Cancer Chemother Pharmacol (1998) 42: 135–141 © Springer-Verlag 1998

ORIGINAL ARTICLE

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Time dependency of hematopoietic growth factor coupled to chronotoxicity of carboplatin

Received: 4 March 1997 / Accepted: 16 November 1997

Abstract A growing body of data suggests that cancer therapy may be improved and toxicity reduced by administration of antineoplastic agents and cytokines at carefully selected times of the day. The time-dependent effects of each of the drugs have been documented, but not their mutual time dependencies. In the present studies we sought to determine the best time for granulocyte colony-stimulating factor (G-CSF) administration after carboplatin treatment. Carboplatin was injected in different groups of ICR mice at four different circadian stages for 5 consecutive days. Mice were synchronized with an alternation of 12 h of light (from 6:00 a.m. to 6:00 p.m.) and 12 h of darkness. After the last injection, peripheral WBCs of three mice from each group were counted every 4 h over a 24-h period. Bone marrow toxicity was estimated with the mean 24-h WBC count. The most severe leukopenia occurred in the group injected at 3:00 p.m. – 9 h after light onset. The second set of experiments evaluated the time-dependent effect of G-CSF when singly injected or given after carboplatin injections for 5 days only at 3:00 p.m. G-CSF was injected into various groups on days 8 and 9 at the same four different circadian stages. On the 10th day after the first injection, peripheral WBCs of three mice from each group were counted every 4 h over a 24-h period. Time-dependent effects were observed when G-CSF was injected as a single agent. When G-CSF was given at various times to the group with the most severe carboplatin-induced leukopenia, peripheral WBC count recovery was monitored at all injection times; it reached its highest level (exceeding even that of the control) when G-CSF was injected at 3:00 a.m. Dosing times of both chemotherapy and growth factor are relevant for optimization of carboplatin's hematologic tolerability.

Key words Carboplatin · G-CSF · Chronotoxicity

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Introduction

A growing body of data suggests that therapeutic effects may be maximized and toxicity may be minimized if drugs are given at carefully selected times of the day [24]. This potential for a marked improvement in the therapeutic index is especially critical for therapies with narrow efficacy-to-toxicity ratios, as is the case for most anticancer treatment regimens [7–9]. Time-dependent toxicity of at least 20 chemotherapeutic agents has been documented in many rodent and some human experimental systems [4, 10, 14, 16, 26, 27]. Time-dependent efficacy of many of these agents, given either singly or in combinations, has been demonstrated in several neoplastic diseases [15, 17, 25].

The platinum complexes are among the most active drugs against a large spectrum of malignancies. However, these anticancer drugs bear serious side effects such as gastrointestinal, neural, and renal toxicities as well as myelosuppression. Carboplatin has proved to be one of the most clinically useful drugs, although myelosuppression, especially thrombocytopenia, is dose-limiting [1]. Experimental data show that the dosing times of carboplatin markedly affect its toxicity and may affect its therapeutic efficacy [2, 3]. The circadian mechanism relevant to platinum-complex treatment of cancer include 24-h changes in the pharmacokinetics in blood and urine and, presumably, in the proliferative activity of bone marrow [2, 18, 28].

The clinical uses for recombinant growth factors are expanding rapidly and impacting both the quality and the cost of health care. The timing of hematopoietic growth-factor therapy with respect to chemotherapy and other cytokines is an important element. In addition, many components of the erythron and myeleron systems

are organized on a 24-h (circadian) time scale in experimental animals and humans.

Circadian-dependent differences have been documented in the concentration of nearly all cellular blood components, including components of the erythroid and myeloid systems (total WBC and neutrophil numbers) [6, 20]. Since hematopoietic cytokines work in part by increasing the number of progenitor cells in the S (DNA synthesis) phase, the concurrent use of hematopoietic growth factors with cytotoxic chemotherapy may result in worsening of the extent and duration of myelotoxicity [31]. The timing of cytokine administration during and around other therapies is important for the achievement of effective cytokine therapy and may even influence the nature of the response (stimulatory or inhibitory).

Circadian timing, i.e., the administration of hematopoietic growth factors at specific times throughout a 24-h period, may also afford certain advantages. The components of the hematopoietic system in animals and humans are coordinated within a 24-h time scale [12, 30, 32]. The growth factor G-CSF (granulocyte colony-stimulating factor) more effectively raises blood neutrophil counts in normal mice when given in a late activity phase. Like the distinct regulation and divergent circadian coordination of erythropoiesis and myelopoiesis, erythropoietin and G-CSF exhibit unique optimal circadian times for administration [13, 23, 32].

