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Circadian Stage Dependence of *cis*-Diamminedichloroplatinum Lethal Toxicity in Rats[1]

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ABSTRACT

Renal physiology is circadian rhythmic. The major toxicity of *cis*-diamminedichloroplatinum (cisplatin) is irreversible renal damage. A single dose of cisplatin (11 mg/kg) was given to groups of standardized female Fischer 344 rats at one of six equispaced circadian stages. A statistically significant effect of time of injection upon tolerance was found by χ^2 analysis. Differences of 3- to 8-fold in survival rate at 50% mortality and a nearly 3-fold difference in long-term survival depended on circadian timing of cisplatin administration. Cisplatin timing resulting in optimal tolerance was similar from study to study.

Additional 0.9% NaCl solution was administered with cisplatin in three experiments and resulted in an increase in overall mean survival time. It also resulted in an amplification of the survival rhythm without changing its timing. The increase in survival resulting from 0.9% NaCl solution loading, when compared to controls receiving cisplatin alone, was also highly time dependent. A 52% improvement in mean survival time was achieved in those animals receiving cisplatin and 0.9% NaCl solution at the most favorable circadian stage, as compared to a 20% improvement when this regimen was administered at an inopportune circadian stage. The safest time for cisplatin administration is near the midactivity span, shortly after the maximum of the circadian rhythm in rectal temperature.

INTRODUCTION

The excretion of water and electrolytes, as well as the clearance of urea and creatinine from the blood, are highly rhythmic within the circadian time scale both in the rat and in human beings (1, 4, 10, 16, 17, 22, 24-26). Cisplatin[4] is an important anticancer agent with a dose-limiting side effect of kidney toxicity (3, 5). This toxicity involves predominantly the proximal tubule but also the distal and collecting tubules (7, 8, 18).

Nephrotoxicity resulting from the administration of cisplatin may be somewhat decreased by concomitant hydration (2). It has been thought that this reduction of cisplatin nephrotoxicity results from decreasing the concentration of cisplatin in the proximal and distal tubular urines (2, 8).

As a follow-up on earlier work (9, 11, 13), we studied cisplatin lethal toxicity in groups of rats by administering a single high dose of cisplatin with or without concomitant hydration at different circadian stages.

MATERIALS AND METHODS

Eleven studies involving the investigation of at least 6 groups of rats at 4-hr intervals during a 24-hr span were performed from February 1978 through April 1979 on a total of 1523 8-week-old 120- to 140-g female Fischer rats. The studies are lettered A through K, with a subscript s representing those in which rats also received i.p. 0.9% NaCl solution hydration concurrent with cisplatin administration (A, B_s, C, D, E, F, G, H_s, I, J, K_s). A 12th study was subsequently performed in October 1980 upon 21 female Lou/Ws1 rats (bred in our laboratories and originally supplied by Dr. Bazin, Louvain, Belgium) to extend some of the findings of Study J.

Chronobiological Standardization. Every animal in each study was singly housed and kept on a standardized lighting regimen consisting of 8 hr of light alternating with 16 hr of darkness for 2 to 3 weeks prior to cisplatin injection. Food and water were freely available to all animals in each study.

Drug Preparation. Cisplatin was supplied by the Investigational Drug Branch of the National Cancer Institute as the purified salt which was kept refrigerated. The reconstituted solution used for injection in all studies was identical to commercially available cisplatin. It was composed of 1 mg *cis*-diamminedichloroplatinum, 9 mg of sodium chloride, and 10 mg of mannitol per ml. The drug was prepared freshly for each injection time point.

Chronobiological Study Design. Animals stratified according to their location in the rhythmometry room were subsequently randomized to a minimum of 6 subgroups. Each subgroup was injected at a different circadian stage. Circadian stages selected for testing were separated by 4 hr. Each rat in each subgroup was weighed and its rectal temperature was measured before being given an i.p. injection of a single dose of 11 mg/kg of cisplatin per kg. Cages were inspected for dead animals twice a day initially and every 4 hr after deaths began occurring frequently.

