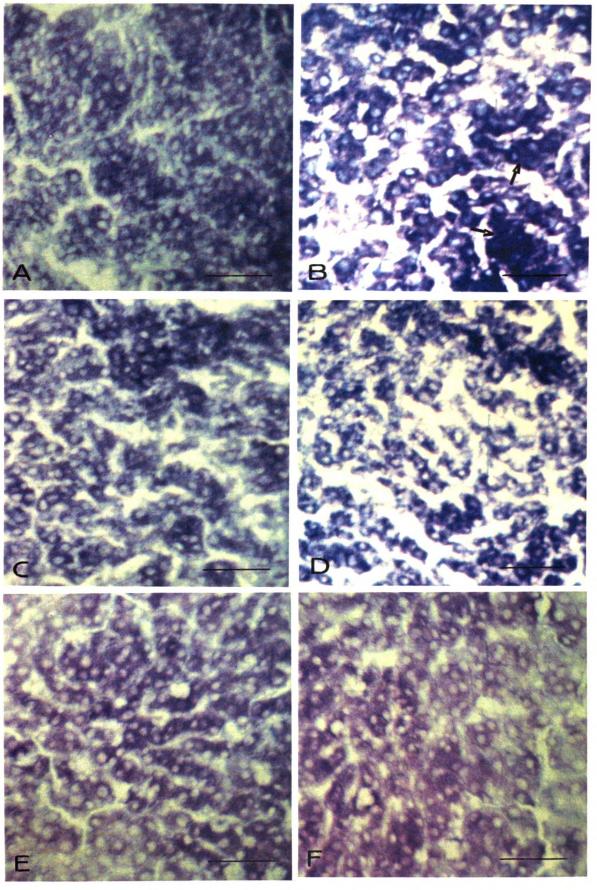
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Figure 7. Light micrographs of normal and treated animal kidneys showing localization of nonspecific lipase activity. (A) Lipase distribution in normal kidney tubule section and (B) cross-section after cisplatin treatment demonstrating a significant increase in lipase activity compared to normal (arrows). (C) CDDP plus calcium treatment showing a minimal decline in lipase localization, from that of normal and (D) carboplatin treatment demonstrating a slight decline in lipase activity from that of the normal kidney tubules. (E) Lipase reaction product after CDDP plus taxol treatment demonstrating a decrease in enzyme activity compared to normal. (F) Section of taxol treated kidney showing slightly more lipase activity than CDDP plus taxol treatment but less than normal. Original magnifications: A-F: X 420. Bars = 40 μm.

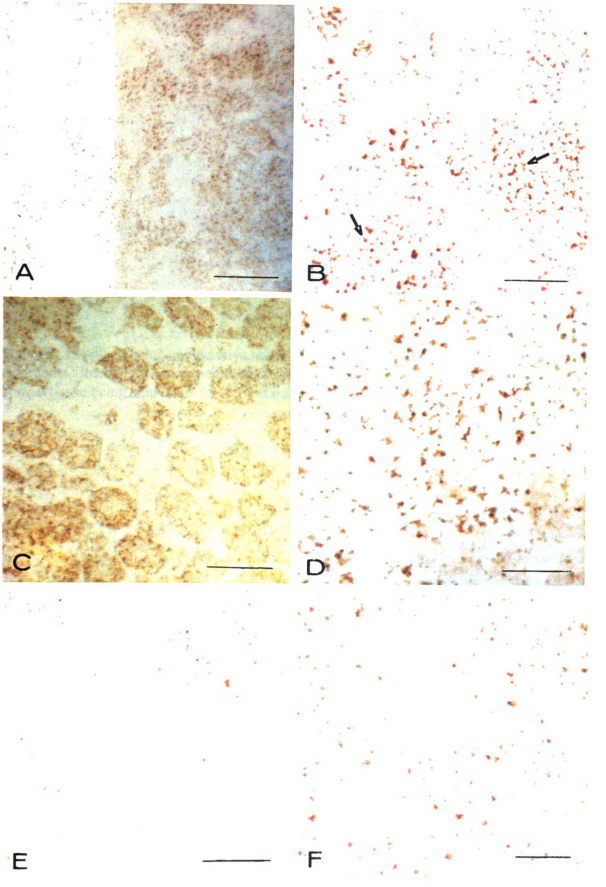
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Figure 8. Graph showing comparative nonspecific lipase activity for the various treatments. Intensity measurements are converted to percentages (%) and based on normal tissue being equivalent to 100% staining intensity.