On the basis of these data, the present study, designed for animal model systems, aimed to assess the optimal daily time for carboplatin administration with respect to minimal bone marrow depletion as evaluated by peripheral WBC counts and to suggest the optimal circadian time for G-CSF administration following carboplatin treatment with regard to the activation of bone marrow and the enhancement of WBC counts in the peripheral blood network.

Materials and methods

Mice

Mature 4- to 5-month-old male ICR mice were housed at eight animals per cage (36 × 32 × 17 cm), with food and water being freely available. The illumination regimen consisted of alternating cycles of 14 h of light:10 h of darkness (LD 14:10). Light onset was at 6:00 a.m. and darkness commenced at 8:00 p.m. This L:D cycle was practiced to mimic the present natural seasonal LD alternation. Mice were exposed to this regimen for at least 2 weeks prior to the initiation of the experiments.

Drugs

The following drugs were used: carboplatin (Abic, Netanya, Israel) at 6.4 mg/kg and G-CSF (Filgrastin-Neupogen, F. Hoffman-La Roche, Switzerland) at 12 μg/kg. Doses were adjusted to mouse body weight on the basis of human therapeutic doses.

Experimental design

Carboplatin was injected i.p. into four groups of mice for 5 consecutive days at either 9:00 a.m., 3:00 p.m., 9:00 p.m., or 3:00 a.m. An untreated group served as the control. Peripheral WBC counts were monitored on the 10th day after the first injection. The WBC counts were monitored as follows: every 4 h (starting at 9:00 a.m.) over a 24-h period, peripheral blood was drawn from 18 mice (3 from each injected group and from the control group) into heparinized tubes. Aliquots of each blood sample were diluted 1:20 (in 3% acetic acid containing 1% gentian violet) and the cells were counted microscopically (Table 1, set 1). In another set of mice the time-dependent effects of G-CSF were analyzed after the results of carboplatin toxicity had become available. The first part of this experiment was the same as that described for the first set except that carboplatin was injected for 5 days only at the hour when the most severe leukopenia had been documented in the first experiment. At 2 days after the last carboplatin injection (on the 8th day) the group was divided into five subgroups and G-CSF was injected s.c. into four of the subgroups for 2 consecutive days at either 9:00 a.m., 3:00 p.m., 9:00 p.m., or 3:00 a.m. On the 10th day after the

Table 1 Experimental design

Set 1: Carboplatin injection[a]

Group injected for 5 days at	Mice (*n*)	Sampling time for WBC count at day 10					
		a.m.	p.m.	p.m.	p.m.	a.m.	a.m.
9:00 a.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
3:00 p.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
9:00 p.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
3:00 a.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
Control	18	9:00	1:00	5:00	9:00	1:00	5:00

Set 2: G-CSF singly and G-CSF with carboplatin injection[b]

Carboplatin injected into 72 mice for 5 days at	G-CSF injected at days 8–9	Mice (*n*)	Sampling time for WBC count on day 10					
			a.m.	p.m.	p.m.	p.m.	a.m.	a.m.
3:00 p.m.	9:00 a.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
	3:00 p.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
	9:00 p.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
	3:00 a.m.	18	9:00	1:00	5:00	9:00	1:00	5:00
	Control	18	9:00	1:00	5:00	9:00	1:00	5:00

[a] Total of 90 mice in each experimental set
[b] Total of 162 mice in each experimental set

first injection of carboplatin, peripheral WBCs of three mice from each group were counted every 4 h over a period of 24-h (Table 1, set 2). The entire experimental procedure was repeated twice.

Statistical analysis

The significance of differences between the groups with regard to the 24-h mean WBC counts was assessed by the χ^2 and *t*-tests. Analysis of variance (ANOVA) and best-fit Cosinor analysis [19] were applied for adjusted periods to determine the significance of time dependency and to elucidate the rhythm's parameters of WBC count. The latter analysis yielded the rhythm's period, its peak hour (acrophase), and the mean (mesor). Phase differences of up to 2 h were not considered as being significant.

