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Circadian Rhythm in Tolerance of Mice for the New Anthracycline Analog 4′-*O*-Tetrahydropyranyl-Adriamycin (THP)*†

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Abstract—*A statistically significant circadian rhythm in tolerance of 226 male B6D2F1 mice synchronized with LD 12:12 for 4′-O-tetrahydropyranyl-adriamycin (THP) was demonstrated. Four intravenous dosages (18, 25, 32 and 40 mg/kg) and six different dosing times (3, 7, 10, 14, 19 and 23 hr after light onset — HALO) were compared. Survival rate, body weight loss and leukopenia depended on both the dose and time of injection. The overall survival rate varied between 83% (light — rest span) and 56% (dark — activity span) ($\chi^2 = 17$; d.f. = 2; $P < 0.001$). Maximal body weight loss occurred 4-5 days after drug injection. Total leukocyte counts were determined on these days. Both body weight loss and leukopenia were reduced by ~100% in those mice injected in their late rest span (7-10 HALO) as compared to those treated in the middle of their activity span (19 HALO). Circadian rhythms in day-60 survival rate, body weight loss and leukopenia were statistically validated by cosinor analysis, with estimated peak times (acrophases) occurring respectively at 7:30, 9:20 and 8:40 HALO. Minor cardiac lesions consisting of diffuse vacuolization and loss of muscular striation were observed in histologic sections from 3/32 hearts (16 controls, 16 treated). All three corresponded to THP given at 19 (2/2 mice) or 23 HALO (1/4 mice). Thus lethal, hematologic and possibly cardiac tolerance for THP were largely optimized by administering the drug to mice in their late span (7-10 HALO).*

INTRODUCTION

THE CIRCADIAN time at which an anticancer drug is administered to rodents largely influences both its tolerance and its antitumor effect [1-5]. More specifically, this has been shown for two anthracyclines, daunorubicin and adriamycin [6-8]. Both hematologic and cardiac toxicities of adriamycin were minimized in rodents by injecting this drug at an appropriate circadian time. However, the times of optimal tolerance of the bone marrow and that of the heart appeared to differ by approximately 6 hr [8].

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Anthracycline analogs with potentially less or no cardiac toxicity as compared to adriamycin are being developed, among them THP [9]. This new anthracycline has demonstrated a promising antitumor activity in clinical phase II trials [10]. The aim of the present studies was to document any effect of dosing time upon tolerance of mice for THP in order to provide guidelines for a further optimization of its therapeutic index. Mortality, body weight loss, leukopenia and microscopic heart lesions were used as toxicity endpoints.

MATERIALS AND METHODS

Animals and synchronization

Three studies were performed between 16 May and 8 December 1983. A total of 226 male B6D2F1 mice (C.N.S.E.A.L., C.N.R.S., Orleans-la-Source,

France) were used. They were housed three per cage, with food and water freely available. Three weeks before each study, mice were randomly distributed into one of six groups (studies 1 and 2) or one of two groups (study 3). Each group was housed on a different shelf of an autonomous chronobiologic animal facility (E.S.I.-Flufrance, Arcueil, France). Each facility had six sound-proof, temperature-controlled compartments, each one having its own programmable lighting regimen. They were constantly provided with filtrated air delivered at an adjustable rate (80 l/min in these studies). All mice were synchronized with a lighting regimen consisting of an alternation of 12 hr of light (L) and 12 hr of darkness (D) (LD 12:12).

For convenience, staggered LD regimens were used so that different circadian stages of mice were explored at similar clock hours. For example, in studies 1 and 2 all six groups were injected between 10:00 hr and 12:00 hr. With such a procedure, six circadian stages were explored: 3, 6, 10, 14, 19 and 23 hr after light onset (HALO). The adequate synchronization of mice kept under such conditions for 3+ weeks is a general property of biological rhythms [11]. In the present studies it was verified by assessing the circadian rhythmicities in body weight and circulating white blood cell (WBC) count in control mice.

Drug

THP was kindly supplied by Roger Bellon Laboratory (Neuilly/Seine, France) in vials containing 10 mg of THP and sodium chloride. The drug was freshly prepared prior to injection by adding the appropriate amount of distilled water in order to achieve the desired concentration. Four dosages were administered (18, 25, 32 or 40 mg/kg body wt) in a fixed fluid volume (5 ml/kg body wt). Hence drug concentrations were respectively: 3.6, 5.0, 6.4 and 8.0 mg/ml. Each mouse was given a single intravenous injection of either drug or saline into the retro-orbital sinus.

