

Circadian Variation of Topoisomerase II- α in Human Rectal Crypt Epithelium: Implications for Reduction of Toxicity of Chemotherapy

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Topoisomerase II-alpha is a target of common chemotherapeutic agents such as doxorubicin and etoposide, which induce DNA damage by altering the activity of this enzyme. We took rectal biopsies at 4-hour intervals over a 24-hour period (seven total) from each of 10 healthy volunteers and examined immunoperoxidase-stained coded anti-topoisomerase II-alpha-stained sections. A significant circadian periodicity was seen in the number of rectal crypt epithelial cell nuclei that were stained ($P = .01$). Mean peak staining was at 7:23 a.m. \pm 45 minutes, and the mean rate of change (difference between peak and trough expression) was 40%. Topoisomerase II-alpha expression in rectal epithelium has a significant circadian variation similar to that of tritiated thymidine incorporation. Although direct confirmation is needed, giving topoisomerase II-targeted chemotherapeutic agents at the proper time of day might reduce their mucositis side effects.

KEY WORDS: Adverse effects, Circadian rhythm, Colon, DNA topoisomerase II, Doxorubicin, Drug therapy, Etoposide, Rectum.

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Circadian rhythms of DNA synthesis and cell proliferation have been documented in human epidermal (1-3), buccal (4), and rectal epithelia (5, 6), and in bone marrow (7, 8). These human studies, and >30 experimental studies in lower animals, have been used to design protocols for time-qualified

delivery of chemotherapeutic agents to tumor-bearing animals and to cancer patients (9, 10). In two prospective randomized clinical trials, in patients with metastatic colorectal cancer, time-of-day-modified 5-fluorouracil, leucovorin and oxaliplatin-based chemotherapy significantly ($P < .00001$) reduced the incidence of severe mucositis (from 76 to 14%) and significantly ($P = .003$) improved the objective response rate (from 29 to 51%), compared with a flat-rate infusion schedule (11, 12). In a third multicenter study, an intensified chronomodulated course of these agents led to an objective response rate of 66% (13). This salutary circadian modified clinical response could be due to circadian variation in nontumor tissue proliferative activity—chemotherapy-induced damage probably would be greatest at the peak time of DNA synthesis (S phase) or mitosis.

DNA topoisomerases link and unlink DNA strands, allowing DNA to twist or untwist as it becomes coiled or uncoiled (14). Topoisomerase II-alpha (topo II- α) is involved in chromosomal condensation and segregation and is essential for untangling intertwined DNA molecules before mitosis (15-17). Topo II- α is needed as cells synthesize new DNA and then separate their newly made chromosomes. The enzyme is synthesized in late G1 and early S-phase, is present in the cell during G2 and in M, and then is degraded as the cell enters G1 (18, 19). Several widely used classes of chemotherapeutic agents, including anthracyclines such as doxorubicin and podophyllotoxins such as etoposide, alter topo II- α and thus induce DNA strand breaks (20, 21). There is *in vitro* evidence that topo II-targeted drugs show a pronounced S-phase toxicity (22). Although there is a single murine study presenting evidence of a circadian variation in both toxicity and activity of topoisomerase I (23), to our knowledge there is no prior evidence of a circadian variation in topo II, either in humans or experimental animals.

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The purpose of this study was to determine whether a circadian, or 24-hour, rhythm of topo II- α expression could be detected in healthy human rectal mucosa. Characterizing this rhythm could lead to safer administration of chemotherapeutic agents using topo II- α .

MATERIALS AND METHODS

Ten 24-hour studies were done on 10 healthy male volunteers, aged 18–27 years (median = 20 y). Subjects denied any history of significant past or current gastrointestinal disease or surgery. The study was approved by the institutional review board of the Department of Veterans Affairs Salt Lake City Health Care System and the University of Utah. Subjects were not allowed to take medications or ingest alcohol for 48 hours before or during the study. Tobacco use was not allowed during the study. Subjects were fasted from solid foods for 12 hours before the study period and during the 24-hour study, except for *ad libitum* tap water and four Coca Colas (total caloric value = 640 Kcal) spaced evenly over the 24 hours. All subjects followed their usual day–night sleep schedule, sleeping between 10:30 p.m. and 6:30 a.m., except when awakened for study procedures. All studies were begun at 6 p.m. on Friday and completed at 6 p.m. on Saturday. Biopsy specimens of rectal mucosa were obtained at 4-hour intervals over each 24-hour study (total of seven samples each). No enemas or other bowel preparation were used. An average of six mucosal biopsies were obtained at each time point using standard-sized disposable biopsy forceps through a flexible sigmoidoscope. All biopsies were taken between 5 and 15 cm from the anal verge. The biopsy specimens for study were immediately placed in neutral buffered formalin. The formalin bottles were coded so that the pathologist (FC) interpreting the slides was blinded as to the timing sequence.

