Chronotherapy as a Potential Strategy to Reduce Ifosfamide-Induced Encephalopathy: A Preclinical Study in a Murine Model

Malek Chennoufi[1], Dorra Dridi[2], Khaled Lasram[3], Nouha Ben Abdeljalil[4], Asma Omezzine [5], Naceur A. Boughattas [1]

Mylene Malek Chennoufi (corresponding author) beyret@diplomats.com
Dorra Dridi dorra.dridi.jeddi@gmail.com
Khaled Lasram khaledlas@yahoo.fr
Nouha Ben Abdeljalil nouhaba@yahoo.fr
Asma Omezzine asmaomezzine2@gmail.com
Naceur A. Boughattas naboughattas@yahoo.fr

1- **Laboratory of pharmacology, Faculty of Medicine, Monastir, Tunisia**
2- **Pasteur Institut of Tunis, Tunisia**
3- **Higher Institut of applied Sciences and Technology of Mahdia, Tunisia**
4- **Laboratory of pathological anatomy, Hospital F.Borguiba, Monastir, Tunisia**
5- **Faculty of Pharmacy, Hospital Sahloul, Sousse, Tunisia**

Abstract

Purpose

Ifosfamide (IFO) is an effective chimiotherapeutic agent for sarcomas and germ-cell tumors but its clinical use is limited by severe toxicities, particularly hemorrhagic cystitis and encephalopathy. We recently demonstrated that IFO displays a circadian rhythm of lethal toxicity at LD50, with survival, body weight loss and core temperature strongly dependent on dosing time *(Chennoufi MM & Boughattas NA, 2025).*

That study established the presence of an intrinsic chronotolerance rhythm, but lethality prevented organ-specific analyses. Here, we extend this work by assessing whether sublethal IFO exposure (LD30).

Methods

160 male Swiss albinos mice were synchronized to a 12:12 h light/dark cycle. IFO LD30 was administered at four circadian times: 1, 7, 13, and 19 Hours After Light Onset (HALO). Endpoints included hematology, hepatic enzymes, histopathology (brain, liver, kidney, and bladder) and neurobehavioral function.

Organ-specific chronotoxicity patterns were compared to the LD50 chronotolerance rhythm previously reported.

Results

Marked circadian rhythms were observed across all endpoints. Dosing at 7 HALO induced the most severe overall toxicity, while administration at 13 HALO significantly reduced hepatic, renal, and bladder injury, encephalopathy-related lesions appeared only at 19 HALO. Hematological suppression and hepatic enzyme elevations also varied by dosing time, with lowest toxicity consistently at 13 HALO. Neurobehavioral impairment followed the same pattern. The optimal tolerance window overlapped with the LD50 peak tolerance found in our earlier study.

Conclusion

Sublethal IFO toxicity is strongly time-of-day dependent. Administration at 13 HALO (early active phase) minimizes brain and multi-organ injury and aligns with the chronotolerance peak previously defined at LD50. These complementary findings support the development of circadian-based IFO chronotherapy to improve clinical safety.

Keywords: Ifosfamide; Chronotherapy; Circadian rhythm; Ifosfamide-induced encephalopathy; Urotoxicity; Murine model.

Declarations

- **Funding:** No specific funding was received for this work.
- **Competing interests:** The authors declare no competing interests.
- **Data availability:** The datasets generated and analyzed during this study are available from the corresponding author on reasonable request.
- **Ethics:** All procedures involving animals were conducted in accordance with the international guidelines for the care and use of laboratory animals. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee of the Faculty of Medicine of Monastir.

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Introduction

Ifosfamide (IFO), developed in the early 1970s as a structural analogue of cyclophosphamide, remains an essential alkylating agent in oncology. Its antitumor activity arises from DNA cross-linking, leading to replication arrest and apoptosis.

For decades, IFO has been widely used for sarcomas, germ-cell tumors and various solid cancers, either alone or combined with other agents **(Zalupski & Baker, 1988; Williams & Wainer, 1999; Furlanut & Franceschi, 2003; Dechant et al., 1991).**
However, its clinical utility has long been limited by dose-dependent toxicities, including encephalopathy, nephrotoxicity, urotoxicity, hepatotoxicity and haematological suppression, for which no fully effective preventive strategy exists.