Results

Carboplatin administration exhibited significant time-dependent toxicity, and the resulting patterns of the parameters (mesors, periods, and acrophases) recorded for all injected and control groups are detailed in Table 2. Figure 1 exhibits the 24-h patterns of the WBC counts of these groups. Figure 2 exhibits the computerized cosine curves. The peripheral levels of WBC counts were reduced in all four injected groups. The least significant change (from control) in the daily mean WBC count was detected in the group injected at 9:00 a.m. The maximal toxic effect (as expressed by the 24-h mean values) was observed in the group injected at 3:00 p.m. (55% of control, $P < 0.001$). There was a significant phase shift (change in acrophases – hour of computed peak activity) of 5 h (as compared with the control) in the group injected at 3:00 p.m. (Table 2).

Table 2 Circadian-dependent peripheral WBC response to carboplatin injection*

Injection time	Mesor ($\times 10^2/mm^3$)	Amplitude ($\times 10^2/mm^3$)	Acrophase (h. min)
Control	86.3 ± 2.54	20.0 ± 3.4	8.5 ± 0.4
9:00 a.m.	68.0 ± 2.72	24.0 ± 2.7	6.2 ± 0.3
3:00 p.m.	47.6 ± 1.64	12.7 ± 2.3	13.2 ± 0.4
9:00 p.m.	64.8 ± 3.50	26.6 ± 4.7	10.5 ± 0.4
3:00 a.m.	59.7 ± 2.62	32.4 ± 5.1	8.4 ± 0.3

* $P < 0.05$ for all parameters presented

Each administration of G-CSF (with no previous injection of carboplatin) given at a different time led to a variation in the mesor. There were clear changes in the number of WBCs when they were measured at the various times of injection (Table 3). The groups injected at 9:00 a.m. and at 3:00 p.m. showed a 12.7% and 10.4% reduction in WBC counts, respectively. No reduction was recorded in the other groups. Table 4 presents the parameters of the 24-h WBC patterns determined in the groups injected with carboplatin at 3:00 p.m. and those determined in the group injected with carboplatin at 3:00 p.m. followed by G-CSF administration at either of four scheduled times. The 24-h WBC patterns determined after G-CSF injections are shown in Fig. 3. Figure 4 shows the computerized cosine curves generated after G-CSF injections. The maximal effect (as expressed by the 24-h mean values) was observed in the group injected at

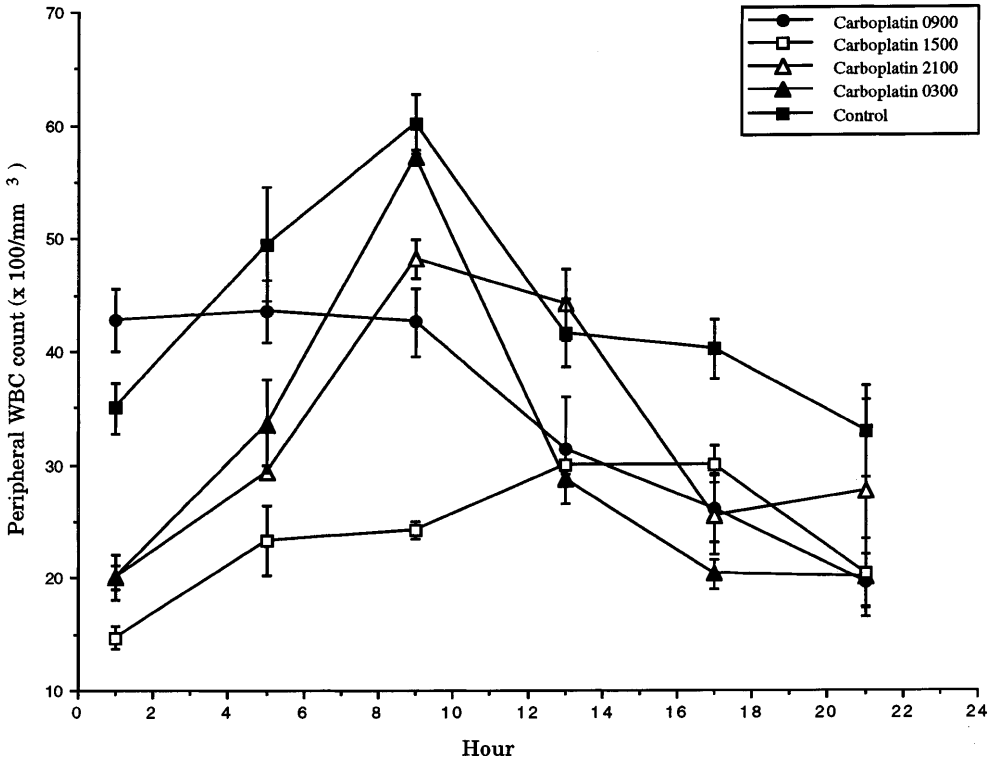

Fig. 1 The 24-h WBC pattern determined in four groups of ICR mice injected with carboplatin as compared with the untreated group. There was a 40% decrease in WBC count ($P < 0.0001$) at 3:00 p.m. (*1500*)