Study designs

The study designs are summarized in Table 1. Toxicity endpoints were as follows: mortality in study 1; mortality, body weight loss and leukopenia in studies 2 and 3. Serial sections of the heart were also prepared for histologic analysis in the two latter studies. For this purpose, surviving mice were randomly selected for sampling 20 days after injection and their hearts removed.

Toxicity endpoints

Mortality was recorded twice a day until day 20, and once a day subsequently up to day 60. The survival rate was computed for each dose, group and study.

Body weight and WBC count were measured 96, 104, 112 and 120 hr after THP dosing in study 2 and 96 and 120 hr after THP injection in study 3. Nine blood samples per THP dose and per injection time were obtained in each of these studies, i.e. each mouse was bled once. A total of 39 blood samples was concomitantly obtained from the control mice. Body weight loss was computed as the percentage difference between post-injection and initial body weight of each mouse. WBC count was determined in 500-μl aspirates of venous blood obtained from the retro-orbital sinus.

The blood was collected in a heparinized 1-ml syringe (Terumo) and diluted in Isoton II (Coultronics). Six drops of Zap-o-globin (Coultronics) were added, and the WBC count measured with a Coulter counter (Coultronics).

Cardiac histopathology

Sections of the hearts of 16 controls and 16 THP-injected mice were analyzed by microscopy

Table 1. *Main characteristics of the three studies investigating the circadian variability in tolerance of male B6D2F1 mice for THP*

Study	Date	No. of mice	Age (weeks)	THP dose (mg/kg)	No. of time-points	Endpoints
1	5/16/83	46	22	18	6	Survival†
2	9/16/83	54	15	25	6	Survival,† weight loss‡
		52	15	32	6	leukopenia,‡ heart histology§
3	12/8/83	18	12	32	2	Survival,† weight loss‖
		17	12	40	2	leukopenia,‖ heart histology§

*A total of 39 additional mice of same sex and ages were used as controls in studies 2 and 3.
†Assessed 60 days after injection.
‡Assessed 96, 104, 112 and 120 hr after injection.
§Assessed 20 days after injection.
‖Assessed 96 and 120 hr after injection.

(including immersion). Hearts were sampled 20 days after injection of saline or 25 mg/kg of THP at 3, 6, 10, 14, 19 or 23 HALO in study 2 (2 mice/drug/time-point), and saline or 32 mg/kg of THP at 10 or 23 HALO in study 3 (2 mice/drug/time-point).

The mice were killed, their hearts removed and allowed to cease beating for approximately 10 min, then fixed in 10% formalin, dehydrated in absolute ethanol and embedded into paraffin. Serial sections were stained with hematein-eosin, which allows a good visualization of muscular striation. Each encoded slide was examined by a histopathologist.

Statistical analysis

Means and one standard error of the mean (S.E.) were computed for each time-point dosage and study. The statistical significance of peak-through differences was validated by the t test for parametric data and for survival rates by the χ^2 test. Time series were also analyzed by the cosinor method [12]. A rhythm was characterized by parameters of the fitted cosine function approximating all data with a period (τ) $\equiv$ 24 hr. The rhythm characteristics estimated by the cosinor are the mesor M (24-hr adjusted mean), the amplitude A (half the difference between the minimum and maximum) and the acrophase ϕ (time of maximum, with time of light onset as ϕ reference). A and ϕ are given with their 95% confidence limits. A rhythm is detected when A differs from zero (non-null amplitude test), with $P < 0.05$; however, A and ϕ may be approximated if $0.05 \leqslant P \leqslant 0.10$.

In all cases the concordance of several statistical methods was required to draw any conclusion.

RESULTS

Survival rates

Deaths occurred between days 4 and 15 following THP dosing. Subsequently, no death occurred until day 60, when each study was terminated. The survival rate differed as a function of dose and dosing time. An increase in dose from 18 up to 40 mg/kg led to a 50% decrease in survival rate (Table 2).