Despite being an "old" chemotherapeutic agent, IFO has experienced a marked resurgence of interest since 2020, prompted by new clinical, pharmacological and mechanistic insights.
Recent high-impact reviews have re-evaluated its metabolism, toxic mechanisms and clinical relevance, particularly focusing on neurotoxicity and the biochemical pathways underlying ifosfamide-induced encephalopathy **(Idle & Beyoğlu, 2023; Torchia et al., 2025; Beyoğlu et al., 2025)**. New clinical alerts and pharmacovigilance reports have also renewed attention to IFO's unpredictable toxicities and the continued lack of effective preventive strategies **(Brink et al., 2020; EMA 2020; Alqahtani et al., 2025)**.
In oncology practice, several modern trials have re-examined IFO-based regimens, including combinations with immunotherapy, regional hyperthermia, and anthracycline-based protocols, showing that IFO remains a cornerstone of current sarcoma management rather than a drug of the past **(Radaelli et al., 2024; Liu et al., 2024; Berclaz et al., 2025; Paudel et al., 2025).** Together, these observations confirm that IFO is undergoing a real renaissance, both clinically and scientifically.
Although chronotherapy of cyclophosphamide has been documented **(Haus et al., 1974; Lévi 1997; Lévi & Schibler, 2010; Fu & Kettner, 2013)**, the circadian determinants of IFO toxicity remain poorly understood, despite its complex metabolism via multiple cytochrome P450 isoenzymes and the recognised vulnerability of the brain to its toxic metabolites. Our previous LD□□ study demonstrated for the first time that IFO lethality follows a robust circadian rhythm, consistent with an intrinsic chronotolerance pattern **(Chennoufi MM & Boughattas NA, 2025)**.
Building on this evidence—and in light of the renewed global interest in IFO safety and optimisation—the present study uses a sublethal LD□□ dose to explore whether multi-organ IFO toxicity also follows circadian variation. We assessed hematological, hepatic, urothelial, neurological behaviour across four circadian dosing times, aiming to identify the 24-h window associated with the lowest global toxicity. Establishing organ-specific chronotoxicity patterns could provide a translational framework for future IFO chronotherapy.

Methods

Animals and synchronization

A total of 160 male Swiss Albinos mice (SIPHAT, Tunis), aged 8–10 weeks (≈25 g body weight), were used. Mice were synchronized for three weeks under controlled conditions. (12:12 light–dark cycle, lights on at 7:00 a.m.or 8:00 pm, room temperature 22 ± 2 °C, food and water ad libitum). Circadian synchronization was verified by body temperature rhythmicity **(Refinetti R. 2004, Waterhous J. et al 2005)**
Two rooms were used to avoid night manipulation :
Room 1 (lights on at 7:00 a.m.)

Room 2 (lights on at 8:00 p.m.)

LD50 and LD30 determination
LD50 and LD30 values were obtained by probit analysis performed in our previous chronotolerance study **(Chennoufi MM & Boughattas NA, 2025)**. The analysis estimated an LD50 of ≈520 mg/kg and a sublethal LD30 of ≈450 mg/kg. These values were used as reference doses in the present work.

LD50 chronotolerance protocol
To evaluate circadian variation in global Ifosfamide tolerance, mice received a single intraperitoneal injection of the LD50 dose at different circadian times (1, 5, 9, 13, 17 and 21 HALO). Animals were monitored for 14 days for survival, body weight variation, and clinical signs. Post-injection body temperature was also recorded. Survival data served to characterize the circadian pattern of Ifosfamide chronotolerance in mice.

LD30 acute toxicity protocol
To assess organ-specific toxicity while avoiding excessive lethality, mice received the LD30-equivalent dose (≈450 mg/kg), estimated from the LD50 probit model. Since all LD30-treated animals were euthanized 24 hours after injection to collect tissues, no survival follow-up was performed for the LD30 group**.** This design enabled the evaluation of acute biochemical, hematological, and histopathological toxicity at four circadian times (1, 7, 13, and 19 HALO).

Endpoints measured
Blood samples were collected for hematological parameters (WBC, lymphocytes, RBC, platelets) and biochemical markers (ALT, AST). Organs (liver, kidney, bladder, brain) were harvested for histopathological examination with blinded scoring. Neurological function was assessed using the wire test before sacrifice.

Ethical approval
All procedures complied with institutional guidelines for animal research. The study protocol was approved by the Institutional Animal Ethics Committee of the Faculty of Medicine of Monastir.