Four biopsies per time point were fixed in neutral buffered formalin and paraffin embedded. Six 4- μ m sections per slide were cut. The slides underwent diethanolamine-based pressure cooker antigen retrieval with Trilogy (Cell Marque, Austin, TX) using the manufacturer's procedure. After treatment with 0.03% hydrogen peroxide for 10 minutes, the slides were incubated with mouse monoclonal anti-topoisomerase II- α (7 μ g/mL for 20 min; 24), and DAKO Envision secondary antibody (20 min), all at 37° C. The slides were stained with diaminobenzidine and counterstained with hematoxylin. The number of staining nuclei per crypt were counted. Only properly oriented crypts in which the lower two thirds of the crypts were present and not tangentially cut were evaluated. A mean of 13.5 crypts was counted per time point. Two subjects each had one excluded data point because of a lack of well-oriented crypts; the other eight subjects had all seven data points.

Statistical Analysis

The data from all subjects were included in the analysis. All data from all time points except two, as mentioned above, were included (Table 1). To normalize the data, each raw data series for each individual, and for the group ($n = 10$), was analyzed for a circadian rhythm by the least-squares fit of a 24-hour cosine; group rhythm characteristics were summarized by population-mean cosinor values (25, 26). The rhythm characteristics include the mesor (middle value of the fitted cosine; the rhythm adjusted mean value), the amplitude (half the difference between the minimum and maximum values of the fitted cosine function), and the high point or acrophase (time of peak value in the fitted cosine function). The standard error (SE) and 95% confidence interval (CI) estimates were also computed. A P value for the rejection of the zero-amplitude (no

TABLE 1. Topo II-Alpha Expressed as Stained Cells/Crypt

Subject No.	Time (24-h Clock)															
	18:00 % of		22:00 % of		2:00 % of		6:00 % of		10:00 % of		14:00 % of		18:00 % of		All Times	
	Raw	Mean	Raw	Mean	Raw	Mean	Raw	Mean	Raw	Mean	Raw	Mean	Raw	Mean	Mean	SEM
1	4.31	89.9	3.75	78.2	4.69	97.8	5.17	107.8	4.77	99.5	5.54	115.6	5.33	111.2	4.79	0.25
2	2.88	85.5	2.67	79.2	3.23	95.8	4.27	126.7	3.33	98.8	3.71	110.1	3.50	103.9	3.37	0.22
3	3.86	77.1	4.43	88.4	4.93	98.4	6.61	132.0	5.20	103.8	5.20	103.8	4.83	96.4	5.01	0.35
4	2.79	92.3	2.67	88.3	3.61	119.4	3.29	108.8	3.88	128.4	2.69	89.0	2.23	73.8	3.02	0.24
5	3.50	95.1	2.25	61.1	3.21	87.2	4.00	108.7	3.58	97.2	4.17	113.3	5.06	137.4	3.68	0.36
6	5.40	79.1	5.61	82.2	7.00	102.5	8.00	117.2	8.85	129.6	ND	ND	6.10	89.4	6.83	0.62
7	4.50	86.8	4.60	88.7	7.45	143.7	6.00	115.7	ND	ND	4.16	80.2	4.40	84.9	5.19	0.58
8	2.69	73.1	2.15	58.4	4.25	115.5	5.87	159.5	5.45	148.1	1.91	51.9	3.44	93.5	3.68	0.64
9	3.30	71.1	4.19	90.3	5.25	113.1	6.86	147.8	4.85	104.5	4.36	94.0	3.67	79.1	4.64	0.48
10	3.83	84.9	4.62	102.4	3.92	86.9	5.00	100.8	6.93	153.6	3.83	84.9	3.46	76.7	4.51	0.49
Mean	3.71	83.5	3.69	81.7	4.75	106.0	5.51	123.5	5.20	118.2	3.95	93.6	4.20	94.6	4.47	
SEM	0.29	2.70	0.40	4.48	0.49	5.77	0.48	6.00	0.62	7.84	0.40	7.14	0.38	6.40	0.37	

ND, inadequate tissue sample.