Survival End Points. Survival was evaluated by (*a*) percentage of survivors within each subgroup when 50% overall mortality was reached in each study (*b*) mean survival time of all rats in each subgroup on Day 12 after drug injection; survivors on Day 12 were assigned 288 hr as survival time. (No further drug-related death occurred between 12 and 21 days after cisplatin administration.) Since Studies A and C were truncated at 50% overall mortality, only the first end point is available for analysis in these 2 studies and (c) modified Gehan-Wilcoxon survival analysis was also used to inspect survival as a function of treatment time.

0.9% NaCl Solution Loading. In order to investigate whether additional i.p. 0.9% NaCl solution could improve murine tolerance for cisplatin, each animal in Experiments B_s, H_s, and K_s received an additional i.p. load of 0.9% NaCl solution equal to 3% of its body weight immediately following cisplatin injection.

Lighting Schedule Reversal. Studies D, E, and F were performed concurrently, each in a separate rhythmometry room. The lighting regimens differed among these rooms to facilitate injections, and the animals were allowed a standardization span of 3 weeks.

Nephrotoxicity. Nephrotoxicity was assessed in 2 separate studies

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[4] The abbreviations used are: cisplatin, *cis*-diamminedichloroplatinum; BUN, blood urea nitrogen; HALO, hr after light onset.

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by a rise in BUN,[4] after cisplatin or 0.9% NaCl solution administration. In Study J, subgroups of rats which were given i.p. injections at each circadian stage of a single dose of 11 mg of cisplatin per kg were killed 4.5 days after the midpoint of the 24-hr cisplatin injection span. Their blood was collected; serum was analyzed for urea nitrogen content; and BUN was determined by the urease method.

In another study, 3 groups of 7 female rats, each of which had been standardized with regard to light and darkness, were injected with either 0.9% NaCl solution or 11 mg of cisplatin per kg at either 7 or 19 HALO. Three to 3.5 days after injection, these animals were killed at a single circadian stage (7 HALO). Blood was obtained and serum was analyzed for urea nitrogen content.

Statistical Analysis. For each study, the proportion of survivors in each subgroup was compared by χ^2 analysis when 50% of the animals in that study were dead. A peak test (23) was performed in order to validate any apparent peak in the data when a statistically significant time effect was demonstrated by χ^2 analysis. Survival times were analyzed by analysis of variance. Modified Gehan Wilcoxon survival analysis was used to compare survival of rats injected at one of each of the 6 test times.

Additionally, in each study, the presence of circadian rhythmicity was assessed for rectal temperature, the percentage of survivors when 50% overall mortality had been reached, and the survival times by the single and population mean cosinor methods (10, 12, 14, 15).

Time References. All times are expressed in relation to lighting onset and not clock hours. HALO stands for time in hours after lighting onset.

RESULTS

Marker Rhythmometry. Table 1 summarizes the circadian rhythm characteristics of rectal temperature in each of the experimental groups. A statistically highly significant circadian rhythm in rectal temperature was demonstrated in each study. The timing of high values in rectal temperature was stable and occurred between 15 and 18 HALO. The synchronization of the circadian rhythm in rectal temperature by the lighting regimen is evident in the similar timing of temperature high point (in relation to light onset) for Studies D, E, and F, in which lights were on from 5 a.m. to 1 p.m., 9 a.m. to 5 p.m., and 7 p.m. to 3 a.m., respectively. A statistically significant mean circadian rhythm also was demonstrated by population mean cosinor analysis. Its high point occurs slightly after the middle of the dark span. This corresponds approximately to the middle of the activity span of these nocturnally active mammals. The full extent of predictable variation within each 24-hr span is 1.2°.

Survival Irrespective of Cisplatin Timing. Fifty % overall mortality in each study was reached within 4 to 6 days if no concomitant hydration was given with cisplatin. In the 3 studies in which hydration was given, this end point was reached within 6 to 10 days.