Hematotoxicity evaluation
Animals were sacrificed 24 hours after IFO administration, at the same circadian dosing-time as the injection. Euthanasia was performed by decapitation using a guillotine, in accordance with approved ethical procedures. Blood was collected immediately at decapitation by placing collection tubes directly under the neck, allowing passive flow into pre-labeled tubes without additional manipulation. Complete Blood Counts (CBC) were performed using an automated hematology analyzer. Parameters assessed:

- White blood cell count (WBC, $10^3/mm^3$)
- Platelet count (PLT, $10^3/mm^3$)
- Red blood cell count (RBC, 10□/mm^3)
- Lymphocytes ($10^3/mm^3$)

Hepatotoxicity evaluation

Blood serum was separated by centrifugation and analyzed for hepatic transaminases:

- **Aspartate transaminase (AST, IU/L)**
- **Alanine transaminase (ALT, IU/L)**
  Enzyme activity was determined using colorimetric commercial kits (Spinreact, Spain) following the manufacturer's instructions.

Histopathology

After blood collection at decapitation, organs including liver, kidney, bladder, and brain were promptly dissected and placed in 10% buffered formalin. Tissue harvest followed immediately after sampling to minimize post-mortem changes, and then embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin-eosin. Lesions were scored semi-quantitatively (0–5) by a blinded pathologist:

- **Bladder:** mucosal erosion, hemorrhage, edema.
- **Liver:** hepatocyte necrosis, inflammation, fatty change.
- **Brain:** neuronal degeneration, edema, vacuolization.
- **Kidney:** tubular epithelial degeneration, cytoplasmic vacuolization, interstitial edema, and inflammatory cell infiltration.

Neurotoxicity functional test (wire test)

Neurobehavioral performance was assessed at day 7 post-treatment using the **wire suspension test**. Each mouse was placed on a horizontal wire (2 mm diameter, 40 cm length, 30 cm above a padded surface). Latency to fall (maximum 60 seconds) was recorded. Results were averaged per group.

Statistical analysis

Data are presented as mean ± SEM. Comparisons between the four circadian dosing times (1, 7, 13 and 19 HALO) were performed using one-way ANOVA followed by Tukey's multiple comparison test for hematological and biochemical parameters. Histopathological scores (0–5) were compared using the Kruskal–Wallis test followed by Dunn's post hoc test.
Survival analysis at LD50 was reported from our previous chronotolerance study and was not re-analysed in the present work.
Statistical significance was set at $p < 0.05$. All analyses were performed using GraphPad Prism (version X, GraphPad Software, USA).

Results

1- **Animals synchronization**

Animals were synchronized under a 12:12 h light–dark cycle, and circadian entrainment was confirmed by the 24-h rhythm of core body temperature, as previously described in our initial chronotolerance study **(Supplementary Fig. S1)**

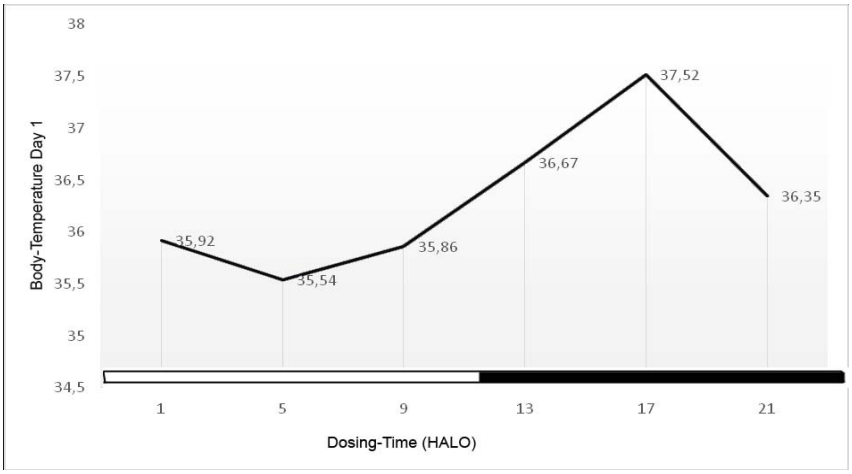

Fig. S1: Circadian variation of rectal temperature in mice before Ifosfamide administration.

2- Determination of LD50 and LD30

The dose–response curve is shown in **Fig. 1**. Mortality (%) increased in a dose-dependent manner, and probit analysis estimated an LD30 ≈ 450 mg/kg and an LD50 ≈ 520 mg/kg.