Whatever the dose given, an effect of the circadian stage of injection was clearly documented. Thus when results from all studies and dosages were pooled, THP dosing during the light (rest) span resulted in an 83% survival rate, as compared to 56% in the groups treated in the dark (activity) span ($\chi^2 = 17$; d.f. $= 2$; $P < 0.001$). An optimal tolerance for THP was achieved when this drug was injected during the light (rest) span (3, 7, 10 HALO) and early dark (activity) span (14 HALO) of the mice. A sudden drop in survival rate occurred when THP was given at 19:00 [middle of the dark (activity) span of mice]. Treatment with 25 or 32 mg/kg THP given at 23:00 HALO resulted in lower survival rates than when these same doses were administered at 10 HALO (Fig. 1). Such results were confirmed by study 3, where treatment with 32 and 40 mg/kg of THP were compared as a function of dosing at 10:00 or 22 HALO (Fig. 2).

In mice receiving the lowest dose (18 mg/kg), the survival rate varied between 100% (treatment at 10 HALO, and also at 03:0, 14:00 and 23:00 HALO) and 75% (treatment at 19 HALO) ($\chi^2 = 6.7$; d.f. $= 5$; $P \simeq 0.20$). The circadian-stage dependence of tolerance of mice for THP, as gauged by survival rate, was statistically validated by both χ^2 test and cosinor ($P < 0.01$) for all three other dosages taken separately. Overall, the acrophase of survival rate as a function of THP dosing time was located at 7:30 HALO, e.g. during the rest span of mice (Fig. 3).

Body weight loss

Effects of dose and treatment time were also statistically validated for this variable (Table 2, Figs 2 and 4). Regardless of dosing time, mice receiving 40 mg/kg THP lost on average 20.7% of their initial body weight, as compared to 17.8% for those injected with 32 mg/kg and 11.8% for those treated with 25 mg/kg. Mean body weight loss was always slightly more pronounced on day 5 than on day 4. For each of the dosages

Table 2. Dose-dependent tolerance for THP in male B6D2F1 mice

Dose (mg/kg)	No. of mice	Survival rate (%) (day 60)	Body weight loss* (%) (days 4-5)	WBC count* (cells/mm³) (days 4-5)
0	39	100	1.2 ± 0.7	7470 ± 440
18	46	93	N.D.†	N.D.
25	54	76	11.8 ± 0.6	1940 ± 176
32	70	57	17.8 ± 0.7	1500 ± 100
40	17	35	20.7 ± 1.4	1510 ± 200

*Mean ± S.E.M.
†N.D., not determined.

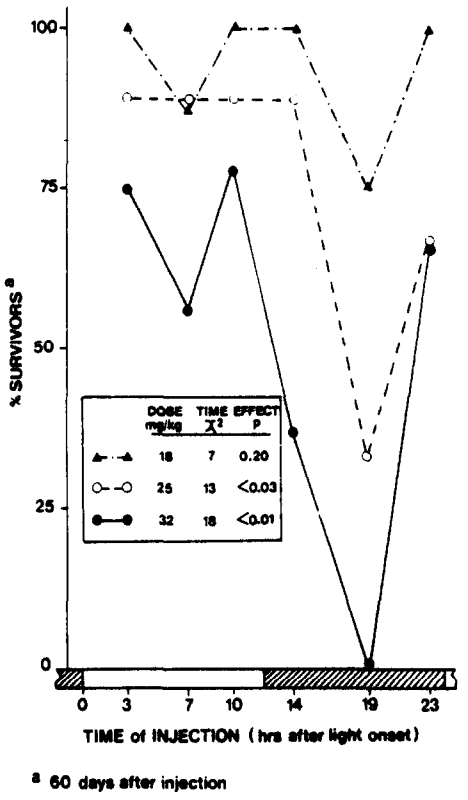

Fig. 1. Day-60 survival rate as a function of dose (18, 25 or 32 mg/kg) and dosing time of THP in male B6D2F1 mice. Six dosing times were explored. Statistically significant time-related differences were documented by χ^2 test all dose levels confounded.

investigated, body weight loss was minimal in the group injected at 10 HALO [light (rest) span] and maximal in the groups treated at 19, 23 or 3 HALO, and peak-trough differences were statistically significant. Irrespective of dose, cosinor analysis also documented a circadian rhythm in body weight loss ($P < 0.001$). The estimated treatment time corresponding to minimal weight loss (acrophase) was located at 9:20 HALO (Fig. 3).