The mean survival time of all rats receiving 11 mg of cisplatin per kg with or without hydration was 160 ± (S.E.) 2 hr. The mean survival time for those receiving no hydration was 152 ± 2 hr as compared with 205 ± 4 hr if hydration was given. This difference of 53 hr (~30%) was statistically significant ($p < 0.001$).

Role of Cisplatin Timing upon Survival Rate at 50% Overall Mortality. In each of these 11 studies, χ^2 analysis revealed statistically significant differences in survival between subgroups injected at different circadian stages (Table 2). Chart 1 depicts survivorship for these 11 studies according to the circadian stage of cisplatin injection. The mean survival rate varied between 35 and 65%, depending on when the animals received the fixed dose of cisplatin. If the results of each study are considered separately, the difference in percentage of survival as a function of injection time was as great as 8-fold in some studies.

Role of Cisplatin Timing upon Survival Time. Irrespective of hydration protocol, the mean survival time of rats also exhibited a marked dependence upon the circadian stage of cisplatin administration ($F = 6.9$, $p < 0.0001$). The longest survival occurred in those rats receiving cisplatin about 17 HALO, *i.e.*, in mid to late activity (Table 3). A similar optimal timing for cisplatin was found relative to light onset in those 3 studies (D, E, and F) performed in August, when rats were standardized on different lighting schedules. At Day 12, after animals had ceased dying of drug toxicity, a > 2-fold difference in survival was found, depending upon injection time (40% *versus* 14% survival) (Chart 2). Modified Gehan-Wilcoxon survival analysis found these differences to be statistically significant ($p < 0.001$).

Combined Role of 0.9% NaCl Solution Load and Cisplatin Timing upon Mean Survival. The longest survival times in those rats receiving 0.9% NaCl solution, as well as in those which did not receive any additional fluid, were achieved when

Table 1

Circadian rhythm in rectal temperature

Rectal temperatures measured in each rat before drug injection at one of 6 circadian stages in 10 studies show well-synchronized circadian rhythms in this variable. The mean circadian rhythm is also statistically significant. The time of highest values (acrophase) occurs near 16.45 HALO, and the extent of predictable daily variation (double amplitude) is 1.2°.

Study	*p*	% of rhythm	No. of rats	Mesor[a]	Amplitude	Acrophase (HALO)
A	0.001	64	110	37.3 ± 0.04°[b]	0.76 ± 0.05°	17.40 (17.08, 18.16)[c]
B_s	0.001	42	120	37.1 ± 0.04	0.66 ± 0.06	16.04 (15.20, 16.45)
D	0.001	45	99	37.6 ± 0.06	0.70 ± 0.08	18.45 (17.36, 19.20)
E	0.001	55	115	37.8 ± 0.05	0.73 ± 0.06	16.30 (15.36, 17.00)
F	0.001	54	115	37.7 ± 0.04	0.58 ± 0.05	16.43 (15.48, 17.04)
G	0.001	51	113	37.5 ± 0.04	0.56 ± 0.05	14.52 (13.59, 15.47)
H_s	0.001	42	110	37.6 ± 0.03	0.42 ± 0.05	15.35 (14.36, 16.43)
I	0.001	59	150	37.2 ± 0.03	0.71 ± 0.05	17.16 (16.36, 17.56)
J	0.001	37	109	37.2 ± 0.05	0.58 ± 0.07	16.44 (15.48, 17.40)
K_s	0.001	29	107	37.3 ± 0.06	0.51 ± 0.08	16.53 (15.24, 17.40)
Mean	0.001	49	1148[d]	37.4 ± 0.02	0.6 ± 0.10	16.44 (15.36, 17.40)

[a] Rhythm-adjusted 24-hr mean.
[b] Mean ± S.E.
[c] 95% confidence limits.
[d] Number of studies.