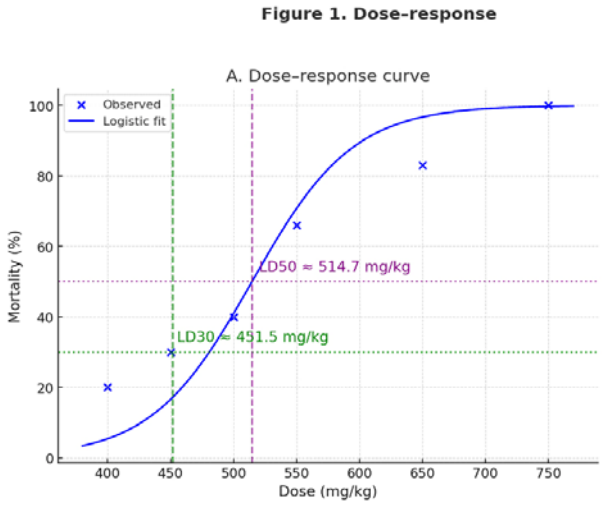

Fig.1. Dose–response and circadian survival after ifosfamide in mice.
Dose–response curve of ifosfamide–induced mortality in male Swiss albino mice.

Mortality (%) increased in a dose-dependent manner, and probit analysis estimated an LD30 ≈ 450 mg/kg and an LD50 ≈ 520 mg/kg.

3- Survival after IFO. LD50

Circadian variation in survival following LD50 Ifosfamide administration (520 mg/kg, i.p.) is presented in Fig. S2.
Survival exhibited clear dosing-time dependence, with the highest survival at 13 HALO (71.4%) and the lowest at 9 HALO (21.4%).

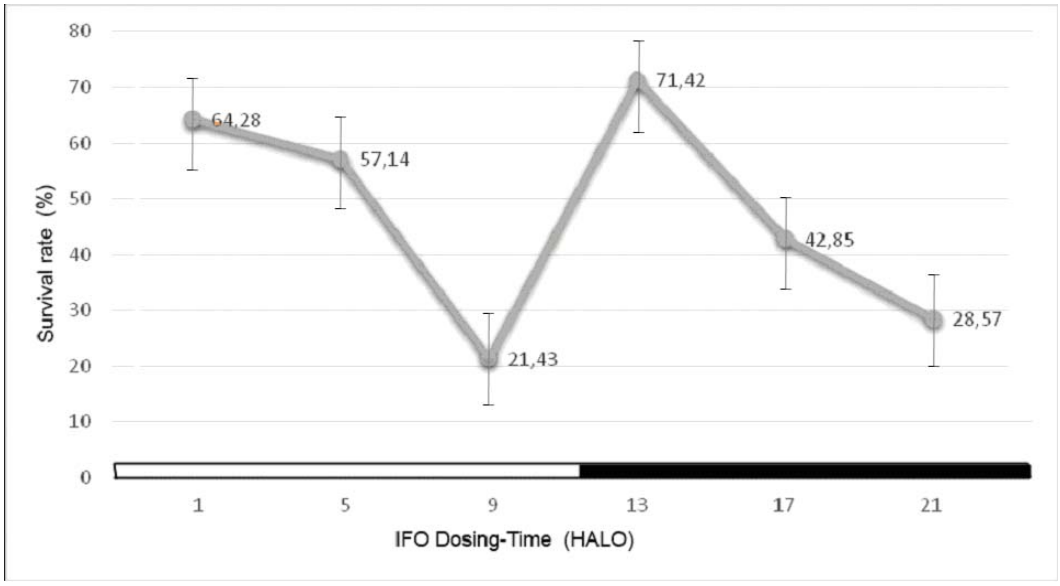

Fig. S2: Circadian variation in survival after a single LD50 Ifosfamide injection (520 mg/kg, i.p.)

4- Impact of IFO. on body core temperature

Circadian variation in rectal temperature before and after LD50 Ifosfamide administration is shown in Supplementary Fig. S3.
Body temperature measured at day 1 and day 14 showed pronounced circadian variation. The greatest post-injection decrease occurred at 7 HALO, whereas values at 13 HALO showed partial preservation of thermal stability.