Lipase Activity of Normal and Treated Kidney and Liver Tissues

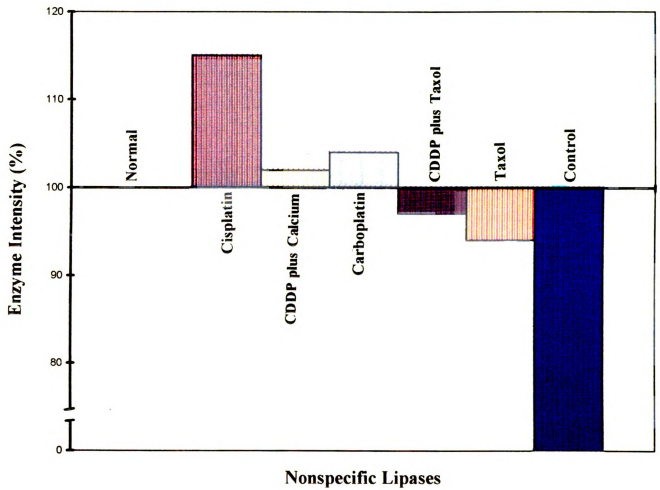

Figure 9. Light micrographs of normal and treated rat livers showing localization of nonspecific lipase activity. (A) Lipase distribution in normal liver hepatocytes and (B) cisplatin treatment demonstrating a significant increase in lipase activity (arrows). (C) CDDP plus calcium treatment showing a minimal decline in lipase localization compared to normal and (D) carboplatin treatment demonstrating a slight decline in lipase activity from that of the normal liver hepatocytes. Note the cylindrical-shaped blood vessel (bv). (E) Lipase reaction product after CDDP plus taxol treatment demonstrating a slight decrease in enzyme activity as compared to normal. (F) Cross-section of taxol treated liver showing less lipase activity than normal as well as less than in CDDP plus taxol liver hepatocytes. Original magnifications: A-F: X 420. Bars = 40 μm.

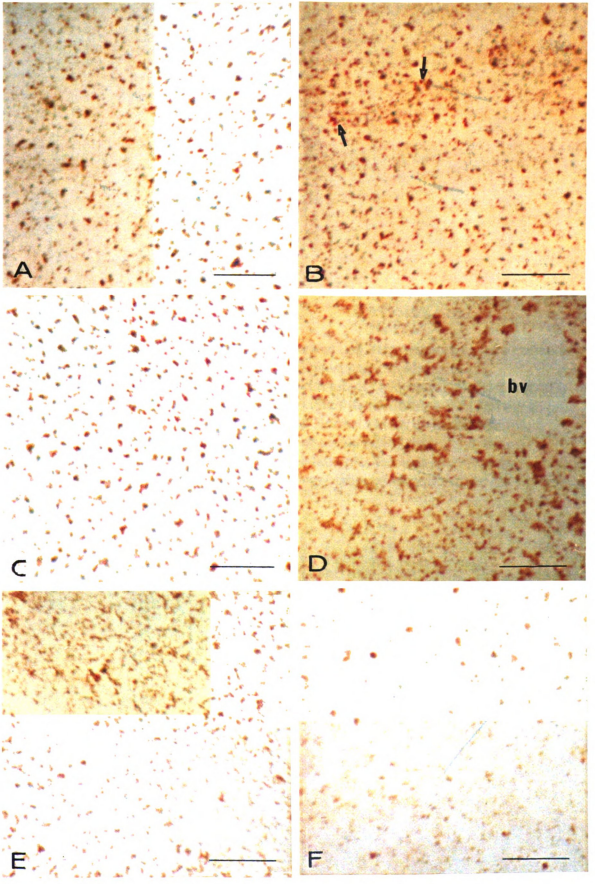
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Figure 10. Light micrographs of normal and treated dog kidneys showing localization of Isocitrate Dehydrogenase (IDH) and Glucose-6-phosphate dehydrogenase (G-6-PDH). (A) Normal kidney cross-section with tubules stained for IDH activity. (B) Cross-section of cisplatin plus calcium treated kidney demonstrating IDH activity slightly less than that of the normal tissue sections. (C) Normal kidney cross-section demonstrating G-6-PDH activity in cytoplasm of tubule cells. Note the unstained glomerulus (g). (D) G-6-PDH activity after cisplatin plus calcium treatment showing enzymatic levels similar to normal tissues (arrow). Original magnifications: A-D: X 260. Bars = 40 μm.