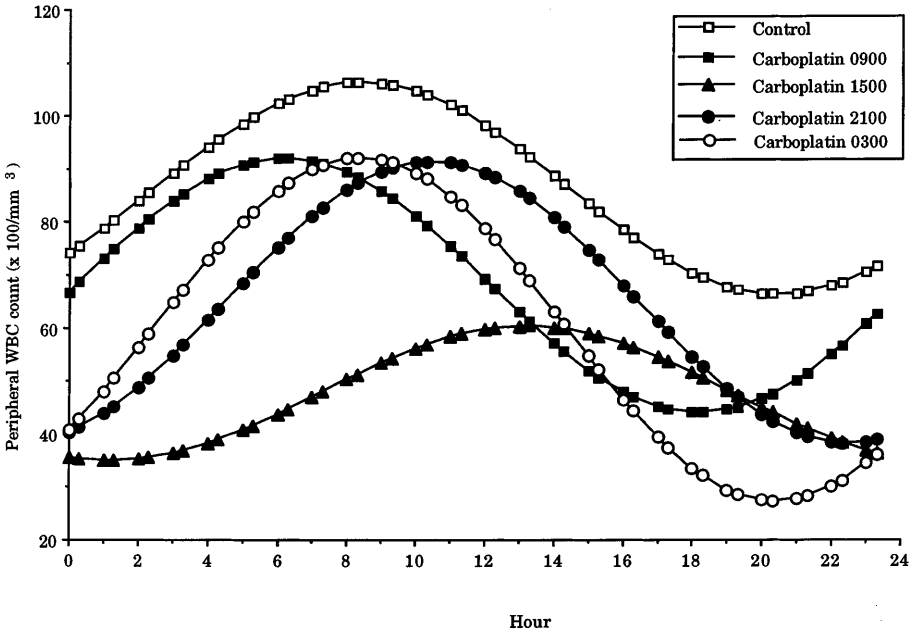

Fig. 2 The 24-h pattern of computerized cosine curves generated for WBC counts determined in ICR mice injected with carboplatin as compared with the untreated group

Table 3 Circadian-dependent peripheral WBC response to G-CSF injection*

Injection time	Mesor ($\times 10^2/mm^3$)	Amplitude ($\times 10^2/mm^3$)	Acrophase (h. min)
Control	89.0 ± 12.7	24.4 ± 17.9	10.5 ± 2.5
9:00 a.m.	77.7 ± 9.4	10.0 ± 12.9	1.2 ± 5.1
3:00 p.m.	79.8 ± 11.0	23.9 ± 15.9	15.5 ± 2.2
9:00 p.m.	88.9 ± 13.2	14.9 ± 18.6	5.5 ± 4.3
3:00 a.m	83.0 ± 13.0	26.0 ± 18.6	10.4 ± 2.4

* $P < 0.05$ for all parameters presented

Table 4 Circadian-dependent peripheral WBC response to carboplatin injected at 3:00 p.m. followed by G-CSF injection at various times (*NS* Not significant)

Injection time	Mesor ($\times 10^2/mm^3$)	Amplitude ($\times 10^2/mm^3$)	Acrophase (h. min)
Control	86.3 ± 2.54*	20.0 ± 3.4*	8.5 ± 0.4
CBP	49.0 ± 11.4*	13.1 ± 14.8*	1.1 ± 4.4
GCSF 9:00 a.m.	75.3 ± 14.7*	21.4 ± 20.2*	13.2 ± 3.5
GCSF 3:00 p.m.	78.0 ± 11.2*	28.0 ± 15.8*	11.3 ± 2.1
GCSF 9:00 p.m.	79.2 ± 17.2*	NS	NS
GCSF 3:00 a.m.	96.0 ± 13.0*	24.3 ± 17.8*	12.3 ± 2.4

* $P < 0.05$

3:00 a.m. (111.2% of control, $P = 0.0002$, and 195.9% of carboplatin singly, $P = 0.00001$). No change in acrophase or amplitude was observed in the group injected at 3:00 a.m.