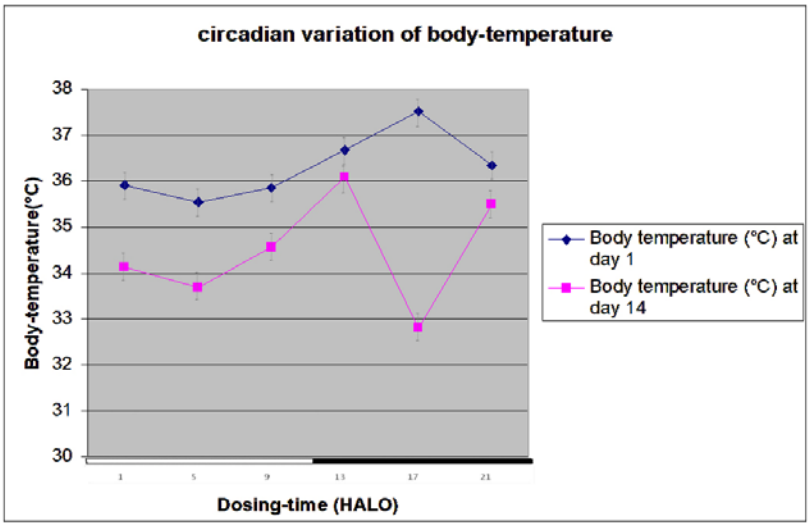

Fig. S3: Circadian variation of rectal temperature after LD50 Ifosfamide administration.

5- Hematotoxicity

Circadian variation in hematological toxicity following LD30 IFO administration is presented in Fig. 2.

Ifosfamide induced significant circadian variations in hematological parameters compared with controls.

- **White blood cells (WBC)**

A marked leukopenia was observed at all dosing times, with the greatest suppression at **7 HALO (1.03 ×10³/mm³)** and **19 HALO (0.7 ×10³/mm³)** compared to controls (13 and 4.76 ×10³/mm³, respectively). The least decrease was recorded at **13 HALO (1.9 ×10³/mm³)** (Fig. 2A).

- **Lymphocytes**

Significant circadian variations were also observed: Highest suppression at **7 HALO** and **19 HALO,** Partial preservation at **13 HALO** (Fig. 2B).

- **Platelets (PLT)**

Thrombocytopenia was significant at **7 HALO (455 ×10³/mm³)**, while **13 HALO (683.6 ×10³/mm³)** showed partial recovery. Interestingly, **19 HALO** exhibited paradoxical thrombocytosis (874.6 ×10³/mm³) (Fig. 2D).

- **Red blood cells (RBC):** A severe drop was observed at **19 HALO (3.07 ×10□/mm³)**, whereas values at **1 and 13 HALO** were closer to controls (6.51 and 6.01 ×10□/mm³) (Fig. 2C).

These findings demonstrate that hematotoxicity, particularly leukopenia and thrombocytopenia, was most pronounced at 7 and 19 HALO, while 13 HALO represented the most protective timing.

6- Hepatic chronotoxicity

Ifosfamide caused significant alterations in transaminase levels with circadian dependency.

- **AST levels** peaked at **7 HALO (975.25 IU/L)** and **19 HALO (959.6 IU/L)**, whereas lower increases were recorded at **1 and 13 HALO (894.2 and 887 IU/L, respectively)** compared to controls (Table 2).
- **ALT levels** were markedly elevated at **19 HALO (136.8 IU/L)**, followed by **7 and 1 HALO (~120 IU/L)**. The lowest increase was observed at **13 HALO (116.7 IU/L)**.

Overall, liver toxicity was maximal when IFO was administered at 7 and 19 HALO, while 13 HALO again showed relative protection.