Circulating WBC count

On average, regardless of timing of treatment, the circulating WBC count dropped from 7470 cells/mm³ in control mice to 1940 cells/mm³ in mice dosed with 25 mg/kg and 1500 cells/mm³ in mice dosed with 32 mg/kg. No difference was apparent between mice injected with 32 mg/kg and those given 40 mg/kg on day 4 or 5 (Table 2). A time-related effect was documented in mice receiving 25 and 32 mg/kg THP (Table 3). The highest mean WBC counts were observed in the groups treated at 7 and 14 HALO and the lowest in those treated at 19 HALO. No statistically significant difference in mean WBC count was documented between the group injected at 10 HALO and that treated at 23 HALO, at any of the three dosages investigated (25, 32 or 40 mg/kg). In study 3, WBC counts (± 1 S.E.M.) of mice

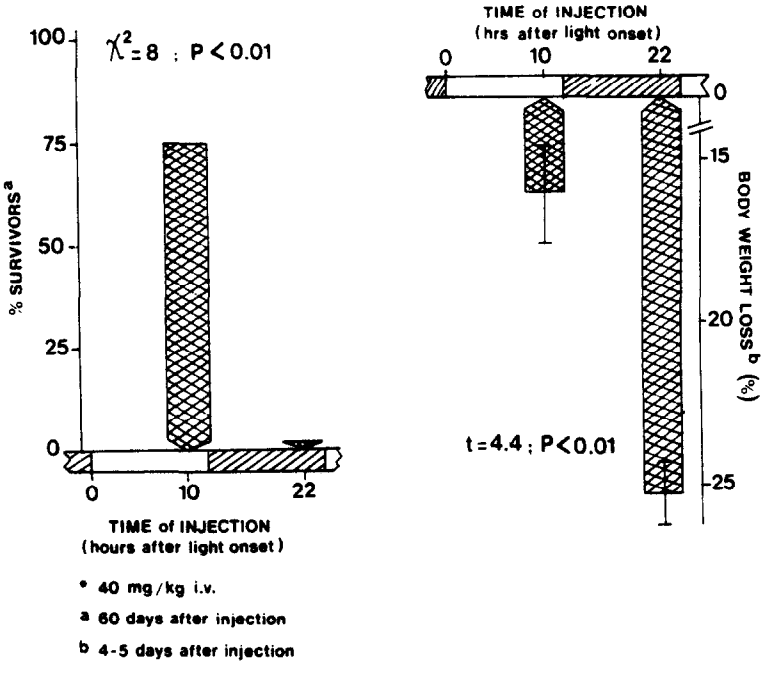

Fig. 2. Survival rate on day 60 (left) and body weight loss on days 4–5 (right) resulting from a single intravenous injection of 40 mg/kg of THP to male B6D2F1 mice at 10 or 23 hr after light onset (HALO). Differences between the two groups were statistically validated for both variables.