Discussion

The extent of toxicity of many drugs varies as a function of the time of administration [21]. In most tumor-bearing animals given chemotherapy the time of least toxicity coincided with or was close to the time of optimal antitumor activity. There are compelling experimental data suggesting that the therapeutic index of commonly applied anticancer agents can be improved by optimal circadian timing of the treatment. The carboplatin chronotoxicity detailed in this study has also been documented by other investigators [2, 11]. The time dependency of carboplatin toxicity was illustrated in the 24-h rhythm of WBC counts (Fig. 1). The significant circadian rhythm and differences in the severity of leukopenia measured in the injected mice as compared to the non-injected animals clearly exhibited a time dependency (Table 2, Fig. 1). The 24-h mean WBC counts revealed a significant time dependency, and the severest leukopenia was obtained when carboplatin was injected at 3:00 p.m. (55% of control, $P < 0.0001$). It should be noted that the method of assessment (24-h mean WBC count) is in good agreement with the more simple time-qualified relative WBC count decrease or the more cumbersome histologic assessment of bone marrow necrosis [2, 3].

Earlier studies have demonstrated that if the subcutaneous route is employed, lower doses of G-CSF are required for equal hematopoietic effects with superior efficacy in raising peripheral blood cell counts [5, 29]. However, secondary interaction of the exogenous hematopoietic cytokines with endogenous cytokines or hormones may not determine all toxic or therapeutic outcomes. Time-dependent effects were observed when G-CSF was injected as a single agent (Table 3). When G-CSF was given at 9:00 a.m. and at 3:00 p.m. and WBC counts were measured on the day following that of the final injection, it not only failed to increase the WBC count but actually depressed it. A parallel circadian variation in G-CSF administration has been studied and shown by Wood et al. [32]. It should be noted that we

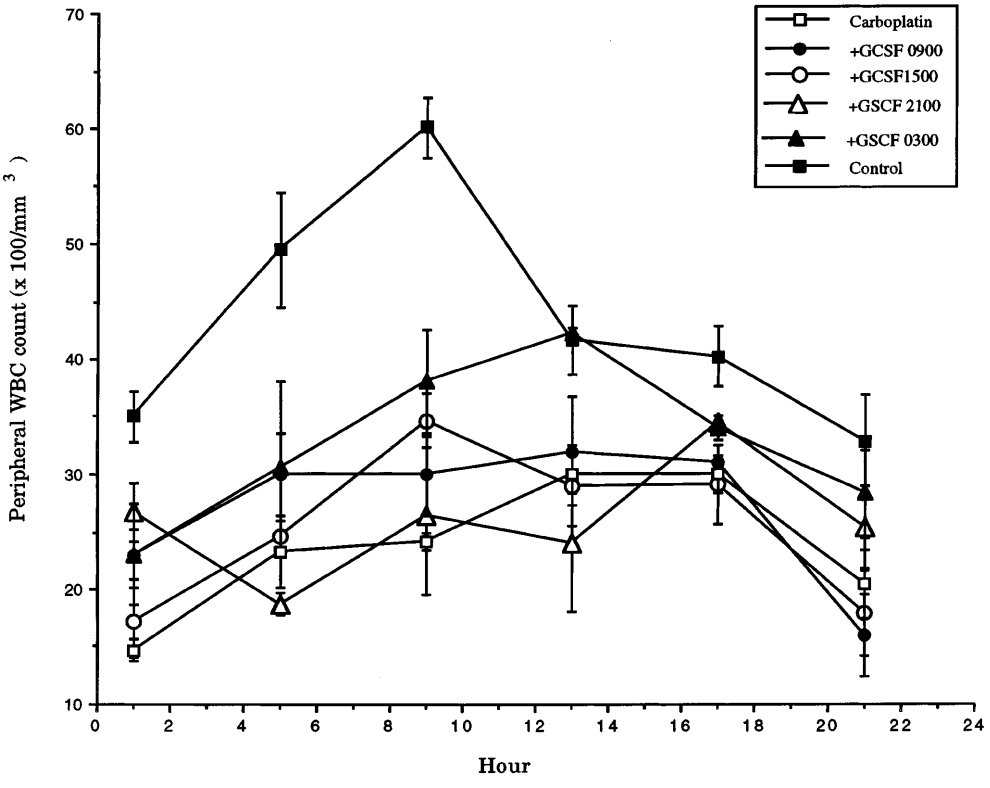

Fig. 3 The 24-h WBC pattern determined in groups of ICR mice injected with carboplatin at 3:00 p.m. (maximal leukopenia hour) followed by G-CSF injection at various times as compared with singly injected carboplatin and with the untreated control group. The differences between the 3:00 p.m. (*+GCSF 0300*) configuration and those of carboplatin given singly and of the control were statistically significant ($P < 0.00001$ and $P = 0.0002$, respectively)