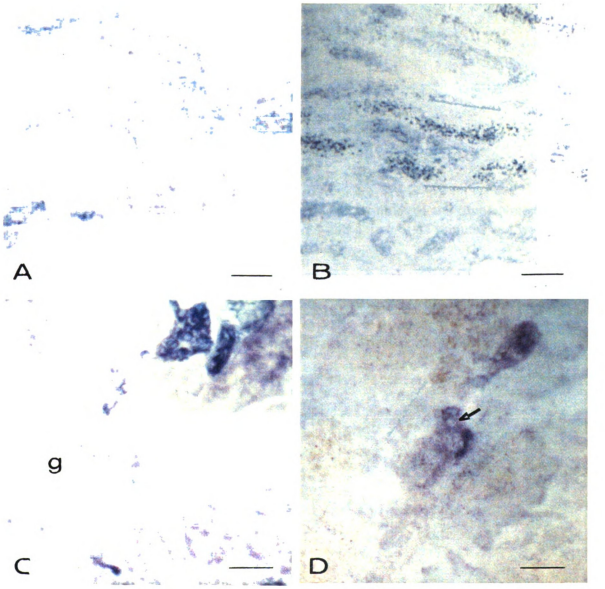
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Figure 11. Graph showing variable intensity levels at variable pH and time. Note that optimal staining is considered to be at a pH of 7.1 and a time of 45 minutes and was assigned a value representing 100% staining intensity.

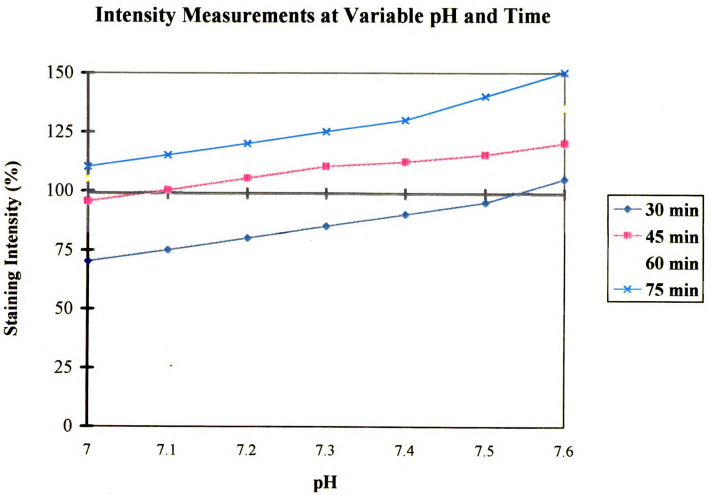
Intensity Measurements at Variable pH and Time
Staining Intensity (%)
150
125
100
75
50
25
0
7
7.1
7.2
7.3
7.4
7.5
7.6
pH
30 min
45 min
60 min
75 min

Figure 12. Graph showing ionized calcium levels during cisplatin treatment cycle (21 days) adjusted for a normal physiological pH of 7.4.