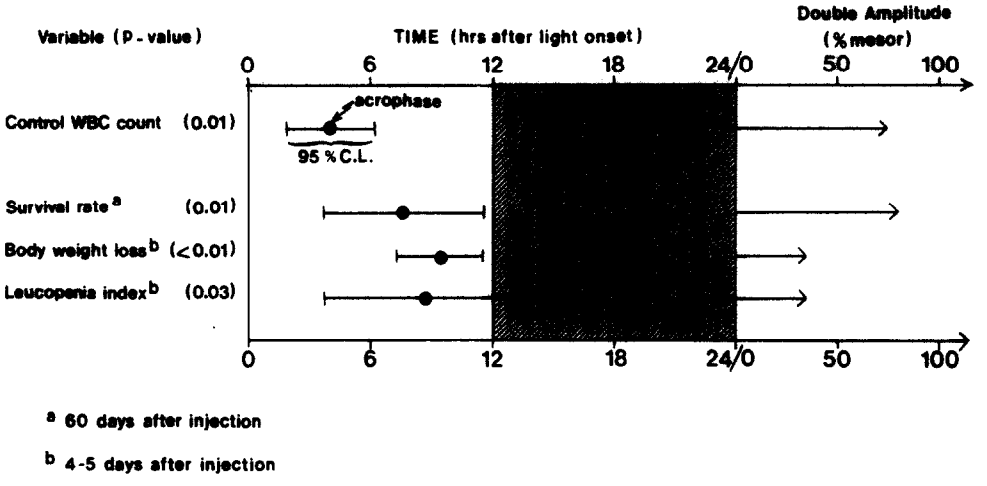

Fig. 3. Acrophase chart of chronotolerance for THP in male B6D2F1 mice. Black dots correspond to the location in time of the acrophase, and the horizontal line represents its 95% confidence interval. The extent of the double amplitude (expressed as % of the 24-hr mean or mesor) is indicated by the length of the vector, on the right of the figure. These two rhythm characteristics (as well as the P value of the detection of a circadian rhythm) are given for (a) the peripheral leukocyte count of controls which may serve as a reference rhythm, and (b) the survival rate, body weight loss and the leukopenia index of THP-injected mice.

receiving 40 mg/kg of THP at 10 or 22 HALO were respectively 1450 ± 270 and 1590 ± 300 cells/mm³ (N.S.).

A circadian rhythm in WBC count was demonstrated and statistically validated in control mice both by *t* test between values at peak (10020 ± 590 at 6 HALO) and trough (5290 ± 860 at 18 HALO; $t = 4.5$; $P < 0.001$) and by cosinor ($P < 0.001$; mesor ± S.E.M.: 7490 ± 380; double amplitude ± S.D.: 5200 ± 1390; acrophase ± S.D.: 04:00 HALO ± 2:10 hr). Such rhythm characteristics were similar to those already reported by Haus *et al.* [13]. Therefore mean WBC counts for each dosing time were also expressed as percentages of their corresponding controls. Mean WBC count was approximately twice higher in the group treated at 7 HALO as that injected at 19 HALO (Fig. 5).

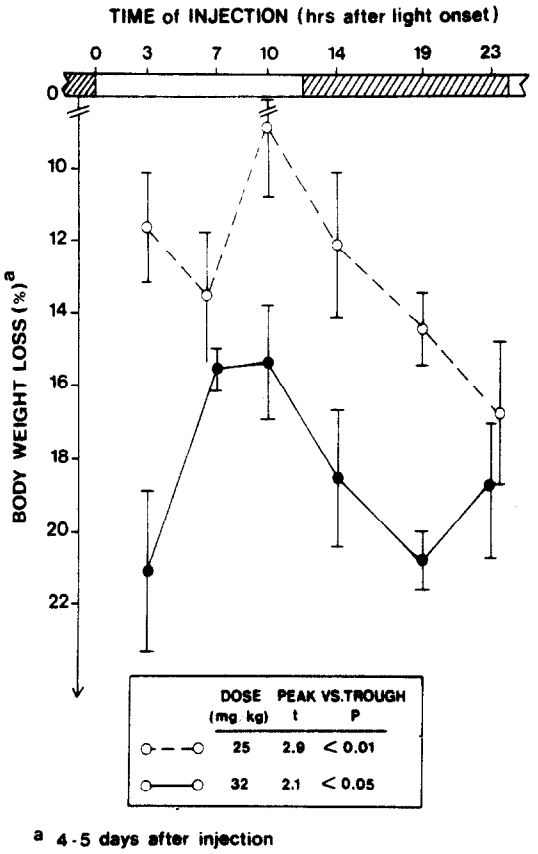

Fig. 4. Body weight loss (on days 4-5) as a function of dose (25 or 32 mg/kg) and dosing time of THP in male B6D2F1 mice. Statistically significant differences between mean values at least and largest body weight loss were validated by t test at each dose level.

Cosinor analysis was performed on data expressed as a percentage of the 24-hr mean at each dose level to minimize interseries differences. A statistically significant rhythm in this index of leukopenia was demonstrated ($P < 0.03$). Least leukopenia, as estimated by the acrophase, corresponded to THP dosing at 8:40 HALO (Fig. 3).

Cardiac histology

Cardiac lesions were observed in only 3/32 mice. All three belonged to treated groups. Lesions consisted of diffuse vacuolization and loss of muscular striation. Such lesions corresponded to treatment given at 19 HALO for 2/2 mice (study 2) and at 23 HALO for 1/4 mice (study 3).