Table 2

Murine chronotolerance for 11 mg cisplatin per kg as gauged by percentage of survivors at 50% overall mortality

Percentage of survival at each of 6 circadian stages was analyzed for each of the 11 studies when 50% mortality was reached in that study. χ^2 analysis was used to test the data for the effect of injection time upon survival.

		% of survival							
Date	Study	1[a] hr	5 hr	9 hr	13 hr	17 hr	21 hr	χ^2	p
2/25/78	A	43.8	40.0	61.1	85.0	11.1	50.0	13.84	<0.05
3/23/78	B_s	61.1	50.0	35.3	23.5	66.6	66.6	11.09	≈0.05
5/18/78	C	87.5	37.5	16.7	50.0	70.8	45.8	29.91	<0.001
8/17/78	D	52.2	29.2	43.4	62.7	65.2	26.1	29.13	<0.001
	E	47.4	21.7	47.8	65.2	65.2	52.2	23.09	<0.001
	F	41.7	21.8	16.7	60.0	54.1	58.3	16.28	<0.025
11/03/78	G	30.0	66.7	11.1	30.0	77.8	66.7	15.537	<0.01
	H_s	52.6	57.9	31.6	31.6	84.2	47.4	14.601	<0.025
3/08/79	I	52.0	52.0	72.0	32.0	36.6	56.0	10.48	≈0.06
4/01/79	J	58.8	29.4	31.2	33.3	93.7	55.5	18.2	<0.001
	K_s	33.3	37.5	38.9	50.0	83.3	72.2	15.4	<0.001

[a] Time of cis-platin (HALO) (±1 hr). Rats standardized in light for 8 hr and dark for 16 hr.

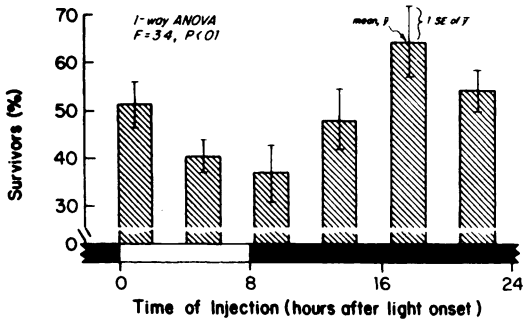

Chart 1. Percentage of survival (at 50% overall mortality) in 11 studies involving 1523 rats treated i.p. with 11 mg of cisplatin per kg at one of 6 circadian times. *ANOVA*, analysis of variance.

Table 3

Survival time after a single i.p. administration of 11 mg cisplatin per kg at one of 6 circadian stages

Data from 1275 rats in the 9 studies evaluable for this end point. Since no deaths occurred after Day 12 in any study, survivors were assigned a survival time of 288 hr.

Time of cis-platin (HALO)	Survival time[a] (hr)
1	155 ± 5[b]
5	151 ± 6
9	149 ± 6
13	152 ± 4
17	186 ± 6
21	168 ± 6

[a] Results from one-way analysis of variance: $F = 6.9$, $df = 5,1269$, $p < 0.0001$.
[b] Mean ± S.E.

cisplatin was given at 17 HALO (Chart 3). A statistically significant effect of both cisplatin timing and 0.9% NaCl solution load was demonstrated by a 2-way analysis of variance (for time effect, $F = 7.8$, $p < 0.001$; for hydration effect, $F = 6.5$, $p < 0.001$). An interaction between the effect of cisplatin timing and that of 0.9% NaCl solution load was also demonstrated ($F = 2.6$, $p < 0.03$).

The improvement in mean survival time which results from the administration of concurrent 0.9% NaCl solution was highly dependent upon treatment timing. Mean survival time was improved by 20% (from 140 ± 8 to 171 ± 1 hr) if the combined treatment was given near 9 HALO (early activity) and by 52% (from 162 ± 6 to 253 ± 7 hr) if this same treatment was given at 17 HALO (mid to late activity) (Chart 3).