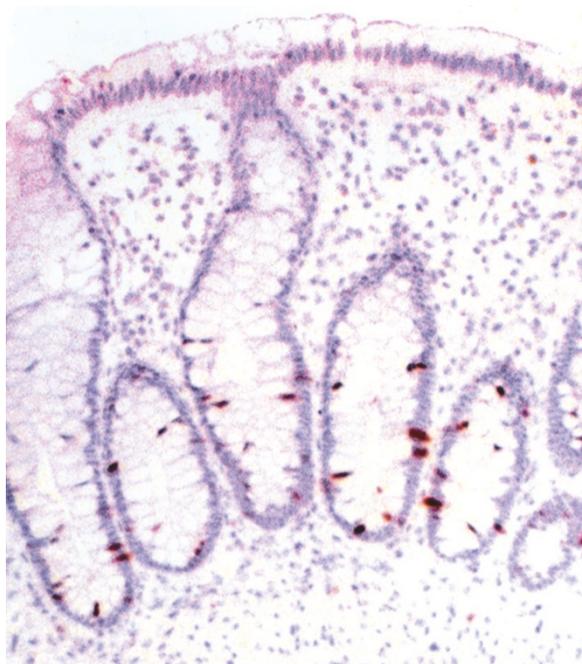


FIGURE 1. Immunostaining for topoisomerase II- α in rectal crypts at highest time point (6 a.m.), showing about 5.4 staining nuclei per crypt.

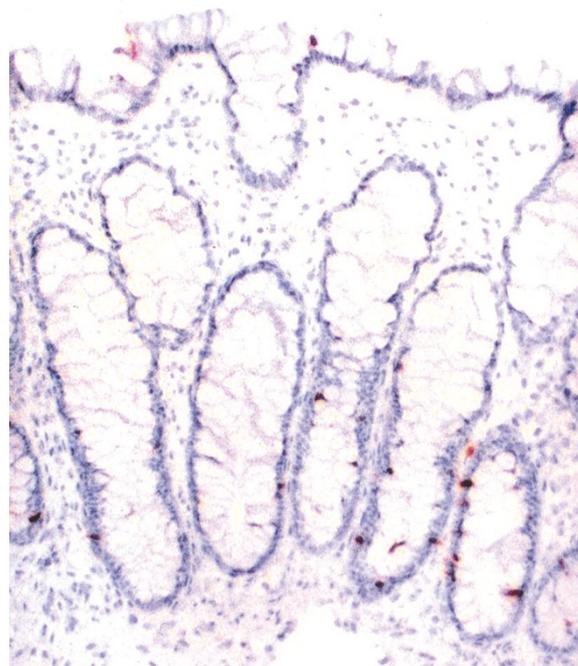


FIGURE 2. Immunostaining for topoisomerase II- α in rectal crypts at a low time point (6 p.m.) showing about 3.4 staining nuclei per crypt.

rhythm) assumption was determined for each individual series and for the group. Rhythm detection is considered statistically significant when $P \leq .05$. The range of change between high and low raw and normalized values was also calculated and subjected to paired t -test analysis.

RESULTS

Similar to the case in a prior study (27), topo II- α staining was found in epithelial cell nuclei in the bottom one-third to one-half of the crypts (Figs. 1, 2). There was no significant epithelial cell staining in the superficial portions of the crypts or surface epithelium. There were more cells staining in the crypts in the morning samples (Fig. 1) than in the evening ones (Fig. 2). The stained cells per crypt data (expressed in number of topo II- α -stained nuclei per rectal crypt) and rate of change values are listed in Table 1 and graphed in Figure 3. The mean rate of change (lowest to highest value) was 40% ($P = .000375$).

The cosinor rhythm analysis of the raw and normalized data for each individual, and for the group, is tabulated in Table 2. The rhythm plots are shown in Figure 3. A significant circadian rhythm in nuclear topo II- α expression in rectal crypt mucosa was found in both the raw ($P = .02$) and normalized ($P = .01$) values. The amplitude of the normalized data curve was 19.43%. The time of peak value (acrophase) of the normalized group data series was calculated at 07:23 a.m. \pm 45 minutes. Analysis

of the data for each subject showed acrophase values varying from 03:41 a.m. to 1:00 p.m.

DISCUSSION

These data show a significant rhythm in topo II- α nuclear staining of rectal crypt cells in healthy male subjects. The acrophase, or time of highest value, occurred at 07:23 a.m., near the end of the sleep phase in the usual sleep-wake schedule. Although the acrophase did vary among the different subjects, it does appear that topo II- α expression is least in the early evening.