Ionized Calcium Levels during Cisplatin Treatment Cycle (21 days)
adjusted for a pH of 7.4

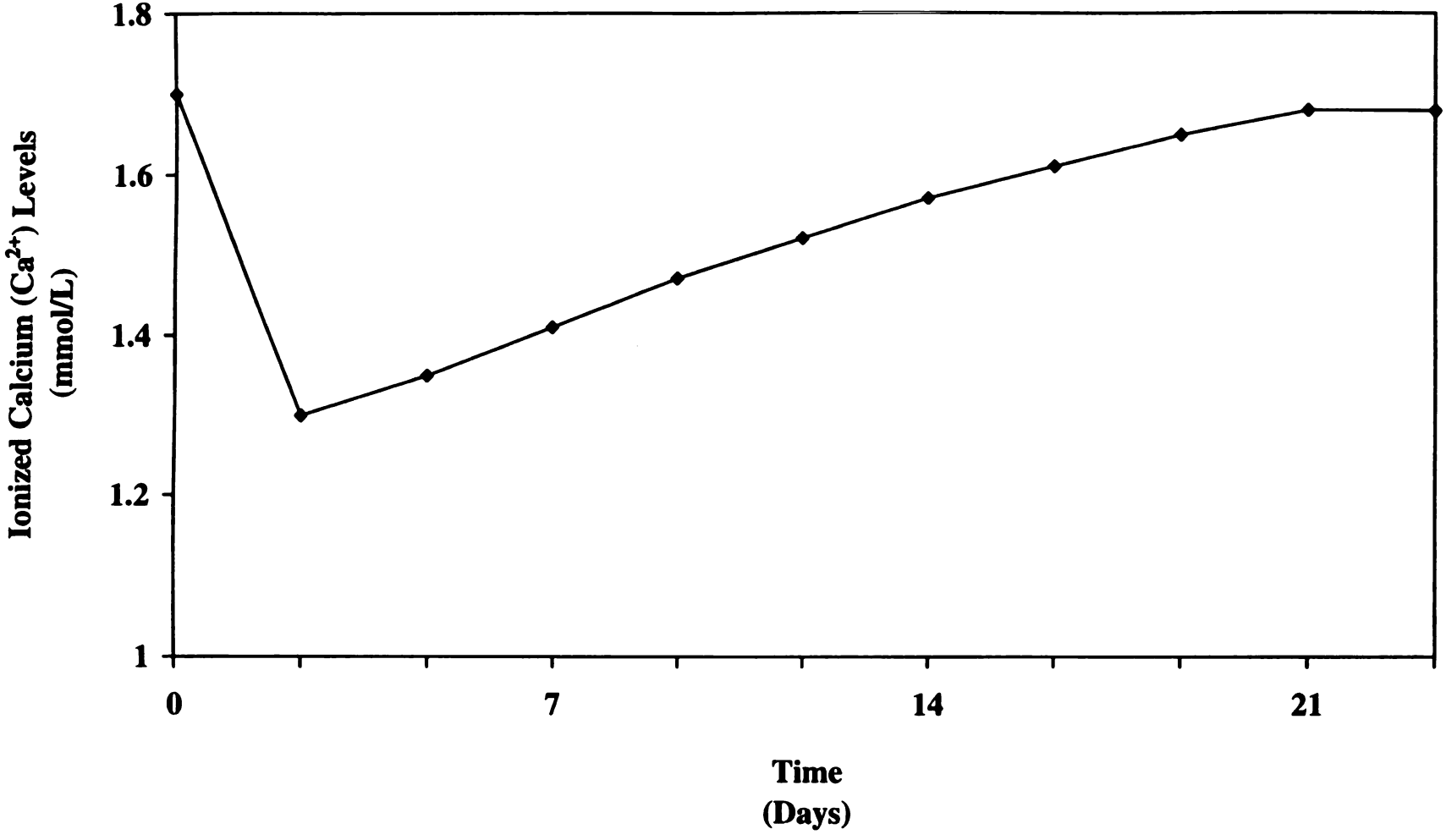

Table 2. Average enzyme histochemical staining intensity for normal, treated, and control tissues[a]