Circadian Rhythm Analysis. Single cosinor analysis was

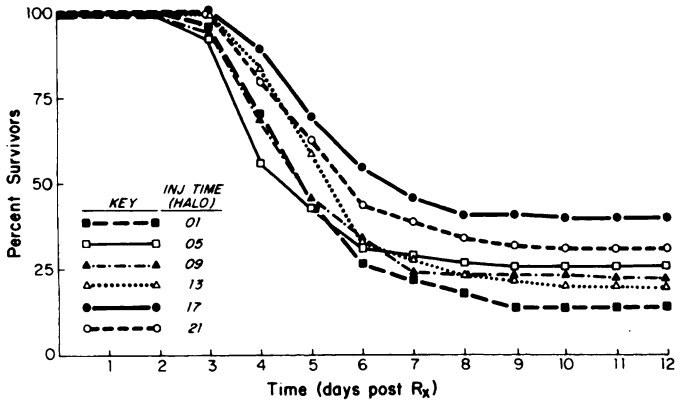

Chart 2. Percentage of survival over the 12-day span during which rats were at risk for death from 11 mg of cisplatin per kg. Each *line* represents survival of animals treated at a single circadian stage. *INJ.*, injection, R_x, drug.

used to test for and quantify circadian variation in susceptibility to cisplatin. Such a rhythm was demonstrable with either the percentage of survivors (when 50% of the animals in each study were dead) or the mean survival time analyzed as a function of injection time (Table 4). The amplitudes of the rhythms in these 2 end points are substantial, and their timing is similar.

Nephrotoxicity. Table 5 describes the rise in BUN in Profile J. The BUN rose from a mean of 19 ± 1 mg/dl in controls to a mean of 306 ± 12 mg/dl in cisplatin-injected rats. Cisplatin injection at midrest, 4 HALO, resulted in a BUN of 385 ± 26 mg/dl, while injection at mid to late activity resulted in BUN values of 242 ± 25 mg/dl. An effect of injection time upon rise in BUN was documented by a one-way analysis of variance ($F = 3.6$, $df = 5,92$, $p < 0.005$).

In the study in which cisplatin toxicity was assessed after administration at one of 2 circadian stages, the BUN was 17 ± 1 mg/dl in control rats sampled at 7 HALO. At the same circadian stage, the BUN was 63 ± 3 mg/dl in rats treated with cisplatin at 19 HALO and 122 ± 3 mg/dl in rats treated at 7 HALO. This difference in BUN as a function of cisplatin timing was statistically significant ($t = 14.1$, $p < 0.001$) (Chart 4).

DISCUSSION

Circadian rhythms have been demonstrated in every carefully measured renal function. The ability of the kidney to concen-

trate and excrete hydrogen ions and trace metals and to clear the blood of urea, creatinine, and inulin are highly circadian stage dependent (1, 4, 10, 22, 26). Circadian rhythms have also been well documented for renal cytokinetics (6, 16). We have shown recently that the urinary activity of a key proximal tubular enzyme, β-*N*-acetylglucosaminidase, participates in a circadian rhythm with high amplitude (19), and others have shown that this is true for many other renal tubular enzymes (20, 21).

In the studies reported here, a clear-cut time effect was present with regard to the lethality of cisplatin, whether or not concurrent hydration was used to minimize cisplatin toxicity.

When additional 0.9% NaCl solution was administered with cisplatin, survival as measured by all mortality-related parameters was increased. The fluid-induced increase in survival, however, was highly dependent upon the circadian stage of administration of the 0.9% NaCl solution ($p < 0.001$). These data indicate that the value of hydration-based renal "rescues" may well be circadian stage dependent. They further suggest that forced diuresis cannot overcome the rhythms which are responsible for the observed circadian rhythm in cisplatin nephrotoxicity and lethality.