The 7:23 a.m. acrophase for topo II- α staining is near the acrophase value of 07:04 a.m. of incorporation of tritiated thymidine (^3H TdR) into rectal tissue biopsy samples obtained over the circadian period in a study reported from our institution (5) using very similar study conditions. Although the uptake of ^3H TdR may not be precisely synonymous with DNA synthesis (28), the correspondence in acrophase values in the two studies, employing different markers, support the view that both are markers of cellular proliferation. In another human rectal biopsy study, using ^3H TdR radioautography techniques, a circadian rhythm in ^3H TdR labeling was found in the basal cells of rectal crypts with an acrophase value of 01:28 a.m. (6). The lack of precise agreement in acrophase values between the two ^3H TdR-based circadian rectal biopsy studies may relate to the different ^3H TdR techniques employed in the two studies and the limitations of

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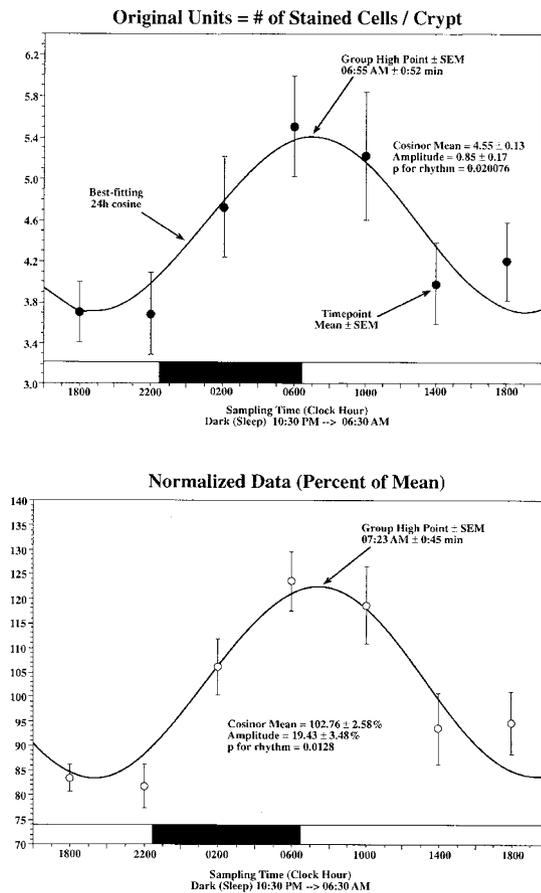


FIGURE 3. Circadian expression of topoisomerase II- α in rectal crypts of 10 healthy men. Values expressed in raw numbers of mean stained cells per crypt in the upper curve and normalized as a percentage of the 24-hour rhythm adjusted mean in the lower curve. Each point represents the mean \pm the standard error of the mean. All times are local clock time.

[³H]TdR incorporation techniques in general (28). Bjarnason *et al.* (4, 29) recently reported significant circadian variation in the expression of cell-cycle and cellular clock proteins and thymidylate synthase activity (an S-phase indicator) in human oral (buccal) epithelium but did not study topoisomerase. These studies suggested an S-phase acrophase in early afternoon. Scheving *et al.* (30) showed, in rodent gut, significant circadian variation in [³H]TdR incorporation in mucosal epithelia throughout the entire gastrointestinal tract. Although the phasing of the rhythms in different regions of the gastrointestinal tract was fairly similar, the acrophase for tongue epithelium preceded that of colonic epithelium by about 3 hours. Ohdo *et al.* (23) studied topoisomerase I activity and the toxicity of a topo I-targeted agent. In mice, they found that bone marrow topo I activity, as well as the greatest weight loss from the topo I-targeted agent, irinotecan, occurred near the end of the active cycle or early in the rest cycle, a time also matching the greatest S-phase fraction.

To our knowledge, this is the first evidence for a circadian variation in topo II- α expression. This finding may have implications for cancer chemotherapy. Although there was significant variation in the timing of the acrophase among our subjects, it appears that topo II- α expression is generally least in the early evening. Topoisomerase-mediated damage is most extensive in S phase (22). As previously mentioned, tritiated thymidine incorporation, an S-phase marker, had a very similar acrophase in a prior study from our institution under similar conditions (5). Thus, early evening, when both S-phase fraction and topo II- α expression are minimal, should be the best time to administer the relevant chemotherapeutic agents to minimize mucositis toxicity. However, direct confirmation of this is needed.