Treatment	SDH	GDH	HBDH	MDH	IDH	LDH	G6PDH	Lipase
Normal	++++	++++	++++	++++	++++	++++	+++	+++
Cisplatin	+	+	+	+	+	+	+++++	+++++
Cisplatin Plus Calcium	+++	+++	+++	+++	+++	+++	+++	++++
Carboplatin	++	++	++	++	++	++	++++	++++
Cisplatin Plus Taxol	+++	+++	+++	+++	+++	+++	++++	++
Taxol	+++	+++	+++	+++	+++	+++	++++	++
Control	-	-	-	-	-	-	-	-

[a] +++++, very intense reaction; ++++, intense reaction; +++, moderate reaction; ++, poor reaction; +, very poor reaction; -, no reaction.

Table 3. Gray scale values indicating average enzymatic staining intensity for normal, treated, and control tissues[a]

Treatment	MDH	G6PDH	Lipase
Normal	112.0 ± 6.0	105.0 ± 6.0	199.0 ± 7.0
Cisplatin	135.0 ± 7.0	94.0 ± 5.0	179.0 ± 9.0
Cisplatin Plus Calcium	95.0 ± 6.0	126.0 ± 7.0	202.0 ± 8.0
Carboplatin	110.0 ± 8.0	97.0 ± 5.0	189.0 ± 7.0
Cisplatin Plus Taxol	98.0 ± 6.0	99.0 ± 6.0	204.0 ± 7.0
Taxol	94.0 ± 5.0	97.0 ± 5.0	209.0 ± 6.0
Control	240.0 ± 7.0	240.0 ± 7.0	240.0 ± 7.0

[a] Gray scale values range from 0 - 256. 0 represents maximum staining intensity and 256 represents the minimum staining intensity. Note that MDH represents SDH, GDH, HBDH, IDH, and LDH activity. Values are means ± SEM.

DISCUSSION

Platinum, a heavy metal, is of great interest for its wide spectrum of antitumor activity (Rozencweig et al., 1977). Specifically, cisplatin, a platinum based compound, is one of the most successful and widely used anticancer agents. However, it is associated with many toxicities, the most severe being the nephrotoxicity (Leonard et al., 1971) as a result of 90% of the drug being removed by renal mechanisms (Casper et al., 1979; Jacobs et al., 1980). Cisplatin's mechanism of action is not fully elucidated, however, it is accepted that upon administration, cisplatin is absorbed by the blood supply and then by nature of its lipophilicity, it enters cells by passive diffusion (Powis and Hacker, 1991). Inside the cell, under low chloride ion concentration, cisplatin hydrolyzes to form aqua complexes (Jones et al., 1991; Mistry et al., 1989) which then migrate and bind to proteins and DNA, exerting their effects by preventing replication and transcription and repair mechanisms have been shown to reverse this process, giving additional support to this explanation (Andrews and Howell, 1990). However, this does not explain the dose-limiting nephrotoxicity associated with this anticancer agent. Presently, we have learned to combat the toxicities associated with cisplatin treatment, through diuresis, slow infusion rates, antioxidants and dose-scheduling but the cause of these toxicities is still

elusive and thus the absolute prevention of the toxicities remains impossible without further clarification.

Ade et al.(1996) have shown that organometals bind to a plasma membrane receptor causing nonselective ionic channels to open, resulting in calcium influx from extracellular stores. This extracellular calcium seems to be required for metal stimulation. This influx of calcium then activates phospholipase C through stimulation of a G protein. Concurrently, it is thought that this calcium influx also activates phospholipase A_2 which translocates from the cytoplasm to the membrane. This lipase activation results in extensive membrane breakdown and the generation of toxic metabolites, such as prostaglandins, through an increase in arachidonic acid levels as well as activation of kinases, proteases, and endonucleases. This cascade leads to altered membrane permeability, cell signaling, and cytoskeletal organization in addition to compromised mitochondrial function and DNA fragmentation that ultimately results in further activation of degradative processes, culminating in cell death (Orrenius and Nicotera, 1994). Thus, it is hypothesized, that cisplatin may act similarly by initially binding to the plasma membrane, causing a significant rise in cytosolic calcium levels that initiates cell cytotoxicity which is further exacerbated by cisplatin's diffusion inside the cell where the parent compound hydrolyzes to its toxic form. Ultimately, it seems that cisplatin's toxicities result, primarily, from its disruption of intracellular calcium homeostasis and from its ability to mimic calcium.