The nephrotoxicity of a lower dose of cisplatin has been documented elsewhere (19). In Study J, surviving rats were killed when 50% overall mortality had been reached, and the

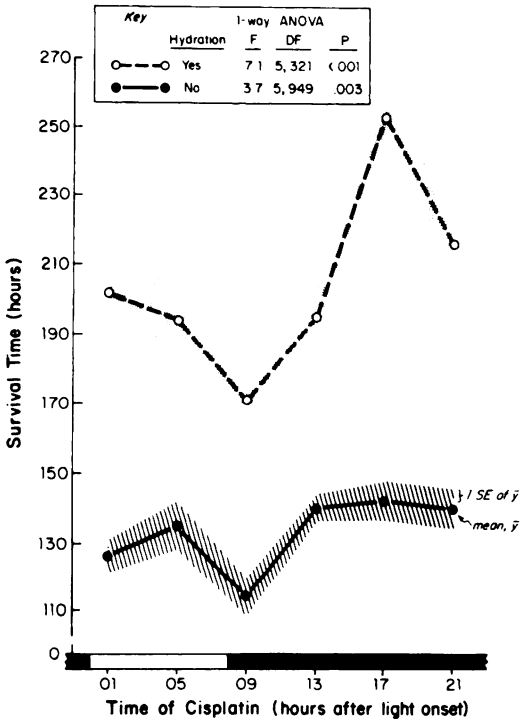

Chart 3. Survival times after cisplatin. *Top curve*, survival times, for 3 experiments in which additional 0.9% NaCl solution load (3% body weight) was given with 11 mg cisplatin per kg. *Bottom curve*, survival times for 8 studies in which cisplatin (11 mg/kg) was given without additional hydration. *ANOVA*, analysis of variance.

Table 5

BUN 4.5 days after a single i.p. administration of 11 mg cisplatin per kg at one of 6 circadian stages

Data from rats in Study J, each of which received the same dose of cisplatin at one of 6 circadian stages. In animals which received no cisplatin, the BUN was 19 ± 1 mg/dl.

Time of cisplatin (HALO)	BUN[a] (mg/dl)
1	327 ± 36[b]
5	385 ± 26
9	337 ± 25
13	245 ± 38
17	242 ± 25
21	308 ± 29

[a] Results from one-way analysis of variance: $F = 3.6$, $df = 5,92$, $p < 0.005$.
[b] Mean ± S.E.

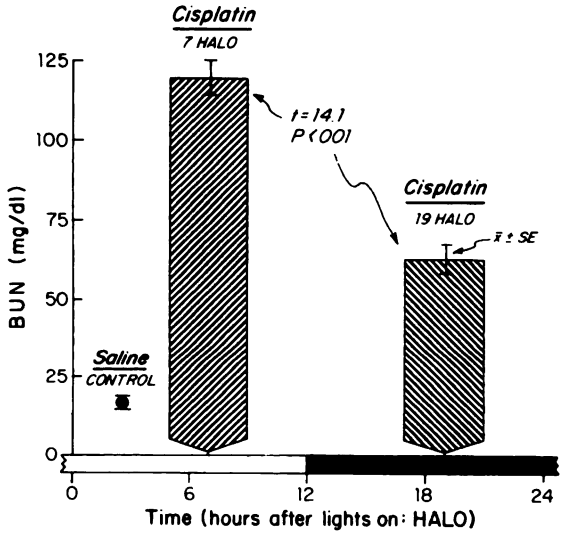

Chart 4. BUN obtained at a single circadian stage (7 HALO) approximately 3 days after administration of 0.9% NaCl solution or 11 mg of cisplatin per kg at either 7 or 19 HALO.

Table 4

Results from single cosinor analysis of survival rate (at 50% overall mortality) and of survival times

p value from an *F* test of the zero-amplitude hypothesis. Circadian rhythm characteristics include the mesor (rhythm-adjusted 24-hr mean), the double amplitude (total extent of variability attributable to the rhythm), and the acrophase (timing of the maximum of the rhythm). Cosinor analysis (14) was used to test whether or not the time effect, demonstrated by χ^2 analysis for survival rate and by analysis of variance for mean survival time, was circadian rhythmic and to estimate parameters of this rhythm.