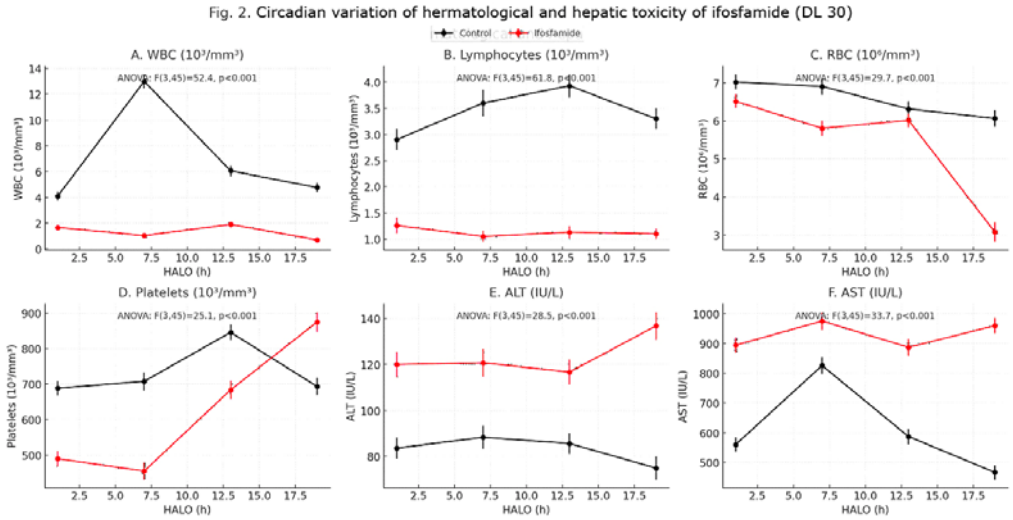

Fig. 2. Circadian variation of hematological and hepatic toxicities of ifosfamide (LD30).
Hematological and biochemical parameters were measured at 1, 7, 13, and 19 HALO (hours after light onset) in control and ifosfamide-treated mice (n = 6–8 per group).
(A) White blood cells (WBC, 10³/mm³): Ifosfamide induced a marked leukopenia at all dosing times, most pronounced at 7 and 19 HALO.
(B) Lymphocytes (10³/mm³): Severe lymphopenia was observed, with significant circadian variation and nadirs at 7 and 19 HALO.

(C) Red blood cells (RBC, $10^3/mm^3$): A profound decrease was recorded at 19 HALO compared with other times.
(D) Platelets ($10^3/mm^3$): Thrombocytopenia was significant at 7 HALO, partial recovery occurred at 13 HALO, while paradoxical thrombocytosis appeared at 19 HALO.
(E) Alanine aminotransferase (ALT, IU/L) and (F) Aspartate aminotransferase (AST, IU/L): Hepatic enzyme elevations displayed significant circadian dependency, peaking at 7 and 19 HALO, whereas 13 HALO showed relative protection.
Data are presented as mean ± SEM. One-way ANOVA revealed significant circadian variation for all endpoints (WBC: $F(4,45)= 52.4$, $p < 0.001$; lymphocytes: $F(4,45) = 61.8$, $p < 0.001$; RBC: $F(4,45) = 29.7$, $p < 0.001$; platelets: $F(4,45)= 25.1$, $p < 0.001$; ALT: $F(4,45)= 28.5$, $p < 0.001$; AST: $F(4,45)= 33.7$, $p < 0.001$).

7- Histopathology

Histopathological evaluation confirmed biochemical data: the bladder and liver were the main targets of IFO toxicity, with worsening effects at 7 and 19 HALO (Fig. 3).

- **Bladder:** Severe urothelial damage was observed at all times, with scores ranging from 2.25 to 3.5. Lesions were most severe at **19 HALO (3.5)**. No lesions were observed in controls (Fig. 3A).
- **Liver:** Significant hepatocellular necrosis and inflammation were present at all dosing times, with highest scores at **7 HALO (5.66)** and lowest at **13 HALO (4.33)**. (Fig. 3B).
- **Brain:** Brain lesions remained minimal except for a sharp increase at 19 HALO (Fig. 3C).
- **Kidney:** Kidney damage also showed a strong dosing-time dependence, with the highest lesion scores recorded at 19 HALO and near-baseline values at 1 and 13 HALO (Fig. 3D).

Figure 3. Histopathological Scores Across Circadian Times

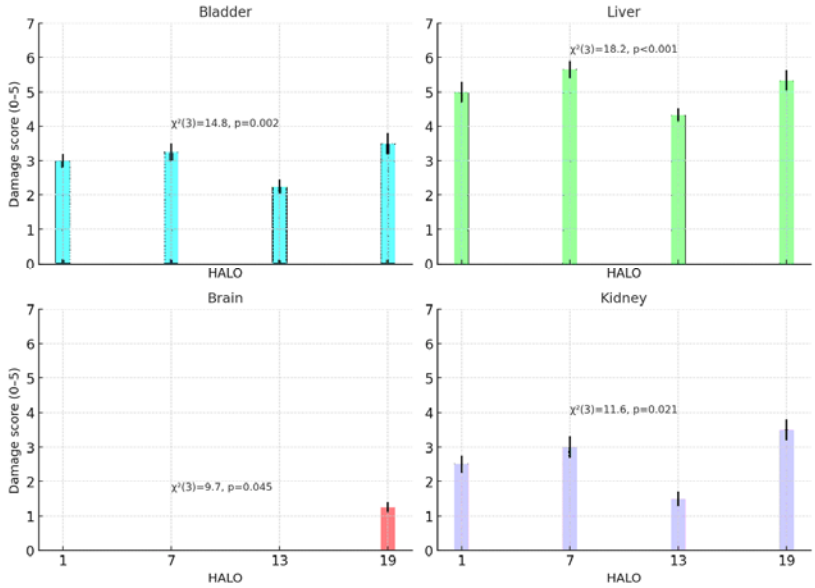

fig3. Circadian variation of ifosfamide-induced histopathological lesions in mice.
Semi-quantitative damage scores (mean ± SEM, n = 6–8 per group) for bladder, liver, kidney, and brain across four dosing times (1, 7, 13, and 19 HALO). Lesion severity varied significantly with dosing time (Kruskal–Wallis:
(A) bladder Chi-square (3) = 14.8, $p = 0.002$; (B) liver Chi-square (3) = 18.2, $p < 0.001$; (C) brain Chi-square (3) = 9.7, $p = 0.045$); (D) kidney Chi-square (3) = 11.6, $p = 0.021$.