The initial event in cisplatin toxicity is likely the increase in intracellular calcium levels from extracellular stores. This rise in intracellular Ca^{2+} disrupts normal signal

transduction, inhibits response to hormones and growth factors, compromises mitochondrial function and cytoskeletal organization and activates degradative processes (Orrenius and Nicotera, 1994). However, it possible that some of these toxicities may be responsible for its antitumor activity. For instance, altered cytoskeletal organization results in depolymerization of microtubules and microfilaments and excessive nuclear calcium levels results in DNA fragmentation (Orrenius and Nicotera, 1994).

Similar to ionized lead (Pb^{2+}), it seems that cisplatin's hydrolysis to *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$ worsens the various cytotoxicities by inhibiting sulphydryl-containing enzymes, specifically mitochondrial dehydrogenases, by mimicry of calcium (Ca^{2+}). This ability of *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$ to mimic endogenous Ca^{2+} is probably the most important reason for the vast and severe toxicities associated with cisplatin treatment. Specifically, earlier studies have shown that cisplatin binds to Ca^{2+}-dependent sulphydryl groups on proteins and results in platinum's modification of protein chemical structure and function and a decline in glutathione (GSH) levels (Zhang and Lindup, 1994). As glutathione is depleted, the cell becomes more vulnerable to toxic compounds because mitochondrial GSH seems to be essential in the regulation of inner mitochondrial permeability and enzyme function by keeping sulphydryl groups (-SH) in the reduced state. When the SH-groups, of enzymes, are not maintained in a reduced form, they become inactivated (Bogin et al.. 1994). NADPH, which also helps to maintain reduced sulphydryl groups, also declines with CDDP treatment, so consequently, the depletion of glutathione (GSH) and NADPH seemingly results in the displacement of calcium, by hydrolyzed cisplatin, and the inhibition of dehydrogenase enzymes, essential rate-

controlling enzymes in the tricarboxylic acid cycle. As a consequence of this biochemical disruption of the TCA cycle, NADH stores are depleted and the uncoupling of oxidative phosphorylation and the electron transport chain results in hydroxyl radical formation and a consequent state of oxidative stress (Zhang and Lindup, 1993). These free radicals attack polyunsaturated lipids and proteins and initiate lipid peroxidation. This process becomes autocatalytic and causes severe damage to membrane integrity and progressive disorder (Tyler, 1992). This lipid peroxidation, in conjunction with inhibition of sulphydryl groups, leads to further loss of membrane integrity, potential, and permeability which further exacerbates the resultant toxicity of cisplatin therapy (Zhang and Lindup, 1994). For instance, Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+} ATPase are affected by inhibition of their essential sulphydryl groups (Orrenius et al., 1992) and this loss of mitochondrial function and metabolism, thus creating a state of anoxia and additional ionic imbalance (K+ efflux; Na+ and Cl- influx), cell swelling, and ultimately cell lysis (Batzer and Aggarwal, 1986). Further, this binding of heavy metals, such as platinum, to -SH groups also induces a rapid release of calcium from intracellular storage organelles (Abramson and Salama, 1989) and ATPase is unable to uptake calcium and restore equilibrium (Binet and Volfin, 1977) thus worsening the disruption of calcium homeostasis initiated by the earlier influx of extracellular calcium.