End point	Hydration	No. of rats	*p*	Mesor	Double amplitude	Acrophase (HALO)
				%		
Survival rate at 50% overall mortality	No	1197	0.06	48 ± 3[a]	10	18.50
	Yes	326	<0.01	51 ± 3	34 (8, 60)[b]	19.50 (16.40, 23.10)[b]
	Pool	1523	<0.002	49 ± 2	24 (8, 38)	19.10 (16.20, 22.00)
				hr		
Survival time	No	949	<0.005	144 ± 2	22 (15, 30)	18.00 (15.50, 20.00)
	Yes	326	<0.001	205 ± 4	64 (52, 76)	18.50 (17.20, 20.00)
	Pool	1275	<0.001	159 ± 2	32 (26, 38)	18.40 (17.20, 20.00)

[a] Mean ± S.E.
[b] 95% confidence limits.

BUN was determined in their sera. It was found that the BUN rise, resulting from cisplatin, was circadian stage dependent.

The circadian stage dependence of nephrotoxicity is demonstrated clearly in Chart 4 and again in Table 5. BUN values obtained from the rats at either Day 3 (several days prior to any cisplatin mortality) or Day 4.5 (when rats had begun to die rapidly) show that differential nephrotoxicity may result from drug timing. We have shown elsewhere that the nonlethal nephrotoxicity of cisplatin at therapeutic dosage is highly circadian stage dependent (13, 19).

When the study design stipulates multiple injection times and a single killing time for BUN sampling, there must necessarily be an inconstant interval between injection and killing. The interval in the smaller study varied between 72 and 84 hr (maximum, 12 hr), while in Study J the interval varied between 92 and 114 hr (maximum, 22 hr). Results of studies by Kociba and Sleight (18), who followed BUN values serially after a single 12-mg/kg injection of cisplatin, suggest that this 22-hr interval could not alone be responsible for the degree of difference in BUN across groups (Table 5). It is also clear that the pattern of BUN rise and fall across groups could not result from a progressively lengthening interval between injection and killing.

In each study and by every end point studied, the most favorable time for cisplatin administration is during the activity span of the animals. In 2 of the 11 studies, however, the optimal time for tolerance differed from that in the other 9 studies. The results of these 2 studies are consistent with one another. Both of them were performed during late February and early March, 1 year apart. This finding suggests a circannual (about yearly) effect upon the circadian rhythm in cisplatin lethal toxicity.

Since the circadian timing of rectal temperature in these 2 studies did not differ from those in studies performed at all other circannual stages, the circadian rhythm in murine cisplatin lethality apparently does not exhibit a constant phase relationship with the circadian rhythm in rectal temperature throughout the year. This possibility has led us to search for a marker rhythm with a more consistent phase relationship to cisplatin toxicity rhythms. Candidate marker rhythms include urinary volume, urinary electrolytes, and urinary β-*N*-acetylglucosaminidase activity.

In summary, cisplatin lethal toxicity as measured by survival rate, survival time, and the Krushal-Wallis *K* sample modification of the Gehan Wilcoxon survival analysis can be substantially reduced by administration of this drug in the mid- to late-activity span. The additional concurrent administration of 0.9% NaCl solution is most effective in decreasing cisplatin toxicity if the fluid is given at this same optimal circadian stage, whereas the 0.9% NaCl solution load results in statistically significantly less benefit if used at the circadian stage associated with lower cisplatin tolerance. Nephrotoxicity resulting from this high dose (11 mg/kg), as well as from a totally nonlethal therapeutic dose (5 mg/kg) of cisplatin, follows precisely the same pattern of time dependence (19). There may be an effect of season upon the circadian rhythm in cisplatin-induced lethality.

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