TABLE 2. Circadian Parameters for Topo II-Alpha Nuclear Expression: Raw and Normalized Data for Each Subject

Subject No.	Raw Data				Normalized Data			
	Stained cells, No./Crypt (Mean \pm SEM)	Amp \pm SEM	Acrophase (Peak Time) \pm SEM	P Value	Mean \pm SEM	Amp \pm SEM	Acrophase \pm SEM	P Value
1	4.81 \pm 0.23	0.55 \pm 0.34	11:15 \pm 2:06	0.37	100.3 \pm 4.8	11.48 \pm 7.11	11:50 \pm 2:06	0.37
2	3.42 \pm 0.18	0.48 \pm 0.26	08:52 \pm 2:03	0.28	101.5 \pm 5.4	14.25 \pm 7.62	08:53 \pm 2:03	0.28
3	5.13 \pm 0.23	0.88 \pm 0.31	07:20 \pm 1:29	0.11	102.3 \pm 4.6	17.51 \pm 6.21	07:19 \pm 1:29	0.11
4	3.11 \pm 0.15	0.62 \pm 0.20	06:31 \pm 1:23	0.09	102.9 \pm 5.0	20.41 \pm 6.59	06:31 \pm 1:23	0.09
5	3.66 \pm 0.34	0.69 \pm 0.51	13:00 \pm 2:31	0.47	99.3 \pm 6.2	18.61 \pm 13.74	13:00 \pm 2:31	0.47
6	7.08 \pm 0.17	1.62 \pm 0.22	08:37 \pm 0:32	0.01	103.7 \pm 2.4	23.85 \pm 3.22	08:38 \pm 0:32	0.01
7	5.35 \pm 0.35	1.34 \pm 0.53	03:41 \pm 1:36	0.10	103.2 \pm 6.8	25.90 \pm 10.25	03:41 \pm 1:36	0.10
8	3.91 \pm 0.41	1.64 \pm 0.54	06:38 \pm 1:24	0.09	106.3 \pm 11.0	44.60 \pm 14.64	06:38 \pm 1:24	0.09
9	4.84 \pm 0.20	1.83 \pm 0.26	05:48 \pm 0:49	0.02	104.2 \pm 4.3	29.65 \pm 5.63	05:48 \pm 0:49	0.02
10	4.64 \pm 0.40	1.20 \pm 0.55	08:23 \pm 1:59	0.25	102.8 \pm 8.8	24.26 \pm 12.14	08:23 \pm 1:59	0.25
Group mean \pm SEM	4.55 \pm 0.13	0.85 \pm 0.17	06:55 \pm 0:52	0.02	102.8 \pm 2.6	19.43 \pm 3.48	07:23 \pm 0:45	0.01

Acrophase data are in time of day on a 24-hour clock. Amp, amplitude; SEM, standard error of the mean.

However, the timing of the maximal amount of topo II- α and S phase in normal tissues is but one of several considerations in optimizing the timing and dosage of chemotherapy. Some tumors might also have circadian rhythms (which could be different than the normal tissues), and metabolism and/or clearance of these agents could also vary during the day. Doxorubicin is the only topo II agent whose efficacy has been extensively studied in humans on a circadian basis. Alone or in combination with other agents, doxorubicin has been found to be least toxic and potentially best when given in the early morning (31–33). This could be because, in humans, doxorubicin clearance is fastest in the morning (34), whereas the related drug epirubicin has slower clearance in the morning (35), and etoposide clearance has no diurnal variation (36).

In mice and rats, the early studies found that intraperitoneal doxorubicin is less toxic when given in the early active phase (37, 38). However, the optimal timing varies with the route of administration (intraperitoneal *versus* intravenous; 39). A recent murine study found that intravenous doxorubicin was least toxic in the midrest phase, and, in combination with docetaxel and in consideration of a diurnal variation in efficacy, doses at either the beginning of the rest phase or early in the activity phase were optimal (40). For daunorubicin (41), THP-doxorubicin (42, 43), and epirubicin (44), the toxicity is least in the early rest phase, whereas etoposide toxicity is least in the late rest phase (45). Thus, in rodent models, all four anthracycline drugs tested (but not etoposide) were least toxic in the early to mid rest phase, fairly similar to our findings in human rectum.

In summary, we found a significant circadian variation in staining for topo II- α staining in the rectal epithelium of healthy subjects, with the acrophase (peak staining) at 7:23 a.m. \pm 45 minutes, near the acrophase for tritiated thymidine incorporation. We speculate that appropriately timed doses of the relevant chemotherapeutic agents, when topo II- α expression and S phase are minimal, might reduce the mucositis side effects. However, other factors such as the circadian rhythms in the drug clearance rate and in the tumor sensitivity must also be considered. Further work is needed to directly study these issues.

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