Concurrent with hydrolyzed cisplatin's binding to Ca^{2+}-dependent sulphydryl containing enzymes, is its competitive binding at calmodulin binding sites. This molecular mimicry activates the calmodulin complex in an unregulated manner (Jarve and Aggarwal, 1996) which may further inactivate Ca^{2+}-sensitive dehydrogenases by

acting in conjunction with the elevated cytoplasmic calcium, which itself acts as a second messenger. This induction of hypermetabolism is a cellular means of trying to compensate for increased energy needs as result of mitochondrial damage but this also leads to the additional activation of unregulated Ca^{2+}-dependent degradative enzymes such as phospholipases (Tyler, 1992) which in turn may alter cytoskeletal organization and membrane permeability. Alkaline phosphatase, Na+/K+-ATPase, and Ca^{2+}/Mg^{2+}-ATPase have been shown to be stripped from their membranes and released into the urine (Aggarwal, 1993) as an indirect result of cisplatin treatment, further altering ionic homeostasis. Bound dehydrogenases may also be stripped from their mitochondrial membrane by the hyperactivated lipases and nonmembrane bound, soluble dehydrogenases, may be lost by damage to the tubules which results in leaky epithelium allowing unregulated movement into and out of cells. Both events further impair ATP formation.

However, glucose-6-phosphate dehydrogenase is seemingly enhanced like lipase activity due to cisplatin treatment. It seems that elevation of glucose-6-phosphate dehydrogenase activity occurs because of its unique and critical function in NADPH generation and maintenance of reduced sulphydryl groups as part of the pentose phosphate pathway (Raven, 1994). Furthermore, if the pentose phosphate pathway is carried through to completion, there is the degradation of glucose to glyceraldehyde-3-phosphate, which can be further oxidized to produce ATP. Glucose-6-phosphate dehydrogenase catalyzes the conversion of β-D-glucose-6-phosphate to 6-phosphoglucono-1,5-lactone and NADP is reduced in the process (Raven, 1994). Thus,

glucose-6-phosphate dehydrogenase has a dual function in being the rate determining step in the pentose phosphate pathway. Its lack of essential sulphydryl groups seems to limit its own inhibition and allows for increased activity that may be a means for cells to adapt to the effects of cisplatin by inducing systems, such as glucose-6-phosphate dehydrogenase, that produce NADPH to compensate for the decrease of free sulphydryl groups (Bogin et al, 1994). This appears to be a cytoprotective mechanism against cisplatin toxicity.

The initial rise in intracellular calcium is transient but it dulls the cells responses to hormones and other trophic stimuli and eventually, cisplatin's binding to proteins and competition with calcium for calcium binding sites, induces persistent hypocalcemia (Brenner, 1996). Hypocalcemia continues the disruption of calcium homeostasis and thus, Ca^{2+}-dependent enzymes such as the dehydrogenases, are inhibited, enhancing the cisplatin-induced cytotoxicities. However, calcium supplementation seems to modulate cisplatin-induced toxicities. Modified membrane permeability and regulation, due to treatment, seems to allow exogenous calcium to enter the cells and reestablish calcium homeostasis by competing with hydrolyzed cisplatin for binding sites on Ca^{2+}-dependent enzymes, allowing for normal enzymatic activity and reduced organ toxicity. We have demonstrated this in the kidney where calcium supplementation maintained normal serum calcium levels as well normal blood urea nitrogen (BUN) and creatinine levels.

Carboplatin (CBDCA), an analogue of cisplatin, has been touted as an effective alternative to cisplatin treatment. Depending on the type of cancer, carboplatin has been shown to be equivalent or slightly superior to cisplatin in randomized trials (Alberts et al.,

1990). However, there are also many toxicities also associated with its use. Thrombocytopenia is dose-limiting but leukopenia and anemia also occur but to a lesser degree (Calvert et al., 1982). Interestingly, CBDCA nephrotoxicity is less severe and studies have shown that calcium levels are only rarely decreased with its use (Canetta, et al., 1985). In this study, dehydrogenase enzyme levels showed less decline in activity and tissue morphology showed less disruption than that demonstrated with cisplatin treatment, suggesting decreased nephrotoxicity associated with CBDCA. This seems to be a result of carboplatin's chemical structure and its consequent slow hydrolysis, which results in the need for clinical dosages 10 times that of cisplatin (Harstruck et al., 1989) for similar therapeutic success. Although lipase activity is still slightly activated by treatment, it is probable that carboplatin's slow hydrolysis limits its binding to dehydrogenase enzymes and allows endogenous protective mechanisms, such as GSH, to combat any potential toxicity associated with its use.