8- Neurotoxicity (wire test)

Performance in the wire suspension test was significantly impaired at **19 HALO**, with the shortest latency to fall (≈12 s), compared to **13 HALO (≈40 s)** and controls (≈55 s). Neurological impairment was minimal at 1 and 13 HALO (Fig. 4).

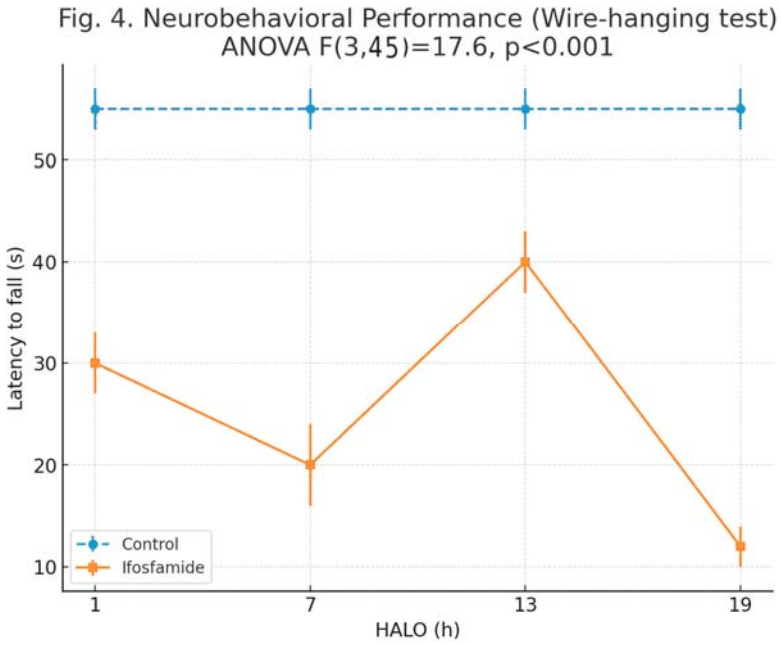

Fig. 4. Neurobehavioral performance in the wire-hanging test after ifosfamide administration (LD30) in mice.
Latency to fall (mean ± SEM, n = 6–8 per group) was recorded at 1, 7, 13, and 19 HALO. Ifosfamide-treated mice exhibited significant circadian variation in neuromuscular coordination (ANOVA F (3,45) = 17.6, p < 0.001). The shortest latency to fall, indicating maximal impairment, was observed at 19 HALO (~12 s), while the longest latency was at 13 HALO (~40 s), suggesting partial preservation of motor performance. Control mice maintained stable latencies (~55 s) across all time points.

Discussion

The present study demonstrates that Ifosfamide (IFO) toxicity exhibits a marked circadian variation across multiple organs—including the liver, kidney, bladder and brain—as well as hematological and neurobehavioral endpoints. These findings extend our previous LD50 chronotolerance study, which established a robust circadian rhythm in

IFO lethality, with optimal tolerance at 13 HALO and maximal vulnerability around 7 and 19 HALO. Here, using a sublethal LD30-equivalent dose, we show that acute multi-organ toxicity follows the same temporal pattern.

Hematological alterations revealed a strong time-of-day dependence. Leukopenia and lymphopenia were most pronounced at 7 and 19 HALO, whereas counts were better preserved at 1 and 13 HALO. Platelets also exhibited a circadian pattern, with significant thrombocytopenia at 7 HALO, partial recovery at 13 HALO, and paradoxical thrombocytosis at 19 HALO. These variations may reflect the circadian rhythmicity of bone marrow proliferation, immune cell trafficking, and the temporal regulation of drug metabolism pathways.

Hepatic toxicity showed a similar rhythmic pattern. Both ALT and AST peaked at 7 HALO, consistent with histopathological findings of marked hepatocellular necrosis and inflammation. The lowest enzyme elevations and histological damage were observed at 13 HALO, suggesting that hepatic detoxification capacity is maximally aligned with IFO metabolism during this phase.