DISCUSSION-CONCLUSION

Optimal tolerance for THP was achieved when this drug was given in the late rest span of mice (7-10 HALO). Whatever the index chosen to gauge tolerance — survival rate, body weight loss,

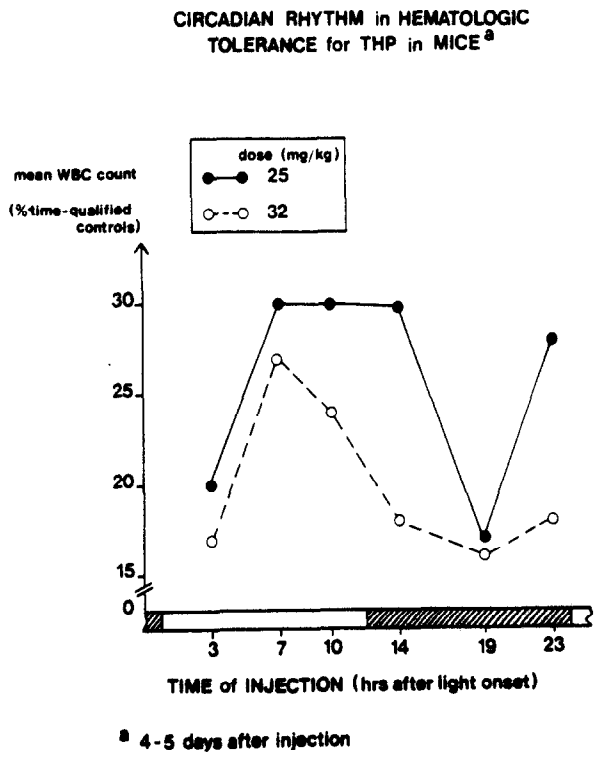

Fig. 5. Peripheral white blood cell (WBC) count 4-5 days after THP dosing (25 and 32 mg/kg). Mean WBC count of mice treated at a given circadian stage are expressed as a percentage of its corresponding mean control (time-qualified reference value). A circadian rhythm in this index of leukopenia was validated by cosinor (cf. Fig. 3).

leukopenia — similar results were obtained and statistically validated by two methods (Fig. 3). Furthermore, the time-effect was documented for three dosages (25, 32 and 40 mg/kg). Bone marrow suppression seemed to constitute the main mechanism of the lethal toxicity of THP. The bone marrow suppression induced by other cytostatics such as arabinosyl cytosine [14], melphalan [15] and X-rays [16] was also minimal when these agents were given in the late rest span of nocturnally active rodents.

Since these agents, including THP, differ with regard to their pharmacokinetics but were best tolerated by mice at similar times, one may hypothesize that the circadian rhythm in the intrinsic susceptibility of bone marrow cells (and presumably stem cells) is the prime mechanism of such a time-related tolerance. The demonstration of a circadian rhythm in the proliferative capacity of murine totipotent and committed stem cells [17, 18] further supports this hypothesis. The present data suggest that the circadian rhythm in the pharmacodynamic effects of a medication may be accounted for by the rhythmic susceptibility of target biosystems rather than by a circadian rhythm in drug pharmacokinetics [19].

The relevance of the mouse model to predict the optimal dosing time of adriamycin in cancer patients has already been documented [20, 21]. Since the peak times in bone-marrow-related tolerance of mice are similar for adriamycin and THP, the dosing time associated with an optimal hematologic tolerance for THP in cancer patients might be 06:00 hr, as for adriamycin.

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Table 3. Circadian changes in circulating WBC count (time-point mean ± 1 S.E.M.) with reference to time of a single intravenous injection of saline (0) or THP in one of three dosages

Dose of THP (mg/kg)	Time of injection (hr after light onset) 3	7	10	14	19	23	*t* test* *t*	*P*
0	8940 ± 950	7700 ±1070	6100 ± 800	8180 ± 990	7280 ±1170	7300 ±1400	2.3	<0.05
25	1790 ± 170	2340 ± 630	1820 ± 340	2370 ± 630	1220 ± 120	2020 ± 240	1.8	<0.05
32	1520 ± 310	2050 ± 340	1460 ± 250	1460 ± 140	1200 ± 170	1320 ± 200	2.3	<0.02
40	-	-	1450 ± 270	-	-	1590 ± 300	-	N.S.

*Comparing underlined peak (solid line) and through (dotted line) values.

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