Another alternative to cisplatin anticancer treatment is cisplatin plus taxol combined therapy. Taxol (paclitaxel) is a diterpene plant product with no heavy metal constituents (Wani et al., 1971). Further, it is highly active in numerous preclinical tumor models (Rose et al., 1992). But again, taxol treatment is associated with a number of toxicities such as hypersensitivity (Weiss et al., 1990), alopecia (Wiernik et al., 1987) and emesis (Brown et al., 1991). Taxol's combination with cisplatin has proven extremely effective against breast cancer with success rates approaching 100% (Donehower and Rowinsky 1992) and nephrotoxicity that is limited. Dehydrogenase inhibition is minimal and lipase activity is approximately normal. There are four possible explanations for this

minimal nephrotoxicity. First, taxol binds extensively to proteins but lacks the structural design allowing it to mimic calcium and thus, its effects on dehydrogenases are limited. Second, taxol is eliminated by hepatic conversion, by the cytochrome P450 system, (Rowinsky and Donehower, 1991) which minimizes its nephrotoxicity due to limited renal exposure. Third, in combination therapy, the maximum tolerated dosage (MTD) of the two chemotherapeutic agents is approximately 50% of their individual dosages. Consequently, this decrease in cisplatin levels leads to less dehydrogenase inhibition, less mitochondrial disruption and thus, less overall cytotoxicity. Lastly, it has recently been shown that taxol and cisplatin combination therapy is schedule dependent. Addition or even synergism occurs when taxol is given approximately 24 hours prior to cisplatin treatment but when cisplatin is given prior to taxol or if they are administered at the same time, strong antagonistic interactions are observed (Vanhoefer et al., 1995). Consequently, all of these factors are probably responsible for the appreciable decline in nephrotoxicity; the most significant being the schedule dependency of combination therapy as in this study. Nonetheless, clinical success of cisplatin plus taxol combination therapy is a viable alternative to cisplatin treatment alone.

Hepatotoxicity from the various treatments, especially cisplatin, is minimal because of the liver's ability to maintain calcium homeostasis through sequestration of calcium in the sarcoplasmic reticulum and the abundant mitochondria in the liver. Also, the liver contains large glutathione (GSH) stores which can protect against hydrolyzed cisplatin (Bellomo et al., 1983). Further, accumulation of the compounds in the liver is less than that in the kidney (Wolf and Manaka, 1977). However, the slight toxicities

(enzyme and morphological alterations) that do occur are seemingly modulated by calcium supplementation.

In conclusion, from this study it seems clear that cisplatin's disruption of calcium homeostasis and metabolism to reactive metabolites, that mimic endogenous elements, initiates primary events such as lipid peroxidation, changes in thiol status and enzyme inhibition. These events damage the cells of the kidney tissues and secondary events occur. Such events include: changes in membrane structure, mitochondrial damage, inhibition of mitochondrial function, depletion of ATP and other cofactors, and further changes in Ca^{2+} levels. These result in final manifestations or tertiary events such as apoptosis and tissue necrosis (Timbrell, 1991). Simply-stated, cisplatin's disruption of calcium homeostasis and inactivation of dehydrogenase enzymes, in conjunction with lipase activity, results in the considerable decrease in dehydrogenase enzymatic activity which is directly responsible for ATP disruption and indirectly responsible for most of the associated toxicities that result from this disruption. Thus, it seems that elevated calcium levels, via calcium supplementation, may act as another means of cyto-protection, along with endogenous glucose-6-phosphate dehydrogenase, by competing for binding sites with hydrolyzed cisplatin and thus preventing cisplatin from initiating a cascade of cytotoxic events.

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