Renal and urothelial injuries also varied significantly across the circadian cycle. Bladder damage, including erosions, hemorrhage and edema, was most severe at 19 HALO, whereas kidney lesions (tubular degeneration, vacuolization and interstitial edema) peaked at the same time point. These converging results are consistent with the susceptibility of renal tubular cells to chloroacetaldehyde, a toxic IFO metabolite known to follow circadian variation in formation.

Brain toxicity was relatively mild except at 19 HALO, where neuronal degeneration and vacuolization became prominent. This increased vulnerability coincided with the poorest performance in the wire-hanging test, indicating a functional correlate of histopathological damage. The alignment of metabolic stress, toxic metabolite accumulation, and reduced antioxidant capacity during this phase may contribute to heightened neurological sensitivity.

These results are particularly relevant in view of recent publications questioning the consistency of Ifosfamide-induced encephalopathy. In their review entitled *"Ifosfamide-induced encephalopathy: Myth or Reality?"*, Torchia et al. (2025) highlighted the heterogeneity of clinical presentations and the absence of reliable predictive markers. Our findings provide experimental evidence that neurological vulnerability to IFO is **not a myth**, but a **real, measurable, and time-dependent phenomenon**.
The marked increase in neuronal lesions and motor impairment at 19 HALO strongly supports the existence of a circadian window of susceptibility, offering a physiological explanation for the variability observed in patients.

Taken together, these findings demonstrate that IFO-induced toxicity is not constant throughout the 24-h cycle but instead follows a reproducible circadian pattern. The optimal tolerance window consistently occurred at **13 HALO**, reinforcing the notion that aligning IFO administration with circadian physiology could significantly reduce adverse

effects. This concept is consistent with earlier chronotherapy studies on other alkylating agents, including cyclophosphamide.

The translational implication is that timing optimization may improve the therapeutic index of IFO in clinical settings, particularly given the drug's narrow safety margin and variability in neurotoxicity. Future clinical studies are warranted to determine whether administering IFO at patient-specific circadian phases can reduce encephalopathy, urotoxicity and systemic toxicity while maintaining antitumor efficacy.

Clinical implications

Taken together, these results highlight that IFO toxicity is not only dose-dependent but also time-dependent. The **worst toxicity at 7 and 19 HALO** underscores the need to avoid dosing during these circadian phases. Conversely, **13 HALO emerged as the most protective time**, consistent with our earlier survival study. For humans, this corresponds approximately to the beginning of the **daytime activity period**, reinforcing the rationale for daytime administration in clinical protocols (Table 1).

While mesna reduces urotoxicity and methylene blue can mitigate encephalopathy in some cases, no strategy fully prevents IFO's major toxicities. Our findings suggest that **chronotherapy may provide an additional, non-pharmacological approach to improve tolerability**, allowing safer administration of IFO in oncology, particularly in pediatric protocols where it remains widely used.

HALO (Mice)	Circadian phase (Mice)	Corresponding HALO (Humans)	Circadian phase (Humans)	Clinical implication
1 HALO	Early rest phase	13 HALO (~6 p.m.)	Early rest phase	Intermediate toxicity
7 HALO	Late-rest phase	19 HALO (~12 a.m.)	Late-rest phase	High toxicity
13 HALO	Early active phase	1 HALO (~6 a.m.)	Early active phase	Best tolerance
19 HALO	Late active phase	7 HALO (~2 p.m.)	Late active phase	High toxicity

Table 1. Correspondence between circadian dosing time (HALO) in mice and humans after LD30 ifosfamide in mice.

Conclusion

This study demonstrates that ifosfamide (IFO) exhibits marked **circadian variation in organ-specific toxicity**. Hematological suppression, hepatic enzyme elevation, urothelial lesions and neurotoxicity were all significantly influenced by dosing time. The most

severe toxic effects occurred at **7 and 19 HALO**, while the **least toxicity was consistently observed at 13 HALO**.

These findings extend our previous work on IFO chronotolerance by highlighting the **specific targets of circadian toxicity**—bone marrow, liver, bladder and brain pathways. Importantly, neurological impairment, a hallmark of ifosfamide-induced encephalopathy (IIE), was minimized when treatment was given at 13 HALO.

Given that IIE remains unpredictable and lacks an effective preventive or curative strategy, chronotherapy emerges as a promising, non-pharmacological approach to improve IFO tolerability. Optimizing administration schedules could reduce systemic and organ-specific toxicities, thereby enhancing the therapeutic index of IFO in clinical oncology, particularly in pediatric protocols where it continues to play a crucial role.

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