used human rG-CSF rather than murine rG-CSF. Therefore, immune reaction to this human peptide might occur in nonimmunosuppressed animals. However, a recent study dealing with the effect of rG-CSF on murine bone marrow has substantiated the present results, and the phase relationship between rhythms of peripheral WBC counts and those of the bone marrow components are discussed in that paper [22].

The timing of cytokine administration during and/or after other therapies is important for the achievement of optimally effective cytokine therapy and may influence the nature of the response (stimulatory or inhibitory). Circadian timing, i.e., administration of hematopoietic growth factors at specific times throughout a 24-h period, may afford advantages in peripheral reuptake of WBC counts. In the present study, when G-CSF was given at various times to the group in which carboplatin induced the most severe leukopenia, a recovery of peripheral WBC counts was monitored at all injection times, reaching its highest level (exceeding even that of the control) when G-CSF was injected at 3:00 a.m. (Table 4, Fig. 2). The results obtained using various

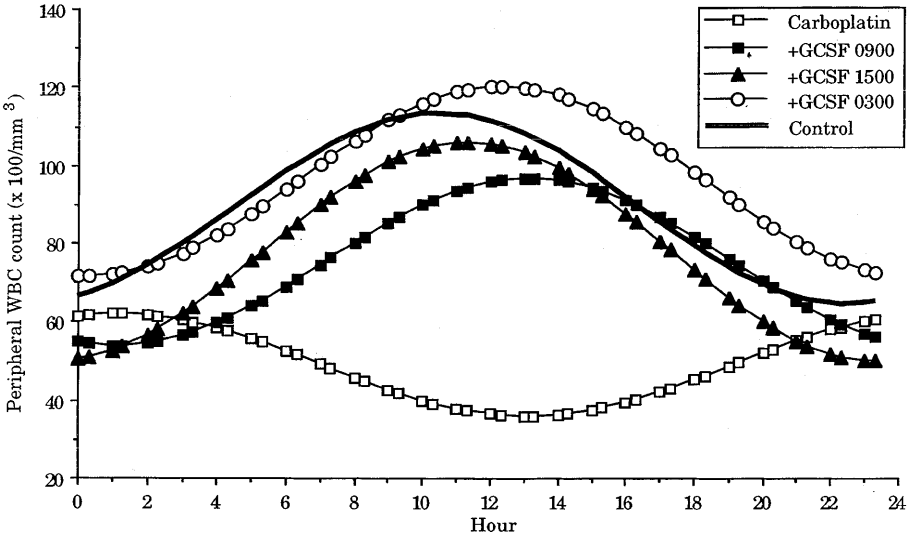

Fig. 4 The 24-h pattern of computerized cosine curves generated for WBC counts determined in ICR mice injected with carboplatin at 3:00 p.m. followed by G-CSF injection at various times as compared with mice singly injected with carboplatin at 3:00 p.m. and with the untreated control group

treatment strategies, such as those described in the present report, show that G-CSF may be given after carboplatin at 3:00 a.m. (highest recovery after carboplatin injection), whereas carboplatin has to be injected at 9:00 a.m., the time at which the 24-h mean WBC counts revealed the minimal level of leukopenia, which may stem from direct chronotoxicity or from the effect of other related rhythms such as those seen in changes in cell distribution.

In view of the present results, drafters of a protocol designed for consecutive cancer treatments based on chemotherapy given concomitantly with or prior to hematopoietic growth factors must consider the following: (a) the intensity of toxic effects (immediate and late) is dependent upon drug administration, and (b) in variables that exhibit a rhythm (diagnostic ones and the nature of the target tissue, e.g., bone marrow) the time of drug injection may also affect the endogenous rhythm, at certain times leading to a reduction and in others to an increase in the efficacy of chronotherapeutic treatments.

It is too early to focus on one of the rhythm's parameters (mesor, amplitude, acrophase) as the most diagnostic one to achieve the least toxicity. These effects deserve further exploration for better specification of the respective optimal circadian timing of the two medications given in sequence.

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